ANALYTICAL STUDIES ON DIAZEPAM

by

Carol J. Barbour

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Department of Pharmacy

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ABSTRACT

Assay methods have been developed which are specific for the analysis of diazepam in the presence of formulation excipients, closely related degradation products and manufacturing impurities. The methods developed used difference ultraviolet spectrophotometry and HPLC.

These methods have been applied to the analysis of diazepam in formulations and to investigate the reaction kinetics of the acid hydrolysis of diazepam.

From analysis of both fresh and stored samples of formulations, it was seen that solid dosage forms showed no degradation, but degradation products were detected in liquid formulations.

Advantages and disadvantages were seen for both techniques and criteria were established for the choice of method.

The reaction kinetics of the degradation of diazepam was studied, using HPLC and GC. Previous workers had investigated the acid hydrolysis of diazepam using non-specific analytical methods. An attempt was made to repeat their work using the specific chromatographic methods described. From initial work, it appears that the results obtained are similar to those previously generated. Further work is required to investigate this more fully.

INDEX

FOR DIAZEPAM

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

The quality of medicines is controlled by a variety of official publications such as the British Pharmacopoeia and the United States Pharmacopeia. These contain monographs for both drug substances and formulated medicines with specifications which must be met. In general, these are not as strict as the limits applied by pharmaceutical companies for the medicines they produce. The specifications set by the official bodies are check specifications which the product must comply with throughout its shelf-life, while those set by manufacturers are release specifications which must be tighter than the check specifications to ensure compliance at the end of the product's shelf-life.

The monographs found in official publications include a description of the drug or product, tests for identity, physical constants, a quantitative assay of the pure chemical entity (for drug substances) or the principal active constituent(s) (for medicines) and also limit tests to exclude excessive contamination.

In these official monographs, the assay method is generally a non-specific one, the tests for identity and impurities serving to confirm the identity and purity of the sample and to complement the precision of the non-specific assay. However, there are many occasions in the development of a drug or medicine where a specific assay is required for the drug in the presence of decomposition products.

A great deal of information is required on a drug substance, both in its pure form and in formulations before submission to the Licensing Authorities can be made for Product Licences. This information is required to provide chemical, pharmaceutical and analytical data to define the purity, potency and stability of the drug and its dosage forms to avoid any variation in the product which might be of biological significance.

The stability of the drug substance is of great importance and knowledge of this for both drug and formulations is required. The stability of the drug on exposure to heat, light, moisture, pH change are all important and have a bearing on formulation to minimise the degradation of the product and to allow prediction of the shelf-life.

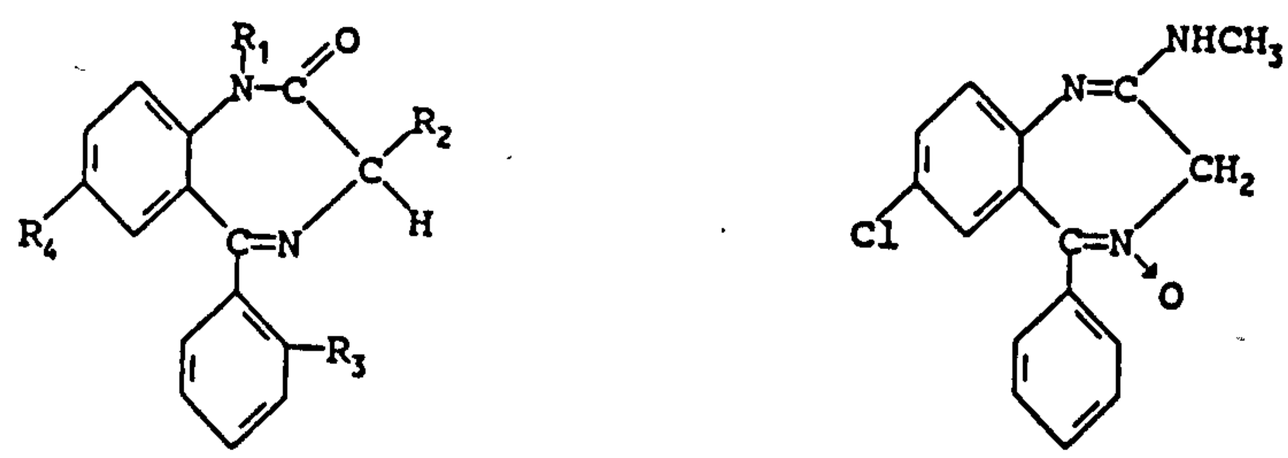
Tests for pharmaceuticals must be suitable for detection of the active drug moiety in the presence of many potential interferents including synthetic intermediates, impurities of manufacture, decomposition products and excipients present in the formulation developed.

The requirements of a stability-indicating assay are speed, ease of performance, sensitivity, selectivity and robustness and methods should be assessed using these criteria.

1.2 DIAZEPAM AND RELATED COMPOUNDS

1.2.1 1,4-Benzodiazepines

Diazepam is a widely used tranquiliser, a member of the 1,4-benzodiazepine series of drugs. This class of drug is used as tranquilisers, sleep inducers and muscle relaxants. A number of molecules of this type have been synthesised and are in clinical use, Fig. 1.1 shows some typical structures.



(a) (b)

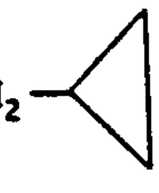
	R_1	R_2	R_3	R_4
Diazepam	CH_3	H	H	Cl
Oxazepam	H	OH	H	Cl
Nitrazepam	H	H	H	NO_2
Lorazepam	H	OH	Cl	Cl
Flurazepam	$-(CH_2)_2N(C_2H_5)_2$	H	F	Cl
Prazepam	$-CH_2-$ 	H	H	Cl
Flunitrazepam	CH_3	H	F	NO_2
Clonazepam	H	H	Cl	NO_2

Fig. 1.1 (a) General structure
(b) Structure of chlordiazepoxide

Many of the compounds are interrelated, with metabolism of some members producing other related drugs or active metabolites, eg the metabolism of diazepam, Fig. 1.2, gives oxazepam as one of

the metabolites.

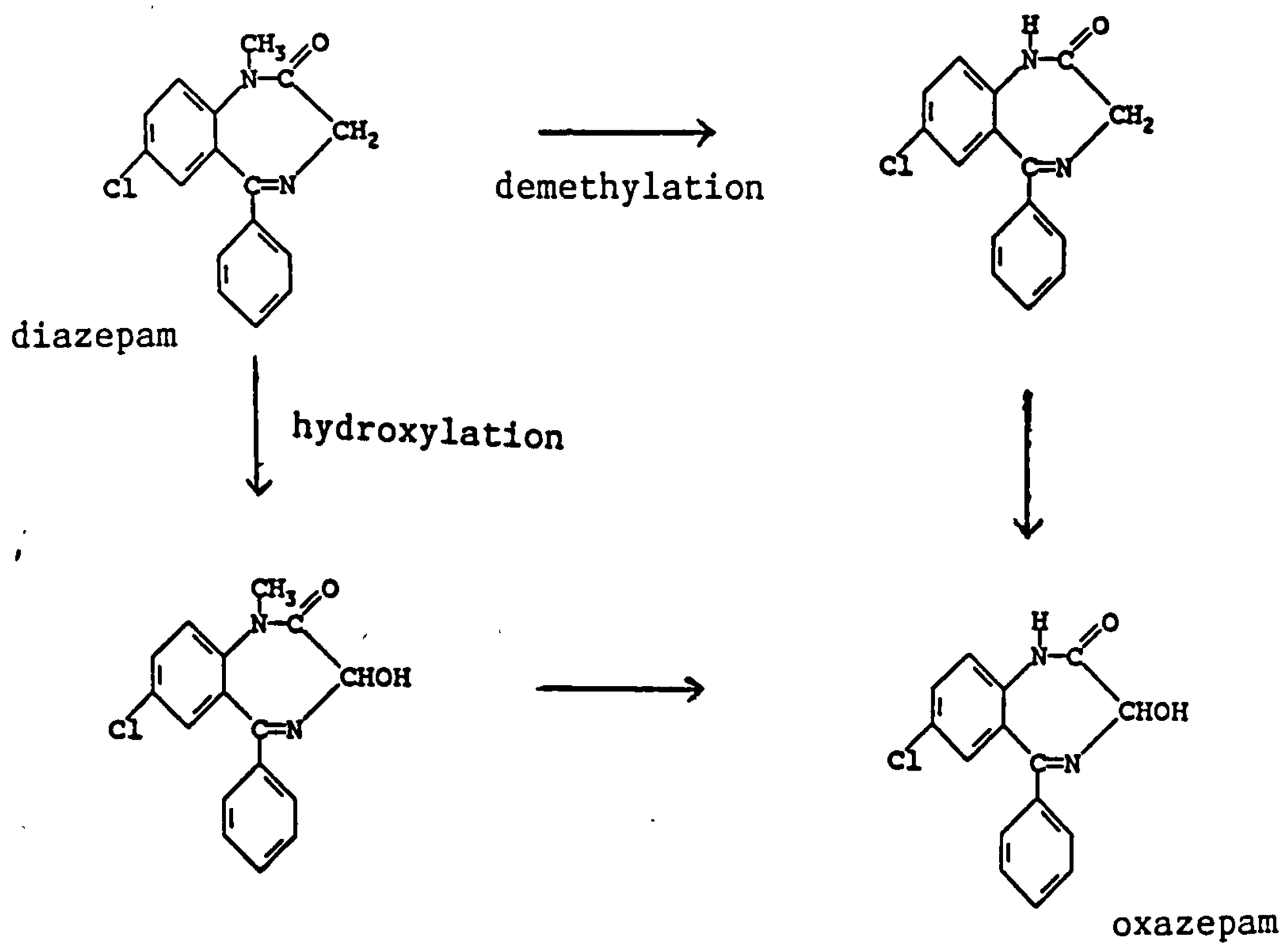


Fig. 1.2 Metabolic pathway of diazepam

1.2.2 Physical properties of diazepam

Diazepam, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 1.3) has a molecular weight of 284.75 and an empirical formula, $C_{16}H_{13}ClN_2O$.

Diazepam is synthesised by a number of routes, including that from 2-methylamino-5-chlorobenzophenone (MACB) and ethylglycinate (Fig. 1.3) and occurs as an off-white to yellow, practically odourless crystalline powder. One reported synthetic precursor of diazepam is desmethyl diazepam, (7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one) which is almost identical in structure to diazepam but lacks the methyl group on

N-1.

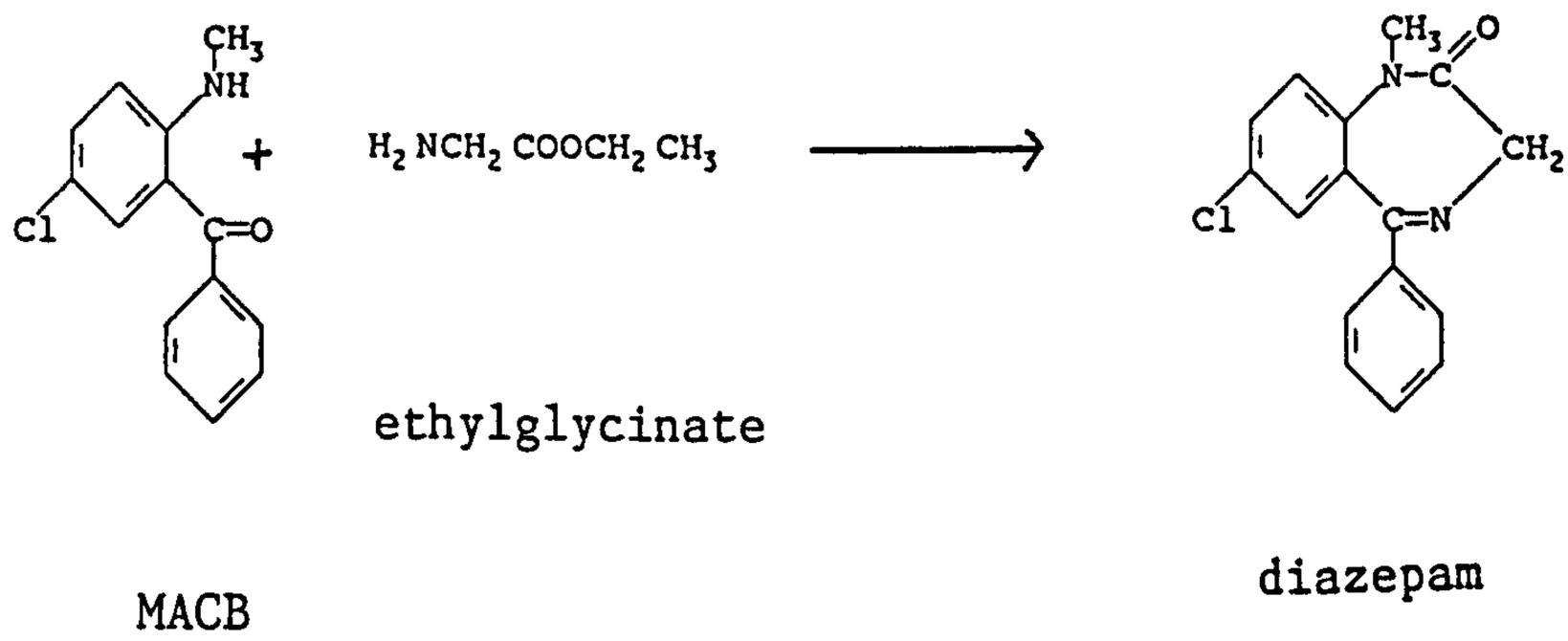
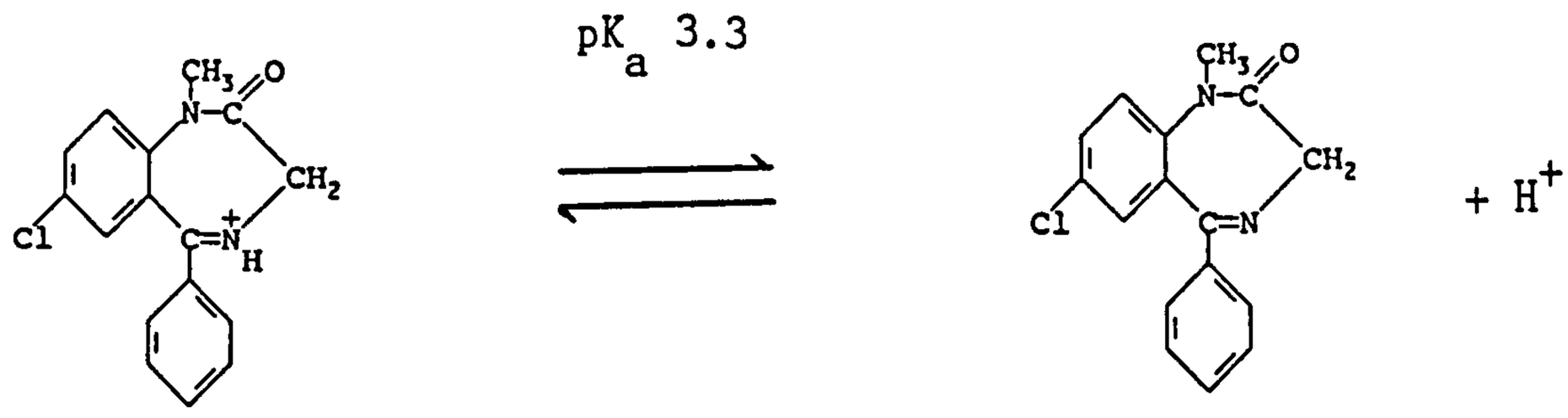


Fig. 1.3 Synthetic pathway of diazepam

Solubility characteristics indicate that diazepam is poorly soluble in water (0.05 mg/ml), more soluble in ethanol and methanol (41 and 49 mg/ml) and very soluble in chloroform (more than 500 mg/ml) [Florey, (1972)].

The dissociation constant has been determined spectrophotometrically and this has been quoted as a pK_a of 3.4 by Newton and Kluza (1978), and as 3.3 by Barrett et al. (1973). Barrett and co-workers predicted that the ionisation occurs at the N-4 atom rather than the N-1 atom on the basis of the spectral changes seen for a number of drugs of this group, Fig. 1.4.



pH 0-2 pH 5-12

Fig. 1.4 Protonation of diazepam

1.2.3 Stability of 1,4-benzodiazepines

The hydrolysis scheme for diazepam proposed by Nakano et al. (1979) is shown in Fig. 1.5.

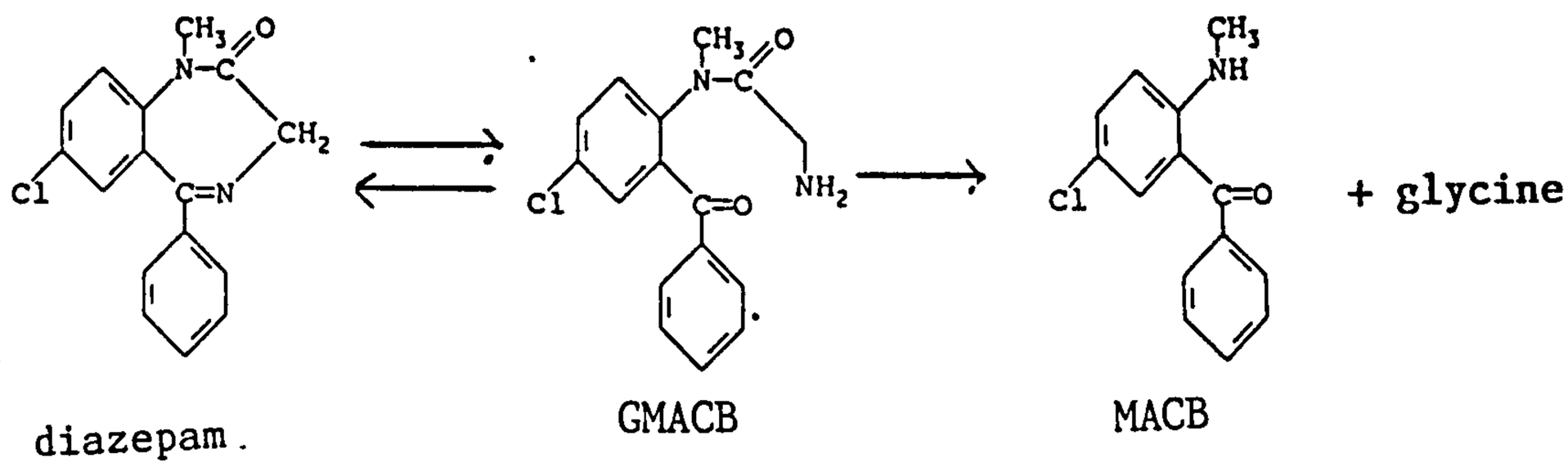


Fig. 1.5 Degradation scheme for diazepam

A general hydrolysis scheme for 1,4-benzodiazepines postulated by Cartensen et al. (1971) is shown in Fig. 1.6. In this scheme, the reaction to form the acridone derivative is facilitated when R_2 is a halogen, in particular fluorine.

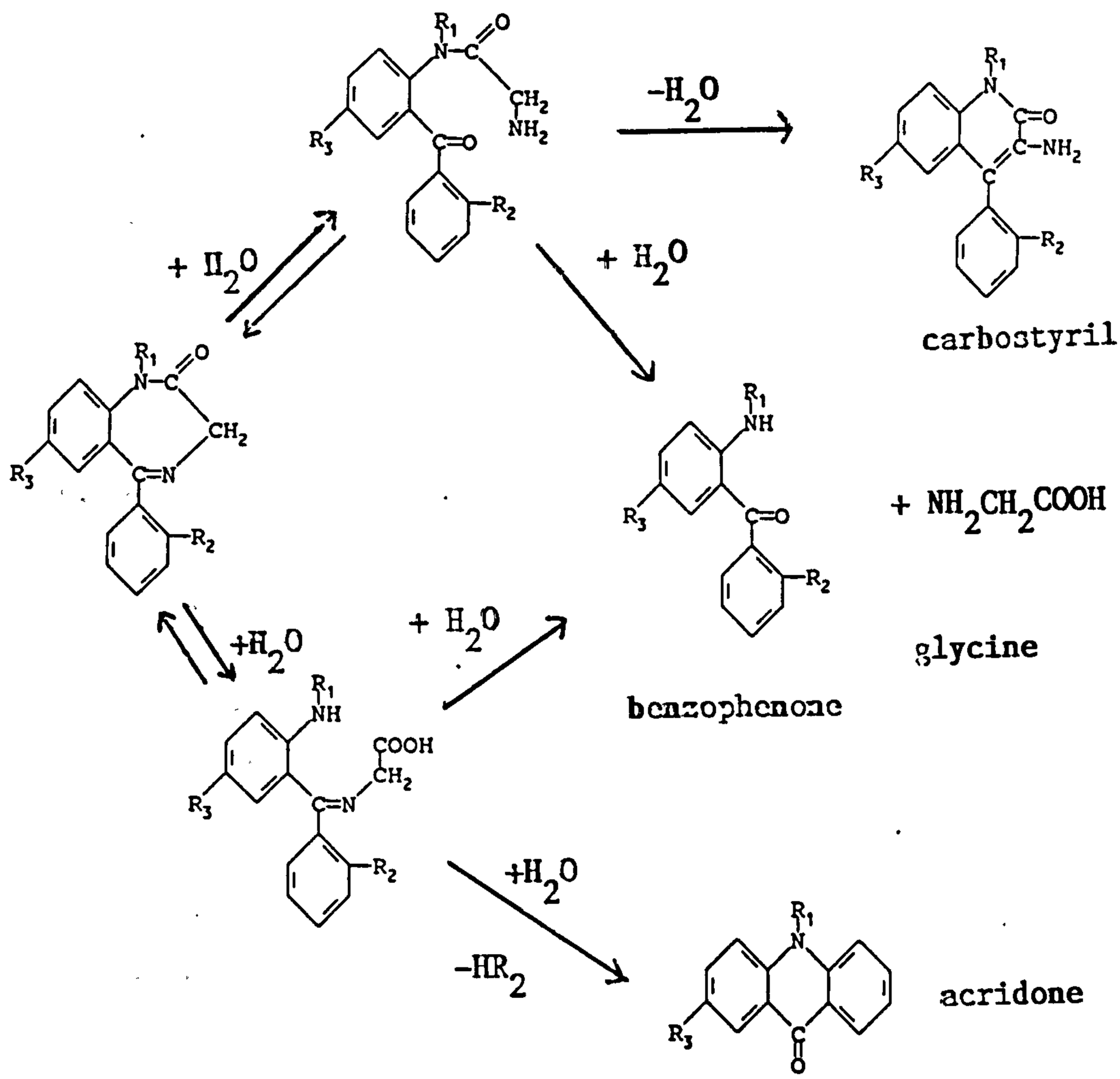


Fig. 1.6 General degradation scheme for benzodiazepines

In this scheme, the point at which cleavage of the ring occurs depends on the substitution of the benzodiazepine involved. Han et al., (1976, 1977a and 1977b) followed the reactions of chlordiazepoxide, demoxepam, diazepam, oxazepam and nitrazepam and discussed the positions of cleavage for these molecules. Demoxepam is predominantly cleaved at the amide linkage, oxazepam would also be preferentially cleaved at this linkage while for diazepam, methyl substitution at the amide nitrogen (N-1) makes initial azomethine hydrolysis the preferred pathway, (R_1 = CH_3, R_2 = H and R_3 = Cl for diazepam in Fig. 1.6). This methyl substitution also makes diazepam one of

the more stable members of the series. In the case of nitrazepam, the reaction was reported to proceed via hydrolysis of the azomethine bond. As indicated by the schematic diagram, this stage in the reaction is reversible, with recyclisation occurring depending on the conditions present.

The further degradation of these open-ring compounds to carbostyril, benzophenone and acridone derivatives are dependent on temperature and pH. At room temperature, benzophenone derivatives are favoured with carbostyril and acridone derivatives present at 10% and 1% respectively of the benzophenone content. As the temperature increases, the incidence of these other compounds increases. As can be seen from the proposed routes, studies of the reaction kinetics of hydrolysis would be very complicated with both sequential reactions and parallel reactions occurring simultaneously. Due to the low concentration of any product other than benzophenone, and the rate determining steps of conversion to benzophenone being much larger than those to carbostyril or acridone derivatives, the overall stability of diazepam was judged by Cartensen et al. (1971) by monitoring the benzophenone formation.

Chlordiazepoxide has a different basic structure from that of the other 1,4-benzodiazepines discussed here, and a different initial step in its degradation pathway, Fig. 1.7. The first stage in the hydrolysis of chlordiazepoxide is the conversion to demoxepam, a lactam, which then reacts in a similar way to the other compounds discussed above.

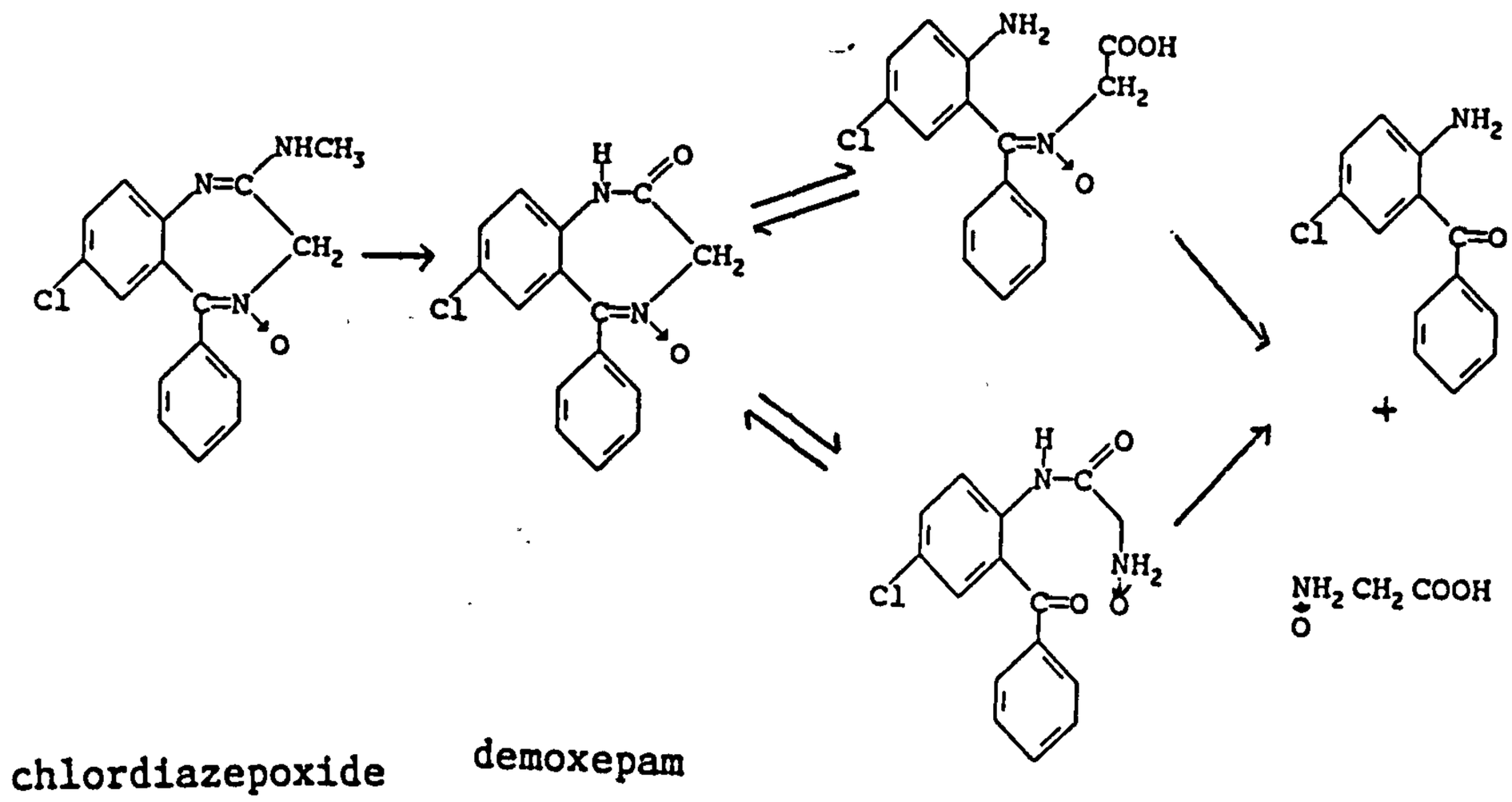


Fig. 1.7 Degradation scheme for chlordiazepoxide

All the steps in the degradation reactions were found to be first-order reactions.

The degradation of chlordiazepoxide has been followed by both Maulding et al. (1975) and Han et al. (1976). As might be expected from the similar structures of these compounds, interference in the ultraviolet spectrophotometric determination occurs. Maulding and co-workers used two techniques to follow the degradation of chlodiazepoxide to demoxepam, which allowed separation of chlordiazepoxide from the reaction mixture before its spectrophotometric quantitation. The methods used were TLC and extraction of the reaction mixture. In the case of TLC, chlordiazepoxide was separated from other compounds by TLC then extracted from the silica after development of the plate. The content of starting product was then measured by spectrophotometric measurement of the recovered material. In the second method, the samples taken from the reaction were

extracted with methylene chloride to remove any demoxepam and benzophenone before measurement. These determinations only followed the initial degradation to demoxepam and not the further reaction to benzophenone, although this material was present in the acidic hydrolysis solutions. The reaction was studied at a number of pH values and with different concentrations of buffer present.

Han et al. (1976) described the complete kinetics of the hydrolysis of chlordiazepoxide, from parent molecule to benzophenone derivative, thus extending the study performed by Maulding et al. The effect of ionic strength and temperature on the rate constants measured were reported. In this study the absorbance of the reaction mixture was measured initially, at appropriate time intervals through the reaction and at t_∞. The difference in absorbance between these points was then plotted against time as described in Appendix I. Sequential reactions were found to occur and a number of rate constants were calculated, each with a different slope, related to the reaction step being monitored. Simplification in the determination of one reaction step was possible by choice of wavelength monitored. An isosbestic point of one reaction stage was chosen to monitor a second reaction stage eliminating interference from the first reaction step. However, problems of interfering results from different reactions resulted in the need for mathematical treatment of the results to isolate each stage in the reaction.

The studies performed by Han et al. were conducted at elevated temperature with benzophenones and quinolines

identified in the reaction mixture. Studies have also been performed on 1,4-benzodiazepines using body temperature to investigate possible effects on bioavailability of these drugs due to the reversible ring-opening reaction described [Nakano et al., (1979)].

These workers investigated the hydrolytic reaction of diazepam in acid solution at body temperature using spectrophotometry. It was found that the azomethine bond was broken to give an open-ring compound which was in equilibrium with protonated diazepam. The rate constant for ring-closure was found to be much greater than for ring-opening, resulting in a larger concentration of diazepam than open-ring material at equilibrium. The cyclization reaction rate was shown to be pH dependent, with immediate ring-closure at pH 7, and very fast closure at values greater that the pK_a of diazepam. At high temperatures, the further degradation to benzophenone is expected to occur, with the potential for quinoline formation if the concentration of water present is low, or the temperature high. At body temperature, the reversible reaction from diazepam to the open-ring compound was expected to be the major reaction. The workers have tentatively identified the open-ring intermediate as 2-glycyl(methyl)amino-5-chlorbenzophenone (GMACB) and have used an authentic sample of this material to confirm the ring-closure to diazepam. Further work was performed on nitrazepam [Inotsume and Nakano, (1980)] and alprazolam [Cho et al., (1983)] which were also found to undergo this ring-opening in acid with recyclization when the solutions were neutralized. This reversible nature and lack of extractibility of

the open-ring form into organic solvents have made isolation difficult.

Although this reaction was investigated to detect possible effects on bioavailability of the drugs, it is not thought likely to adversely affect bioavailability. On administration of the drug orally, GMACB may form in the acidic environment of the stomach, and would not be absorbed. However, on passage of the stomach contents into the small intestine, the neutralisation would result in reversion of the ring-opened material to parent molecule and absorption would be possible.

This pH-dependent ring-opening has been used in a recent marketed benzodiazepine product, midazolam, an i/v sedative. This is formulated as a salt in aqueous solution. At pH values lower than pH 4 it undergoes ring-opening and is highly water soluble, at pH values greater than pH 4, it undergoes ring-closure to give a lipid soluble compound, Fig. 1.8.

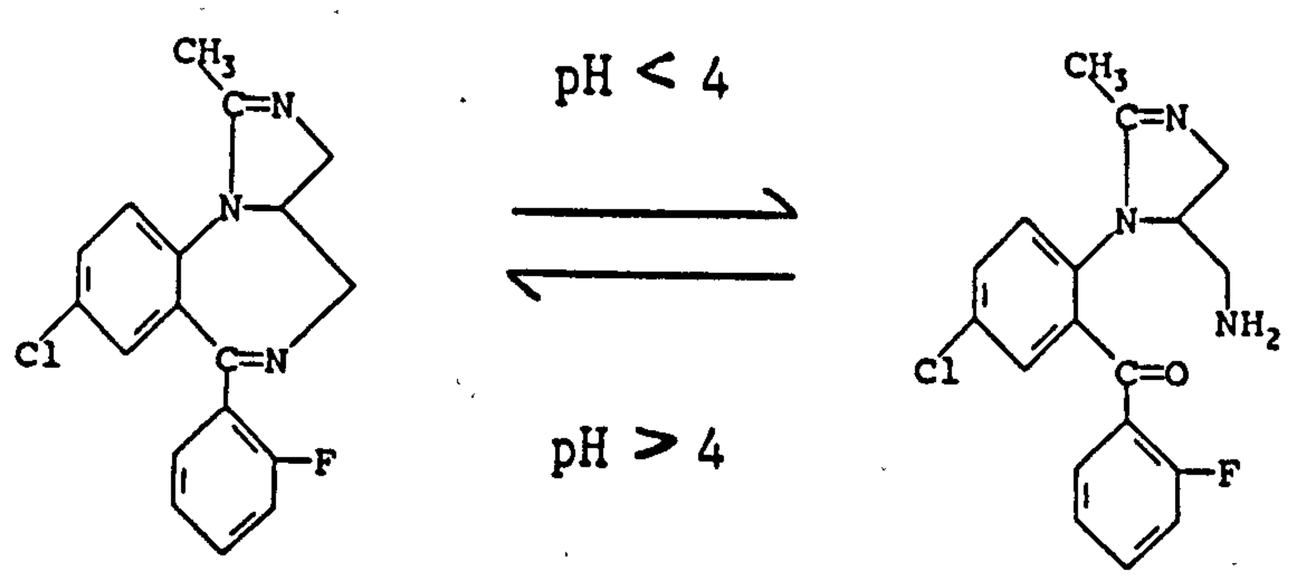


Fig. 1.8 Ring-opening reaction of midazolam

The ring-open form is stable in aqueous solution and

has a solubility dependent on the pH of solution. It is formulated in the ring-open form, but once injected into the body, the ring closes at physiological pH and stays closed to enhance the lipid solubility of the drug and provide a fast onset of action after i/v administration, [Dundee, (1979)].

Work has also been performed on the solid state stability of nitrazepam, [Genton and Kesselring, (1977)]. In this study, the decomposition of nitrazepam in microcrystalline cellulose was studied by extraction of nitrazepam and degradation products, separation of these by TLC and measurement of each by diffuse reflectance measurement. The effect of temperature and humidity was studied by following the decrease of nitrazepam and the increase of 2-amino-5-nitrobenzophenone and 3-amino-6-nitro-4-phenyl-2(1H)-quinolone. The reaction scheme found was as described in Fig. 1.6 with a reversible reaction to an open-ring intermediate being followed with the further reaction to the benzophenone product (by addition of water) or to the quinolone (by loss of water). The main product is benzophenone in aqueous solution and quinolone in solid state, with the ratio depending on the availability of water. The direct formation of the quinolone derivative without intermediates was only seen at high temperatures, at or above the fusion temperature. Further unidentified compounds were observed in samples stored at high temperature and humidity, which were thought to be breakdown products of the benzophenone and quinolone products.

In addition to hydrolytic breakdown, a second route for degradation is photochemical decomposition. This

has been studied for diazepam [Cornelissen et al., (1978)]. These workers irradiated a solution of diazepam in methanol for 17 hours. The solution was then chromatographed to separate the various compounds obtained and these were identified, after isolation, by NMR and MS. The compounds isolated are shown in Fig. 1.9 and were found to fall into three main groups, benzophenones, 4-phenylquinazolinones and 4-phenylquinazolines. The percentage of each type of compound found was dependent on the solvent used, the concentration of solution and the intensity and wavelength of light used.

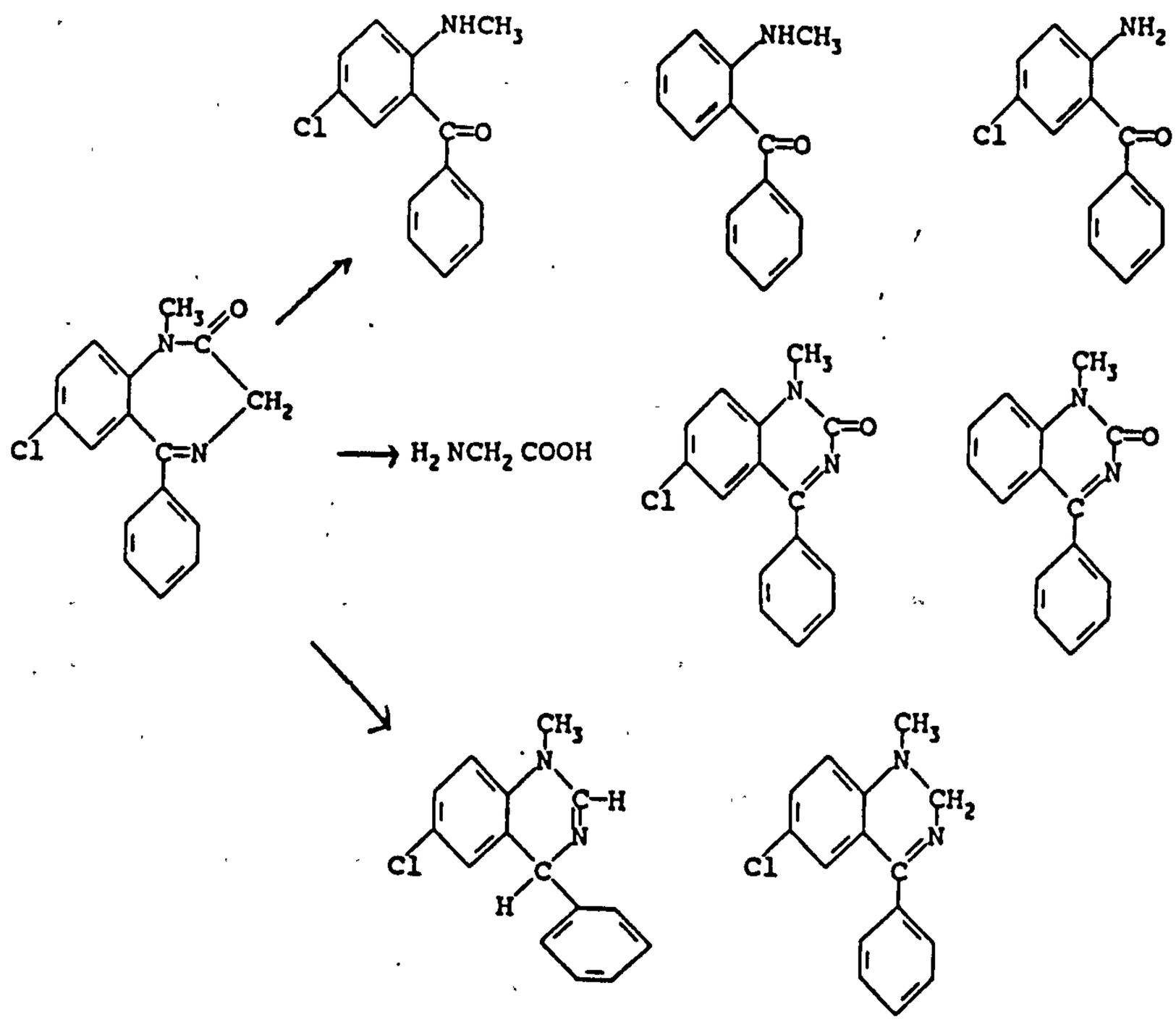


Fig. 1.9 Photochemical decomposition of diazepam

Diazepam has been produced in a number of different formulations, both liquid and solid. In the case of solid formulations ie tablets, capsules and

suppositories, very low moisture content is present and these formulations have been shown to undergo minimal decomposition after storage for extended periods of time [Connors et al., (1979)]. As the major route for degradation is hydrolysis, the liquid dosage forms ie syrup and injection would be expected to show more decomposition. The formulations produced for these liquid preparations optimise the conditions for stability. The pH-rate profile for hydrolysis of diazepam indicates maximum stability of diazepam in aqueous solution occurs at approximately pH 5, the pH of the syrup formulation. The use of a mixed solvent system containing propylene glycol, ethanol, benzyl alcohol and water shows enhanced stability and solubility over that of a simple aqueous solution. This type of mixed solvent system is used for Valium injection, thus enhancing the stability of diazepam.

1.2.4 Analytical methods for assay of diazepam and other benzodiazepines

As might be expected for a drug substance which has been so widely used, a great number of analytical methods, using a variety of techniques, have been published. These methods have been developed for use in four main areas,

(i) for use in elucidation of the metabolism of the drugs and their toxicology during initial development of the drugs
(ii) for checking the purity and specification of the drugs during manufacture of the dosage forms, and on storage of these for stability determination

(iii) for studying tissue and body fluid levels of the drug in clinical use for pharmacokinetic studies

(iv) measuring tissue and body fluid levels during forensic examination, eg after overdosage.

As a result of these different sample matrices, the requirements of the analytical methods depend on the application they are to be used for, the information required and the other materials likely to be present in the sample to be analysed, eg whether degradation products, impurities, metabolites, co-formulated drugs, or excipients are likely to be present.

Two reviews have been published on the analysis of 1,4-benzodiazepines. One of these deals with all types of analytical methods as applied to measurement of the drugs in body fluids [Clifford and Smyth, (1974)], while the second describes the application of chromatographic methods [Hailey, (1974)]. In the years since these have been published, a number of other analytical methods have been developed, using new and improved analytical techniques.

In general, the major requirements for metabolic study assays are sensitivity and specificity. For the assay of active ingredient in formulations with this group of drugs, sensitivity is not a problem although specificity is still important.

For analysis of some members of the series, particularily for metabolic or forensic studies, the response of the drug substance is poor and does not give the sensitivity required. Also for analysis using

GC, not all the compounds are thermally stable. As a result a number of assay methods have been developed where the parent drugs are converted to the benzophenone derivatives by the use of a hydrolytic reaction before measurement. This has both advantages and disadvantages, as specificity can be lost with several parent molecules and metabolites converting to the same benzophenone, preventing the determination of each individually. However, this can increase the sensitivity for the assay of levels of drug and related compounds in toxicology studies. A review has been published of analytical methods developed using hydrolysis [Gasparic and Zimak, (1983)].

1.2.4.1 Titrimetric methods

The use of non-aqueous titration is described in both the British Pharmacopoeia and the United States Pharmacopeia [B.P., (1980); U.S.P., (1980)]. In both, a suitable weight of diazepam is dissolved in acetic anhydride and is titrated with 0.1 M perchloric acid in glacial acetic acid. This type of method provides a very precise measurement, but is non-selective with the possibility of related compounds interfering in the determination. These methods are generally used for drug substance assay and are used in conjuction with other tests in the monograph. Thus the lack of specificity is compensated for by the use of identity tests and TLC tests for determination of related substances. The official methods also lack sensitivity, requiring solutions of approximately 10 mg/ml. However, as the method is only applied to drug substance, this does not pose a problem.

1.2.4.2 Thin-layer chromatography methods

These methods have been used as semi-quantitative methods for estimation of impurities or degradation products. A number of different methods of detection have been used; UV spectrophotometry [B.P., (1980); U.S.P., (1980)], hydrolysis of the compound and reaction with reagent on the plate [Gasparic and Zimak, (1983)].

Wouters et al., (1979) have developed systems for the identification of 19 benzodiazepine derivatives using three different chromatographic systems, while Bakavoli et al. (1984) have used two-dimensional TLC with hydrolysis before the second development to identify 12 benzodiazepines.

More accurate measurement of the quantity of drug present has used densitometric measurement [Grijalba et al., (1979)]. In this work, a number of different densitometric methods of quantitation were used, some without internal standard and some with the incluion of oxazepam as internal standard. Accuracy and precision were claimed for the method, and it was proposed as a means of quantitative analysis of diazepam which would eliminate interference from any of its degradation products.

1.2.4.3 UV and visible spectrophotometry

This technique has been widely used for analysis of members of the group of drugs, and UV spectrophotometry is used in the current official method for analysis of diazepam in formulations of the

British Pharmacopoiea [B.P., (1980)]. These methods involve preparation of solutions of diazepam in methanolic sulphuric acid by extraction from tablets and capsules and measurement of the absorbance at the λ_{max} of 284 nm. For diazepam injection, chloroform is used to extract diazepam from the injection, the extract is evaporated, the residue dissolved in 0.05 M methanolic sulphuric acid and the UV absorbance measured at the λ_{max} of 368 nm. The method used by the United States Pharmacopeia was also spectrophotometric, but this type of method is non-specific for diazepam in the presence of degradation products [Newton, (1978)]. The current method in the United States Pharmacopeia uses HPLC, Section 1.2.4.4.

Spectrophotometric methods for the determination of benzodiazepines in body fluids are normally not sensitive enough. To improve the sensitivity and specificity of the measurement, a number of different methods of modification have been used.

In benzodiazepines where the N-1 atom is not substituted, eg nitrazepam, hydrolysis of the parent molecule to its benzophenone product leaves a primary amine group which can then undergo a diazotization and coupling reaction with reagents such as Bratton-Marshall reagent (N-(1-naphthyl)ethylenediamine) to produce highly coloured azo dyes, Fig. 1.10.

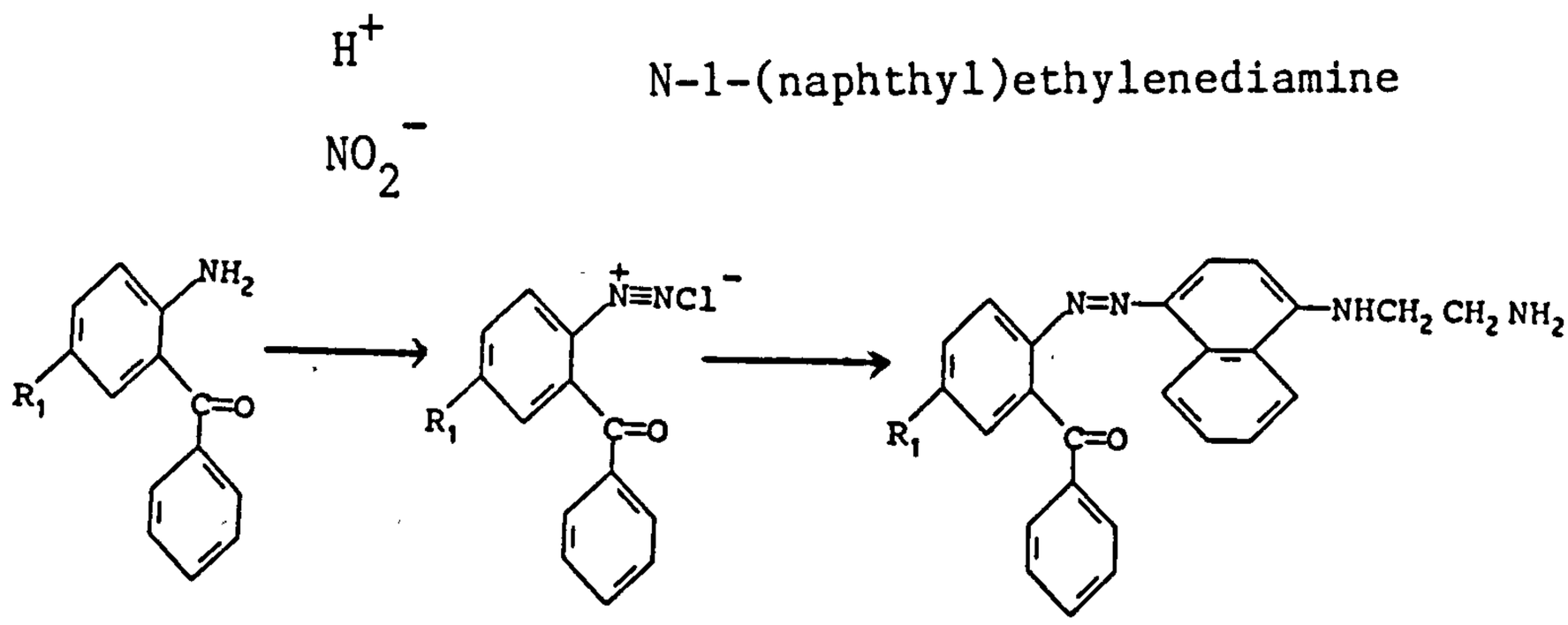


Fig. 1.10 Diazotization and coupling reaction

This type of colour reaction has also been used for detection in TLC [Gasparic and Zimak, (1983)].

In the case of diazepam, as it is substituted in the N-1 position, no colour reaction is possible. However, MACB is intensly yellow in colour and can be determined itself without prior reaction. This forms the basis for a method proposed by Baggi et al., (1975). In this method, which was applied both to pure drug and formulations, diazepam was dissolved in ethanol or extracted into ethanol from formulations. 6 M hydrochloric acid was added and the mixture was heated for 1 hour in boiling water. The solutions were then cooled and extracted with chloroform and the absorbance of the yellow solution obtained was measured at 410 nm. The colour produced gave a linear response and good recovery was obtained. No interference was found from commonly used excipients. However, the method would not be stability-indicating as MACB present in the formulation before hydrolysis would also be measured. If two determinations were

made on the samples, one before hydrolysis and one after hydrolysis, it may be possible to develop a stability-indicating assay from this method, by subtraction of the MACB measured before hydrolysis from that measured after hydrolysis.

A colorimetric method for determination of nitrazepam has been reported [Sanghavi and Jivani, (1979)] which uses the change in absorbance of nitrazepam in the presence of a strong base. This deprotonation results in a shift in absorbance to give a yellow anion with λ_{max} of 355 nm, Fig. 1.11.

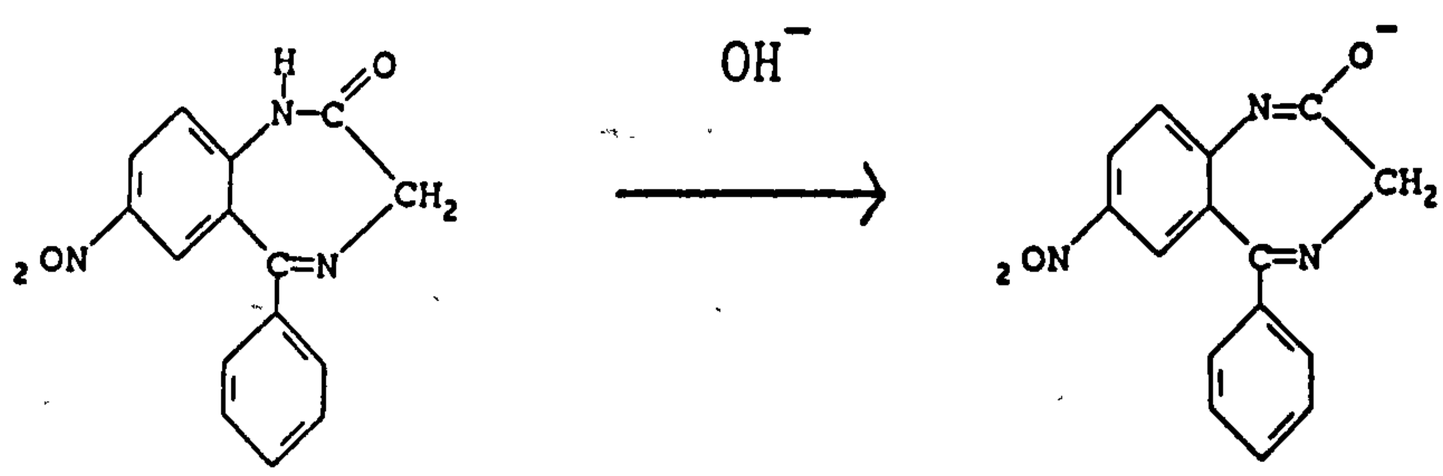


Fig. 1.11 Deprotonation of nitrazepam

Ethanolic solutions of nitrazepam were mixed with 0.5 % sodium hydroxide and left for 10 minutes to allow the reaction to occur, the solutions were then diluted and the absorbance measured. A number of drugs including promazine, sodium phenobarbitone and chlorpromazine

hydrochloride were tested and found not to interfere in the reaction. Recoveries of known weights of nitrazepam from formulations were satisfactory.

It has been shown that MACB will interfere with determination of diazepam using UV absorbance measurements of diazepam in acidified alcohol at 284 nm [B.P., (1980); U.S.P., (1980)]. Newton, (1978) proposed a minor alteration to the official assay to correct the problem. In this work it was shown that while the UV spectra of MACB in ethanol and acidified ethanol are similar, the corresponding spectra for diazepam differ. Thus if the extracted diazepam was redissolved in both ethanol and acidified ethanol, the content of MACB could be measured from the ethanolic solution at 405 nm without interference from diazepam. The acidified ethanol solution could then be measured at the normal wavelength of 284 nm to maximise sensitivity for diazepam, and the appropriate corrections made for the presence of MACB. This method was found to be unaffected by the presence of glycine or excipients used in the sample examined.

Kapp et al. (1979) have proposed a similar modification to the UV assay of diazepam to give specificity in the presence of MACB. In this case, the samples and standards are be prepared in 5% hydrochloric acid in methanol and the UV determination made at 315 nm. At this wavelength, there is very little absorbance from MACB in this solvent, so little interference is seen from MACB. However, the wavelength is not on a λ_{max} for diazepam but on the spectral slope. This will reduce the sensitivity of the measurement.

A stability indicating assay has been reported for prazepam, [Chafetz and Gaglia, (1967)]. This involves the measurement of the protonated drug in acidic methanol at 365 nm. At that wavelength, the interference from the benzophenone degradation product was less than 5 % of the absorptivity of the parent drug. It was suggested by the authors that this method would also be applicable to diazepam due to the similarity in structures of both these molecules.

Other techniques have been applied to improve the specificity of the UV measurement using derivative and difference spectrophotometry and orthogonal polynomials. The use of these techniques to improve the specificity of analytical methods has increased over recent years. They allow the use of a rapid, simple technique for analysis ie UV spectrophotometry, but with only a slight increase in either the sample preparation complexity, or the analysis of the spectral data obtained, give an increased specificity without the requirement of solvent extraction or isolation of the material being measured.

1.2.4.3.1 Difference spectrophotometry

Difference spectrophotometry has been used for a number of years and has been applied to a variety of compounds [Doyle and Fazzari,(1974)]. The major requirement of this method is that it is possible to change the absorptivity of the analyte in some way without affecting that of the excipients, degradation products or co-formulated drugs. This modification in

the absorption properties of the chromophore is performed by any suitable method such as hydrogenation or complex formation, but can also be as a result of a change in the ionisation of the analyte. For drug substances which are basic, acidic or amphoteric, the use of pH change to give a spectral shift is very frequently used.

The use of difference spectophotometry eliminates not only specific interference from degradation products and co-formulated drugs, but also non-specific interference from the formulation matrix. The method is particularily suited to quantitative work because not only is the concentration of a compound proportional to its absorbance, but also its difference absorbance. In order to effect the alteration of spectral properties, two equimolar solutions are subjected to treatment. These solutions are then measured with one in the reference beam and the other in the sample beam of the spectrophotometer. The difference absorbance measured can then be related to the concentration of analyte present, and providing the correct conditions are chosen, this will be free from interference from the other absorbing compounds.

If the spectral change is obtained by pH alteration, the pH values chosen require to be at least 2 pH units above and below the pK_a value of the compound being analysed to ensure that the compound exists almost entirely in the ionised and molecular forms. Compounds such as paracetamol [Elsayed et al., (1979)], oxyphenbutazone [Amer et al., (1980)], indomethacin [Hassan and Shaaban, (1982)] and ampicillin and cloxacillin [Davidson and Stenlake,

(1974)] have been analysed using pH-induced difference spectrophotometry.

Other reactions which have been used include oxidation, for determination of phenothiazine drugs in the presence of their oxidation products [Davidson, (1976)], and reaction of drugs containing 1,2-diphenolic groups with either boric acid or germanium dioxide reagent [Davidson, (1984b and c)].

Difference spectrophotometry has been applied to the analysis of 1,4-benzodiazepines. Chlordiazepoxide and demoxepam have been determined in chlordiazepoxide formulations [Davidson, (1984a)]. In this work, spectrophotometric methods specific for both compounds in the presence of each other and 2-amino-5-chlorobenzophenone were described. For determination of chlordiazepoxide (pK_a 4.6), the pH values used were pH 3 and 8, for determination of demoxepam (pK_a 10.6), pH 8 and 13 were used.

Other workers, after the present work had started, [Abdel-Hamid et al., (1984)] reported difference spectrophotometric methods for determination of clonazepam, diazepam and medazepam in formulations. These methods generated difference spectra using solutions in pH 1 and 13, enabling the determination of drug content without interference from excipients.

1.2.4.3.2 Orthogonal polynomials

A method of elimination of interference in spectrophotometric measurments which has been used more widely recently is orthogonal or polynomial

functions. This mathematical concept was introduced by Glenn (1963). In this technique, the absorbances measured at a set of equally spaced wavelengths are used to calculate the coefficient of a suitable polynomial. The polynomial normally used is quadratic, although cubic or higher polynomials can be used. The calculated coefficient of the polynomial is proportional to concentration and so can be used to calculate the concentration of analyte present.

The choice of polynomial type, the number of wavelengths used, the interval between wavelengths and the actual wavelengths used can all be varied. Thus provided the correct choice of these conditions is made, the technique can be used to eliminate non-linear irrelevant absorption, or absorption from co-formulated drugs. In these cases, the polynomial is selected to give a value of zero for the coefficient of the interfering absorbance.

Calculation packages are now available on computer which allow the repeated calculations to be performed in order to establish the best conditions to use. A number of drugs have been determined in formulations by this technique, including thiamine hydrochloride [Wahbi, (1981)] and nifuroxine and furazolidine [Hassan et al., (1981)]. Methods have also been published for benzodiazepines, by Korany and Haller for oxazepam and dipyridamole (1982), for diazepam and chlordiazepoxide [Bedair et al., (1984)], and for nitrazepam, prazepam and chlorazepate potassium [El-Yazbi et al., (1986)]. The technique allowed measurement of the drugs without interference from any degradation products present.

1.2.4.3.3 Derivative spectrophotometry

Derivative spectrophotometry has also seen a large increase in applications over the past few years. The theoretical background has been known for a long time, but only recently has suitable equipment been available for easy generation of derivative spectra, [Traveset et al.,(1980); Fell, (1980)]. Rapid development of electronic and microcomputer technology in recent years has provided refined, relatively low noise devices which are available commercially and permit routine recording of high quality derivative spectra. The most commonly used derivatives for analytical work are second and fourth order, with higher orders, the signal-to-noise ratio decreases, and noise becomes a problem.

The technique has advantages over zero order spectra, by providing enhanced resolution between overlapping bands, and preferentially removing interference. Broad absorbance spectra such as the absorbance from irrelevant interference is discriminated against in derivative spectra while narrow bands will be increased in size. Thus it may be possible to eliminate irrelevant interference by this technique. Suitable conditions can generally be chosen where the amplitude of the analyte is proportional to concentration and interference has been removed, providing a specific technique.

Some examples of the technique are described by Traveset et al. (1980). Other compounds examined include phenol and aromatic alcohols [Fell, (1978)]

and sulphoxide in degraded chlorpromazine formulations, [Fell and Davidson, (1980)]. Members of the benzodiazepine series of drugs have also been analysed by this technique [Martinez and Paz Gimenez, (1981); Abdel-Hamid et al., (1984)].

Advantages of derivative spectroscopy include the accurate determination of the absorption maxima of the compound of interest, better resolution of spectra, quantitative determination of material in the presence of turbidity and the qualitative determination of a compound in the presence of more than one absorbing component. Martinez and Paz Gimenez have used the technique to determine diazepam, nitrazepam, chlordiazepoxide and chlorazepate in toxicology samples. Blood, urine and stomach contents were made alkaline and extracted with ether. These were then redissolved in 0.05 M sulphuric acid and analysed. At the selected wavelengths, linearity was observed for the four benzodiazepines investigated and the amount of drug in the sample analysed was measured by comparison with calibration curves.

In the work presented by Abdel-Hamid and co-workers, second derivative and differential second derivatives were used for the quantitative estimation of clonazepam, diazepam and medazepam in pharmaceutical formulations. The method was used to eliminate interference from excipients in the formulations.

1.2.4.4 Spectrofluorimetric methods

Fluorimetry is a more sensitive method than spectrophotometry for determination of compounds. However, one problem is that not all compounds fluoresce. Maness and Yakatan (1975) have suggested that fluorescence methods can provide very useful assays for benzodiazepines, but that care has to be taken in the control of conditions to ensure reproducibility.

Braun et al. (1968) reported the intense fluorescence produced by chlordiazepoxide, diazepam, nitrazepam and oxazepam when an alcoholic solution of these was diluted in sulphuric, phosphoric or perchloric acids. This fluorescence was shown to be proportional to the amount of benzodiazepine present, and to be specific in excitation and emission wavelengths for each benzodiazepine. The acid hydrolysis products of the benzodiazepines were found not to exhibit fluorescence under these conditions. Thus the use of fluorescence produced in this way would provide a specific assay for the benzodiazepines discussed. This would also be sensitive, with limits of detection of 5 ng/ml for oxazepam and 100 ng/ml for the other three compounds.

This method of generation of fluoresence was applied to the quantitative determination of chlordiazepoxide, diazepam and oxazepam in pharmaceutical formulations. The method was compared with a polarographic technique, and both proved satisfactory with spectrofluorimetry proving much more sensitive for determination of oxazepam. It was found that the methods were not specific for determination of

chlordiazepoxide in the presence of co-formulated drugs such as clidinium bromide [Caille et al., (1970)].

Fluorescence was induced for nitrazepam using the reaction of its hydrolysis products with o-phthalaldehyde [Reider, (1973)] and for the hydrolysis products when reacted with fluorescamine [Stewart and Williamson, (1976)].

1.2.4.5 Gas chromatography methods

A number of GC methods have been developed and used, in particular for the determination of benzodiazepines and their metabolites in body fluids. For most of these determinations, electron-capture detectors (ECD) or nitrogen-phosphorus detectors are often used to increase the sensitivity.

Initially, methods were determined for diazepam using hydrolysis of the parent molecule to its benzophenone derivative prior to analysis to provide a more volatile derivative. De Silva et al. (1964) produced a method using hydrolysed diazepam with Carbowax 20M as the stationary phase and ECD. One disadvantage of this method was the lack of specificity for intact drug substance, with the same derivative formed on hydrolysis of different metabolites. However, a ten-fold increase in sensitivity was seen.

With further developments in the technique of GC, it has proved possible to separate the intact benzodiazepines, allowing the generation of stability-indicating assays, such as that developed by Kapp et

al. (1979) using 3% SE-30 on Chromosorb W with a flame-ionisation detector (FID) and temperature programming. In this study, oxazepam was used as an internal standard and the method was found capable of separating both the intact benzodiazepines and their corresponding benzophenone derivatives. A GC method has also been developed by Black et al. (1981) using a 2 mm i.d. column packed with 3% Poly I-110 on Gas Chrom Q, with FID. This method has been used for the routine evaluation of the drug content, content uniformity, impurities and identity (by retention time) for diazepam, chlordiazepoxide and flurazepam formulations using dicyclohexylphthalate as internal standard.

Further developments in GC technology have led to the use of wall coated open tubular columns of very narrow diameter which give very high performance with greater efficiency and short elution times. This type of column with SE-30 stationary phase (0.4 μm thick) has been used for the routine determination of diazepam and other drugs in serum [Debruyne et al., (1980)]. Use of this type of column plus nitrogen-selective detector allowed great selectivity and sensitivity with a minimum detectable concentration of 50 ng/ml from a 100 μl sample of serum.

A number of benzodiazepines which are not ammenable to GC determination on packed column systems due to thermal lability, require derivatisation or conversion to benzophenones before analysis. The use of capillary columns has made direct determination easier without any apparent thermal degradation. However a study has been undertaken [Joyce et al., (1984)] which

suggests that not all the benzodiazepines are satisfactorily chromatographed on capillary columns, with some undergoing decomposition with only partial resolution of the decomposition product eg nitrazepam. The output from the GC was fed into a MS to allow determination of the identity of the compounds as they were eluted.

A method was developed for the determination of diazepam in intravenous admixtures using GC with 3% OV-1 on Gas Chrom Q for separation and nitrogen-phosphorus detection, with prazepam as internal standard, [Raymond and De Gennero, (1986)]. This allowed accurate measurement of diazepam with simple sample preparation, without the potential interference of benzophenone (MACB) if degradation had occurred.

1.2.4.6 High performance liquid chromatography methods

The introduction of HPLC as a technique about 15 years ago provided a potentially very useful technique, particularily for the separation of closely related compounds such as different benzodiazepines, or benzodiazepines and related compounds such as metabolites and degradation products or synthetic impurities. In recent years, improvements in columns, packing materials and detectors have been made, and the number of published applications has increased dramatically, many methods being published for use in pharmacokinetic and toxicology applications in addition to those applied to pharmaceuticals.

Methods have been developed for the parent benzodiazepines and also for the hydrolysis products of the parent molecules. This allows increased sensitivity but decreases the specificity, [Baggi and Chatterjea, (1986); Gasparic and Zimak, (1983)].

Due to the characteristics of the benzodiazepines, a number of detectors have been used. Most methods have used UV spectrophotometry, but fluorimetry and also amperometric methods have also been used [Lund et al., (1979)].

An early method developed for chlordiazepoxide used normal phase HPLC with two mobile phases [Butterfield et al., (1977)]. Reverse phase methods for determination of chlordiazepoxide in the presence of amitriptyline hydrochloride [Burke and Sokoloff, (1980)] and in the presence of degradation products [Ali, (1980)] have been reported using octadecyl silane (ODS) columns and mobile phase mixtures of 0.01 M sodium lauryl sulphate in tetrahydrofuran: methanol:pH 2.5 Britton Robinson buffer (120:30:150) and acetonitrile:water:potassium bromide (65 ml:35 ml:100 mg) respectively. Columns containing ODS packing material are frequently used for reverse phase chromatography. This stationary phase is made of silica to which a layer of non-polar C_{18} functional groups has been chemically bonded. A much more simple method has been developed recently for the analysis of chlordiazepoxide and chlordiazepoxide hydrochloride and related impurities in tablet and capsule formulations. This method uses a 10 μm μBondapak C_{18} column with a methanol:water mobile phase [Roberts and Delaney, (1984)].

A number of ODS columns prepared by different manufacturers including μBondapak C_{18}, Partisil ODS-3 and Zorbax ODS were tested for use in a stability-indicating assay for oxazepam in tablets and capsules. The mobile phase used was methanol:water:acetic acid (60:40:1) with spectrophotometric detection at 254 nm [Reif and De Angelis, (1983)]. Samples of oxazepam were hydrolysed in acid, alkali and buffer and these solutions were injected onto the HPLC systems. Degradation products were isolated and identified to validate method specificity for all degradation products reported in literature or seen in the stressed samples. The degradation determined in dosage forms had a limit of detection of 0.1%. Separation of all the degradation products tested was achieved on the different columns tested with minor variations in the resolution of some of the impurities on different columns.

Gordon and co-workers (1986) have developed a method for the determination of temazepam and its major degradation products in soft gelatin capsules by reverse phase HPLC using methanol:water (60:40) as mobile phase and C_{18} packing material.

Determination of diazepam by HPLC has been performed for tablets [Emery and Kwotko, (1979)] and injection [Smith and Nuessle, (1982a and b)]. The method developed for tablets used methanol:water (65:35) as mobile phase, with a μBondapak C_{18} column and detection at 254 nm. Diazepam was extracted from the tablets using methanol, and an internal standard of benzene was added for quantitative purposes. The

assay was stability-indicating with carbostyril and MACB both being separated, but was very slow, requiring 30 min to effect separation. A limit of detection of 0.1% was seen for the degradation products.

The assay published for diazepam and its major degradation product, MACB, in injection formulations was developed for a study of the stability of prepacked syringes containing diazepam, [Smith and Nuessle,(1982 a and b). The method used a mobile phase of methanol:water (65:35) as in the paper by Emery and Kwotko, but the column was a Spherisorb C_6, 5 μm column. The internal standard used was biphenyl and with a flow rate of 1.5 ml/min, chromatography was complete in 6 min. The method was tested for compatibility with the excipients present in the injection formulation and no interference was seen from benzoic acid or benzyl alcohol. As biphenyl was found to co-elute with carbostyril, the method was only applicable to samples where no carbostyril was present, and no carbostyril was found in this study.

The method for analysis of diazepam formulations in the United States Pharmacopeia (1985) uses HPLC. A column packed with ODS packing material is used for separation, with a mobile phase of methanol:water (65:35 or 70:30 depending on the formulation). Internal standardisation is used, with the standard used depending on the formulation.

With further advances in equipment, a number of systems are available for separation and identification of benzodiazepines, particularily

useful for forensic applications. Noggle and Clark (1979) used HPLC, UV and IR to identify a number of these related compounds, while Chiarotti et al. (1986) record TLC, GC and HPLC data for nineteen benzodiazepines. Gill et al. (1986) have reported the use of ODS and silica packing materials to obtain retention data for twenty-one benzodiazepine drugs and their metabolites.

1.2.4.7 Polarographic methods

Studies of the polarographic behaviour of some benzodiazepines and metabolites of these, including diazepam and desmethyl diazepam have been made [Barrett et al., (1974)]. Determination of the pK_a of these compounds and study of their behaviour allowed differentiation of mixtures of them after extraction of both parent molecules and metabolites into solvent as neutrally charged molecules. Diazepam and desmethyl diazepam are polarographically reduced at the dropping mercury electrode. A mixture of diazepam and desmethyl diazepam in 0.1 M sodium hydroxide was extracted with light petroleum (40-60°) for 5 min. Polarography of the aqueous layer gave waves at -1.32 and -1.65 V corresponding to stock desmethyl diazepam, while evaporation of the organic layer with redissolution in 1 M sodium hydroxide with 5% methanol gave a wave at -1.2 V corresponding to standard diazepam. Even if both compounds are extracted using pH 7, the different polarographic behaviour of each means that with dissolution in sodium hydroxide before measurement, they can be polarographically differentiated.

Due to the different reaction potentials of a number of benzodiazepines, including diazepam, oxazepam and nitrazepam, and their hydrolysis products, it has proved possible to determine simultaneously the parent molecule and its hydrolysis product [Smyth and Groves, (1982)]. The mechanism of hydrolysis of a number of benzodiazepines in dilute acid solution (0.01, 0.1 and 1 M hydrochloric acid and 0.05 and 0.5 M sulphuric acid) has been studied using differential pulse polarography, DPP.

Both d.c. polarography and DPP have been applied to analysis of formulations containing nitrazepam [Mishra and Gode, (1985); Lannigan et al., (1982)] with satisfactory results being obtained for tablets and capsules without interference detected for commonly used excipients. The use of DPP for an extemporaneously prepared suspension of tablets required the use of a standard additions method due to interference from excipients in the suspending agent.

2 AIM OF PROJECT

Although diazepam is one of the more stable members of the 1,4-benzodiazepine series of molecules, it does undergo hydrolysis. At the time of starting this work, analysis of diazepam formulations of the British Pharmacopoeia and the United States Pharmacopeia was performed by direct UV spectrophotometry. The United States Pharmacopeial method was shown by Newton (1978) to be non-specific for diazepam in the presence of its major degradation product, MACB. A stability-indicating assay using HPLC had been published by Emery and Kowtko (1979), but this was slow and took 30 min to chromatograph each sample. The aim of the project was to develop a specific, stability-indicating assay for diazepam in formulations, using difference UV spectrophotometry. It was also intended that an improved HPLC assay would be developed to obtain faster separation. These methods were then to be tested against the direct UV assay method of the British Pharmacopoeia to allow comparison of all three methods.

A number of workers have studied the reaction kinetics of the hydrolysis of diazepam, following the degradation reaction with non-specific analytical methods. It was intended that the stability-indicating methods developed for formulated products should be used to study the hydrolysis reaction of diazepam.

3 MATERIALS AND EQUIPMENT

3.1 REAGENTS

Roche	Diazepam, carbostyril, 2-methylamino-5-chlorobenzophenone (MACB), 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepine-2-one, erythrosine dye, raspberry flavouring, Valium tablets 5 mg, capsules 5 mg, suppositories 10 mg, injection 20 mg/4 ml, syrup 2 mg/5 ml
Rathburn	Methanol and chloroform (HPLC-grade)
Griffin	Citric acid, potassium dihydrogen orthophosphate, potassium chloride, disodium tetraborate decahydrate. Each of the reagents was of analytical reagent grade quality.
Sigma	Tris(hydroxy)methylaminomethane (reagent grade quality), stearic acid (Grade II quality).
BDH	Glycine, sodium acetate trihydrate, disodium hydrogen orthophosphate (anhydrous), 98% m/m sulphuric acid, sodium hydroxide, anhydrous sodium sulphate. Each of these reagents was

	AnalaR grade.
BDH	Benzyl alcohol, sodium benzoate, benzoic acid, caffeine, diethyl phthalate. Each of these reagents was of laboratory reagent grade.
Koch-Light	Di-(2-ethylhexyl)-phthalate (puriss)
Kerfoot	Diazepam tablet 2 mg
BDH	Methanol (distilled), chloroform, petroleum ether, cyclohexane. All these solvents were reagent grade.
Water	Distilled, and deionised (Milli-Q reagent water system).

3.2 SOLUTIONS

Universal buffer solution

Citric acid	21.01 g	(0.1 M)
Potassium dihydrogen orthophosphate	13.61 g	(0.1 M)
Sodium tetraborate	19.07 g	(0.05 M)
Tris(hydroxy)methylaminomethane	12.11 g	(0.1 M)
Potassium chloride	7.46 g	(0.1 M)
Water to	1000 ml	

Buffers of the required pH were prepared by placing 50 ml of the above buffer solution in a 200 ml calibrated flask, adding the specified volume of 0.4 M hydrochloric acid or 0.4 M sodium hydroxide and diluting to volume with water, [Davies,(1959)].

Glycine buffers

25 ml 0.2 M glycine + x ml 0.2 M hydrochloric acid to 100ml with water

[Documenta Geigy Scientific Tables,(1975)]

Potassium chloride buffers

25 ml 0.2 M potassium chloride + x ml 0.2 M

hydrochloric acid to 100 ml with water

[Documenta Geigy Scientific Tables,(1975)]

Glycine buffer pH 2.6 (x5 strength)

250 ml 1 M glycine + 121.0 ml 1 M hydrochloric acid to 1000 ml with water

[Dawson et al.,(1969)]

Acetate buffer pH 5.4 (x5 strength)

860 ml 1 M sodium acetate + 140 ml 1 M acetic acid

[Dawson et al.,(1969)]

When solutions were prepared in these buffers, the appropriate volume of buffer solution was added depending on the size of flask being used and the solution was then made to volume with water after the other components of the solution had been added. This ensured that the same final strength of buffer solution was present in each solution prepared.

Mixed phosphate buffer pH 7.0

Dissolve 0.50 g anhydrous disodium hydrogen

orthophosphate and 0.301 g potassium dihydrogen orthophosphate in sufficient water to produce 1000 ml, [B.P.,(1980)]

Diazepam injection

This was prepared using the formula in Smith and Neussle (1982a).

Diazepam	5	mg/ml
Propylene glycol	40	%
Ethanol	10	%
Sodium benzoate	4.88	%
Benzoic acid	0.12	%
Benzyl alcohol	1.5	%
Water for injection to make	100	%

0.05 M methanolic sulphuric acid

This solution was prepared by adding slowly 5.0 g (2.72 ml) of 98% m/m sulphuric acid to methanol and making to 1000 ml with methanol. After preparation, the solution was allowed to stand for 24 h before use, [B.P.,(1980)]

0.05 M ethanolic sulphuric acid

This solution was prepared as for 0.05 M methanolic sulphuric acid, but using ethanol, [B.P.,(1980)]

3.3 EQUIPMENT

3.3.1 General work

Microangle centrifuge
MSE minor 'S' centrifuge
Stuart wrist action flask shaker
Kerry ultrasonic bath
PAAR DMA45 calculating digital density meter
Corning pH meter Model 12 (calibrated on buffer solutions prepared from BDH tablets)
Whatman filter paper, No 1 and No 42
Millipore filters 0.45 μm

3.3.2 Ultraviolet spectrophotometry

Perkin-Elmer 552 double-beam UV-visible spectrophotometer with microprocessor control. For recording spectra, a Perkin-Elmer 561 recorder was used, in series with the spectrophotometer.

1, 2 and 4 cm silica cells were used.

3.3.3 Gas liquid chromatography

Pye-Unicam 340 with Phillips PM single pen recorder
Perkin-Elmer F33 with Perkin-Elmer 56 recorder
Perkin-Elmer F11

For some work a Pye-Unicam CDP1 computing integrator was used for peak area and height measurement.

Injections were made using a 1 μl Hamilton syringe. Nitrogen was used as the carrier gas and detection was by flame ionisation detector.

3.3.4 High performance liquid chromatography

Three HPLC systems were used for different parts of the work.

1.	Columns	30 cm stainless steel column and 10 cm flexible Radpak column, Z-module compression unit. Both columns were packed with μBondapak C_{18} packing material (Waters Associates). A guard column containing the same packing material was used with the Radpak column.
	Pumps	Pye-Unicam LC-XPD, Waters M6000A
	Injectors	Kontron MS1660 sampler, WISP 710 A/B autoinjector
	Detector	Pye LC-UV detector
	Recorders	Servoscribe RE511, Linseis LSA

	Computer	DEC PDP11 with a commercial chromatography package called MULTICHROM, VG Laboratory Instruments Ltd.
2.	Column	30 cm stainless steel column, packed with 5 μm Spherisorb ODS.
	Pump	Altex 100A reciprocating pump
	Injector	20 μl Rheodyne manual loop
	Flowcell	Altex spectrophotometer flowcell
	Detector	Hitachi 100-10 spectrophotometer
	Recorder	Linseis

All this equipment was fed power through an Advance Voltstat Harmonic Neutralised supply to prevent interference from fluctuation in supply.

3.	Column	As in system 2
	Pump	ACS Ltd HPLC Pump Series 300
	Injector	Negretti and Zambra Model M190 syringe loading sample injector with 20 μl loop
	Detector	LDC Spectromonitor III Model

1204A

Recorder Linseis

4 METHODS USED IN THE ANALYSIS OF FORMULATIONS OF DIAZEPAM

4.1 DEVELOPMENT AND VALIDATION OF A DIFFERENCE UV SPECTROPHOTOMETRIC ASSAY FOR DIAZEPAM

4.1.1 Method development

A stock solution of diazepam in methanol was prepared (125 μg/ml). Two 2.0 ml portions of this were diluted to 25 ml with pH 5.3 phosphate buffer and 0.1 M hydrochloric acid, to give a final concentration of 10 μg/ml. The difference absorption spectrum of these solutions was recorded with the pH 1 solution in the sample beam and the pH 5.3 solution in the reference beam of the spectrophotometer.

To investigate the effect of pH on the absorptivity of diazepam, further dilutions of the stock solution were made as above, with a series of buffer solutions between pH 1.3 and pH 11.2, 0.1 M hydrochloric acid and 0.1 M sodium hydroxide. The absorbance of each of these solutions was measured at 290 nm against the appropriate blank solution and the absorptivity of diazepam at each pH was calculated.

A stock solution of MACB (2-methylamino-5-chlorobenzophenone) in methanol was prepared (40 μg/ml). 2.0 ml portions of this were diluted to 25 ml with the same buffers, acidic and alkaline solutions as used above, to give a final concentration of 3 μg/ml. The absorbance of these solutions was measured at 290 nm against the appropriate blank

solution (using 4 cm pathlength cells) and the absorptivity of MACB at each pH was calculated.

Separate solutions of diazepam and MACB in methanol were prepared in duplicate (200 μg/ml and 50 μg/ml respectively). 2.0 ml of each of these solutions were diluted to 25 ml using pH 2.6 (glycine) and pH 5.4 (acetate) buffers. The UV absorbance of each of these dilutions was measured at 290 nm against an appropriate blank solution and the difference absorptivity for both diazepam and MACB was calculated.

Solutions of desmethyl diazepam and carbostyril in methanol were prepared in duplicate (75 and 50 μg/ml respectively). 2.0 ml of each of these were diluted to 25 ml using pH 2.6 and pH 5.4 buffers and the UV absorbances of the resulting solutions were measured at 290 nm. The difference molar absorptivity was calculated.

A solution containing the intermediate hydrolysis product of diazepam, (2-glycyl(methyl)amino-5-chlorobenzophenone, GMACB) in 0.1 M hydrochloric acid was prepared as described in Section 5.2.1. This solution was extracted with chloroform to remove any diazepam and MACB present and leave a solution containing only GMACB in 0.1 M hydrochloric acid. 10.0 ml samples of this solution were added to volumetric flasks containing 1.0 ml 1 M sodium hydroxide and 2.0 ml methanol (to match the methanol content of other solutions prepared). These flasks were made to volume with pH 2.6 and pH 5.4 buffer solutions and the absorbance of each solution was

measured at 290 nm, at zero time against the appropriate blank solution. The absorbance spectrum of each solution was also recorded. These measurements were repeated on each solution at 10 minute intervals after preparation.

4.1.2 Assay method

A stock solution of diazepam in methanol was prepared and equal portions of this solution were diluted in buffer solutions at pH 2.6 and pH 5.4 to give a final methanol concentration of 8% v/v. (The buffer solutions were added as a 5X strength solution and the flasks made to volume with water).

The absorbance of each of these solutions and appropriate blank solutions was measured at 290 nm. The difference absorbance at 290 nm was used to calculate the content of diazepam present by reference to a value of $\Delta A(1\%,1cm)$ for standard diazepam, (obtained by measurement of a standard solution of diazepam using the same batch of buffer solutions).

4.1.3 Validation of the assay method

4.1.3.1 Rectilinearity

A stock solution of diazepam in methanol was prepared (1000 μg/ml) and 2.0, 4.0, 6.0, 8.0 and 10.0 ml portions of this were diluted with methanol to 20 ml. This gave a series of solutions containing a range of concentrations of diazepam (Table 4.1). These were further diluted by taking two 2.0 ml portions to 25 ml with buffer solutions of pH 2.6 and pH 5.4. The

absorbance of each solution was measured at 290 nm using each of the spectral bandwidth settings on the spectrophotometer (0.5, 1.0, 2.0 and 4.0 nm).

Table 4.1 Preparation of solutions for study of rectilinearity at 290 nm

Volume of stock diazepam solution (ml)	Approximate concentration of diazepam (μg/ml)
2.0	100
4.0	200
6.0	300
8.0	400
10.0	500

4.1.3.2 Assessment of the specificity of the method

Solutions of diazepam and MACB in methanol were prepared (410 and 325 μg/ml respectively) to give equimolar concentrations of each compound. Mixtures of these solutions were prepared as described in Table 4.2 and diluted to 25 ml with methanol.

Table 4.2 Determination of interference from MACB (high concentrations)

Volume of diazepam	(ml)	0	2.0	4.0	6.0	8.0	10.0
Volume of MACB	(ml)	10.0	8.0	6.0	4.0	2.0	0

Final volume of all the solutions was 25 ml, using methanol as diluent.

These mixtures were analysed by the general method for difference UV spectrophotometric analysis (Section 4.1.2). Each intermediate dilution was further diluted with methanol (5.0 ml to 10.0 ml) and then

diluted in pH 2.6 and pH 5.4 buffers. The difference absorbance of each pair of solutions was measured at 290 nm and the content of diazepam calculated. The measured content of diazepam was then compared to the actual content and the recovery of diazepam calculated.

A second experiment was performed in the same way using lower concentrations of MACB. Stock solutions of diazepam and MACB were prepared (650 and 430 μg/ml respectively) and the solution of MACB was further diluted with methanol (5.0 ml to 20 ml). This gave a standard solution containing 108 μg/ml. Mixtures were prepared from these solutions (Table 4.3) diluting each to 25 ml with methanol. These solutions were further diluted with buffer as described in the general method (Section 4.1.2) and the difference absorbance of each pair of solutions measured at 290 nm. The content and recovery of diazepam were calculated.

Table 4.3 Determination of interference from MACB (low concentrations)

Volume of diazepam (ml)	10.0	9.0	8.0	7.0	6.0
Volume of MACB 1 (ml)	0	1.0	0	0	0
Volume of MACB 2 (ml)	0	0	1.0	3.0	5.0

A stock solution of diazepam in methanol was prepared (800 μg/ml). This was further diluted to give solutions each containing 160 μg/ml diazepam. A stock solution of MACB in methanol was made (360 μg/ml) and a series of dilutions of this were prepared in methanol to give solutions containing 30 and 2.3 μg of

MACB/ml. Different quantities of these solutions were mixed in 25 ml volumetric flasks to give a series of solutions, each containing the same concentration of diazepam, but with varying concentrations of MACB (Table 4.4).

Table 4.4 Effect of MACB on absorptivity of diazepam; preparation of solutions with constant concentration of diazepam and variable concentration of diazepam.

Volume of diazepam	Volume of MACB (2.3 μg/ml)	(30 μg/ml)	(360 μg/ml)
(ml)	(ml)	(ml)	(ml)
5.0	0	0	0
5.0	2.0	0	0
5.0	10.0	0	0
5.0	0	2.0	0
5.0	0	6.0	0
5.0	0	12.0	0
5.0	0	0	2.0
5.0	0	0	3.0

These solutions were diluted in buffer as described in the general method and then analysed by the difference UV absorption method. The measured value of absorptivity (ϵ) was calculated from the known concentrations of diazepam and these values were plotted against the concentration of MACB in each solution.

4.1.3.3 Precision of measurement

Two standard mixtures of diazepam and MACB were

prepared in methanol, one containing 300 μg/ml of diazepam and 3 μg/ml of MACB and the other containing 290 μg/ml of diazepam and 28 μg/ml of MACB, (approximately 1 and 10% m/m of MACB respectively).

Both mixtures were assayed ten times using the general method described in Section 4.1.2. The content of diazepam in each was calculated using equation (4.1). The measured content of diazepam was compared with the true content of diazepam and the variation in the replicate determinations was analysed statistically.

$$\text{Content of diazepam} = \frac{\text{Difference absorbance measured}}{\Delta A(1\%, 1\text{cm}) \times b} \qquad (4.1)$$

where b = the pathlength of the cell (cm)

4.1.3.4 Assessment of the effect of methanol concentration

A stock solution of diazepam was prepared (2 mg/ml) in methanol. 2.0 ml portions of this solution were transferred to 10 ml volumetric flasks and increasing quantities of methanol were added. The volumes used were 0, 1.0, 2.0, 3.0, 7.0 and 8.0 ml. The solutions were diluted to 10 ml and then two 2.0 ml portions of each of these solutions were diluted to 25 ml with pH 2.6 and pH 5.4 buffer solutions to give a series of solutions containing from 1.6 to 8.0% v/v methanol. The difference absorbance of these solutions was measured at 360 nm.

4.1.4 Development of difference UV spectrophotometric methods for analysis of dosage forms

4.1.4.1 Tablets

4.1.4.1.1 Method development

Ten tablets were weighed and powdered. A quantity of the powder equivalent to 8 mg diazepam was accurately weighed into a 25 ml volumetric flask. After addition of 20 ml methanol, the flask was shaken for 15 minutes using a mechanical shaker. The solution was made to volume with methanol and filtered, discarding the first 5 ml of filtrate. Two 2.0 ml portions of the later filtrate were diluted to 25 ml with pH 2.6 and pH 5.4 buffer solutions respectively. The absorbance difference at 290 nm was measured and the content of diazepam in the tablets calculated using equation (4.2).

$$\text{Content of diazepam (mg/dosage form)} = \frac{\Delta A_{sam} \times DF \times W_{av}}{\Delta A(1\%,1cm) \times W_{sam}} \qquad (4.2)$$

where DF = dilution factor for the sample preparation

W_{av} = average weight of dosage form, in mg

W_{sam} = weight of sample, in mg

ΔA_{sam} = difference absorbance of sample solution at 290nm

and $\Delta A(1\%,1cm)$ is the difference $A(1\%,1cm)$ for diazepam at 290 nm measured using the same batch of buffer solutions

Turbidity was seen in these buffer solutions which interfered with the determination of diazepam. This was thought to be due to magnesium stearate, a tabletting excipient likely to be present in the

formulation. A solution of magnesium stearate in methanol was prepared (27 μg/ml) and further diluted, two 2.0 ml portions to 25 ml with pH 2.6 and pH 5.4 buffer solutions respectively. This concentration of magnesium stearate was chosen to give a solution with an equivalent concentration to that obtained by extraction from the tablets, (assuming the concentration in the tablets to be 1%). The absorbance difference at 290 nm was measured. This showed that magnesium stearate may interfere. In order that any magnesium stearate present was removed before analysis, 0.01 M hydrochloric acid was used to extract diazepam from the tablet debris.

As diazepam is subject to acid hydrolysis, the stability of diazepam in 0.01 M hydrochloric acid was investigated. Three stock solutions were prepared. Approximately 20 mg of diazepam was accurately weighed in duplicate, one portion was dissolved in 100 ml of 0.01 M hydrochloric acid and the second in 100 ml methanol. A third solution of diazepam was prepared by dissolving approximately 10 mg of diazepam, accurately weighed, in 100 ml of 0.05 M methanolic sulphuric acid. Each solution of diazepam was diluted and analysed at set time intervals; zero, 15, 30, 85, 120 and 480 minutes and 24 hours after initial solution preparation. The following dilutions were prepared; the solution in 0.05 M methanolic sulphuric acid was diluted by taking 2.0 ml and diluting to 25 ml with the same solvent and the solutions of diazepam in methanol and in 0.01 M hydrochloric acid were diluted by taking 2.0 ml and making to 25 ml with pH 2.6 and pH 5.4 buffer solutions. The UV absorbance of the dilutions in 0.05 M methanolic sulphuric acid was

measured at 284 nm, and the difference absorbance for the dilutions of the solutions in methanol and in 0.01 M hydrochloric acid was measured at 290 nm.

Diazepam was found to be less stable in 0.01 M hydrochloric acid than in methanol or 0.05 M methanolic sulphuric acid. The assay procedure adopted therefore used methanol for extraction of diazepam from the tablets. 0.01 M hydrochloric acid was added after extraction to cause precipitation of the magnesium stearate immediately before filtration and dilution with buffer solutions.

To ensure that the buffering capacity of the solutions measured was adequate when 0.01 M hydrochloric acid was present, duplicate solutions of diazepam (800 μg/ml) were prepared in methanol and also in 20.0 ml methanol made to a final volume of 100 ml with 0.01 M hydrochloric acid. 2.0 ml portions of the methanolic solution and 10.0 ml portions of the methanolic hydrochloric acid solution were diluted in pH 2.6 and pH 5.4 buffer solutions. This kept the methanol content in the final solutions constant, (Section 4.1.3.4). The difference absorbance of these pairs of solutions was measured at 290 nm and the $\Delta A(1\%,1cm)$ calculated using equation (4.1).

To check for absence of interference from excipients the difference absorption spectra of sample and standard solutions were superimposed. A blank difference absorption spectrum was also generated from methanol diluted in the buffer solutions. The isosbestic points, ie wavelengths with zero ΔA owing to the equal absorbance of the pH 2.6 and pH 5.4

solutions were compared for the standard and sample spectra.

To investigate the reproducibility of the assay procedure, 50 tablets from a batch of Kerfoot 2 mg diazepam tablets were powdered. Ten separate samples of this powder were accurately weighed into 100 ml volumetric flasks, and analysed as described in Section 4.1.4.1.2.

4.1.4.1.2 Assay method

The average weight per tablet was calculated by weighing 10 tablets, which were then powdered. Quantities of powder equivalent to approximately 8 mg of diazepam were accurately weighed into 100 ml volumetric flasks, 20.0 ml of methanol were added and the flasks were shaken for 15 min. Each flask was then made to volume with 0.01 M hydrochloric acid and immediately filtered using a Buchner funnel supporting Whatmans No 1 filter paper. The first 10 ml of filtrate was discarded and 10.0 ml portions of later filtrate were diluted to 25 ml with pH 2.6 and pH 5.4 buffer solutions. The absorbances of the resultant solutions were measured at 290 nm, and the difference absorbance corrected for the absorbance due to buffer blanks. The diazepam content of the tablets was calculated using equation (4.2).

4.1.4.2 Capsules

4.1.4.2.1 Method development

As the formulation for capsules contains similar excipients as tablets, including magnesium stearate, the assay developed was similar to that for tablets.

Sample solutions prepared by the developed method (Section 4.1.4.2.2) were tested for excipient interference. The isosbestic points in the difference spectrum of the sample solution were compared with those in the difference spectrum of the standard solution of diazepam, obtained using the same buffer solutions.

4.1.4.2.2 Assay method

The average content of powder in each capsule was determined by weighing 10 capsules when full and also when emptied of their contents. Any powder clinging to the capsule shells was removed by compressed air before weighing.

A quantity of bulked capsule content, equivalent to approximately 8 mg of diazepam was accurately weighed into a 100 ml volumetric flask. The method described for tablets was then followed (Section 4.1.4.1.2).

The content of diazepam in the capsules was calculated using equation (4.2).

4.1.4.3 Suppositories

4.1.4.3.1 Method development

Very little development was required for analysis of this formulation. Sample solutions obtained using the developed method (Section 4.1.4.3.2) were tested for excipient interference by comparison of the isosbestic points as described earlier.

4.1.4.3.2 Assay method

One 10 mg suppository was accurately weighed into a 100 ml flask, 20.0 ml of methanol were added and the flask contents subjected to ultrasonic treatment for 5 minutes. The solution was made to volume with water and 10.0 ml portions of this solution were diluted to 25 ml with pH 2.6 and pH 5.4 buffer solutions. The difference absorbance of these solutions was measured at 290 nm.

The content of diazepam in the suppositories was calculated using equation (4.2).

4.1.4.4 Injection

4.1.4.4.1 Method development

To investigate possible interference from excipients in the formulated injection, solutions were prepared of sodium benzoate, and benzyl alcohol. Known weights of each (approximately 100 mg) were dissolved in 20.0 ml methanol in 100 ml volumetric flasks and made to

volume with 0.01 M hydrochloric acid. These solutions were diluted with pH 2.6 and pH 5.4 buffer solutions to give final concentrations of approximately 0.04% m/v. The absorbance of each solution was measured at 290 nm. The difference A(1%,1cm) was calculated for each excipient using equation (4.1).

To measure the content of diazepam in the formulation, 2.0 ml portions of injection solution were diluted in buffer solutions pH 2.6 and pH 5.4. The difference absorbance of each pair of solutions was measured at 290 nm and the concentration of diazepam present calculated, using equation (4.3).

$$\text{Content of diazepam (mg/ml)} = \frac{\Delta A_{sam} \times DF}{\Delta A(1\%,1cm)} \qquad (4.3)$$

where ΔA_{sam} = the difference of the sample solution
DF = the dilution factor used in the sample preparation
and $\Delta A(1\%,1cm)$ is the difference A(1%,1cm) of diazepam

As a small difference absorbance was measured for sodium benzoate at 290 nm, the difference absorbance measurement and the difference A(1%,1cm) was also calculated for each solution of the excipients at 360 nm, a secondary maximum in the difference absorption spectrum of diazepam.

The rectilinearity of the detector response to diazepam was investigated at 360 nm by preparation of a stock solution of diazepam in methanol (760 μg/ml). This solution was diluted to 10 ml with methanol as described in Table 4.5. 2.0 ml portions of these solutions were diluted to 25 ml with pH 2.6 and pH 5.4

buffer solutions and the difference absorbances of the resulting solutions were measured at 360 nm.

Table 4.5 Preparation of solutions for study of rectilinearity at 360 nm

Volume of stock diazepam solution (ml)	Approximate concentration of diazepam (μg/ml)
2.0	12
4.0	24
6.0	37
8.0	49
10.0	61

To assess the interference from MACB at 360 nm, stock solutions of MACB in methanol were prepared, (1000 μg/ml, accurately weighed, in duplicate). These solutions were diluted, 2.0 ml to 25 ml, to give solutions of 80 μg/ml MACB in methanol. 2.0 ml portions of these solutions were diluted to 25 ml with pH 2.6 and pH 5.4 buffer and the difference absorbance measured at 360 nm.

Further investigation of the interference from MACB in the difference UV assay method was performed. Stock solutions of diazepam and MACB in methanol (200 μg/ml) were prepared and diluted as described in Table 4.6. These mixtures contained the same molar concentration of both components, but the proportions of each component varied.

Table 4.6 Preparation of solutions for the determination of interference from MACB at 360 nm

Volume of diazepam	(ml)	10.0	8.0	6.0	4.0	2.0	0
Volume of MACB	(ml)	0	2.0	4.0	6.0	8.0	10.0

These volumes of diazepam and MACB were mixed in 25 ml volumetric flasks and made to volume with methanol. 2.0 ml portions of these mixtures were diluted to 25 ml with pH 2.6 and pH 5.4 buffer solutions and the difference absorbance of the solutions were measured at 360 nm.

After it was established that no interference occurred at 360 nm from either MACB or excipients in the formulation, diazepam injection was assayed using the difference absorbance measurement at 360 nm. The contents of two 4 ml ampoules were bulked and three 2.0 ml portions of the solution transferred to separate 10 ml volumetric flasks and made to volume with methanol. Two 2.0 ml portions of these dilutions were transferred to 25 ml volumetric flasks and made to volume with pH 2.6 and pH 5.4 buffer solutions. The difference absorbance was measured at 360 nm and the concentration of diazepam calculated from equation (4.3).

A synthetic injection formulation was prepared (Section 3.2), containing the excipients at approximately the concentration present in the marketed injection, [Smith and Nuessle,(1982a)]. Accurately weighed quantities of diazepam were added to this prepared mixture and also to solutions of each

individual excipient at the same concentration as in the formulation.

2.0 ml portions of these solutions were sampled, diluted to pH 2.6 and pH 5.4 using buffer solutions and the difference absorbance of each of these pairs of solutions measured at 290 and 360 nm. The observed $\Delta A(1\%,1cm)$ of diazepam was calculated using equation (4.1).

From the investigation of the effect of the excipients on the absorptivity of diazepam, it appeared that some interference was present from sodium benzoate. Approximately 50 mg of diazepam was dissolved in 10.0 ml methanol then made to volume (50 ml) with water. 2.0 ml portions of this solution were transferred to 25 ml volumetric flasks. A solution of sodium benzoate in water (0.63% m/v) was added to the volumetric flasks (Table 4.7). Blank solutions containing sodium benzoate only, at the various concentrations used, were also prepared. Each sample was diluted to volume with pH 2.6 and pH 5.4 buffer solutions and the difference UV absorbance of each sample measured at 360 nm.

The analytical concentration of diazepam in all the above solutions was constant at 0.0754 mg/ml. The difference absorbance was plotted against the concentration of sodium benzoate.

Table 4.7 Investigation of the effect of sodium benzoate concentration on the absorptivity of diazepam

Volume of sodium benzoate solution (0.63% m/v) added (ml)	Analytical concentration (mg/ml)
0	0
1.0	0.252
2.0	0.504
3.0	0.756
4.0	1.008
5.0	1.260

Sodium benzoate was shown to affect the absorptivity of diazepam. Subsequently an extraction of diazepam in chloroform at pH 7 was performed. 2.0 ml of injection (20 mg/4 ml) was added to 20 ml pH 7.0 buffer solution (mixed phosphate buffer pH 7.0 B.P.). This solution was extracted with four 20 ml volumes of chloroform and each extract was passed through 5 g of anhydrous sodium sulphate, supported on a sintered glass funnel. The filtrate was collected in a 100 ml volumetric flask and made to volume. The chloroform was then evaporated to dryness under nitrogen, the residue redissolved in methanol and made to 10 ml. Two 2.0 ml portions of this methanolic solution were then diluted to 25 ml with pH 2.6 and pH 5.4 buffer solutions. The absorbance was measured at 290 and 360 nm and the concentration of diazepam in the injection was calculated from equation (4.3).

The solutions in buffer after chloroform extraction showed turbidity. Investigation into the cause of this turbidity was made. A standard solution of

diazepam was prepared in methanol (0.3 mg/ml) and diluted 2.0 ml to 25 ml with pH 2.6 and pH 5.4 buffer solutions. Two 2.0 ml portions of methanol were also made to volume with pH 2.6 and pH 5.4 buffer solutions in the same way. A standard solution of diazepam in chloroform was prepared (0.1 mg/ml) and 30.0 ml of this was evaporated to dryness under nitrogen. The residue was redissolved in 10.0 ml methanol, and two 2.0 ml portions of this solution were diluted to 25 ml with pH 2.6 and pH 5.4 buffer solutions. A 2.0 ml sample of the prepared injection was added to 20 ml pH 7.0 buffer and extracted with four 20 ml portions of chloroform. These were collected and bulked after passing through sodium sulphate and made to 100 ml with chloroform. A 30.0 ml portion of this solution was evaporated to dryness under nitrogen, the residue redissolved in 10.0 ml methanol and two 2.0 ml samples of this solution diluted to 25 ml with pH 2.6 and pH 5.4 buffer solutions. The synthetic injection was analysed in duplicate as described above but using HPLC grade chloroform for extraction.

From the work described above, turbidity was seen in the samples prepared using reagent grade chloroform but not in those prepared using HPLC grade chloroform. Thus all subsequent work was performed using HPLC grade chloroform.

Low recoveries were obtained for extracts from the synthetic injection solution. Investigation into the steps in sample preparation where losses of diazepam could occur were made. Two further extractions were made of the prepared injection. After the chloroform extracts were bulked and made to volume, two 30.0 ml

portions of chloroform were taken from each sample solution and treated as before. A solution of diazepam in chloroform was prepared (0.1 mg/ml) and three 30.0 ml portions were taken from this, evaporated to dryness and the residue redissolved in methanol (10.0 ml). The methanolic solution was diluted in pH 2.6 and pH 5.4 buffer (2.0 ml in 25 ml) and the difference absorbance of these solutions measured at 290 nm. A sample of synthetic injection was extracted into chloroform as described in Section 4.1.4.4.2. Two samples of the chloroform extract were evaporated to dryness, one of 30.0 ml which was treated as for the difference absorbance measurement (Section 4.1.4.4.2), and the second of 10.0 ml which was treated as for the direct UV measurement (Section 4.2.3.4.2). A solution of diazepam in methanol was prepared at the same concentration as the synthetic injection. 2.0 ml of this solution was mixed with phosphate buffer and extracted with chloroform as for the injection solution.

This work showed good reproducibility for evaporation of a solution of diazepam in chloroform and redissolution of the residue, either in methanol for measurement by the difference UV assay method, or in 0.05 M ethanolic sulphuric acid for measurement by the direct UV assay of the British Pharmacopoeia. The recovery of diazepam from the extraction process was low however, whether the diazepam was extracted from a solution in methanol, or mixed with the excipients of the formulation. It was concluded that either diazepam was very unstable in chloroform, or poor extraction was responsible for the low recovery.

To investigate the stability of diazepam in chloroform, a 0.1 mg/ml solution of diazepam in chloroform was prepared. The UV absorbance of this solution was measured at the λ_{max} for diazepam in chloroform, 312 nm. A 25 ml and a 50 ml volumetric flask were filled with this solution. The 25 ml volumetric flask was left in natural daylight for 60 hours, and the 50 ml flask placed in the dark for the same period of time. The level of solution in each flask was checked to ensure no evaporation had occurred on storage. The UV absorbance of each solution was measured at 312 nm.

As no loss of diazepam occurred on storage, further investigation of the extraction process was performed. Three types of standard solution were prepared in duplicate; diazepam in chloroform (100 μg/ml), diazepam in methanol (300 μg/ml) and diazepam in 0.05 M ethanolic sulphuric acid (40 μg/ml). A batch of synthetic injection was also made. Two 2.0 ml samples were taken from the prepared injection and added to two separators. pH 7.0 buffer was added and diazepam extracted from these solutions using four 20 ml portions of chloroform. The extracts were filtered through anhydrous sodium sulphate supported on filter paper, collected, bulked and made to 100 ml with chloroform. The UV absorbance of these solutions and the duplicate standard solutions prepared in chloroform were measured at 312 nm. Two 10.0 ml portions and two 30.0 ml portions were taken from each of the two chloroform extracts and from the two standard solutions in chloroform and evaporated to dryness under nitrogen. The residues from the 10.0 ml portions were each redissolved in 0.05 M ethanolic

sulphuric acid as described in Section 4.2.3.4.2, and the UV absorbance of these solutions measured at 368 nm. The UV absorbance of the two standard solutions prepared in 0.05 M ethanolic sulphuric acid were also measured at the same wavelength. The residues from the 30.0 ml samples were redissolved in methanol and diluted in buffer as described in Section 4.1.4.4.2. The difference absorbance of these solutions and the standard solutions in methanol (prepared in the same way) were measured at 290 nm. By using the values for difference A(1%,1cm) and A(1%,1cm) calculated for the standard solutions, the content of diazepam in the synthetic injection samples and the standard chloroform samples was calculated and compared to the nominal content. A 2.0 ml sample of standard diazepam (0.5 mg/ml in methanol) was extracted with four 20 ml portions of chloroform from pH 7.0 buffer. The extracts were bulked and made to 100 ml with chloroform without filtering through anhydrous sodium sulphate. The absorbance of this solution was measured at 312 nm.

This work showed the loss of diazepam occurred at the filtration stage. When the chloroform extract was bulked without filtration, 100% recovery was obtained, but in each sample where filtration through anhydrous sodium sulphate was performed, the recovery was low.

To investigate whether the same recovery problem occurred with the use of sintered glass funnels to support the anhydrous sodium sulphate, the following experiment was performed. A stock solution of diazepam in chloroform was prepared (0.483 mg/ml) and subjected to different filtration treatments. 20.0 ml

of the stock solution (equivalent to approximately 10 mg diazepam) was passed through anhydrous sodium sulphate supported by filter paper (Whatmans No 1). The sodium sulphate and filter paper were rinsed with four 20 ml portions of chloroform and the bulked solution was made to 100 ml with chloroform. 20.0 ml of the stock solution was filtered through anhydrous sodium sulphate suspended on a sintered glass funnel (size 1). The sodium sulphate and sintered glass funnel were rinsed with four 20 ml portions of chloroform and the bulked solution made to 100 ml with chloroform. 10.0 ml of the stock solution was transferred to a 50 ml volumetric flask, 1.0 ml pH 7.0 buffer solution was added and the solution made to volume with chloroform. 10.0 ml of stock solution was transferred to a 50 ml volumetric flask and made to volume with chloroform. The UV absorbance of all four solutions were measured at 312 nm.

The use of sintered glass funnels to support the anhydrous sodium sulphate was shown to have no effect on the recovery of diazepam in chloroform. A sample of injection mixture with a known amount of diazepam present was assayed to ensure that no problem occurred when excipients were present. 2.0 ml of injection was added to a separator containing 20 ml pH 7.0 phosphate buffer and mixed. This was extracted with four 20 ml portions of chloroform, passed through anhydrous sodium sulphate supported on a sintered glass funnel (size No 1) and collected in a 100 ml volumetric flask. The filter and sodium sulphate were rinsed with chloroform and the bulked extracts made to volume with chloroform. Two 10.0 ml and two 30.0 ml portions of this solution were evaporated to dryness

under nitrogen and the residues redissolved in 0.05 M ethanolic sulphuric acid for the 10.0 ml portions and in methanol for the 30.0 ml portions. The methanolic solutions were treated as described in Section 4.1.4.4.2. The absorbances of these solutions were measured at the appropriate wavelengths and the content of diazepam in the prepared injection calculated using equations (4.3) and (4.7).

4.1.4.4.2 Assay method

A 2.0 ml portion of injection was added to a separating funnel containing 20 ml pH 7.0 phosphate buffer and mixed. This solution was extracted with four 20 ml portions of chloroform, which were then filtered by passing through anhydrous sodium sulphate supported on a sintered glass funnel (size No 1) and collected in a 100 ml volumetric flask. The filter and sodium sulphate were rinsed with chloroform and the bulked extracts made to volume with chloroform. Two 30.0 ml samples of this solution were evaporated to dryness under nitrogen, the residues redissolved in methanol and diluted to 10 ml. Two 2.0 ml samples of the methanolic solution were diluted to 25 ml with pH 2.6 and pH 5.4 buffer solutions and the difference UV absorbance of these solutions measured at 290 nm.

4.1.4.5. Syrup

4.1.4.5.1 Method development

The weight per ml of the syrup was determined, as described in Section 4.2.3.5. A sample of approximately 6.0 g (equivalent to 2 mg of diazepam)

was accurately weighed into a 10 ml volumetric flask and made to volume with methanol. Two 2.0 ml portions of the methanolic solution were transferred to 25 ml volumetric flasks and made to volume with pH 2.6 and pH 5.4 buffer solutions. The difference spectrum of these solutions was generated and superimposed on the difference spectrum of diazepam in methanol treated in the same way.

Examination of the difference spectra, showed interference, and so the excipients likely to be present in the formulation were also examined. A list of excipients present in the formulation sold in France was available [Dictionnaire Vidal,(1987)] and included erythrosine dye, flavouring, aluminium magnesium stearate, sorbitol, sodium benzoate, lactic acid, glycerol, polyoxyethylene monostearate with some other ingredients. Solutions of erythrosine and the flavouring used were prepared in methanol at concentrations of approximately 0.1 mg/ml. 2.0 ml portions were taken and diluted to 25 ml with pH 2.6 and pH 5.4 buffer solutions and the difference spectrum of each pair of solutions was generated. The dye used was found to interfere in the spectroscopic measurement, as did sodium benzoate (Section 4.1.4.4.1). Therefore both of these components had to be removed from the sample before any analytical measurement could be made.

To extract diazepam from the formulation, a method was used similar to that for diazepam injection. A 6.0 g portion of syrup was accurately weighed into a separating funnel, 20 ml of pH 7.0 buffer solution was added and the resultant mixture extracted with 20 ml

chloroform for 2 min. The chloroform layer was dried by passing through anhydrous sodium sulphate and this was rinsed with fresh chloroform. The chloroform extract and rinsings were bulked in a 25 ml volumetric flask and made to volume with chloroform. A 20.0 ml portion of this was evaporated to dryness, redissolved in methanol (10.0 ml) and two 2.0 ml samples of the methanolic solution were diluted with pH 2.6 and pH 5.4 buffer solutions. The difference spectrum of these solutions was generated.

When pH 7.0 buffer solution was used for dilution prior to extraction, the solution in chloroform was seen to be pink. Interference was also seen in the difference spectrum generated. The effect of pH on the extraction of the dye was therefore investigated. A stock solution of dye was prepared (0.2 mg/ml in water) and this was diluted (2.0 ml to 25 ml) with pH 7.0 and pH 9.2 buffer solutions and 0.1 M sodium hydroxide. The absorbance of each solution was measured at 524 nm and 10.0 ml of each added to separating funnels and extracted with 20.0 ml chloroform. The absorbance of the remaining aqueous solutions were measured at 524 nm and compared with the original absorbances.

As the pH of the aqueous phase increased it was found that the quantity of dye extracted by chloroform decreased. The extraction process for diazepam syrup was therefore modified and repeated. Approximately 6.0 g of syrup was accurately weighed into a separating funnel, 20 ml of 0.1 M sodium hydroxide were added and the mixture was extracted with 20 ml chloroform. The chloroform extract was treated as before and the

difference absorption spectrum compared with that obtained from treatment of a solution of diazepam in methanol.

After extraction from pH 13 solution, although no dye material was transferred, interference was still seen from some component in the mixture. The measured content of diazepam was also found to be lower than expected, from knowledge of the nominal content of diazepam.

The stability of diazepam in alkali was investigated to ensure that no degradation occurred resulting in the low content measured. A solution of diazepam in methanol was prepared (5 mg/ml) and 2.0 ml of this solution was added to 20 ml 0.1 M sodium hydroxide in a separating funnel. The mixture was extracted with 20 ml chloroform. The chloroform extract was dried and redissolved in methanol as described above and the difference absorbance of the final solutions in pH 2.6 and pH 5.4 buffer were measured at 290 nm. The content of diazepam in the syrup was calculated using equation (4.4).

$$\text{Content of diazepam (mg/ml)} = \frac{\Delta A_{sam} \times Wt/ml \times DF}{\Delta A(1\%,1cm) \times W_{sam}} \qquad (4.4)$$

where ΔA_{sam} = the difference absorbance of samples
Wt/ml = the weight per ml of syrup
DF = the dilution factor used
W_{sam} = the weight of sample taken
and $\Delta A(1\%,1cm)$ is the difference absorptivity of diazepam

In an attempt to improve the extraction process, approximately 6.0 g of syrup was diluted with 20 ml methanol. After shaking, the solution was centrifuged to sediment the flocculant precipitate formed. The supernatant liquid was decanted into a separating funnel, the tube and remaining pellet rinsed with fresh methanol and the rinsings added to the separating funnel. 20 ml 0.1 M sodium hydroxide was added to the methanolic solution, the mixture was extracted with 20 ml chloroform and the extract treated as described above. It was found that some interference was present in the aqueous solutions, resulting in turbidity when the redissolved residue was diluted in the buffer solutions.

Approximately 6.0 g of syrup was accurately weighed and diluted with methanol as described above. The solution obtained after centrifugation and rinsing was bulked in a separating funnel and extracted with petroleum ether (60-80). The petroleum ether layer was decanted and evaporated to dryness. 20 ml 0.1 M sodium hydroxide was added to the methanolic layer and the mixture extracted with 20 ml chloroform. The chloroform extract was evaporated, the residue was redissolved in methanol and this solution was diluted with pH 2.6 and pH 5.4 buffer solutions as described above. The difference spectrum of these solutions was generated and the difference absorbance at 290 nm was measured. The content of diazepam was calculated using equation (4.4).

4.1.4.5.2 Assay method

No satisfactory method was developed for the analysis

of diazepam in Valium syrup using difference spectrophotometry.

4.2 DEVELOPMENT AND EVALUATION OF A DIRECT UV SPECTROPHOTOMETRIC ASSAY FOR DIAZEPAM

4.2.1 Basis of the method

The assay methods used for tablets, capsules and injections are based on those described in the B.P. 1980 and 1982 Addendum. The methods used for diazepam suppositories and syrup were those used by Roche QC laboratories for analysis of their products.

These methods involve sample preparation to give solutions of diazepam in 0.05 M methanolic or 0.05 M ethanolic sulphuric acid and measurement of the UV absorbance of these solutions at 284 nm or 368 nm. The choice of wavelength and solvent was dependent on the formulation being analysed.

Subsequent to this work being performed, the official method for the assay of diazepam injection changed to use 0.05 M methanolic sulphuric acid instead of 0.05 M ethanolic sulphuric acid [Addendum to B.P.1980,(1982)]. The A(1%,1cm) value at 284 nm was also altered from 446 to 450.

4.2.2 Selectivity of the method

Newton(1978), has shown that MACB, the major degradation product of diazepam has a significant UV absorbance at the wavelength of measurement for diazepam. Work was performed to assess the level of interference from MACB in the Pharmacopoeial method to allow direct comparison with the difference UV spectrophotometric method developed in Section 4.1.1.

Standard solutions of diazepam and MACB were prepared in 0.05 M methanolic sulphuric acid to contain 10 and 8.5 μg/ml respectively (approximately equimolar concentrations of each, 35 μmol/l). The absorption spectra of these solutions, obtained using a blank solution of 0.05 M methanolic sulphuric acid, were superimposed. The absorbance values of each solution were measured at 241.4 and 284 nm, the λ_{max} of diazepam when measured under these conditions. The molar absorptivities of both compounds were calculated at each wavelength using equation (4.5).

$$\text{Molar absorptivity} = \frac{\text{Measured absorbance}}{\text{Molar concentration x pathlength}} \qquad (4.5)$$

The samples prepared in Table 4.2 (Section 4.1.3.2) were analysed by direct UV spectrophotometric measurement. The methanolic mixtures were prepared by taking 2.0 ml portions of each solution and diluting to 25 ml with 0.05 M methanolic sulphuric acid. The absorbance of these solutions was measured at 284 nm using a blank solution of 0.05 M methanolic sulphuric acid. The diazepam content of each solution was calculated using an A(1%,1cm) value measured by subjecting duplicate solutions of diazepam reference substance to the same dilution and measurement process. The measured content of diazepam was then compared to the known content to calculate the recovery of diazepam.

The sample solutions prepared in Table 4.3 (Section

4.1.3.2) were analysed by direct UV spectrophotometric measurement. 2.0 ml portions of each solution were diluted to 25 ml using 0.05 M methanolic sulphuric acid and the absorbance measured at 284 nm against a blank solution of 0.05 M methanolic sulphuric acid. The content of diazepam was then calculated as above and compared with the known content.

The standard mixtures of diazepam and MACB prepared as described in Table 4.4 (Section 4.1.3.3), containing 1 and 10% m/m MACB in methanol were analysed using the method described for direct UV spectrophotometric measurement. 2.0 ml portions were diluted to 25 ml using 0.05 M methanolic sulphuric acid and the UV absorbance of the resulting solutions was measured at 284 nm. The content of diazepam in each solution was calculated for each of ten replicate measurements and the results analysed statistically.

4.2.3 Development of direct UV spectrophotometric methods for analysis of formulations

4.2.3.1 Tablets

4.2.3.1.1 Method development

The average weight of each tablet was calculated by weighing 20 tablets and taking the mean. These tablets were then powdered and mixed.

A quantity of powdered tablet equivalent to 10 mg diazepam was accurately weighed into a 100 ml volumetric flask, 5 ml of water added, the solutions were mixed and allowed to stand for 15 minutes. 50 ml

of 0.05 M methanolic sulphuric acid was added and the flask was shaken for 15 minutes using a mechanical shaker. The suspension was made to volume with 0.05 M methanolic sulphuric acid and then filtered through Whatmans No 42 filter paper. The first 10 ml of filtrate was discarded and 10.0 ml of the collected filtrate was diluted to 100 ml with the same diluent. The absorbance of this solution was measured at 284 nm in a 1 cm silica cell against 0.05 M methanolic sulphuric acid.

The diazepam content of the tablets was calculated using equation (4.6).

$$\text{Content of diazepam (mg/dosage form)} = \frac{A_{sam} \times DF \times W_{av}}{450 \times W_{sam}} \qquad (4.6)$$

where W_{av} = the average weight of the dosage form, in mg

W_{sam} = the weight of sample taken, in mg

A_{sam} = the absorbance of the sample solution at 284nm

and 450 is the A(1%,1cm) for diazepam in 0.05 M methanolic sulphuric acid at the maximum of 284 nm, quoted by the B.P., (1980), Addendum, 1982.

The precision of this method was assessed by repeating it ten times on separate portions of powdered tablet.

Analysis of samples from the same batch of tablets by both difference UV and direct UV spectrophotometric assay showed that a higher measured % nominal concentration was obtained using the direct UV spectrophotometric method. The cause of this

difference was investigated by checking for interference from tablet excipients. A stock solution of diazepam in 0.05 M methanolic sulphuric acid was prepared and diluted with the same solvent to give five standard solutions of varying concentrations (5.39, 10.78, 16.17, 21.56 and 26.95 μg/ml). The spectra of these solutions were recorded, using a solution of diazepam extracted from diazepam 2 mg tablets as the reference solution. The concentration of diazepam was calculated by extrapolation from a graph of absorbance versus concentration of diazepam. A standard solution of the same concentration was then prepared. The spectrum was recorded for both the sample solution and the standard solution of the same concentration of diazepam, using one as the reference and one as the sample solution. Any difference in absorbance between these two solutions would be due to irrelevant absorption from tablet excipients, all diazepam absorbance having been eliminated by the reference solution.

As very little interference from tablet excipients was found by this procedure, further investigations were made to elucidate the cause of the difference in the results between the two assay methods. A solution of diazepam in 0.05 M methanolic sulphuric acid was prepared at the same analytical concentration as the test solutions. Approximately 10 mg of diazepam was accurately weighed into a 100 ml flask and dissolved and diluted in 0.05 M methanolic sulphuric acid. This stock solution was subjected to a variety of treatments. A 5.0 ml sample of the solution was diluted to 50 ml with the same solvent, and samples of the solution were filtered through Whatmans No 1 and

No 42 filter papers before dilution of the early and late filtrates from both filter papers, (5.0 ml to 50 ml with the same solvent). The absorbance of each dilution was measured at 284 nm and the A(1%,1cm) values calculated. A blank solution of 0.05 M methanolic sulphuric acid was subjected to the same treatments and the absorbance of this solution measured for each condition.

The change in weight of the solution during filtration was monitored to investigate if evaporation was occurring. A beaker, filter and filter paper were placed on a top-pan balance. The absorbance of a diluted stock solution of diazepam was measured and the solution was then filtered (samples of both No 1 and No 42 filter paper were used). The absorbance of the solution was measured again after filtration. The weight of the filtration apparatus plus solution was measured both before and after filtration.

To investigate if any weight loss or absorbance change occurred during centrifugation, standard solutions of diazepam in 0.05 M methanolic sulphuric acid were prepared. Approximately 10 mg samples were accurately weighed, in duplicate, and dissolved in solvent. Portions of these solutions were filtered through No 42 filter paper, and then centrifuged (using a tube sealed with Nesco film to prevent evaporation). The weight of the centrifuge tube with solution was measured before and after centrifugation. The solutions after treatment were diluted and the absorbance values measured at 284 nm. These measurements were used to calculate the A(1%,1cm) value for diazepam.

4.2.3.1.2 Assay method

The average weight of each tablet was calculated by weighing 10 tablets and taking the mean. These tablets were then powdered and mixed. A quantity of powdered tablet equivalent to 10 mg diazepam was accurately weighed into a 100 ml volumetric flask, 5 ml of water added, mixed and allowed to stand for 15 min. 50 ml of 0.05 M methanolic sulphuric acid was added and the flask shaken for 15 min using a mechanical shaker. The extract was made to volume with 0.05 M methanolic sulphuric acid and then filtered through Whatmans No 42 filter paper. The first 10 ml of filtrate was discarded and 10.0 ml of the collected filtrate was diluted to 100 ml with the same diluent. The absorbance of this solution was measured at the λ_{max} of 284 nm in a 1 cm silica cell with 0.05 M methanolic sulphuric acid as the blank solution. The diazepam content of the tablets was calculated using equation (4.6).

4.2.3.2 Capsules

4.2.3.2.1 Method development

No method development was required for this formulation as the method used was similar to that developed for tablets.

4.2.3.2.2 Assay method

A quantity of bulked capsule content equivalent to 10 mg of diazepam was accurately weighed into a 100 ml

volumetric flask before proceeding as in Section 4.2.3.1.2. The diazepam content of the capsules was calculated using equation (4.6).

4.2.3.3 Suppositories

4.2.3.3.1 Method development

No method development was performed for this formulation. The method used was that developed by Roche QC laboratories.

4.2.3.3.2 Assay method

Six suppositories were weighed individually and the average weight of each was calculated. One suppository, containing a stated weight of 10 mg diazepam was accurately weighed and transferred to a 100 ml volumetric flask. 50 ml of 0.05 M methanolic sulphuric acid was added, and the flask contents were subjected to ultrasonic treatment for 5 minutes to dissolve the suppository. The solution was then made to volume, and 10.0 ml diluted to 100 ml with the same solvent. The absorbance of the solution was measured at 284 nm against a blank solution of 0.05 M methanolic sulphuric acid. The content of diazepam was calculated from equation (4.6).

4.2.3.4 Injection

4.2.3.4.1 Method development

The method development for the direct UV spectrophotometric assay was performed at the same

time as the method development for the difference UV spectrophotometric assay, (Section 4.1.4.4.1).

4.2.3.4.2 Assay method

20 ml of mixed phosphate buffer pH 7.0 was added to a volume of injection equivalent to 10 mg diazepam and this solution was extracted with four quantities of 20 ml of chloroform. Each extract was passed through 5 g of anhydrous sodium sulphate, supported on a sintered glass funnel, then bulked and diluted to 100 ml with chloroform. A 10.0 ml portion of this solution was evaporated to dryness under nitrogen and sufficient 0.05 M ethanolic sulphuric acid was added to a volume of 25 ml. The resulting solution was thoroughly mixed and its absorbance was measured at 368 nm. The diazepam content of the injection was calculated using equation (4.7).

$$\text{Content of diazepam (mg/ml)} = \frac{A_{sam}}{151} \qquad (4.7)$$

where A_{sam} = is the absorbance of the sample solution at 368 nm

and 151 is the A(1%,1cm) for diazepam in 0.05 M ethanolic sulphuric acid at the maximum of 368 nm

4.2.3.5 Syrup

4.2.3.5.1 Method development

No method development was necessary for this formulation. The method used was supplied by Roche QC

laboratories.

4.2.3.5.2 Assay method

A Paar DMA45 calculating digital density meter was used to determine the weight per ml of the syrup batches. The sample cell of the digital density meter was flushed with acetone and dried prior to use and the instrument calibrated using air and water. The cell was filled with each batch of syrup, care being taken to ensure that no bubbles formed in the section of tube used for measurement. The instrument automatically calculated the density of syrup in the sample cell. (The method of measurement was by monitoring the change in the natural frequency of the harmonic oscillations of the hollow tube when it had been filled with syrup).

The syrup was shaken thoroughly and approximately 3 g were accurately weighed into a 150 ml separating funnel. 25 ml of a 1:1 mixture of 1 M sodium hydroxide and methanol was added and the mixture shaken vigorously for 2 minutes. This mixture was extracted with five 25 ml portions of chloroform, by shaking vigorously for 2 minutes and filtering the extracts through anhydrous sodium sulphate into a 250 ml round-bottom flask. The bulked extracts were then evaporated to dryness using a rotary evaporator. The residue was dissolved in 25.0 ml 0.05 M methanolic sulphuric acid and filtered. The first 5 ml of filtrate was discarded and the absorbance of the remaining clear filtrate was measured at 368 nm, with 0.05 M methanolic sulphuric acid in the reference

cell. The concentration of diazepam in the sample was calculated using equation (4.8).

$$\text{Content of diazepam (mg/ml)} = \frac{A_{sam} \times Wt/ml \times DF}{151 \times W_{sam}} \qquad (4.8)$$

where A_{sam} = absorbance of sample solution at 368 nm

Wt/ml = weight per ml of sample, in g

DF = dilution factor used in the sample preparation

and 151 is the A(1%,1cm) for diazepam in 0.05 M methanolic sulphuric acid at the maximum at 368 nm

4.3 DEVELOPMENT AND VALIDATION OF AN HPLC METHOD FOR THE ANALYSIS OF DIAZEPAM

4.3.1 Method development

Separate solutions of diazepam, carbostyril and MACB in methanol (200, 20 and 20 μg/ml respectively) and a mixture containing all three substances (40, 8 and 8 μg/ml respectively) were prepared.

Injections were made on two different columns using a mobile phase containing methanol:water (65:35). Both columns were filled with the same packing material, μBondapak C_{18}. One column was a 30 cm stainless steel column and the second a 10 cm flexible walled Radpak column, with 2000 psi applied externally using a Z-module compression unit.

Solutions of excipients and manufacturing impurities likely to interfere in the assay method were prepared in methanol. Compounds used were sodium benzoate, benzyl alcohol and 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (a synthetic precursor of diazepam, desmethyl diazepam) at 2000, 600 and 20 μg/ml respectively. A mixture was also prepared containing the above three components in addition to diazepam, carbostyril and MACB.

The mixture described above containing diazepam, excipients, manufacturing impurities and degradation products was chromatographed using mobile phase mixtures containing various proportions of methanol:water from 65:35 to 80:20. Injections were made in each of these mobile phases to investigate the

best conditions for separation.

The mixture used to determine suitable mobile phase composition was also injected with different wavelengths of detection, (230 and 254 nm).

4.3.2 Validation of chromatographic conditions

4.3.2.1 Chromatographic conditions

A list of the chromatographic conditions used are described below

Column	10 cm x 8 mm Radpak, with 10 μm μBondapak C18 packing material
Mobile phase	Methanol:water (72:28)
Flow rate	3 ml/min
Detection	Spectrophotometric at 254 nm
Detector attenuation	Typically 0.32 aufs
Chart speed	Typically 200 mm/hr
Injection volume	20 μl by automatic injection

The column was compressed externally to 2000 psi using a Z-module. The mobile phase was filtered and degassed before use by filtering through a Millipore filter (0.45 μm) under vacuum. The typical back-pressure on the column was approximately 800 psi (depending on the age of the guard column used).

An external standard solution was used for quantitative analysis and consisted of a methanolic solution of diazepam, carbosytril and MACB containing approximately 100, 5 and 5 μg/ml respectively. These solutions were prepared fresh daily, in duplicate, from accurate weighings of each component.

4.3.2.2 Precision of injection

Standard solutions containing diazepam, carbostyril and MACB, at approximately 100, 5 and 5 μg/ml were prepared in methanol. At least six replicate injections were made from a single vial of standard solution using both the Kontron MS1660 sampler and the Wisp 710 A/B autoinjector.

4.3.2.3 Stability of solutions

A standard solution and a test extract from Valium capsules were prepared and the content of diazepam, carbostyril and MACB in the test extract was measured. The solutions were left in clear, stoppered glassware for 24 h and the content of diazepam, carbostyril and MACB in each solution was measured against a fresh standard solution.

4.3.2.4 Linearity of response

A stock mixture of diazepam, carbostyril and MACB in methanol was prepared (680, 60 and 120 μg/ml respectively). Dilutions of this solution were made in methanol to give seven solutions containing diazepam at concentrations from 16 to 167% of 0.1

mg/ml, the analytical concentration for diazepam. The content of carbostyril and MACB in these solutions varied from 1 to 15% and 2 to 30% respectively, of the analytical concentration of diazepam.

These solutions were injected singly in sequence, firstly in descending and then in ascending order of concentration. The peak heights and areas obtained for each solution were averaged and plotted against concentration to determine the linearity of the response.

4.3.3 Development of HPLC methods for analysis of formulations

4.3.3.1 Tablets

4.3.3.1.1 Method development

No method development was performed for this formulation. The extraction method used was developed for the capsule formulation and found to be suitable for tablets.

4.3.3.1.2 Assay method

The average weight per tablet was calculated from the weight of 10 tablets, which were then powdered. Quantities of powder equivalent to approximately 10 mg of diazepam were accurately weighed into 100 ml volumetric flasks, 5 ml of water added and the flask contents allowed to stand for 5 min. 50 ml of methanol was added and each flask shaken for 25 min using a mechanical shaker. The contents were then

diluted to volume with methanol. The solution was thoroughly mixed and a portion of this suspension centrifuged using a microangle centrifuge. The supernatant liquid was injected onto an HPLC system as described in Section 4.3.2. A standard solution containing diazepam, carbostyril and MACB was injected at the same time under the same conditions and the peak areas compared to those in the sample chromatogram. The content of diazepam and any degradation products present were calculated using equation (4.9). The peak areas for sample and standard solutions were measured and the content of diazepam, carbostyril and MACB were calculated.

$$\text{Content of diazepam (mg/dosage form)} = \frac{A_{sam} \times W_{std} \times W_{av}}{A_{std} \times W_{sam}} \qquad (4.9)$$

where A_{sam} = peak area of sample

A_{std} = peak area of standard

W_{std} = weight of standard, mg (in 100 ml flask)

W_{sam} = weight of sample, in g

W_{av} = average weight of dosage form, g

4.3.3.2 Capsules

4.3.3.2.1 Method development

The best extraction method was investigated by varying the volume of methanol and the length of time of extraction.

The powder removed from the capsule shells was bulked

and mixed. A weight equivalent to approximately 10 mg diazepam was accurately weighed into 100 ml volumetric flasks and 5 ml of water were added to each flask. The contents of the flask were extracted and assayed as described for the tablet assay in Section 4.3.3.1.1. The peak areas for diazepam and any degradation products present were measured and the content of each calculated using equation (4.9).

The content of diazepam extracted using methanol or 0.05 M methanolic sulphuric acid as the extraction solvent was compared. Samples of bulked powder from the capsules were extracted as described in Section 4.2.3.2.1. The solution obtained after extraction was diluted as described in the direct UV spectrophotometric assay and the UV absorbance of this solution was measured. In addition the solution in 0.05 M methanolic sulphuric acid was injected onto the chromatographic system and the content of diazepam calculated using equation (4.9). (A solution of diazepam and its degradation products in 0.05 M methanolic sulphuric acid was used as a standard in this instance to ensure that no difference in chromatography resulted from the change of solvent).

4.3.3.2.2 Assay method

The method used was that used for tablets except that a quantity of bulked capsule contents equivalent to approximately 10 mg of diazepam was accurately weighed into a 100 ml volumetric flask before proceeding as described in Section 4.3.3.1.2.

4.3.3.3 Suppositories

4.3.3.3.1 Method development

No method development was performed on this formulation.

4.3.3.3.2 Assay method

Six suppositories were weighed individually and this weight was used to calculate the average weight of suppository. For the assay, one suppository was weighed and transferred to a 100 ml volumetric flask. 50 ml of methanol was added and the flask contents subjected to ultrasonic treatment for 5 min to disperse the suppository. The solution was made to volume with methanol and a portion assayed by HPLC. The content of diazepam and its degradation products were calculated using equation (4.9).

4.3.3.4 Injection

4.3.3.4.1 Method development

Sodium benzoate and benzyl alcohol, excipients present in Valium injection were tested for interference in the assay (Section 4.3.1).

4.3.3.4.2 Assay method

Samples of injection were prepared for analysis by dilution of 2.0 ml of injection (nominal concentration of diazepam, 5 mg/ml), to 100 ml with methanol. The content of diazepam and its degradation products in the injection were calculated using

equation (4.10).

$$\text{Content of diazepam (mg/ml)} = \frac{A_{sam} \times W_{std} \times 0.5}{A_{std}} \qquad (4.10)$$

where A_{sam} = peak area of sample

A_{std} = peak area of standard

W_{std} = weight of standard, mg (in 100 ml flask)

4.3.3.5 Syrup

4.3.3.5.1 Method development

No method development was performed on this formulation.

4.3.3.5.2 Assay method

The density of each batch of syrup was measured using a Paar DMA45 digital density meter as described in Section 4.2.3.5. Quantities of syrup containing approximately 2 mg diazepam were accurately weighed into 20 ml volumetric flasks and 10 ml of methanol added. The flasks were shaken for 15 min using a mechanical shaker, made to volume, shaken well and a sample centrifuged using a microangle centrifuge. A

portion of the clear supernatant liquid was analysed by HPLC. The content of diazepam and its degradation products in the syrup were calculated using equation (4.11).

$$\text{Content of diazepam (mg/ml)} = \frac{A_{sam} \times W_{std} \times Wt/ml \times 0.2}{A_{std} \times W_{sam}} \qquad (4.11)$$

where A_{sam} = peak area of sample

A_{std} = peak area of standard

W_{std} = weight of relevant standard, mg (in 100 ml flask)

Wt/ml = weight per ml of sample, g

W_{sam} = weight of sample, g

4.4 APPLICATION OF DIFFERENCE UV SPECTROPHOTOMETRIC, UV SPECTROPHOTOMETRIC AND HPLC ASSAY PROCEDURES TO DOSAGE FORMS

4.4.1 Tablets

The methods described in Sections 4.1.4.1.2, 4.2.3.1.2 and 4.3.3.1.2 were applied to samples of Valium tablet (5 mg). Three batches were assayed, one which had been recently made and two which had been stored for 7 years at 25°C and 35°C.

4.4.2 Capsules

The methods described in Sections 4.1.4.2.2, 4.2.3.2.2 and 4.3.3.2.2 were applied to samples of Valium capsule (5 mg). A freshly prepared batch and two batches stored for 6 years at 25°C and 35°C were assayed.

4.4.3 Suppositories

Using the methods described in Sections 4.1.4.3.2, 4.2.3.3.2 and 4.3.3.3.2, batches of Valium suppository (10mg) were assayed. One batch of material was freshly prepared, and the other two samples analysed had been stored for 3 years at 25°C and 35°C.

4.4.4 Injection

The methods described in Sections 4.1.4.4.2, 4.2.3.4.2 and 4.3.3.4.2 were used to analyse samples of Valium injection (20 mg/4 ml). These samples were both freshly prepared and aged as described for

tablets, (Section 4.4.1).

4.4.5 Syrup

The methods described in sections 4.2.3.5.2 and 4.3.3.5.2 were applied to samples of Valium syrup. The samples analysed were both fresh and aged by storage at 25°C for 7 years.

5 METHODS USED IN THE STUDY OF THE KINETICS OF THE HYDROLYSIS REACTION OF DIAZEPAM

5.1. DEVELOPMENT AND VALIDATION OF A GAS CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF DIAZEPAM AND MACB

5.1.1 Initial development

Individual solutions of diazepam and MACB were prepared in methanol at a concentration of approximately 1 mg/ml. These solutions were injected onto a 2 m column packed with 3% OV-17 on Gas Chrom Q. The injections (1 μl volume) were made manually using a Hamilton syringe.

Injections of methanol, ethanol and chloroform were made to determine the best solvent for use. This was considered to be the solvent with the least tailing solvent front. A solution was prepared containing diazepam and MACB in chloroform at a concentration of approximately 1 mg/ml each. This mixture was injected onto the chromatographic system. The chromatographic conditions were adjusted until satisfactory separation of both components was obtained. The conditions selected are given below:

Column	2 m, 3% OV-17 on Gas Chrom Q
Oven temperature	275°C
Detector temperature	300°C
Injector temperature	300°C

Carrier gas	Nitrogen	(30 ml/min)
Detection	Flame ionisation	

During the GC work, different instruments were used, two Perkin Elmer gas chromatographs (F11 and F33) and a Pye Unicam 304 chromatograph. Due to the specific orientation of the inlet and outlet ports, these instruments all required different columns. All columns used were packed with 3% OV-17 stationary phase. The oven, detector and injector temperatures together with the carrier gas flow rate were adjusted as necessary to optimise the sensitivity and reproducibility. These changes will be specified where relevant.

5.1.2 Choice of internal standard

Solutions of a number of compounds, selected on the basis of their Kovat's indices [Moffat,(1975)], containing approximately 1 mg/ml in chloroform were prepared. These compounds included benztropine mesylate and some benzodiazepines i.e. nitrazepam, oxazepam and chlordiazepoxide. Some pure chemicals were also injected, e.g. long chain (C_{22}, C_{24} and C_{36}) alkanes and di-(2-ethylhexyl) phthalate (DEHP).

5.1.3 Development of the assay procedure

The assay method involved extraction of diazepam and MACB from an aqueous solution, either at pH 1.0 or pH

7.0, into chloroform. A 2.0 ml portion of internal standard solution in chloroform was added and the mixture evaporated to dryness. The residue was redissolved in chloroform (1 ml) and injected onto the chromatographic system described in Section 5.1.1.

5.1.3.1 Investigation of the extraction stage

A solution of diazepam and MACB in ethanol was prepared (17 and 15 mg/ml respectively). Three 2.0 ml portions were diluted with 498 ml of water, 498 ml of 0.1 M hydrochloric acid / 0.9 M sodium chloride solution and also with chloroform to 50 ml after the addition of 10 ml internal standard solution (5 mg/ml DEHP in chloroform). The chloroform solution was used as the standard for the GC assay. The concentrations of each component in this solution were 0.7, 0.6 and 1.0 mg/ml respectively for diazepam, MACB and DEHP.

Portions (10.0 ml) of the water and acidic solutions were transferred to a separating funnel, containing 1.0 ml water or 1 M sodium hydroxide (for the water and acidic solutions respectively) and 10 ml of pH 7.0 buffer. The samples were immediately extracted for 2 min with 20 ml chloroform. The experiment was repeated with duplicate and triplicate extractions with 20 ml of chloroform. The chloroform extracts were pooled where appropriate and 2.0 ml of a diluted internal standard solution was added (0.5 mg/ml DEHP in chloroform). The mixtures were evaporated to dryness and then redissolved in 1 ml of chloroform.

Replicate injections were made of each sample solution

and of the standard mixture. Peak areas were measured and the appropriate ratios calculated. The efficiency of the single, duplicate and triplicate extractions were calculated by reference to the standard solution prepared and injected at the same time.

5.1.3.2 Investigation of the stability of diazepam in acid and water

In Section 5.1.3.1 the extractions were performed immediately after addition of diazepam to the diluting solvent. To see if any loss of diazepam occurs in acid or water, the extraction stage was repeated for a sample taken from water at 75 min and from acid at 120 min after dilution. These samples were shaken with one 20 ml portion of chloroform to extract the diazepam and the chloroform was treated as described in Section 5.1.3. Injections of the final solution were made onto the chromatographic system, together with injections of the standard mixture containing diazepam at the concentration of the original dilution. Peak areas and peak heights were measured and the ratios calculated. The concentration of diazepam extracted from these older solutions was calculated by reference to the standard solution injected at the same time. These values were compared with the concentration measured for the samples which had been extracted immediately after dilution of the ethanolic solution (Section 5.1.3.1).

5.1.3.3 Investigation of the stability of diazepam in chloroform

The chloroform solutions obtained by extraction of the aqueous and acidic samples (Section 5.1.3.1) were retained and reinjected 16 and 17 days after they had first been injected. The standard mixture of diazepam, MACB and DEHP described in Section 5.1.3.1 was also injected. The peak areas and heights from these injections were measured and the ratios calculated. The concentration of diazepam and MACB in these solutions was calculated by reference to the standard mixture injected at the same time. These concentrations were compared for each solution to allow examination of the effect of age on the solutions.

5.1.4 Validation of the assay method

5.1.4.1 Precision of injection

The standard mixture prepared containing diazepam, MACB and DEHP in chloroform at concentrations of 0.7, 0.6 and 1.0 mg/ml respectively (Section 5.1.3.1) was used. Replicate injections of 1 μl of this solution were made and the peak height ratios of diazepam and MACB to the internal standard calculated. The detector output was also fed to a benchtop integrator and the area of each peak measured. The peak area ratios were calculated. The precisions of the peak height ratios and the peak area ratios were calculated statistically using the relative standard deviation (RSD).

5.1.4.2 Reproducibility of extraction

Seven 10.0 ml samples of the acidic solution described in Section 5.1.3.1 were neutralised and buffered in different separating funnels using 1 ml of 1 M sodium hydroxide and 10 ml of pH 7.0 buffer. Each sample was then extracted with 20 ml of chloroform for 2 minutes and the internal standard solution was added to the organic layer. The chloroform was evaporated and the residue was redissolved in chloroform prior to injection. The peak area ratios for diazepam and MACB to the internal standard were calculated for each of the extracts and the reproducibility of extraction was assessed by comparison of these results.

The extraction of diazepam from both pH 7 and pH 1 solutions was investigated. A solution of diazepam in ethanol was prepared (4 mg/ml) and diluted in 0.1 M hydrochloric acid/ 0.9 M sodium chloride solution (2.0 ml to 100 ml). Six 10.0 ml samples of the acidic dilution were extracted into chloroform as described above, but without prior neutralisation. Internal standard was added to the extracts, these were evaporated to dryness and the residues redissolved in chloroform. These samples were injected onto the chromatographic system described in Section 5.1.1. The peak area ratio of diazepam to internal standard was calculated for each extract and the reproducibility of the extraction process assessed by comparison of the results.

The acidic solution described in the previous

paragraph was allowed to degrade and further samples from it were extracted into chloroform. Six samples were extracted as above from acid and four samples were neutralised by the addition of 0.1 M sodium hydroxide and 10 ml of pH 7.0 buffer before extraction into chloroform. Internal standard solution was added to each of the extracts and these were evaporated and redissolved in chloroform as described above. These samples were then injected onto the chromatographic system and the peak heights obtained treated as described for peak areas in the previous paragraph.

5.1.4.3 Investigation of the linearity of the assay procedure

Solutions of diazepam and MACB in ethanol were prepared containing 7.5 and 6.5 mg/ml respectively. These were diluted to 500 ml with 0.1 M hydrochloric acid as shown in Table 5.1.

Table 5.1 Preparation of solutions for study of rectilinearity of assay procedure

Volume of diazepam solution (ml)	Concentration of diazepam in chloroform injected (mg/ml)	Volume of MACB solution (ml)	Concentration of MACB in chloroform injected (mg/ml)
5.0	0.75	1.0	0.13
4.0	0.60	2.0	0.26
3.0	0.45	3.0	0.39
2.0	0.30	4.0	0.52
1.0	0.15	5.0	0.65

Immediately after the addition of diazepam and MACB to

each volumetric flask, the flask was vigorously shaken and a 10.0 ml sample transferred to a separating funnel. The samples were neutralised and buffered with 1 ml of 1 M sodium hydroxide and 10 ml of pH 7.0 buffer. These were then extracted with 20 ml of chloroform for 2 min. The extract was mixed with 2.0 ml internal standard solution (0.5 mg/ml DEHP in chloroform) and evaporated to dryness. The residue was redissolved in 1.0 ml chloroform and 1 μl portions repeatedly injected onto the chromatographic system. This procedure was repeated for each solution, the peak areas measured and the ratios calculated. The peak areas were plotted against the concentration of diazepam and MACB and the linearity of response was assessed statistically.

5.2 USE OF GC TO STUDY THE EXTRACTION PROCEDURE

5.2.1 Preparation of hydrolysed diazepam

A solution of diazepam in ethanol (40 mg/ml) was diluted, 2.0 ml to 100 ml with 0.1 M hydrochloric acid. The resultant mixture was allowed to stand at room temperature in daylight for 5 days to allow hydrolysis to occur and equilibrium to be established between diazepam and the intermediate hydrolysis products (Fig. 1.5). From work performed using UV and difference UV spectrophotometry (Section 5.4.1) it had been established that 5 days was sufficient for equilibrium to be established.

5.2.2 Investigation of extraction characteristics

A 20.0 ml sample of the equilibrated hydrolysis solution (Section 5.2.1) was extracted with three successive 20 ml quantities of chloroform which were each kept separate. 2.0 ml of internal standard solution (0.5 mg/ml DEHP in chloroform) were added to each chloroform extract. These extracts were evaporated and the residue was redissolved in 1 ml chloroform (Section 5.1.3).

A further 10.0 ml sample of the hydrolysis solution was extracted with 20 ml chloroform. 1 ml of 1 M sodium hydroxide and 10 ml of pH 7 buffer solution were added to the extracted acidic phase to neutralise it and a further extraction was made with chloroform. Internal standard solution was added and the solutions were evaporated and redissolved as described above.

10.0 ml of hydrolysis solution was neutralised prior to any extraction by addition of 1 ml of 1 M sodium hydroxide and 10 ml of pH 7 buffer solution. This was then extracted with 20 ml chloroform which had internal standard added and was evaporated as described above.

All the redissolved chloroform extracts were injected onto the gas chromatographic system, conditions as described in Section 5.1.1. The peak heights were measured and the peak height ratios calculated. The effect of extraction from aqueous solutions at pH 1 and pH 7 and the effect of extracting from pH 7 after extraction from pH 1 were compared.

5.3 APPLICATION OF THE GC METHOD TO STUDY THE KINETICS OF HYDROLYSIS OF DIAZEPAM

5.3.1 Preparation of the reaction mixture

The acid hydrolysis reaction was carried out at 70°C in a reaction mixture similar to that used by Han, Yakatan and Maness(1977a). 2.0 ml of a solution of diazepam in ethanol (approximate concentration 17 mg/ml) was added to a round-bottom, two-neck flask containing 498 ml of a 0.1 M hydrochloric acid/ 0.9 M sodium chloride solution, (pre-heated to 70°C in a water bath). A condenser was fitted in one neck of the flask and the other neck was fitted with a stopper which could be removed for sampling the contents.

5.3.2 Analysis of the reaction mixture

At time intervals (10, 20, 30, 40, 50, 60, 80, 100, 120, 150, 180, 210, 140, 300 and 360 minutes), a sample was transferred from the reaction mixture (Section 5.3.1) to a test-tube and cooled rapidly in ice water to stop further reaction. A 10.0 ml sample of this solution was then transferred to a separating funnel, 1 ml of 1 M sodium hydroxide was added to neutralise the solution and 10 ml of pH 7.0 buffer were added to control the pH. This mixture was then extracted with 20 ml chloroform for 2 min. The organic layer was collected and 2.0 ml of internal standard solution added (0.5 mg/ml DEHP in chloroform). This mixture was evaporated to dryness and the residue was redissolved in chloroform and injected onto the chromatographic system (Section

5.1.1).

The procedure described above was repeated on a second reaction mixture. In this case after sampling the reaction mixture into test-tubes at the appropriate time intervals, the 10.0 ml sample was transferred to a separating funnel and extracted with chloroform. There was no addition of sodium hydroxide or pH 7.0 buffer solution, so that no alteration was made to the pH of the solution.

5.4 APPLICATION OF DIFFERENCE UV AND DIRECT UV SPECTROPHOTOMETRIC METHODS TO STUDY THE KINETICS OF HYDROLYSIS OF DIAZEPAM

5.4.1 Hydrolysis reaction studied at room temperature

A stock solution of diazepam in ethanol was prepared (8 mg/ml) and this was diluted 10.0 ml to 500 ml with 0.1 M hydrochloric acid. Immediately after preparation of this acidic dilution, and on successive days for one week, a number of samples were taken and extracted and diluted as described below. This provided five sets of solutions, for direct UV and difference UV absorbance measurement. The acidic solution was left at room temperature, in daylight, between sampling. A flow chart showing the preparation and relationship of each solution is given in Fig. 5.1.

Solution 1 was prepared as follows. 2.0 ml of the acidic solution together with 2.0 ml of methanol was diluted to 25 ml with 0.1 M hydrochloric acid. A further two 2.0 ml portions of the acidic solution were transferred to 25 ml volumetric flasks. 2.0 ml of methanol and 0.2 ml of 1 M sodium hydroxide solution were added to each and made to volume with pH 2.6 and pH 5.4 buffer solutions (solutions 1A and 1B).

10.0 ml of the acidic solution was extracted with 10 ml of chloroform. Three samples from the acid fraction were treated as described above to prepare solution 2 and solutions 2A and 2B. The chloroform layer was evaporated to dryness and the residue was

Fig 5.1 Preparation of dilutions from the solution of diazepam in 0.1 M hydrochloric acid

Solution of diazepam in acid

DIRECT DILUTION

1 Dilution in acid
1A Dilution in pH 2.6 buffer
1B Dilution in pH 5.4 buffer

EXTRACTED WITH CHLOROFORM

Acidic layer diluted

2 Dilution in acid
2A Dilution in pH 2.6 buffer
2B Dilution in pH 5.4 buffer

Chloroform layer evaporated, redissolved then diluted

3 Dilution in acid
3A Dilution in pH 2.6 buffer
3B Dilution in pH 5.4 buffer

EXTRACTED WITH CHLOROFORM

Acidic layer, pH adjusted to pH 7, then extracted with a second portion of chloroform

Neutral layer diluted

4 Dilution in acid
4A Dilution in pH 2.6 buffer
4B Dilution in pH 5.4 buffer

Chloroform layer evaporated, redissolved then diluted

5 Dilution in acid
5A Dilution in pH 2.6 buffer
5B Dilution in pH 5.4 buffer

Chloroform layer discarded

redissolved in 10.0 ml methanol. 2.0 ml of the methanolic solution was diluted to 25 ml with 0.1 M hydrochloric acid, (solution 3). A further two 2.0 ml portions of this methanolic solution were added to 25 ml volumetric flasks, 0.2 ml of 1 M sodium hydroxide solution were added to each and they were made to volume with pH 2.6 and pH 5.4 buffer solutions (solutions 3A and 3B).

A further 10.0 ml of the acidic solution was extracted with chloroform. After removing the organic layer, the acidic solution was neutralised by the addition of a further 1 ml of 1 M sodium hydroxide and 10.0 ml of pH 7 buffer. This neutral solution was extracted with a fresh 10 ml portion of chloroform. The neutral aqueous phase was treated as described for the acidic solution, except that the addition of 0.2 ml of 1 M sodium hydroxide was omitted, (solutions 4, 4A and 4B).

The chloroform layer was evaporated to dryness and the residue was redissolved in 10.0 ml of methanol. This solution was treated as described for the preparation of solution 3, to give solutions 5, 5A and 5B.

Absorbance measurements were made by direct UV spectrophotometry at 238, 255 and 284 nm for solutions 1, 2, 3, 4 and 5 against a corresponding blank solution. The UV spectra of each of these solutions were also recorded. The difference absorption spectra were recorded for each pair of solutions A and B with the pH 2.6 solution in the sample beam and the pH 5.4 solution in the reference beam (solutions A and B respectively for each set). The difference absorbance of these solutions were measured at 255 and 290 nm.

5.5. DEVELOPMENT AND VALIDATION OF AN HPLC METHOD FOR THE ANALYSIS OF DIAZEPAM, MACB AND GMACB

5.5.1 Development of method

The method used for the study of the kinetic reaction was developed from that used for analysis of diazepam in formulations. A 30 cm stainless steel column packed with 5 μm Spherisorb ODS packing material was used.

5.5.1.1 Choice of conditions

A number of mobile phase mixtures were prepared using methanol : buffer. The proportion of methanol varied from 65% to 75% and the buffer solutions used were acetate (0.005 M) and phosphate (0.1 M). The pH of the mixtures were measured and the mobile phase pH adjusted using acetic acid and orthophosphoric acid as appropriate. A simple mobile phase mixture containing only methanol and 0.1 M sulphuric acid was also prepared (75:25).

A solution containing a mixture of diazepam, MACB and carbostyril (approximate concentrations 76, 14 and 65 μg/ml respectively in methanol) was injected onto the HPLC systems. Each different mobile phase was allowed to equilibrate and the separation and retention times of the compounds obtained. Carbostyril was included in the mixture injected as it is a potential degradation product, although it is usually present at very much lower levels than MACB. A solution of diazepam in acid was prepared by dissolving approximately 10 mg of

diazepam to 100 ml with 0.1 M hydrochloric acid. This solution was allowed to hydrolyse at room temperature (Section 5.2.1). This degraded sample of diazepam was also injected onto the chromatographic systems.

5.5.1.2 Chromatographic conditions

The chromatographic conditions selected for the stability study are listed below:

Column	30 cm x 4.6 mm stainless steel column, packed with 5 μm Spherisorb ODS
Mobile phase	Methanol:0.1 M sodium dihydrogen orthophosphate (75:25). Adjusted to pH 2.8 with orthophosphoric acid.
Flow rate	1.2 ml/min (1.0 ml/min)
Pressure	Typically 3000 psi (2000 psi)
Injection volume	20 μl by Rheodyne valve, manual injection
Detection	UV detection at 254 nm

The values for flow rate and pressure shown in brackets were those used on System 3.

The mobile phase was degassed by boiling the solution under reflux.

The absorbance was recorded using a chart recorder. The peak heights were measured for each sample, and used to calculate the change in relative concentration with time.

5.5.2 Validation of conditions

5.5.2.1 Precision of injection

Replicate injections were made of a standard solution of diazepam in methanol (approximately 125 μg/ml). The peak height was measured on each injection system used and these values were analysed statistically to determine the precision of injection.

A standard solution of diazepam in methanol was prepared (approximately 2.5 mg/ml) and this was diluted in 0.1 M hydrochloric acid to give a solution containing approximately 125 μg/ml of diazepam. This solution was allowed to reach equilibrium between diazepam and GMACB and replicate injections made of the degraded mixture. The peak heights for diazepam, MACB and GMACB from these injections were measured and analysed statistically to determine the precision of injection.

5.5.2.2 Measurement of response against concentration

Stock solutions of diazepam and MACB were prepared in

methanol (approximately 580 and 61 μg/ml respectively). These were diluted as shown in Table 5.2. Duplicate injections were made of each dilution prepared and the mean response plotted against the concentration of diazepam and MACB.

Table 5.2 Preparation of solutions for study of rectilinearity of detector response

Concentration of diazepam (μg/ml)	Concentration of MACB (μg/ml)
23.23	2.45
46.46	4.89
69.68	7.34
92.91	9.78
116.14	12.23

A solution containing only GMACB was prepared in 0.1 M hydrochloric acid as follows. Diazepam was dissolved in 0.1 M hydrochloric acid (0.11 mg/ml) and allowed to degrade for 5 days at room temperature in daylight. Any diazepam and MACB left after the reaction had reached equilibrium were extracted with chloroform. The resultant solution of GMACB in 0.1 M hydrochloric acid was diluted further by taking 2.0, 4.0, 6.0 and 8.0 ml of solution and diluting to 10 ml with 0.1 M hydrochloric acid. These dilutions were all injected in duplicate onto the HPLC system, together with the original extracted solution. The peak height of the GMACB peak was measured. The approximate concentration of GMACB present in the solution after degradation was calculated from the initial concentration of diazepam and the mean response from

each solution was plotted against this.

5.5.2.3 Investigation of the ring-closure of GMACB during analysis

Mixtures of methanol:buffer (75:25) were prepared and their pH adjusted to a range of values between 1.7 and 7.4, (Table 5.3).

Table 5.3 Preparation of mobile phases of different apparent pH

Buffer used	Acid used for adjustment of final pH	Apparent final pH
0.1 M hydrochloric acid	-	1.7
0.1 M phosphate	orthophosphoric acid	2.8
0.1 M phosphate	orthophosphoric acid	3.2
0.1 M phosphate	orthophosphoric acid	3.5
acetate pH 5.4	-	5.8
phosphate pH 7.0	-	7.4

A degraded mixture containing diazepam, MACB and GMACB in 0.1 M hydrochloric acid was extracted with chloroform to remove the diazepam and MACB. The initial concentration of diazepam was 0.5 mg/ml before degradation, but the solution had not been allowed to equilibrate, so the concentration of GMACB present was not known. Samples of the solution after extraction were diluted in the mobile phase solutions at pH 1.7 and pH 3.2. These dilutions were injected onto the chromatographic system (Section 5.5.1.2) at zero time and at time intervals of up to 25 mins after dilution. The pH of the mobile phase used in the

chromatographic system was pH 3.2. Portions of this solution were also diluted with the mobile phase solutions (4 ml to 25 ml) and the UV absorbance of these were monitored with time.

A further degraded mixture of diazepam with MACB and GMACB was prepared but was allowed to reach equilibrium. The initial concentration of diazepam in this solution was 0.5 mg/ml. This solution was diluted with the prepared mobile phases described above (2 ml to 10 ml) and the dilutions injected onto the HPLC system with mobile phase at pH 3.2 in the chromatographic system. Portions of this solution were also diluted in these mobile phase solutions and the UV absorbance of the dilutions monitored with time.

5.6 APPLICATION OF THE HPLC METHOD TO STUDY THE KINETICS OF HYDROLYSIS OF DIAZEPAM

5.6.1 Preparation of the reaction mixture

The reaction conditions used were similar to those used by Nakano et al.(1979). A flask containing 95 ml of 0.1 M hydrochloric acid was placed in a water bath at 37°C. A stock solution of diazepam in ethanol was prepared containing approximately 2.5 mg/ml diazepam. At zero time, 5.0 ml of this stock solution was added to the pre-warmed acid, the flask contents were well mixed and a portion of the mixture sampled into a test-tube then plunged into ice-water to quench the reaction. The reaction mixture was sampled at 15 minute intervals and from each portion sampled, a 20 μl volume was injected directly onto the HPLC system.

5.6.2 Analysis of the reaction mixture

The sample of the reaction mixture was injected directly onto the chromatographic system described in Section 5.5.1.2. The injections of sample solution were separated by injections of a standard solution of diazepam in methanol, containing approximately 125 μg/ml of diazepam. The peak heights were measured and those of each sample injection adjusted by reference to the peak heights of the standard injections made immediately before and after the sample. The concentration of diazepam was not calculated from these results but the percentage of diazepam remaining at any stage in the reaction was calculated from the initial value for peak height. These values were used to calculate the equilibrium constants as described in Appendix I.

5.7 FURTHER CHARACTERISATION OF THE INTERMEDIATE HYDROLYSIS PRODUCT OF DIAZEPAM

5.7.1 Thin-layer chromatography

5.7.1.1 Chromatographic conditions

Plates precoated with silica gel GF_{254}, 0.25 μm, were used for this work. The plates were spotted with sample and standard solutions and allowed to dry in air. These were then developed in a tank lined with chromatography paper and saturated with mobile phase vapour for at least one hour prior to development. Mobile phase systems used were toluene:ethyl acetate:glacial acetic acid (80:18:2 and 80:15:5 by volume).

After development, the plates were removed from the tank, dried and the spots were visualised under UV light.

5.7.1.2 Preparation of standard and sample solutions

Standard solutions of diazepam, MACB and carbostyril in 0.1 M hydrochloric acid/ethanol (50:50) and in chloroform were prepared (all at approximately 2 mg/ml). A solution of glycine in 0.1 M hydrochloric acid/ethanol (50:50) at about the same concentration was also prepared.

5 μl portions of the standard solutions in chloroform and also the standard solutions in 0.1 M hydrochloric

acid/ethanol (50:50) were applied to separate plates. These plates were developed in both mobile phase mixtures and the R_f values for each spot calculated after visualisation of the developed plates. Subsequent work was performed using the mobile phase mixtures with solvent proportions 80:18:2 for development.

A solution of diazepam hydrolysed in 0.1 M hydrochloric acid was prepared, (Section 5.2.1) and 20.0 ml of this was extracted with two portions, each of 10 ml chloroform. After the extractions, 2 ml of 1 M sodium hydroxide and 10 ml of pH 7 buffer solution were added to the aqueous layer and the neutral mixture was extracted with a further 10 ml of chloroform. All three chloroform extracts were evaporated and the residue of each was redissolved in 1 ml of chloroform.

A plate was spotted with standard diazepam, carbostyril and MACB solutions in chloroform and also the solutions obtained from extraction of the hydrolysed solution of diazepam. This was developed in mobile phase and visualised.

The hydrolysis mixture was concentrated for direct application of the acidic solution to TLC plates. Two methods were used, to concentrate the material. Firstly, 10 ml of acidic solution was evaporated to dryness on a hotplate, and secondly 10 ml of the acidic solution was evaporated to dryness under vacuum using a water bath at a temperature not exceeding 37°C. The residues from both of these methods were redissolved in 1 ml of a mixture of 0.1 M hydrochloric

acid/ethanol (50:50).

Standard solutions of diazepam, carbostyril, MACB and glycine were spotted onto a TLC plate (5 μl of each) together with 10 μl spots of the concentrated hydrolysis mixtures prepared by both methods of evaporation. Two spots were applied of each of the mixtures, and 10 μl of concentrated ammonium hydroxide was applied to a spot from each solution and allowed to dry before development of the plate.

5.7.1.3 Two-dimensional chromatography

The ethanolic hydrochloric acid solutions obtained after evaporation of the hydrolysis mixture were spotted at the lower left-hand corner of two 20 x 20 cm precoated silica gel GF_{254} plates (10 μl). A mixture of diazepam, carbostyril and MACB standards in the same solvent was applied to the lower right-hand corner of each plate. After drying, the plates were developed and visualised under UV light. The plates were turned through 90°, a further portion of the mixture of standards was applied to the new baseline and 10 μl of concentrated ammonium hydroxide applied to the original sample origin before the plate was redeveloped in the same mobile phase system.

5.7.2 <u>Infra-red spectroscopy</u>

5.7.2.1 Preparation of discs

Diazepam and MACB separately were mixed with potassium

bromide in an agate mortar to give a 0.5% mixture. These were ground together with an agate pestle then transferred to a die press. All equipment used in the preparation of discs including the potassium bromide was stored in an oven to keep it dry. The powders were then compressed at 8 tons per square inch for 5 min. The discs were removed from the press, inserted into a holder and placed in the beam of the IR instrument. Scans were made over the IR range, (4000 to 600 cm^{-1}).

Solutions were prepared of diazepam and MACB in chloroform at about 1 mg/ml. 1 ml samples of these were added to approximately 200 mg of potassium bromide and ground together while evaporating the chloroform under a stream of air. The powders were compressed into discs as described above.

A 10 ml sample of hydrolysed diazepam solution in 0.1 M hydrochloric acid, which had been allowed to reach equilibrium at room temperature (Section 5.2.1) was extracted with 20 ml chloroform. The chloroform was evaporated, the residue redissolved in 1 ml of chloroform and this solution mixed with approximately 200 mg of potassium bromide, while drying as above. A disc was prepared from this material as described previously.

The acidic solution remaining after this extraction was neutralised by the addition of 1 ml of 1 M sodium hydroxide and 10 ml of pH 7 buffer solution. A further 20 ml of chloroform was then added. The sample solution was extracted a second time and the chloroform solution obtained from this extraction was prepared for IR analysis as described above.

6 RESULTS AND DISCUSSION FOR ANALYSIS OF FORMULATIONS

6.1 DEVELOPMENT AND VALIDATION OF A DIFFERENCE ABSORBANCE UV SPECTROPHOTOMETRIC METHOD

6.1.1 Development of a difference UV spectrophotometric method

The individual spectra and the difference spectrum generated for equimolar solutions (10 μg/ml) of diazepam in pH 5.3 buffer solution and in 0.1 M hydrochloric acid were recorded and are shown in Fig. 6.1. The difference spectrum was generated with the acidic solution in the sample beam and the solution in buffer in the reference beam. The difference spectrum was generated under these conditions of pH in order to maintain at least 2 pH units above and below the pK_a of diazepam, (pK_a 3.3, [Newton and Kluza,(1978)]). Solutions with pH of two pH units on either side of the pK_a value were selected to ensure that at least 99% of the species present were in the molecular or protonated forms respectively. A λ_{max} was seen at 290 nm where the largest difference absorbance for these solutions occurs. A secondary λ_{max} at 360 nm in the difference spectrum was also observed.

Equimolar solutions (3 μg/ml) of MACB (2-methylamino-5-chlorobenzophenone), the major degradation product of diazepam, were prepared in the same solvents as above. The difference spectrum of these solutions were generated and it was observed that a change in the benzophenone spectrum occurred over this pH range.

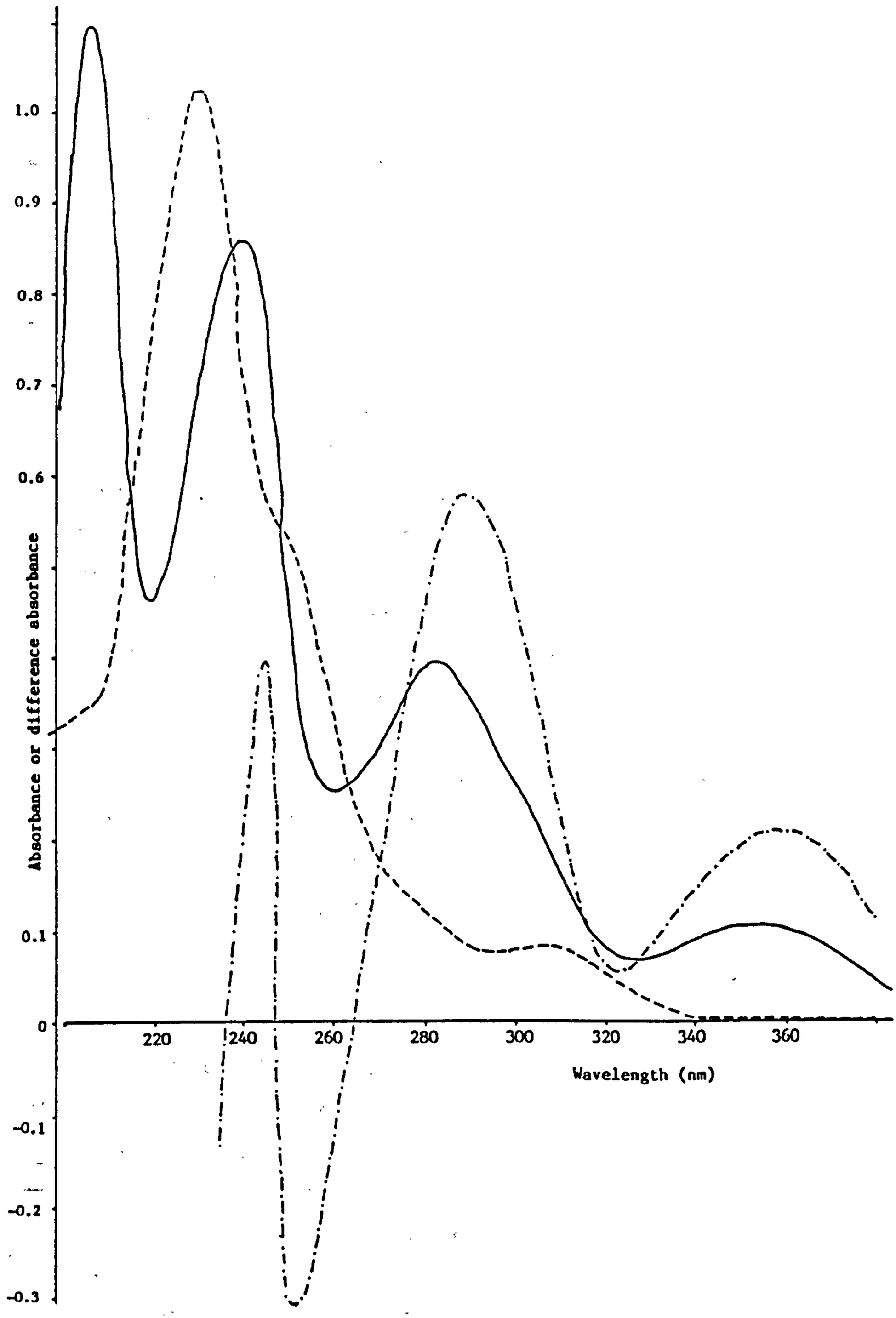


Fig. 6.1 Individual and difference spectra of diazepam

——— Absorption spectrum of diazepam in 0.1 M hydrochloric acid

- - - - - Absorption spectrum of diazepam in pH 5.3 buffer solution

-·-·-·- Difference absorption spectrum of diazepam, pH 1 vs pH 5.3

This showed that if MACB was present in samples being analysed, a difference absorbance would be measured due to its presence, giving interference in the measurement of diazepam, (Fig. 6.2).

The absorbance of dilutions of stock solutions of diazepam and MACB in a range of buffer solutions were plotted against the pH of solution. The pH-absorbance profiles for both diazepam and MACB obtained in this way are shown in Fig. 6.3. The absorbance measurements were made at 290 nm, the λ_{max} in the difference absorbance spectrum. The graph plotted for diazepam formed a sigmoidal curve, the mid-point of the inflection occurring at pH 3.3, corresponding to the quoted pK_a for diazepam.

For optimum sensitivity and robustness the pH values used should be at most pH 1.3 and at least pH 5.3. Both these values give absorbance measurements lying on the horizontal portions of the graph for diazepam (Fig. 6.3). However, MACB shows a change of absorbance over these pH values, and the selectivity of the assay would be reduced if these values were used. Newton (1979) has quoted a pK_a value for MACB of 1.45 which agrees with the absorbance profile obtained (Fig. 6.3). (A true sigmoidal curve was not seen due to lack of results at pH values less than 2 pH units from the pK_a value).

After examination of the data in Fig. 6.3, pH values of 2.6 and 5.4 were selected for use in the assay. Fig. 6.4 shows the individual spectra of diazepam at pH 2.6 and pH 5.4 and their difference spectrum. These values reduced the sensitivity (by reducing the

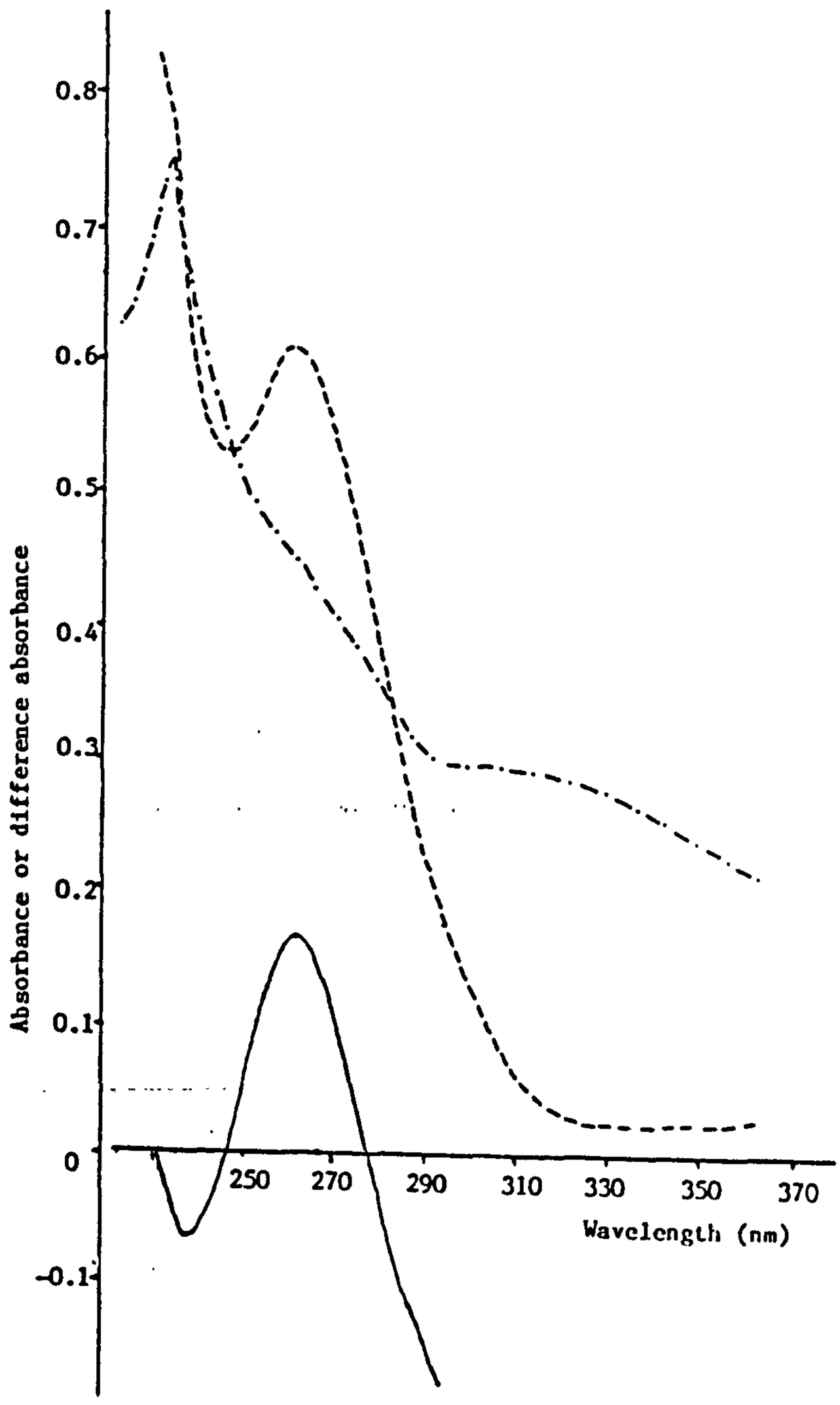


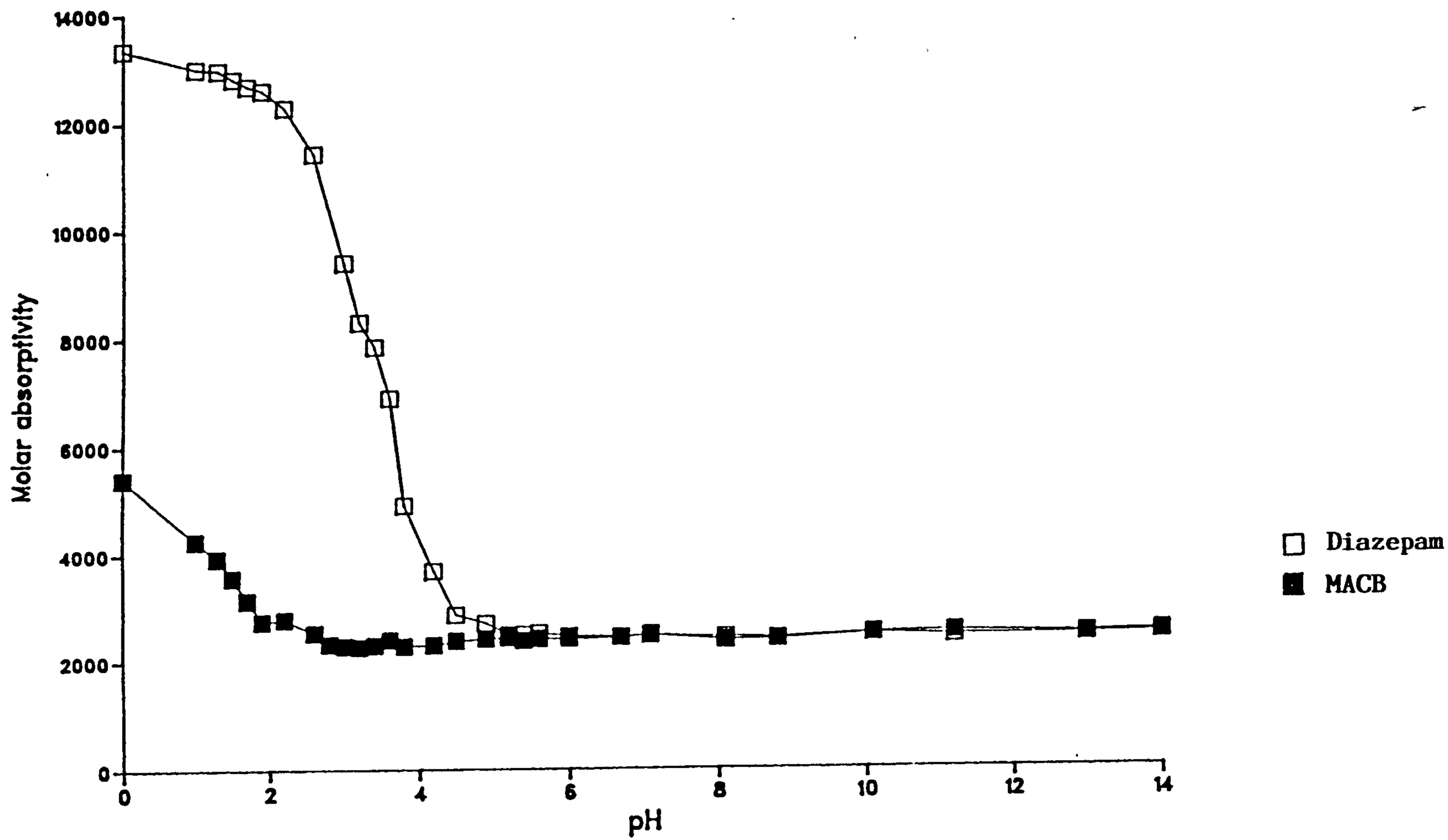
Fig. 6.2 Individual and difference spectra of MACB

----- Absorption spectrum of MACB in 0.1 M hydrochloric acid

- -·-·- Absorption spectrum of MACB in pH 5.3 buffer solution

——— Difference absorption spectrum of MACB, pH 1 vs pH 5.3

Fig 6.3 pH–absorbance profile for diazepam and MACB at 290 nm



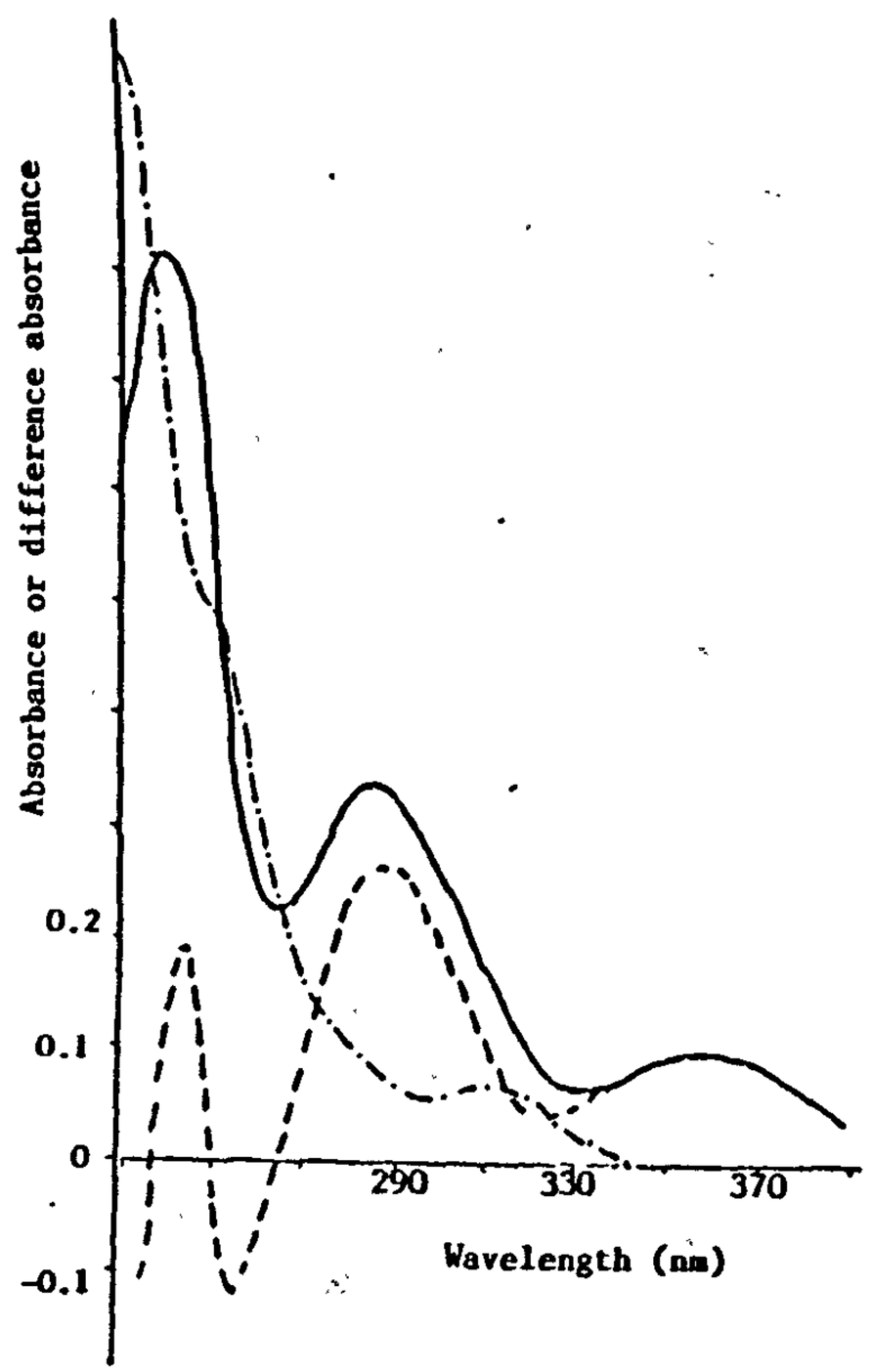


Fig. 6.4 (a) Individual and difference spectra of diazepam

——— Absorption spectrum of diazepam in pH 2.6 buffer

-·-·- Absorption spectrum of diazepam in pH 5.4 buffer

- - - - Difference absorption spectrum of diazepam, pH 2.6 vs pH 5.4

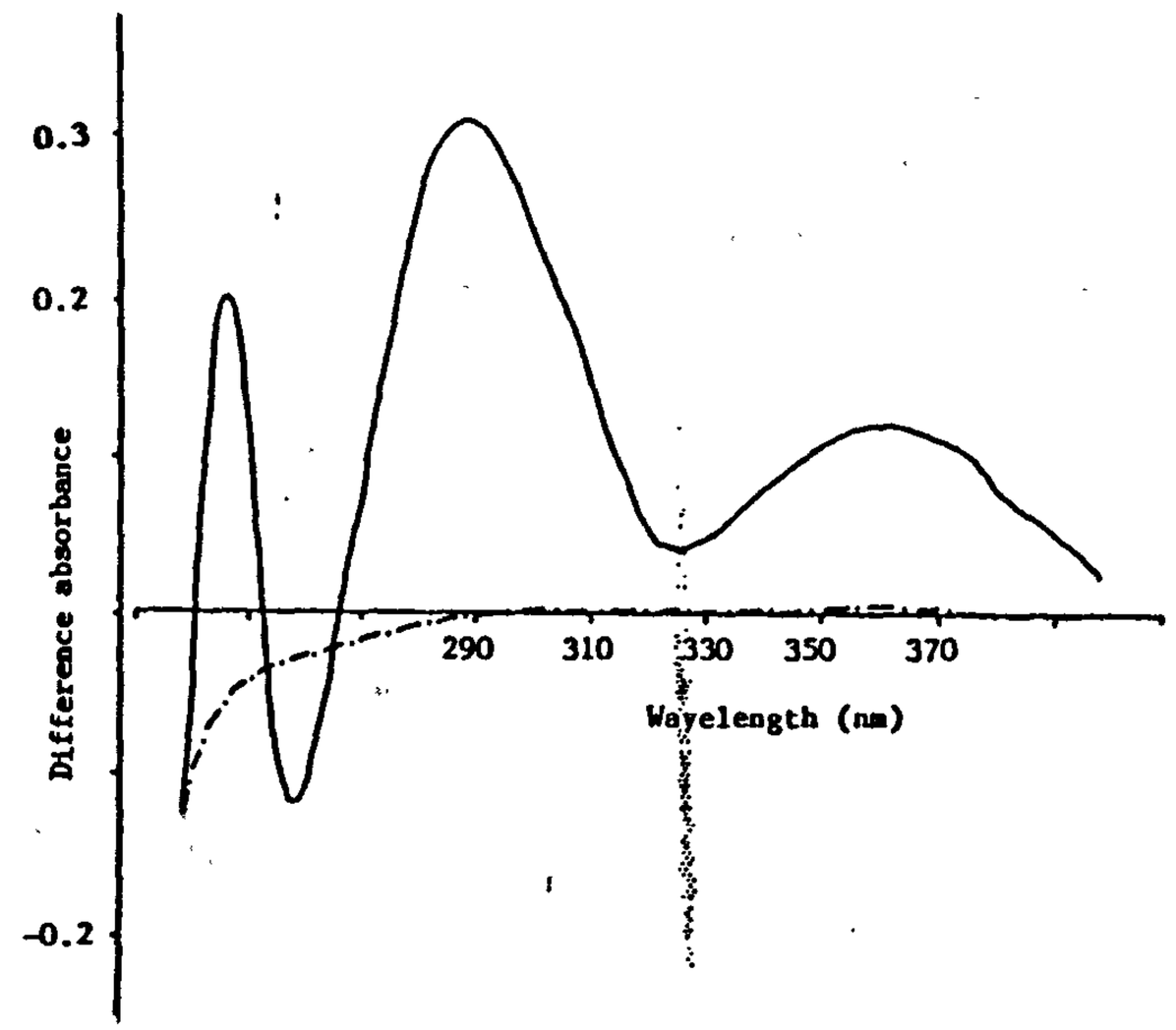


Fig. 6.4 (b) Difference spectra of diazepam and MACB; pH 2.6 vs pH 5.4

——— Difference absorption spectrum of diazepam

- - - - Difference absorption spectrum of MACB

maximum difference absorbance measured) but at the same time maintained the specificity by reducing the contribution from MACB. By using these pH values to obtain the difference absorbance spectra, typical difference A(1%,1cm) values were obtained of 320 for diazepam and of 6 for MACB. Thus less than 2% interference would occur due to an equimolar concentration of MACB.

The robustness of the assay was also reduced by the use of these pH values. The use of pH 2.6 buffer introduces a potential source of imprecision since it is on the inflection of the graph, at a point where the absorbance of diazepam changes rapidly. Thus, small changes in the pH of the buffer solutions could result in large changes in the ratio of ionised to nonionised species present and therefore in large changes in the measured absorbance. To reduce any error from this source during the experimental work, standard solutions of diazepam were included in each series of assays. This ensured that any difference in the measured ΔA (1%,1cm) due to differences in the pH of the buffer solutions could be detected and compensated for.

In addition to MACB, there are other compounds which might be expected to be present. These were tested for interference in the difference absorbance measurement under the pH conditions selected. The compounds tested were desmethyl diazepam (Fig. 1.2), a manufacturing impurity of diazepam, carbostyril and GMACB (2-glycyl(methyl)amino-5-chlorobenzophenone) two degradation products of diazepam (Fig. 1.5). The difference UV spectra for desmethyl diazepam and

carbostyril were recorded using pH 2.6 and pH 5.4 buffer solutions and these can be seen in Fig. 6.5. No interference was seen from carbostyril. However, a very similar difference spectrum was found for desmethyl diazepam, with a ΔA (1%,1cm) of 421. This value is higher than that obtained for diazepam under the same conditions. As the structure of desmethyl diazepam is similar to that of diazepam, (Section 1.2), it would be expected that the UV absorption spectrum would also be very similar. Therefore the presence of desmethyl diazepam will interfere with the determination of diazepam.

Prior to release of diazepam drug substance for manufacture of dosage forms, stringent testing is carried out. It is anticipated that only very low levels (if any) of this manufacturing impurity would be present. The B.P. monograph for Diazepam [B.P.,(1980)] has a test for related substances by thin layer chromatography and the limit for this test is 0.1% of drug related substance. The lack of specificity for diazepam in the presence of desmethyl diazepam does not therefore preclude the use of this method as the contribution from desmethyl diazepam is likely to be extremely small.

The stability of diazepam has been investigated by a number of workers. This is discussed in Section 1.2.3. It has been proposed that the hydrolysis of diazepam proceeds by a reversible reaction through an intermediate which exists only in acidic solution, this intermediate has been identified as GMACB by Nakano et al.(1979). GMACB is reported to exist in equilibrium with diazepam, and this equilibrium is

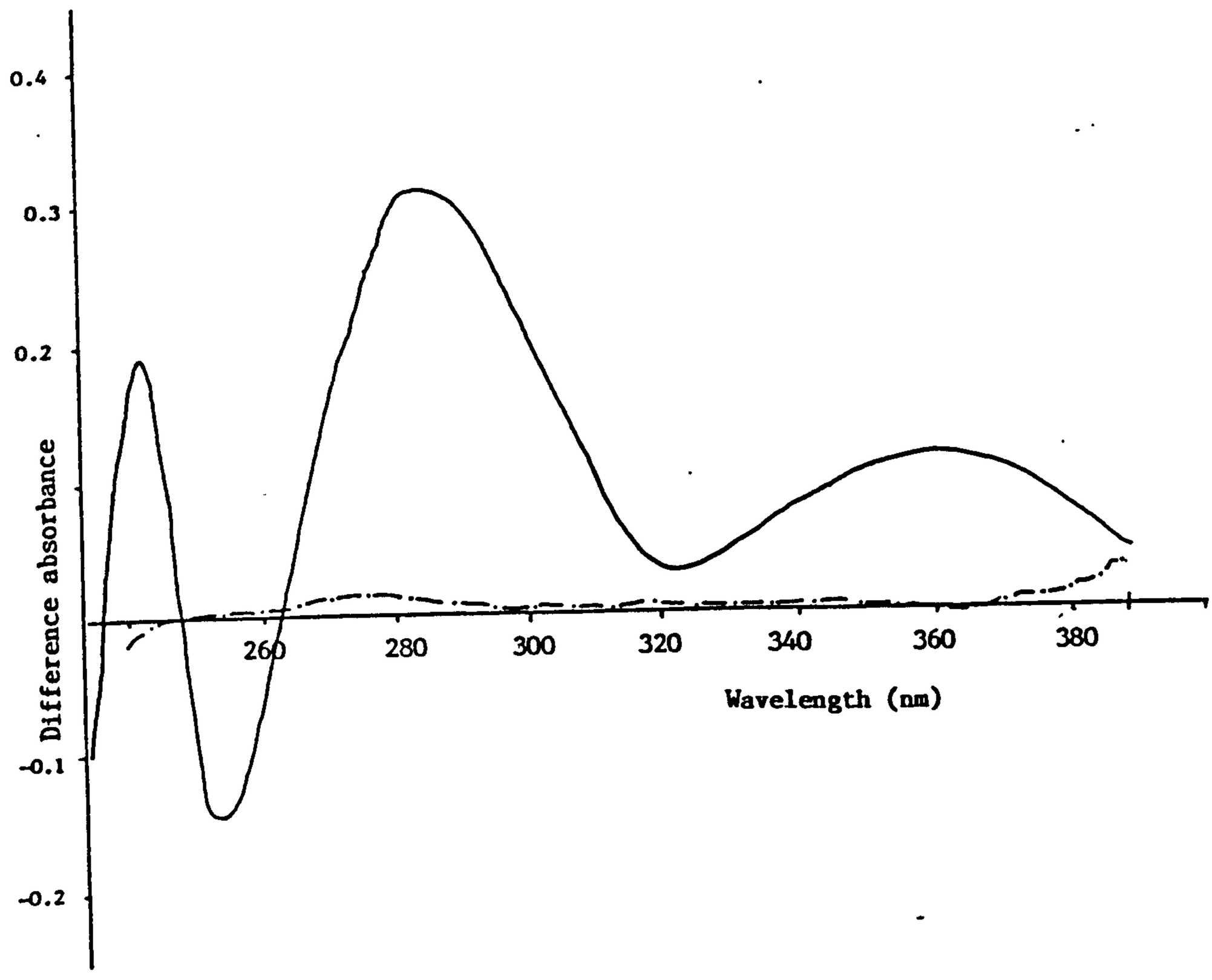


Fig. 6.5 Difference absorption spectra of desmethyl diazepam and carbostyril; pH 2.6 vs pH 5.4

——— Desmethyl diazepam

-·-·-·- Carbostyril

thought to be pH dependent. At low pH values, GMACB is present but as the pH increases, especially above the pK_a of diazepam, the reaction moves toward diazepam, with almost immediate and total recyclization of GMACB to diazepam at pH 7.0. The generation of the difference UV spectrum depends on the use of solutions of different pH values. It was therefore anticipated that the change in pH would result in alteration of the established equilibrium. A solution was prepared containing only the intermediate (GMACB). Dilutions of the acidic solution containing GMACB were prepared by addition of the acidic sample to flasks containing both the pH 2.6 and 5.4 buffer solutions and sodium hydroxide to neutralise the acid. The results for the absorbance of the pH 2.6 and pH 5.4 solutions are recorded in Table 6.1.

Table 6.1 Absorbance change in solutions of GMACB with time, measurements made at 290 nm

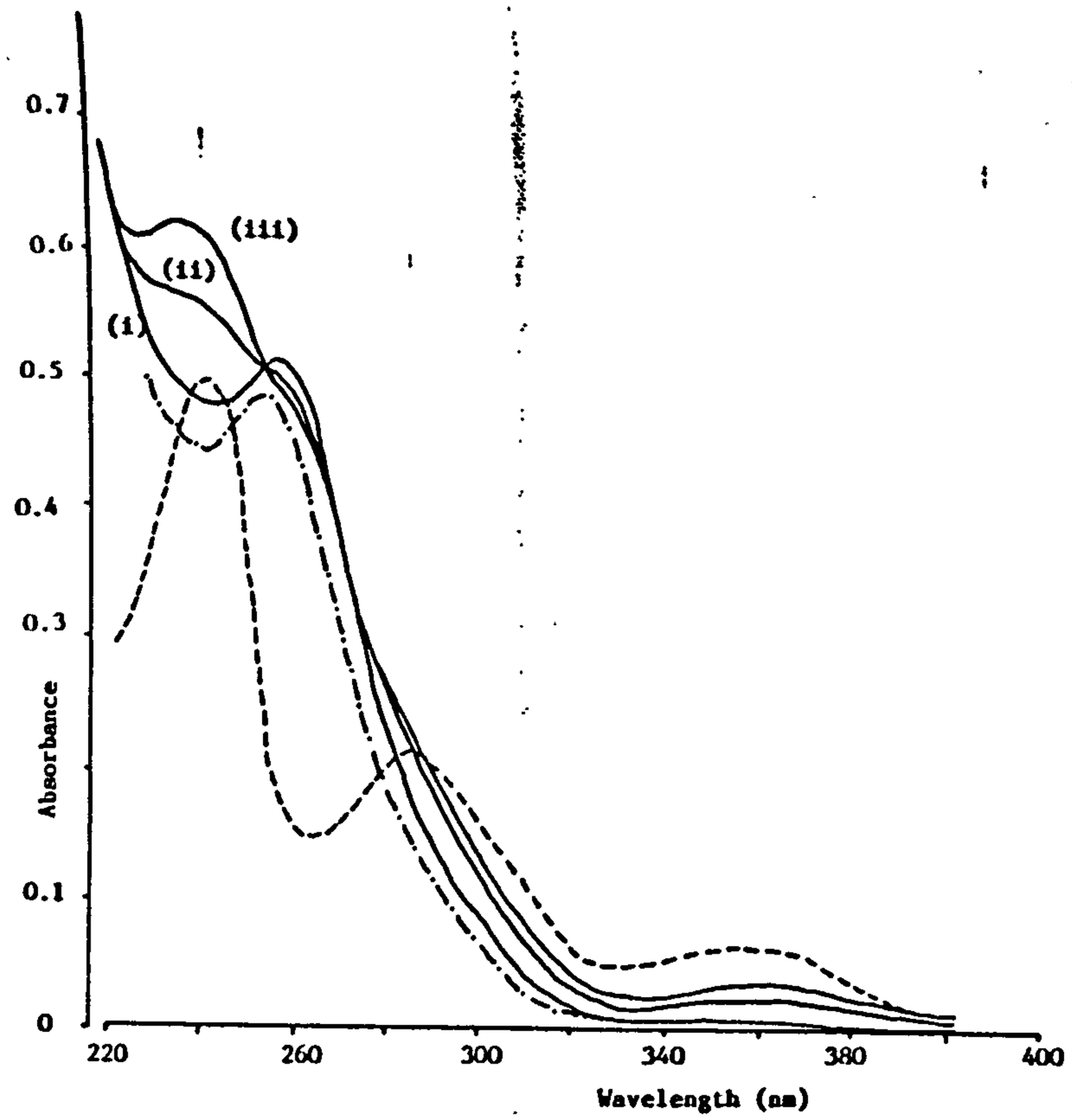
Time (minutes after preparation)	pH of solution	
	pH 2.6	pH 5.4
0	1.002	0.427
10	1.011	0.397
20	1.032 *	0.387
30	1.037	0.385

* measurement of the pH 2.6 solution was 25 minutes after preparation

From these results it can be seen that there is a change in the UV absorbance of each solution with time after preparation. The rate of change of the pH 5.4

solution is faster than that of the pH 2.6 solution. This is due to GMACB recyclizing to diazepam at a faster rate in pH 5.4 than in pH 2.6 buffer. The UV spectra of these solutions, (Fig 6.6), show that the spectra of GMACB is different to that of diazepam and that as the recyclization reaction proceeds, the mixture contains more diazepam. The absorbance of diazepam at pH 2.6 is approximately four times greater than that at pH 5.4. Thus although the recyclization reaction at pH 5.4 occurs more rapidly, the slower change occurring at pH 2.6 is more readily seen due to the greater absorptivity of the diazepam at this pH.

The pH conditions chosen for the generation of the difference spectra, provide a method specific for diazepam in the presence of carbostyril and MACB, the major degradation products of diazepam. Due to the pH-dependent nature of GMACB, the method is subject to interference from this compound and so would not be suitable as a stability-indicating assay for the study of the acid hydrolysed degradation of diazepam where GMACB would be present. In commercially available diazepam formulations, no GMACB would be present. The liquid dosage forms available are formulated at pH values greater than pH 3.3, and in fact more closely approach pH 7.0 (for both the syrup and injection). Thus, although the difference spectophotometric method developed is valid for the selective assay of diazepam in formulated products, it cannot be used for the assay of diazepam in kinetic studies of reaction mixtures as both the open and closed ring species may be present and interference from GMACB could occur.



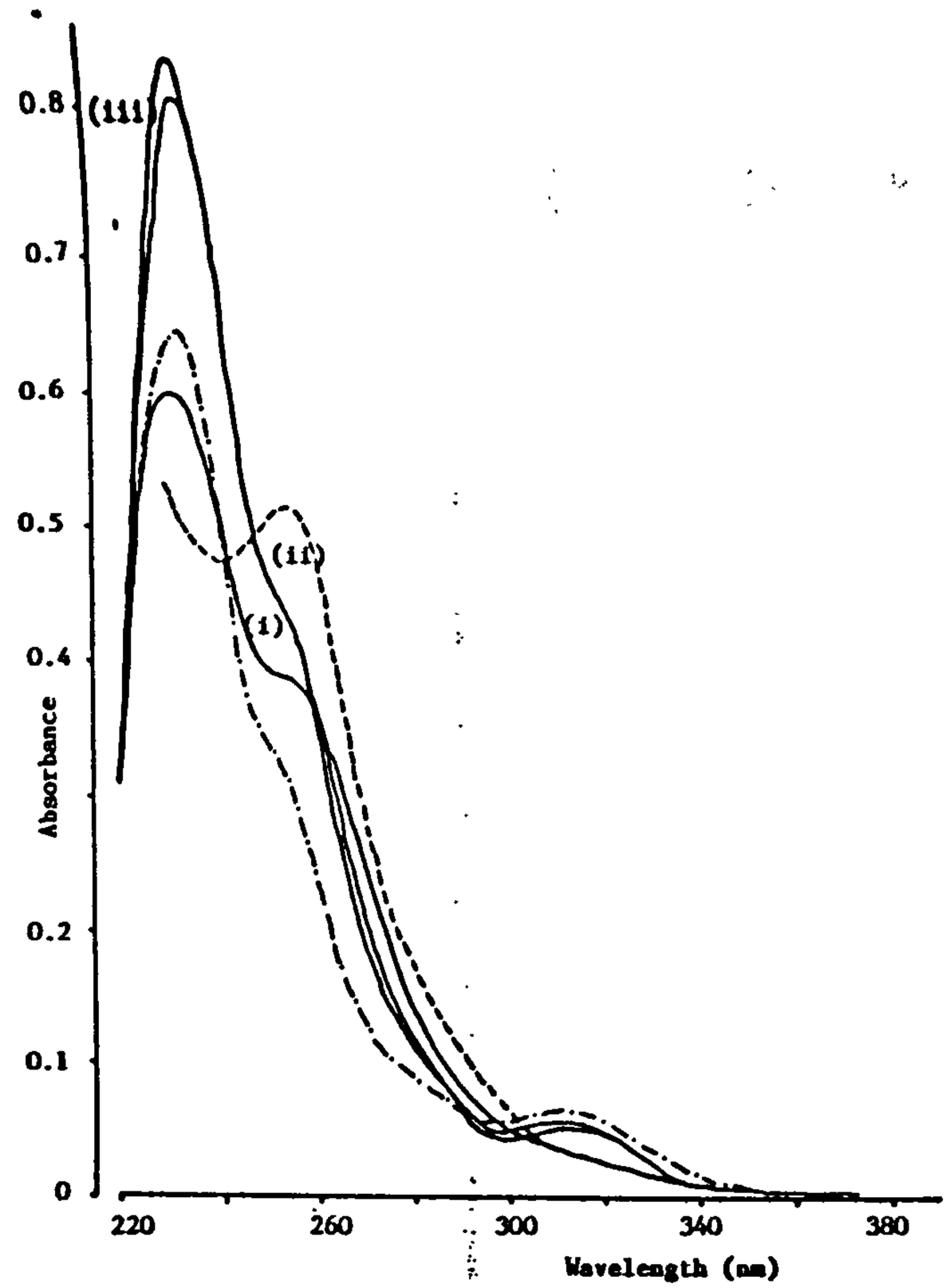


Fig. 6.6 Change in absorbance of GMACB with time

(a) in pH 2.6 buffer

-·-·-·- Absorption spectrum of GMACB in 0.1 M hydrochloric acid

- - - - - Absorption spectrum of diazepam in pH 2.6 buffer

——— Absorption spectra of GMACB in pH 2.6 buffer, at various time intervals
(i) zero time
(ii) 20 min
(iii) 30 min

(b) in pH 5.4 buffer

- - - - Absorption spectrum of GMACB in 0.1 M hydrochloric acid

-·-·-·- Absorption spectrum of diazepam in pH 5.4 buffer

——— Absorption spectra of GMACB in pH 5.4 buffer, at various time intervals
(i) zero time
(ii) 20 min
(iii) 30 min

6.1.2 Validation of the difference UV spectrophotometric method

6.1.2.1 Rectilinearity of response

The rectilinearity of the ΔA_{290} and diazepam concentration was assessed. A graphical plot was made of the difference absorbance measured against concentration, for a series of dilutions of a stock solution of diazepam, (Fig. 6.7).

A straight line was obtained at all three of the four instrument bandwidth settings tested, over the concentration range 8 to 40 μg/ml (ie 25 to 125% of the nominal assay concentration). An intercept value of approximately 0.4% of the analytical concentration was obtained for all three lines plotted.

The slope of the graphs for each slit width setting were identical. At 0.5 nm, the most narrow bandwidth setting available on the instrument, insufficient light was transmitted to give precise readings. At all the other bandwidth settings, very little difference was observed in the measured result although the widest setting of 4.0 nm gave a slightly reduced response, this is so small that it cannot be seen in Fig. 6.7, but is indicated by the slight differences in the $\Delta A(1\%,1cm)$ values obtained from the slope of the lines in Table 6.2 . This lowering of measured response was thought to be due to the reduction of spectral purity of the light, in relation to the relatively narrow band width of the difference absorption band. The linear regression statistics are shown in Table 6.2. On the basis of these results a

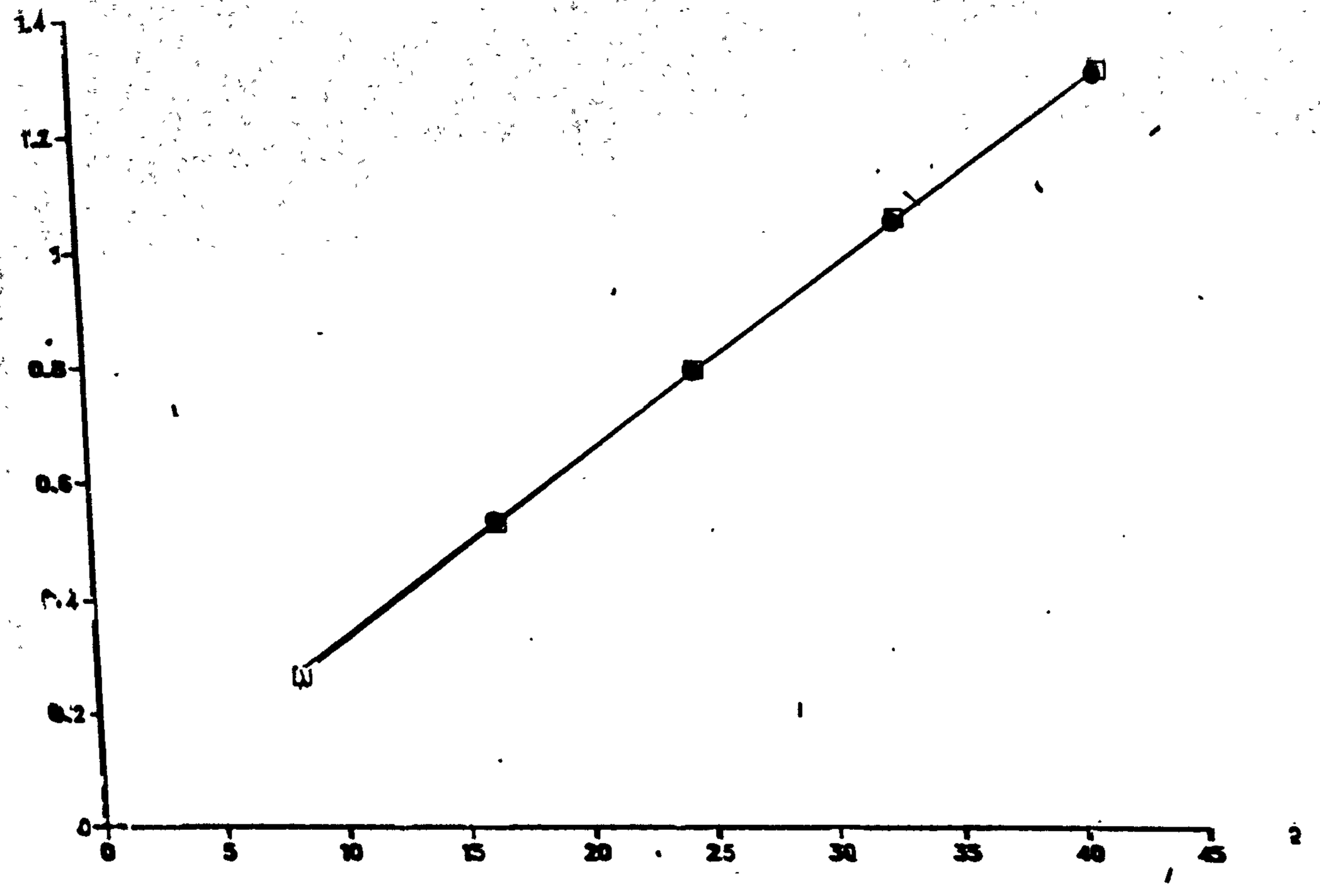

1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
40
45

Fig 6.7 Rectilinearity of ΔA_{290} at different spectral bandwidth (S.B.W.) settings to diazepam concentration

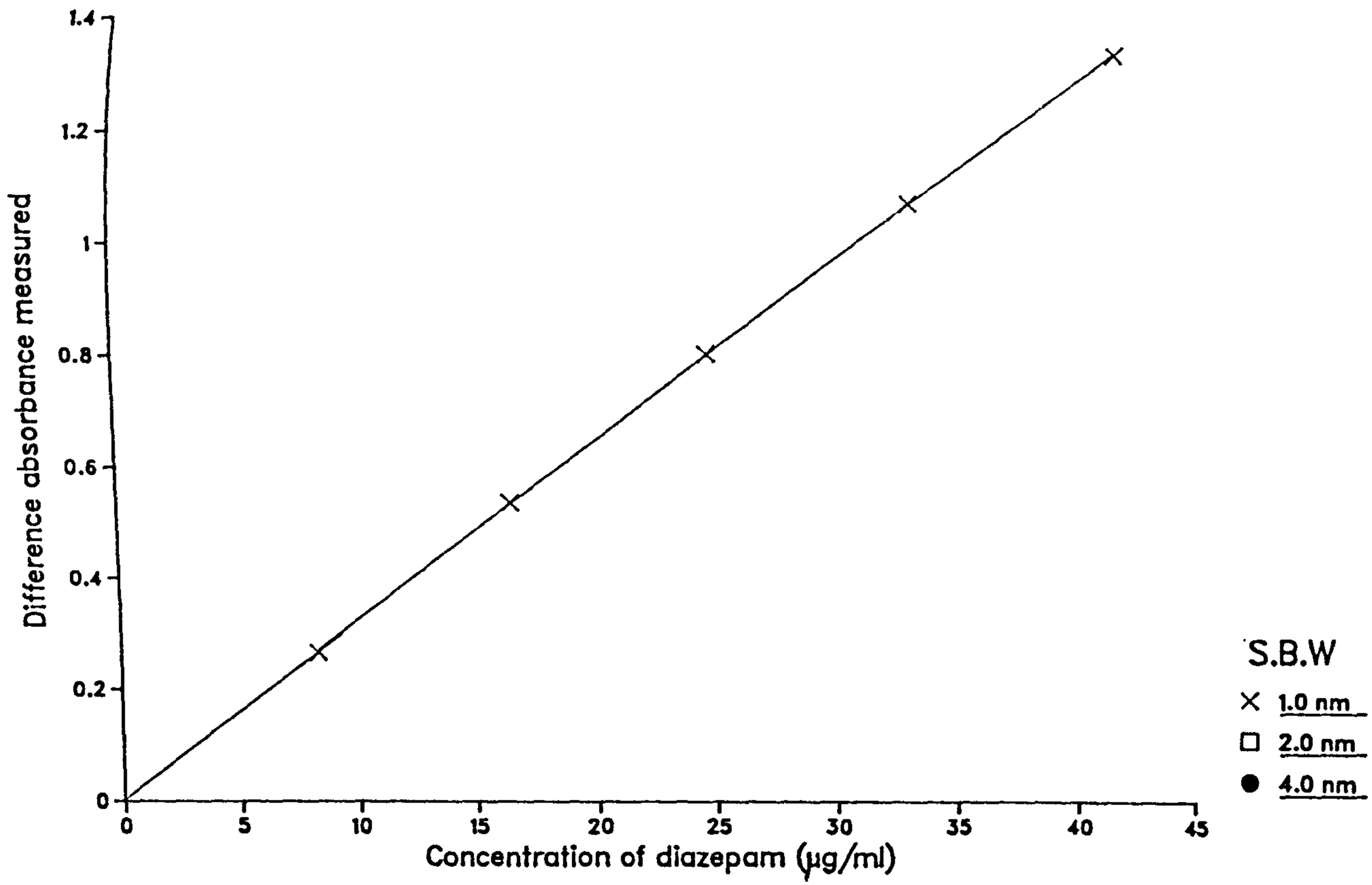
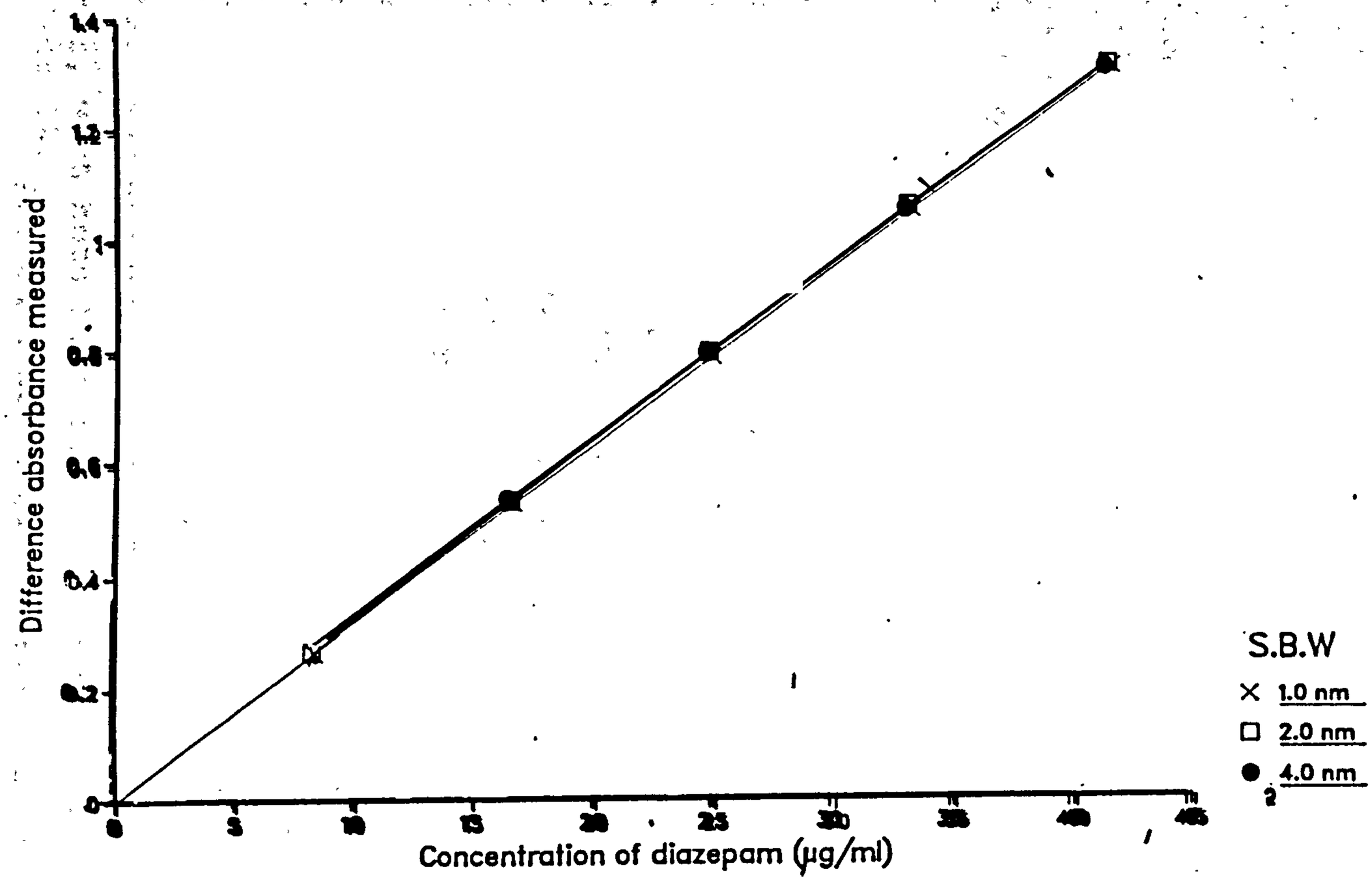


Fig 6.7 Rectilinearity of ΔA_{290} at different spectral bandwidth (S.B.W.) settings to diazepam concentration



setting of 2.0 nm was selected for further work. This was considered to be the optimum slit width setting, providing a compromise between sensitivity and precision.

Table 6.2 Effect of instrument spectral band width (SBW) setting

SBW setting (nm)	Linearity equation	Correlation coefficent
1.0	Y = 0.00232 + 319.6 X	0.999
2.0	Y = 0.00305 + 319.2 X	0.999
4.0	Y = 0.00307 + 317.8 X	0.999

where X = concentration of diazepam (% m/v)

and Y = measured absorbance

6.1.2.2 Assessment of the specificity of the method

In Section 6.1.1 the results indicate that under the conditions chosen for the difference absorbance measurement no interference is seen from carbostyril but there is some interference from MACB. To assess the specificity of the method for measurement of diazepam in the presence of MACB, a number of solutions were prepared containing both diazepam and MACB. These solutions were analysed for diazepam content, using dilutions of these solutions in pH 2.6 and pH 5.4 buffers to generate difference spectra. Tables 4.2 and 4.3 describe the preparation of these solutions, with both high and low amounts of MACB present. The measured content of diazepam in these

solutions are listed in Tables 6.3 and 6.4. In Table 6.3, the solutions contained 0 to 100% MACB while in Table 6.4 the solutions contained 0 to 14% MACB, more realistic concentrations of MACB which might be encountered in practice.

Table 6.3 Measured content of diazepam in prepared mixtures, using the difference absorbance method at 290 nm for measurement

Concentration of diazepam ($\times 10^{5}$ moll^{-1})	Concentration of MACB ($\times 10^{5}$ moll^{-1})	% MACB	Measured diazepam ($\times 10^{5}$ moll^{-1})	% recovery
2.310	0	0	2.321	100.1
1.855	0.423	18.6	1.868	100.7
1.391	0.846	37.8	1.437	103.3
0.928	1.269	57.8	0.961	103.6
0.464	1.693	78.5	0.486	104.7
0	2.116	100	0.033	

Table 6.4 Measured content of diazepam in prepared mixtures containing up to 14% MACB, using the difference absorbance method at 290 nm for measurement

Concentration of diazepam ($\times 10^{5}$ moll^{-1})	Concentration of MACB ($\times 10^{5}$ moll^{-1})	% MACB	Measured diazepam ($\times 10^{5}$ moll^{-1})	%recovery
7.278	0	0	7.269	99.9
6.550	0.559	7.9	6.539	99.8
5.822	0.140	2.4	5.737	98.5
5.095	0.420	7.6	5.080	99.7
4.367	0.700	13.8	4.400	100.8

From these results no interference is seen when MACB is present at up to 14% (expressed as a percentage of

the total absorbing species present). As the content of MACB increases, a small amount of interference is seen. The concentration of diazepam in these two series of solutions was different due to the poor aqueous solubility of MACB (at higher concentrations MACB precipitated in the buffer solutions). Thus at low levels of MACB the assay is specific for the measurement of diazepam.

The selectivity of the assay was investigated further. A series of solutions were prepared containing a constant concentration of diazepam but with increasing concentrations of MACB (up to 25% of the diazepam concentration, Table 4.4). These solutions were measured by difference spectrophotometry at 290 nm and the difference absorbance used to calculate the $\Delta A(1\%,1cm)$ value. The graph obtained on plotting the calculated $\Delta A(1\%,1cm)$ against the proportion of MACB present in the solutions is shown in Fig. 6.8. The calculated $\Delta A(1\%,1cm)$ was unaffected when the concentration of MACB was increased to 25%. The relative standard deviation was calculated for the results obtained for the $\Delta A(1\%,1cm)$ and was found to be 0.5%. The mean $\Delta A(1\%,1cm)$ value was 325 and there was no trend in the result when compared with the MACB content in each solution.

6.1.2.3 Precision of measurement

Ten replicate difference UV spectrophotometric assays were performed on each of two bulk mixtures of diazepam and MACB in methanol (1% and 10% MACB). The results of these measurements are shown in Table 6.5.

Fig 6.8 Effect of increasing concentration of MACB on the measured difference A 1%, 1cm

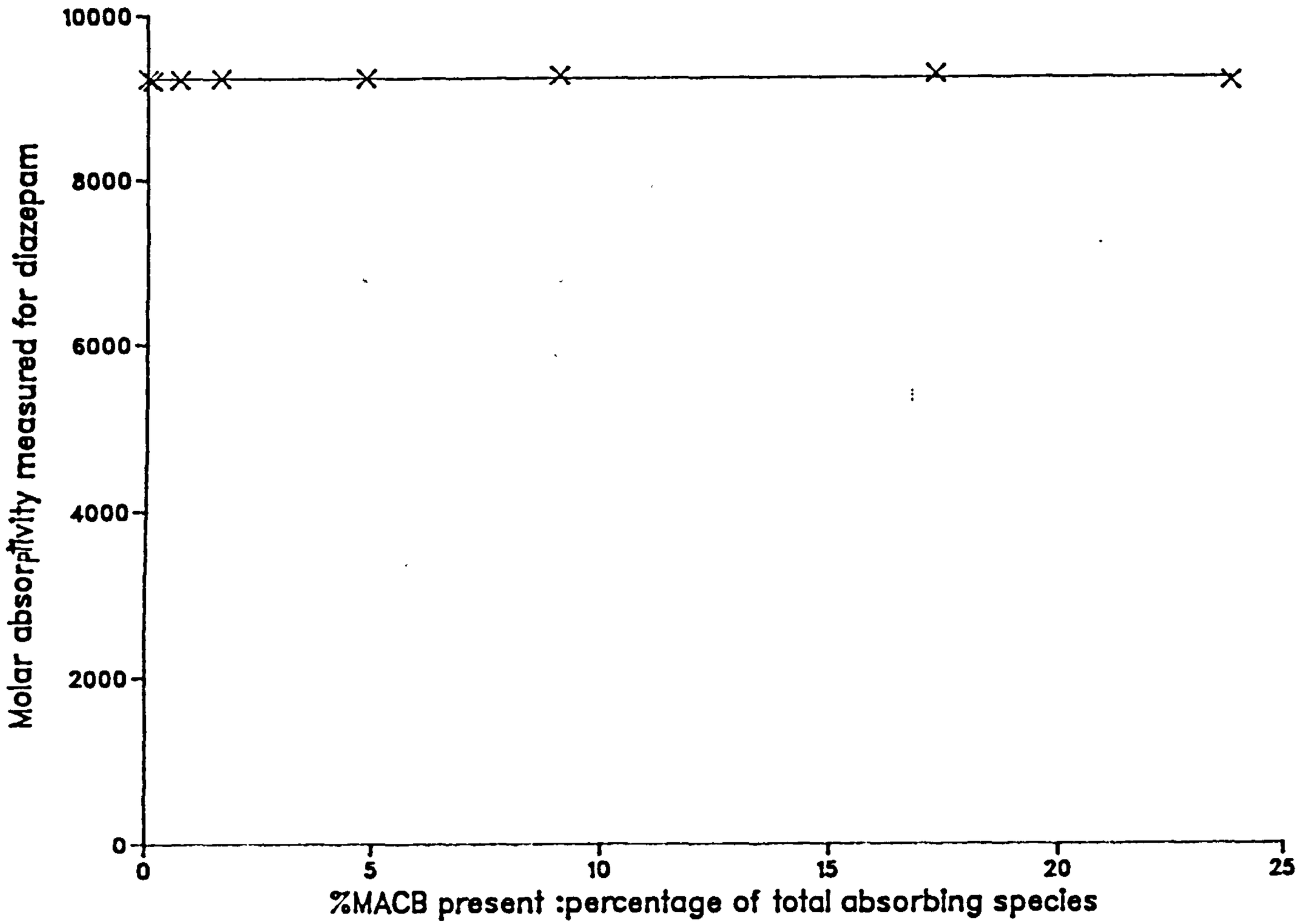


Table 6.5 Content of diazepam in bulk prepared mixtures measured using the difference absorbance method at 290 nm

MACB content 1%				MACB content 10.2%			
Measured content of diazepam (μg/ml)	Average (n = 10)	Actual content (μg/ml)	% recovery	Measured content of diazepam (μg/ml)	Average (n = 10)	Actual content (μg/ml)	% recovery
24.93				23.01			
25.01				22.96			
24.89				23.24			
24.90				23.23			
24.98				23.24			
24.92	24.95	25.35	98.4	23.20	23.14	23.11	100.1
24.97				23.15			
24.95				23.15			
24.93				23.11			
25.03				23.12			
	sd 0.046				sd 0.096		
	RSD 0.19%				RSD 0.41%		

The RSD obtained on replicate analysis of these solutions is satisfactory, especially as a number of stages are involved in the solution preparation, each of which will have errors involved. When the % recovery of the actual content of diazepam in these solutions is considered, there is close agreement with the known content.

6.1.2.4 Effect of methanol concentration on absorptivity of diazepam

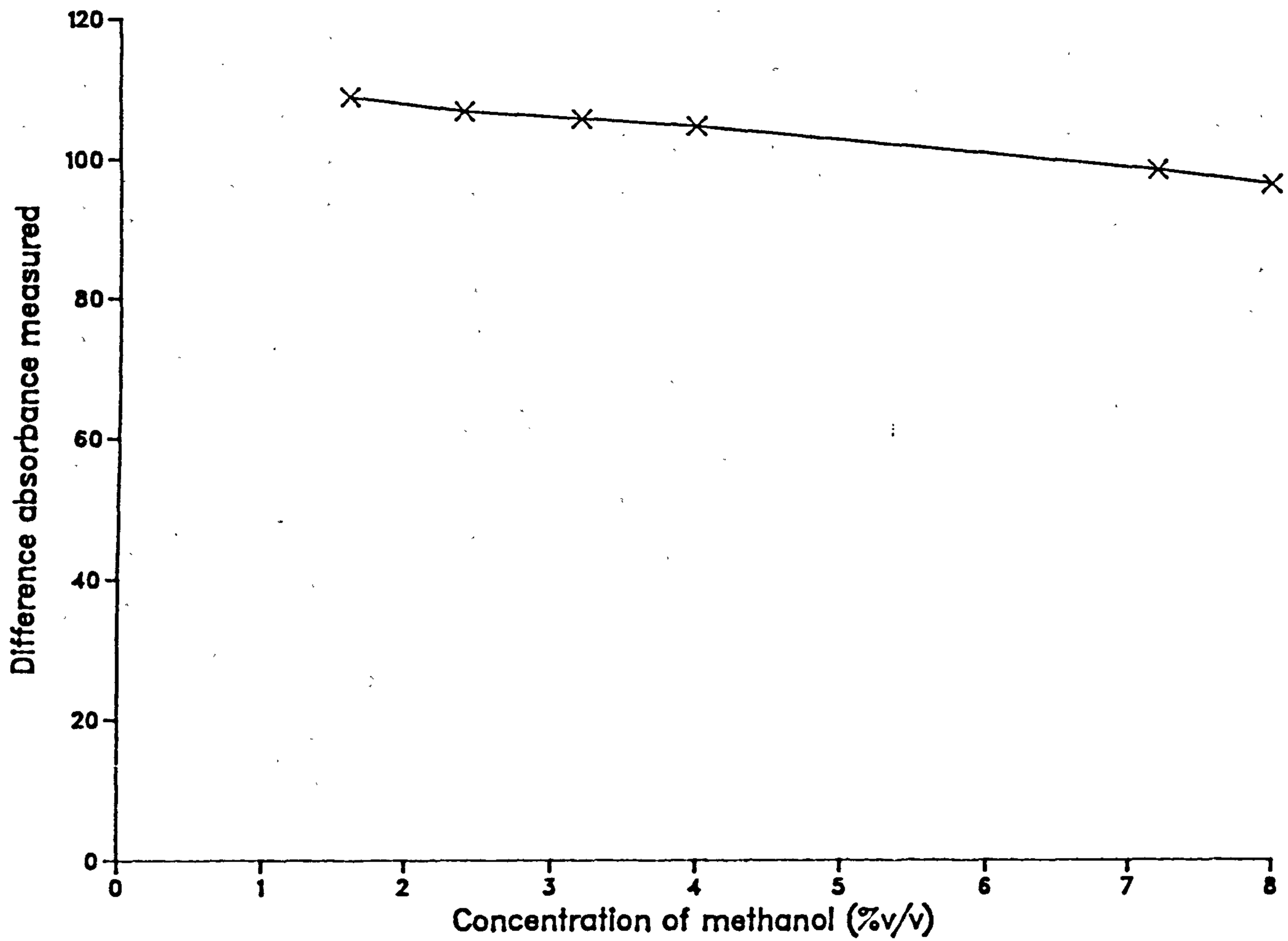
A series of solutions was prepared containing the same concentration of diazepam but with increasing concentrations of methanol. The difference absorbance at 360 nm, the secondary λ_{max} in the difference

spectrum of diazepam was measured for these solutions and plotted against the % methanol in the final solution. The graph showed that a relationship exists between these (Fig. 6.9) and that as the methanol concentration in the solution increases, the absorbance of diazepam decreases. The change is small, but is large enough to demonstrate the importance of treating the sample and standard solutions in the same way, as any difference between the methanol content in the sample and standard would result in an alteration in absorptivity and be a source of error.

The difference in the absorptivity as the methanol content increases is proposed to be due to the use of a mixed solvent system. Albert and Serjeant (1971) discuss this problem, and give an example of alteration of pK_a of substances when dissolved in ethanol. As the ethanol content increases the pK_a of the compound decreases. Difference spectrophotometry involves the alteration of pH to values above and below the pK_a to give different states of ionisation of the compound being investigated. Therefore, any alteration of the pK_a of that compound due to the presence of different amounts of ethanol (or methanol) would result in alteration of the degree of ionisation of the compound in the buffer solutions and so affect the measured absorbance.

In the assay methods developed, the problem can be avoided by maintaining constant concentrations of methanol in both the sample and standard solutions.

Fig 6.9 Effect of methanol concentration on difference absorptivity measured



6.1.3 Development of difference UV spectrophotometric methods for analysis of formulations

Section 6.1.1 describes the establishment of satisfactory pH conditions to generate a difference spectrum for diazepam, together with suitable spectrophotometric conditions to measure it. The sample preparation steps required to generate these spectra were then investigated for each type of formulation. The aim was to provide a stability-indicating assay for diazepam in the presence of its two major degradation products, MACB and carbostyril.

6.1.3.1 Tablets

The initial analysis of the tablet formulation involved extraction of diazepam from the powdered tablet debris into methanol. This methanolic solution (after filtration) was diluted into pH 2.6 and pH 5.4 buffer solutions and the difference absorbance at 290 nm measured. Assay of samples of Kerfoot diazepam tablets (2 mg), by this procedure, gave results which were higher than expected, approximately 105% of the nominal content. The aqueous buffer solutions prepared from dilution of the tablet extract were seen to be slightly turbid prior to UV measurement, although the methanolic solution had been clear. One advantage of the use of difference UV spectrophotometry is the removal of interference from irrelevant background interference (Section 1.2.4.3.1). This is only true however if the interference is at the same level in the sample solutions at the two pH values. In this

case, visual examination of the two solutions suggested that the level of precipitate in the pH 2.6 solution was higher than that in the pH 5.4 solution used in the reference cell. This would be expected to give a high absorbance and consequently a falsely high level of diazepam.

The turbidity was thought to be due to the presence of some material in the tablet which is soluble in methanol, but insoluble in water. The material would be freely extracted from the tablet debris, but would then precipitate out when the methanol content was reduced. The higher degree of precipitation in the pH 2.6 solution suggests that the material might have different solubilities at the two pH values used. Magnesium stearate is present in some tablet formulations as a lubricant, and it was thought that this may be responsible for the interference. Magnesium stearate is soluble in methanol [Handbook of Pharmaceutical Excipients,(1986)] so will be extracted from the tablet debris.

The level of magnesium stearate in tablet formulations is normally quite low. A solution of magnesium stearate was prepared in methanol and diluted in buffer solutions as in the tablet assay. On preparation of these dilutions, turbidity was seen in both the aqueous buffer solutions. Measurement of the difference absorbance of these solutions at 290 nm showed that there was a variation, with a higher degree of turbidity in the pH 2.6 solution. This was equivalent to a difference A(1%,1cm) of 11, which is approximately 3% of the difference A(1%,1cm) value measured for diazepam. It was concluded that

magnesium stearate in the formulation is responsible for the high recovery obtained in the assay of tablets.

If the extraction of diazepam from the tablet debris was performed in aqueous acid rather than methanol and if the theory described above is correct, then the compound interfering in the assay would precipitate in acid. Thus on filtration to remove the tablet debris, it would also be removed, and the methanolic solution could be diluted in pH 2.6 and pH 5.4 buffer solutions without precipitation occurring.

As diazepam is hydrolysed by acid, the stability of diazepam under these conditions had to be investigated. Also, the efficiency of its extraction into acidic solutions had to be investigated as it is poorly water soluble. The effect of acid on the pH of the buffer solutions being used also had to be examined to check that there was sufficient buffering strength. It was considered that, as the buffers used did not have a high buffer capacity they may vary in pH with the addition of acid. As discussed in Section 6.1.1, any variation in pH of the diazepam solution being used to generate the difference spectrum could result in errors being introduced as the method is sensitive to pH change.

The stability of diazepam in 0.01 M hydrochloric acid, in methanol, and in 0.05 M methanolic sulphuric acid was monitored by measuring the absorbances of prepared solutions. The results from this work are shown in Table 6.6.

Table 6.6 Stability of diazepam

Time after preparation (h)	A(1%,1cm) in 0.05 M methanolic sulphuric acid	Time after preparation (h)	ΔA(1%,1cm) in methanol	Time after preparation (h)	ΔA(1%,1cm) in 0.01 M hydrochloric acid
0	452	0	322	0	328
0.75	446	0.42	320	0.42	320
1.50	452	1.42	317	1.42	305
2.08	443	1.92	315	1.92	304
4.08	446	4.83	315	4.92	278
5.08	442	5.83	315	5.83	280
23.92	440	24.83	313	24.92	248

Very little change was seen in the absorbance of diazepam in 0.05 M methanolic sulphuric acid, measured at 284 nm, and the difference absorbance of diazepam in methanol, measured at 290 nm (after dilution of the methanolic solution in pH 2.6 and pH 5.4 buffer solutions). A decrease in difference absorbance was found for diazepam in 0.01 M hydrochloric acid indicating that diazepam is degraded by 0.01 M hydrochloric acid at room temperature.

In order to extract the drug from powdered tablets, the powder must be shaken with the chosen solvent for a period of time. In the case of diazepam, it is left to stand for 15 minutes then shaken for 15 minutes [B.P.,(1980)]. If 0.01 M hydrochloric acid is used as the extracting solvent, degradation of diazepam would occur over this period of time resulting in inaccuracy of measurement.

A modification of the method was made to use methanol for extraction, then the extract was diluted with 0.01 M hydrochloric acid immediately before filtration, followed by dilution in buffer solutions for the measurement of the difference absorbance. On filtration using this modification, the interfering excipient was removed together with the other solid debris and on further dilution with pH 2.6 and pH 5.4 buffer solutions the solutions remained clear. When the methanol was mixed with acid, heat is produced which results in expansion of the liquid and so may lead to errors when the contents of the flasks are made to volume. This is important as the solutions cannot be left to cool slowly due to potential degradation of diazepam in acid solution. Therefore the flasks were cooled by placing them in cold water to improve the dissipation of heat generated.

To assess the reproducibility of the assay method, replicate assays were performed on separate weighings of tablet powder from a batch of Kerfoot diazepam tablets (2 mg), Table 6.7. The measured diazepam content of the tablets was calculated and compared with the result obtained by direct spectrophotometric measurement, (Section 6.2.2.1).

Table 6.7 Replicate difference spectrophotometric assays performed on crushed, bulked Kerfoot 2 mg diazepam tablets

	Measured content of diazepam (mg/tab)	
	2.010	
	1.984	
	2.002	
	2.006	
	2.006	
	2.000	
	2.012	
	2.010	
	2.009	
Average	2.004	(100.2% nominal content)
RSD	0.43%	

The assay method gave precise values, although the assay result was lower than that for the same batch of tablets using the direct spectrophotometric assay method, (Section 6.2.2.1).

The relative standard deviation (RSD) for the difference method was lower than that for the direct UV method. This would not have been expected due to the greater number of preparation stages in the difference method where errors could be introduced. However, in the direct method, the measured concentration was influenced by the length of time required for the filtration of samples (Section 6.2.2.1).

Isosbestic points are wavelengths in the spectra of a compound where the absorbance of its ionised and non-ionised forms are the same. In the difference spectrum of the compound the isosbestic points are wavelengths where the difference absorbance is zero. If there is only one absorbing species present in the sample, then the isosbestic points will occur at the same wavelengths as those of the standard. However, if there are absorbing species present which have different absorbances under the same conditions, the presence of these interferents will shift the isosbestic points. Thus co-incident isosbestic points in the difference spectra of the standard and sample solutions are reasonable evidence of the lack of interference in the sample solution.

The difference spectra of the standard solution of diazepam and sample solutions extracted from diazepam tablets were superimposed (Fig. 6.10). The isosbestic points in these two spectra were identical, confirming that there is no interference from the tablet excipients, using the modified extraction procedure.

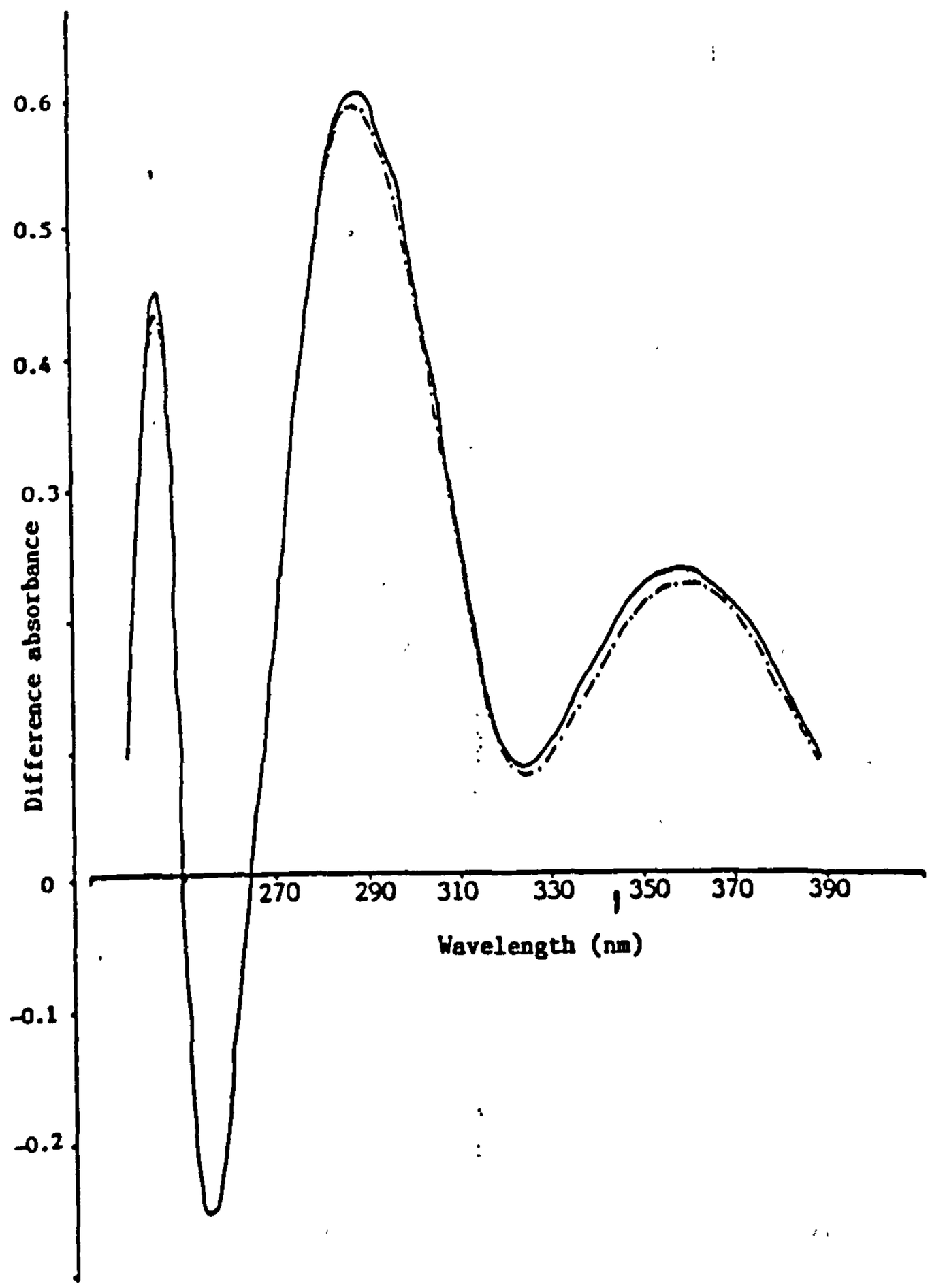


Fig. 6.10 Difference absorption spectra, pH 2.6 vs pH 5.4, for diazepam and an extract from Valium tablets

——— Difference absorption spectrum of diazepam

—·—·— Difference absorption spectrum of a tablet extract

6.1.3.2 Capsules

The method used was modified from that used for the tablet assay. A suitable weight of the powdered contents of the capsules was treated in the same way as the crushed tablet samples. The problem in the tablet assay due to turbidity of the samples was anticipated with the capsules as magnesium stearate is a likely excipient in the capsule formulation.

The difference spectra generated for standard solutions and sample solutions extracted from diazepam capsules were superimposed. The isosbestic points were identical in the two spectra confirming that no interference occurred from the capsule excipients.

6.1.3.3 Suppositories

No problems were encountered in development of the assay method used for the suppository formulation. The sample preparation was modified from that of the drug substance, the sample solution was obtained by dissolving the suppositories in methanol.

Difference spectra were generated for standard and sample solutions. No interference was seen from the suppository excipients with the isosbestic points in both spectra at identical wavelengths.

6.1.3.4 Injection

Solutions of each of the excipients present in the injection were prepared and their difference absorbance in pH 2.6 and pH 5.4 buffer solutions measured at 290 nm. It was found that sodium benzoate exhibited a small difference absorbance at 290 nm and so would interfere slightly in the assay (Fig. 6.11).

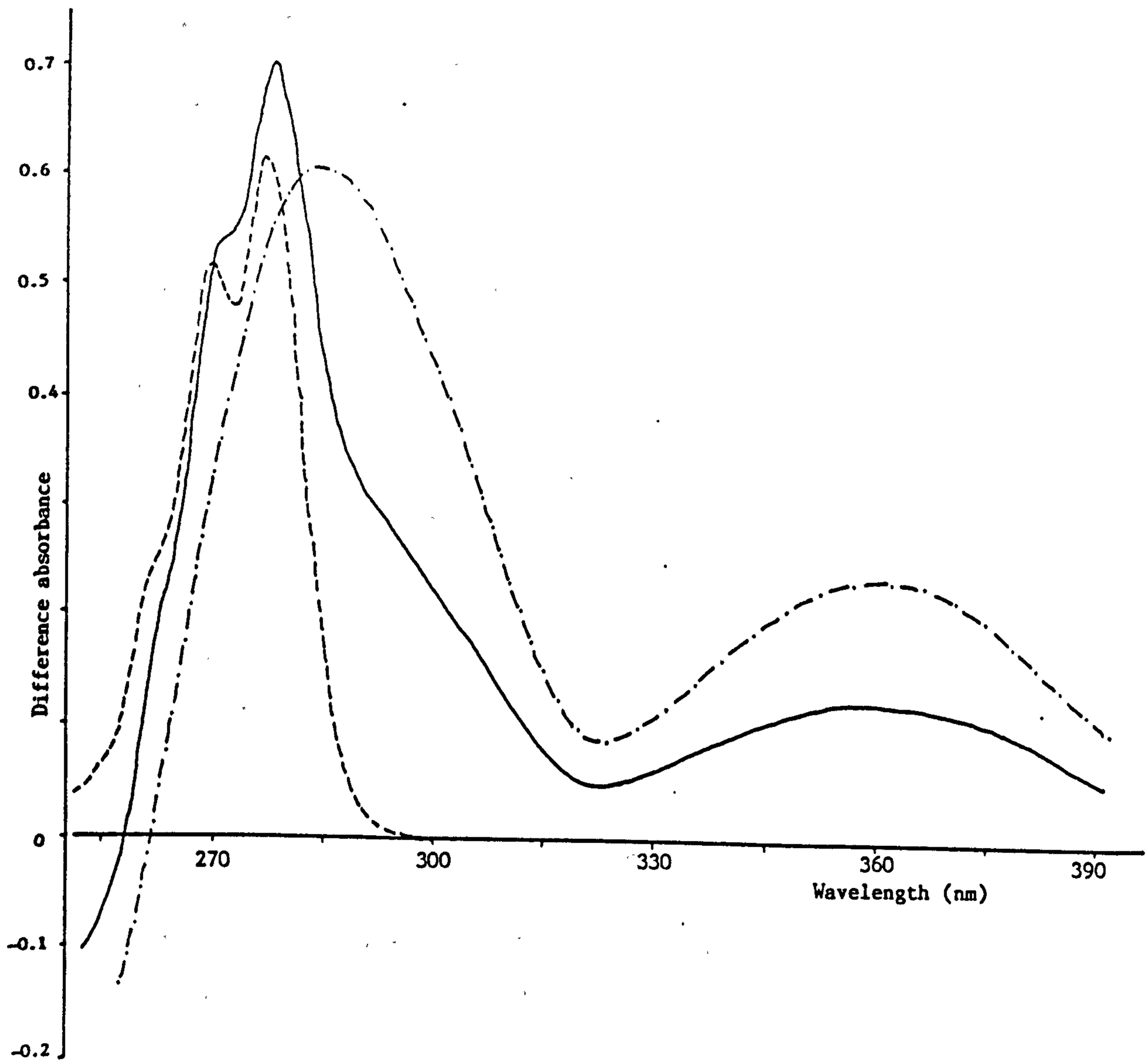


Fig. 6.11 Difference absorption spectra of sodium benzoate, diazepam and a dilution of Valium injection; pH 2.6 vs pH 5.4

—·—·— Diazepam

- - - - Sodium benzoate

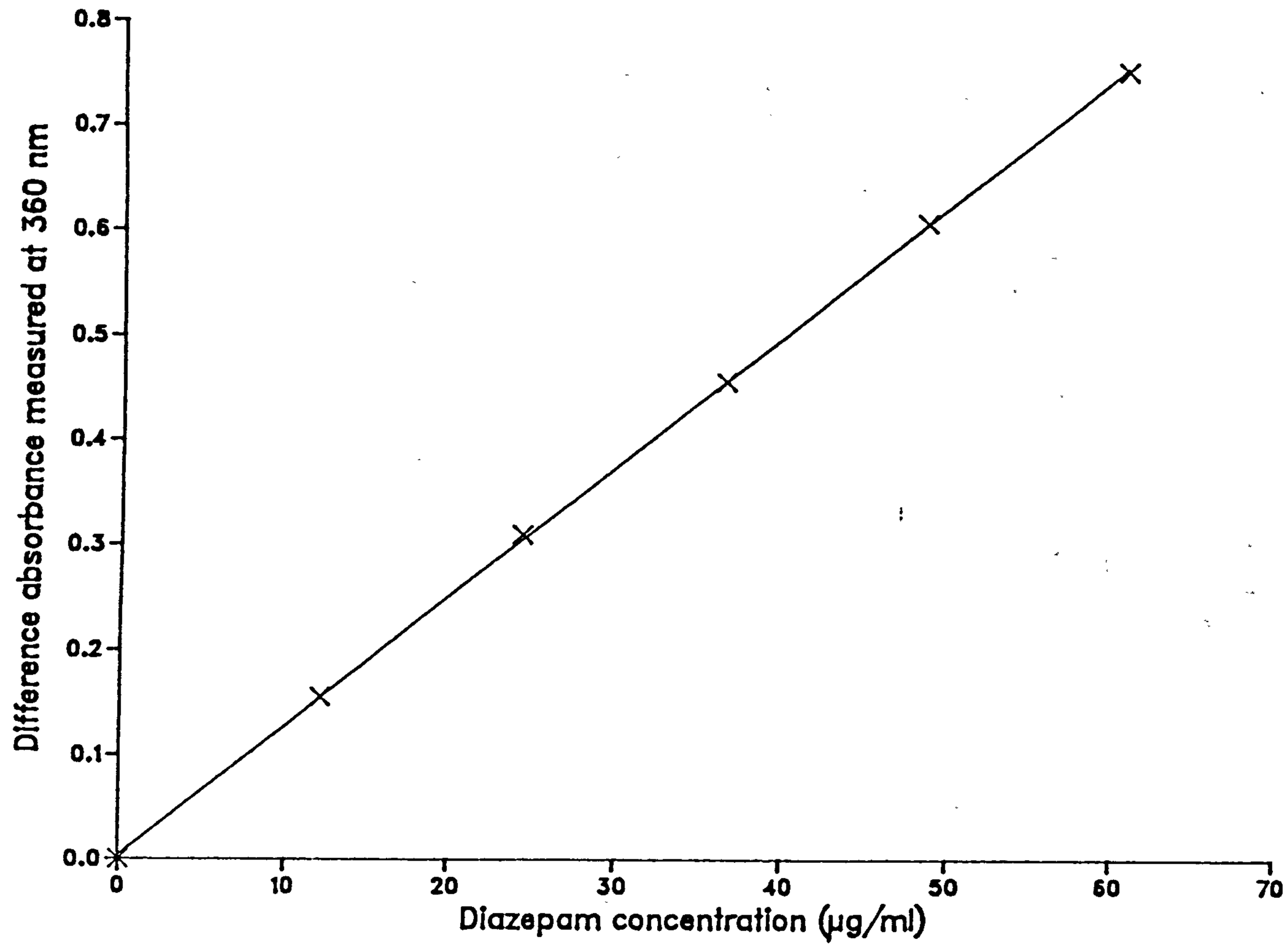
—— Valium injection

The pK_a of benzoic acid is 4.19 [Handbook of Pharmaceutical Excipients,(1986)], therefore it would be expected that some difference in its absorbance would occur in solutions of the pH values used. However, at 360 nm, the other λ_{max} in the difference spectrum of diazepam, sodium benzoate exhibited no absorbance or difference absorbance. Thus despite the reduction in sensitivity due to the lower $\Delta A(1\%,1cm)$ at 360 nm (96 compared with 320 at 290 nm), 360 nm was considered to be the most suitable wavelength to use. By using a series of solutions containing different concentrations of diazepam, the rectilinearity of the difference absorbance at 360 nm was measured (Fig. 6.12). The difference absorbance was also measured for solutions containing mixtures of diazepam and MACB. This confirmed that no interference was present from MACB at 360 nm (Table 6.8). As the concentration of MACB increased, the accuracy of the measured ΔA_{360} decreased. However, as the difference absorbances of the solutions containing 60% and 80% MACB were 0.029 and 0.012, the errors at the high concentrations of MACB were not surprising.

Table 6.8 Content of diazepam measured in prepared mixtures using the difference absorbance method at 360 nm

Concentration of diazepam ($\times 10^5$ moll^{-1})	Concentration of MACB ($\times 10^5$ moll^{-1})	% MACB	Measured diazepam ($\times 10^5$ moll^{-1})	%recovery
2.346	0	0	2.328	99.2
1.877	0.484	20.0	1.872	99.7
1.407	0.960	40.6	1.430	101.6
0.938	1.454	60.8	1.032	110.0
0.469	1.939	80.5	0.416	88.7
0	2.424	100.0	0	

Fig 6.12 Rectilinearity of response to diazepam at 360 nm



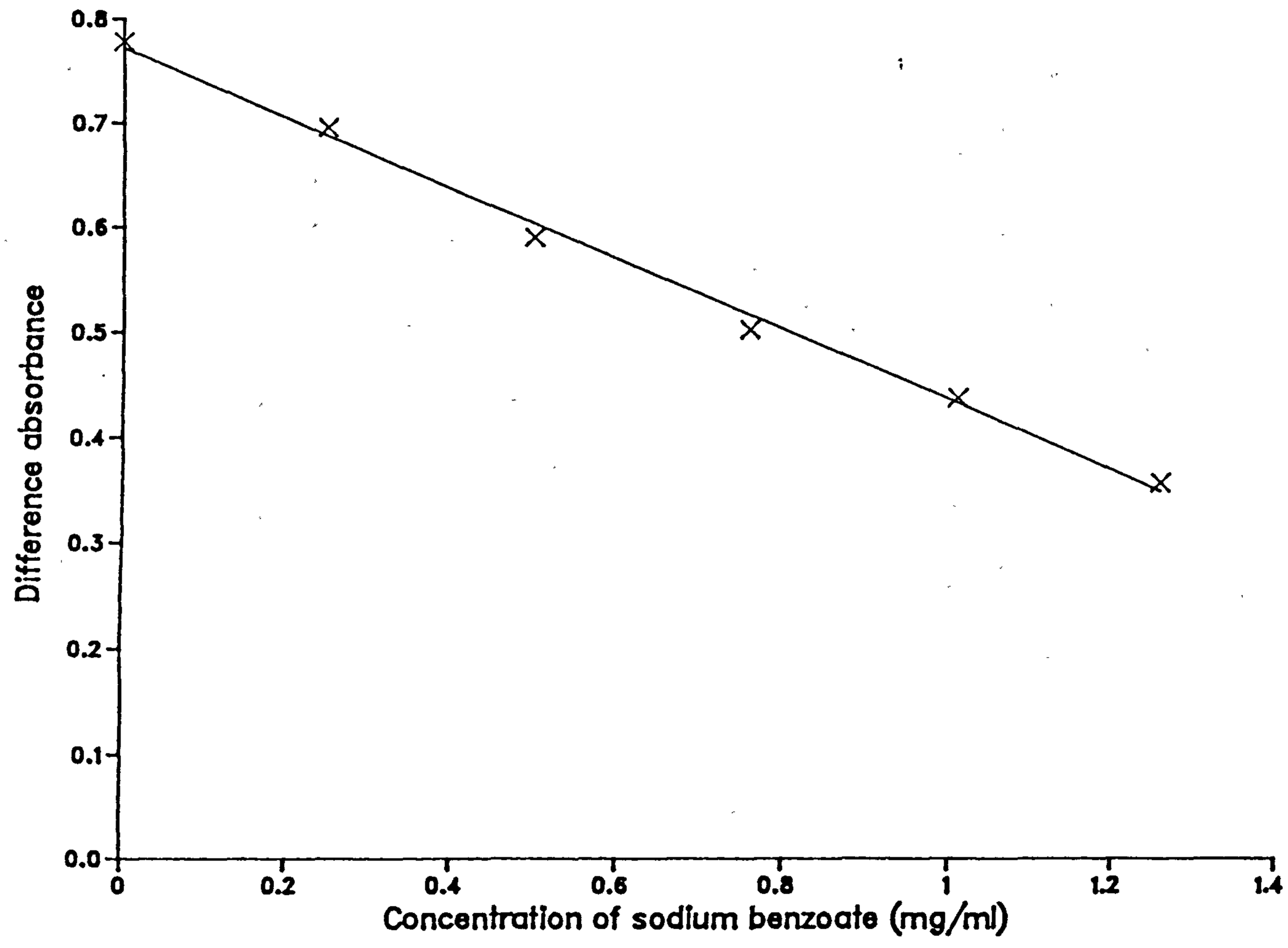
Attempts were made to measure the diazepam content of the injection using simple dilution of the injection sample in the two buffer solutions. Replicate determinations on a solution obtained by bulking the contents from several ampoules gave a measured content of 79.3% of the nominal content. A known weight of diazepam was added to a solution containing the excipients present in the injection, at the levels present in the formulation. The formulation of the injection is described in the paper by Smith and Nuessle (1982a), and is reproduced in Section 3. This was analysed in the same way as the proprietary injection and the measured content of diazepam was found to be 76.5% of the known concentration. Thus a similar reduction of the content of diazepam occurred in the prepared sample as that measured in the proprietary sample. This suggested that some problem with interference was present, rather than the injection sample merely containing low concentrations of diazepam. Each of the excipients were tested for interference in the determination of diazepam. Known weights of diazepam were added to solutions containing each individual excipient. The % recovery of diazepam from each of these solutions was calculated (Table 6.9). From these results, the interference was shown to be due to the presence of sodium benzoate.

Table 6.9 Recovery of diazepam in the presence of injection excipients

Excipient	Concentration of diazepam (μg/ml) Added	Found		% recovery
Benzyl alcohol (290 μg/ml)	71.0	72.2		101.7
	86.2	87.4		101.4
			Mean	101.5
Propylene glycol (6.6 mg/ml)	70.4	71.3		101.3
	69.3	69.8		100.2
			Mean	101.0
Ethanol (1.4 mg/ml)	87.0	88.5		101.7
	93.5	95.2		101.8
			Mean	101.8
Sodium benzoate (760 μg/ml)	76.9	59.4		77.2
	82.2	62.3		75.8
			Mean	76.5

To investigate this further, the difference absorbance at 360 nm was measured for a series of solutions containing the same concentration of diazepam, but with increasing sodium benzoate concentrations. On plotting the difference absorbance against concentration of sodium benzoate, a straight-line graph was obtained (Fig. 6.13) with a decrease in absorbance as the sodium benzoate concentration increases. At 360 nm, sodium benzoate exhibits no difference absorbance, therefore no interference was anticipated at this wavelength. As there is a linear relationship between the content of sodium benzoate and the difference absorbance for the same

Fig 6.13 The effect of sodium benzoate on the difference absorbance of diazepam at 360nm



concentration of diazepam, it appears that some interaction occurs between the two compounds.

The ability of some salts including sodium salicylate, sodium benzoate and sodium p-aminosalicylate to increase the solubility of some poorly water soluble drugs has been investigated by a number of workers. These salts are called hydrotropes, and their action has been defined as 'the increase of solubility of a solute by a third substance which is not highly surface active, or at least which does not form micelles at low concentrations' [Florence and Attwood,(1983)]. The method by which these salts act has been proposed as an electrostatic force of the "donor-acceptor" type [Higuchi and Drubulis,(1961)]. It has also been proposed as being due to a combination of a number of factors including complex formation between the solubilized and solubilizing molecules and also a salting-in effect of the solubilizing molecules due to decreasing the activity coefficient of the water insoluble material [Ueda,(1966 a,b and c)]. Other workers have proposed that the mechanism involves change in water structure [Saleh et al.,(1980)], or molecular aggregation of the solubilizing molecule with inclusion of the poorly soluble molecule into these aggregates where a donor-acceptor interaction then stabilises the inclusion [Badwan et al.,(1983)]. Drugs which have been solubilised in this way include caffeine and theophylline, as well as benzodiazepines including diazepam [Saleh et al.,(1980), Badwan et al.,(1980),(1983)]. The physico-chemical nature of these complexes formed have been studied by PMR, for the theobromine:sodium benzoate interaction [Nishijo

and Yonetani,(1982)]. Results from this work suggest that the complex formed in this case is by vertical stacking or plane-to-plane stacking. A similar form of interaction has been proposed for the complex formed between various benzodiazepines (including diazepam) and sodium salicylate. In this case the authors suggest that as both the benzodiazepine derivatives and sodium salicylate are planar molecules, the sodium salicylate will form a stack of self-associated monomers. The planar benzodiazepines will therefore be included in the stack and so be solubilized [Badwan et al.,(1983)]. In the papers by Ueda (1966), the visible spectra of the drugs tested were measured at a variety of concentrations of sodium p-aminosalicylate, at constant pH. These spectra show significant differences and this is attributed to the special molecular interactions between the solubilized and solubilizing components, (sodium p-aminosalicylate has no absorption in the region over 370 nm, in which region change was seen). A similar change in absorption was seen when other compounds similar to sodium benzoate were used. These absorbance changes were dependent on the salt concentration and type of salt used, with the largest changes occurring with sodium sulphate and sodium benzoate [Ueda,(1966c)].

From Fig. 6.13, it can be seen that there is a relationship between the concentration of diazepam and sodium benzoate. This could be due to sodium benzoate complexing with diazepam thus increasing its solubility, but also altering its absorptivity at the wavelength used for measurement. The change in absorbance is quite marked. At the expected molar

ratio of diazepam and sodium benzoate in the injection solution used for analysis, the decrease in absorbance is of the same order in both the proprietary injection, and the synthetic injection.

To eliminate this interference from sodium benzoate, diazepam must be extracted from the injection prior to the assay. The method used for extraction of diazepam was that described in the B.P. (1980) for diazepam injection. This involved diluting a known volume of the injection with pH 7.0 buffer solution and extracting this solution with chloroform. Under these conditions, diazepam extracts into the chloroform, leaving the sodium benzoate in the aqueous layer. The chloroform extract was dried, the residue redissolved in methanol and diluted with pH 2.6 and pH 5.4 buffer solutions.

Initial attempts were made to measure the concentration of diazepam using this method, with measurement of the difference absorbance of the solutions at both 290 nm and 360 nm. The results at the two wavelengths were different suggesting that there was still some interference present. The dilutions of the methanol solutions in buffer appeared slightly opalescent. The difference absorbance ratios (ΔA at 290 nm/ΔA at 360 nm) were not the same as that of standard diazepam in methanol diluted in buffer. Solutions of diazepam were therefore prepared in reagent-grade chloroform and HPLC-grade chloroform and these were treated in the same way as the chloroform extracts from the injection. No opalescence was seen in the dilutions prepared when HPLC-grade chloroform was used. From the difference

absorbance ratios of these solutions (Table 6.10) it appears that some contaminant is present in the reagent-grade chloroform that increases the ΔA at 290 nm. This contaminant is soluble in chloroform and methanol but not in aqueous buffer and precipitates in the buffer solutions. As in the case of the tablet interferent, thought to be magnesium stearate (Section 6.1.3.1), the use of difference absorbance measurements would have eliminated this interference, if the turbidity had occurred to the same degree in both the pH 2.6 and pH 5.4 solutions. The interference in the difference absorbance ratio indicated that the level of turbidity was not the same in the two solutions. Consequently work performed after this finding used HPLC-grade chloroform.

Table 6.10 Difference absorbance ratios measured for diazepam in chloroform and methanol

Solution	Difference absorbance ratio (290 nm/360 nm)
Diazepam in methanol	2.57
Diazepam in reagent grade chloroform	2.71
Diazepam in HPLC grade chloroform	2.58

A known weight of diazepam was dissolved in a solution containing the injection excipients and this solution was assayed to measure the recovery of diazepam. Two samples of this solution were analysed and the difference absorbance of the buffer solutions were measured at 290 nm and 360 nm.

The concentration calculated from sample 1 was 11.32 and

11.18 μg/ml at 290 and 360 nm respectively, and that for sample 2 was 10.13 and 10.04 μg/ml at 290 and 360 nm. Both samples showed good agreement between the duplicate measurements of the same solution at 290 and 360 nm, but poor agreement between the duplicate extractions (91.5% recovery from sample 1 and 82.0% recovery from sample 2). In both cases the recovery was low suggesting that there was a problem with the assay method.

A further known weight of diazepam was dissolved in the injection excipient solution and two portions of this were extracted with chloroform as before. After the chloroform extracts had been bulked and made to volume, duplicate samples from each were analysed. Two sets of results were generated and the measured concentration of diazepam from each solution is shown in Table 6.11. In this table solution 1 and 2 refer to the two portions of the initial sample and two samples from each of these solutions were treated further to give solutions in buffer for difference absorbance measurement. The absorbance measurements were made at 290 nm due to the greater sensitivity at this wavelength.

Table 6.11 Recovery of diazepam from a synthetic injection preparation

Solution	%recovery
1 a	74.8
1 b	74.2
2 a	85.3
2 b	85.6

As good agreement was obtained with the replicates obtained by treatment of the chloroform solution after extraction, the low recovery must be attributed to the extraction stage. Replicate pipetting from this chloroform solution and evaporation and redissolution of the residue gave good reproducibility of recovery.

It was felt that the use of sintered glass funnels may have been responsible for the problems. Different surface areas or pore size could result in the retention of different quantities of diazepam. The use of filter funnels with filter paper was thought to provide an easier way of reproducing the same filtration conditions. Four portions were taken from a further known weight of diazepam added to a solution of injection excipients and were extracted with chloroform. The chloroform extracts were dried by filtering them through anhydrous sodium sulphate supported on filter paper (Whatmans No 1) rather than on sintered glass funnels. The difference absorbance of solutions prepared in this way showed better agreement but low % recoveries (85.7, 84.0, 88.3 and 86.8% for the four replicates).

The difference absorbance spectra of the aqueous phase remaining after extraction was checked and found to contain sodium benzoate, but no diazepam. All the diazepam was removed from the aqueous phase by extraction, thus poor extraction was not responsible for the low recoveries found.

The low recoveries could be due to the breakdown of diazepam in chloroform. Although this was thought to

be very unlikely, it was investigated by preparing a solution of diazepam in chloroform and measuring its UV absorbance. The solution was then divided into two portions in volumetric flasks, with the volumes in each known. The flasks were left for 60 hours, one in darkness and the other in natural light and the volumes in each were checked and the UV absorbance of each measured. No significant level of evaporation had occurred over 60 hours, and there was very little difference in the absorbance measured. The initial absorbance was 0.929 and that after storage both in darkness and in natural light was 0.933. It was concluded that degradation of diazepam in chloroform solution was not responsible for the low recoveries measured.

A solution of diazepam in chloroform was prepared and three portions were evaporated. The residues after evaporation were treated as in the assay. The difference absorbance was measured at 290 nm for the buffer solutions prepared from these samples. The content of diazepam calculated was 26.61, 26.48 and 26.35 μg/ml and is equivalent to a mean recovery of 100.5%. This confirms that the problem of low recovery was not due to loss of diazepam on evaporation, or degradation of diazepam due to the heat used for evaporation of the chloroform.

Standard solutions of diazepam were prepared in chloroform, in 0.05 M ethanolic sulphuric acid (the solvent used in the redissolution of the chloroform residue in the assay method of the B.P. (1980)) and in methanol (for dilution in pH 2.6 and pH 5.4 buffer solutions). The A(1%,1cm) of diazepam in chloroform

at 312 nm was 78, in 0.05 M ethanolic sulphuric acid at 368nm was 148 and the difference A(1%,1cm) of diazepam in pH 2.6 and pH 5.4 at 290 nm was 316. Subsequent to this work, in the Addendum (1982) to the B.P. 1980, 0.05 M methanolic sulphuric acid was substituted for 0.05 M ethanolic sulphuric acid for redissolution of the residue.

A known weight of diazepam was added to a solution of excipients used in the injection. Two samples of this solution and treated as in the assay procedure. The diazepam content of the chloroform solution was measured after filtration, but prior to evaporation, and also after evaporation and redissolution in two different solvents. For one set of sample solutions, the residues were redissolved in 0.05 M ethanolic sulphuric acid and the UV absorbance of the solutions obtained were measured at 368 nm (as in the direct UV assay, Section 4.2.3.4.2). In the second set of solutions, the residues were redissolved in methanol then further diluted with pH 2.6 and pH 5.4 buffer solutions and the difference absorbance of these solutions measured. From these results, the final concentration of diazepam in solution and the % recovery was calculated. The standard chloroform solutions were also evaporated, without any filtration stage, and the residues from these extractions measured by the direct UV method and the difference absorbance method. The results are seen in Table 6.12.

Table 6.12 Investigation of extraction and filtration processes

Solution	% recovery	Mean recovery
Diazepam in chloroform solution	85.2, 85.3	85.3
Diazepam in 0.05 M ethanolic sulphuric acid	97.3, 98.4	97.9
Prepared injection in 0.05 M ethanolic sulphuric acid	83.0, 84.3	83.7
Diazepam in buffer solutions	98.4, 99.6	99.0
Prepared injection in buffer solutions	83.9, 84.3	84.1

It can be seen that in both cases, the losses occur at the extraction or filtration stages. The evaporation and redissolution can be performed quantitatively on the standard chloroform solution to give almost 100% recovery. The concentration measured after evaporation of the solutions from the injection extracts are also very similar, with no further loss of diazepam occurring.

A portion of the solution of diazepam with excipients was extracted into chloroform and the individual extracts were bulked and made to volume without filtration. The absorbance of this solution was measured and the concentration of diazepam calculated. This was found to be 107.2 μg/ml, equivalent to 101.9% recovery of the known concentration of diazepam. Thus it appears that diazepam can be extracted quantitatively into chloroform, and that any loss occurs during the filtration stage.

A solution of diazepam in chloroform was prepared and

this solution was treated in a number of ways. A portion of the solution was diluted in chloroform, and another portion diluted to the same extent, but with the addition of a small quantity of pH 7.0 buffer solution. This was intended to mimic the situation where the chloroform is not dried through anhydrous sodium sulphate prior to dilution. Further portions of the chloroform solution were dried through anhydrous sodium sulphate supported on either filter paper or sintered glass funnels, or filter paper alone. In each of these cases the filtration equipment was rinsed with chloroform before the filtrate and rinsings were made to volume. The absorbance of the solutions prepared in this way was measured at 312 nm, the λ_{max} of diazepam in chloroform, and the content of diazepam in each was calculated. The % recovery obtained from each treatment is shown in Table 6.13.

Table 6.13 Effect of various filtration methods on % recovery of diazepam

Solution and filtration method	% recovery
Diluted solution of diazepam in chloroform	100.7
Chloroform solution with 1 ml of buffer added	102.9
Chloroform solution filtered through filter paper	95.7
Chloroform solution filtered through sodium sulphate on filter paper	95.6
Chloroform solution filtered through sodium sulphate on a sintered glass funnel	100.5

The use of filter paper either to support sodium sulphate or on its own, results in a reduction in the concentration of diazepam in the final solution

although this was not as large as observed for the prepared injection. The diazepam must be strongly bound to the filter paper as even rinsing the filter paper with 80 ml of chloroform does not give 100% recovery. In the following work, the sodium sulphate was supported on sintered glass funnels (No 1) since no decrease in the measured diazepam content on filtration was seen using this method, when the sinter funnel size was standardised. The value obtained for diazepam when buffer was added to the final solution was higher than the others. On mixing the chloroform and water, an emulsion is formed and the higher result is probably due to light scattering rather than being a true reflection of the measured absorbance.

6.1.3.5 Syrup

By definition, a syrup is a liquid oral dosage preparation in which the vehicle is a concentrated aqueous solution of sucrose or some other sugar. It can be either a clear solution or a suspension. Valium syrup is a raspberry flavoured, pink coloured viscous suspension with a labelled content of diazepam of 2 mg/5 ml.

The weight per ml of the syrup was determined as 1.21 g/ml, thus the ratio of excipients to drug was very high, (approximately 6 g of syrup containing 2 mg of diazepam). The formulation is also known to contain a large number of excipients.

Dilution of the syrup with methanol produced a flocculant precipitate, with a clear sparkling

supernatant liquid. This liquid was pink in colour owing to the presence of the colouring agents, but on dilution in pH 2.6 and pH 5.4 buffer solutions a change in colour was noticed. The pH 2.6 solution became colourless, but the pH 5.4 solution remained pink. A slight turbidity was seen in both solutions. When the difference spectrum of the sample was superimposed on that of a standard solution of diazepam, differences were seen (Fig. 6.14). No isosbestic points were seen in the difference spectrum of the sample, and the λ_{max} was shifted from 290 nm to a lower wavelength. This was probably due to the many excipients present in the formulation. Samples of the dye and flavouring, at pharmaceutical concentrations, were subjected to the same treatment as the syrup to investigate which of them was responsible for the interference. The difference spectrum generated for the dye showed interference at 290 nm, the wavelength used for measurement of the difference absorbance. No interference was given by the flavouring agent (Fig. 6.15). On examination of the difference spectrum generated from the syrup dilution, (Fig. 6.14) it could be seen that the dyestuff gave a small difference absorbance at 290 nm, but the major interference was from sodium benzoate, also present in the formulation (Fig. 6.11). As previous work had shown that this interferes at 290 and 360 nm, (Section 6.1.3.4), it was necessary to extract diazepam from sodium benzoate, before an accurate measurement of the content of diazepam in the syrup could be made.

When the extraction method developed for the injection formulation was used, (Section 4.1.4.4.2), it was found that interference still occurred (Fig. 6.16).

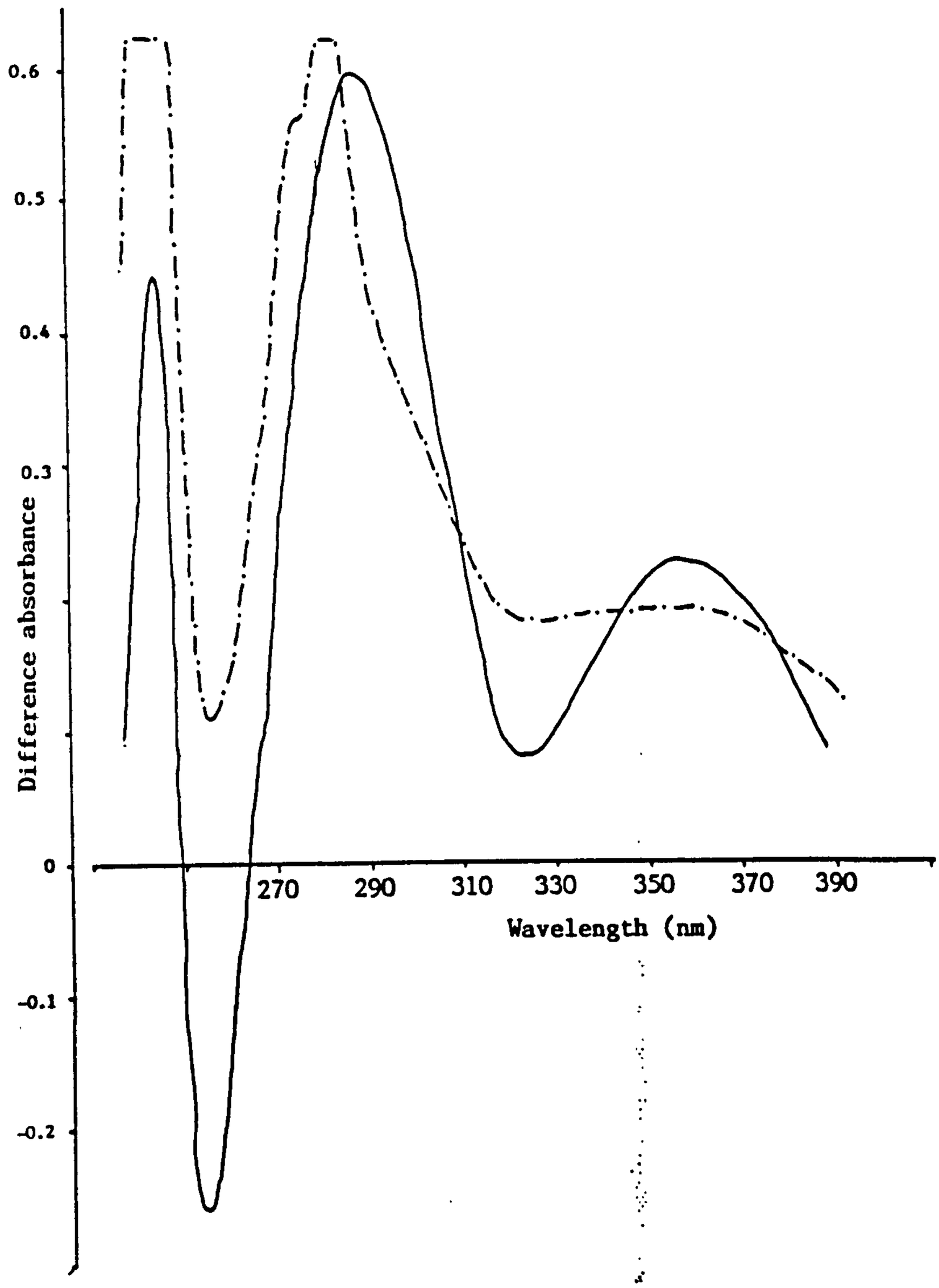


Fig. 6.14 Difference absorption spectra, pH 2.6 vs pH 5.4, for diazepam and a dilution of Valium syrup

——— Difference absorption spectrum of diazepam

—·—·— Difference absorption spectrum of a dilution of syrup

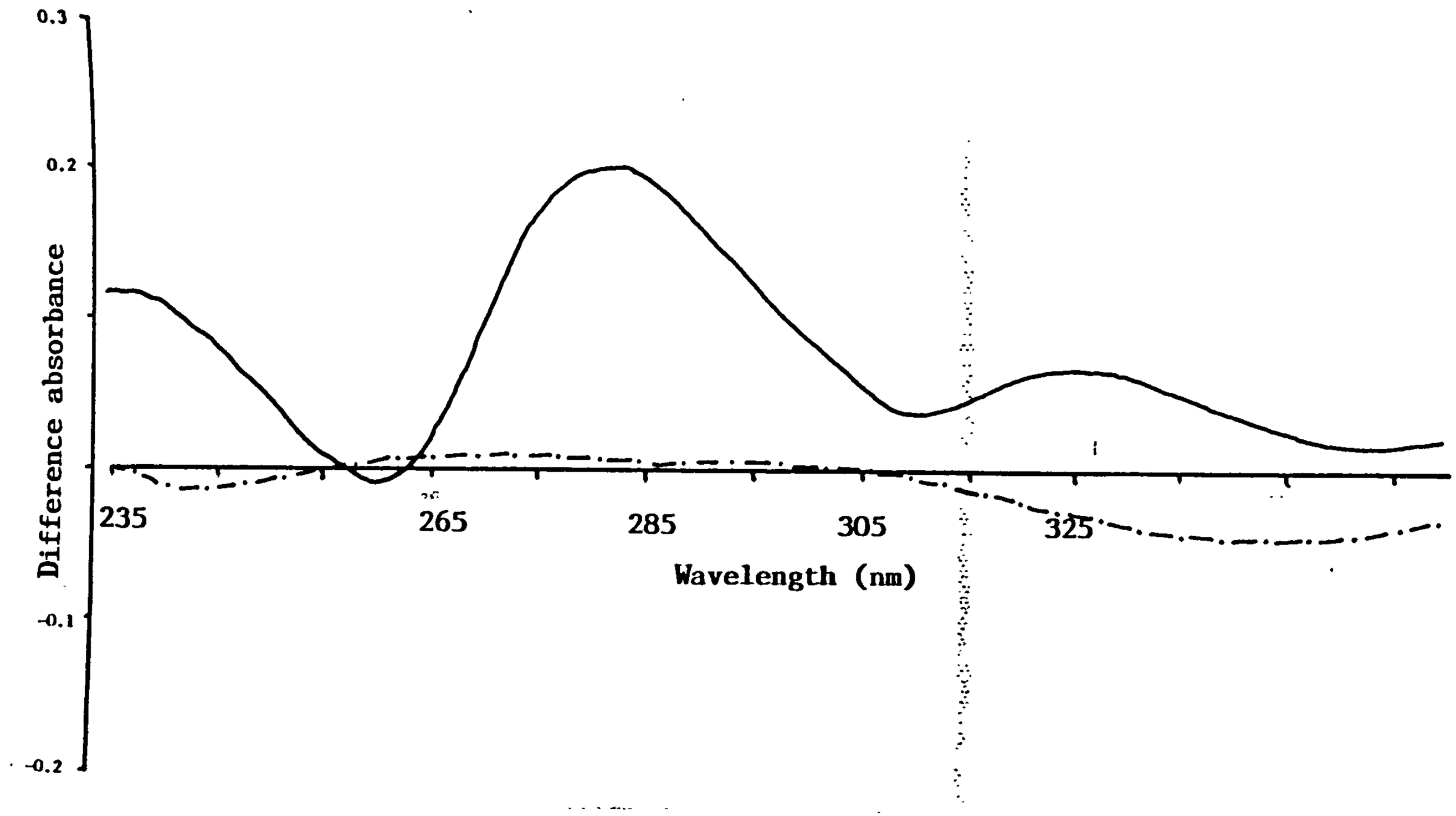


Fig. 6.15 Difference absorption spectra, pH 2.6 vs pH 5.4, for the flavouring agent and dye used in Valium syrup

——— Difference absorption spectrum of erythrosine dye

—·—·— Difference absorption spectrum of the flavouring agent

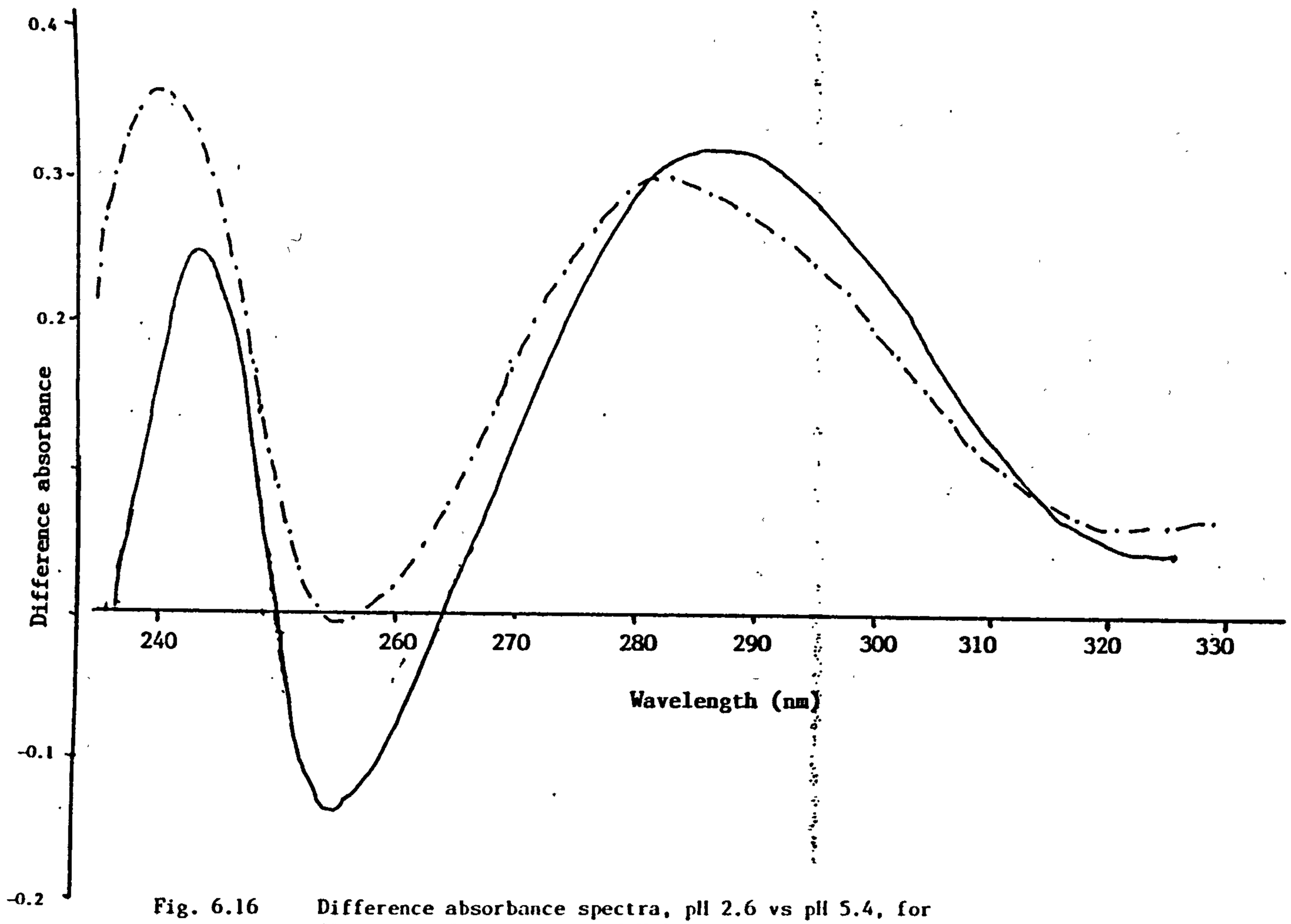


Fig. 6.16 Difference absorbance spectra, pH 2.6 vs pH 5.4, for diazepam and an extract of Valium syrup

——— Difference absorption spectrum of diazepam

-·-·- -- Difference absorption spectrum of an extract of Valium syrup

The chloroform extract was pink in appearance and the difference absorbance spectrum showed areas similar to those in the difference spectrum of the dye.

The dye used was erythrosine which is soluble in water and alcohol, but forms a precipitate in acid and alkali [Merck Index,(1968)]. The effect of pH on the extraction of the dye into chloroform was examined to obtain conditions where the dye was retained in the aqueous layer. The dye has a λ_{max} of 524 nm in aqueous solution. Dilutions of a solution of dye were made at different pH values and the absorbance measured at 524 nm before and after extraction with chloroform. These measurements showed a reduction in absorbance after extraction, proving that some dye still transferred into the chloroform layer. However, this effect was reduced when pH 13.0 solution was used, Table 6.14.

Table 6.14 Effect of pH on the extraction of erythrosine dye

pH of solution	Absorbance at 524 nm		% reduction
	Before extraction	After extraction	
7.0	1.686	1.517	11.1%
9.2	1.683	1.640	2.6%
13.0	1.681	1.644	2.3%

The procedure for extraction of diazepam from syrup into chloroform was repeated but, 0.1 M sodium hydroxide was added to the syrup prior to extraction. The difference absorbance of the solutions were recorded (Fig. 6.17) showed that some interference

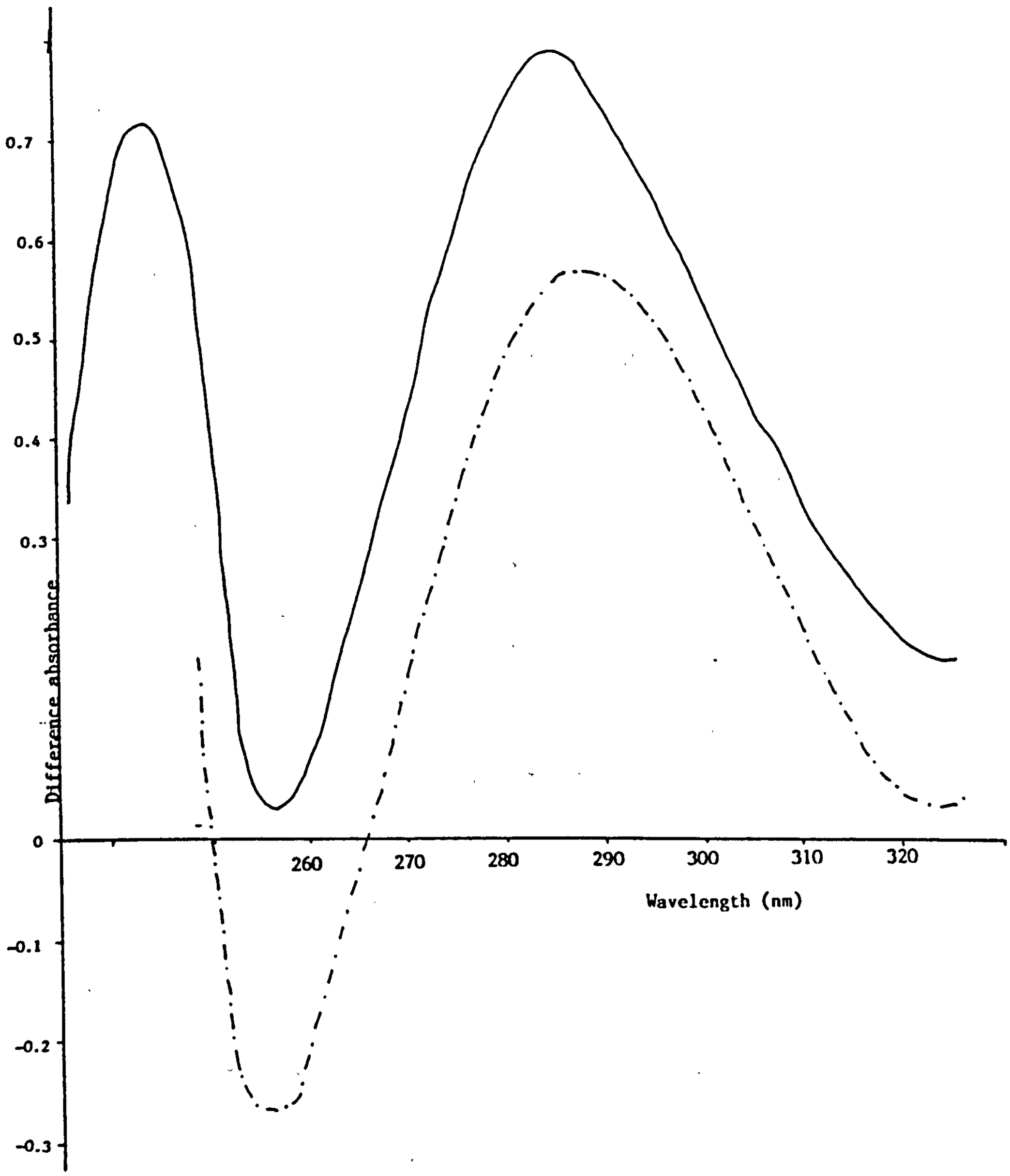


Fig. 6.17 Difference absorbance spectra, pH 2.6 vs pH 5.4, for diazepam and an extract of Valium syrup

—·—·—·— Difference absorption spectrum of diazepam

——— Difference absorption spectrum of an extract of Valium syrup

The spectra appear slightly different with differences in the wavelength at which the maximum difference occurs. There is obviously some other component in the sample which is interfering with the determination.

was still present. The concentration of diazepam measured in the extract using this method was only 43.6% of the nominal content. This suggested that either the diazepam was not fully extracted, or that it was not stable in alkali. Investigation of the extraction of diazepam in methanol from 0.1 M sodium hydroxide into chloroform showed that in the absence of syrup excipients, 103.8% recovery was obtained.

The low measured content of diazepam suggested that poor extraction of diazepam into the chloroform was occurring. This was thought to be due to poor solubility of diazepam in the aqueous phase. If the diazepam in the syrup was not completely dissolved in alkali, then the partitioning into chloroform would require a longer period. Thus the sample of syrup was first diluted with methanol to ensure complete dissolution of diazepam before extraction. However, on dilution of the syrup with methanol, a flocculant precipitate formed (thought to be due to sugars in the formulation) which proved to be difficult to filter and was very sticky. When the mixture was centrifuged however, a firm pellet was formed below a clear supernatant liquid. The supernatant liquid and rinsings were mixed with 0.1 M sodium hydroxide and extracted with chloroform. In this way, the dye was retained in the aqueous layer together with the sodium benzoate. On evaporation of the chloroform layer, a waxy material was seen which dissolved in methanol. Preparation of the pH 2.6 and pH 5.4 dilutions from this methanolic solution showed some turbidity and interference was seen in the difference spectrum generated.

This interference was thought to be due to material soluble in methanol which was insoluble in aqueous solution. It is possible that this is the waxy material seen on evaporation of the chloroform layer. One likely compound is an emulsifying agent which may be present in the syrup to aid the stability of the suspension.

A further extraction stage was included to attempt to remove this material. Diazepam is known to be freely soluble in chloroform, less soluble in methanol but even less so in petroleum ether (water, 0.05 ; petroleum ether, 0.9 ; methanol, 49 ; chloroform, >500 mg/ml, [Florey,(1972)]. However, if the methanol soluble material could be extracted into some other solvent which would not remove diazepam, it may be possible to separate both components without loss of diazepam, before extraction into chloroform. After centrifugation of the methanolic dilution of syrup, the solution was extracted with petroleum ether. The methanol layer remaining was mixed with 0.1 M sodium hydroxide and further extracted with chloroform. On examination of the residue after evaporation of the petroleum ether extract, a waxy substance was seen which had a similar appearance and odour to that previously obtained from the chloroform extract. Redissolution of the residue left from the chloroform extract in methanol, and further dilution of this in buffer gave clear solutions. The difference spectrum generated from these solutions showed no interference, (Fig. 6.18). However, a low concentration of diazepam was measured when compared with the nominal content, (90.8%).

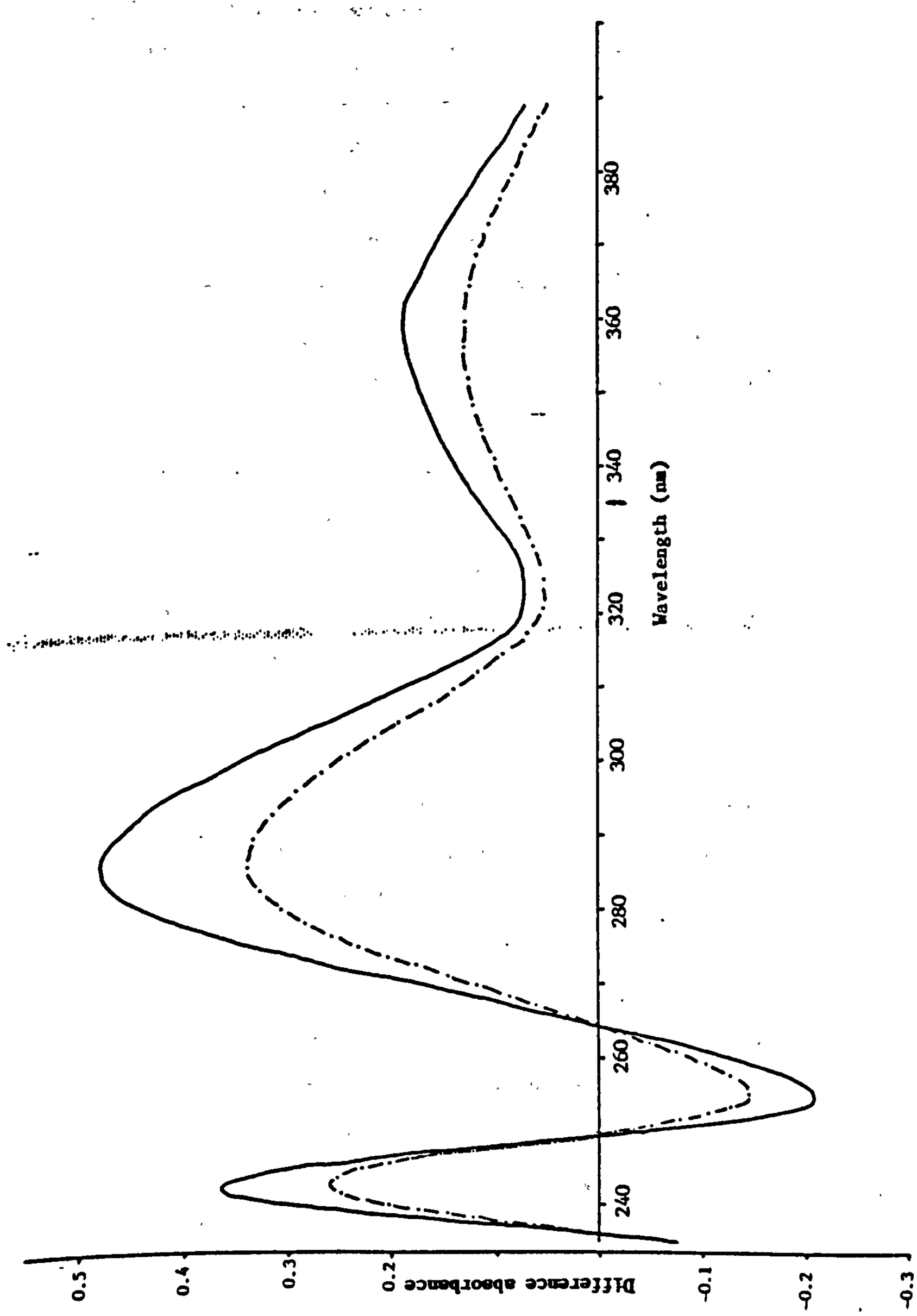


Fig. 6.18 Difference absorption spectra, pH 2.6 vs pH 5.4, for diazepam and an extract of Valium syrup

——— Difference absorption spectrum of diazepam

—·—·— Difference absorption spectrum of an extract from Valium syrup

This low value is probably a reflection of the complexity of the extraction procedure. As diazepam has some solubility in petroleum ether, it is possible that some could be lost at that extraction stage. Also, methanol and petroleum ether have some degree of mutual solubility, resulting in a potential loss of methanol containing diazepam into the petroleum ether layer. Other areas where some diazepam may be lost include the stages where methanol is transferred from the centrifuge tube to the separating funnel and where chloroform is separated from methanol after extraction. Although diazepam has been shown to extract almost completely into choroform on one extraction, in the case of formulations there may be some binding to excipients, requiring more than one extraction with chloroform to remove the diazepam completely.

Due to the difficulties experienced and discussed above, no difference UV spectrophotometric method was developed for Valium syrup. On using the method developed by Roche for the direct UV determination of diazepam in the syrup, (Section 4.2.3.5.2), it was seen to comprise steps similar to those investigated for the difference method. In the direct method, a mixture of methanol and 0.1 M sodium hydroxide were used to dissolve the syrup prior to extraction. This ensured that no dye material was transferred to the chloroform layer as the dye interferes with the direct UV measurement. Use of an extraction procedure also meant that no interference from sodium benzoate would occur. As methanolic sulphuric acid is used for redissolution of the residue after extraction, no problem occurs due to the poor aqueous solubility of

the other material found to interfere in the difference UV method of analysis. Thus no other areas were possible where modifications would provide any improvements to the assay, allowing it to be used for the measurement of diazepam in syrup.

6.2 DEVELOPMENT AND EVALUATION OF A DIRECT UV SPECTROPHOTOMETRIC METHOD

Although the method used for assay of diazepam was that described in the B.P. (1980) for the assay of diazepam tablets and capsules, some experiments were carried out to cross-validate the difference absorbance UV method and the direct absorbance method. Newton (1978) has commented on the lack of specificity of the official method for diazepam in the presence of MACB, referring to the assay method of the USP XIX. This method was a spectrophotometric determination similar to that used in the B.P. (1980). Newton suggests a modification to this assay to allow for estimation of the MACB content of the formulation. However, since this paper was published, the official method of the USP has been modified [U.S.P.,(1985)] and now uses an HPLC method which is stability-indicating. As the difference absorbance method was developed to improve the specificity of UV measurement, it was important to determine how significant the interference from MACB was by both methods. The performance of both methods were therefore compared in the presence of significant levels of MACB.

6.2.1 Evaluation of the direct UV spectrophotometric method

Solutions containing diazepam and MACB, at approximately equimolar concentrations were prepared in 0.05 M methanolic sulphuric acid as described in Section 4.2.1. The UV absorption spectrum of each of these solutions was recorded and is shown in Fig.

6.19. The molar absorptivities of both compounds were calculated at 241.4 and 284 nm, the wavelengths at which diazepam exhibits absorption maxima, under the conditions used. These results are shown in Table 6.15.

Table 6.15 Molar absorptivities of MACB and diazepam in 0.05 M methanolic sulphuric acid

Compound	Wavelength	
	241.4 nm	284 nm
Diazepam	28972	12797
MACB	16422	3622

From Table 6.15, it can be seen that the contribution due to MACB when present in equimolar amounts is 36.2% at 241.4 nm and 22.1% at 284 nm. Thus for each 1% m/m of MACB present in diazepam, the absorbance of the diazepam at 284 nm would be increased by 0.33% over the true absorbance.

Solutions of diazepam and MACB of constant total molar concentration, in which the content of diazepam varied from 0 to 100% were prepared as described in Table 4.2, (Section 4.1.3.2). These were analysed by the direct UV spectrophotometric assay. The apparent concentration of diazepam in these solutions was calculated using the A(1%,1cm) value for diazepam of 450 (Addendum 1982 to B.P. 1980), and the results are shown in Table 6.16.

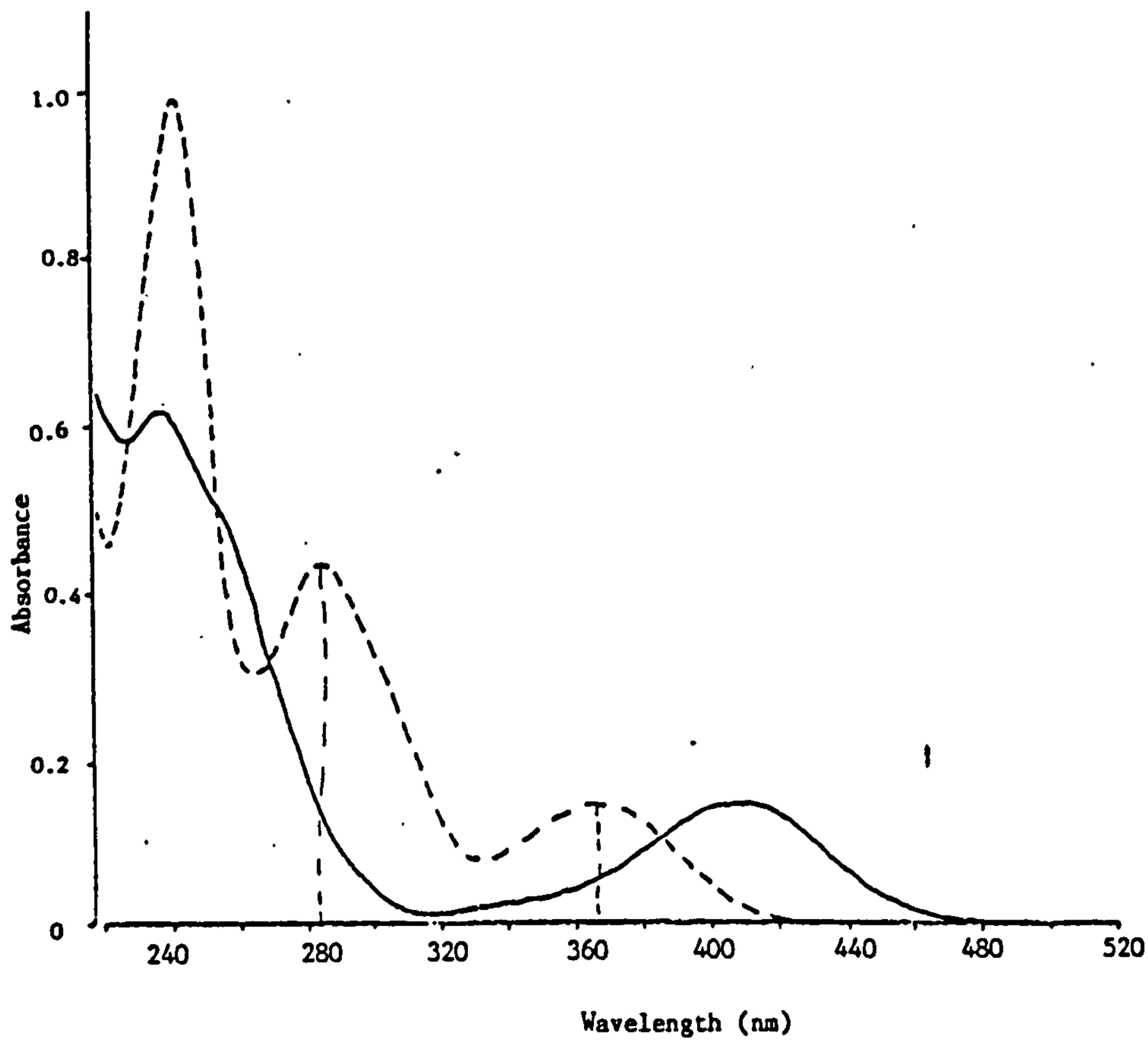


Fig. 6.19 Absorption spectra of diazepam and MACB

---- Absorption spectrum of diazepam (3.5×10^{-5} M)

——— Absorption spectrum of MACB

in 0.5% m/v sulphuric acid in methanol

Table 6.16 Content of diazepam in prepared mixtures, using direct UV measurement at 284 nm

Concentration of diazepam (μg/ml)	Concentration of MACB (μg/ml)	Mole fraction of MACB (as %)	Measured diazepam (μg/ml)	% recovery
13.21	0	0	13.40	101.4
10.56	2.08	18.6	11.49	108.8
7.92	4.16	37.8	9.58	121.0
5.28	6.24	57.8	7.47	141.5
2.64	8.32	78.5	5.48	207.6
0	10.40	100	3.46	

From these results, it can be seen that MACB has a significant UV absorbance under the conditions used to assay diazepam. This gives rise to interference in the assay of diazepam. The molar absorptivity of MACB calculated from these results was 3675, which agrees well with the value of 3622 reported in Table 6.15.

Comparison of the results for % recovery by both UV methods of analysis carried out on the same solutions shows that much greater interference is present in the direct method of measurement. However the concentration of MACB used in these solutions was abnormally high. The experiment was therefore repeated using mixtures with a much lower proportion of MACB. The results from these measurements, (Table 6.17) show that even at lower concentrations of MACB,

interference occurs. From Table 6.16, it can be calculated that 1% MACB would be expected to give a 0.3% contribution to the absorbance of diazepam, in agreement with the level of interference calculated from the molar absorbance in Table 6.15. The results in Table 6.17, where MACB is present at lower concentration levels, also confirm that interference occurs in the direct method of measurement.

Table 6.17 Content of diazepam in prepared mixtures, with MACB present at up to 14% of the total absorbing species using direct UV measurement at 284 nm

Concentration of diazepam (μg/ml)	Concentration of MACB (μg/ml)	Mole fraction of MACB (as %)	Measured diazepam (μg/ml)	% recovery
10.36	0	0	10.56	101.9
9.33	0.69	7.9	9.78	104.8
8.29	0.17	2.3	8.51	102.7
7.25	0.52	7.6	7.58	104.5
6.22	0.86	13.8	6.63	106.6

The standard mixtures of diazepam containing 1% and 10% of MACB prepared as described in Section 4.1.3.3 and analysed by the difference UV method, (Table 6.5) were also analysed by direct UV measurement at 284 nm. The results of these measurements are given in Table 6.18. Ten replicate determinations were made on each solution and the results were analysed statistically.

Despite the larger number of manipulations performed in the difference UV method, the precision obtained for ten replicate determinations compared favourably with that obtained from the direct UV measurement.

For both sets of solutions, the precision of the difference UV method was found to be better than that of the direct UV method at the 1% significance level when the results were subjected to F-tests. The recovery of diazepam was higher in both sets of solutions when measured by the direct UV method confirming the lack of specificity of this method for analysis of diazepam in the presence of MACB.

Table 6.18 Content of diazepam in mixtures containing 1% and 10% MACB using direct UV measurement at 284 nm

MACB content 1%				MACB content 10.2%			
Measured content of diazepam (μg/ml)	Average (n = 10)	Actual content (μg/ml)	% recovery	Measured content of diazepam (μg/ml)	Average (n = 10)	Actual content (μg/ml)	% recovery
25.42				23.62			
25.59				22.96			
25.59				23.71			
25.57				23.93			
25.61				23.98			
25.56	25.64	25.35	101.1	23.90	23.86	23.11	103.2
25.64				23.86			
25.91				23.87			
25.71				23.97			
25.75				23.97			
	sd 0.131				sd 0.125		
	RSD 0.51%				RSD 0.52%		

6.2.2 Development of direct UV spectrophotometric methods for analysis of formulations

6.2.2.1 Tablets

Kerfoot diazepam tablets (2 mg) were assayed ten times using the direct spectrophotometric method described in Section 4.2.3.1.2. The results of the assays are show in Table 6.19.

These results gave a slightly high measured content of diazepam per tablet, with respect to the nominal content. There was some scatter, but the relative standard deviation was satisfactory. These tablets were also analysed by the difference UV assay method and the results are given in Table 6.7. The precision of the two methods was compared and the difference UV method was found to give better precision than the direct UV method at the 5% significance level, but not at the 1% significance level.

Table 6.19 Precision of replicate direct spectrophotometric assays performed on crushed, bulked Kerfoot 2 mg diazepam tablets

	Measured content of diazepam (mg/tab)	
	2.041	
	2.038	
	2.043	
	2.041	
	2.034	
	2.070	
	2.054	
	2.055	
	2.089	
	2.088	
Average	2.055	(102.8% nominal content)
RSD	1.0%	

On comparing the assay results obtained from one batch of tablets, by using two methods of analysis, it was found that the direct UV measurement results were higher than the difference UV results. As it is known that the difference spectrophotometric procedure can be used to eliminate excipient interference, the amount of interference from excipients in the direct UV assay was investigated to see if this was the reason for the higher results. As described in Section 4.2.3.1.1, a graph of absorbance versus concentration of diazepam was generated from a series of standard solutions of diazepam and the concentration of diazepam in the tablets was calculated by extrapolation from this. On eliminating the interference from diazepam in the absorbance measurement by recording the UV absorbance spectrum of an extract from the tablets against the exact concentration of diazepam present, the absorbance at 284 nm was found to be zero. No interference was seen from absorption by excipients, and no reason for the higher measured content of diazepam could be demonstrated.

The effect of filtration on the A(1%,1cm) value of diazepam in 0.05 M methanolic sulphuric acid at 284 nm was investigated. The A(1%,1cm) values were calculated from absorbance readings taken after the diazepam solutions had been subjected to a number of treatments. The results are shown in Table 6.20. A stock solution of diazepam was prepared in 0.05 M methanolic sulphuric acid and this was diluted with the same solvent to a suitable concentration for measurement. Further samples of this stock solution

were filtered through filter paper (both No 1 and No 42) and both filtrates collected. Portions of the filtrate from the first 20 ml filtered and also the later filtrate were diluted separately, to give a suitable concentration of diazepam for measurement.

Table 6.20 Effect of filtration on measured A(1%,1cm) value

Solution treatment before measurement	Measured A(1%,1cm) at 284 nm
Original dilution	453,451
Filtration, No1 (dilution of first 20 ml)	461,465
Filtration, No1 (dilution of later filtrate)	464,466
Filtration, No42 (dilution of first 20 ml)	459,458
Filtration, No42 (dilution of later filtrate)	465,462

It can be seen from these results that a significant increase in the measured A(1%,1cm) value occurs as a result of filtration. This was thought to be due to two causes; either the solution picked up extraneous material from the filter paper which absorbs in the region of interest, or concentration of the solution occurred by evaporation of the methanolic solvent during filtration.

Previous workers have found increased UV absorption after filtration of distilled water, [Chiou and Smith,(1970)]. The amounts of UV absorbing species removed from Whatmans No 4 filter paper was found by these workers to be small, and large increases in absorbance were only seen at wavelengths below 230 nm. Therefore this was unlikely to be the cause of the increase in absorbance. Also, if some extraneous

material was extracted from the paper, it would be expected to appear in the early filtrate only, with later filtrates free from interference. The A(1%,1cm) values given in Table 6.20 show that the value obtained from the later filtrate is higher than that from the early filtrate.

In this assay, a large proportion of methanol is used, and so if filtration is prolonged, then evaporation could occur. This would then account for the increase in the measured A(1%,1cm) between the early and later filtrates.

Two sets of filtration apparatus, with No 1 and No 42 filter paper, were set up on top pan balances. Solutions containing diazepam in 0.05 M methanolic sulphuric acid were prepared and filtered using both sets of apparatus. Dilutions of the solution were made before and after filtration in the same solvent and the A(1%,1cm) were calculated. The weight of solution added to the filtration equipment was also measured before and after filtration. The results of the weight loss and increase in A(1%,1cm) during filtration are shown in Table 6.21.

Table 6.21 Loss of weight during filtration and its effect on the absorbance measured at 284 nm

Filter paper	Weight of solution before filtration (g)	Weight of solution after filtration (g)	Loss of weight (%)	A before filtration	A after filtration	Increase in absorbance (%)
No 1	9.78	9.50	2.9	0.894	0.919	2.7
No 42	27.46	26.23	4.5	0.960	0.988	2.8

The increase in A(1%,1cm) measured on filtration is similar to the decrease in solution weight. Thus the increased A(1%,1cm) value (Table 6.20) appears to be due to concentration of the solution during filtration as a result of evaporation. From this, if the B.P. value of A(1%,1cm) for diazepam is used in the calculation of results, a systematic error of 3% would be introduced. This may explain the higher recoveries obtained for tablets when measured by the direct spectrophotometric method compared with the difference absorbance method.

Prior to analysis of the solutions obtained by extraction of diazepam tablets, some method of separation is required to remove the insoluble tablet excipients. Centrifugation and filtration of a stock solution of diazepam were compared and the results of this are shown in Table 6.22.

Table 6.22 A(1%,1cm) values for diazepam after filtration and centrifugation

Treatment of solution	Measured A(1%,1cm)	% increase
No treatment	453	
Filtration, fluted filter paper for 10 minutes	473	4.4
Filtration, non-fluted filter paper for 10 minutes	466	2.9
Centrifugation	453	0

No weight loss occurred on centrifugation and it appears that this would provide an effective method of reducing the systematic error introduced by evaporation. However, when a suspension of powdered

tablet was treated in this way, the tablet debris was too fine to be spun down using the centrifuge available. Thus the only way of removing the tablet excipients was to filter the solutions.

6.2.2.2 Capsules

The method for capsules was modified from that used for the tablet assay, with an appropriate quantity of powder taken from a mixture of powder from a number of capsules.

6.2.2.3 Suppositories

The method for suppositories was modified from that used for the tablets and capsules by dissolving one suppository in 0.05 M methanolic sulphuric acid and diluting to an appropriate concentration in the same solvent.

6.2.2.4 Injection

The method described in the B.P.(1980) for diazepam injection was used to analyse injections.
Investigation into the method of drying the chloroform extract was performed at the same time as the work described for the difference UV spectrophotometric method. The results are detailed in Section 6.1.3.4. The use of sintered glass funnels for supporting the sodium sulphate was selected after this.

Subsequent to this work being performed, the Addendum, 1982 to the B.P. (1980) altered the solvent used for

redissolution of the residue from 0.05 M ethanolic sulphuric acid to 0.05 M methanolic sulphuric acid.

6.2.2.5 Syrup

The method used here was that developed and used by the QC laboratories at Roche. No further work was performed on development.

6.3 DEVELOPMENT AND VALIDATION OF AN HPLC METHOD FOR THE ANALYSIS OF DIAZEPAM

6.3.1 Development of the general method

Previous workers [Emery and Kowtko,(1979)] reported the analysis of diazepam in tablets by HPLC, using a reverse-phase system with μBondapak packing material. Since this work, recent advances in equipment have led to the introduction of short, thick flexible walled columns which are compressed externally, as an alternative to conventional stainless steel columns. These short columns appear to have a number of advantages. One of these is the high flow rate of mobile phases which can be used compared with that for stainless steel columns, thus reducing analysis time. As both types of column, packed with the same stationary phase were available at the time of the work presented here, a comparison was made.

Separate solutions were prepared containing diazepam, MACB and carbostyril and also a solution containing a mixture of all three compounds. These compounds were chromatographed using both columns, (Fig. 6.20). Good baseline separation and symmetrical peak shape were achieved in both cases, but the later eluting peaks appeared to suffer less band broadening using the radial compression column, despite a longer retention time.

With the 30 cm stainless steel column, flow rates of 2 ml/min were used resulting in back pressures of over 1000 psi. The Radpak radial compression column

resulted in back pressures of approximately 300 psi for the same flow rates.

The Radpak column was selected for use in further work because of the better peak shape. Alteration of the mobile phase composition and flow rate allowed reduction of the time required for analysis from 24 min (Fig. 6.21), to 10 min. By increasing the methanol content of the mobile phase, it was possible to reduce the retention time of the components of the mixture while still maintaining separation. This faster analysis has the added advantage of increased sensitivity by producing sharper peaks.

The potential interference from formulation excipients and compounds related to diazepam was investigated. A solution was prepared containing sodium benzoate and benzyl alcohol (excipients in formulations), carbostyril and MACB (potential degradation products) and diazepam and 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (desmethyl diazepam, a synthetic precursor of diazepam), and injected onto the chromatographic system.

Desmethyl diazepam was found to interfere with the diazepam peak when the mobile phase methanol:water, 80:20 was used. In order to achieve separation of diazepam from this, it was necessary to use a mobile phase containing 72:28 methanol:water. This increased the retention time for all components of the mixture and gave a final analysis time of 11 min, (Fig. 6.22).

Initial work used 254 nm as the wavelength of detection. By alterating the wavelength to 230 nm, it

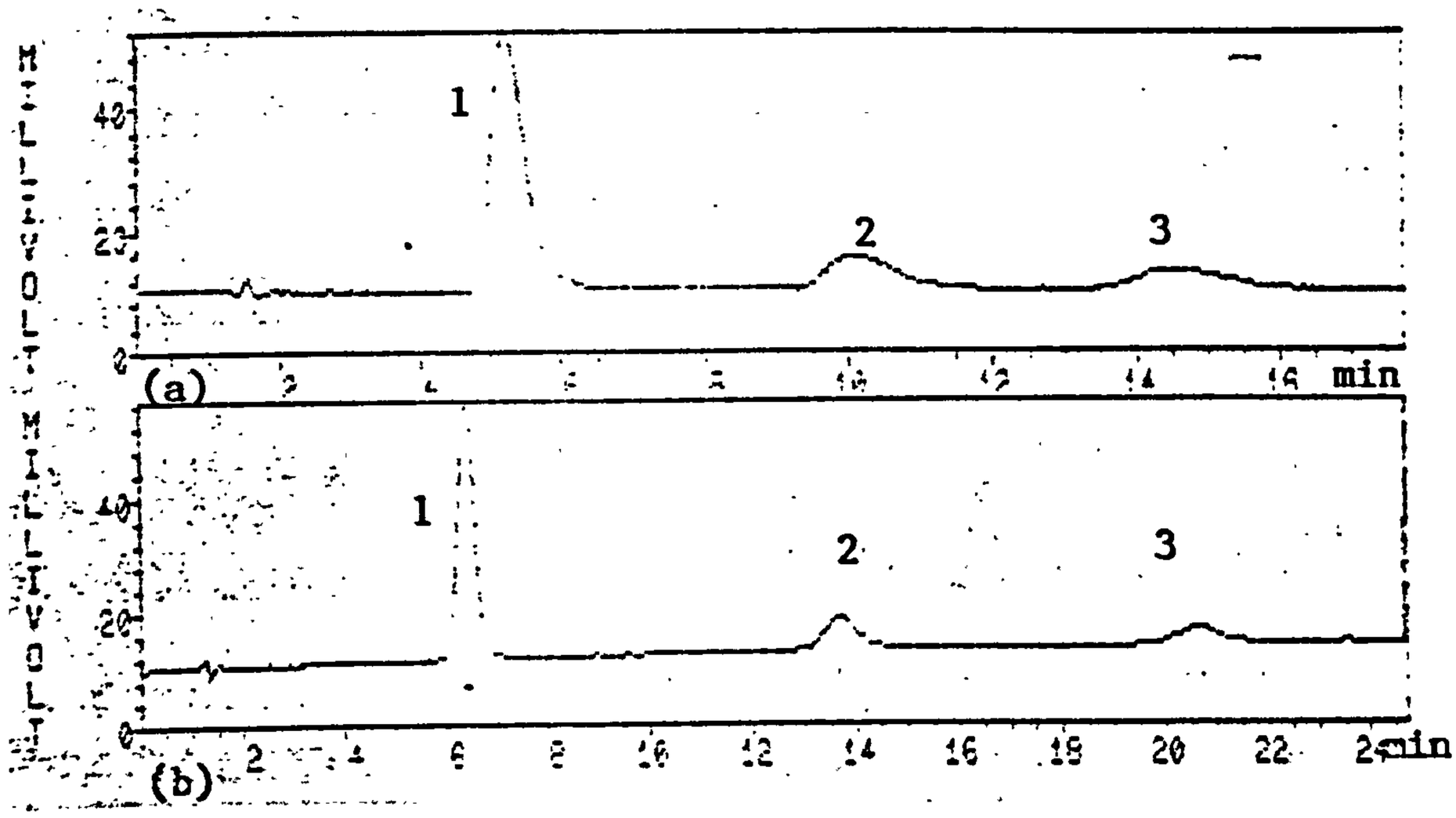


Fig. 6.20 Separation of diazepam, carbostyril and MACB on a conventional column and on a Radpak column

(a) conventional column

(b) Radpak column

where peak 1 is diazepam, peak 2 is carbostyril and peak 3 is MACB

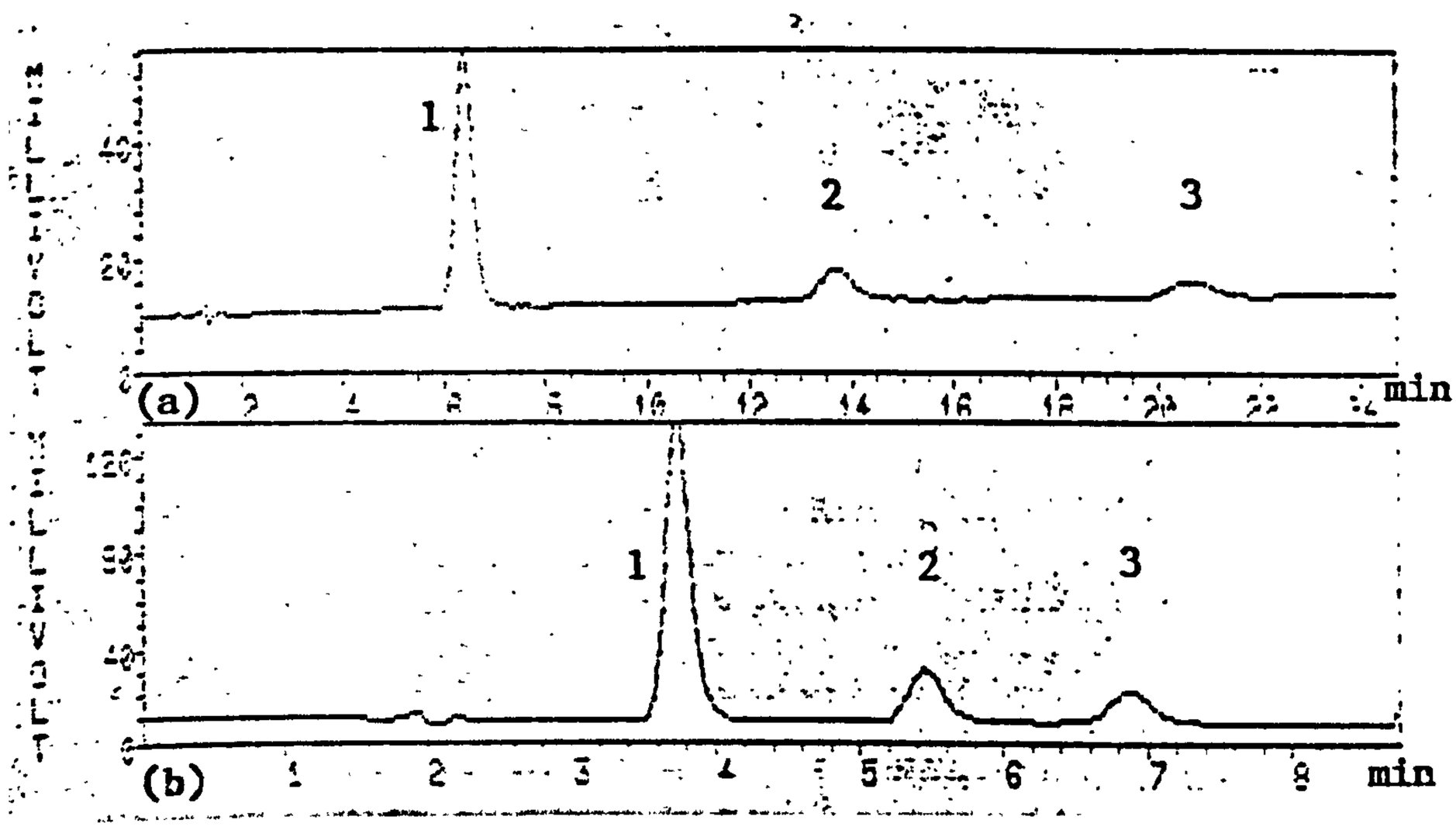


Fig. 6.21 Effect of alteration in mobile phase composition on the HPLC separation of the standard mixture

(a) 65:35 methanol:water
(b) 80:20 methanol:water

where peak 1 is diazepam, peak 2 is carbostyril and peak 3 is MACB

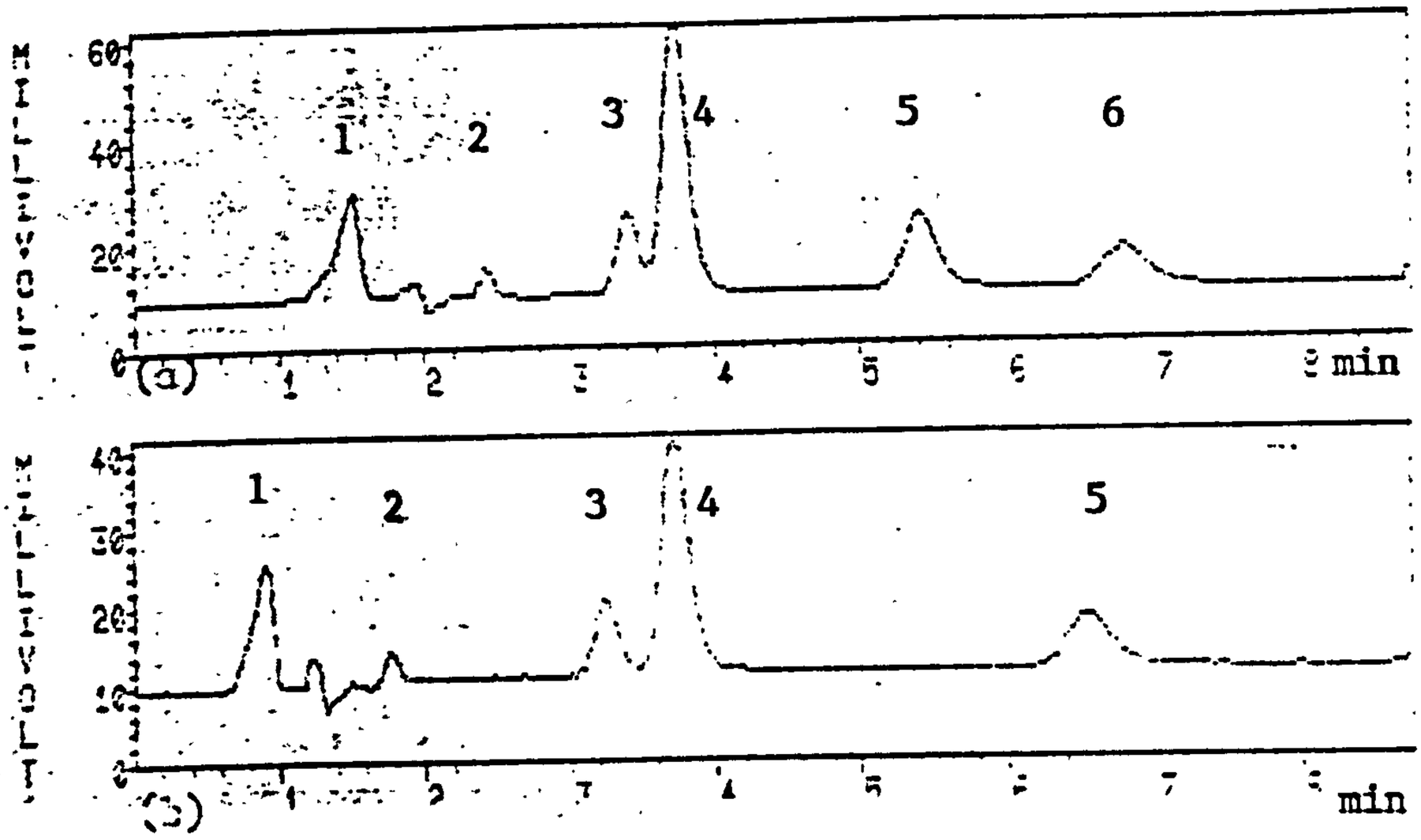


Fig. 6.22 Effect of alteration of mobile phase composition on the HPLC separation of diazepam and desmethyl diazepam

(a) 76:24 methanol:water

(b) 72:28 methanol:water

where peak 1 is sodium benzoate, peak 2 is benzyl alcohol, peak 3 is desmethyl diazepam, peak 4 is diazepam, peak 5 is carbostyril and peak 6 is MACB

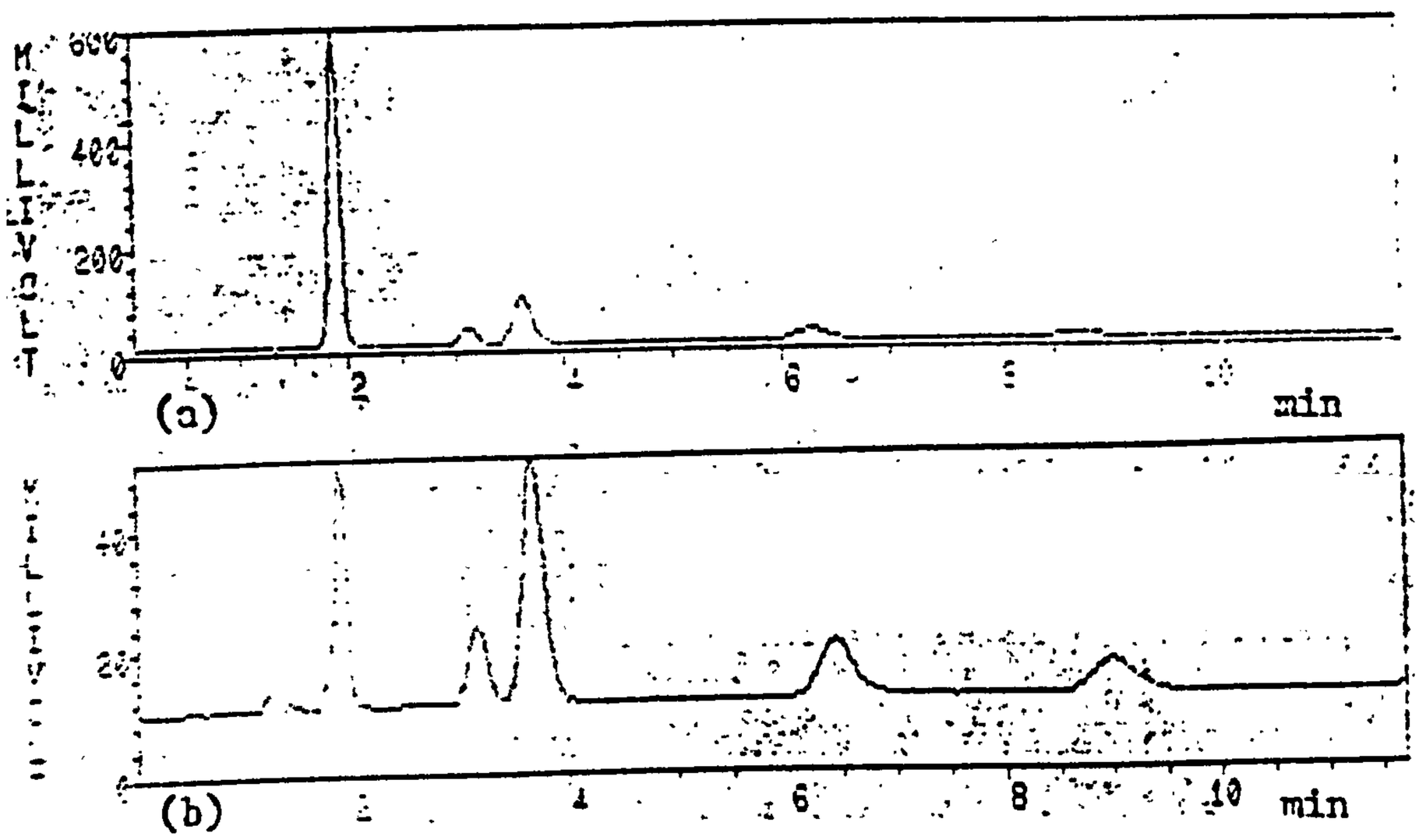


Fig. 6.23 Effect of wavelength of detection

(a) 230 nm
(b) 254 nm

was possible to increase the sensitivity of the assay for diazepam. However, MACB shows maximum absorbance at 255 nm, and alteration of the wavelength of detection away from this resulted in a reduction in the sensitivity of detection for degradation products, (Fig. 6.23).

6.3.2 Validation of the method

The two injection systems used in this work, the Kontron MS1660 sampler and Wisp 710 A/B autoinjector, are loop injector systems. Each has a rinsing procedure to flush the system with ethanol between injections to prevent contamination due to carry-over. The automatic injectors were expected to have a high degree of reproducibility and as a result an external standard method was used rather than an internal standard method. Provided satisfactory reproducibility of injection can be achieved, the error involved in an assay procedure is reduced by external standardisation. With internal standardisation, two measurements have to be made for each sample, and two compounds have to be accurately mixed to prepare each sample. Thus there are more sources of errors than for a single measurement. External standardisation also considerably simplifies the sample preparation. Sample solutions were bracketed by calibration standards to compensate for any variation in response factor which might occur during the automated assay.

Duplicate injections were made of each solution and averaged for calculation purposes. However, before comparisons between different solutions could be made,

it was necessary to demonstrate the precision of injection of the autosamplers. Replicate injections were made of a single solution by each autoinjector (different standard mixtures were used for each autoinjector) and the output from the detector was processed by the computer software. The results are recorded in Tables 6.23 and 6.24.

Table 6.23 Precision of replicate injections using a Kontron MS1660 sampler (concentrations of diazepam, carbostyril and MACB; 97.0, 6.5 and 4.2 μg/ml respectively)

	Detector response					
	Height			Area		
	Diazepam	Carbostyril	MACB	Diazepam	Carbostyril	MACB
	182903	7403	4050	2704671	181307	125718
	184414	7480	4154	2722403	177296	132254
	181789	7431	4082	2676541	179710	127379
	185146	7579	4179	2712086	176184	129904
	186031	7525	4197	2707469	176644	126800
	188366	7552	4241	2705741	178905	129338
Average	184775	7495	4150	2704818	178341	128566
RSD	1.3%	0.9%	1.7%	0.6%	1.1%	1.9%

Table 6.24 Precision of replicate injections using a Wisp 710 A/B autoinjector (concentrations of diazepam, carbostyril and MACB were 111.0, 5.4 and 5.4 μg/ml respectively)

	Detector response					
	Height			Area		
	Diazepam	Carbostyril	MACB	Diazepam	Carbostyril	MACB
	222594	7624		2900136	160534	
	220656	7704	5761	2886351	160878	153088
	227492	7725	5865	2893189	160436	154224
	223891	7754	5981	2894495	160264	159709
	227080	7876	6050	2910725	160830	156506
	227461	8019	6103	2876827	160638	156878
	231414	8009	6137	2905568	163097	155399
	228301			2896698		
Average	226111	7816	5983	2895499	160954	155967
RSD	1.4%	1.8%	2.2%	0.4%	0.6%	1.4%

These results show that good precision is obtained using both auto-samplers. The peak area was selected as the measured value to be used in future work as this showed less variation than peak height.

Test and standard solutions for analyses were prepared in methanol and so were expected to be stable. The stability of these solutions was investigated by measuring the content of diazepam in samples extracted from capsules, and then repeating the analysis (against fresh calibration standards) after the solutions were left exposed to natural daylight for 24 h.

The results are given in Table 6.25. Over the 24 h period there was no detected change in the measured concentration of diazepam, carbostyril and MACB in the methanolic solutions. Due to automation of the analysis, up to 12 h can occur between sample preparation and measurement. As the solutions have been shown to be stable over this time period, no problem was anticipated due to the lengthy analysis time.

Table 6.25 Stability of standard and sample solutions

STANDARD

Compound	Initial content (μg/ml)	Measured content after 24 h (μg/ml)	% recovery
Diazepam	99.15	101.70	102.6
Carbostyril	5.62	5.70	101.4
MACB	0.434	0.445	102.5

SAMPLE

	Content of diazepam extracted from capsules	
	Initial measurement of extract (mg/capsule)	Measurement of extract after 24 h (mg/capsule)
Sample 1	4.96	4.96
Sample 2	4.91	4.93

The linearity of the detector response was tested over the working concentration range for diazepam and for lower concentration ranges for its degradation products. A graph was plotted of concentration against response, for both height and area, and the correlation coefficient calculated using linear regression analysis. The linear regression statistics are shown in Table 6.26 and the graphs in Fig 6.24. A linear relationship was observed between concentration and response, both with peak height and peak area. The response obtained from peak area measurement was larger that that from peak height and was used to provide increased sensitivity in later work.

Table 6.26 Linearity of detector response
(a) by peak height
(b) by peak area

	Compound	Linearity equation	Correlation coefficent
(a)	Diazepam	Y = 970.6 + 1875.6 X	1.000
	Carbostyril	Y = -299.0 + 1419.4 X	1.000
	MACB	Y = 366.3 + 995.5 X	1.000
(b)	Diazepam	Y = 20839 + 25164 X	1.000
	Carbostyril	Y = -10116 + 31400 X	0.999
	MACB	Y = 4634 + 28803 X	0.999

where X = concentration of diazepam (μg/ml)
and Y = peak height or peak area

Fig 6.24a(i) Linearity of detector response (peak height) to diazepam in the HPLC assay

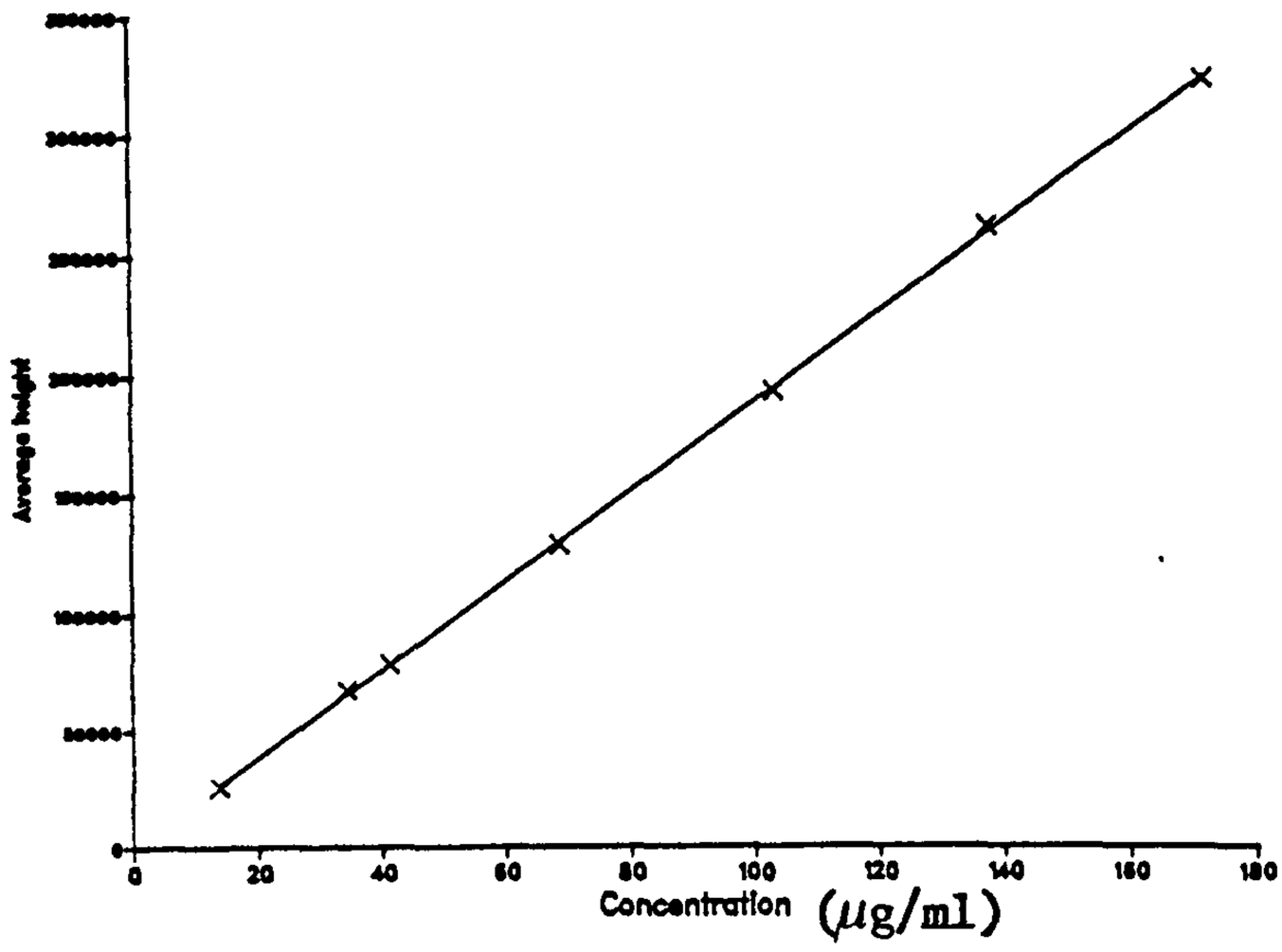


Fig 6.24(i) Linearity of detector response (peak-area) to diazepam in the HPLC assay

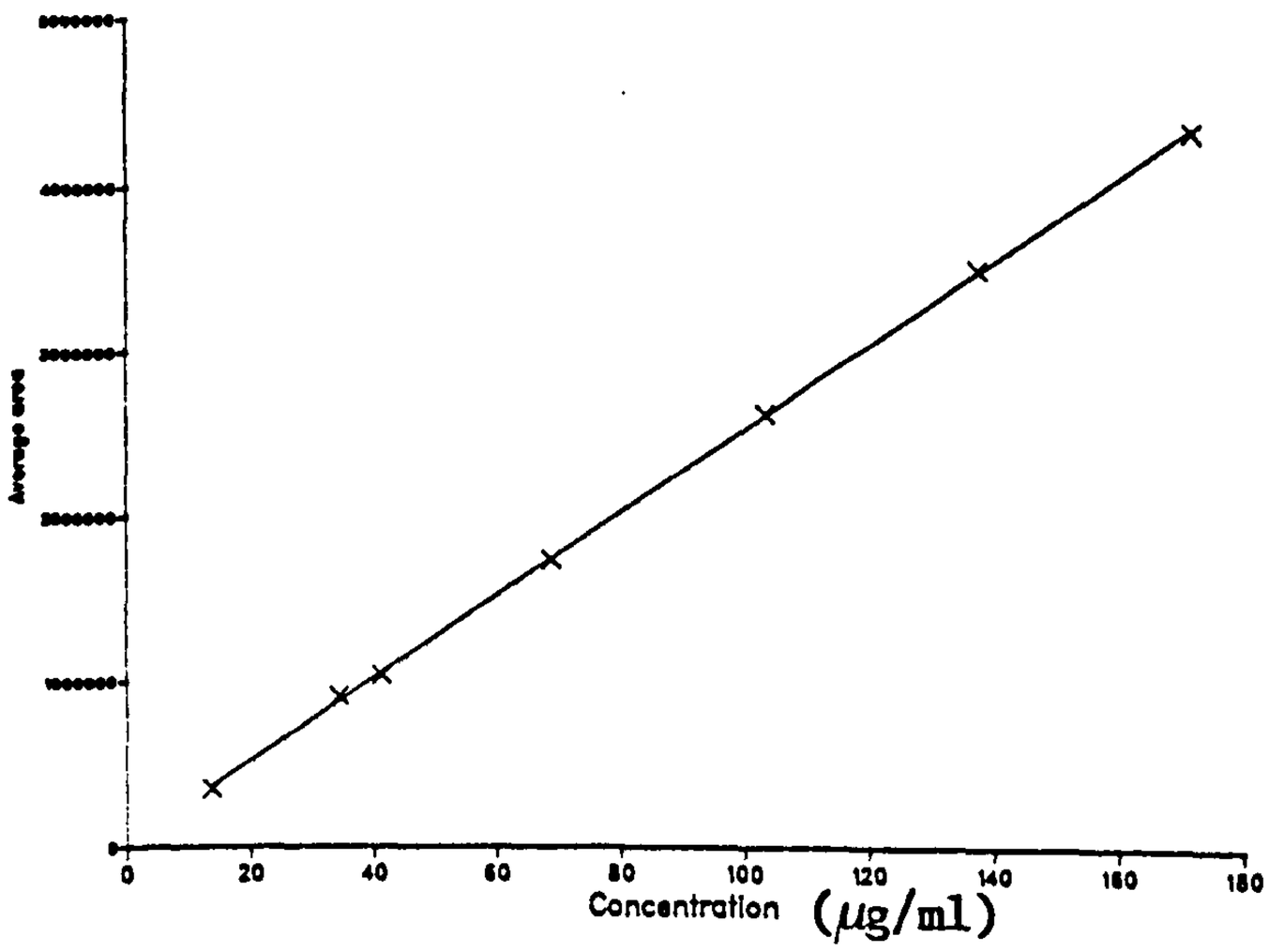


Fig 6.24a(ii) Linearity of detector response (peak-height) to carbostyril in the HPLC assay

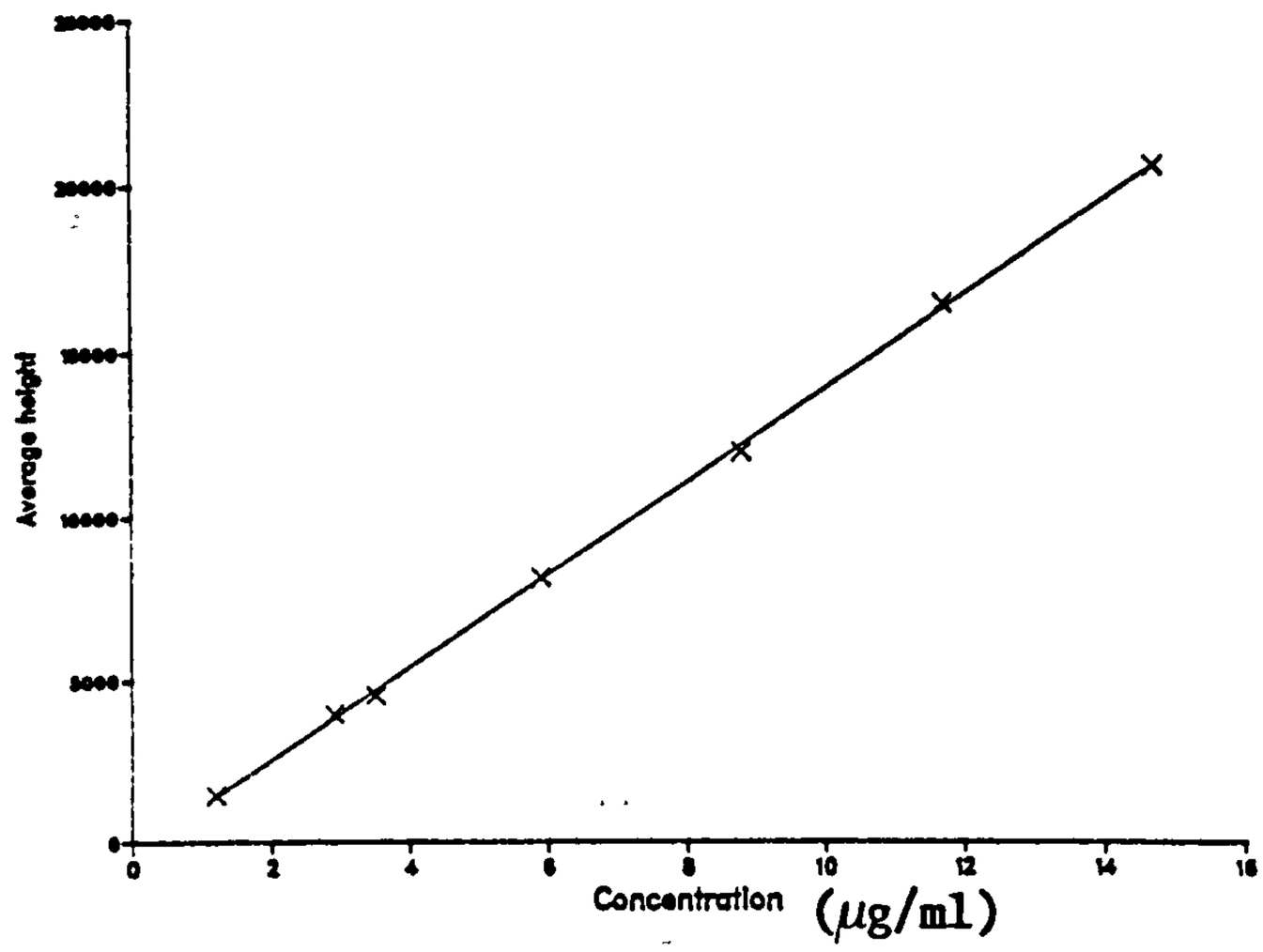


Fig 6.24(ii) Linearity of detector response (peak-area) to carbostyril in the HPLC assay

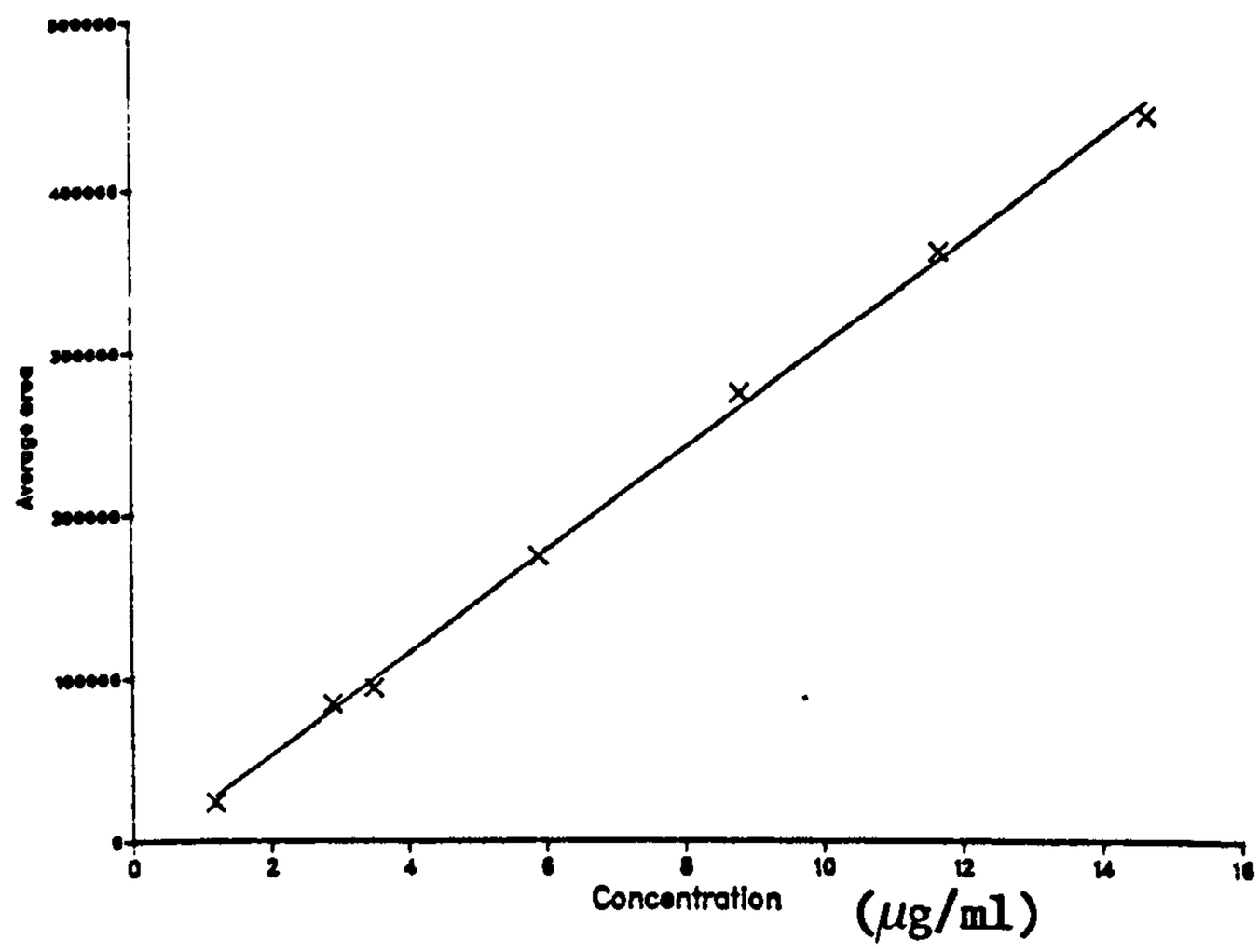


Fig 6.24a(iii) Linearity of detector response (peak-height) to MACB in the HPLC assay

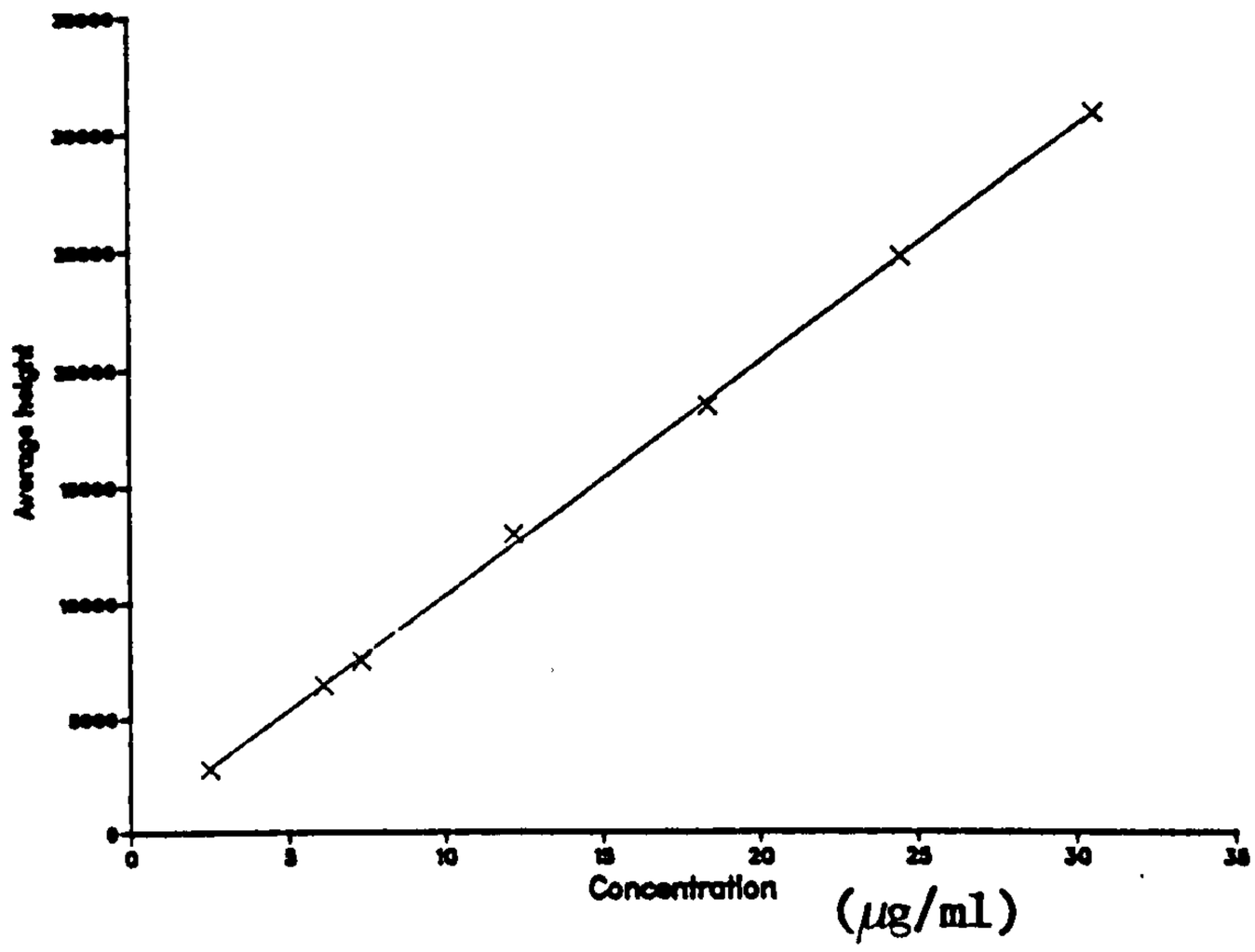
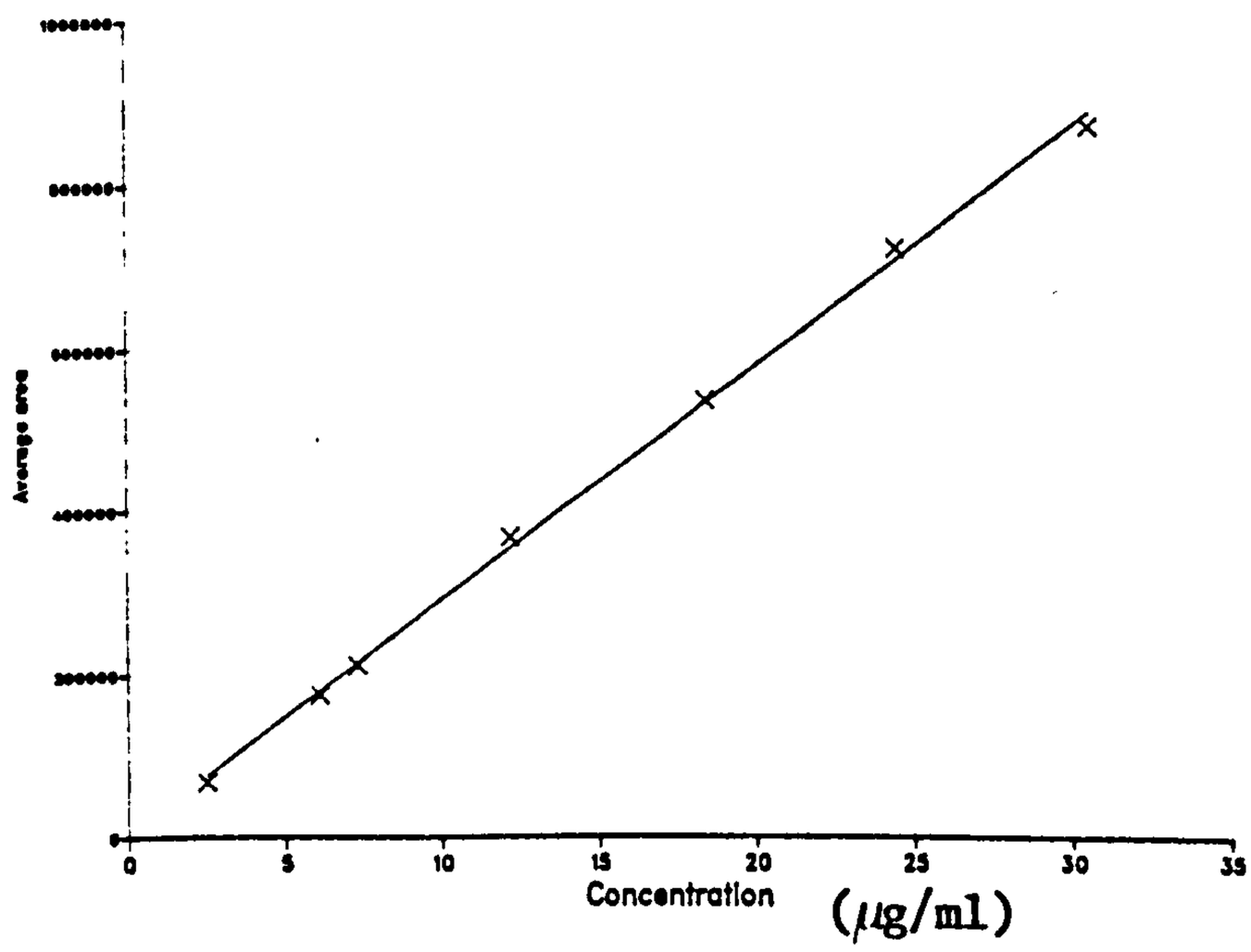


Fig 6.24(iii) Linearity of detector response (peak-area) to MACB in the HPLC assay



6.3.3 Development of the HPLC methods for analysis of formulations

6.3.3.1 Tablets

The HPLC method described in Section 4.3.3.1.1 was used for the analysis of tablets. Validation of the efficiency of extraction of diazepam from solid dosage forms was performed on capsules, and the results were assumed to be applicable also to the tablets.

6.3.3.2 Capsules

The extraction procedure used to prepare the test solutions involved dispersing a known weight of powder from the capsules in water, adding methanol, shaking and then making to volume with methanol. Variations in the initial volume of methanol, the length of time of shaking and the method of shaking were made to see if this had any effect on the recovery of diazepam (Section 4.3.3.2.1). The results are recorded in Table 6.27.

Table 6.27 Recovery of diazepam from capsules by various extraction methods

Volume of methanol used for extraction (ml)	Length of ultrasonic treatment (min)	Shaking time (min)	Number of replicates	Average recovery (mg/capsule)	% RSD
40	-	15	6	4.87	2.1
45	-	15	2	4.81	
45	5	25	2	4.83	
50	-	25	6	5.00	1.8
70	-	25	6	4.84	0.9

Ultrasonic treatment was used to improve the dispersion of powder in solution to ensure that all particles were wetted. It was thought that this might increase the recovery of diazepam by eliminating large clumps of non-wetted powder from which it may not be extracted. However, no increase in the recovery was obtained by this method.

On replicate extractions, the RSD values were found to be low, showing good precision. Little difference was seen for the recovery measured by each different extraction method, suggesting that all diazepam was being removed in each case.

The solvent used for extraction was also varied. The official assay method for diazepam capsules [B.P.,(1980)] uses 0.05 M methanolic sulphuric acid as solvent. Samples of capsule were extracted with both methanol and methanolic sulphuric acid to investigate if this had any effect on the recovery of diazepam from the formulation. As both extracts were injected directly onto the chromatographic system, the effect of the different solvents on the HPLC separation of a standard mixture was also examined. The chromatograms obtained for injection of calibration standards in methanol and in 0.05 M methanolic sulphuric acid are shown (Fig. 6.25). A small difference in retention time was noticed for injections made in the different solvents, although complete separation of the three components was achieved. This difference in chromatography is often seen when solutions are injected onto HPLC systems in different solvents from the mobile phase. It is normally due to some re-equilibration occurring when the compounds mix with the mobile phase.

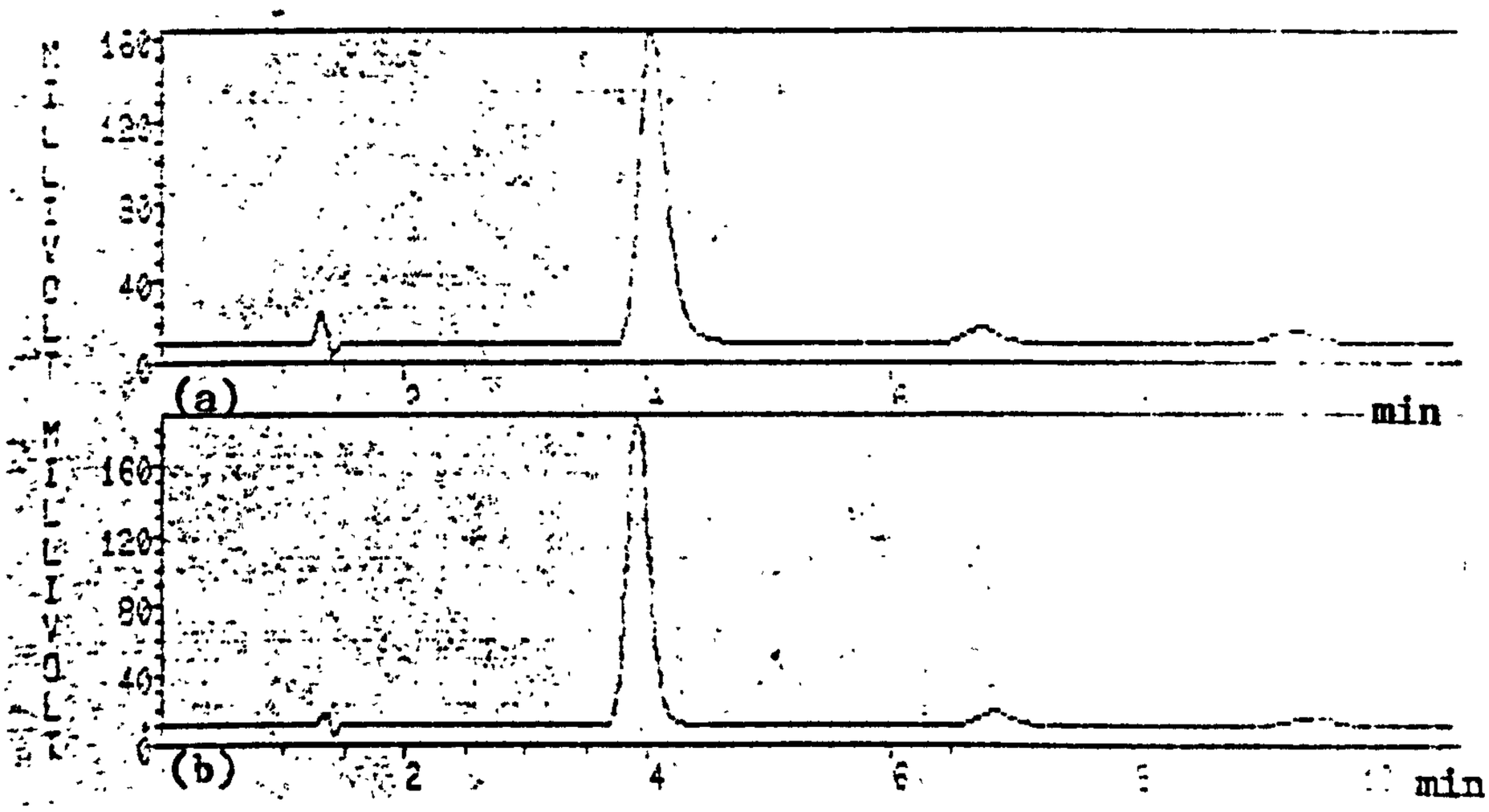


Fig. 6.25 Comparison of separation obtained when the standard is prepared in

(a) 0.05 M methanolic sulphuric acid

(b) methanol

6.3.3.3 Suppositories

No investigative work was required to develop an assay for this formulation. The suppository was dissolved in methanol and a dilution of this made. The resultant solution was injected onto the HPLC system.

6.3.3.4 Injection

No investigative work was required to develop the HPLC assay. Dilution of the injection with methanol provided a satisfactory solution for injection onto the HPLC system.

6.3.3.5 Syrup

Valium syrup contains only 2 mg diazepam in 5 ml, ie 2 mg of active drug in approximately 6 g of formulation. The formulation is complicated, containing a large number of excipients. Sample solutions of excipients which were thought likely to interfere were prepared and these were injected onto the HPLC system under the same conditions as diazepam, carbostyril and MACB, at concentrations similar to those expected in final dilutions of the formulation.

Chromatograms obtained for the flavouring and dye are shown in Fig. 6.26. The chromatogram of the extract from the syrup is also shown, which has a large peak at the solvent front due to components in the dye and flavouring, and to sodium benzoate. A small peak was seen in the chromatogram of the flavouring which had a retention time of 6.5 min. This peak has a similar retention time to that of carbostyril (6 min) and if it had been larger, might have interfered with its quantitation. However, as the peak is very small and is completely resolved, no overlap occurs. The flavouring peak does not have any significant effect on the measurement of carbostyril.

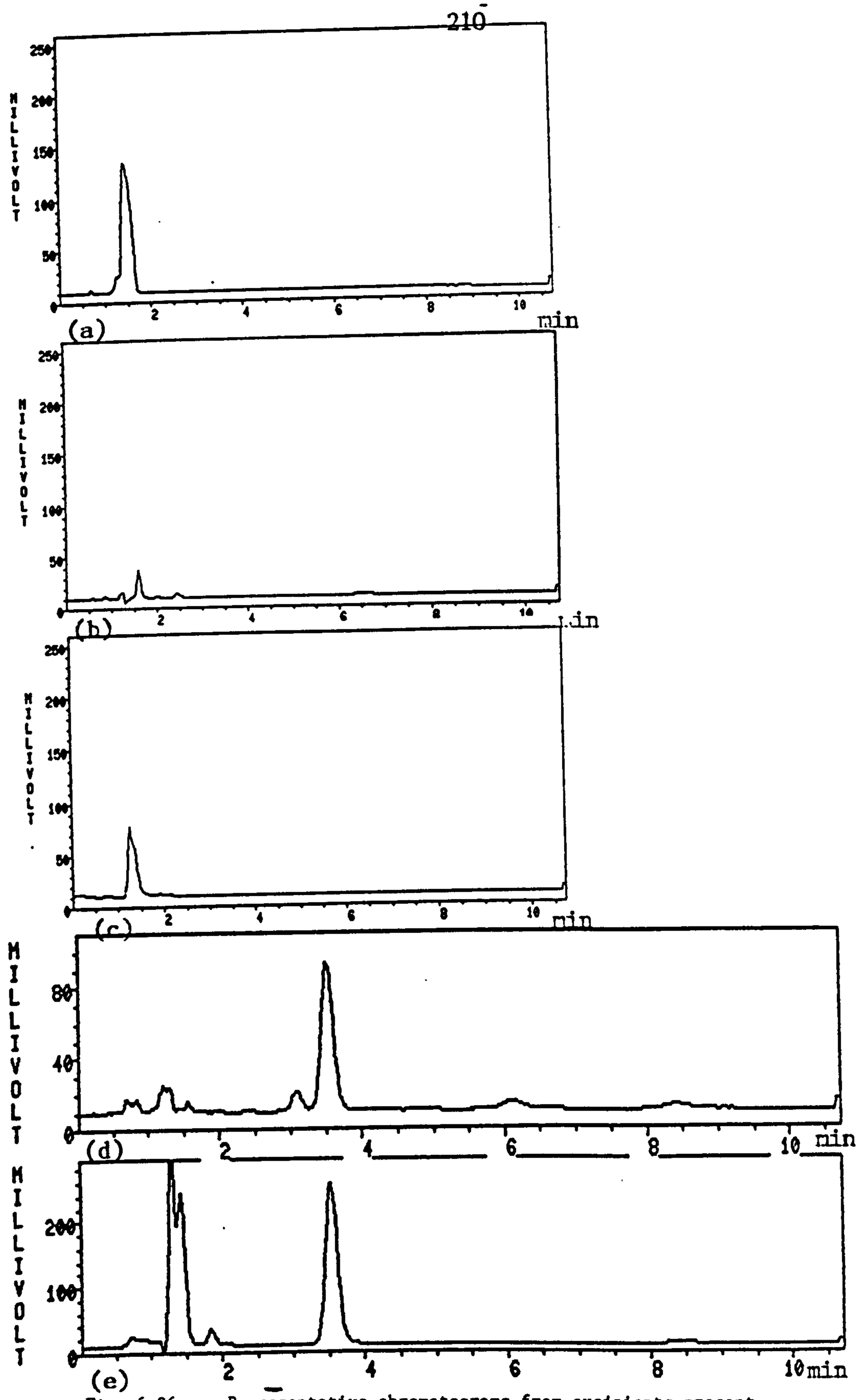


Fig. 6.26 Representative chromatograms from excipients present in the syrup formulation, a mixture of these and from a dilution of Valium syrup

(a) sodium benzoate
(b) erythrosine dye
(c) raspberry flavouring
(d) a mixture containing the above excipients plus diazepam, carbostyril, MACB and desmethyl diazepam
(e) a dilution of Valium syrup

When using methanol to dilute the syrup and extract the diazepam, it was found that the sugars which are present at high concentrations in the syrup formulation precipitated. The clear supernatant solution remaining was used for the HPLC assay.

6.4 APPLICATION OF DIFFERENCE UV SPECTROPHOTOMETRIC, DIRECT UV SPECTROPHOTOMETRIC AND HPLC METHODS TO DOSAGE FORMS

6.4.1 Tablets

The results from analysis of Valium tablets (5 mg) are shown in Table 6.28.

Table 6.28 Content of diazepam in fresh and aged samples of Valium tablets, 5 mg, measured by difference and direct UV spectrophotometry and HPLC

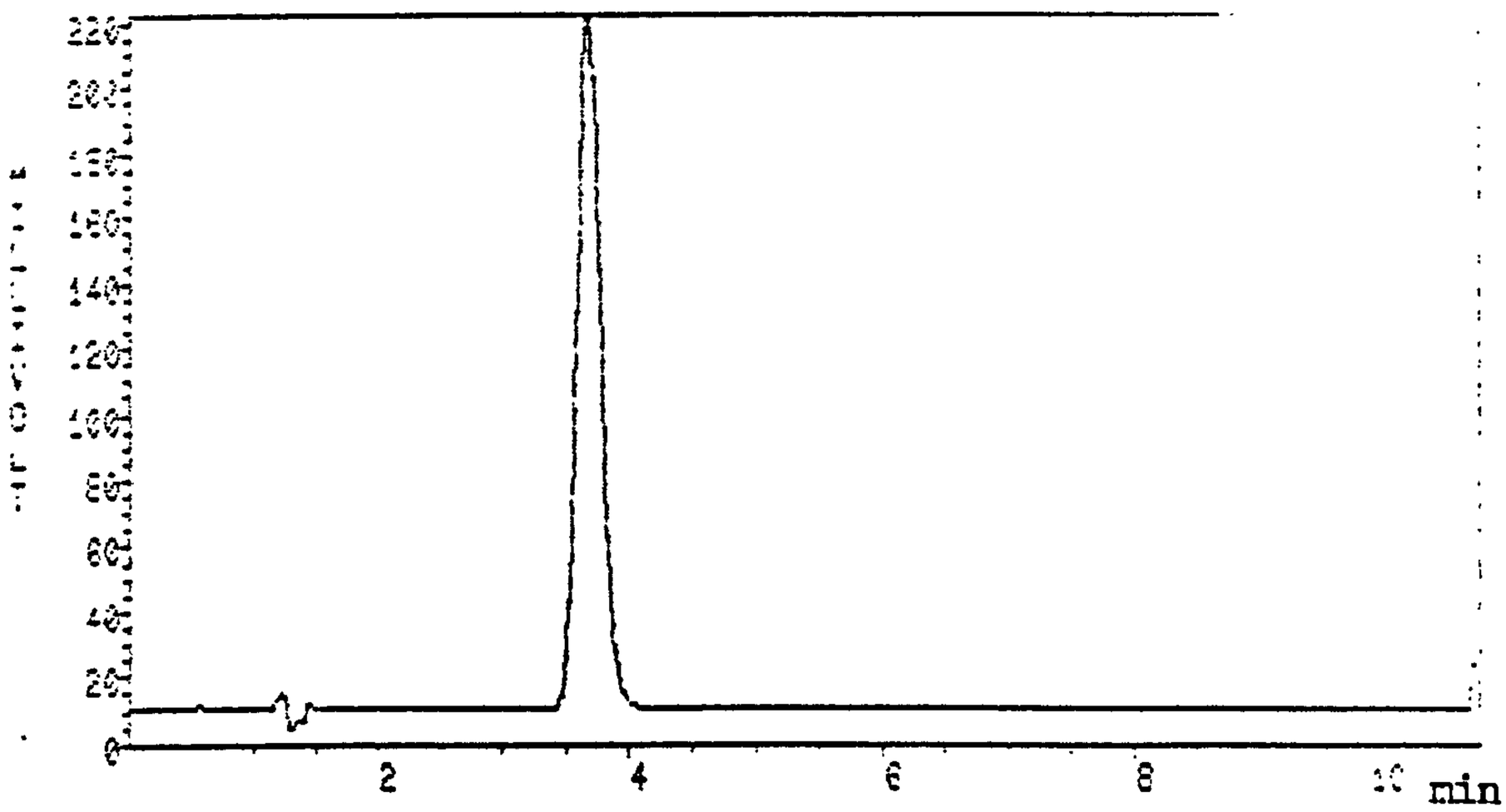
Storage conditions and age	Measured content of diazepam (mg/tablet)	Average content	% Nominal content
Difference UV spectrophotometric method			
Fresh	4.98, 4.98	4.98	99.6
7 years, 25°C	5.12, 5.13	5.13	102.6
7 years, 35°C	4.96, 4.95	4.96	99.2
UV spectrophotometric method			
Fresh	5.12, 5.09	5.11	102.2
7 years, 25°C	5.21, 5.19	5.20	104.0
7 years, 35°C	5.05, 5.02	5.04	100.8
HPLC method			
Fresh	4.90, 4.96, 4.93 4.99, 4.99, 4.80	4.93 *	98.6
7 years, 25°C	4.93, 4.89	4.91	98.2
7 years, 35°C	4.85, 4.92	4.89	97.8

* RSD 1.5% (n=6)

The difference UV spectra of the tablet extracts were superimposed on that of standard diazepam to check if any other absorbing species were present which would

result in a shift of the isosbestic points. The isosbestic points were co-incident (Fig. 6.10) indicating the absence of interference from other components in the tablet formulation.

By comparing the results of these assays, it was observed that the results obtained for the UV spectrophotometric method were higher than those for the difference UV method and HPLC method. This can be explained in part by the systematic error introduced by filtration as discussed in Section 6.1.3.1. Filtration of the solutions was shown to result in increased absorptivity due to concentration occurring by evaporation, (an increase of about 3%). This increase is of the same order as the difference measured between the difference UV method and the direct UV method. The difference UV method and the HPLC method also show different results. Analysing six samples by the HPLC method, an RSD of 1.5%. was obtained. Although this is acceptable, it is higher than would ideally be wanted for an HPLC assay procedure. This may explain the difference between the results obtained using the HPLC and difference UV methods. No degradation products were seen in the HPLC chromatograms of the fresh tablets or those stored at 25°C. In those stored at 35°C, some carbostyril was found, (Fig. 6.27). However, the amount of carbostyril was very low, equivalent to 0.004 mg/tablet or 0.08% of the nominal diazepam content. As no MACB was found, even in the stored samples, there would have been no interference from this substance in the direct UV measurement.



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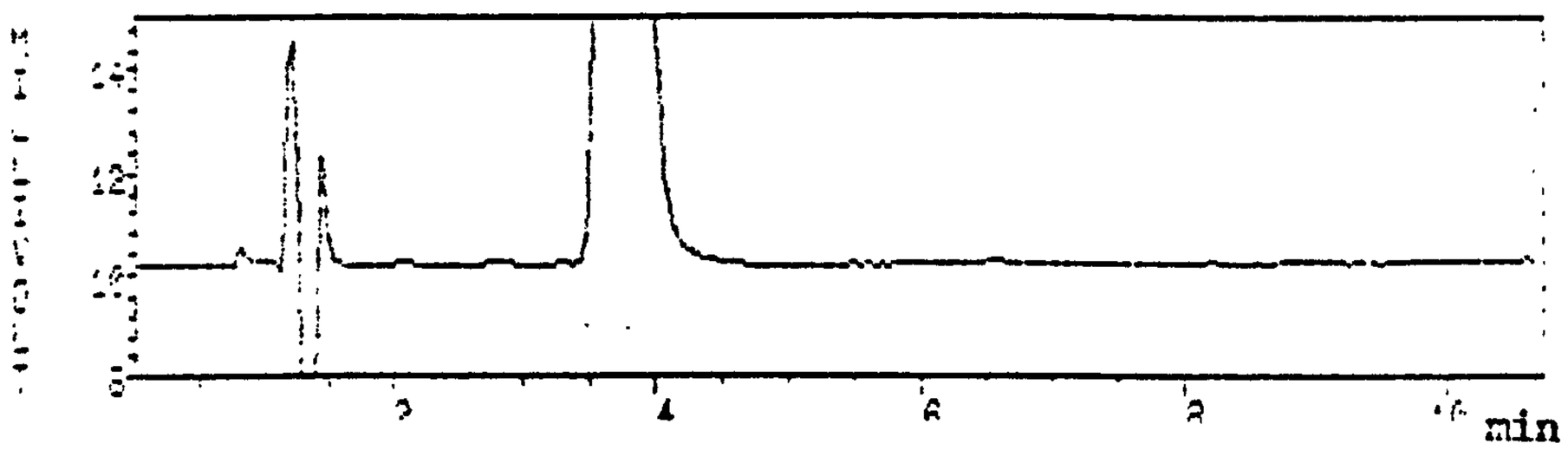

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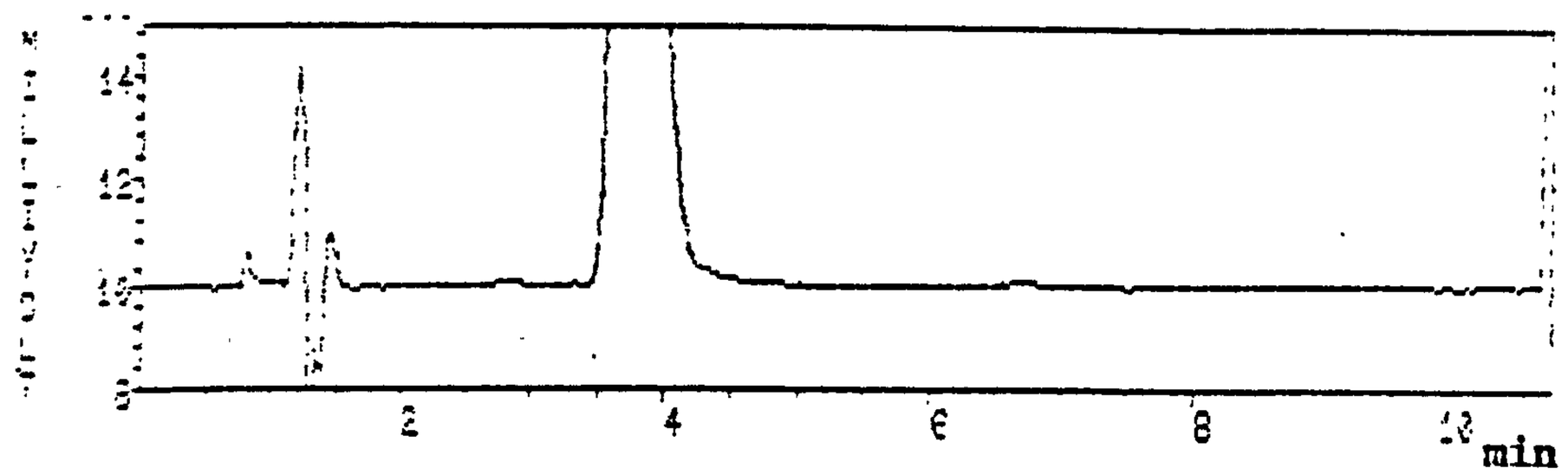


(c)

Fig. 6.27 Representative chromatograms from samples of Valium tablets,

(a) Fresh sample
(b) sample stored at 25°C
(c) sample stored at 35°C

6.4.2 Capsules

The results of the analysis of Valium capsules, (5 mg) are shown in Table 6.29.

Table 6.29 Content of diazepam in fresh and aged samples of Valium capsules, 5 mg, measured by difference and direct UV spectrophotometry and HPLC

Storage conditions and age	Measured content of diazepam (mg/capsule)	Average content	% Nominal content
Difference UV spectrophotometric method			
Fresh	4.80, 4.83	4.82	96.4
6 years, 25°C	4.86, 4.90	4.88	97.6
6 years, 35°C	4.86, 4.83	4.85	97.0
UV spectrophotometric method			
Fresh	4.86, 4.86	4.86	97.2
6 years, 25°C	4.92, 4.98	4.95	99.0
6 years, 35°C	4.94, 4.91	4.93	98.6
HPLC method			
Fresh	4.96, 4.94, 4.93 5.01, 4.98, 5.19	5.00 *	100.0
6 years, 25°C	4.94, 4.97	4.96	99.2
6 years, 35°C	4.80, 4.80	4.80	96.0

* RSD 1.9% (n=6)

The isosbestic points in the difference spectra of sample and standard solutions of diazepam were found to coincide in the capsule assay. The recovery from the UV spectrophotometric method was slightly higher than that for the difference UV spectrophotometric method. However, the results obtained from all three techniques were very similar. The values measured by HPLC for the capsules stored at elevated temperature were lower. On examination of the chromatographic traces there was no evidence of degradation products

or manufacturing impurities, (Fig. 6.28). Consequently the explanation for these slightly lower values is unknown. The RSD measured for the six replicate determinations was higher than expected (1.9%) and this suggests that there is some variation present in the assay method.

6.4.3 Suppositories

As this formulation has been on the market for a shorter time than the others samples had been stored for only 3 years. These were assayed by the three methods and the results for measurement of Valium suppository, (10 mg) are shown in Table 6.30. Two individual suppositories were analysed to provide duplicate determinations and the content of diazepam expressed in terms of concentration per g of suppository. This was to ensure that the differences measured between duplicate determinations was due to the different weights of sample taken and not to poor reproducibility of the techniques. An alternative approach would have been to slice each suppository into small pieces, which could then have been mixed to provide a bulked sample for assay.

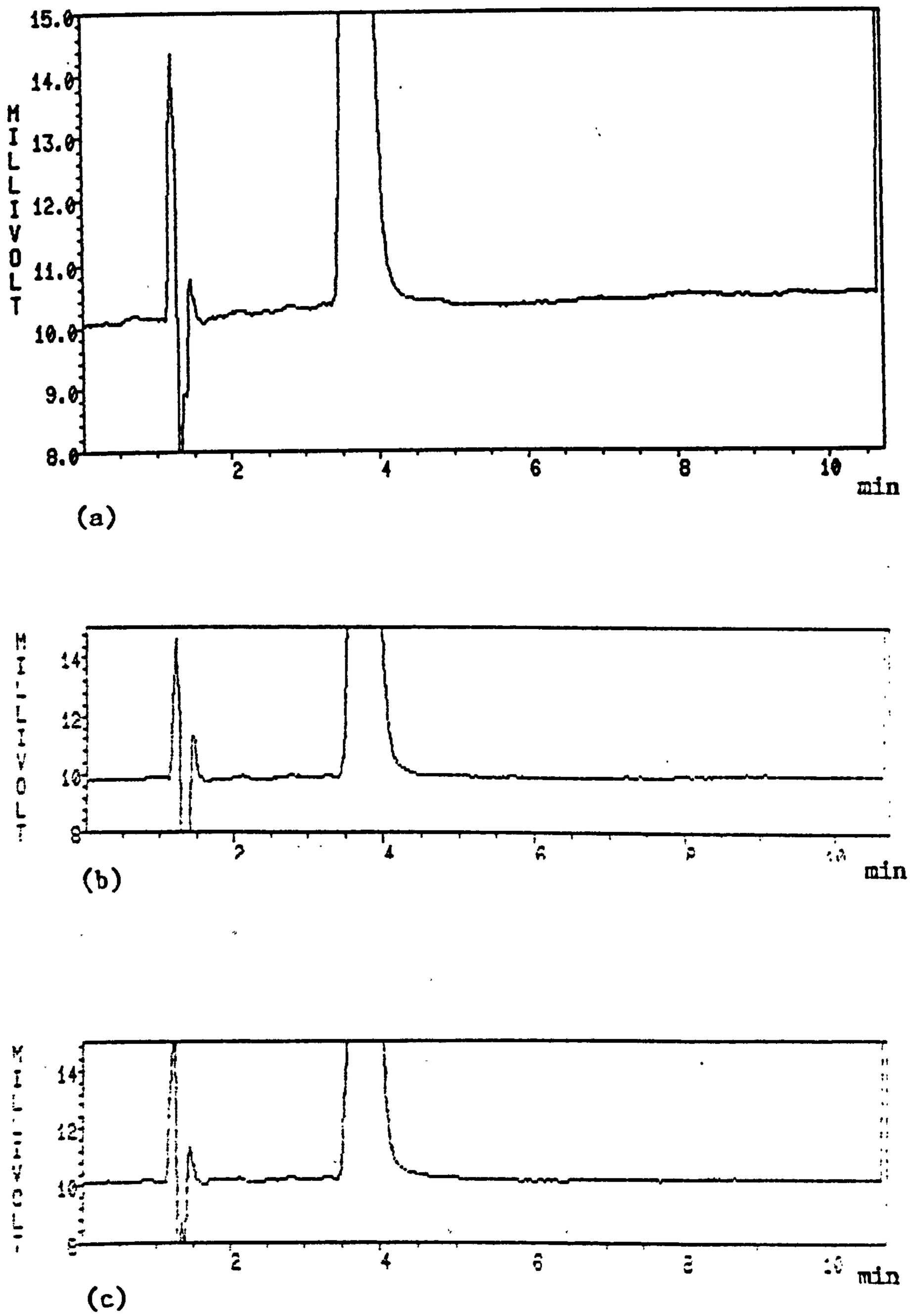


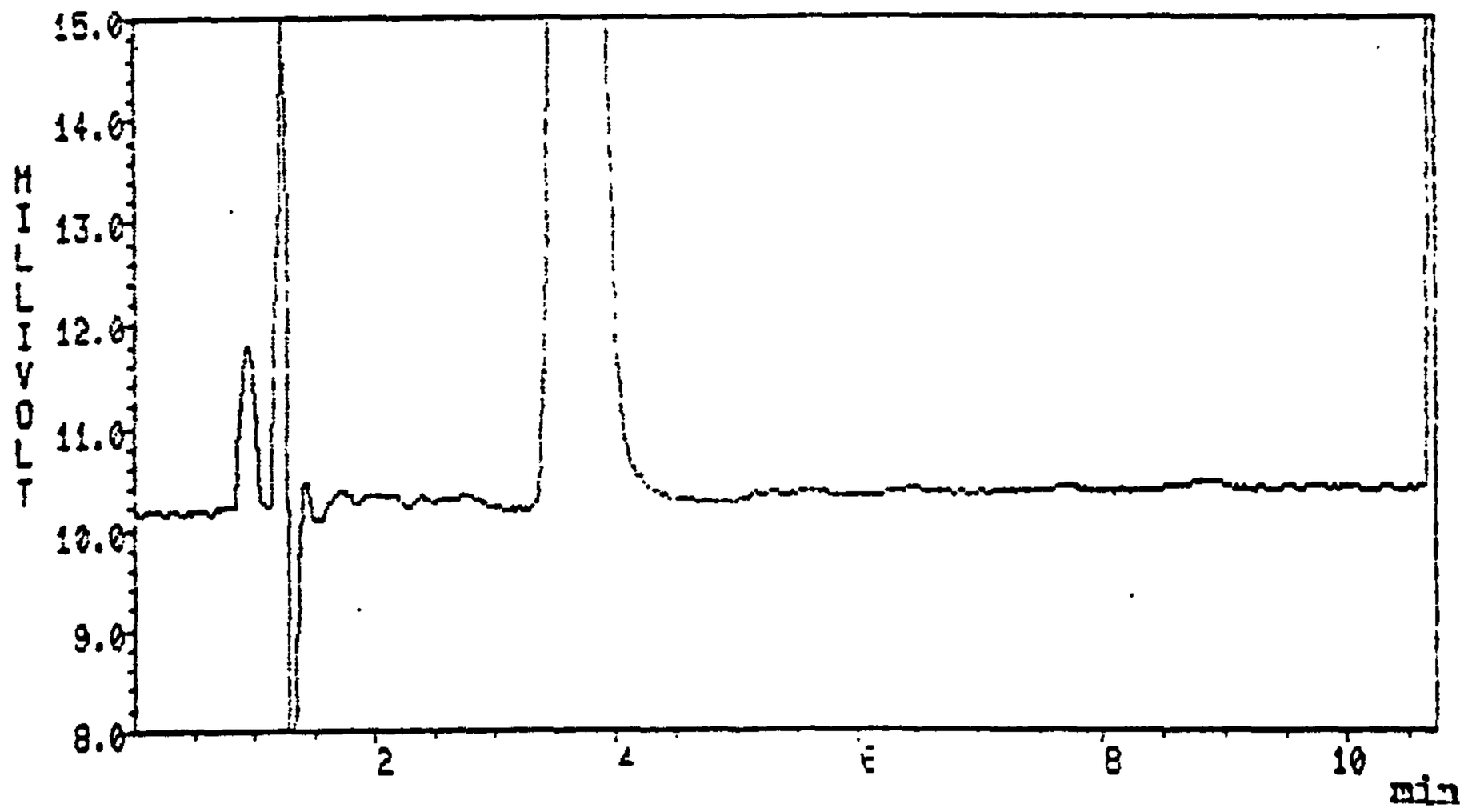
Fig. 6.28 Representative chromatograms from samples of Valium capsules,

(a) Fresh sample
(b) sample stored at 25°C
(c) sample stored at 35°C

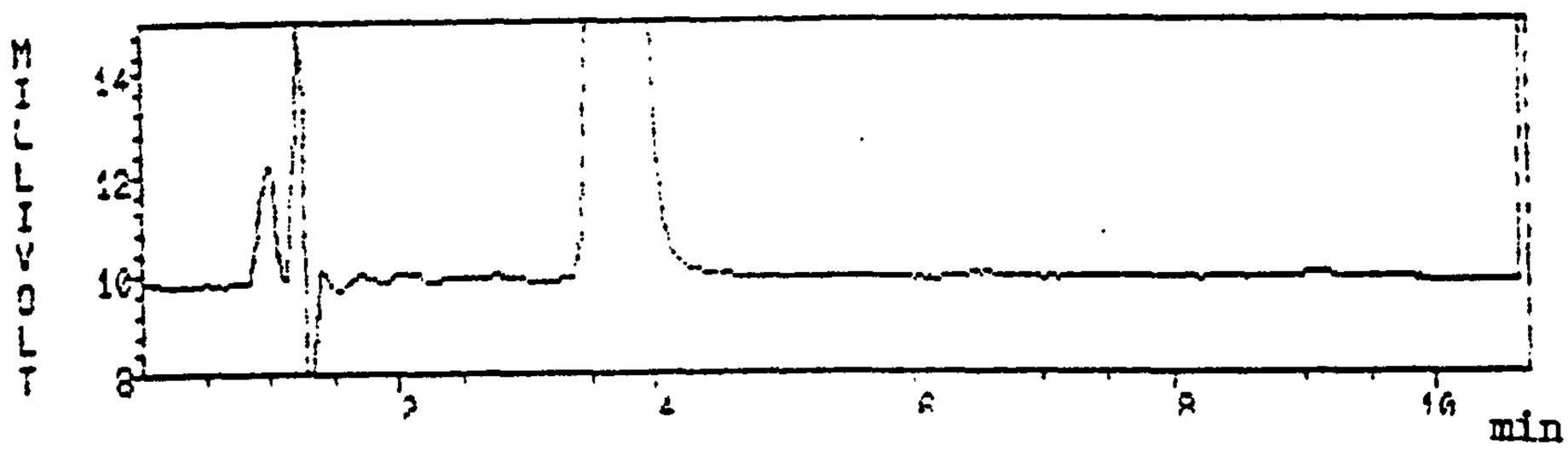
Table 6.30 Content of diazepam in fresh and aged samples of Valium suppository, 10 mg, measured by difference and direct UV spectrophotometry and HPLC

Storage conditions and age	Measured content of diazepam (mg/suppository)	Measured content of diazepam (mg/g suppository)	Average content of diazepam (mg/g suppository)
Difference UV spectrophotometric method			
Fresh	9.73, 9.87	3.72, 3.71	3.72
3 years, 25°C	9.72, 9.82	3.70, 3.74	3.72
3 years, 35°C	9.75, 9.75	3.70, 3.71	3.71
UV spectrophotometric method			
Fresh	10.14, 10.04	3.80, 3.80	3.80
3 years, 25°C	10.29, 10.18	3.88, 3.85	3.87
3 years, 35°C	10.18, 10.20	3.83, 3.88	3.86
HPLC method			
Fresh	9.81, 9.95	3.74, 3.79	3.77
3 years, 25°C	10.13, 9.96	3.80, 3.81	3.81
3 years, 35°C	10.00, 9.90	3.80, 3.76	3.78

The isosbestic points were again determined for the sample and standards in the difference UV assay and were found to coincide. Thus no interference was detected in the assay method. The results obtained in the UV spectrophotometric method were slightly higher than by the other two methods, but degradation products were not detected by HPLC to account for this, (Fig. 6.29). This difference may have been due to irrelevant absorption from formulation excipients in the direct UV spectrophotometric method, although these were not tested.



(a)



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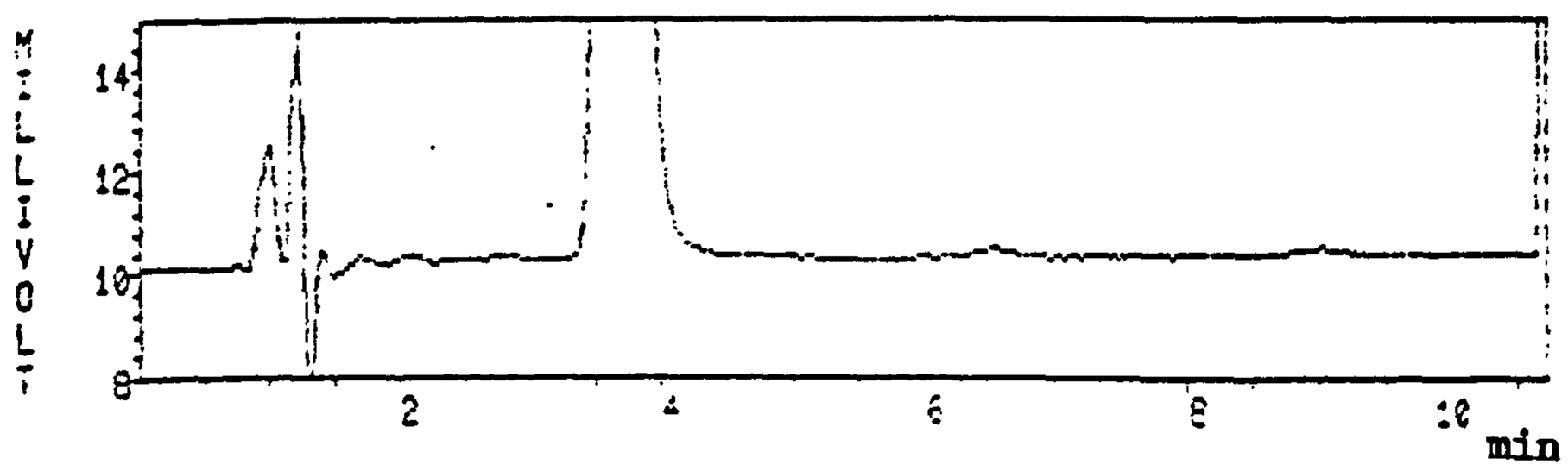


(c)

Fig. 6.29 Representative chromatograms from samples of Valium suppositories,

(a) Fresh sample
(b) sample stored at 25°C
(c) sample stored at 35°C

6.4.4 Injection

The three assay methods were applied to samples of Valium injection (20 mg/4 ml) both fresh and stored samples. The results of these assays are shown in Table 6.31. The measured content of diazepam was found to decrease with time, particularily in the samples stored at 35°C.

Table 6.31 Content of diazepam in fresh and aged samples of Valium injection, 20 mg/4 ml, measured by difference and direct UV spectrophotometry and HPLC

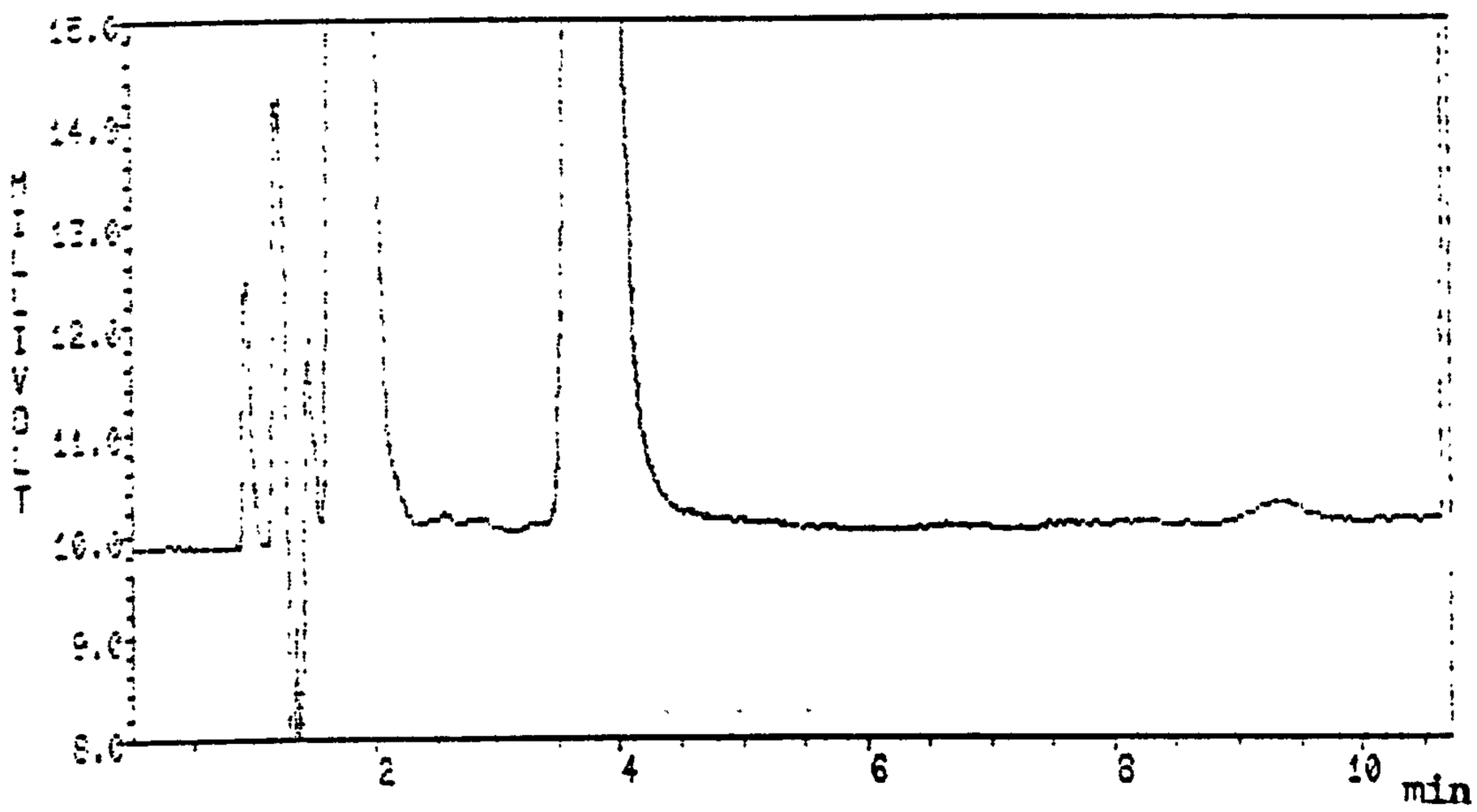
Storage conditions and age	Measured content of diazepam (mg/ml)	Average content (mg/ml)	% Nominal content
Difference UV spectrophotometric method			
Fresh	5.06, 5.02, 4.99	5.02	100.4
7 years, 25°C	5.03, 4.98, 5.01	5.01	100.2
7 years, 35°C	4.89, 4.88 4.84, 4.80	4.85	97.0
UV spectrophotometric method			
Fresh	5.00, 4.98	4.99	99.8
7 years, 25°C	4.90, 4.88	4.89	97.8
7 years, 35°C	4.78, 4.83	4.81	96.2
HPLC method			
Fresh	5.03, 5.02	5.03	100.6
7 years, 25°C	5.08, 5.13	5.11	102.2
7 years, 35°C	4.81, 4.80	4.81	96.2

The presence of carbostyril and MACB was detected in the HPLC chromatograms of the stored samples. In the injection stored for 7 years at 25°C, 0.066 mg/ml MACB was measured (1.3% of the nominal content of diazepam). The sample stored for the same period of

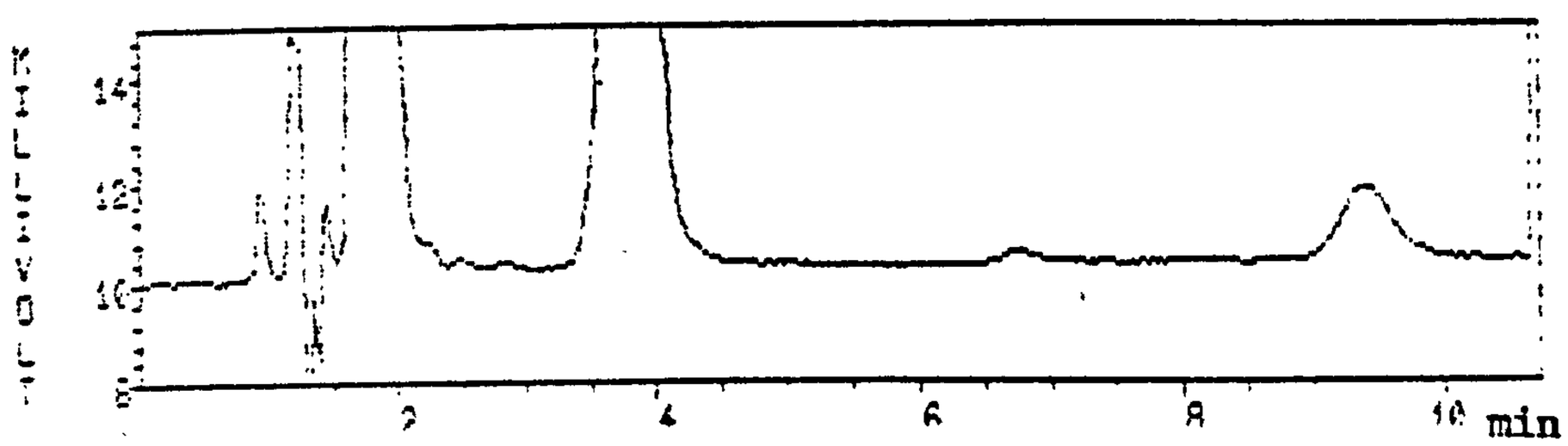
time at 35°C showed 0.025 mg/ml carbostyril and 0.245 mg/ml MACB (0.5% and 4.9% respectively of the nominal diazepam content), Fig. 6.30.

No interference was detected on examination of the isosbestic points of sample and standard diazepam in the difference spectrophotometric assay, Fig. 6.31. However there was no significant difference in results obtained by the direct UV spectrophotometric assay and the difference UV spectrophotometric assay. As a large amount of MACB was present in the samples of stored injection, it might have been expected that this would affect the results using the direct measurement. At 368 nm, the contribution to the measured absorbance from MACB would be approximately 25%, if present in equimolar quantities (Fig. 6.19). Thus with almost 5% present (measured by the HPLC assay), an increase of at least 1% in the measured absorbance would have been expected.

The content of diazepam measured in the injection was found to be similar on measurement by all three methods of analysis for the fresh samples and for those stored for 7 years at 35°C although the results from the samples stored for 7 years at 25°C were varied, from 97.8% to 102.2%. As MACB and carbostyril were found to be present in some samples, it had been expected that differences in the content of diazepam measured by these different techniques would have been found. The absence of a significant difference may be due to the errors involved in the measurements. Lack of reproducibility of extraction and loss of diazepam at this stage may mask any differences which might otherwise be seen. More



(a)



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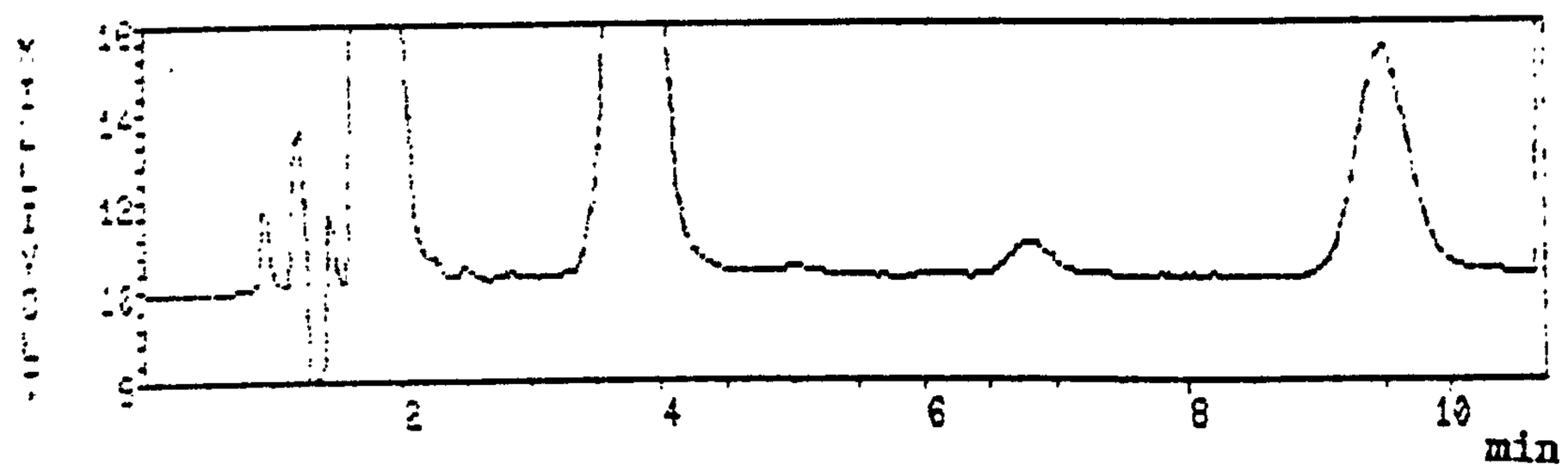


(c)

Fig. 6.30 Representative chromatograms from samples of Valium injection,

(a) Fresh sample
(b) sample stored at 25°C
(c) sample stored at 35°C

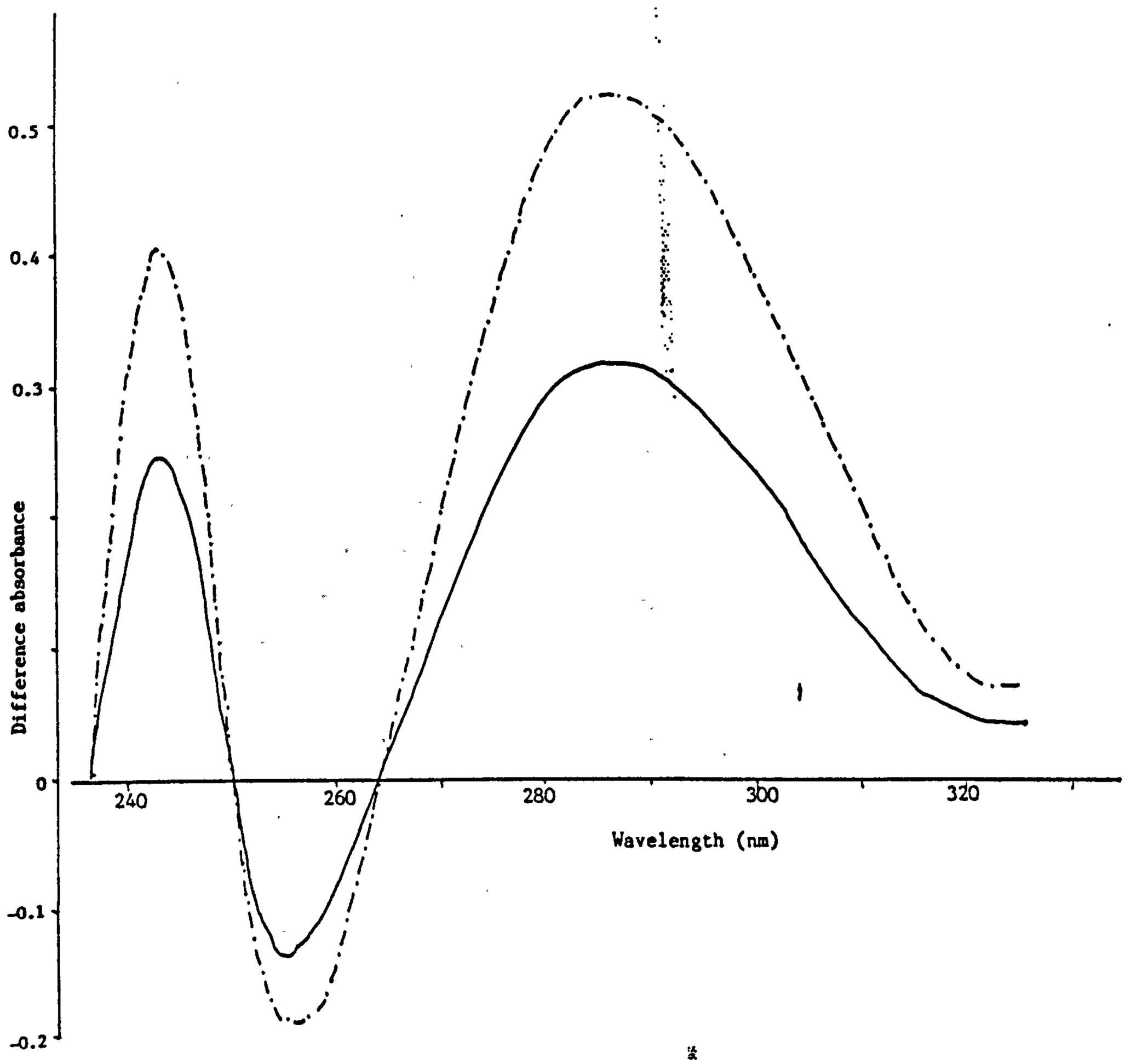


Fig. 6.31 Difference absorption spectrum, pH 2.6 vs pH 5.4, for diazepam and a dilution of Valium injection

—·—·—·— Difference absorption spectrum for diazepam

——— Difference absorption spectrum for a dilution of Valium injection

samples require to be analysed by each method to provide a larger sample to assess the variability of each technique and allow comparison of the techniques to be made.

The increase in MACB measured in stored samples, and the presence of carbostyril in the elevated temperature samples agree with previous results [Cartensen et al.,(1971)]. This paper reports finding much higher concentrations of MACB than carbostyril, especially at low temperatures. For the HPLC assay, the reduction in diazepam content measured can be related to the increase in the content of degradation products. Increased levels of degradation products had been expected in the injection formulation. As the injection is presented as a liquid, hydrolysis is more likely to occur than in the solid dosage forms, although the vehicle is a mixed solvent with propylene glycol in addition to water, which has a protective effect, [Connors et al.,(1979)].

6.4.5 Syrup

A satisfactory difference UV method for this formulation proved extremely difficult to develop. Therefore there are no results available on the content of diazepam in syrup using difference UV spectrophotometry. Results obtained from analysis of samples of Valium syrup (2 mg/5 ml) by direct UV spectrophotometry and HPLC are quoted in Table 6.32.

Table 6.32 Content of diazepam in fresh and aged samples of Valium syrup 2 mg/5 ml, measured by direct UV spectrophotometry and HPLC

Storage conditions and age	Measured content of diazepam (mg/ml)	Average content (mg/ml)	% Nominal content
UV spectrophotometric method			
Fresh	0.430, 0.431	0.431	107.8
7 years, 25°C	0.389, 0.385	0.387	96.8
HPLC method			
Fresh	0.443, 0.437, 0.435 0.445, 0.447, 0.437	0.441 *	110.3
7 years, 25°C	0.387, 0.395 0.404, 0.403	0.397 **	99.3

* RSD 1.1% (n=6)
** RSD 2.0% (n=4)

Both MACB and carbostyril were found in the syrup which had been stored for 7 years. The levels detected were 0.0025 mg/ml carbostyril and 0.0060 mg/ml MACB (0.63 and 1.5% of the nominal content of diazepam), Fig. 6.32.

For the syrup formulations the initial content measured was high, when expressed as a percentage of the nominal content. However, to allow for decomposition during storage the manufacturing formula contains an overage, 0.44 mg/ml (equivalent to 110% of the labelled content). This agrees with the results obtained in Table 6.32 for the initial samples, but the recovery from the aged sample is less than expected from the level of degradation products found. Possible reasons for this may be that other degradation products which are not detected are

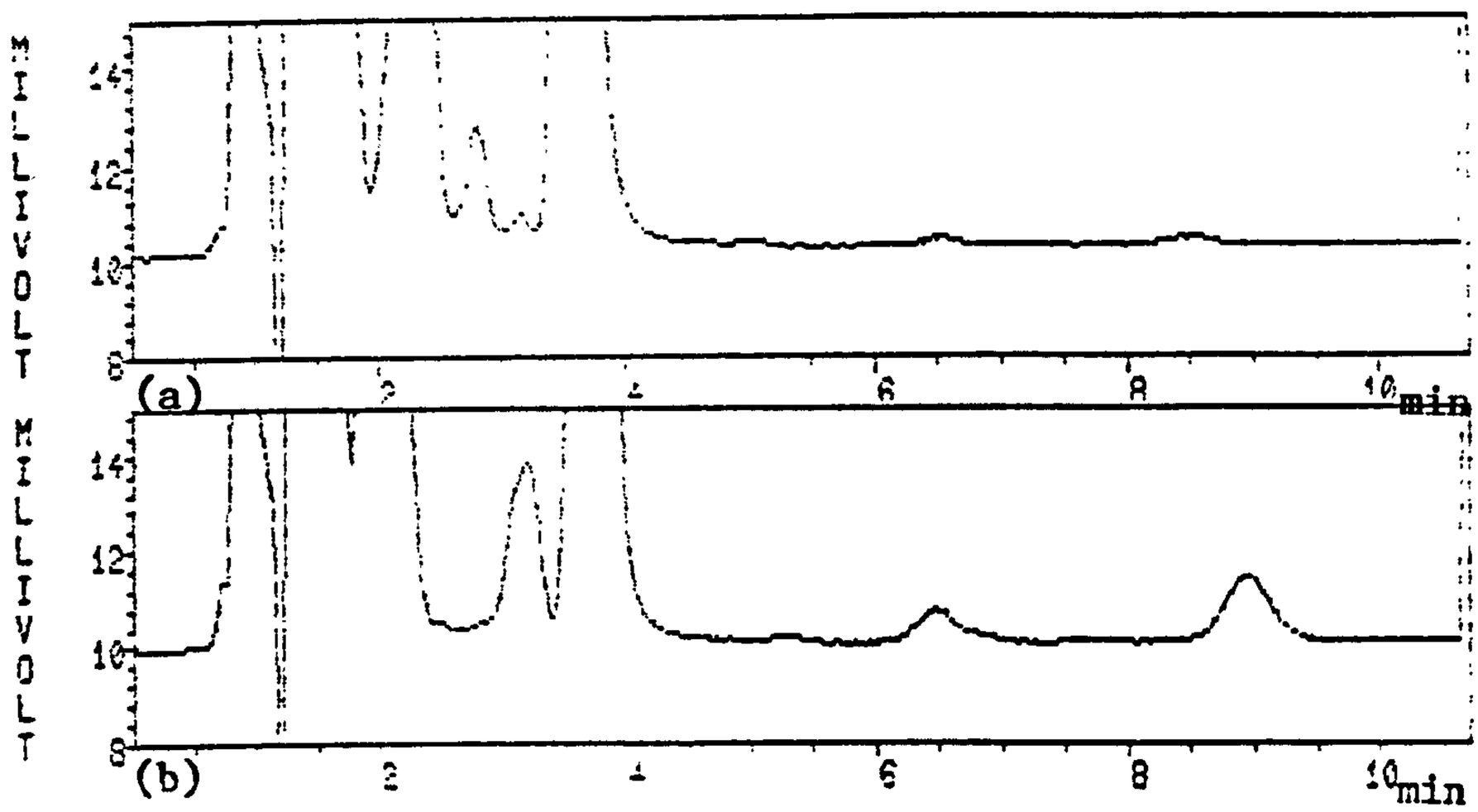


Fig. 6.32 Representative chromatograms from samples of Valium syrup,

(a) Fresh sample

(b) sample stored at 25°C

present or that some reaction occurs between the syrup excipients and diazepam on storage. This could result in poor recovery of diazepam from the aged samples. Further work would be required to investigate this.

7 RESULTS AND DISCUSSION OF THE KINETICS OF THE HYDROLYSIS REACTION OF DIAZEPAM

7.1 RESULTS OF THE DEVELOPMENT AND VALIDATION OF A GAS CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF DIAZEPAM AND MACB

While the Δ A UV spectrophotometric assay was found to be specific for diazepam in the presence of MACB, it was not possible to measure both diazepam and MACB by this method. Thus a GC method was developed to allow analysis of mixtures containing both MACB and diazepam.

7.1.1 Initial Development

A column packed with 3% OV-17 on Gas Chrom Q was used for separation of diazepam and MACB, at an column oven temperature of 275°C. A previous publication had reported the use of a similar chromatographic system to this for the routine evaluation of benzodiazepine drugs, for analysis of the benzophenone content of tablets [Black et al.,(1981)]. Injections of a mixture of the two components in methanol were made and the chromatographic conditions optimised to obtain separation. The methanol solvent front tailed badly and interfered with the peak due to MACB, injections of chloroform (Fig. 7.1) indicated that chloroform gave a less tailing solvent front and consequently was selected for use in subsequent work. To maximise the sensitivity of the assay for diazepam and MACB, the use of a system with the shortest possible run time was required. With faster elution of the components, sharper, more easily detectable peaks

Fig. 7.1 Tailing in gas-liquid chromatography from methanol and chloroform

(a) mixture of MACB and benztropine mesylate in methanol
(b) chloroform

would be obtained. For increased accuracy and reproducibility it was necessary to ensure a level baseline. This would give a minimum of interference in the determination of peak height and area. However, to maximise the peak height a compromise was required, resulting in the peak due to MACB being eluted on the tail of the solvent front. The optimum conditions found are specified in the method, (Section 5.1.1) and are reproduced below:

Column	2 m, 3% OV-17 on Gas Chrom Q
Oven temperature	275°C
Detector temperature	300°C
Injector temperature	300°C
Carrier gas	Nitrogen (30 ml/min)
Detection	Flame ionisation

When samples were chromatographed under these conditions, the retention times for MACB and diazepam were 2.2 and 6.2 minutes respectively.

7.1.2 Choice of Internal Standard

As injectors for GC lack precision, quantitative work demands the use of an internal standard. Ideally an internal standard should be stable, have a retention time close to the peaks of interest, but be completely resolved from these and be pure and readily available.

Also, in this particular case, it should ideally elute between MACB and diazepam and be soluble in chloroform.

From a publication by Moffat(1975) the Kovat's indices of related benzodiazepine compounds on a column packed with SE-30 (a silicone polymer with 100% methyl residues) were as follows:- diazepam 2410, chlordiazepoxide 2790, oxazepam 2335, nitrazepam 2675. These compounds and some other chemical and drug substances were tested on the column used for this work, packed with OV-17 (similar to SE-30, containing 50% methyl residues and 50% phenyl residues), and their suitability as an internal standard was assessed. The compounds used were all readily available in a pure state (AnalaR, Sigma or B.P. grades). Chlordiazepoxide had a very long retention time and a poor peak shape, but both oxazepam and nitrazepam eluted very quickly, on the tail of the solvent front. The long chain alkanes were also unsatisfactory with C_{22}, docosane, and C_{24}, tetracosane, eluting in less than 1.5 min, again on the solvent front, and C_{36}, N-hexatriacontane, taking around 32 min. The retention times of other compounds which gave better peak shape and more suitable retention times, together with diazepam and MACB and the retention times relative to diazepam are listed in Table 7.1. On a day-to-day basis the GC system conditions can vary even when the instrument settings have been maintained. This makes it difficult to compare retention times obtained on different days. The use of Kovat's indices or relative retention times overcomes these variations.

Table 7.1 Retention times of compounds injected onto the chromatographic system

Compound	Retention time (min)	Relative retention time (RRT)
MACB	2.2	0.36
Diazepam	6.2	1.00
Benzotropine mesylate	3.4	0.56
Harmine	3.8	0.59
Di-(2-ethylhexyl)phthalate (DEHP)	4.1	0.56

The retention time of benztropine mesylate was satisfactory, but after preliminary investigations the compound was found to be unsuitable. On injecting a mixture containing diazepam, benztropine mesylate and MACB several times, Fig. 7.2, it was found that the peak height and peak area ratios between MACB and diazepam were reproducible, but those of MACB and diazepam to benztropine mesylate were not, Table 7.2. This lack of reproducibility suggests that variation in the volatilisation of benztropine mesylate was occurring.

Table 7.2 Replicate injection of a mixture containing benzotropine mesylate as internal standard

	Peak height ratios			Peak area ratios		
	MACB/BM	DIAZEPAM/BM	MACB/DIAZEPAM	MACB/BM	DIAZEPAM/BM	MACB/DIAZEPAM
	1.149	0.731	1.572	0.749	0.905	0.826
	1.171	0.752	1.552	0.760	0.925	0.822
	1.227	0.790	1.552	0.806	1.001	0.806
	1.123	0.734	1.529	0.716	0.857	0.836
	1.253	0.820	1.528	0.834	1.067	0.782
	1.232	0.810	1.522	0.830	1.030	0.806
Average	1.193	0.773	1.543	0.783	0.964	0.813
RSD	4.4%	5.0%	1.3%	6.1%	8.4%	2.4%

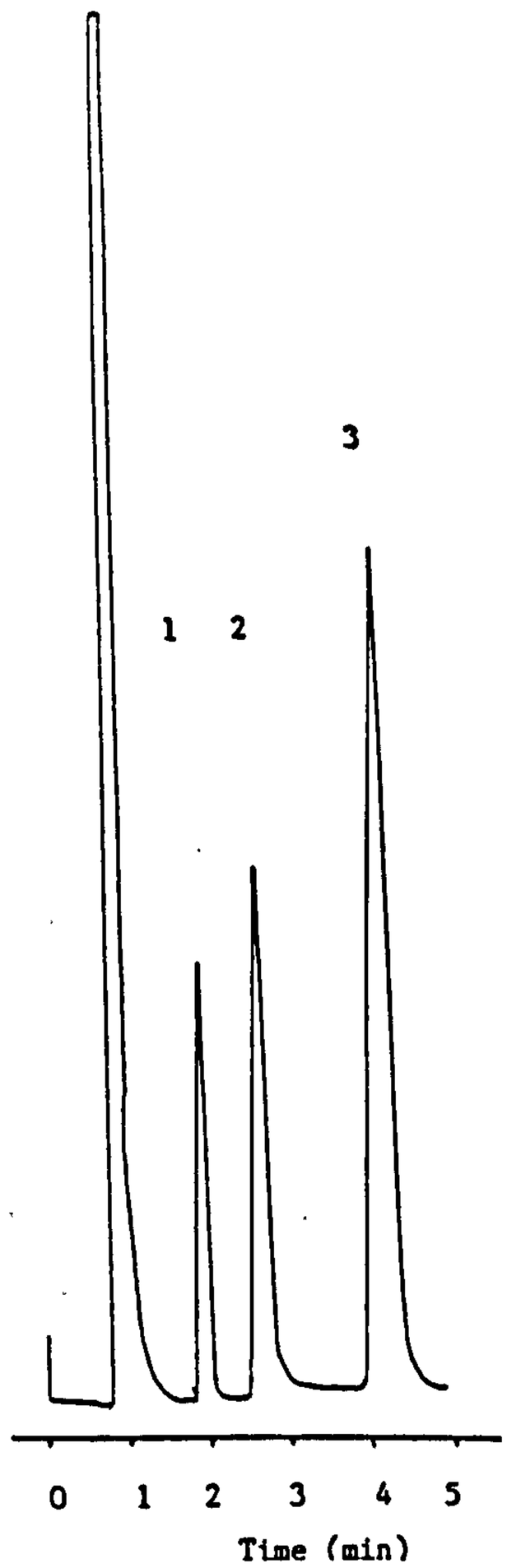


Fig. 7.2 Gas-liquid chromatography of MACB, benztropine mesylate and diazepam, where peak 1 is MACB, peak 2 is benztropine mesylate and peak 3 is diazepam

Di-(2-ethylhexyl)phthalate (DEHP) was found to be the most suitable of the other compunds tested. Injection of a mixture of MACB, diazepam and DEHP showed that all three compounds were adequately separated (Fig. 7.3). Thus, DEHP was chosen as the internal standard for the gas chromatographic assay of diazepam and MACB.

The choice of internal standard (IS) concentration to be used in the sample preparation was investigated. The assay method was to be used to analyse samples from a solution of diazepam undergoing hydrolysis, which is prepared and treated as described in Section 5.3.2. For the purposes of calculation of the rate constant of the hydrolysis reaction, the absolute concentrations of diazepam are not required. The initial value at zero time (t_o) was identified as 100% diazepam, the amounts present as the reaction proceeds being calculated relative to this. The initial concentration of diazepam in the solution subjected to hydrolysis was approximately 0.7 mg/ml and if all of this degraded to MACB, then there would be 0.6 mg/ml MACB present in the solution at the end of the reaction. Solutions containing these concentrations of diazepam and MACB were prepared and injected onto the GC system. The peak heights obtained were compared to those from injections of varying concentrations of the IS (DEHP). A concentration of 1 mg/ml DEHP gave a peak height similar to that of MACB (0.6 mg/ml) and was selected as the concentration of IS to be used. As peak height ratios were used, (measured on the chromatogram obtained by using a chart recorder), it was important that all three peaks

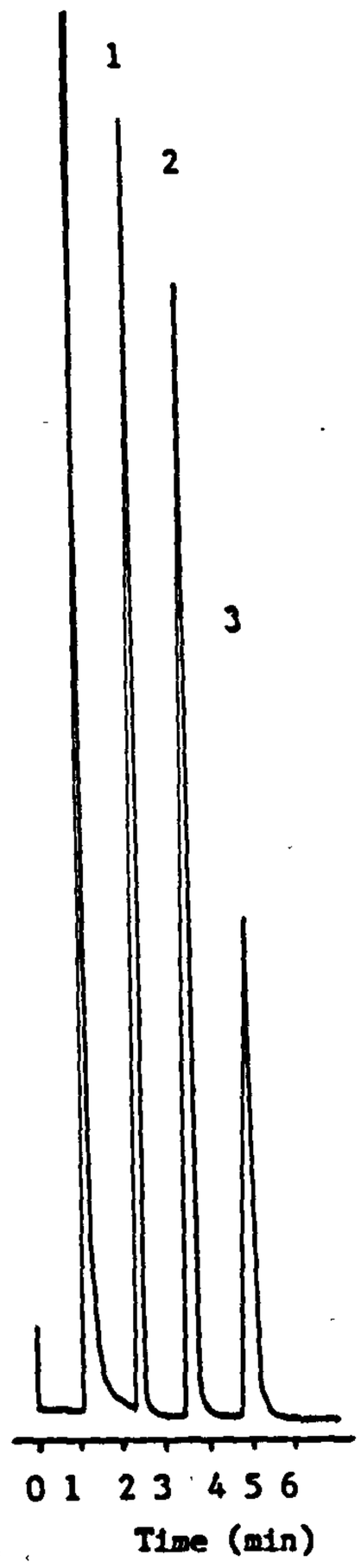


Fig. 7.3 Gas-liquid chromatograms of MACB, DEHP and diazepam, where peak 1 is MACB, peak 2 is DEHP and peak 3 is diazepam

could be measured using the same attenuation setting, and that the largest peaks on this setting were at almost full scale deflection. In this way it was hoped that any error introduced by measurement of the peak height would be minimised.

7.1.3 Development of the assay method

7.1.3.1 Investigation of the extraction stage

To ensure that the extraction of diazepam and MACB from the reaction mixture was quantitative under the conditions used, a solution of diazepam and MACB was diluted in water, and in a mixture of 0.1 M hydrochloric acid and 0.9 M sodium chloride (Section 5.1.3.1). Samples from each of the water and acid dilutions were extracted either once, twice or three times with chloroform. At least six replicate injections were made of each of the extracts and the peak height and area ratios were calculated. The mean and relative standard deviation of these were calculated, and are given in Table 7.3. A further dilution of the ethanolic solution containing diazepam and MACB was made in chloroform, with IS added to give a standard mixture of all three components. This solution was injected repeatedly and the peak height and peak area ratios measured. These were used for comparison with the ratios obtained in the chromatograms of the extracts to allow calculation of the recovery of diazepam and MACB by the extraction procedure.

The first extraction with 20 ml of chloroform gave almost complete extraction of diazepam from the acid

phase. The large relative standard deviation (RSD) for injections of the same extract indicated that some variation occurred between the replicates. Although there was a slight increase in the quantity of diazepam recovered from acid when further volumes of chloroform were used for extraction there was no significant improvement in the precision. Thus in subsequent work, unless otherwise stated, a single extraction with chloroform was made and it was assumed that 100% recovery of diazepam was obtained.

In the aqueous and acidic dilutions of diazepam, yellow crystals were visible and were thought to be due to MACB. The solubility of MACB in water is low and so despite initial solubility of this in ethanol, on dilution of this with water or acid, some precipitation of the MACB may occur. On sampling of these solutions, less than 100% recovery would be expected.

Comparison of the results obtained by peak height ratio (PHR) and peak area ratio (PAR) shows that a similar result is obtained by each method of calculation. The variation between replicate injections of the same solution is due to errors from a number of sources. These include variation in the injection technique used. This may result in the sample being injected at different points in the injector block which may not be at constant temperature throughout and thus cause different degrees of volatilisation. Also the needle may be left in contact with the injector for different lengths of time. Some error will also be introduced by the measurement of the area or height of the peak.

Table 7.3 Efficiency of extraction process

Type of aqueous phase	Number of extracts	Number of injections	Peak area ratio MACB:DEHP	RSD (%)	%recovery	Peak area ratio DIAZEPAM:DEHP	RSD (%)	%recovery
Water	1	6	0.435	3.3	88.4	0.481	1.7	95.1
Water	2	7	0.440	2.1	89.4	0.481	1.2	95.1
Water	3	7	0.408	6.0	82.9	0.486	1.7	96.0
Acid	1	6	0.431	1.0	87.6	0.503	1.4	99.4
Acid	2	6	0.434	2.0	88.2	0.505	0.8	99.8
Acid	3	6	0.375	2.2	76.2	0.508	0.6	100.4
Standard mixture		10	0.492	1.6		0.506	1.0	
Type of aqueous phase	**Number of extracts**	**Number of injections**	**Peak height ratio MACB:DEHP**	**RSD (%)**	**%recovery**	**Peak height ratio DIAZEPAM:DEHP**	**RSD (%)**	**%recovery**
Water	1	6	0.761	1.6	84.6	0.342	1.9	93.4
Water	2	7	0.794	0.7	88.3	0.342	0.4	93.4
Water	3	7	0.719	1.3	80.0	0.352	1.5	96.2
Acid	1	6	0.791	0.9	88.0	0.364	1.9	99.5
Acid	2	6	0.817	2.3	90.9	0.364	0.8	99.5
Acid	3	6	0.704	4.0	78.3	0.365	1.0	99.7
Standard mixture		10	0.899	1.4		0.366	0.4	

Table 7.4 Stability of diazepam and MACB in acid and water

Aqueous phase	Number of extracts	Time of sampling (min)	MACB:DEHP Peak area ratio	RSD (%)	%recovery	DIAZEPAM:DEHP Peak area ratio	RSD (%)	%recovery
Water	1	Initial	0.435	3.3	88.4	0.481	1.7	95.1
Water	1	75	0.439	1.6	89.2	0.471	0.8	93.1
Acid	1	Initial	0.431	1.0	87.6	0.503	1.4	99.4
Acid	1	120	0.296	1.0	60.2	0.487	2.0	96.2
Standard mixture			0.492	1.6		0.506	1.0	

Due to the many areas where errors can be introduced, the method used to measure the peak height or area will be only a small part of the total error. Where possible PAR was used because of the convenience of automatic measurement by a bench-top integrator. This was not possible for all measurements. The results reported in Table 7.3 were generated using both PAR and PHR for the same set of injections. From these results no difference in precision was seen between the two methods of measurement and for samples where PAR could not be measured, PHR measurement was used.

7.1.3.2 Investigation of the stability of diazepam in acid and water

During the study of the hydrolytic degradation of diazepam in acid, samples from the reaction mixture were taken at time intervals and suitable solutions were prepared in chloroform for injection onto the gas chromatograph. As several minutes would elapse between sampling and extracting the reaction mixture, the stability of diazepam and MACB in acid, at room temperature, was studied. This was important to ensure that no further degradation occurred in cold acid after sampling the reaction mixture. If degradation had continued, interference in the study of the hydrolysis reaction would have resulted.

Samples of the solutions of MACB and diazepam in acid and water (Section 5.1.3.1) were extracted at time intervals after initial preparation of the solutions. These extracts were injected onto the GC system, together with the extracts prepared from samples taken

immediately after preparation of the solutions. The % recovery of MACB and diazepam at various times after preparation of the solutions in acid and water (Section 5.1.3.1) was measured by comparison of the PHR and PAR obtained from injections of the standard mixture and extracts, Table 7.4.

These results show that some decrease in the concentration of diazepam occurs in acid and water at room temperature after an hour, although this was very small. As the samples were extracted immediately after cooling in the reactions followed, this small decrease was not expected to cause any errors in the study of the hydrolysis reaction. The cooling stage was performed very quickly, and no further degradation of diazepam should occur after sampling and before extraction.

The recovery measured for MACB left in acid was lower than for MACB in water. A yellow precipitate was seen in the dilutions of ethanolic solutions of diazepam and MACB in both water and acid. When the dilutions were left to stand before further sampling, yellow crystals were seen to grow in the acidic solution. It was proposed that on standing, crystal growth had occurred, which reduced the concentration of MACB in solution and gave a low recovery on extraction of the solution into chloroform.

As MACB is poorly soluble in aqueous or acidic solutions, when present it may precipitate out of solution. For example, as the hydrolysis of diazepam in acid proceeds, crystals of MACB become visible as the reaction progresses. As these crystals are freely

soluble in the chloroform used for extraction, any variations due to sampling will affect the reproducibility of the assay. Thus difficulty was experienced in sampling portions of the reaction mixture with the same amount of MACB present and poor reproducibility in the measurement of MACB resulted.

7.1.3.3 Investigation of the stability of diazepam in chloroform

In following the hydrolysis reaction of diazepam by GC, Section 7.3, a standard mixture of diazepam, MACB and DEHP in chloroform was injected at the start of the reaction, and the PAR or PHR for diazepam:DEHP was assigned a value of 100% diazepam. Subsequent samples were quantified against this to monitor the decrease in diazepam content with time. As the samples take 9 minutes to be eluted from the GC column, and as replicate injections were made at each sample time, the samples in chloroform are left for some time before injection. By injecting samples of fresh standard mixture and samples of extracts in chloroform (prepared from the aqueous and acidic solutions used to investigate the extraction procedure and stability of diazepam in 0.1 M hydrochloric acid and water solutions (Section 5.1.3.3)), it was possible to check the stability of diazepam in chloroform over a period of 17 days. The results are shown in Table 7.5.

Table 7.5 Stability of MACB and diazepam in chloroform

MACB

Aqueous phase	Number of extracts	Initial content	RSD (%)	%recovery * After 16 days	RSD (%)	After 17 days	RSD (%)
Acid	1	87.6	1.0	84.1	0.8	85.1	0.5
Acid	2	88.2	2.0	85.1	1.5	86.9	3.3
Acid	3	76.2	2.2	74.8	0.9	84.3	14.0
Acid	1 (after 120 min in acid)	60.2	1.0	58.4	2.3	58.5	1.0

DIAZEPAM

Aqueous phase	Number of extracts	Initial content	RSD (%)	%recovery * After 16 days	RSD (%)	After 17 days	RSD (%)
Acid	1	99.4	1.4	99.2	0.4	98.8	0.8
Acid	2	99.8	0.8	100.8	0.9	99.8	0.9
Acid	3	100.4	0.6	100.8	1.1	100.0	1.9
Acid	1 (after 120 min in acid)	96.2	2.0	98.6	0.5	97.5	1.2

* %recovery calculated with reference to a fresh standard mixture of diazepam and MACB

These results indicate no difference in the concentration of MACB and diazepam over this time period. Slight variation occurred in the results measured on different days, but the differences are no greater than might be expected due to experimental error. With a relative standard deviation of 1.0% on replicate injections made of a solution on the same day, then injections of the same solution on different days would show wider variation. Thus diazepam is considered to be sufficiently stable in chloroform. Results reported in Section 6.1.3.4 confirm the stability of diazepam in chloroform over the time

periods of interest.

7.1.4 Validation of the assay method

7.1.4.1 Precision of injection

The standard mixture containing diazepam, MACB and DEHP was injected repeatedly and the peak areas and heights measured. The peak heights were measured using a ruler, as the vertical distance from the tangent drawn across the base of the peak to the apex of the peak (Fig. 7.4). The peak areas were measured by bench-top integrator. The results are shown in Table 7.6. Due to the small size of the diazepam peak height compared to those of the other two peaks, the attenuation control was altered during some injections to increase the sensitivity of the measurements for diazepam, to see if any improvement in precision could be obtained. Although it was possible to alter the attenuation control, it was difficult to perform this at exactly the same stage in each injection. As the peaks were tailing, errors were introduced as the change in attenuation caused a slight shift in baseline. The higher relative standard deviation obtained when this was done suggests that greater variability is introduced. In subsequent work, the attenuation control was therefore not altered. In some instances a bench-top integrator was used and the peak areas measured rather than peak height.

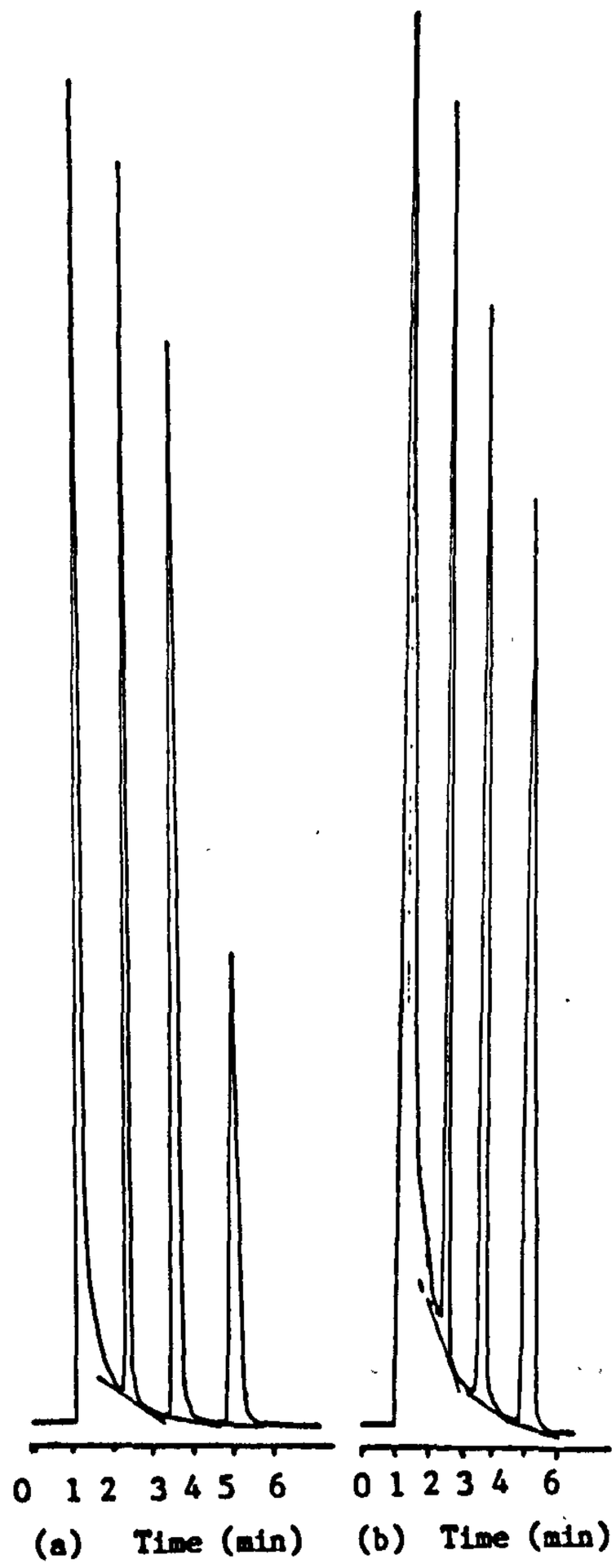


Fig. 7.4 Gas-liquid chromatography of MACB, DEHP and diazepam

(a) without alteration of attenuation
(b) with alteration in attenuation

Table 7.6 Precision of injection

	MACB:DEHP		DIAZEPAM:DEHP	
	Peak area ratio	Peak height ratio	Peak area ratio	Peak height ratio
Mean	0.592	1.138	0.582	0.427
RSD	1.9%	3.3%	1.0%	1.0%
n	11	11	16	16
		AFTER ATTENUATION CHANGE		
Mean	0.589	1.140	0.582	0.852
RSD	1.9%	4.5%	1.7%	1.5%
n	10	10	16	16

The relative standard deviation of the PHR for the MACB peak was large. This was thought to be because the peak for MACB occurs just after the solvent, and the tailing from the solvent occurs to a variable extent.

The relative standard deviation of 1.0% for the PHR and PAR of diazepam was higher than ideal. No improvement could be made with the equipment used, despite attempts to alter the injection technique used.

7.1.4.2 Reproducibility of extraction

The precision of injection of a mixture of diazepam, MACB and DEHP was found to be adequate for measurement of the concentration of diazepam. For the assay procedure, diazepam was extracted from the reaction mixture and then injected onto the GC system. The reproducibility with which this extraction could be performed was assessed by diluting an ethanolic

solution of diazepam and MACB in 0.1 M hydrochloric acid/0.9 M sodium chloride solution. Seven samples of this dilution were extracted with chloroform (Section 5.1.4.2), after neutralisation of the samples with 1 M sodium hydroxide and pH 7.0 buffer solution, evaporated to dryness and redissolved in chloroform containing DEHP. At least eight replicate injections were made of each of these solutions and the mean peak area ratio for diazepam:internal standard and MACB:internal standard was calculated for each sample, Table 7.7.

Table 7.7 Reproducibility of extraction of diazepam and MACB from pH 7 solution

Extract	DIAZEPAM:DEHP PAR	RSD %	MACB:DEHP PAR	RSD %
1	0.450	1.0	0.294	2.3
2	0.450	0.5	0.365	1.5
3	0.445	2.2	0.356	1.3
4	0.444	2.5	0.388	3.5
5	0.447	1.1	0.357	3.4
6	0.445	1.1	0.377	1.3
7	0.438	1.7	0.367	0.9
Mean	0.446	0.9	0.358	8.5

These results show that the reproducibility of extraction of diazepam from the portions of acidic solution after neutralisation is very good. The variation between samples is no greater than that for the repeated injection of the same solution, suggesting that no extra error is introduced at the extraction stage.

The variation in the recovery of MACB from repeated

extracts of the same solution was much larger than for diazepam. The solution used for extraction had crystals of MACB present and this was thought to be responsible for the greater variation.

A second dilution of diazepam in 0.1 M hydrochloric acid/ 0.9 M sodium chloride was prepared and six portions of this solution were extracted without alteration of the pH of solution. The extracts were treated as for those extracted from pH 7 solution and were injected onto the chromatographic system. Each extract was injected at least five times and the mean peak area ratio for diazepam:internal standard was calculated for each sample, Table 7.8.

Table 7.8 Reproducibility of extraction of diazepam from pH 1 solution

Extract	DIAZEPAM:DEHP PAR	RSD %
1	0.455	3.7
2	0.489	5.0
3	0.472	1.7
4	0.488	6.1
5	0.436	1.6
6	0.513	3.4
Mean	0.476	5.7

These results suggest that the reproducibility of extraction from acidic solution is more variable than from pH 7 solution with a much larger RSD seen for these results. However, the reproducibility of injection of each individual solution is much larger, and the variability between the individual extracts is no greater than that between the injections of each

solution. Thus before any conclusion can be drawn, these solutions would have to be reinjected when the chromatographic system was giving better reproducibility of injection.

A variety of changes were made to try and improve the reproducibility of injection, but despite changes in temperature of the injector and use of different lengths of needle for injection there was no improvement in the precision.

A solution of diazepam in 0.1 M hydrochloric acid/0.9 M sodium chloride was prepared and allowed to reach equilibrium between diazepam and its ring-open hydrolysis product, GMACB. This solution was extracted repeatedly, both from the acidic solution, and the acidic solution which had been neutralised by the addition of 1 M sodium hydroxide and pH 7.0 buffer solution before extraction. At least four extractions were made from each pH, and at least three injections were made of each extract. The peak area ratio for diazepam:internal standard was calculated for each injection and these are shown in Table 7.9.

Table 7.9 Comparison of extraction from pH 1 and pH 7

Extract	pH	DIAZEPAM:DEHP PAR	RSD %
1	1	0.657	1.7
2	1	0.677	0.5
3	1	0.684	1.1
4	1	0.684	0.5
5	1	0.682	0.6
6	1	0.692	0.6
1	7	0.914	1.5
2	7	0.938	0.1
3	7	0.926	0.6
4	7	0.927	1.3

pH 1	Mean	0.679	RSD	1.8%
PH 7	Mean	0.926	RSD	1.1%

Considering the variation on repeated injection of the same solution, it appears that there is no difference between the reproducibility of the extraction process from pH 1 and pH 7. The amount of diazepam extracted from pH 7 was larger, confirming that some recyclization of the ring-opened intermediate hydrolysis product of diazepam was occurring with increase in the pH of solution.

7.1.4.3 Linearity of assay procedure

Five solutions were prepared containing mixtures of diazepam and MACB in ethanol. These solutions were diluted in 0.1 M hydrochloric acid/0.9 M sodium

chloride solution and samples of each dilution were extracted with chloroform immediately after preparation. The chloroform extracts were evaporated and the residue redissolved in chloroform. These solutions were each injected onto the GC system at least five times. The mean peak area ratios were plotted against the concentration of diazepam and MACB in the injection solution. The extraction of diazepam and MACB from the acidic solutions was assumed to be 100%. Thus it was possible to calculate the concentration of both components present in the samples injected. The mean values are shown in Table 7.10, and the graphs in Fig. 7.5.

Table 7.10 Mean peak areas measured for linearity samples

Concentration of diazepam (mg/ml) (X)	Mean PAR DIAZEPAM:DEHP (Y)	RSD %	Concentration of MACB (mg/ml) (X)	Mean PAR MACB:DEHP (Y)	RSD %
0.75	0.546	0.7	0.13	0.087	4.0
0.60	0.435	1.3	0.26	0.166	1.7
0.45	0.325	1.0	0.39	0.273	3.4
0.30	0.215	1.4	0.52	0.364	3.7
0.15	0.100	11.9	0.65	0.430	3.0

Compound	Linearity equation	Correlation coefficent
MACB	Y = -0.0012 + 0.68 X	0.997
Diazepam	Y = -0.0095 + 0.74 X	1.000

In this study, MACB gave a straight line, with no significant variation in the individual results. However, the acidic solution from which it was extracted had been prepared immediately before extraction by addition of an ethanolic solution of

Fig 7.5a Linearity of gc assay for diazepam

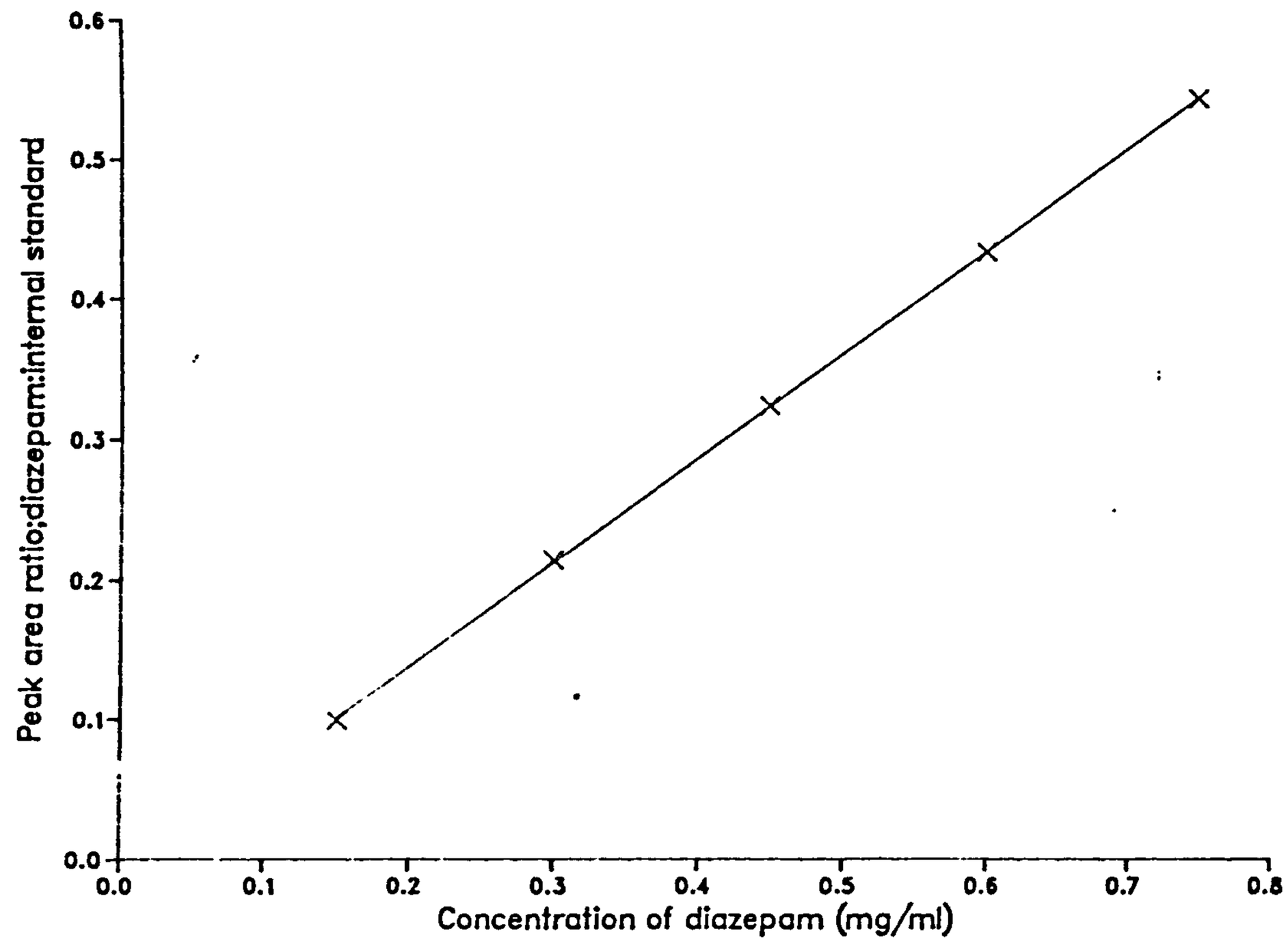
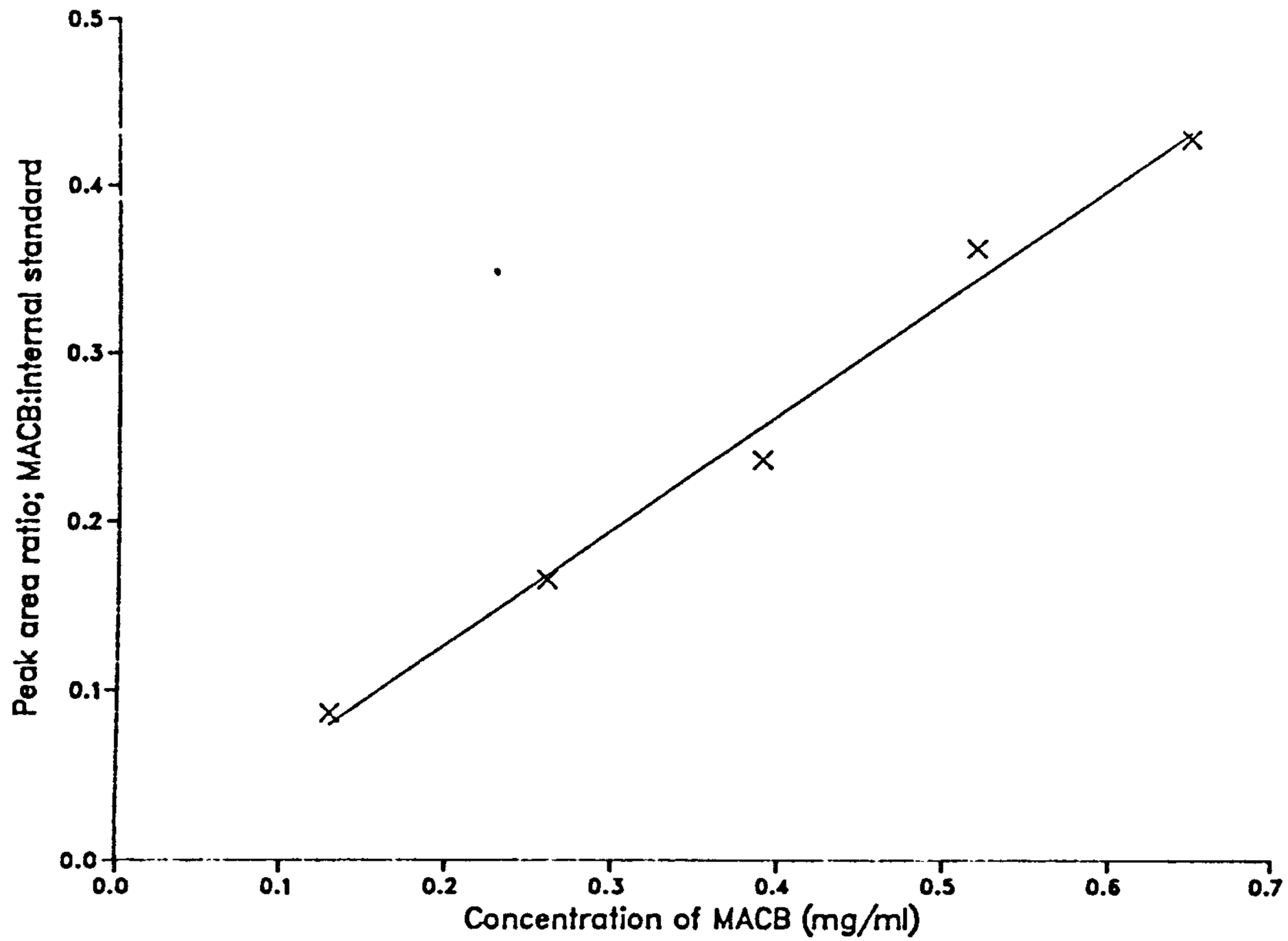


Fig 7.5b Linearity of gc assay for MACB



MACB. Although crystallisation from solution occurred, the crystals were very small, giving a cloudy suspension rather than larger more clearly defined crystals. As the sample for extraction was removed immediately after shaking the suspension, the fine particles would be uniformly dispersed throughout the volume of liquid and a representative sample taken.

7.2 USE OF GAS CHROMATOGRAPHY TO STUDY THE EXTRACTION OF DIAZEPAM, MACB AND GMACB

7.2.1 Preparation of hydrolysed diazepam

A solution of diazepam in ethanol was diluted with 0.1 M hydrochloric acid and the resultant solution left at room temperature to allow hydrolysis to occur. The solution was left for at least five days, and at the end of that time an equilibrium between the components in the mixture was established. The system is not a true equilibrium as the GMACB formed continues to degrade in an irreversible reaction to form MACB. However, this second reaction is very slow at room temperature and very little MACB is formed. The presence of MACB was detected by the formation of yellow crystals in the reaction mixture.

7.2.2 Investigation of the extraction characteristics of GMACB

A solution of diazepam, MACB and GMACB was prepared as described in Section 5.2.1. A portion of this acidic solution was extracted with chloroform, internal standard was added and this extract was injected onto the chromatographic system described in Section 5.1.1. Injection of this extract showed that both diazepam and MACB were extracted into chloroform. Injections of a second and third chloroform extract from this solution were also made and no MACB was detected in either extract. A small peak corresponding to diazepam was found in both. The peak height ratios for diazepam:internal standard were calculated for each

injection, Table 7.11. The solution of diazepam initially prepared contained approximately 8 mg/ml, a much more concentrated solution of diazepam than was normally injected onto the GC system (the typical injections were made of solutions containing approximately 1 mg/ml). As a result the peak height due to diazepam was very much larger than that due to the internal standard, and the peak height ratios determined were likely to be inaccurate due to the large difference in the heights measured. To provide a large quantity of the intermediate in the hydrolysis reaction, a large concentration of diazepam was required to be present initially. As the content of diazepam present after hydrolysis was not known accurately, dilution of the solution before injection was not performed, and the peak height ratios obtained in Table 7.11 were used to give an estimate of the ratio between diazepam and GMACB.

Table 7.11 Diazepam remaining after successive extractions with chloroform

Extraction	DIAZEPAM:DEHP PHR
Extraction from pH 1	9.57
Second extraction from pH 1	0.08
Third extraction from pH 1	0.08
Extraction from pH 1	4.67
Extraction from pH 7 after pH 1	1.41
Extraction from pH 7	6.33

Most of the diazepam was removed from the hydrolysed solution by the first extraction with chloroform. Two

subsequent extractions removed only a further 0.8% of that removed by the initial extraction. When the extraction procedure was performed on fresh solutions of diazepam in acid it was shown that 99.5% efficiency was obtained for the extraction (Section 7.1.3.1). It is proposed that this small quantity extracted on subsequent extractions of the hydrolysed solution, is diazepam formed from GMACB (the open-ring intermediate) as it readjusts in acid solution to re-establish the equilibrium disturbed by the extraction.

On extraction of a further sample of hydrolysed diazepam in acid at pH 1, (prepared on a separate occasion with a different concentration of diazepam), peaks with retention times corresponding to diazepam and MACB were obtained (in addition to the internal standard peak). On alteration of the pH of this solution to pH 7 by the addition of sodium hydroxide and buffer solution, and re-extraction with chloroform, a greater amount of diazepam was measured. A further sample of this hydrolysed diazepam in acid was adjusted to pH 7 as above, before extraction with chloroform, to convert GMACB to diazepam before extraction, to ensure extraction of the total content of diazepam present. Peaks corresponding to diazepam and MACB were seen, in addition to the internal standard peak.

The peak height ratios in Table 7.11 suggest that approximately one-third of the diazepam in 0.1 M hydrochloric acid solution at equilibrium is present as the open-ring intermediate (this has also been shown by UV spectrophotometry, Section 7.4.1). The ratios also suggest that the intermediate product can

be converted to diazepam by adjustment of the pH of solution to pH 7 to force the equilibrium towards diazepam. This is confirmed by the chromatographic evidence, only one peak being seen at the same retention time as diazepam, with no evidence of a split peak. There is a loss of approximately 4% between the diazepam measured after pH 7 extraction and that measured after pH 1 extraction plus subsequent pH 7 extraction. This is assumed to be due to errors in the experimental procedures (such as poor precision of injection) and the large number of manipulations involved.

The MACB content in the chloroform extracts of the hydrolysed solution of diazepam at pH 1 and pH 7 were almost the same. This would be expected as MACB is stable over the pH range.

These results support the theory that in the hydrolysis of diazepam there are two species which are in equilibrium, that the equilibrium is pH dependent, and that only one of the species is extractable into chloroform. The extracted compound has the same retention time as diazepam, both on extraction from pH 1 and pH 7. In this work, the identity of the compounds in the samples injected was based on comparison of the retention times of the unknown samples with the retention times of standard materials. The retention times for these peaks were measured from the chromatograms. The errors involved in this measurement might result in two compounds with slight differences in retention time being identified as the same compound. On injection of the solution extracted from pH 7, only one peak was seen. This

supports the theoretical reaction which has been proposed, and that the increase in pH causes the formation of diazepam, only one compound being present in the sample solution injected.

7.3 APPLICATION OF THE GAS CHROMATOGRAPHIC METHOD TO STUDY THE KINETICS OF THE HYDROLYSIS OF DIAZEPAM

7.3.1 Preparation of the reaction mixture

The reaction mixture was prepared as described in Section 5.3.1. The water bath was set to control the temperature of the reaction mixture, but a thermometer was also used to measure the temperature of the solution within the reaction vessel. The temperature was found to be 72°C, slightly higher than that used by Han et al., (1977a).

7.3.2 Analysis of the reaction mixture

The reaction mixture described was prepared and sampled at the stated time intervals (Section 5.3.2). The samples were extracted and at least three injections made of each extract. The mean peak area ratio for diazepam:internal standard was calculated for each sample. The value obtained at t_0 was assumed to be equivalent to 100% diazepam and the values obtained for other time points were divided by this to give the % diazepam remaining at each time point. These values were used to calculate the rate of reaction using the equations described in Appendix I.

Three separate experiments were carried out. The first hydrolysis reaction studied was followed by neutralizing the reaction mixture before extraction of diazepam and MACB. A further two hydrolysis reactions were followed with extraction of the reaction mixture

from pH 1, ie without neutralisation. The % diazepam remaining with time is shown in Table 7.12.

Table 7.12 Decrease in diazepam content in 0.1 M hydrochloric acid/ 0.9 M sodium chloride solutions

Time (min)	% Diazepam remaining after extraction from		
	pH 7	pH 1	pH 1
0	100.0	100.0	100.0
10	98.3	80.1	83.4
20	95.6	69.6	71.0
30	95.2	65.7	66.8
40	94.6	62.2	62.9
50	93.4	60.0	62.4
60	93.4	59.5	58.9
80	93.2	58.3	58.5
100	94.6	57.9	55.8
120	91.4	57.7	54.6
150	91.1	56.7	
180	90.2	56.7	55.4
210	87.8	56.3	55.2
240	85.3	56.7	52.1
300	87.3	55.2	
345			51.4
360	87.3	52.2	
420			49.2

From these results, a difference can be seen between the measured content of diazepam extracted from the acidic reaction mixture, and from a sample of the reaction mixture which had been neutralised. In both cases where the extractions were made from the reaction mixture, there was very good agreement, but when the solution was neutralised, the content of

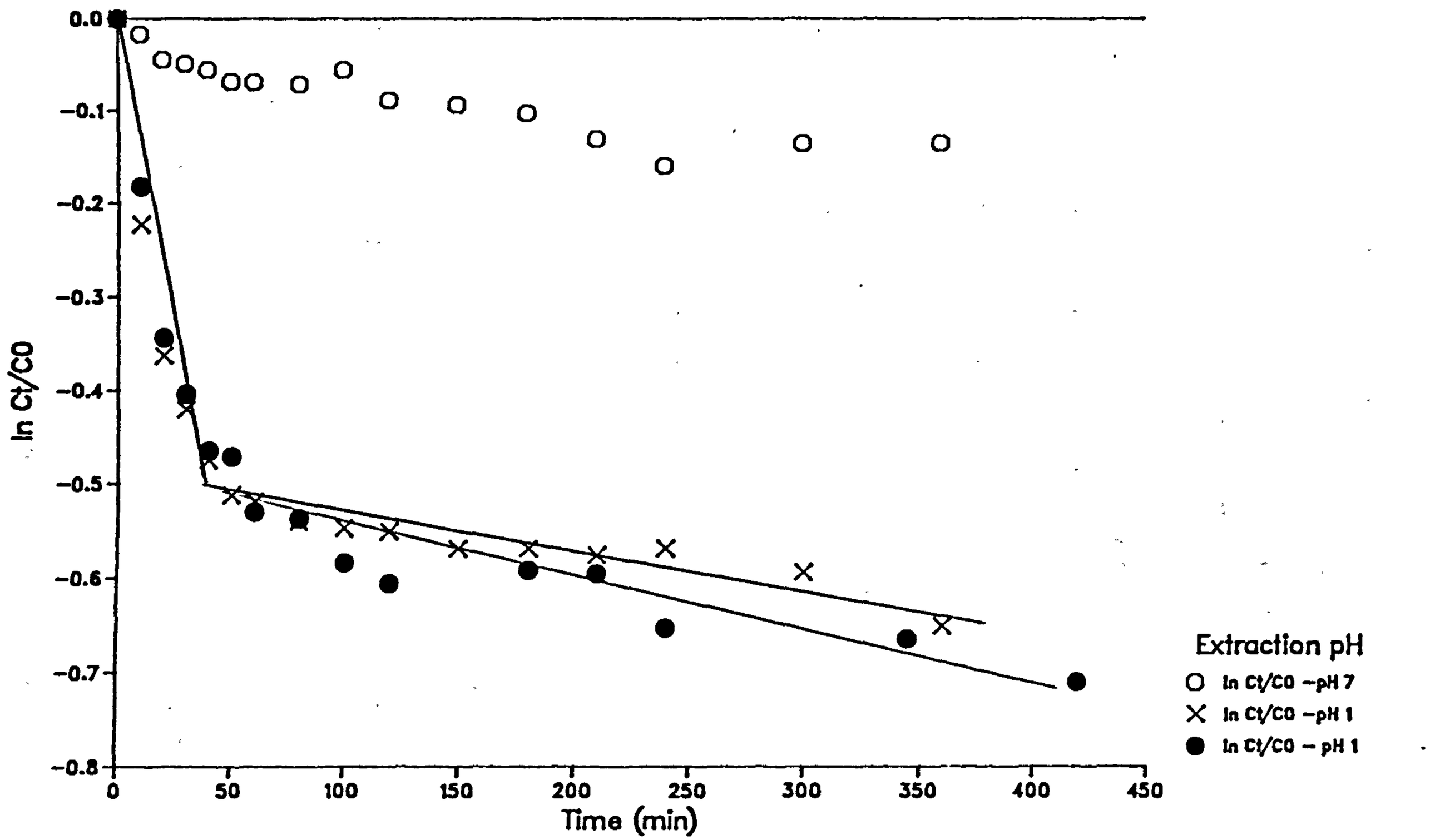
diazepam measured was higher. This confirms the findings of Nakano et al.,(1979) where it was proposed that GMACB present in the reaction mixture is present in equilibrium with diazepam. On alteration of the pH to pH 7, the GMACB reverts to diazepam and is extracted, resulting in a higher measured content than when pH 1 is used for extraction.

On studying the hydrolysis reaction of diazepam by UV spectrophotometry, Han and co-workers (1977a) found that at pH values below the pK_a of diazepam, the spectral changes seen indicated a two-step reaction, both steps being first order, but that at pH values above the pK_a of diazepam, a single step reaction from diazepam to MACB was occurring. In the two-step reaction, the initial stage involved hydrolysis of diazepam to an intermediate, a reversible reaction, with a second slower stage of hydrolysis of this intermediate to MACB and glycine (Section 1.2.3).

The rate constants for the reactions were calculated by Han et al. using plots of the logarithm of differential absorbance measurements at time t, and time infinity (A_t-A_∞), and these were found to give two linear sections for the two-step reaction with the slope of the last linear segment equal to the negative of the slowest rate constant.

As all three components in the reaction mixture have related structures, a degree of similarity in their absorbance spectra was anticipated, with a consequent risk of interference in the assay. A similar study of the hydrolysis of chlordiazepoxide reported by Maulding and co-workers (1975) made use of either

Fig 7.6 Plot of the decrease in diazepam concentration with time during hydrolysis. Reaction followed by GC



quantitative TLC or extraction to isolate chlordiazepoxide from its co-absorbing degradation products before analysis of its content in the reaction mixture. (In this work, no open-ring intermediate was found. However, the TLC conditions and the extraction method used for sample preparation would not be expected to allow measurement of this). By using TLC for measurement, the $A_t - A_\infty$ term was simplified to A_t as A_∞ became zero.

When GC was used as the method of measurement, it was possible to isolate diazepam and measure the change in concentration of diazepam with time, unaffected by the presence of the other UV absorbing species present in the reaction mixture. As diazepam was isolated, it was also possible to simplify the equations used for calculation of the rate constant as at t_∞, no diazepam would be present.

A plot of ln (C_o/C_t) against time was made for the three reactions followed, see Fig. 7.6. From these graphs, linear relationships were seen confirming the reactions occurring to be first-order reactions. When diazepam was extracted from the reaction mixture without alteration of pH, a two-phase reaction was seen, but when the pH of the sample was adjusted to pH 7 before extraction, only one stage was seen.

As the equilibrium between diazepam and GMACB is pH dependent, it appears that when pH 7 is used for extraction, any GMACB present is converted back to diazepam, providing a direct method of measurement of the overall reaction from diazepam to MACB. When the

pH of the reaction mixture was not altered before extraction, the diazepam which had converted to GMACB would not be extracted, thus the fast moving slope initially indicates the rate of equilibrium establishment (although it is affected by the second reaction occurring), and the second slow moving slope gives the reaction rate for the conversion of diazepam to MACB.

After calculation of k_2 from these graphs, it was seen that the results obtained were similar for extraction from both pH values, and of the same order as those obtained by Han et al., see Table 7.13.

Table 7.13 Reaction constants for the hydrolysis reaction, followed by GC

pH 7 extraction	3.71×10^{-4} min^{-1}	
pH 1 extraction	3.45×10^{-4}	
	4.51×10^{-4} min^{-1}	Mean 3.89×10^{-4} min^{-1}
Han et al.	2.06×10^{-4} min^{-1}	

It appears that the developed GC method provides a suitable assay method for study of the hydrolytic degradation of diazepam, allowing measurement of the separated reaction stages. The reaction of diazepam to GMACB could be followed by the differential extraction procedure used in Section 5.4 to follow the rise in GMACB levels. To allow full advantage of the technique, a more precise method of injection and quantitation of resultant height or area would be required however, as only small changes are seen with time, which are difficult to measure accurately when poor precision of injection is obtained.

7.4 APPLICATION OF DIFFERENCE UV AND DIRECT UV SPECTROPHOTOMETRIC METHODS TO STUDY THE KINETICS OF HYDROLYSIS OF DIAZEPAM

7.4.1 Hydrolysis reaction studied at room temperature

From the results obtained by GC (Section 7.2.2) it appeared that the hydrolysis reaction for diazepam proceeds via the intermediate proposed by Nakano et al. To gain a better understanding of the length of time required for equilibrium to be established and to enable some of the open-ring intermediate to be isolated, the reaction was followed by UV spectrophotometry. The measurements were made by both difference UV and direct UV spectrophotometry and the results analysed making some initial assumptions. It was known from work performed by direct UV measurements (Section 6.2.1) that MACB interfered in the measurement of diazepam at 284 nm. The difference UV assay, however, had been shown to be specific for diazepam in the presence of up to 30% MACB. As the proposed structure for GMACB was similar to that for diazepam, it was anticipated it would be UV absorbing and would interfere in a direct UV spectrophotometric measurement. As the formation of this compound was governed by an equilibrium reaction which was pH dependent, it was also thought likely that it would interfere in the difference UV assay.

From work performed on the extraction characteristics of the components of the mixture (Sections 7.1.3.1, 7.1.3.4 and 7.2.2) it was known that diazepam and

MACB would extract into chloroform at pH 1 and pH 7. However, the open-ring intermediate would not extract into chloroform from pH 1 and would reverse to form diazepam on neutralisation of the solution to pH 7.

On the basis of these assumptions, various fractions were sampled and extracted from pH 1, from pH 7 and from pH 1 followed by pH 7. Analysis of the solutions left after these extractions showed that the rate at which the open-ring intermediate was formed could be monitored.

At each time point a sample of the acidic solution was analysed by both UV methods, (solutions 1, 1A and 1B). From the result of this analysis, at the initial time point, both methods would measure the concentration of diazepam present in the sample. At later time points, the sample would contain diazepam, MACB and GMACB as the hydrolysis reaction proceeded. Thus at later time points, the diazepam content would not be accurately measured by either method as the other components of the mixture would interfere.

A second sample of the reaction mixture was extracted with chloroform. The acidic portion remaining after extraction was analysed by both UV methods, (solutions 2, 2A and 2B). In these solutions, only GMACB was expected to be present, as both diazepam and MACB would be extracted into the chloroform layer. Thus analysis of these solutions at the zero time point gave no absorbance. At later time points, the UV and difference UV spectra of this species was obtained. This assumed that the rate of reversal of diazepam at pH 1 is slow and that insufficient diazepam reformed

before measurement to interfere. As no A(1%,1cm) had been obtained for GMACB, no calculation of its content could be made.

The chloroform layer remaining after extraction from the acidic solution was evaporated and redissolved in methanolic sulphuric acid and methanol for analysis by the direct and difference UV methods (solutions 3, 3A and 3B). At the initial time point both methods would give the concentration of diazepam present. As the reaction proceeds, the direct UV determination is subject to interference from MACB which was extracted into the chloroform layer.

The acidic sample extracted by chloroform was adjusted to pH 7 by the addition of alkali and buffer solution and then re-extracted by a further sample of chloroform. The residue from this after evaporation was redissolved as above and analysed by both UV methods (solutions 4, 4A and 4B). At the initial time point, these samples were expected to show zero absorbance, as the diazepam present would all have been extracted in the initial extraction from pH 1. At later time points however, the absorbance measured was due to diazepam formed from GMACB present in the acidic solution, which reverts to diazepam after pH change.

A further acidic sample of the reaction mixture was neutralised by the addition of alkali and buffer and the solution at pH 7 extracted with chloroform. The residue from this extraction was redissolved in methanolic sulphuric acid and methanol and analysed by the UV methods (solutions 5, 5A and 5B). From

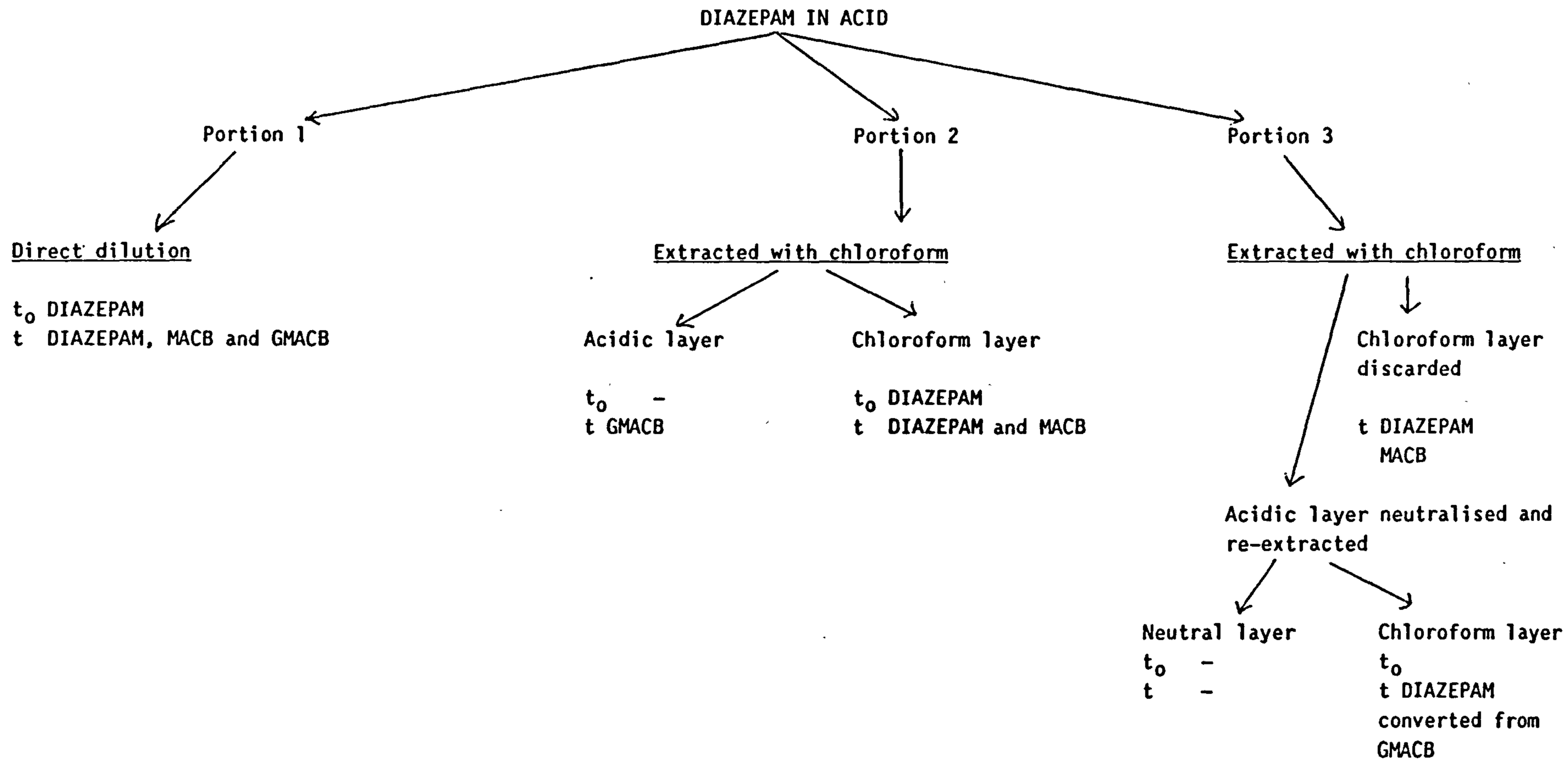
these extractions it was expected that all the GMACB present would be converted to diazepam. These solutions would contain diazepam initially but at later points in the study, would also contain MACB. Thus initially, the total content of diazepam could be measured but at later stages a slight reduction in this would be seen using the difference method. As the MACB concentration present increased, this would interfere in the direct UV method of analysis.

Direct UV absorbance measurements were made at 238, 255 and 284 nm (to allow calculation of absorbance ratios in addition to concentration of diazepam) and the difference measurements made at 255 and 290 nm.

The distribution of the three components between these solutions prepared for analysis is summarised in a flow diagram (Fig. 7.7).

Examination of the measurements showed that even at zero time when no MACB or GMACB was expected to be present, small absorbance measurements were made from solutions 2A-2B and solution 2, and also solutions 5A-5B and solution 5. No absorbance was measured for solutions 4A-4B and solution 4 at zero time, although small measurements were made from some of these solutions at later time points. The absorbances measured for solutions 4A-4B and 4 were assumed to be due to some small amount of carry-over during the extraction step of the sample preparation, while those in the initial 2 and 5 solutions were assumed to be due to the equilibrium reaction occurring very rapidly immediately, with some GMACB being formed in the short time between dilution of the diazepam and extraction of the dilution.

Fig 7.7 Distribution of diazepam, GMACB and MACB in the UV study



t_o = zero time
t = later time points

As the reaction proceeded, the absorbances measured for solutions 2A-2B and 2 increased, this was most noticeable from the measurement at 255 nm in solution 2 (the λ_{max} for GMACB, see Fig. 7.13). The content of GMACB present could not be calculated directly from these measurements, but was calculated using the increase in diazepam content measured for solutions 5A-5B and 5. In these solutions, any diazepam measured had been converted from GMACB present in the reaction mixture before analysis.

The diazepam content of solutions 1A-1B, 3A-3B and 5A-5B and also solutions 1,3 and 5 was calculated as a percentage of the initial concentration present for each of the six time points studied. This calculation assumed that the absorbance measured in each case was totally due to diazepam. This was not true in all cases and will be discussed further later. The results of these calculations are shown in Table 7.14.

Table 7.14 Percentage of diazepam measured in each set of solutions
(a) from difference absorbance measurements
(b) from direct UV absorbance measurements

(a)	Time after sample preparation (hr)	Solutions			
		1A-1B	3A-3B	5A-5B	(3A-3B)+(5A-5B)
	0	96.5	95.8	3.3	99.1
	24	85.1	77.2	21.1	98.3
	48	79.9	73.4	28.3	101.7
	72	77.9	70.0	27.0	97.0
	96	80.9	70.2	27.8	98.0
	168	81.9	71.2	26.6	97.8

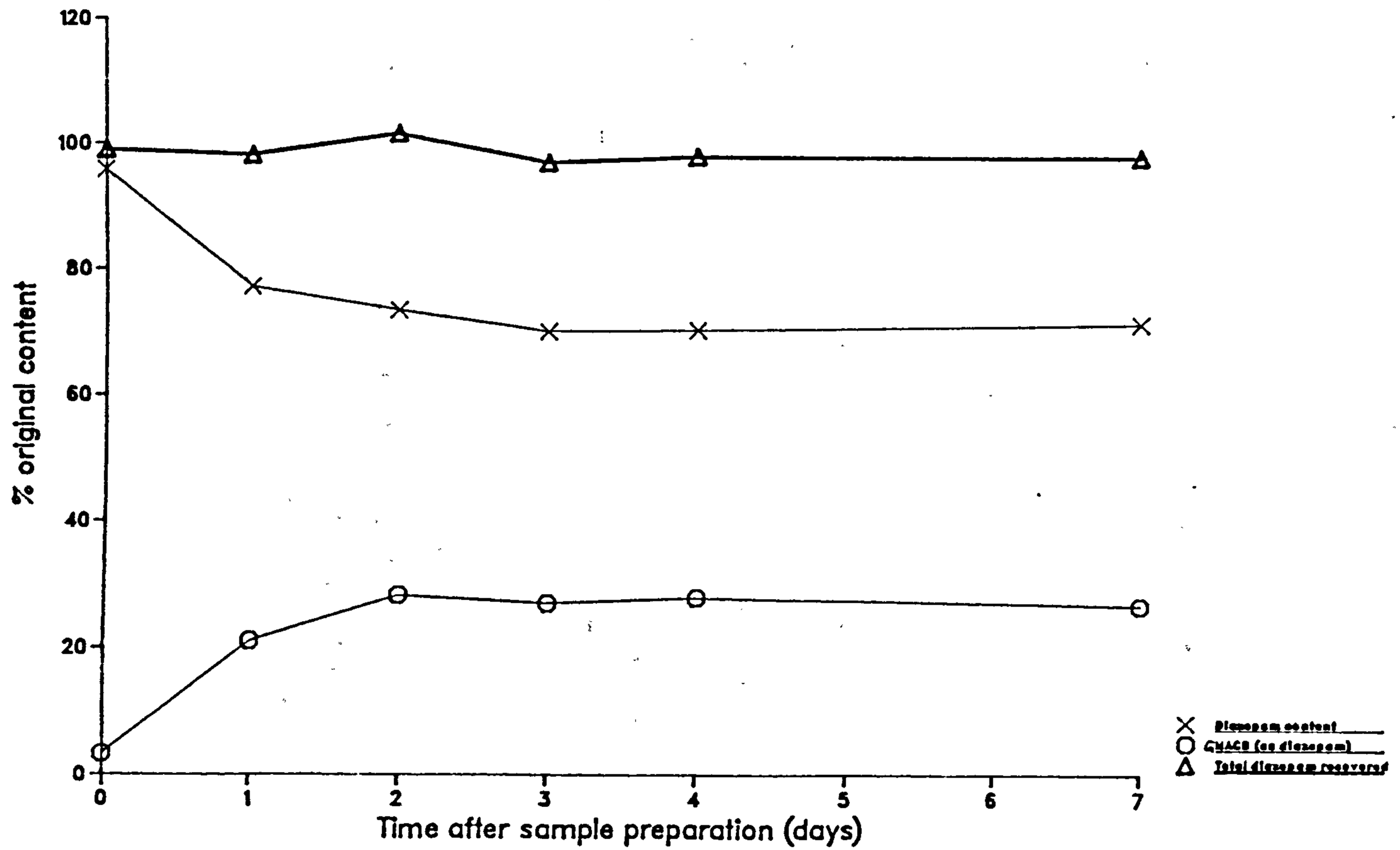
(b)	Time after sample preparation (hr)	Solution			
		1	3	5	(3+5)
	0	101.0	96.6	2.7	99.3
	24	82.9	76.0	18.4	94.4
	48	79.6	72.4	19.3	91.7
	72	79.4	70.1	18.8	88.9
	96	78.6	69.8	19.2	89.0
	168	79.6	70.1	17.9	88.0

In these solutions it was expected that at zero time, the values for solutions 1A-1B and 1 should give similar measured concentrations of diazepam. At time points after this, neither solutions 1A-1B not 1 were expected to give results which were free from interference as MACB and GMACB were anticipated in these solutions. MACB was known to interfere in the direct UV measurement (Section 6.2.1) and GMACB was known to interfere in the difference UV measurement (Section 6.1.1). Measurements made of solutions 3A-3B would be specific for diazepam in the presence of MACB, but the measurements made on solution 3 using direct UV would be affected by the presence of MACB as the reaction proceeded. The acidic solution became yellow as the reaction continued, this was likely to be due to the presence of MACB in the solution. The quantity of MACB formed was expected to be low due to the very slow speed of the reaction, especially at room temperature. In solutions 5A-5B and 5, no interference was expected by either method as only diazepam should have been present in these.

From these solutions, it was expected that addition of the measured diazepam in solutions 3A-3B and 5A-5B should give the total diazepam present, this is represented graphically in Fig. 7.8.

On examination of Table 7.14, it can be seen from solutions 3A-3B, that the diazepam content dropped rapidly, but then reached equilibrium. At the same time, the amount of GMACB present, measured as diazepam, rose quickly initially then became steady. Equilibrium was established quickly and a plateau appeared to have been reached within 5 days, where the proportion of GMACB present was approximately 30%. A small decrease in the content of diazepam measured was seen with time when that present as GMACB and diazepam were combined (the final column in Table 7.14). This

Fig 7.8 Establishment of equilibrium between diazepam and GMACB in hydrochloric acid at room temperature, followed by difference UV measurement at 290 nm



decrease was assumed to be due to MACB formation. Comparison of the content of diazepam measured from solutions 1A-1B and 3A-3B confirmed that GMACB interfered in the determination of diazepam using difference spectrophotometry.

On examination of the measured content of diazepam using direct UV measurement, it was found that the presence of GMACB and MACB affected the measurements, with different results seen in solutions 1 and 3, which were also different from solutions 1A-1B and 3A-3B. However, it was also found that the results obtained from measurement of solution 5 were lower than for solution 5A-5B. This was not expected as only diazepam converted from GMACB should have been present in these solutions, and the same content would have been expected to be measured by both methods. Examination of the UV spectra generated for the direct UV measurement showed slight differences for the absorbance ratio at 255 nm and 284 nm in this solution compared with that of standard diazepam and may explain the different content measured. No explaination was found for this alteration in spectral characteristics, and no change was seen in the difference spectrum generated from this sample when compared with that of diazepam. The chromatographic evidence reported supports the reversal of the open-ring intermediate to diazepam.

7.5 RESULTS OF THE DEVELOPMENT AND VALIDATION OF AN HPLC METHOD FOR THE ANALYSIS OF DIAZEPAM, MACB AND GMACB

7.5.1 Development of the method

An assay has been developed by HPLC for diazepam, MACB and carbostyril (Section 6.3.1) and also by GC for diazepam and MACB (Section 7.1.3). However, it was not possible to determine directly the content of the open-ring intermediate by either of these methods. The different extraction characteristics of diazepam and GMACB, and the conversion of GMACB to diazepam with pH means that a double extraction stage could be used to obtain this information. This is a time-consuming process, with many steps where error can be introduced in the sample preparation. With the choice of a more acidic mobile phase, it was hoped that an HPLC method could be developed which would effect separation of all the components of the hydrolytic reaction. This would allow the rate of that reaction to be followed more easily.

7.5.1.1 Choice of conditions

The need for a mobile phase of low pH to maintain the equilibrium between diazepam and GMACB, required the use of an acidic buffer solution in the mobile phase. Phosphate and acetate buffers were therefore used and it was found that although separation of diazepam, MACB and carbostyril could be achieved with both mobile phase mixtures, phosphate was required for separation of GMACB and removal of the sample solvent

interference.

Mixtures of diazepam, carbostyril and MACB in methanol and of diazepam, MACB and GMACB in 0.1 M hydrochloric acid (prepared by allowing a solution of diazepam in acid to degrade to equilibrium at 37°C) were injected onto an HPLC system. The mobile phase was methanol:acetate buffer (65:35) with the apparent pH of the final mixture brought to pH 3.1 by the addition of acetic acid. An injection was also made of 0.1 M hydrochloric acid, the sample solvent. The chromatograms obtained are shown in Fig. 7.9.

The solution of diazepam which had been allowed to degrade to equilibrium in 0.1 M hydrochloric acid was injected. Mobile phase mixtures were prepared from methanol:phosphate buffer (75:25) with an apparent pH of 2.8, obtained by addition of orthophosphoric acid and also methanol and orthophosphoric acid (75:25) without any buffer present giving a measured pH of 2.4. The chromatograms obtained from injection of degraded diazepam into these mobile phases are shown in Fig. 7.10. It can be seen from these chromatograms that poor separation is obtained using the mobile phase prepared with acetate buffer. Much improved separation is achieved using phosphate and this suggests that the chromatography may involve some ion-pairing to the phosphate counter ion present.

The identity of each peak in the chromatogram was confirmed by the injection of standard solutions injected under the same conditions. The retention times of these are shown in Table 7.15. The standard solution of GMACB was obtained by extracting a portion

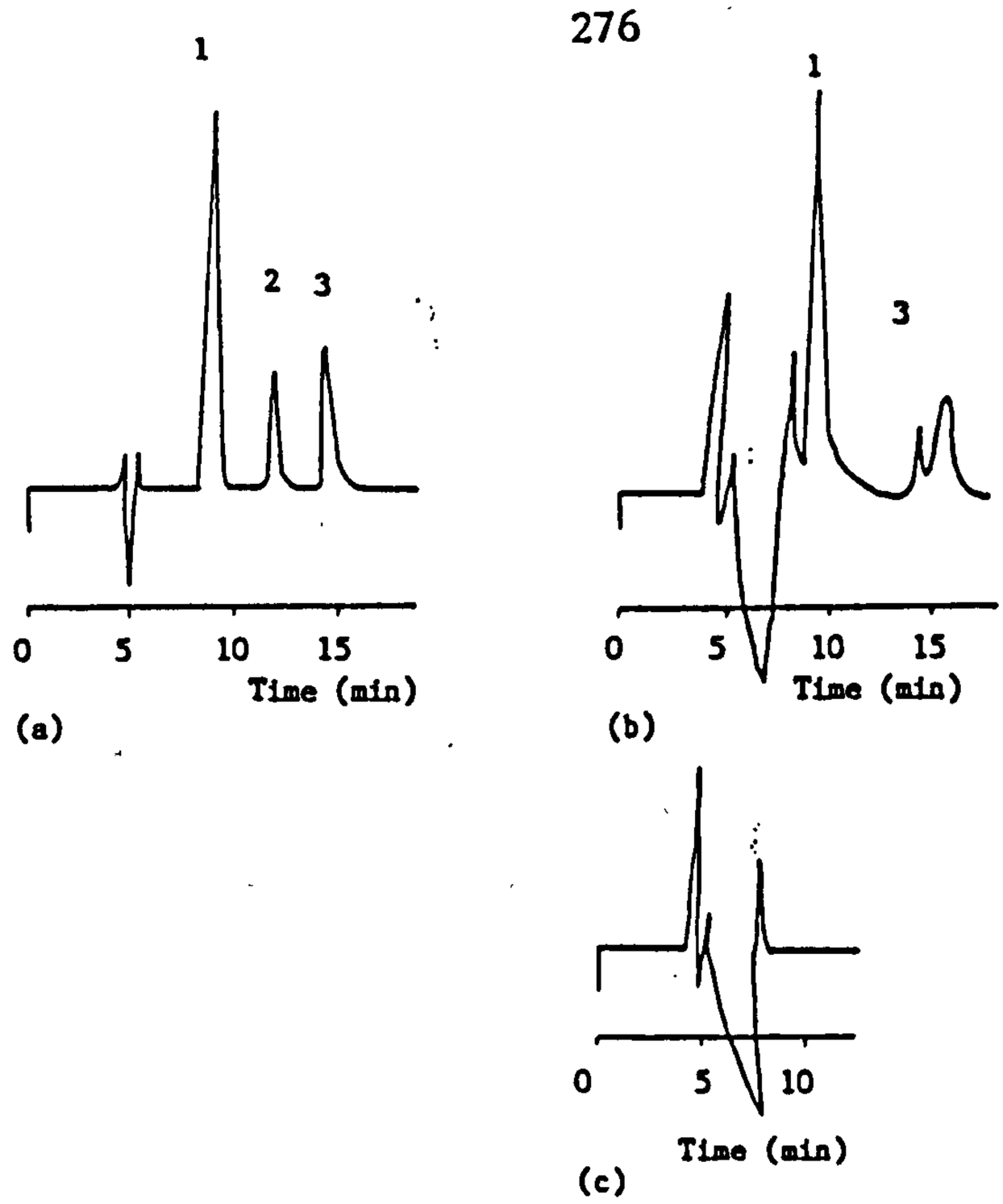


Fig. 7.9 Chromatograms of solutions on a system with a mobile phase of methanol:acetate buffer (65:35), pH 3.1

(a) diazepam, carbostyril and MACB in methanol
(b) degraded diazepam in 0.1 M hydrochloric acid
(c) 0.1 M hydrochloric acid

where peak 1 is diazepam, peak 2 is carbostyril and peak 3 is MACB

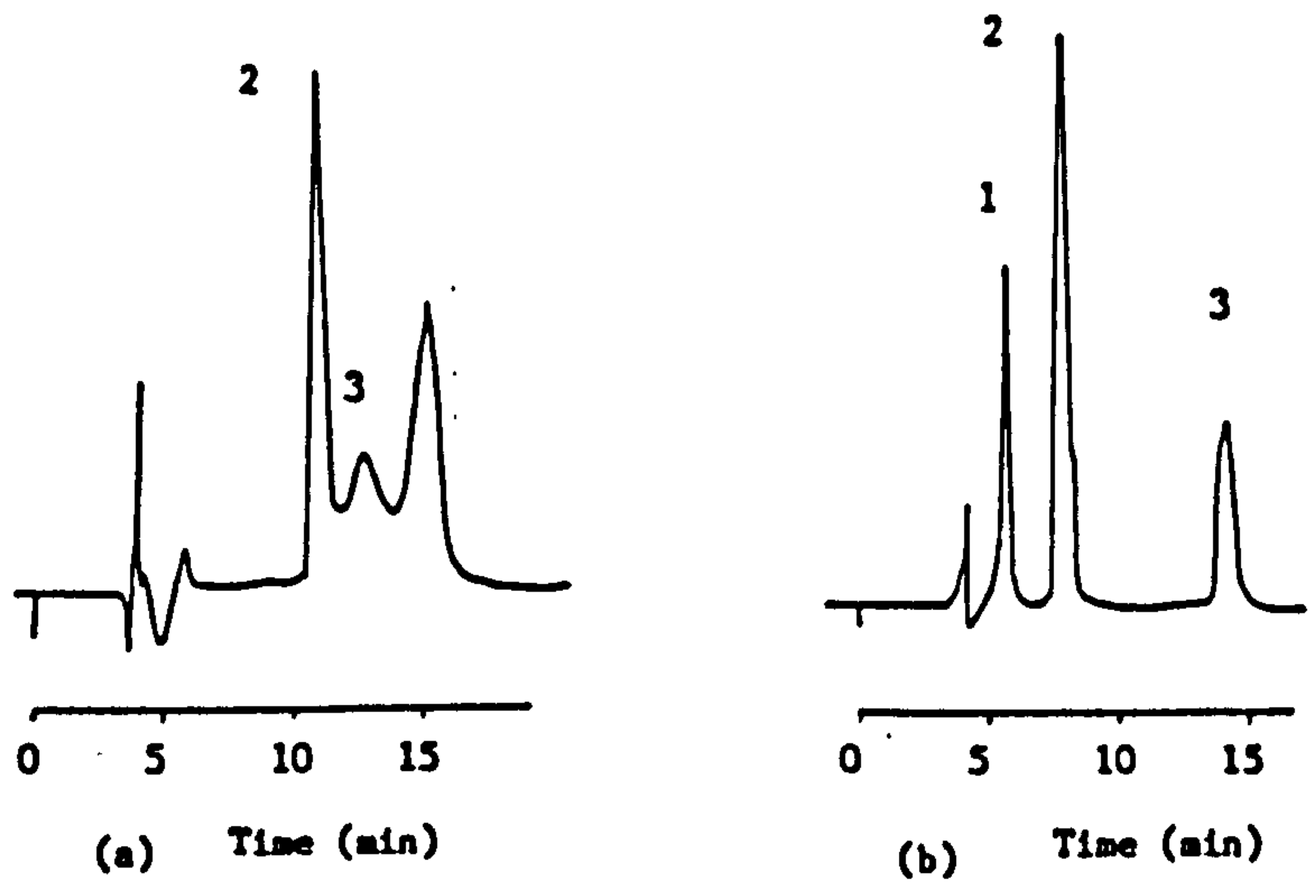


Fig. 7.10 Chromatograms of degraded diazepam in 0.1 M hydrochloric acid with mobile phase mixtures

(a) 75:25 methanol:orthophosphoric acid (pH 2.4)
(b) 75:25 methanol:phosphate buffer (pH 2.8)

where peak 1 is CMACB, peak 2 is diazepam and peak 3 is MACB

of a hydrolysed solution of diazepam in 0.1 M hydrochloric acid with chloroform. After hydrolysis, the acidic solution contains an equilibrium mixture of diazepam, MACB and GMACB. On extraction of this mixture with chloroform, the diazepam and MACB are extracted into the chloroform, leaving only GMACB in the acidic phase. The acidic phase after extraction was injected directly onto the chromatographic system to obtain the retention time of GMACB. Although all the diazepam is extracted, as there is an equilibrium between diazepam and GMACB, this will result in some reversion of the GMACB to diazepam after the extraction. However, on immediate injection after extraction very little of GMACB was found to have reverted. The rate of this reversal was studied for different mobile phase mixtures (Section 7.5.2.3).

Table 7.15 Retention times of compounds of interest; mobile phase methanol:phosphate buffer (75:25) with pH adjusted to pH 2.8

Compound	Retention time (min)	
	Flow rates	
	1.0 ml/min	1.2 ml/min
GMACB	4.8	3.5
Diazepam	6.3	4.5
Carbostyril	12.9	8.1
MACB	15.6	9.9

When a faster flow rate was used, the retention time of the compounds was decreased. This was advantageous for two reasons. Firstly it gave a shorter analysis time allowing more injections of the reaction mixture to be made and secondly it meant that the peaks obtained for each of the components of the mixture

were sharper. This increased the sensitivity of their determination.

7.5.2 Validation of conditions

7.5.2.1 Precision of injection

Replicate injections of a solution containing diazepam in methanol (0.2 mg/ml), were made using a manual Rheodyne valve fitted with a 20 μl loop (HPLC system 2, Section 3.3). Very poor reproducibility of injection was obtained using this system. A second solution of diazepam in methanol of approximately the same concentration was prepared and injected on to a second system (HPLC system 3, Section 3.3). In this system a Negretti and Zambra syringe loading sample injector was used with a 20 μl loop. The peak heights obtained were measured and the results analysed statistically. These results are given in Table 7.16.

Table 7.16 Precision of injection

	Detector response height (mm)	
	System 2	System 3
	196.0	128.6
	194.0	127.0
	195.3	128.0
	193.3	126.5
	197.0	127.4
	192.5	126.8
	204.2	124.4
	204.2	124.8
		125.8
		129.8
		129.8
		129.5
		126.8
		128.0
Mean	197.1	127.4
sd	4.6	1.7
RSD	2.4%	1.3%

Although the relative standard deviation of the injections in System 2 appeared to be poor all of the work reported was performed using this system. To confirm that the Rheodyne valve used in System 2 was responsible for the high relative standard deviation, rather than some fault in the assay method, some injections were made on System 3. When System 3 was used, the relative standard deviation for replicate injections was much lower as were the peak heights measured. The lower peak heights measured may have been due to slight differences in the concentration of diazepam used and also to the sensitivity of the different detectors.

A solution of diazepam (1 mg/ml) in 0.1 M hydrochloric acid was allowed to degrade to equilibrium at room temperature for at least five days. (In this case it was left for four weeks before injections were made, resulting in a higher concentration of MACB). The acidic solution was injected onto the HPLC system repeatedly and the peak height measured for each component. The reproducibility of injection and the ratio of the diazepam and GMACB peaks were calculated. The results are shown in Table 7.17.

Table 7.17 Reproducibility of injection of a degraded sample of diazepam

	Peak height (mm)			PHR
	GMACB	DIAZEPAM	MACB	GMACB:DIAZEPAM
	32.0	113.0	14.3	0.283
	33.5	116.3	17.7	0.288
	31.8	109.6	7.5	0.290
	32.2	114.0	4.5	0.282
	31.8	113.0	5.5	0.281
Mean	32.3	113.2	9.9	0.285
sd	0.7	2.4	5.8	0.004
RSD	2.2%	2.1%	58.6%	1.4%

No change was detected in the equilibrium of the degraded mixture between injections. The precision of peak heights of both GMACB and diazepam is acceptable but the relative standard deviation for MACB is very high. The solution injected in this experiment had been left for sufficient time to allow crystallisation of MACB. The greater variation in peak height for MACB is thought to be due to both inhomogeneity of the samples and also to the low concentration present in solution in the degraded mixture. As the peak height for MACB is very much smaller than those for diazepam and GMACB, the scatter of the measurement of this peak height is much greater. As the MACB is present in the hydrochloric acid solution in quantities greater than its solubility (confirmed by the yellow crystals visible in the solution) this may result in difficulty in obtaining uniform samples of the solution.

7.5.2.2 Linearity of response

A series of solutions of diazepam and MACB were prepared as described in Table 5.2. The average peak height from duplicate injections of each solution was plotted against the concentration of diazepam and MACB (Fig. 7.11). The correlation coefficients of these lines were calculated using standard statistical

Fig 7.11a Linearity of detector response to diazepam

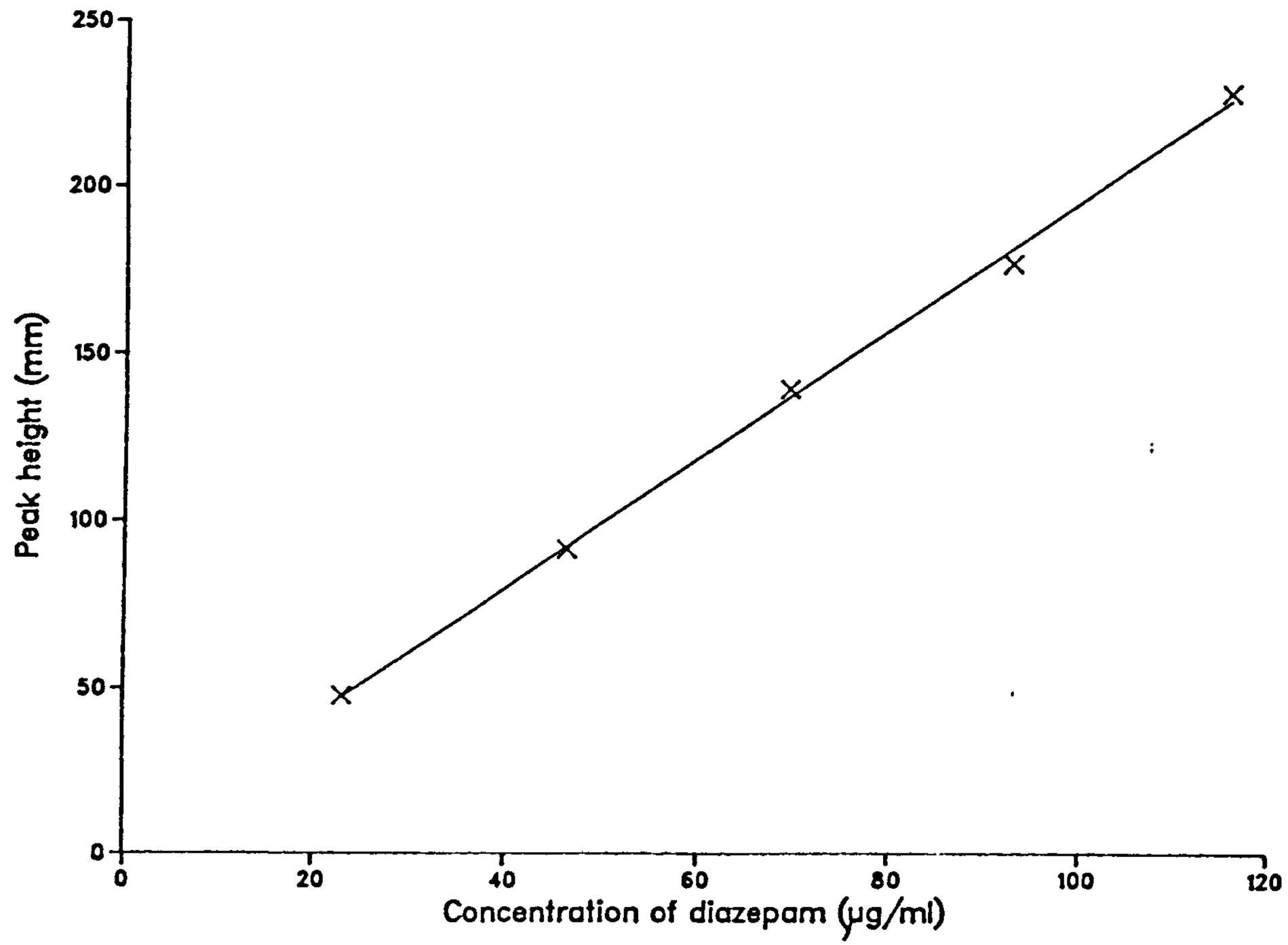
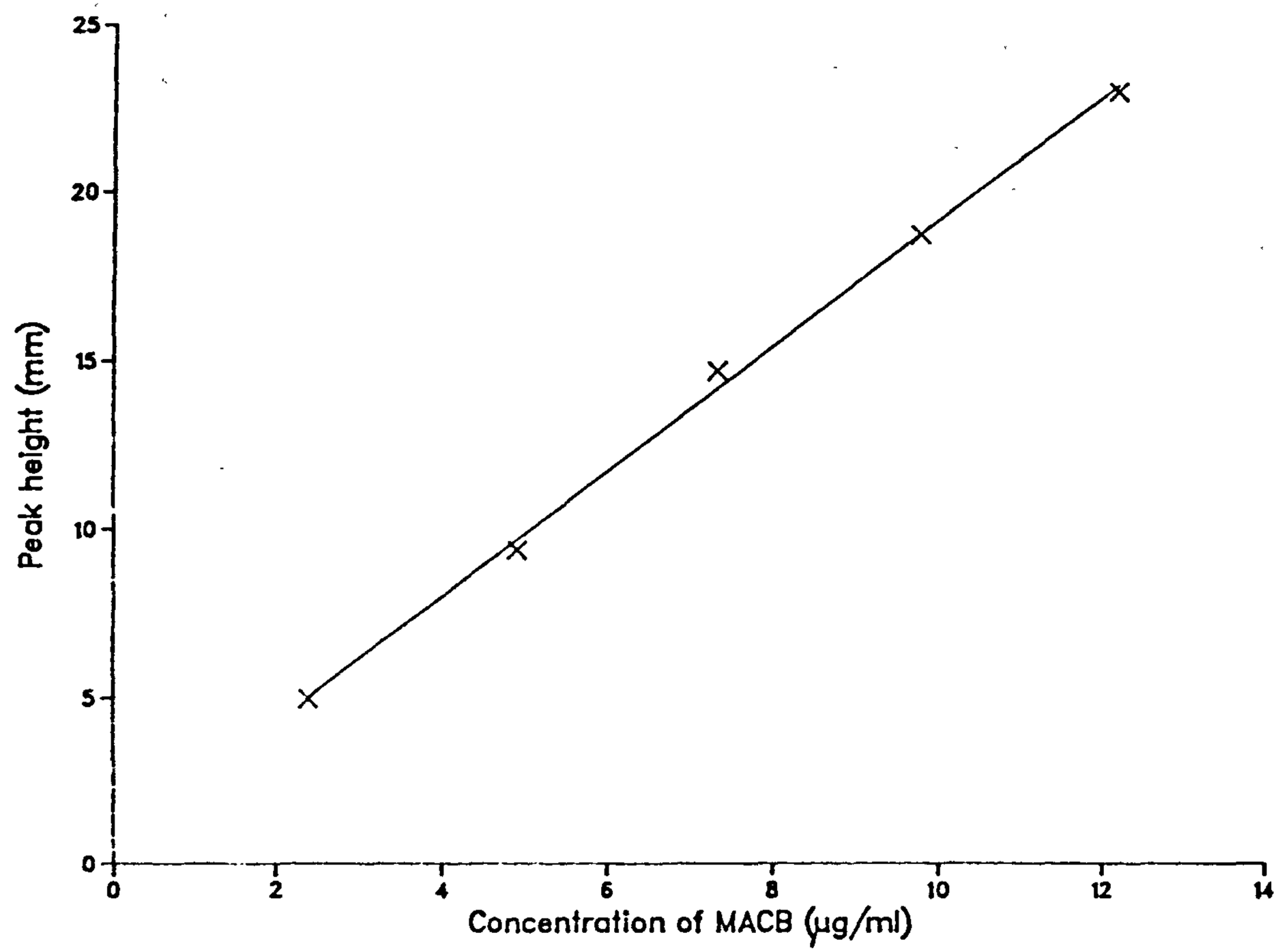


Fig 7.11b Linearity of detector response to MACB



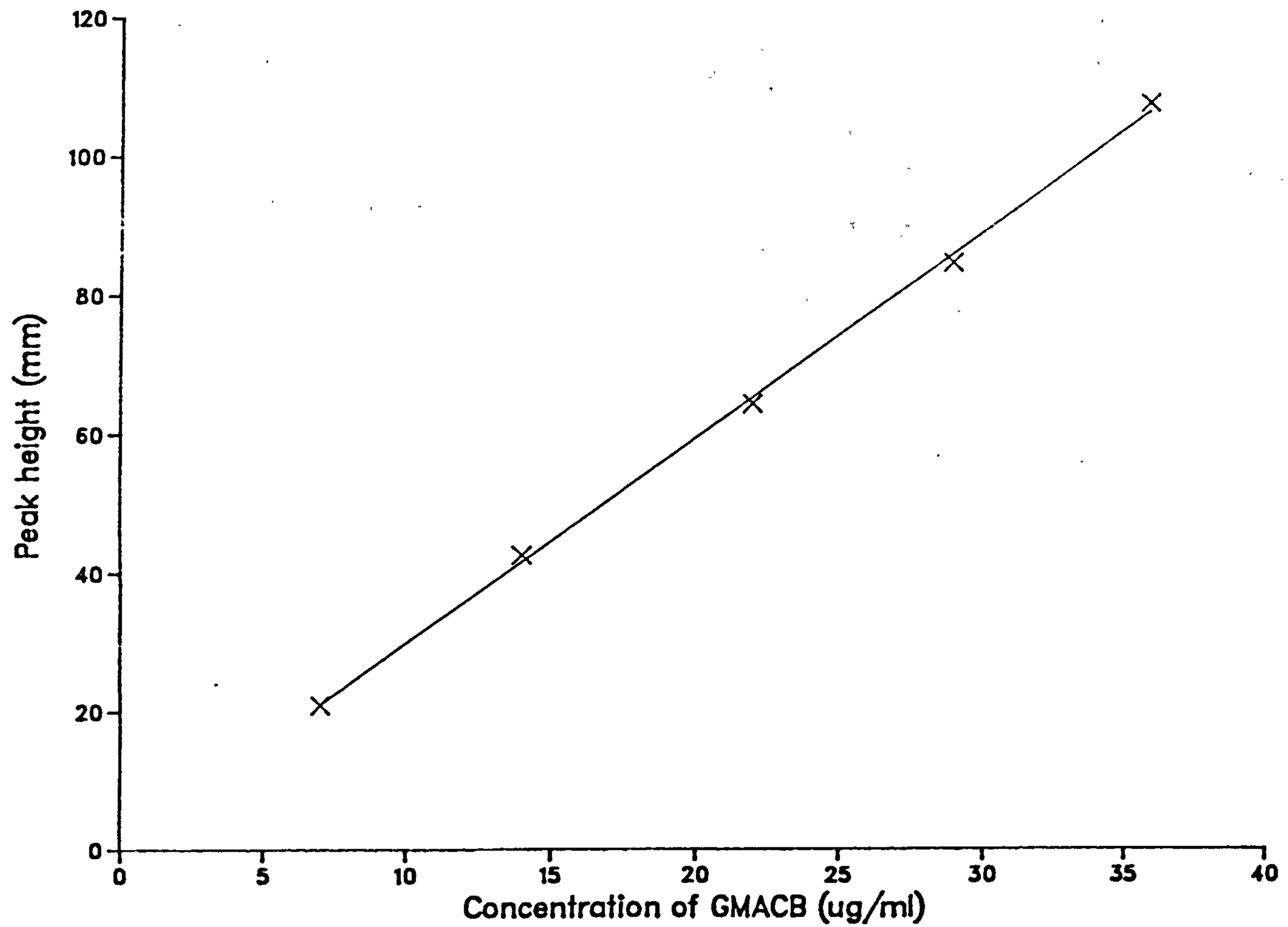
equations. A linear relationship was found for both compounds in the concentration ranges tested. The correlation coefficients for diazepam and MACB were found to be 0.999 and 0.999 respectively.

For GMACB the concentration of the compound present in the solutions was not known in absolute terms. However, as the reaction had proceeded to equilibrium it was expected to be approximately one-third of the initial concentration of diazepam (0.11 mg/ml). Thus the concentration of GMACB was approximately 0.04mg/ml in the most concentrated solution injected. The measured height was plotted against the expected concentration of GMACB. A correlation coefficient of 0.9999 was found in this case, again indicating that the response was linear over the concentration range investigated (Fig. 7.12). The solutions injected from the reaction mixture, (Section 7.6) contained approximately 125 μg/ml of diazepam initially. Thus the solution at equilibrium would contain levels of GMACB within the concentration range checked for linearity.

7.5.2.3 Investigation of the ring-closure of GMACB during analysis

The equilibrium reaction proposed by Nakano et al. and shown in Fig. 1.5 is pH dependent. Instant reversal from GMACB to diazepam is reported to occur at pH 7 and has been confirmed by the GC studies (Section 7.1.3.1). Although GMACB exists in solution at low pH, it is possible that some ring-closure to diazepam would occur at pH 2.8, the pH of the mobile phase. Thus investigation of the equilibrium of the mixture

Fig 7.12 Linearity of detector response to GMACB



at various pH values around pH 3.3 was made to see if any change in equilibrium occurred which may prevent the use of HPLC to measure the concentration of diazepam and GMACB.

An extracted solution of a degraded mixture of diazepam which, if the assumptions of Nakano et al. are correct, should have contained only GMACB, was diluted with mobile phase mixtures containing methanol:buffer (75:25) at a variety of different pH values (pH 1.7, 2.8, 3.5, 5.8 and 7.4). These pH values were the apparent final pH of the mobile phase mixtures.

The UV spectrum of GMACB shows a λ_{max} at 255nm while diazepam exhibits a λ_{min} at 240 nm, (Fig. 6.1 and Fig. 7.13). The absorption spectra of the solutions at different pH values were recorded and the absorbances at 240 and 255 nm were measured, at set time intervals after preparation. A standard solution of diazepam (0.24 mg/ml) was also diluted in these mobile phases for comparison with the spectra of the equilibrium mixture solutions. The absorbance measurements are given in Table 7.18.

These results and the spectra obtained, (Fig. 7.14) show that no change in spectral properties of GMACB occurred in pH 1.7 solution. The spectra of this solution did not match that of the standard solution of diazepam in methanol under the same pH conditions. For the solution at pH 2.8, the spectra at each time interval appeared similar, but the absorbances at 240 and 255 nm altered slightly, as did the absorbance ratio of both wavelengths. This

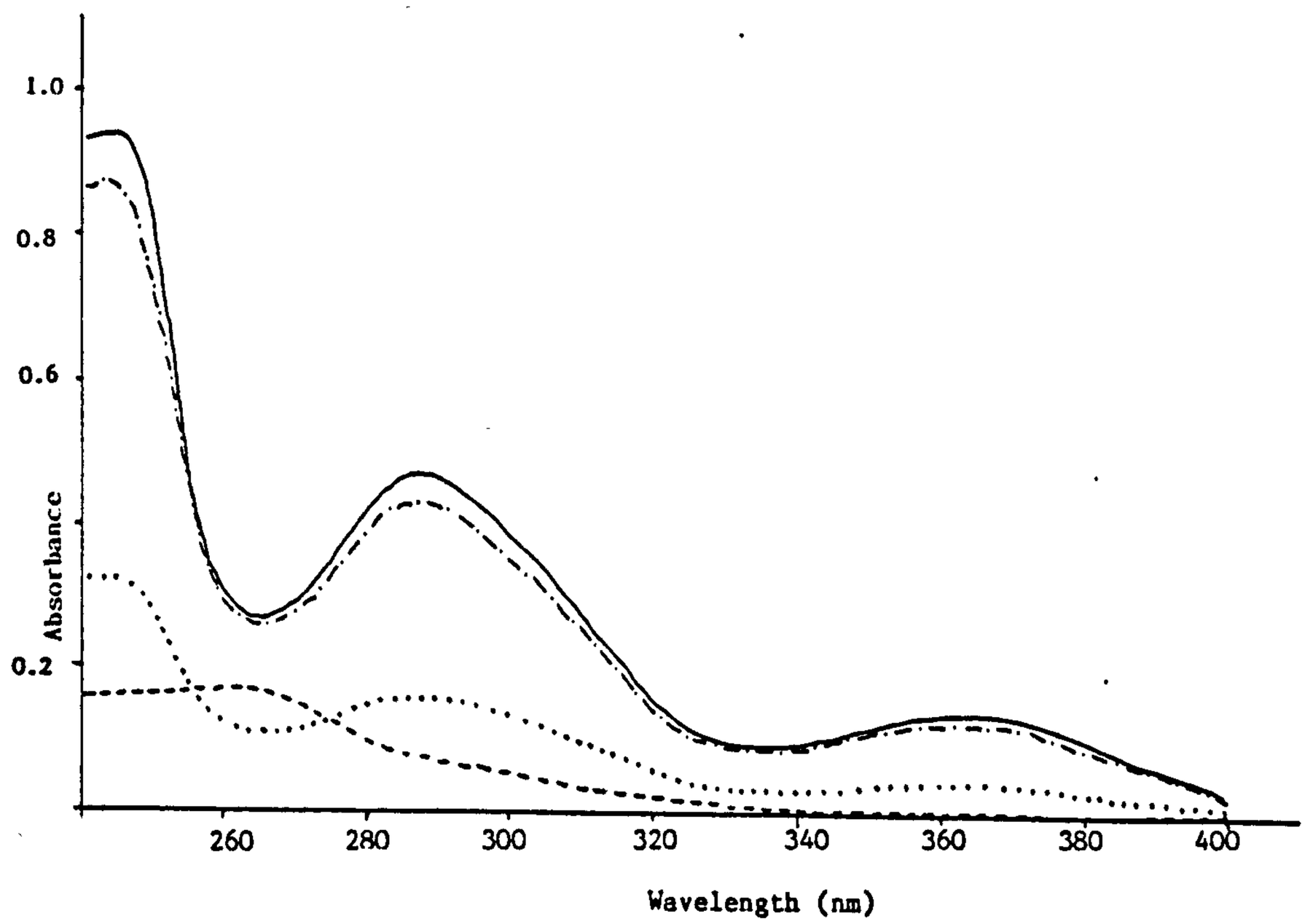


Fig. 7.13 Absorption spectra of diazepam and GMACB from a degraded solution of diazepam in 0.1 M hydrochloric acid

——— Absorption spectrum of diazepam in methanol

—·—·— Absorption spectrum of the extract from the solution at pH 1, sample redissolved in methanol

- - - - - Absorption spectrum of the acidic solution left after extraction

····· Absorption spectrum of an extract from the aqueous phase after extraction and neutralisation, sample redissolved in methanol

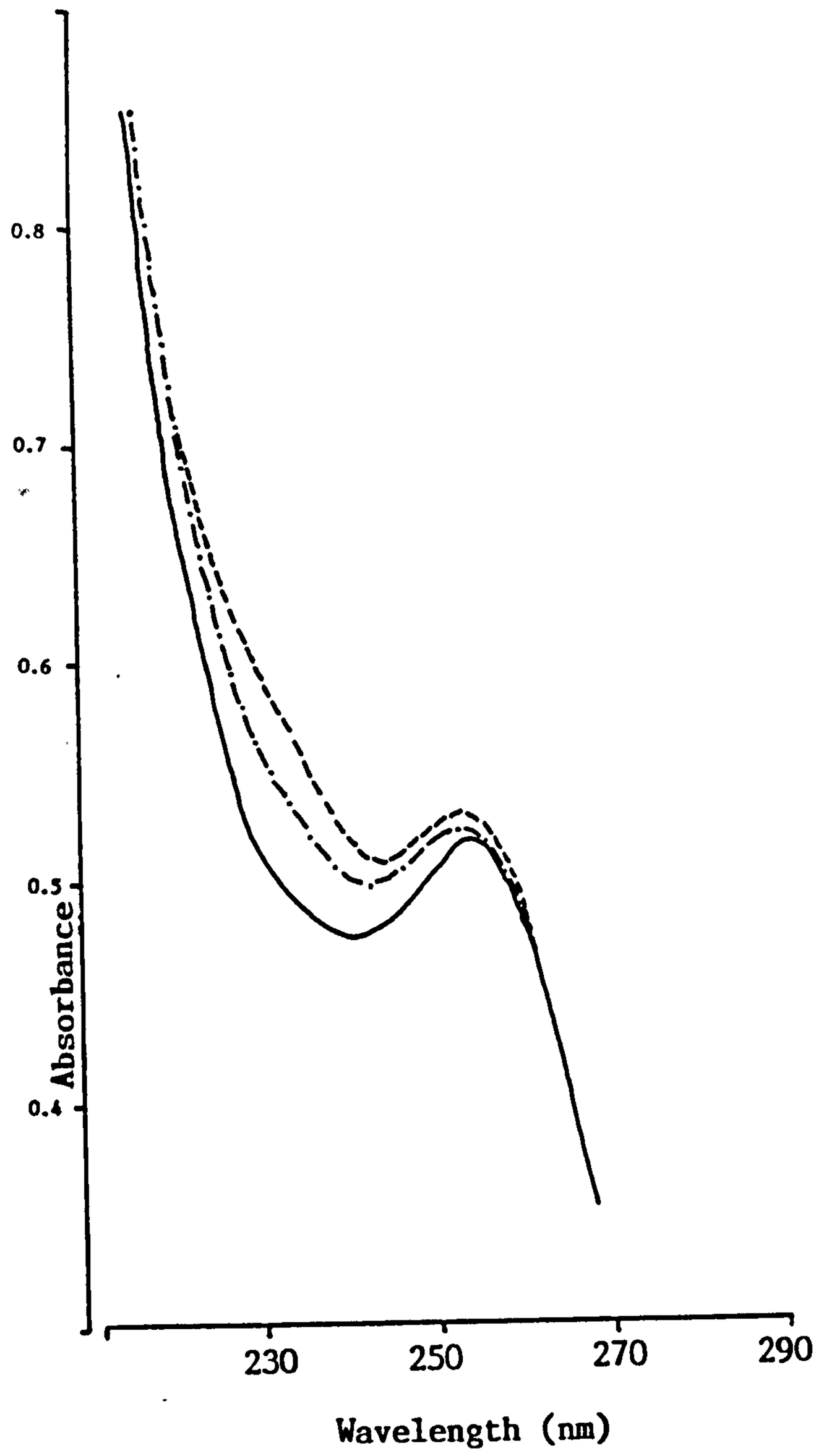


Fig. 7.14 Change in the absorbance spectrum of GMACB with time, at pH 3.5. Spectra generated at pH 1.7 and pH 9.3 were superimposed on each other.

———— Absorbance spectrum of GMACB at zero time

—·—·— Absorbance spectrum of GMACB after 5 min

- - - - - - Absorbance spectrum of GMACB after 10 min

Table 7.18 Investigation of ring-closure at different pH values, monitoring the absorbance at 240 nm and 255 nm for dilutions of a solution of GMACB and a standard solution of diazepam

Time after dilution (min)	Wavelength 240 nm	255 nm	Ratio 240 nm/255 nm
pH 1.7			
0	0.451	0.523	0.862
5	0.452	0.523	0.864
10	0.454	0.524	0.866
15	0.457	0.526	0.869
Diazepam	0.486	0.186	2.613
pH 2.8			
0	0.480	0.547	0.878
5	0.535	0.589	0.908
10	0.486	0.531	0.915
15	0.536	0.562	0.954
Diazepam	0.344	0.152	2.263
pH 3.5			
0	0.477	0.558	0.855
5	0.512	0.574	0.892
10	0.545	0.586	0.930
15	0.545	0.567	0.961
Diazepam	0.270	0.151	1.788
pH 6.8			
0	0.598	0.581	1.029
5	0.799	0.602	1.327
10	0.864	0.607	1.423
15	0.890	0.611	1.457
Diazepam	0.379	0.252	1.504
pH 9.3			
0	0.487	0.569	0.856
5	0.488	0.570	0.856
10	0.490	0.570	0.860
15	0.491	0.568	0.864
Diazepam	0.382	0.262	1.458

variation in absorbance ratio suggests that some change occurs with time.

At pH 6.8 the absorbance of the solution changes rapidly to match that of standard diazepam. At pH 9.3 there appears to be no change in the spectral properties of the diluted solution, suggesting that the reversal to diazepam does not occur under these conditions.

Nakano proposes that at pH values above the pK_a of diazepam (pH 3.3), the ring-closure occurs very rapidly to form diazepam and that at pH 7.4 the reaction is instantaneous. The ring-closure gives a compound with a UV spectrum corresponding to that of diazepam. The results in Table 7.18 appear to confirm this for the lower pH solutions. However, the response of the solution at pH 9.3 cannot be explained and further work is required to resolve the problem. The UV spectrum obtained for the species formed after the ring-closure is not identical to that of diazepam, but no explanation has found for the difference seen. Further work was performed, reported in Section 7.7, where other attempts were made to identify this species. No alternative has been found for the identity of the compound formed after neutralisation.

A solution of hydrolysed diazepam in 0.1 M hydrochloric acid (initial concentration 1 mg/ml) at equilibrium at room temperature was extracted with chloroform. The solution remaining after extraction was diluted with mobile phase and injected onto the HPLC system. The mobile phase used was

methanol:phosphate buffer (75:25), at pH 3.2. The chromatogram of this solution is shown in Fig. 7.15. Two peaks are seen, one of which corresponds to GMACB and the other to diazepam. From this it appears that immediately after dilution of the solution of GMACB some diazepam is formed.

In a second experiment, injections of GMACB solutions prepared in the same way were made at time intervals after dilution of the solution in pH 1.7 and pH 3.2 mobile phase solutions. The mobile phase used for the chromatography was methanol:phosphate buffer (75:25) at pH 3.2. The peak heights for diazepam and GMACB were measured and the results are given in Table 7.19.

Table 7.19 Investigation of the rate of ring-closure using HPLC for measurement

pH	Time after dilution (min)	Peak height (mm) GMACB	Diazepam	PHR GMACB:DIAZEPAM
1.7	0	15.5	1.5	10.3
	12	16.5	1.8	9.2
	25	15.8	1.5	10.5
3.2	0	14.5	1.5	9.7
	10	13.5	3.0	4.5
	20	12.0	4.5	2.7

Measurable quantities of diazepam were found in both solutions even at zero time. This is due either to incomplete extraction of the diazepam in the first instance or immediate reversion of some of the GMACB to form diazepam. From previous work (GC and UV) it is known that 99.5% of the diazepam can be removed by extraction, and so this is unlikely to be the problem. On following the change in content of GMACB with time, it could be seen that the solution at pH 1.7 does not alter over a 20 minute period whereas that at pH

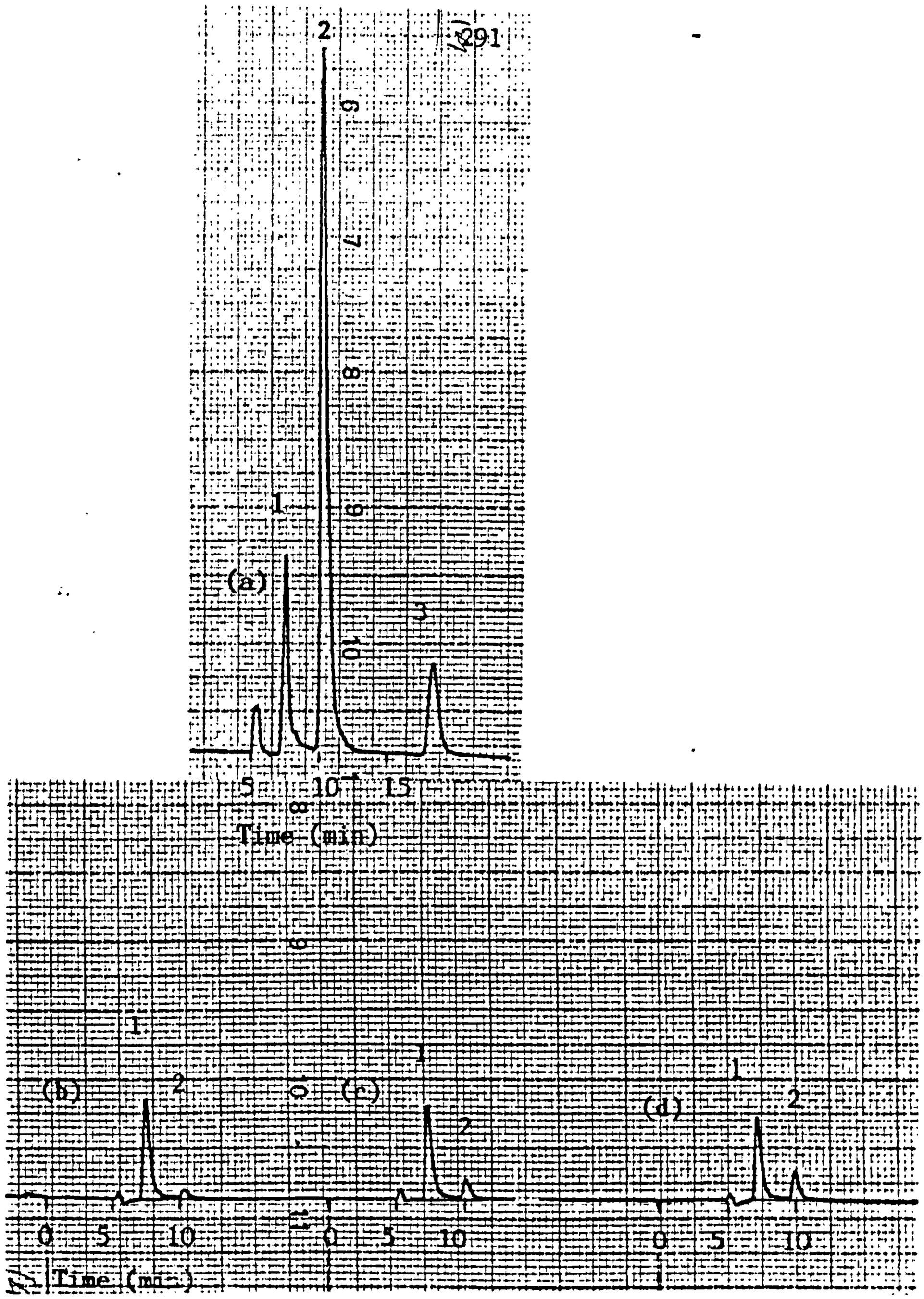


Fig. 7.15 Investigation of the stability of a solution of GMACB in 0.1 M hydrochloric acid, diluted in mobile phase at pH 3.2

(a) chromatogram of degraded mixture

(b) chromatogram of the solution of GMACB diluted in mobile phase at pH 3.2 at zero time

(c) chromatogram of the solution of GMACB after 10 min

(d) chromatogram of the solution of GMACB after 20 min

where peak 1 is GMACB, peak 2 is diazepam and peak 3 is MACB

3.2 does alter with the content of GMACB decreasing and that of diazepam increasing.

These changes suggest that some re-equilibration of GMACB occurs when its pH is altered. The change occurs more quickly at higher values. It appears that there is no measurable change in pH 1.7 solution, but that in pH 3.2 solution the ring-closure occurs at a significant speed. On injection of the solution at pH 1.7 onto the column, the diazepam detected may be due to re-equilibration occurring on the column, once the 20 μl band of pH 1.7 solution injected becomes mixed with the mobile phase at pH 3.2.

The re-equilibration observed in this experiment is a somewhat different situation than would occur in the acidic solutions of diazepam which are being used to follow the rate of hydrolysis. In the work described above, a solution of GMACB was prepared by extraction of diazepam and MACB from the acidic mixture. As GMACB is formed from diazepam in an equilibrium reaction, once the equilibrium has been altered, by removing one of its components, it would be expected that the GMACB would undergo the ring-closure reaction to regain the equilibrium state.

To mimic more closely the situation expected in following the hydrolysis reaction, a solution of equilibrated mixture was diluted with methanol:buffer solutions at a variety of pH values. The pH values used were 1.3, 1.7, 2.8 and 3.2 and these dilutions were injected onto the HPLC system with mobile phase at pH 3.2. Samples were injected at zero time, 10 and 20 minutes after dilution. In this study the peak

heights, particularly for diazepam, were much larger than in the previous experiment (Table 7.19), and consequently the error involved in their measurement was less. The results in Table 7.20 show that as the pH of the solution used as diluent increases, the composition of the mixture appears to alter. However, this change is very much less than that reported for the solution of GMACB alone. At pH 2.8 and pH 3.2 small changes occurred in the diazepam content and the peak height ratio with time, although these were not considered to be significant. As the peak height ratio of diazepam:GMACB was constant when the solutions were left in the mobile phase for 20 min before injection, samples removed from the reaction mixture were expected to remain unaltered during analysis. The dilution of the solution of hydrolysed diazepam with mobile phase at pH 2.8, that occurs on injection into the chromatographic system should not result in any significant alteration of the equilibrium between GMACB and diazepam. Thus the method developed allows the accurate determination by HPLC of all the components in the reaction mixture (diazepam, GMACB and MACB).

Table 7.20 Investigation of the effect of pH on the equilibrium between diazepam and GMACB

pH	Time after dilution (min)	Peak height (mm) GMACB	Peak height (mm) Diazepam	PHR GMACB:DIAZEPAM
1.3	0	32.4	112.0	0.29
	10	32.4	111.0	0.29
	20	32.0	110.0	0.29
1.7	0	33.8	110.8	0.31
	10	31.0	102.8	0.30
	20	31.8	103.8	0.31
2.8	0	31.0	109.2	0.28
	13	31.5	111.8	0.28
	23	31.0	112.0	0.28
3.2	0	28.0	97.0	0.29
	12	27.0	97.5	0.28
	21	26.8	100.0	0.27

7.6 APPLICATION OF THE HPLC METHOD TO STUDY THE KINETICS OF THE HYDROLYSIS OF DIAZEPAM

7.6.1 Preparation of the reaction mixture

The reaction mixture was prepared as described in Section 5.6.1.

7.6.2 Analysis of the reaction mixture

Samples of the reaction mixture taken at specified time intervals were injected onto the HPLC system defined in Section 5.6.2. The peak heights for diazepam and GMACB were measured and used to calculate the rate constants for the hydrolysis reaction of diazepam at 37°C. Although the HPLC traces indicate that a gradual increase in GMACB concentration occurs with time, the peak height of diazepam did not show any significant reduction. Therefore it proved impossible to use these results for the calculation of the rate constant for the hydrolysis of diazepam. The difficulty was thought to be due to the poor reproducibility of injection using the Rheodyne loop on this system (system 2). The possibility of drift in the detector response was also considered and in an attempt to compensate for any drift the work was repeated using injections of the hydrolysis mixture sampled at 20 min intervals. These were alternated with injections of a standard solution of diazepam of the same concentration as that initially present in the hydrolysis reaction. The peak heights of the standard solutions were used to give a measure of any variation in the detector response over the time course of the reaction and the sample peak heights

were adjusted to compensate for this. The adjusted peak height was calculated at each time point and the equations detailed in Appendix I were applied to the results.

By plotting the peak heights of diazepam and GMACB against time, it was possible to determine the stage in the reaction when equilibrium appeared to be established, with no large change occurring in the measured height (Fig. 7.16). The mean peak height for diazepam was calculated once the equilibrium had been reached and this was used in calculation of the rate constants for the reaction.

The value calculated for $\log(P_t-P_\infty/P_o-P_\infty)$ was plotted against time, and for a first-order reaction would give a straight line passing through zero, with slope equal to $-(k_f+k_r)$. The value of K_{eq} was calculated from $(P_o-P_\infty)/P_o$ and was equal to k_f/k_r. The values obtained for the slope and K_{eq} were substituted into the equations and the rate constants for the forward and reverse reactions were calculated. These graphs, plotted for the change in diazepam with time, are shown in Fig. 7.17, and the calculated rate constants are reported in Table 7.21, together with values reported by Nakano et al. (1979).

Table 7.21 Rate constants for the hydrolysis of diazepam at 37°C, followed by HPLC

	Rate constant (h^{-1})		
	k_f (ring-opening)	k_r (ring-closing)	K_{eq}
Diazepam decrease (not bracketed)	0.10	0.20	0.50
Diazepam decrease (bracketed)	0.17	0.34	0.50
Nakano et al. (using a variety of different methods)	0.15	0.30	0.50
	0.14	0.23	0.61
	0.10	0.18	0.55
	0.13	0.24	0.54

Fig 7.16 Peak height of diazepam monitored by HPLC
Study of hydrolysis reaction.

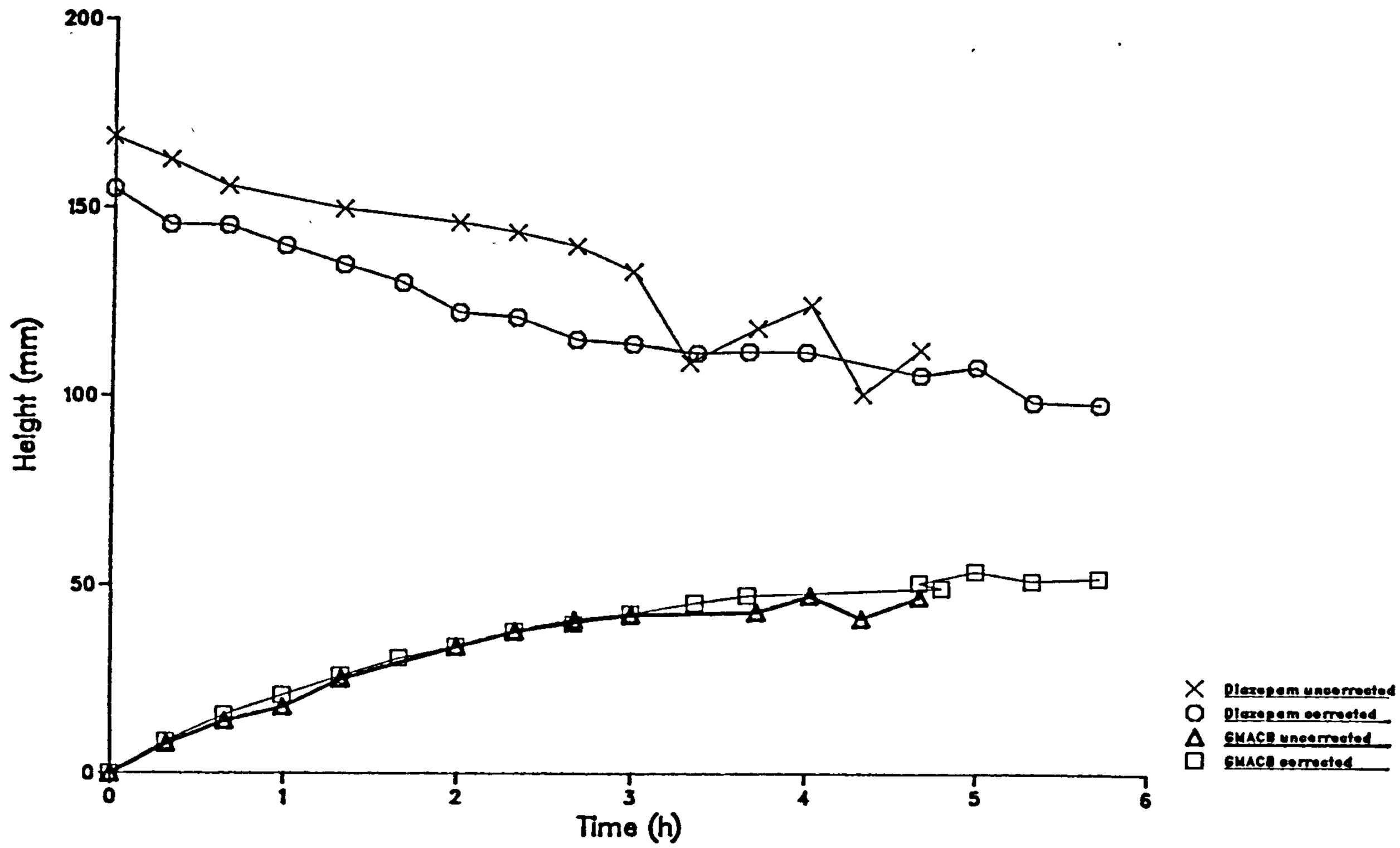
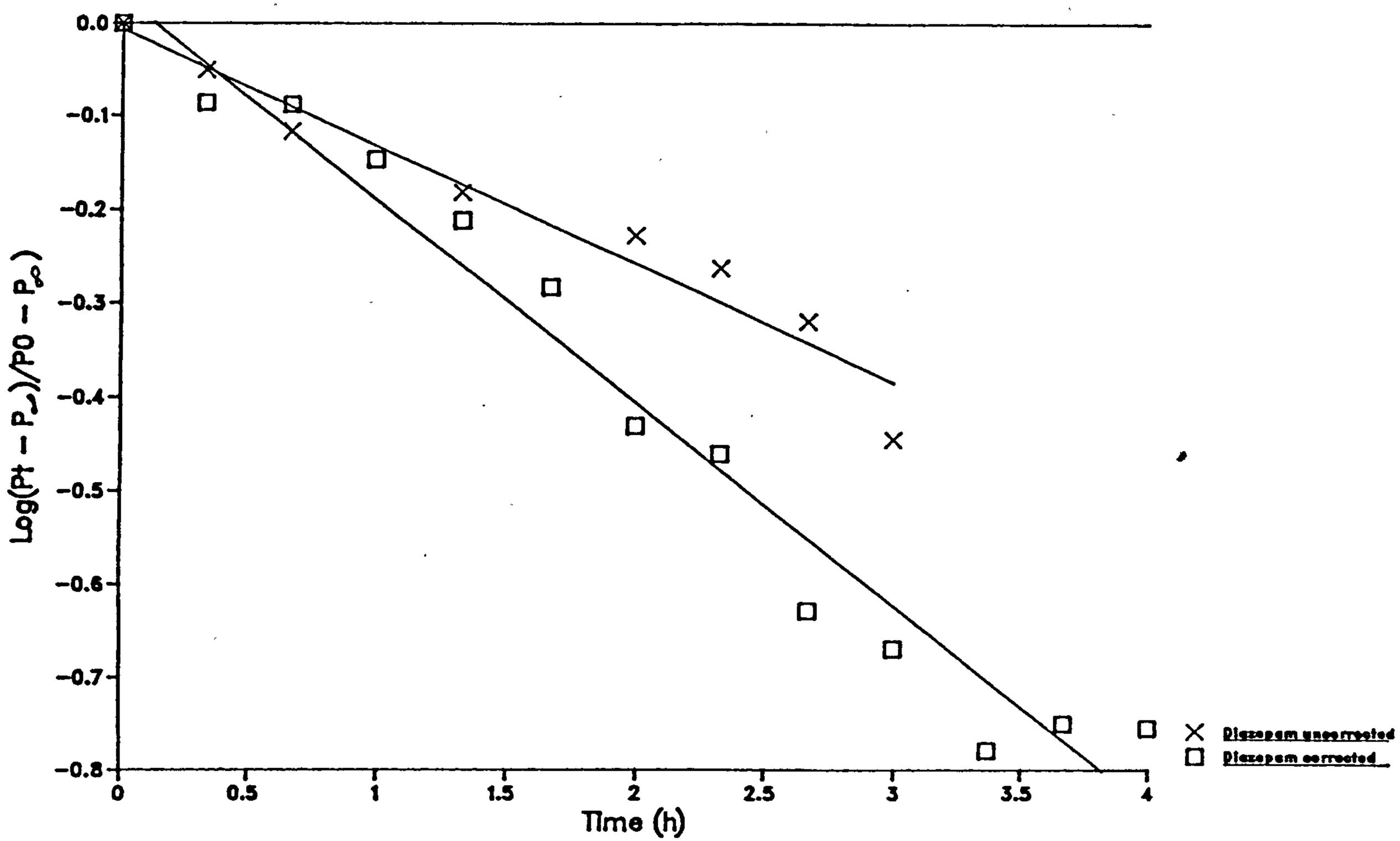


Fig 7.17 Change in concentration of diazepam with time (in 0.1M hydrochloric acid at 37°C). Reaction followed by HPLC measurement



The values obtained by HPLC for the rate constants for the hydrolysis of diazepam in 0.1 M hydrochloric acid at 37°C agree with the values obtained by Nakano et al., using UV measurement. The ratio of GMACB to diazepam also agrees with that seen by GC and UV measurements (Sections 7.3.2 and 7.4.1). A large spread of results was seen for the actual rate constants measured, both by HPLC and UV measurements.

The rate constants quoted by HPLC measurement were calculated from the graphs plotted in Fig. 7.17. The assumption made by Nakano et al. and continued in this work was that the reaction being studied was the first-order reversible reaction between diazepam and GMACB, and that no other species is formed in the reaction. The equations used to calculate the rate constants are only valid if this is true, and under those conditions would give a straight-line logarithmic plot passing through the origin.

As was seen from the GC work, the reversible reaction is only one step in the hydrolysis of diazepam in acid with a further reaction occurring to give MACB. This second reaction is slow, and at 37°C will be slower than seen by GC (Section 7.3.2), however it does occur, and the presence of increasing amounts of MACB was observed with time on the HPLC trace. At the end of the 6 hour period studied, the content of MACB

measured by HPLC was approaching 0.7% of the initial content of diazepam, however, as MACB is poorly soluble in acid, it is possible that more may be present than is actually measured. It is unlikely that the level of MACB would be large enough to cause errors leading to the differences seen between the different reactions followed and it seems more likely that these were due to poor reproducibility of the analytical technique used. In studying the reaction kinetics of the reversible reaction, Nakano et al. followed the reaction for 11 hours at 37°C to ensure that equilibrium was reached. In the work reported here it was not possible to follow the reaction for that length of time, so although the reaction appeared to have reached equilibrium, this may not have been the case, providing a further potential source of error.

The HPLC procedure used in this study is dependent on precise volums of solution being injected onto the system, as no internal standard was used. It would have been difficult to find an internal standard suitable for this study, requiring a compound with suitable retention time on the system used, which was non-reactive, stable, soluble in acid and UV absorbing at the wavelength of detection. With modern HPLC systems, external standardisation is becomming more common, as less errors are introduced in the determinations, provided satisfactory reproducibility of injection can be obtained. In this study, the reproducibility of injection was not as good as required, better precision of injection having been acheived with the injector used in System 3. Unfortunately this system could not be used for

analysis of samples taken from the hydrolysis reaction, although it shows that different equipment would have provided better precision. A further improvement may have been obtained if an automated injection system had been available. No obvious drift was seen on injection of standard solutions between samples over the 6 h period of the study, but these injections were used to calculate the concentration of diazepam using bracketed standards. Any drift present may have been masked by the differences seen between injections due to poor precision of injection.

In the work by Nakano et al.(1979), a number of different rates of forward and reverse reactions were calculated, depending on the way in which the experiment was performed. The results obtained here gave similar reaction rates to those quoted, but obviously a larger number of reactions require to be studied to provide meaningful results. Using a more reproducible injection system, with automated integration of the peaks obtained would be required to improve the precision of the results. Comparison could then be made of with results generated by UV absorbance measurements where more than one absorbing species was present. The use of HPLC provides a method whereby all three components of the reaction mixture can be separated and quantified by one method, allowing calculation of reaction rates from these results without the need for mathematical manipulation of the data generated, or extraction of the different components of the mixture prior to measurement.

7.7 INVESTIGATION OF THE INTERMEDIATE HYDROLYSIS PRODUCT OF DIAZEPAM

Evidence was seen from HPLC, GC and UV to support the formation of an equilibrium between diazepam and an intermediate hydrolysis product. Further work using TLC and IR spectrophotometry is reported confirming this.

7.7.1 Thin-layer chromatography

7.7.1.1 R_f values of spots of interest

Spots of diazepam, carbostyril and MACB were applied to TLC plates in two solvents, and the plates were developed in two mobile phase systems. The R_f values obtained for the spots are shown in Table 7.22.

Table 7.22 R_f values of compounds of interest

Compound	R_f values			
	Solvent system 1*		Solvent system 2*	
	A	B	A	B
Diazepam	0.15	0.17	0.20	0.19
Carbostyril	0.25	0.28	0.28	0.29
MACB	0.60	0.60	0.60	0.60

* Solvent system 1 = TEA (80:18:2)
Solvent system 2 = TEA (80:15:5)

Spotting solvent A = chloroform
Spotting solvent B = hydrochloric acid/ethanol

No large difference was seen between the R_f values obtained using different solvent or mobile phase systems. The mobile phase chosen used solvent

proportions 80:18:2 and the standards applied to the plates were in the same solvent as the samples applied.

The spot seen for MACB was visible as a yellow spot, the spot corresponding to carbostyril showed intense blue fluorescence under UV light. No spot was seen for glycine using UV light for visualisation, a white area was seen on the plate at the origin both before and after development of the plate, suggesting that no movement of glycine occurred in the mobile phase mixtures used, although the plate was not sprayed with ninhydrin reagent to confirm this.

A sample of hydrolysis mixture was extracted with two separate portions of chloroform and the pH of the remaining aqueous solution adjusted to pH 7 before extraction with a third portion of chloroform. These chloroform extracts were concentrated and applied to a TLC plate together with standard diazepam, carbostyril and MACB.

On development of this plate, spots were seen from the first extract with R_f values corresponding to diazepam and MACB. A very small spot corresponding to diazepam was seen in the second extract, and a larger spot with the same R_f value as diazepam was seen in the third extract. These results support those obtained by GC, HPLC and UV (Sections 7.1, 7.5 and 7.6) which suggested that diazepam and MACB were both extracted by chloroform, but that some other compound was also present which was not extracted from acid. On alteration of the pH of the solution after any diazepam present had been extracted, it was found

possible to extract amounts of a compound with an R_f value corresponding to diazepam. This suggested that the non-extracted material was being converted to diazepam by pH change.

Spots of the concentrated hydrolysis mixture obtained by evaporation under vacuum and by heating on a hotplate were applied to a TLC plate and the plate developed. The profile seen from both of these samples was different. The sample evaporated using low temperature and vacuum contained two spots, one with an R_f value corresponding to MACB and the second on the base-line. The samples subjected to greater heat on a hotplate did not show any spot corresponding to MACB but had three low-running spots, one with the same R_f as diazepam, a second with R_f value 0.07 and a small base-line spot. No spot corresponding to carbostyril was seen in either sample.

A different profile was seen from the spots of hydrolysis mixture which had ammonium hydroxide applied before development. For the sample evaporated at low temperature, a spot was seen with an R_f value corresponding to diazepam, the spot corresponding to MACB was still present, and the base-line spot was still seen although it was smaller than in the sample developed without treatment with ammonium hydroxide. A spot with R_f of 0.06 was also seen. For the sample which had been subjected to high temperature evaporation, the application of ammonium hydroxide before development resulted in no obvious change in profile.

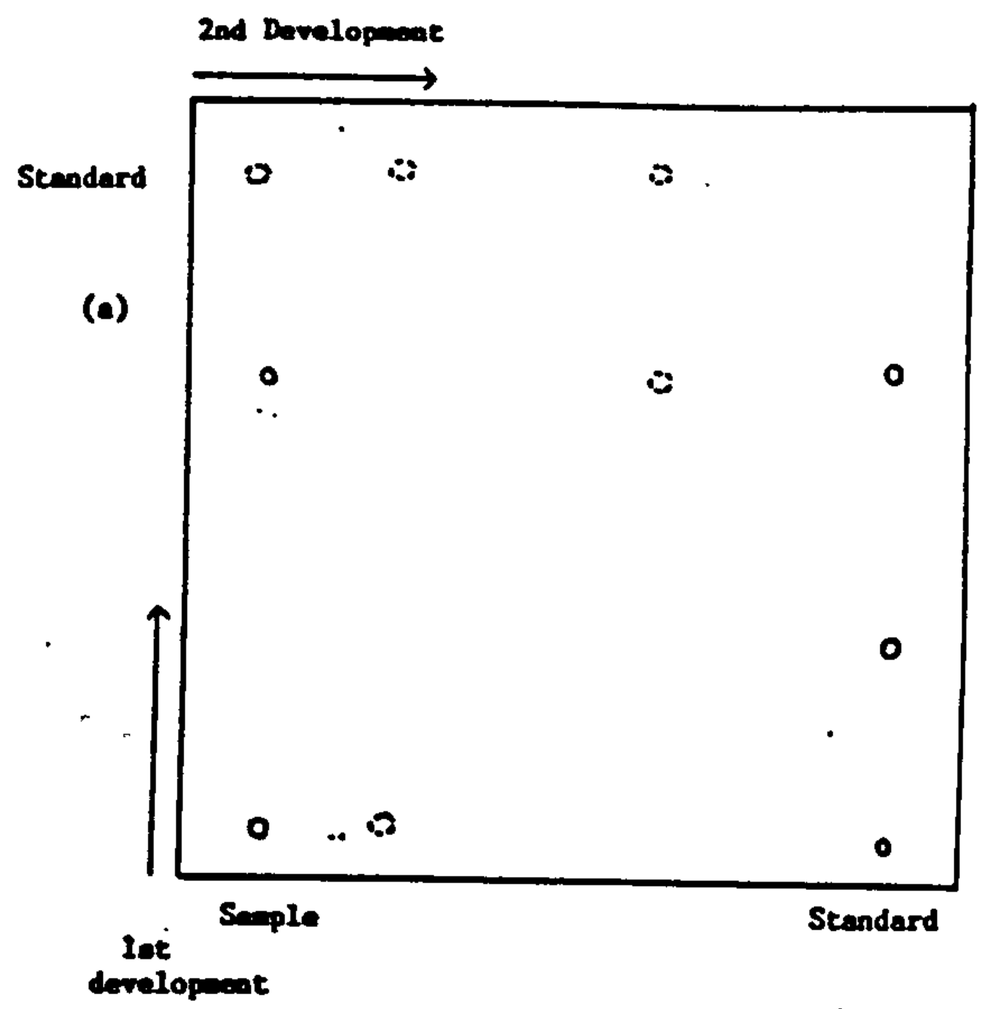
From this work it was postulated that the base-line

spot contained the open-ring intermediate, and that the addition of ammonium hydroxide to this resulted in reversion of GMACB to diazepam. To confirm this, further work was performed using two-dimensional TLC.

7.7.1.2 Two-dimensional plates

Spots of the two concentrated hydrolysis mixtures were applied to two plates and developed in mobile phase as described (Section 5.7.1.3). After drying and visualising, further treatment of each plate was performed by addition of ammonium hydroxide to the origin, and the plates were redeveloped in a second direction. After development in two directions, the plates were visualised again and the spot pattern seen reproduced in Fig. 7.18. A different impurity profile was seen for the samples treated by different amounts of heat.

In the sample evaporated under vacuum, on first development two spots were seen, one at the base-line and one corresponding to MACB. After treatment with ammonium hydroxide and redevelopment of the plate, the spot corresponding to MACB on first development had moved to an R_f value corresdonding to standard MACB. The spot on the base-line was still present but was smaller, and a second spot was seen at an R_f corresponding to diazepam, with some streaking between these two spots. It was proposed that the spot on the base-line contained GMACB which was converted to diazepam by treatment with alkali. The spot remaining on the base-line was thought to be due



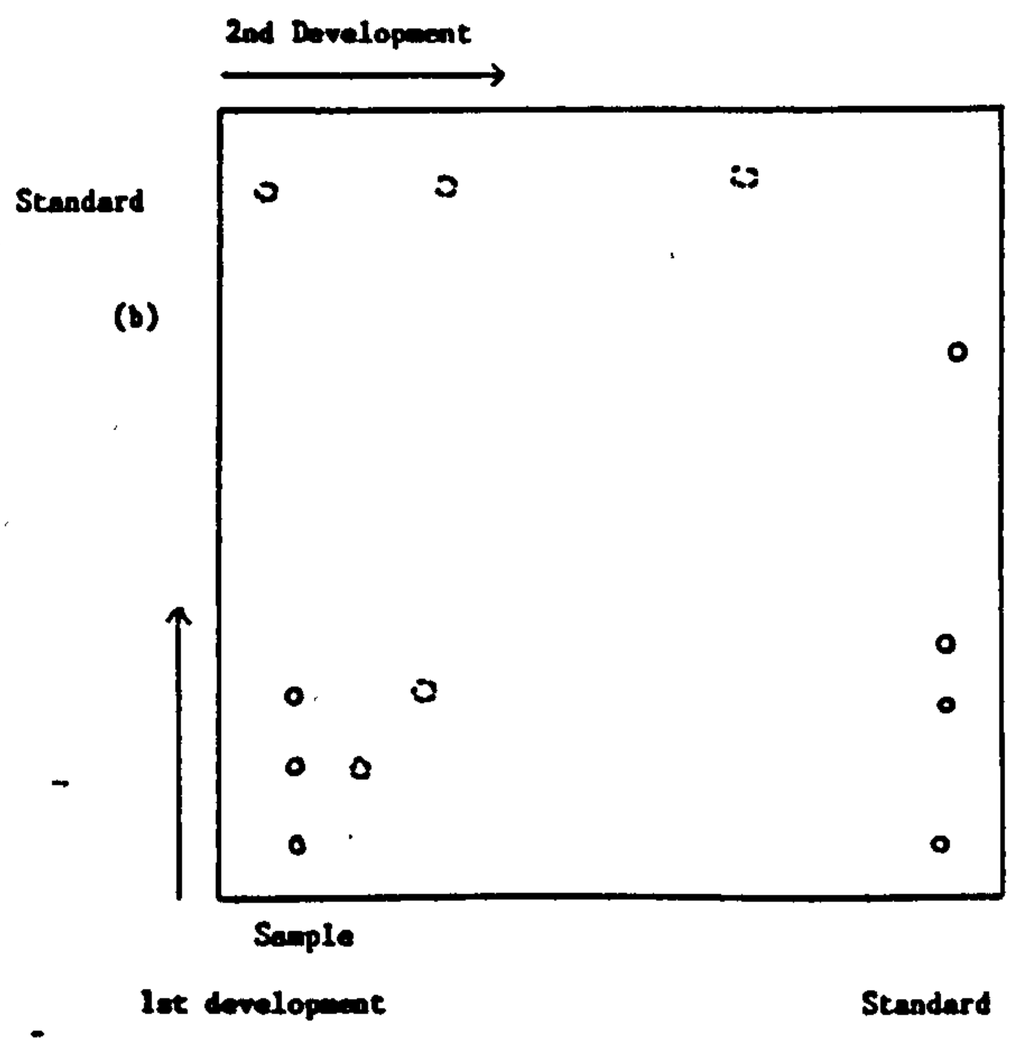


Fig. 7.18 Two-dimensional TLC plates where

o are spots from the first development
◌ are spots from the second development

(a) solution of diazepam in 0.1 M hydrochloric acid, evaporated under vacuum at low temperature

(b) solution of diazepam in 0.1 M hydrochloric acid, evaporated at high temperature on a hot-plate

either to insufficient alkali being added to convert all the GMACB to diazepam, or to some other component on the base-line which was unaffected by the addition of alkali. No spot was seen at R_f 0.06 as was seen when single dimension application of alkali had been performed (Section 7.7.1.1) although the presence of a spot in this region may have been masked by the streak seen.

When the sample of hydrolysis mixture which had been subjected to a greater amount of heat was examined, a spot was seen on the base-line after initial development of the plate. After treatment with alkali, this spot did not show any change and no spot corresponding to diazepam was seen to result from this treatment. Two spots were seen above the base-line spot after initial development, one with an R_f of 0.07 and one with an R_f corresponding to standard diazepam. After redevelopment in the same mobile phase, these spots gave a similar R_f value.

These results support the theory that as diazepam degrades in acid solution it forms an intermediate which is not extracted by chloroform but which runs on the base-line in the chromatographic system used, and can be converted to diazepam by alteration of pH. On gentle heating to evaporate the sample, it appears that the equilibrium for the reaction is forced towards the formation of GMACB, with MACB also present, but with excessive heat, the hydrolysis reaction appears to result in the formation of other degradation products. Reports on the hydrolysis products of diazepam have shown two major degradation products, MACB (with glycine) and carbostyril (a

quinoline derivative), see Section 1.2.3. No spots corresponding to these were seen in the sample evaporated using a hotplate. Workers studying the photochemical decomposition of diazepam have identified three types of compound formed; benzophenones, 4-phenylquinazolinones and 4-phenylquinazolines [Cornelissen et al., (1978)]. These last two types of compound were not available to test against the unidentified spots seen in the sample. As a larger amount of energy was present in the sample heated on the hotplate, a different degradation pathway may have been followed and resulted in some of these compunds being produced.

The isolation of GMACB and other similar intermediates from benzodiazepine drugs has been discussed by Han et al., (1976, 1977 a and b). In this work they used a solvent system containing dioxane:benzene:hexane:7.4 M ammonium hydroxide (45:50:70:5), for separation of the intermediates of hydrolytic degradation. As work reported here has shown the open-ring intermediate to revert to diazepam on treatment with ammonium hydroxide, it is difficult to understand how these workers obtained isolation of the open-ring compounds under these conditions although the work reported was not repeated. The sample preparation stage used by Han and co-workers before application of the sample to the chromatoplate involved extraction of the acidic hydrolysis reaction into chloroform. Work reported here suggests that the open-ring compound formed from diazepam does not extract into chloroform, and this was also reported by other workers [Nakano et al., (1979)], it thus seems unlikely that Han et al. would have extracted any of the intermediate from the

reaction mixture for application to the TLC plate.

7.7.2 Infra-red spectroscopy

7.7.2.1 Comparison of IR spectra

IR spectra were obtained from discs prepared from potassium bromide mixed with solutions of authentic diazepam and MACB in chloroform. IR spectra were also obtained from discs prepared by mixing potassium bromide with chloroform extracts from an acidic solution of hydrolysed diazepam and from the extract obtained after neutralisation and re-extraction of the acidic solution. These spectra are shown in Fig. 7.19.

Comparison of the spectra of MACB and diazepam showed obvious differences. The main difference was the presence of a band in the N-H stretching region 3100-3500 cm^{-1} in the MACB spectrum which was missing in the diazepam spectrum.

Comparison of the spectra obtained from extracts of a solution of diazepam degraded at pH 1 and the same solution after extraction and neutralisation to pH 7, and that for authentic diazepam showed all three to be the same, providing further evidence to support the theory that the open-ring intermediate reverts to diazepam in alkaline conditions. As IR is not a very sensitive technique, the presence of a small amount of MACB in the initial extract from pH 1 was not detected. It is also possible that other compounds of similar structure may have been present. There is no

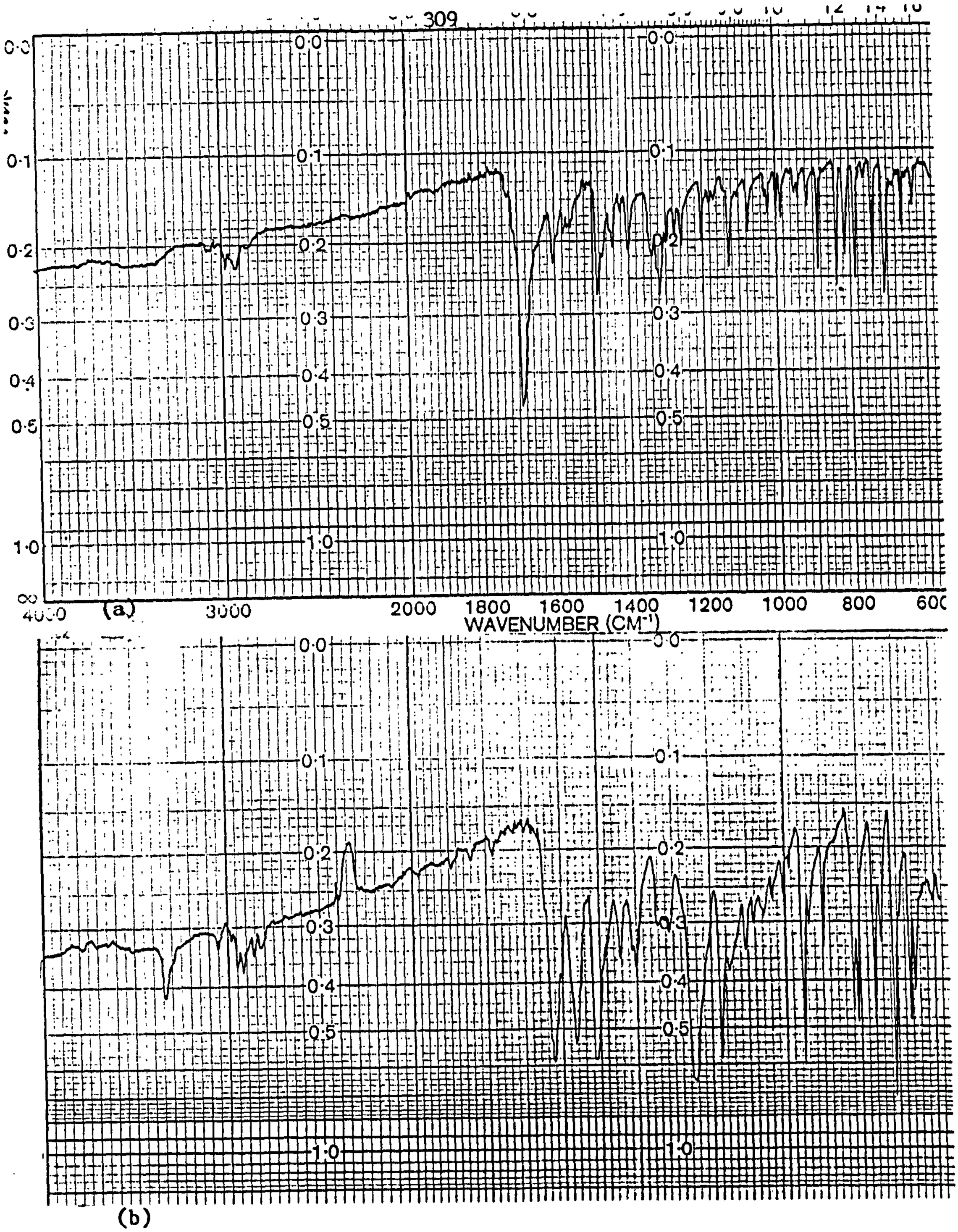


Fig. 7.19(i) IR spectra of diazepam and MACB, from powdered material mixed with potassium bromide

(a) IR spectrum of diazepam
(b) IR spectrum of MACB

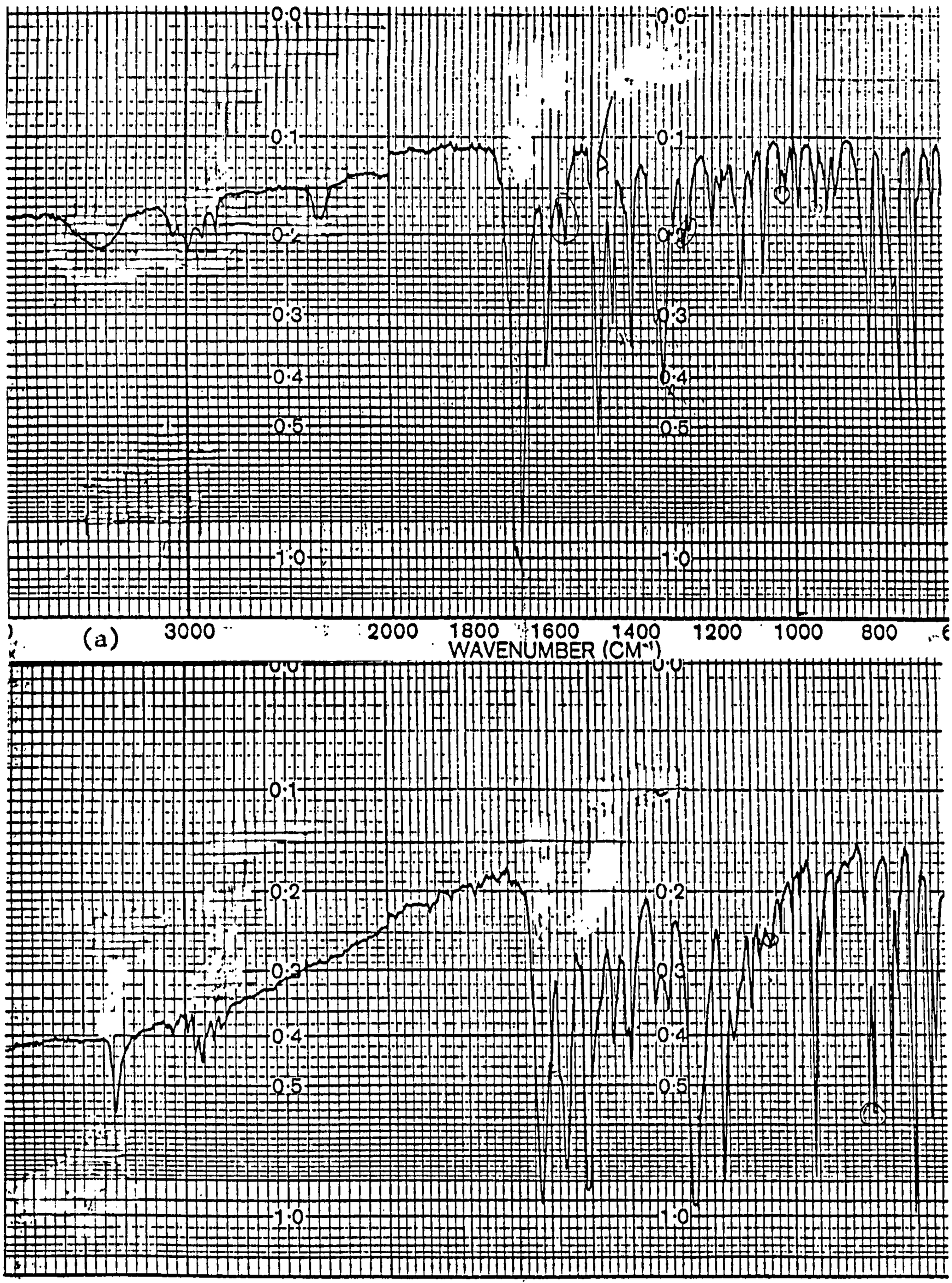


Fig. 7.19(ii) IR spectra of diazepam and MACB, from a solution of each in chloroform, mixed with potassium bromide and evaporated

(a) IR spectrum of diazepam
(b) IR spectrum of MACB

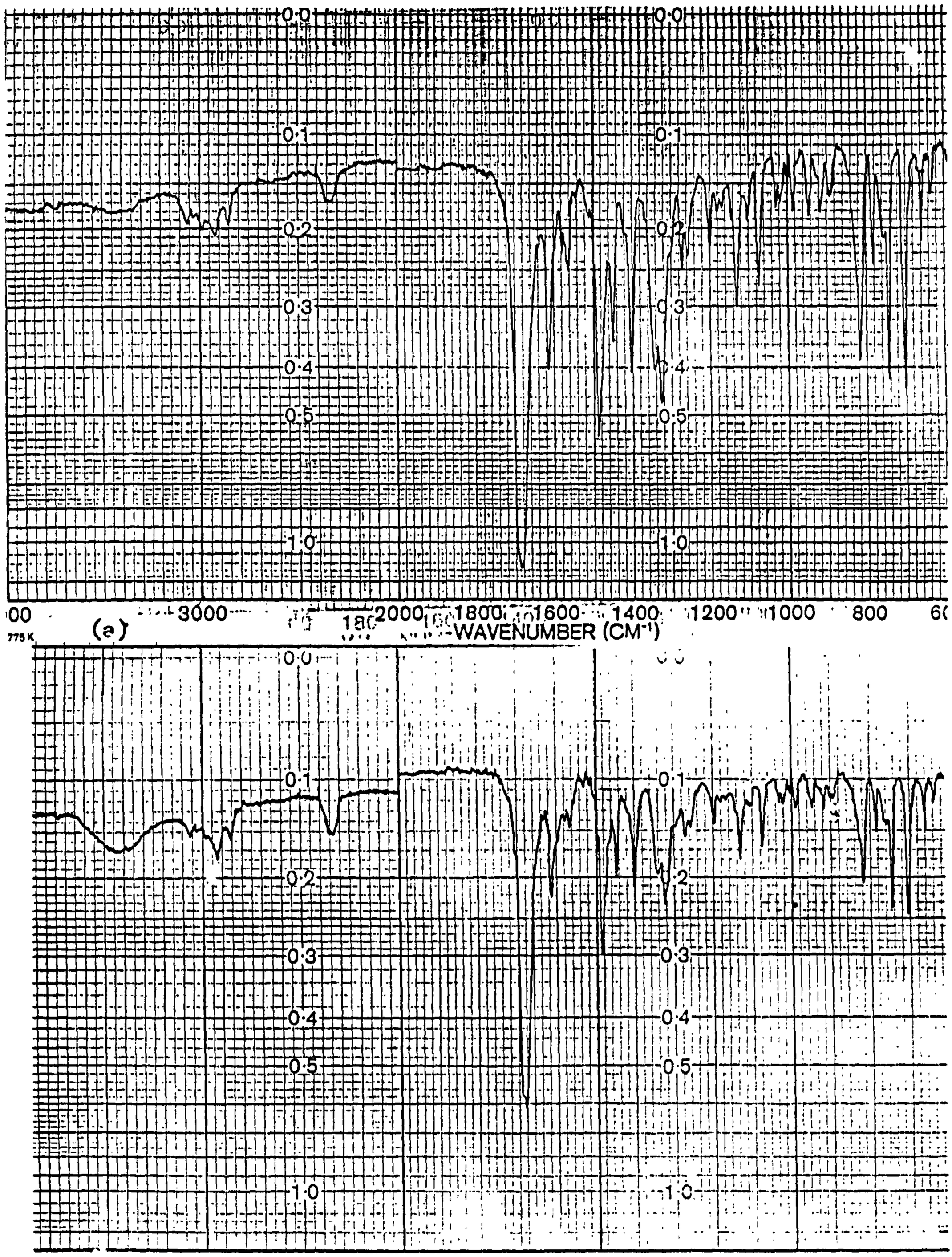


Fig. 7.19(iii) IR spectra of samples extracted from a solution of degraded degraded diazepam in 0.1 M hydrochloric acid

(a) IR spectrum of sample extracted from the acidic solution
(b) IR spectrum of sample extracted from the aqueous solution after initial extraction from pH 1 followed by neutralisation

spectral evidence to suggest that the open-ring intermediate is reverting to some very different compound. However, the technique is not sufficiently sensitive to prove that it is definitely reverting to diazepam.

8 CONCLUSIONS

Stability-indicating assay methods were developed for the analysis of diazepam using difference UV spectrophotometry and HPLC. These methods were used to analyse formulated products and compared with the direct UV spectrophotometric method of the B.P. on samples of commercial products. The ease of operation of the techniques, the information provided by them and the information required in the analysis of the samples are all factors which have to be taken into consideration in assessing the best analytical method for any purpose.

Consideration of the three methods used showed that the HPLC method provided most information about the samples, giving both the assay value for the diazepam content and also information on the presence or absence of degradation products, and the level at which these were present. For solid dosage forms, the extraction process required for sample preparation was the same as in the spectrophotometric methods. The sample preparation was much simplified for the liquid preparations where a simple dilution could be performed for injection onto the HPLC system, compared with the extraction processes required for the UV determinations. The major disadvantage of the HPLC method is the time required for analysis, as duplicate or triplicate injections are made, and numerous standard solutions are injected to monitor drift in the system during the prolonged analysis time.

The developed difference spectrophotometric method

appeared to provide advantages over the current pharmacopoeial method of analysis, providing a specific method for analysis of diazepam in the presence of its degradation products. The sample preparation required is slightly more lengthy than that for direct UV measurement, with two dilutions in different pH solutions required to generate the difference spectrum. From the comparisons of analysis of standard mixtures and formulations by both methods, no decrease in precision was seen for the difference method however, suggesting that no greater error is being introduced by the more complicated sample preparation.

In the case of a relatively stable drug such as diazepam, for solid dosage forms, no advantage is seen for the use of HPLC in preference to UV determination. As the sample work-up required for both methods is similar, no saving would be obtained from this to compensate for the longer analysis time, and no increase in the information generated would be seen. For liquid preparations however, the use of an HPLC method would have advantages, particularily if aged samples are being analysed. The increase in time required for the analysis would be compensated by the decrease in time required for sample preparation, and the information generated from the assay would include quantitation of any degradation products present. The HPLC method developed in this work was recommended for use by Roche for routine testing of batches of Valium Syrup due to the simplified sample preparation when compared to the UV assay method.

In recent years, HPLC methods of analysis have become much more widely used, and in many instances are the method of choice. These systems are ideal if large numbers of samples of the same compound have to be analysed as they can be automated very easily providing 24 h working to compensate for the increased analysis time required in most instances. However, in some instances, if only a few samples are to be analysed, it can be very time-comsuming to set up a suitable chromatographic system and a satisfactory, specific, sensitive UV method such as provided by the difference UV method developed would appear a useful alternative.

Work published by Abdel-Hamid et al.(1984) suggested that the use of pH 1 and pH 14 solutions for generation of the difference spectrum would provide a satisfactory specific assay for benzodiazepines, including diazepam. From results quoted in this work, it has been seen that MACB, the major degradation product described by Newton (1978) in his criticism of the U.S.P. assay method, would interfere with the assay method under these conditions, although at a lesser extent than in the direct UV spectrophotometric method.

The choice of assay method should be influenced by the information required from the assay and the interference likely to be be present.

Although the main area studied was development of specific analytical methods for analysis of diazepam, the stability-indicating assay methods developed were applied to the study of the hydrolytic degradation of

diazepam in acid. A number of workers had studied these reactions, using the common method of measuring the decrease in concentration of diazepam and substituting this change into the relevant equations for calculation of the reaction rate. Some of these measurements were made using non-specific methods involving the use of mathematical corrections to compensate for measurement of the degradation products.

The GC and HPLC methods developed allow measurement of diazepam without interference and should provide improved methods for studying the degradation reactions. A recent review has discussed the improvements possible in the evaluation of stability when decomposition products are monitored rather than parent molecules [Taylor and Shivji,(1987)]. Not only does this allow a more sensitive detection of decomposition but also provides information on the rate constants of individual reactions when parallel or consecutive reactions occur. The HPLC method developed in this work and used to follow the hydrolytic degradation is capable of separating the major degradation products from diazepam and may provide a satisfactory method for closer study of the degradation of diazepam.

The results obtained from the study of hydrolysis of diazepam were rather limited and are described to provide a further application of the analytical methods developed.

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APPENDIX 1

For a simple reaction $A \longrightarrow B$, the first-order rate constant of the reaction was calculated using the equation below.

$$\ln A_t = \ln A_o - kt$$

where the concentration of A at time intervals were measured and substituted into the equation, and k was the rate constant.

If the logarithm of the drug remaining is plotted as a function of time, a linear relationship should be observed for a first-order reaction, with the slope of the line used to calculate the first-order rate constant.

$$\text{slope} = -\frac{k}{2.303}$$

In the series of papers by Han et al., for reaction where more than one reaction step was occurring, time plots of the logarithm of the differential absorbance measurements $(A_t - A_\infty)$ gave lines with different linear segments with different slopes. A mathematical correction was employed on these to differentiate between the different linear segments.

For the reversible reaction $A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B$, studied by Nakano et al., slightly different calculations wer employed.

For this reaction, the rate of decomposition of the reactant was

$$-\frac{d[A]}{dt} = k_1[A] - k_{-1}[B]$$

on integration, this becomes

$$\log \frac{P_t - P_\infty}{P_o - P_\infty} = -\frac{k_1 + k_{-1}}{2.303} t$$

where P_o, P_t and P_∞ are concentrations of fractions of unreacted diazepam at times zero, t and infinity respectively.

Also at equilibrium ,

$$K_{eq} = \frac{k_1}{k_{-1}} = \frac{P_o - P_\infty}{P_\infty}$$

where K_{eq} is the equilibrium constant, and k_1 and k_{-1} are the rate constants for the forward and reverse reactions respectively.