Applications of high performance liquid chromatography in the analysis of basic compounds

A Thesis Presented for the Degree of Doctor of Philosophy in the Faculty of Science at the University of Strathclyde

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Declaration of author's rights

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of degree.

Signed: Date:

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I wish to thank all those who helped me. Without them, I could not have completed my Ph.D thesis.

The Government of Kuwait: to take on their shoulders my scholarship.

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My parents: for their support, my father was my spirit to accomplish this thesis.

My supervisor Dr Dave Watson: I don't know what to say here?? He wasn't my supervisor only, he was a friend, father and brother. Every single drop of sweat on this thesis is dedicated to you Dr Dave.

My Family: Thank you for your patience

Abstract

The development of chromatographic methods for basic drugs can still present problems. Two modes of chromatographic separation were studied in order to assess their performance with regard to the separation of organic bases. Reversed phase chromatography was applied in the assessment of the stability of two extemporaneous formulations of drugs used in hospital for pain relief in palliative care, for which there was no stability information. Three different formulations of the two combinations were assessed. In the case of the diamorphine/clonidine/bupivacaine combination there were no major stability issues apart from the slow hydrolysis of diamorphine to monoacetyl morphine. In a combination containing morphine and levomepromazinemorphine remained stable, but there was a gradual decline in the level of levomepromazine which fell to below 90% of the original concentration after one week.

The theme of chromatographic analysis of basic drugs was continued in the second part of the PhD project where silica based alkyl phases were studied with regard to their ability to retain basic drugs under hydrophilic interaction like conditions. It was observed that with mobile phases containing high levels of organic solvent, for example water/acetonitrile (5:95 % v/v) containing 0.025% w/v ammonium acetate, basic compounds were strongly retained. The strength of the retention of the bases depended on the % of water in the mobile phase and the strength of the ammonium acetate. The novel methods developed were applied to drug impurity profiling and fundamental research on mechanisms was carried out using simple basic test probes.

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butylbenzylamine (TBBA), α-methylbenzylamine (AMBA), benzylamine (BA), dimethylbenzylamine (DMBA), benzyltriethylamine (BTEA), N-methyl-, N-ethyl and N-isopropylbenzylamine (MBA, EBA and PBA) run on an ACE Ph column (150 mm x 3 mm, 3 µm particle size) water/acetonitrile (5:95) containing 0.04% w/v tris acetate pH 6.0. Flow rate 0.8 ml/min.

5.11 Test mixture of basic drugs run on an ACE Ph column (150 mm x 3 mm, 3 µm particle size) water/acetonitrile (5:95) containing 0.04% w/v tris acetate pH 6.0. Flow rate 0.8 ml/min 200

Chapter 1

General Introduction to Stability Testing

1.1 Introduction

Every pharmaceutical product should have a clear shelf life in which the company will ensure the product safety, quality, efficacy, properties and characteristics that the product possesses at the time of manufacture, if it is stored under the conditions stated by the manufacturer [1]. Drug stability can be divided into pre-market and market stability [1]. Drug efficacy and assurance can be evaluated by the clinical trials, which are carried out during pre-market stability stage. Market stability refers to the continuous assurance of the pharmaceutical product for a long term stability monitoring. The stability of pharmaceutical products depends on two major factors, on the one hand, environmental factors such as ambient temperature, light and humidity, and on the other, product related factors such as the chemical and physical properties of the product, dosage form, manufacturing process, container-closure system and packaging properties[2]. Some chemicals are stable under slight changes in the surrounding environmental matters, while others are very sensitive to such changes. For that, some drugs are stable in normal conditions of storage and undergo degradation only under high or low pH, or high temperatures, on the other hand, other drugs are unstable or labile under normal conditions. Drug stability can be evaluated through establishing a stability indicating method in order to test the drug-substance (active pharmaceutical ingredient [API]) [3] and drug product (final pharmaceutical formulation of the drug). Drug substances can be derived from plant, animal and biological sources and chemical synthesis, which can contain impurities. Data on active ingredient substance's chemical and physical stabilities are helpful for designing methods that indicate drug product's

stability and formal stability studies. Drug stability is of a great importance in pharmaceutical sciences. This importance given to this field is because of the following reasons:

A- The newly formed products due to chemical changes in the drug may be more toxic to the patient than the parent drug.

B- As a result of decomposition the effective dose which should be delivered to the patient will be reduced. This may become significant when >5% to 10% of drug content is decomposed.

C- Support drug stability used in clinical and non-clinical studies.

D- To establish an accurate expiry date of the marketed product [2].

Some degradation products are not toxic, while some are very toxic. For example, some antibiotic syrup e.g. amoxicillin syrup, which has β-lactam ring, is unstable and susceptible to hydrolysis, the degradation of the drug content can be up to 10% without harming the patient and without any reduction in the syrup efficacy. Actually, the manufacturer usually adds a small excess of the active ingredient to allow for some degradation. Many penicillins are unstable and are supplied to pharmacies as a dry powders in sealed containers in order to avoid the degradation due to hydrolysis promoted by water If the decomposition products are not toxic to the patient, up to 10% degradation may be acceptable. However, if the degradation products are of high toxicity then it is essential that the level of those products do not harm the patient. For

example, isoniazid, an anti-tuberculosis drug, can be chemically changed due to hydrolysis to hydrazine, which is very toxic. In the British Pharmacopoeial monograph it is indicated that the amount hydrazine in isoniazid should not be more than 0.5mg in each gram (g) of isoniazid. Luckily, the rate of degradation of isoniazid is very low and the 0.5mg /g limit for hydrazine is unlikely to be exceeded. According to The Medicines Act 1968 and subsequent legislation, all medicines are required to have an expiry date that should be agreed with the medicines regulatory agency. The chemical stability of the drug or other components of the medicine is the limiting factor in the setting of the expiry date.

Stability can be examine by establishing a stability indicating method, which should be validated and quantitative, to detect any change of the drug substance and drug product properties with time under defined storage conditions. Stability indicating assay methods can be used to accurately measure the active ingredient peak without interference from other peaks and thus accurately detect and measure the degradants [4].

Stability tests under different conditions (temperature, humidity, and light) will help the manufacturer to determine the retest period of a drug substance, the drug product shelf life under specified conditions and the optimum packaging that will ensure product efficacy and quality in different parts of the world. Stability test conditions should be wide enough to cover the four climatic zones in the world [4,5].

1.2 Summary of stability testing methods in pharmaceutical development

A. Pharmaceutical product development phases

The life cycle of a pharmaceutical product can be divided into four phases

I. Phase 1: safe to use in healthy subjects
II. Phase 2: safe and effective in patients
III. Phase 3: ultimate clinical efficacy studies
IV. Phase 4: post-approval studies for other uses

Stability studies are necessary for the support of every phase during drug development and phase 1 will be supported by evaluating the physical and chemical properties and stability of drug substance. At this phase, stability will help in determining salt selection, drug substance degradation pathways, excipient compatibility and a simple clinical dosage form can be established.

In Phase 2, stability studies will be run longer to support longer clinical studies. The best candidate formulation will be selected in this phase after examining different formulation and packaging materials, therefore, short term stability (6 months maximum) will be initially applied then a longer term stability studies (2-3 years) to help in such selection of candidate. Furthermore, the drug substance will be subjected to stability studies from 2 to 5 years at this phase.

In Phase 3, pharmaceutical dosage form and strength will be determined. During Phase 3, the use of regulatory guidelines is vital for establishing stability studies to support shelf life, specified storage conditions in the targeted market countries.

Phase 4 will appear after the approval and launch of the pharmaceutical product. Within this phase, stability studies will focus on the final product and these studies established to assure that the product continues to be stable under the specified storage conditions and expiry date [6].

B. strategy for stability studies on drug substance and product

1. Stress testing of drug substance:

The stability of a new drug will be investigated in the early stages of the new product life. Stress testing of the active ingredient can help identifying degradation products, the degradation pathways and stability of active substance [7]. Some drugs have shown great promise for further pharmaceutical development but this has had to be terminated when it was discovered that the drug was unstable. Stress testing will be carried out by examining at least one batch of the drug substance and it should be subjected to the following effects :

A. Heat (from 20°C to 60°C)
B. Light (photostability testing)
C. Humidity (60%RH, 75%RH)
D. pH effect (hydrolysis)
E. Air (oxidation)

The design of the stress test will depend on the type of drug product involved. For example, temperature, photostability and humidity tests are recommended for solid

drug substances, while an oxidation test is optional. On the other hand, hydrolysis and oxidation is recommended for solutions and suspensions of drug substances while photostability is optional. In addition, in order to design a degradation experiment for a solid dosage form of drug product, oxidation, photostability, temperature and humidity tests are recommended [7]. Moreover, the nature of drug substance has a great role in designing the stress test. For example, a common reaction between primary and secondary amine drugs with a reducing carbohydrate excipient such as lactose, fructose, dextrose, glucose and maltose can occur. Stress tests for these types of drug substances should include excipient concentration in addition to temperature, humidity and photostability [8].

The following approaches can be used for an active substance:

I. No data on degradation product is needed when the active substance is described in a well-known pharmacopoeial monograph and fully meets the requirements.

II. Two options are available, if the active substance is not described in an official pharmacopoeial monograph:

- Citing published literature can be acceptable to support the projected degradation pathways
- Stress testing should be designed, when no data is available in the scientific literature or official pharmacopoeial monographs.

Stress testing studies will form a vital part of the information supplied to regulatory authorities [9].

2. Common Decomposition Reactions

Hydrolysis of Esters

When ester is treated with water partial conversion into a carboxylic acid occurs. Therefore, if one mole of ethyl acetate is treated with one mole of water at room temperature, 0.33 moles of acetic acid and 0.33 moles of ethanol will formed when the reaction reaches equilibrium (Figure 1.1).

$CH_3COOCH_2CH_3 + H_2O \leftrightarrows CH_3COOH + CH_3CH_2OH$

Ester Acetic acid Ethanol

0.67 mole 0.67 mole 0.33 mole 0.33 mole

Figure 1.1 Ester hydrolysis

The forward reaction is called hydrolysis, while the reverse reaction is termed esterification. To accelerate the reaction in the hydrolysis direction one or more of the following should be applied:

1. Add an excess of water that will drive the reaction in the hydrolysis direction, and if the water is removed from the reaction by any means the reaction will be driven in the esterification direction.
2. Increase the temperature of the reaction (heat)

3. Addition of acid or alkali as catalyst.

Lactides and lactones (Figure 1.2) are cyclic esters and are also susceptible for hydrolysis. Lactones are a part of the structure of many medicines, these medicines should be well stored and the instability should be immediately inspected. Aspirin, a phenolic ester (ROCOR', R= C_6H_5) is more susceptible to hydrolysis than the other alcoholic esters. This is due to the electron withdrawing effect of the benzene ring which destabilises the ester bond. For this reason aspirin cannot be formulated as aqueous dosage form [10].

Hydrolysis of Amides

When the hydroxyl group of a carboxyl function is replaced by amino group the product is called an amide (Figure 1.2).

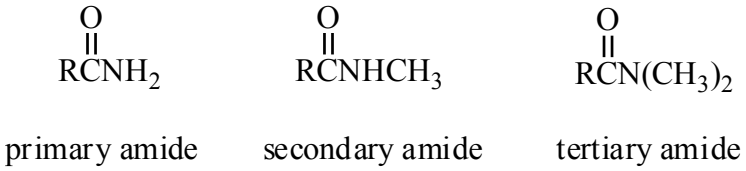

Figure 1.2 Lactones, lactides and amides.

Amides are hydrolyzed in the same way esters by using high temperature and addition of acid or alkali. Amides are more stable than esters and require higher temperatures or more concentrated acid or alkali for them to hydrolyse and thus have a much greater shelf life [10].

Autoxidation

Many pharmaceutical products may lose their stability by autoxidation. Autoxidation is a radical- induced chain type of reaction and is usually initiated because of exposure of the product to light and air. These reactions can be accelerated in the presence of metal ions such as copper and iron, which act as catalysts. Autoxidation can be considered in three stages (figure 1.3).Alkenes, amide and phenol containing drugs susceptible to autoxidation and must be stored in a well closed container of limited capacity under nitrogen and protected from light [11].

Oxidation in amines occurs next to the nitrogen atom. Thus, free organic bases are rarely used in medicines, because they difficult to formulate and highly susceptible to oxidation. The salt form of the free organic bases is always preferred, if the nitrogen is basic enough to form a salt. The salt is easier to be handled, stored, and more stable.

Phenols are a well-known example of a class of compounds that are highly susceptible to oxidation. The stability of phenols is dependent on pH, and their rate of oxidation increases with pH.

Initiation (e.g. shown for an alkane)

R-H → R· + H·

R· + ·O-O· → R-O-O· (peroxide radical)

Propagation

R-O-O· + R-H → R-O-O-H + R· (Hydroperoxide radical)

R· + ·O-O· → R-O-O·

R-O-O· + R-H → and so on

Termination

R· + R· → R-R (Dimer of the drug)

R-O-O· + R· → R-O-O-R (Peroxide compound)

Figure 1.3 The stages of autoxidation

Design of a stable dosage form for a drug substance which is susceptible to oxidation presents a big challenge to the drug formulator in the pharmaceutical industry. Therefore, a number of procedures are used either individually or in combination to overcome the oxidation effect:

1. Exclude oxygen e.g. using hermetically-sealed blister packs. Introduce nitrogen bubbles or helium gas in liquid preparations (water) to eliminate oxygen. Air above liquids in injection vials can be replaced by nitrogen.

2. Protect from light. Use opaque containers or amber-glass dispensing bottles.
3. Use an optimum pH. Investigate the variation of reaction rate against pH and select the pH of maximum stability for the product.
4. Use a chelating agent. As mentioned, the oxidation reactions are catalyzed in the presence of metal ions such as iron and copper. Chelating agents will act by forming a complex with such metal ions. Well known as a strong chelating agent is EDTA (Ethylene diamine tetra acetic acid), which is added to a liquid preparation to reduce oxidation.
5. Antioxidants. There are two types of such inhibitors:

Oxygen scavengers: These scavengers are substances which are more easily oxidized than the susceptible drug. Sulphite, which used in the food and brewing industries, is one of the well know scavengers (Figure 1.4).

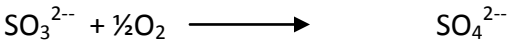

Figure 1.4 Oxidation of sulphite.

Another type of scavenger is ascorbic acid which is readily oxidized to dehydroascorbic acid (Figure 1.5).

Figure 1.5 Oxidation of ascorbic acid.

Chain terminators: Chain terminators are phenolic compounds which will form stable free radicals and terminate the oxidation reaction [9]such as butylated hydroxyl toluene (Figure 1.6).

Figure 1.6 Formation of a radical from butylated hydroxy toluene.

Photostability Testing

Photostability testing is a type of stress testing method which can evaluate drug substance and product stability, efficacy and safety when exposed to light. Photostability testing has an important role in determining the optimum packaging system to protect the drug product from light. This test can be performed by exposing the drug substance to a different type of light and light intensities in a light chamber or

by leaving the drug substance or product in an open area or on a window. Photostability studies should progress until the results clearly show that the drug substance and product is stable. Photostability testing of a drug product and substance should be evaluated to assure that the product is within specification during shelf-life even if it is stored under strong light. Photostability testing can be divided into two parts:

- Forced degradation: to evaluate the material photosensitivity for method development and degradation pathways elucidation
- Confirmatory testing: to provide a detailed information about labelling, packaging and handling the pharmaceutical product.[1,15]

Alkenes need light of relatively low energy to promote photochemical changes. Most alkenes exist in two stable geometrical isomeric forms (cis and trans). Light absorption by alkenes promotes a π electron in the double bond to an anti-bonding π* orbital and an excited single-bonded intermediate is formed. Rotation about the single bond of the alkenes can occur during the life time of the excited species and intermediate can return its ground state as a mixture of the two isomers (Figure 1.7)

Figure 1.7 Isomerisation of fumaric acid as a result of exposure to light.

When fumaric acid is irradiated with light this will result in the conversion of 25% of the fumaric acid into maleic acid. This area is of great importance for pharmaceutical companies and scientists. The geometric isomers resulting from cis/trans isomerisation may have different pharmacological activity or potency. The formulator should make sure to deliver the right drug or the right geometric isomer to the patient, thus, the product should be stable until it is expired.

1.3 Batch and Container Closure System Testing

Stability data on three primary batches should be provided. In case of drug substance, batches should be manufactured to pilot scale by the same synthetic route that simulates the final process to be used for production batches. Drug substance should be packed in a container closure system resemble the packing proposed for storage and distribution, for stability studies evaluation. For drug product, stability studies will carried out on drug products have the same formulation and packaged in the same container as the marketing products. For the drug product, two of the three batches should be at least pilot scale batches [1].

1.4 Test Frequency and Storage Conditions

The stability of drug substance and product should be evaluated under storage conditions by the effect of heat (thermal test) and humidity. The storage conditions and the length of stability test applied should cover storage, transportation and the patient use. The recommendation of storage conditions has been supplied by International Conference of Harmonisation (ICH) as shown in Table 1.1. Moreover, testing for assay, impurities and water content determination is highly recommended for solid dosage forms of drug products. Drug substances and products intended to be stored in refrigerator orfreezer,should be tested under long term storage

conditions in order to obtain real time data, which can be used as an evidence for shelf life determination.

The ICH guidelines for drug substances and products stored in refrigerator and freezer are:

Drug substances and products intended for storage in refrigerator:

- Long term: storage condition at 5 ± 3°C for 12 months.
- Accelerated: storage condition at 25 ± 2°C/60 ± 5% RH for 6 months

Drug substances and products intended for storage in a freezer:

- Long term: storage condition at -20 ± 5°C for 12 months

During the 6 month's testing, any significant change occurs at any time in the accelerated storage condition will require additional evaluation by testing at the intermediate storage condition [9].

Briefly, the general ICH conditions are shown in Table 1.1

Table 1.1ICH guidelines for stability testing [1]

Study	Storage conditions	Minimum period of study
Accelerated	40°C ± 2°C/ 75% RH+/- 5%	6 months
Intermediate	30°C ± 2°C/ 60% RH+/- 5%	6 months
Long term	25°C ± 2°C/ 60% RH+/- 5%	12 months

A significant change is considered to have occurred if:

- If there is a 5% decrease of the assay value compared with the initial assay value.
- Specific degradation products are higher than the specified limit
- pH limits are no longer met
- Dissolution specification limits of 12 capsules or tablets are no longer met
- Physical properties and appearance specifications are no longer met, such as change in (colour, hardness, softening of suppositories and melting cream).

Long term testing should cover a period of 12 months on three primary batches at the time of submission, and continues to cover the shelf life of the drug product. Drug products, which expire within a year or more, a frequent testing at long term storage condition will take place every 3 months during the first year, every 6 months over the second year, and once a year through the estimated shelf life [1].

On the other hand, accelerated testing will take place minimally at three points at 6 months and it should include the initial time and 6 months (initial and end point). If any significant change occurs at the accelerated storage conditions, testing at the intermediate condition for 12 months is recommended [1,2].

1.5 Stability Studies on Marketed Batches

The design of the stability test depends on intended market and climatic conditions where the drug will be used.

Climatic zones can be divided into four different climatic conditions:

- Zone 1: temperate
- Zone 2: subtropical, with possible high humidity
- Zone 3: hot/dry
- Zone 4:hot/humid

Since there are only a few countries in zone one, it is recommended that the stability test is designed for the conditions in climatic zone 2 when the manufacturer intends to market products in climatic zone 1. For countries based in zones 3 and 4, it is advised to design stability studies based on the conditions in zone 4 [2].

The derived climate zones for use in stability studies are given in table 1.2 [11].

Table 1.2 Stability testing conditions for different climatic zones [7]

Climate zone		**Derived storage condition**
Zone1	Temperate	21°C / 45% RH
Zone2	Mediterranean, subtropical	25°C / 60% RH
Zone3	Hot, dry	30°C / 35% RH
Zone4	Hot, humid	30°C / 70% RH

It is possible to simplify the main stability testing objectives as shown in Table 1.3

Table1.3 The main objectives of stability studies [2]

Objective	**Type of study**	**Use**
To select effective formulation and container closure system	Accelerated	Product development
Determine storage conditions and drug shelf life	Accelerated and real-time	Product development and drug registration
To prove the indicated shelf-life	Real-time	Drug registration
To indicate that the formulation and manufacturing process have not been changed in a way affect the drug stability	Accelerated and real-time	Quality assurance

1.6 The Design of Stability Studies

The stability studies of a specific drug should be designed based on the properties and stability characteristics of the drug substance and on the climatic conditions of the intended market zone. Some stability information on drug substance should be collected and analysed before initiating dosage form stability studies.

Testing samples of the final pharmaceutical product should be done for registration purposes. The data of two samples of different production batches of fairly stable active substance, in contrast, three samples of different production batches of limited stability active substance should be a representative of the manufacturing process. To apply this test the active ingredient should be from different batches. Batches will be tested according to a predetermined schedule, and the suggested schedule should be as following:

- A batch every other year if the formulation is considerably stable, otherwise every year.
- A batch every 3-5 years if the formulation stability profile is established.

Testing records should involve detailed information on batch number, production date, patch size, etc.

1.6.1 Accelerated studies

An example of accelerated studies on relatively stable active ingredients is shown in Table 1.4 [2].

Table 1.4 Example of conditions used for accelerated stability testing of products containing relatively stable active ingredients [2]

Storage temperature (°C)	Relative humidity	Study duration
40±2	Zone 4: 75±5	6 months
40±2	Zone 2: 75±5	3 months

For drug products with a limited stability drug substance or a limited availability of stability data, it is highly recommended that the accelerated studies for Zone 2 should be increased to 6 months, or the drug substance can be stored at a temperature of at least 15°C above the actual stated temperature for 6 months, e.g. 45-50°C and 75% RH for zone 4. For solid dosage forms in a semi-permeable package, stability studies under storage conditions of high relative humidity are highly recommended.

1.6.2 Real time studies

Experimental storage condition should be close to the actual stated storage conditions. Studies data of at least 6 months duration should be available at the registration time. Moreover, real-time studies must be continued until the end of shelf-life.

1.6.3 Frequency of testing and evaluation of test results

A frequent product testing procedure in the developmental phase will be considered as being a powerful support in the registration application. Products with considerably high stability will be tested as follows:

- Accelerated studies, at 0, 1, 2, 3 and 6 months
- For real-time studies at 0, 6, and 12 months and then once a year

For continuing the studies, sample is tested every 6 months or every year for a well-established product. Products with a highly stable formulation will be tested after 12 months and then at the end of the shelf life, while products containing less stable drug substance should be tested every 3 months in the first year, every 6 months in the second year, and then once a year [2].

1.7 Analytical methods Used in Stability Testing

Analytical techniques, which can evaluate the important physical, chemical, biological and microbiological properties, should be available to evaluate and test the stability information of a particular product. Analytical methods should be validated with adequate accuracy and precision

1.7.1 High Performance Liquid Chromatography (HPLC) for stability testing

HPLC is a popular technique for use in stability technique due to its highly precision, accuracy and ease of usage. Simple techniques such as planar chromatography (TLC and paper chromatography) can be used for quick impurity and purity tests [12]. New technologies such as chiral chromatography can be used to separate racemic mixtures

into individual enantiomers, which have different therapeutic activities [4] and which may arise as impurities. The HPLC method should be validated with adequate specificity, which is the ability of the analytical method to measure the analyte without any interference from other component in the sample [13]. The HPLC method and column selection will depend on molecular size and solubility of the compound in water. The priority of the HPLC usage in stability studies came from its highly precision, sensitivity, resolution, accuracy and ease of using different types of detector.

1.7.2 Stability indicating method development

Drug delivery and its technologies have been improved in the recent years. The various drug delivery system will depend on the drug characteristics and the indication of usage. Sample preparation plays a vital part in establishing a HPLC stability indicating method, not considering the route of administration. Product stability will be affected by the stability of the excipient, mixture and active substance, thus a stability indicating method should be established for excipient, drug substance and drug product.

Pharmaceutical companies are trying to achieve better patient compliance, through developing and marketing new chemical entities for multiple diseases. Thus, establishing and validating a stability indicating assay for such product is a big challenge. Active ingredient solubility, pK_a value, and dosage strength have a great role in developing sample preparation, stability indicating assay and choosing an analytical method. When the product contains two or more active ingredients, samples must be evaluated and impurities should be quantified, identified and their origin determined.

Failure in identification of impurities and in determining their origin and may lead to over or underestimation of the actual amounts of impurity present.

Stability indicating methods are crucial for analysing active ingredients, impurities and measuring the active ingredient potency at an initial time point and the decrease of potency during storage. Establishing a good stability indicating method will help in measuring the level of, potentially toxic, impurities in a formulation administered to humans. Stability indicating methods should be validated so that they can accurately quantify the drug content, while the assay method can help to calculate drug losses during the manufacturing procedures. Isocratic elution with mobile phase is the best choice for active pharmaceutical ingredient assay due its simplicity and the lack of a need to re-equilibrate to starting conditions at the beginning of a run. Gradient elution, which involves a continuous change in the mobile phase composition over a period of time, may be used for an active pharmaceutical ingredient which contains impurities with different affinities for the separation column thus shortening the analysis time and accommodating a greater variety of compounds in a mixture. This is because the elution strength of the mobile phase is gradually increased with time rather than remaining constant.

1.7.3 Dosage Form Testing

1.7.3.1 Tablets

Dissolution is the process of dissolving a solid substance into a solvent to make a solution. Dissolution test is a measurement of drug solubility over time. Basket method and paddle method are the most commonly used apparatus for dissolution tests. The paddle method is more suitable for immediate release tablets, where sampling typically occurs at 5-15min-intervals for a total of 1-2h [12]. HPLC assay methods should be highly sensitive due to the low drug concentration, linear and have a good chromatographic resolution.

1.7.3.2 Testing of Preservatives in Pharmaceutical Products

The shelf life of a pharmaceutical product can be extended by the use of preservatives, which prevent the microbial growth. A generic HPLC method can be designed for the commonly used preservatives. For instance, butylatedhydroxytoluene (BHT) is a commonly used antioxidant for a solid dosage form. A validated HPLC method for (BHT) can be used for any drug as long as the method has acceptable specificity, precision, accuracy and robustness. Preservatives, such as sodium benzoate, EDTA, sorbic acid and parabens, are very important in liquid and semi-liquid formulations. Preservative tests should be carried out in order to ensure that adequate preservative is included in the drug product and to evaluate preservative stability.

1.8 Protocols for Stability Testing

The initial parts of the stability studies on a pharmaceutical substance and product will be written as a document called a protocol. The protocol consists of two main parts:

1. Tests to be applied
2. Schedule to perform the planned tests

The protocol will be performed on batches used in clinical studies, formulation development, registration, marketed products and to test the compatibility of a product with a vehicle[6]. Moreover, the protocol will show the task of each person in the study team (analysts, formulators and management) and explain their roles to assure successful completion of the important tasks and explain to the team which is the most important type of information to be collected in order to evaluate the product stability. In addition, it will contain the expected data obtained in order to develop a stability profile of a product. Furthermore, the protocol will contain a signature section in which all the team sign to clearly show that the team will follow the written plan and they are agreed on the plan design.

1.8.1 Tests , methods and stability criteria

Tests similar to the tests written in the product specification (the tests will be performed on samples stored in different storage conditions) will be listed in the protocol. Stability tests, which evaluate product quality, potency, identity and purity, will be applied to monitor any changes in product stability. Table 1.5 is an example of the type of stability tests for tablet dosage form included in this section of the protocol. Thus, dissolution,

assay, degradation products and appearance are evaluated by these tests. Test methods used in this section will be the same as the test methods listed in the product specifications. Stability criteria are the limits for each test showing that a product is stable. As the tests and methods, stability criteria should hold to values the same as the specification limit values for a product. More tests may be applied for specific dosage forms such as moisture content for a moisture sensitive product, pH limits, microbiological tests for liquid products and viscosity tests for cream products.

Table 1.5 Tests for a Product in Tablet Form [7]

Test	**Stability criteria**	**Test method**
Assay	90.0-110.0% of label	CT-prod#1-M1
Degradation products	Not more than 0.3% each	CT-prod#1-M2
Dissolution	Q = 80% in 45 min	DM-prod#1-M3
Appearance	No significant difference from a control stored at 5°C	Visual inspection

1.8.2 Stability schedules

The time of all or some of the stability tests applied on a sample in a study can be designed in a schedule form called stability schedules (series of stability test intervals). The stability profile of a product, which can be obtained from the stability schedule

results, will define storage conditions and support the expiration date of the product. A typical example of ICH stability schedule can be found in table 1.6.

Table 1.6 Typical ICH Schedule for Stability of a New Product for Zone 2 [7]

Environment	**Intervals (months)**
25°C/60% RH	3, 6, 9, 12, 18, 24, 36
30°C/60% RH	6, 9, 12
40°C/60% RH	1, 3, 6

1.8.3 Stability Chambers

ICH guidelines recommended for the storage conditions are listed in table 1.7. The guidelines explain conditions for refrigeration, freezer and normal storage. In addition, guidelines indicate stress conditions that are used to identify the degradation mechanisms such as light. Moreover, the effects of cycle temperatures are discussed by the guidelines such as freeze and thaw or hot and cold temperatures. The American FDA recommends examination of liquid formulations in low humidity environments [14].

Table 1.7 Proposed storage guidelines [15,17]

Storage condition	Tolerances	Testing zone	Guidelines
25°C/60% RH	±2°C/±5% RH	Long term (2)	ICH, WHO
30°C/60% RH	±2°C/±5% RH	intermediate	ICH, WHO
40°C/75% RH	±2°C/±5% RH	accelerated	ICH, WHO
21°C/45% RH	±2°C/±5% RH	Long term (1)	WHO
30°C/35% RH	±2°C/±5% RH	Long term (3)	WHO
30°C/70% RH	±2°C/±5% RH	Long term (4)	WHO
2-8°C	Not specified	refrigeration	ICH, WHO
-5 to -20°C	Not specified	freezer	WHO
Below -18°C	Not specified	Deep freezer	WHO
Light	1.2 million lux hours/200 watt hours (ultraviolet)	stress	ICH

Stability chambers should be qualified and meet the manufacturer's specifications at the time of purchase in order to assure installation qualification (proper installation of the chamber). The chambers should be monitored to maintain the proper temperature and humidity within the specified tolerances, this procedure addresses the operation qualification (OQ). The performance of chambers should be continuously checked in

order to verify that the environment is within specified tolerances to confirm the performance qualification (PQ) of the chambers. The protocol should include the responsibilities of the study coordinator and the testing laboratories involved. Responsibilities such as sample amount and provider, sample storage, chamber environment, the way that data are reported should be defined in the protocol and agreed by the study coordinator and laboratory management.

1.9 Stability Reports

The main aim of designing a stability programme is to determine the expiration date of a product and storage statement recommended to ensure product purity, quality and safety in the marketplace. Stability reports should include summary information about the design described in the protocol, procedures and tests involved in the studies with appropriate limits to be evaluated, stability data schedule and results discussion and conclusion. Apart from stability data tables, stability report should include information about the aims of the studies, batch formulation and site of manufacture, description of package and packager, testing schedule, test methods and the stability criteria. Without this information, stability studies will be insufficient.

Stability data tables are a brief format report of the stability study results that help in evaluation and interpret these results. Stability data table should include concise information about product, batch, strength, formula number, drug substance supplier, package, date of manufacture, manufacture site and date on stability as shown in the figure 1.8. Each study should be included in a separate table or multiple tables.

Study Name:_____ Date on Stability:_____
Formula Number:_____ Protocol Number:_____
Lot/Batch No:_____ Manufacturer:_____
Product Name:_____ Manufacturing Site:_____
Package Description:_____ Date of Manufacture:_____
Strength/Concentration:_____ Batch Size:_____
Drug Substance Lot No.:_____

Specifications		Pass	90-110%	Q=75% @ 30min	NMT 0.1%	NMT 0.2%	Pass
Storage	Interval	Appearance	Assay	Dissolution	Impurity 1	Impurity x	Color
	Initial	Pass	99.9%	Mean=83%	ND	ND	Pass
25C/60%	3M	Pass	99.5%	Mean=84%	ND	<0.1%	Pass

Table continues for all storage conditions and intervals.
Impurities are list for each or for totals.
The tests listed here are for example of a data table format.

ND= none detected; NMT=not more than

Figure 1.8 Example of a data table containing information on the stability test for a product [SatinderAhuja, S.S., Handbook of Modern Pharmaceutical Analysis].

Data collected from each stability test should be reviewed and discussed. Notification of any instability should be reported and the reason of such instability should be discussed. Reporting and discussing the drug substance instability and drug product degradation to differentiate between inherited instability versus formulation related instability. Stability data effect on the expiration date and storage statement recommendation must be recorded and consistent with stability guidance documents relevant to the appropriate authority [15, 17].

1.10 Overall Aims

1. Robust and specific methods for two complex extemporaneous formulations containing several basic drugs which are used in palliative care and Edinburgh Western Infirmary will be developed.
2. The potential of HPLC phases based on silica gel bonded to alkyl chains in hydrophilic interaction chromatography (HILIC) mode in the separation of basic drug molecules will be developed.
3. An attempt will be made to better understand the mechanisms involved in the interaction between alkyl bonded silica gel phases and basic drugs.
4. Alkyl bonded silica gel phases will be used in HILIC mode for impurity profiling of some basic drugs.

1.11 References

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Chapter 2

A Study of the Stability of Diamorphine.HCl, Clonidine.HCl and Bupivacaine.HCl in an Extemporaneous Formulation Used In Palliative Care

2.1 Introduction

The Pharmacy Department at the Western General Hospital in Edinburgh prepares an injection in which diamorphine hydrochloride, clonidine hydrochloride, and bupivacaine hydrochloride are freshly mixed and used as a pain killer injection for administration via the spinal route (epidural space or subarachnoid (intrathecal space) for patients with complex pain problems. In managing palliative care patients, it is not uncommon for combinations of two or more medicinal products to be mixed and used [1-16].However, it is recognised that there is a lack of published information regarding the compatibility and stability of drugs in the many of the combinations used in palliative care [17].

From the available published information, it is clear that the stability and compatibility of morphine and its derivatives in combination with other drugs cannot be assumed. The Pharmacy Department at the Western General Hospital in Edinburgh prepares an infusion containing diamorphine, clonidine and bupivacaine for administration via the spinal route (epidural space or subarachnoid (intrathecal) space) for patients with complex pain problems. In July 2009, the MHRA published the outcome of its consultation regarding the legal position of practitioners mixing and administering medicines in palliative care [18] together with the Commission on Human Medicines' recommendations for changes to medicines legislation. The changes proposed apply not only to palliative care but to all clinical areas where the mixing of medicines is accepted practice. The Commission's recommendations have been accepted by Ministers and will a) allow doctors and dentists to direct other to mix, b) allow non-medical prescribers to mix medicines themselves and direct others to mix, and c) allow

nurse and pharmacist, independent prescribers, to prescribe unlicensed medicines for their patients on the same basis as doctors and supplementary prescribers. As the MHRA intends to extend the types of practitioners authorised to be involved in the mixing of medicines and the situations where mixing of medicines is acceptable, there could well be an increase in the number of medicines that are mixed. This will certainly require an assessment of the physical and chemical stability of drugs when medicines are mixed. While many medicines are stable in this situation, without investigation of this, the stability of such combinations cannot be assumed. The aim of this study was to develop an analytical method for the determination of diamorphine hydrochloride, clonidine hydrochloride, and bupivacaine hydrochloride (and also levobupivacaine hydrochloride) in three different combinations stored at 4°C, 22°C, and 37°C.

2.2 Previous Methods Used for the Analysis of the APIs

First it is important to know more about each medication in terms of chemical structure, mode of actions and uses, moreover, it is important to know more about the way that these medicines may be analysed. The chemical structures of the analytes are shown in Figure 2.1.

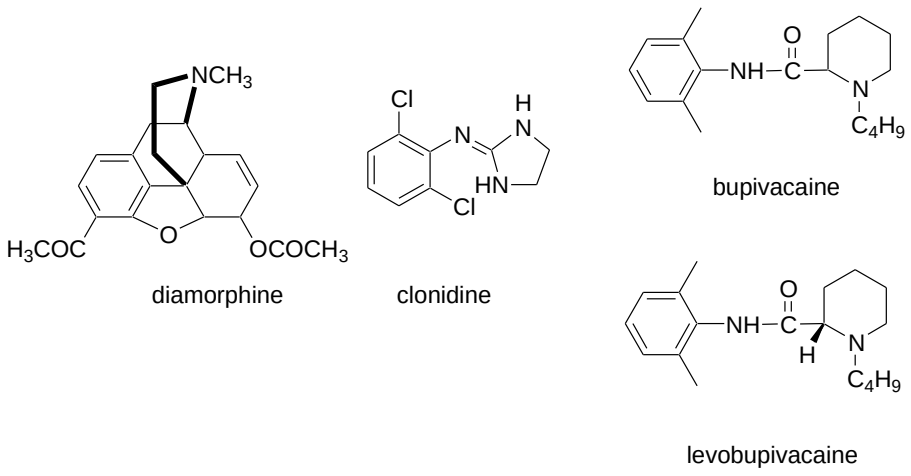

Figure 2.1 Chemical structures of clonidine, diamorphine, bupivacaine and levobupivacaine.

2.2.1 Diamorphine hydrochloride (Heroin)

Diamorphine hydrochloride, also called heroin, is controlled under schedules I and IV of the single convention on Narcotic Drugs [19].Manufacturing, possessing, and selling of heroin is unlawful in most of the world. It has some advantages over morphine, of which it is a pro-drug, since it is more readily water soluble and can thus be prepared freeze dried in ampoules for reconstitution with water for injection. Diamorphine is a semi-synthetic opioid, derived from opium poppy and synthesized from morphine. Heroin is usually synthesized as a white crystalline form which is commonly the hydrochloride salt of diamorphine. Diamorphine is the 3,6-diacetylmorphine (hence the chemical name is diacetyl-morphine). It can be taken by different routes such as intravenously, intramuscularly, subcutaneously, nasally (sniffed), and it can be smoked by inhaling the vapour which results from heating it. Orally, diamorphine undergoes an extensive first

pass effect via deacetylation. Injecting heroin can avoid this effect and may have a rapid onset depending on the injection route. After injecting the diacetylmorphine into the human body, it will rapidly cross the blood-brain barrier, due to the acetyl groups in the heroin. It is much more lipophilic than the morphine itself [20,21]. Heroin is a μ- opioid agonist being active at mu-opioid receptors, which exist in the human brain, spinal cord, and gut. Heroin acts as agonist of four endogenous neurotransmitters, which are β-endorphin, dynorphin, leu-enkephalin, and met-enkephalin. Endogenous neurotransmitters are regularly released in the brain and nerves to reduce the pain during trauma. The body will reduce or sometimes stop the production of these endogenous neurotransmitters as a response to the heroin. This repose to the heroin will create dependence and well-being effects which lead to addiction. High doses of heroin can be fatal, as it can be used for suicide or as a murder weapon. In the brain, diacetylmorphine will be rapidly metabolized and converted to morphine by removal of acetyl groups, thus diacetyl morphine can act as a pro-drug for morphine. Morphine then binds to the opioid receptor to give its effect. Diamorphine can be used for extreme pain relief, as a cough suppressant, anti-diarrheal and can be used to treat pulmonary oedema and heart attacks. There are many side effects associated with diamorphine use such as urinary retention, dry mouth, insomnia, changes in mood, sweating, confusion, loss of appetite, constipation, nausea and vomiting. Diamorphine should be prescribed by the doctor in severe cases only and the usage by the patient should be under his supervision, because heroin has a serious withdrawal effects including diarrhea, nausea, pain and tremor [20,21].

2.2.2 Clonidine Hydrochloride:

Clonidine is imidazoline derivative and has a hypotensive effect. Clonidine acts on the central and peripheral nervous system as an α-adrenergic agonist. It inhibits the bulbar sympathetic cardio-accelerator and vasoconstrictor centres. Thus leading to a decrease in sympathetic out-flow from the brain [22]. It is most effective in the control of severe, burning and shooting pains, if it is used with opiates. Clonidine hydrochloride injection may be given as an epidural injection. The dose of this injection depends on the patient's condition and response to the medication. After prescription of clonidine by a doctor, it cannot be stopped suddenly, because this results in serious side effects such as high blood pressure and stroke. Clonidine hydrochloride has many side effects including dizziness, light-headedness, nausea, constipation, drowsiness, itching and dry mouth [23].

2.2.3 Bupivacaine hydrochloride

Bupivacaine hydrochloride is an amide type, long acting potent local anaesthetic. Bupivacaine binds to specific sodium ion channels in the neuronal membrane reversibly. This action decreases the voltage-dependent membrane permeability to sodium ions and membrane stabilization. Thus, inhibition of depolarization and nerve impulse conduction and reversible loss of sensation will occur. Bupivacaine can be used for a number of different types of anaesthesia including peripheral sympathetic nerve anaesthesia, epidural anaesthesia, infiltration anaesthesia, surgical anaesthesia and obstetric anaesthesia. It is used whenever long anaesthesia effects are needed. The

bupivacaine dose will depend on the therapeutic use.Bupivacaine produces common side effects such as nausea, vomiting, tinnitus, numbness, nervousness, blurred vision, tremor, dizziness and disorientation. Bupivacaine can be used to treat epididymitis and orchitis [24,25].

2.2.4 HPLC Methods Used for the Analysis of Diamorphine

High performance liquid chromatography (HPLC) has been used as a separation and identification technique for heroin. Many papers have been published on the separation or identification of heroin by using HPLC. A simple, rapid and sensitive method was used for the identification of heroin abuse. Urine samples were directly introduced into a system which consisted of a semi-microcolumn HPLC with a column switching system allowing connection to an electrospray ionization mass spectrometer (ESI-MS). After removal of other compounds in the sample matrix using on-line column switching solid phase extraction with a strong cation exchange (SCX) cartridge, heroin is perfectly separated by the use of strong cation exchange and incorporated with ESI mass spectral information [9]. Another HPLC method was developed in order to study diamorphine (0.02 mg/ml) in 250 ml of a 0.15% w/v bupivacaine hydrochloride infusion. Stability was assessed at different temperatures ranging from 7°C to 45°C. At a temperature of 7°C, there was 0.13% reduction in thediamorphine hydrochloride initial concentration per day, while bupivacaine was stable under the same conditions. The diamorphine hydrochloride concentration was reduced by 5% under the same conditions within 14 days. At temperature 25°C, diamorphine showed a reasonable stability

allowingtransport and administration within 24 hours. Diamorphine hydrochloride showed adequate stability at 32 and 45°C. The mixture remained stable for 6 months on frozen storage at -18°C [26]. A HPLC instrument was used to assess the effects of under storage and prolonged in- use conditions on the stability of diamorphine hydrochloride infusion (5 mg/ml) in an ambulatory pump. A Kontron S5CN analytical column was used as a stationary phase in the system. The mobile phase was composed of acetonitrile - 0.01 M phosphate buffer (15:85, v/v) with a pH of 3.5. UV detection was carried out at 274 nm. Accelerated degradation was performed using 1 M hydrochloride acid, 1 M sodium hydroxide, hydrogen peroxide and heat to four different samples, to indicate the stability under such conditions. The diamorphine hydrochloride peak was reduced under each degradation test used [27].

2.2.5 HPLC Methods Used for the Analysis of Clonidine

A HPLC method was used to indicate the stability of clonidine hydrochloride by accelerating its decomposition using heat, oxidation and high pH values. Decomposition of clonidine hydrochloride could be attained by mixing it with 1.0 N hydrochloric acid and boiling for two hours or by exposing to 3% hydrogen peroxide for one hour at room temperature. It was established that the peaks of the decomposition products would not interfere with the intact clonidine peak.

A method to assess the stability of clonidine was carried out using a Hypersil BDS C8 analytical column (150 x 4.6mm i.d.)(5 µm). The mobile phase was consisted of 500 ml of water, 500 ml of methanol, 1.1 g sodium 1-octanesulfonate and 1 ml of sulphuric

acid, and the mobile phase pH was adjusted to 3.0. Injection volume was 15 µl, and UV detection was performed at 220nm and 0.5 AUFS. The retention time of clonidine was 5.8 minutes [28].

Another HPLC method was used for assessing clonidine hydrochloride stability. AZorbax Trimethylsilyl column (250 x 4.6mm i.d.)(5 µm) was used as the stationary phase. A mixture of methanol - phosphoric buffer solution (65:35, v/v) (0.0022 M monobasic potassium phosphate and 0.016 M dibasic sodium phosphate, PH 7.9) was used as the mobile phase. An isocratic run was applied and UV detection was performed at 254nm. Guanabenz acetate was used as an internal standard. The sample was diluted with deionized water. The pH of clonidine hydrochloride solution was adjusted to 12 with 1.0 N sodium hydroxide or to pH 2 with 1.0 N sulphuric acid then heated to 100°C for 30 minutes to accelerate the clonidine decomposition.Clonidine and internal standard retention times were 5.7 and 6.7respectively [29].Both studies showed a linear relationship between the clonidine hydrochloride concentration and the peak height of clonidine hydrochloride.

2.2.6 HPLC Methods Used in the Analysis of Bupivacaine

A HPLC method was used to indicate the stability of epidural opiate solutions in 0.9% sodium chloride infusion bags. ChrompakSpherisorb ODS-2 column (250×4.6mm i.d.)(5 µm) was used as the stationary phase. 0.01 M heptanesulfonic acid and acetonitrile (55:45, v/v) containing 7.7 mM dimethyloctylamine and adjusted to pH 3.5 was used as the mobile phase for the system. The system had a flow rate of 1 ml/min and the UV

detection was performed at 254 nm. The sample was analyzed without any dilution, and 3.5 was the retention time for bupivacaine. Accelerated degradation for bupivacaine was applied to the sample by placing it on the water bath for 2 hours. The bupivacaine peak was completely resolved from its decomposition products peaks [30].

Another HPLC method was used to evaluate bupivacaine hydrochloride injection stability. The injection contained 1.25mg/ml of bupivacaine in 0.9% w/v sodium chloride injection in polypropylene. A Beckman Ultrasphere XL-ODS C18 column (75×4.6mmi.d.)(3 µm) was used as the system stationary phase. Acetonitrile - 0.1 M monobasic potassium phosphate buffer (40:60, v/v) adjusted to pH 5 was used as mobile phase. The system flow rate was 1.5 ml/min and UV detection performed at 254 nm [31]. Another HPLC method was developed to study the stability of bupivacaine hydrochloride and fentanyl citrate in portable pump reservoirs. A Waters Radial-Pak phenyl column (100×4.6 mm i.d.)(4 µm) was used as a stationary phase for the system. Methanol - 0.005 M monobasic potassium phosphate buffer (65:35, v/v) was used as a mobile phase after adjusting the pH to 4.8. A system flow rate of 3 ml/min was used and the UV detection was performed at 210 nm. The sample was analysed without any dilution. Bupivacaine and fentanyl retention times were 7.5 and 11.6 minutes, respectively. Accelerated decomposition for the sample was applied by storing bupivacaine solution (2 mg/ml in 0.3 N hydrochloric acid) at 140°C for 44 hours. Decomposition products peaks were completely resolved from the intact bupivacaine peak. Bupivacaine hydrochloride standard curves were constructed each day from 200 to 2200 µg/ml [32].In another study HPLC was used to assess the stability of an

admixture combining ziconotide with bupivacaine hydrochloride during simulated intrathecal infusion under laboratory conditions at 37°C. HPLC was used to analysean admixture containing ziconotide (25 µg/ml) and bupivacaine hydrochloride (5 mg/ml),which was stored in SynchroMed® II pumps at 37°C and in control vials at either 37 °C or 5°C. Drug concentrations were determined for samples obtained at varying intervals during the 30-day study. Ziconotide and bupivacaine hydrochloride concentrations were measured after 30 days and an average of 86.9% and 99.4% of their initial concentrations were indicated, respectively. Similar degradation rates for both drugs were shown for the control vials. The admixture has shown a reasonable stability (90%) for 22 days and 80% for 45 days (extrapolated) in SynchroMed® II infusion pumps [33].

A study was designed to assess the stability of the admixture of morphine sulfate, bupivacaine hydrochloride, and clonidine hydrochloride which was administered by intrathecal infusion via an implantable pump under simulated clinical use conditions. SynchroMed implantable pumps were filled with an admixture and incubated at 37 ° C for a period of 90 days. Drug admixture was stored in glass vials at 4°C and at 37 °C which served as controls. Samples were collected every 30 days. A Phenomenex Luna C18 (150 x 4.6 mm i.d.)(5 µm) column was used to analyze the drug. The mobile phase was composed of two solutions. Solution A was 39 mM potassium phosphate dibasic reagent at pH 9 and solution B was HPLC-grade methanol. The flow rate was 1 ml/min and 210 nm was the detection wavelength. All drugs showed reasonable stability and the original concentrations remained greater than 96% over 90 days [34].

Another method was developed by using a reversed-phase HPLC technique to measure the concentration of both bupivacaine and ketamine in plasma. Plasma samples (0.5 ml) were prepared using a rapid and simple back-extraction technique. A cyano (CN) column (250×4.6mmi.d.)(5µm) was used. A mixture of methanol:acetonitrile:orthophosphoric acid:0.01 M sodium dihydrogenphosphate (200:80:2:718 v/v/v/v) was used as the mobile phase for the system. UV detection was performed at 215 nm. Although the wavelength selected was short, the wavelength selected provided a good sensitivity for bupivacaine. The method showed anappropriate selectivity to measure bupivacaine and ketamine concentrations in patient plasma samples with no interference from bupivacaine and ketamine metabolites or co-medications administered to patients during surgery including clonidine, lignocaine and midazolam. Moreover, the technique displayed good within-day and between-day repeatability for both ketamine and bupivacaine. Method accuracy was assessed by adding and measuring ketamine and bupivacaine in 10 plasma samples. The technique was shown to have a good agreement between the added and measured concentrations for both ketamine and bupivacaine, thus the assay had acceptable accuracy for ketamine and bupivacaine. Limits of quantification of ketamine and bupivacaine were 10 ng/ml and 125 ng/ml respectively and the limit of detection of ketamine was 1ng/ml and that of bupivacaine was 0.8 ng/ml. Desipramine was used as an internal standard and resolution between both analytes and the internal standard was obtained [35].

A coupled-column liquid chromatographic method was developed to measure the free concentration of ropivacaine and bupivacaine in blood plasma. Ultrafiltration to the

sample following adjustment of the temperature and pH was carried out. The sample was then analyzed directly by the system. The system consisted of one reversed-phase and one ion-exchange column. UV detection was performed at 210 nm. The system was demonstrated to have a high selectivity [36].

2.3 Aims

The Pharmacy Department at the Western General Hospital in Edinburgh uses an extemporaneously prepared infusion containing diamorphine, clonidine, and bupivacaine for administration via the spinal route. This infusion may be used over 72 hours in palliative care. There are three different combinations which are of interest.

The aims of the project will be to:

- Prepare the extemporaneous formulations in the laboratory in a manner suitable for testing.
- Develop a stability indicating HPLC method suitable for assessing the stability of the formulation.
- Examine the stability of the three different combinations used under three sets of storage conditions.
- Examine the degradants formed by forced degradation of the three drugs used in the formulations.

2.4 Materials and Methods

2.4.1 Chemicals

Tris buffer, clonidine and bupivacaine hydrochloride were obtained from Sigma-Aldrich, Dorset, UK. Acetonitrile and methanol were obtained from Fisher Scientific, Leicestershire, UK. HPLC grade water was produced “in house” using a Millipore MilliQ system. The Pharmacy Department at the Western General Hospital in Edinburgh supplied diamorphine hydrochloride injection (Wockhardt UK Ltd, Wrexham, UK), clonidine hydrochloride 150 microgram/ml injection (BoehringerIngelheim Ltd, Bracknell, Berks, UK), bupivacaine hydrochloride 0.5% injection (AstraZeneca, Luton, Beds, UK) and levobupivacaine hydrochloride 0.75% injection (Abbott Labs Ltd, Queensborough, Kent, UK). The Western General Hospital also supplied syringes, sodium chloride 0.9% w/v injection and water for injection (B.Braun, Melsungen, Germany). Three part, polypropylene Luer-Lok^{TM} Syringes (10 ml; 30 ml) were obtained from Becton Dickinson, Cowley, Oxford, UK, and HelapetCombi Caps were obtained from B.Braun, Melsungen, Germany.

Tris buffer was prepared by dissolving 6.592 g of tris free base in 500 ml of water and adjusting to pH 8 with 5 M HCl. The solution was then adjusted to 1000 ml.

2.4.2 Preparation and analysis of diamorphine, clonidine, and bupivacaine admixtures

2.4.2.1 Combination 1:Diamorphine 100 mg, Clonidine 150 µg and Bupivacaine 80 mg per 17 ml.

200 mg of diamorphine hydrochloride for injection was dissolved in bupivacaine hydrochloride 0.5% w/v injection to give a final volume of 32 ml. . Two 1ml ampoules of clonidine (150µg/ml) were added to the bupivacaine hydrochloride/diamorphine hydrochloride solution. Two syringes were filled with 15 ml of the sample reserving 4 ml of the sample for a time zero analysis. At each time point 0.5 ml of the sample was analysed directly without any dilution in order to determine the clonidine hydrochloride peak. A further aliquot of the sample (1 ml) was diluted with HPLC grade water to 50 ml in a volumetric flask in order to determine the diamorphine hydrochloride and bupivacaine hydrochloride content. A blank of the solvent (water) was run in between the diluted and the concentrated sample in order to check for sample carry over. Duplicate syringes were prepared as described above for each set of storage conditions (4ºC; room temperature (*ca* 22 °C); 37°C). Aliquots of the sample were analysed after 4, 24, 48, 96 and 192 hours, withdrawing 2.5 ml of sample at each time point. At each time point analysis was carried out in duplicate from each syringe giving a total of x 4 analyses per time point.

2.4.2.2 Combination 2: Diamorphine 50 mg, Clonidine 150 µg, Bupivacaine 45 mg per 10 ml

100 mg of diamorphine hydrochloride for injection was dissolved in bupivacaine hydrochloride 0.5% w/v injection to give a final volume of 18 ml. Two 1ml ampoules of clonidine hydrochloride (150µg/ml) injection were added to the bupivacaine/diamorphine solution. Two syringes were filled with 8 ml of the sample and 2 ml of the sample was reserved to carry out a time zero analysis. The samples were then stored and analysed as described for combination 1.

2.4.2.3 Combination 3: Diamorphine 650 mg,Clonidine 300 µg and Levobupivacaine 110 mg per 17 ml

The syringes were prepared by mixing enough levobupivacaine to make a total of 29.4ml. 1300mg of diamorphine.HCl were dissolved in levobupivacaine solution; rinsing out the vials of diamorphine with levobupivacaine solution and rinsing it back into the beaker. 4 vials of clonidine.HCl (150µg) were added to the mixture and the vials were rinsed with the mixture solution. Each syringe was filled with 15.7ml of the sample reserving just over 2 ml of the sample to do a time 0 analysis. 1 ml of the sample was directly placed into the auto-sampler for the analysis in order to analyse the clonidine, The other 1 ml that remained was diluted to 100 ml (X100) and then analysed to check diamorphine.HCl and levobupivacaine.HCl. The same procedure was carried out at each time point for the remaining analyses at 4, 24, 48 hours, 4 days and 8 days.

2.4.3 Calibration Solution

A calibration sample check was carried out with each run in order to confirm detector stability from day to day. A solution containing 0.01 mg of clonidine, 0.1 mg of bupivacaine and 0.12 mg/ml of diamorphine was run at 0 min, 4 hrs, 24 hrs, 48 hrs, 96 hrs and 192 hrs. The calibration solution was prepared from stock solutions containing 1 mg/ml of standards for the three compounds in methanol.

2.4.4 Chromatographic Analysis

HPLC analysis was carried out by using a P2000 system supplied by ThermoFisher. The system consisted of an autosampler, a column oven, a binary pump and a variable wavelength UV detector and was controlled by Chromquest software. The column used was a Varian Pursuit C18 column (150 x 4.6 mm i.d.)(5 µm) fitted with a guard column, the column oven temperature was 40°C and the detection wavelength was 245 nm. The following solvent programme was used:

Time (minutes)	Tris buffer	Methanol
0 minute - 6.9 minutes	70%	30%
7 minutes – 15 minutes	30%	70%

2.5 Results

In Figure 2.2 a chromatogram for an undiluted clonidine/diamorphine/ bupivacaine preparation containing 150 µg, 50 mg and 80 mg respectively in 10 ml after storage at room temperature is shown. It can be seen that even in the undiluted sample the clonidine peak is small and it was important to maintain good chromatography to get good precision in the analysis of this peak.

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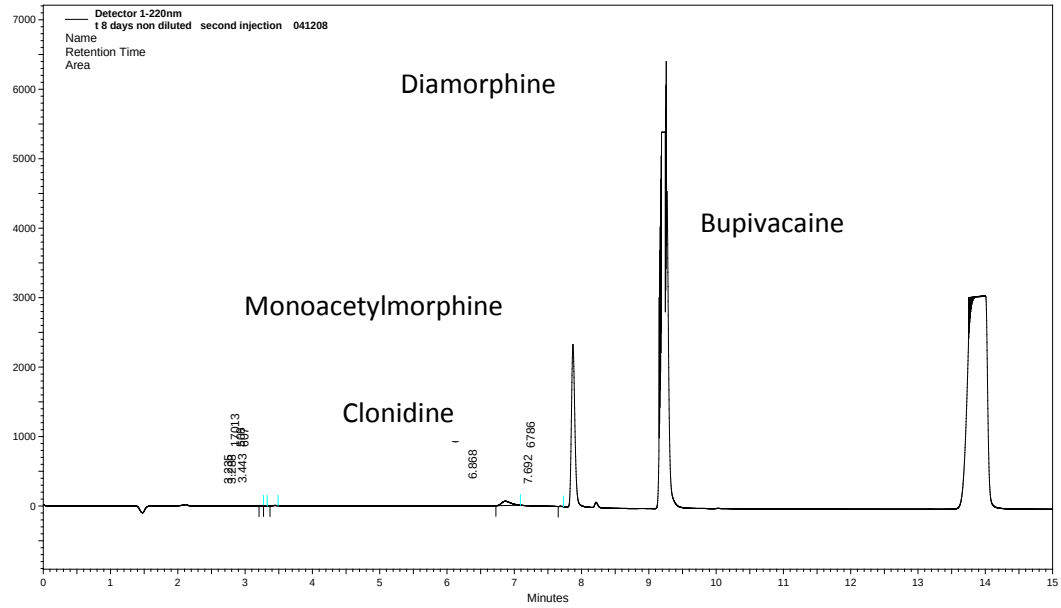

Figure 2.2 Chromatogram of undiluted clonidine, diamorphine, bupivacaine preparation containing 150 µg, 50 mg and 45 mg of each analyte respectively per 10 ml

The mobile phase was buffered at pH 8 since this ensured that clonidine peak and the monacetyl morphine hydrolysis product were well resolved, at lower pH values they ran closer and it took some time to find the optimum pH. The relative increase in retention

of monoacetyl morphine against clonidine as pH rises probably reflects the fact that it is a slightly weaker base than clonidine or the fact that its partition coefficient is inherently higher than that of clonidine and as it becomes less ionised its retention time increases to a greater extent than that of clonidine. The tiny peak at 8.2 minutes is due to the main manufacturing impurity of diamorphine HCl which is acetylcodeine [37]. In Figure 2.3 the chromatogram for the same sample after dilution by x 50 thus bringing the diamorphine and the bupivacaine within the range of the detector is shown. The fall in the baseline in Figure 2.3 is due to the sudden change in % of organic modifier between 6.9 and 7 minutes. It was far enough away from the peaks of interest to have no impact on peak integration.

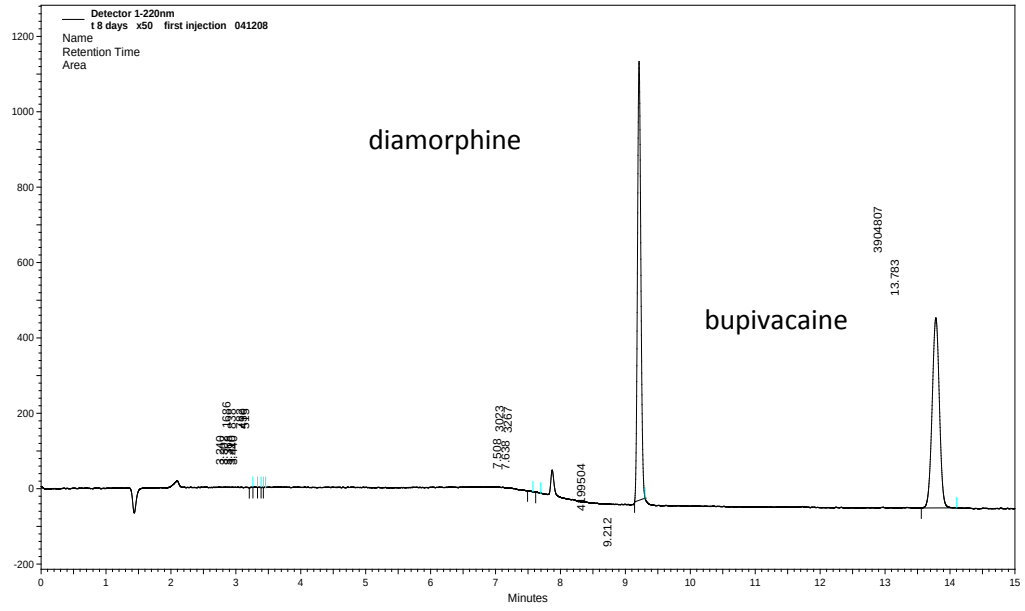

Figure 2.3 Chromatogram of x 50 diluted clonidine, diamorphine, bupivacaine preparation originally containing 150 µg, 50 mg and 45 mg of each analyte respectively per 10 ml.

Since the clonidine concentration was so low compared to the rest of the components in the formulation it had to analysed in the undiluted sample. Thus it was not possible to analyse all the components in a single run since they did not all fit into the dynamic range of the detector in the undiluted and also the high concentrations of diamorphine and bupivacaine overloaded the column resulting in poor peak shape. It was important to ensure that the HPLC column remained in good condition in order to get good peak shape for the bupivacaine peak which tailed very readily if the column performance degraded. The Pursuit column worked very well for this purpose in comparison with other columns which were tried initially and only maintained optimum performance for a few weeks and the peak shape of bupivacaine was maintained provided that the guard column was changed about every two or three weeks.

In order to calculate the % of degradation the original peak area for each component in the formulation was taken to be 100%. In Table 2.1 the stability data for an injection containing clonidine (150 µg), bupivacaine (45 mg) and diamorphine (50 mg) in a 17 ml injection stored at room temperature is shown. The clonidine and bupivacaine were quite stable when stored up to 192 hours. The diamorphine, as might be expected, underwent up to 14 % degradation into monoacetyl morphine. As might be expected the diamorphine degraded more rapidly when the injection was stored at 37˚C with up to 30% degradation over 192 hours (table 2.2). The pattern of degradation was somewhat unusual in that there was an initial rapid degradation followed by a slower rate of degradation. This probably reflects a change in pH with the pH falling due to the release of acetic acid as the diamorphine is hydrolysed to a level where the diamorphine

is more stable. The optimum pH for stability of esters is around pH 4.5 where the alkaline and acid catalysed degradation rates are similar. Tables 2.3-2.5 show the change in pH with time in the formulations. The rate of fall of pH is greatest at the higher temperature reflecting the faster rate of hydrolysis of diamorphine to monoacetylmorphine. In Table 2.6 the stability data for the combination at 4°C is shown. In this case the degradation of diamorphine was surprisingly bit more extensive than at room temperature.

Table 2.1 Stability of a 10 ml extemporaneously prepared injection containing clonidine (150 μg), bupivacaine (45 mg) and diamorphine (50 mg) stored at room temperature (*ca* 22°C)

	Clonidine % remaining			**Diamorphine % remaining**			**Bupivacaine % remaining**		
Time (h)	Syr.1	Syr.2	RSD%	Syr.1	Syr.2	RSD%	Syr. 1	Syr. 2	RSD%
0	100.0	100.0	0%	100.0	100.0	0%	100.0	100.0	0%
4	99.6	100.3	± 0.5%	97.1	99.1	± 1.4%	101.1	100.0	± 0.8%
24	100.9	102.2	± 0.9%	94.4	96.4	± 1.5%	101.0	99.1	± 1.3%
48	100.9	99.7	± 0.9%	93.0	95.1	± 1.6%	101.3	98.6	± 1.9%
96	99.6	100.0	± 0.3%	92.0	93.8	± 1.4%	101.3	98.7	± 1.8%
192	99.9	100.4	± 0.4%	88.4	86.2	± 1.8%	98.8	95.8	± 2.2%

***syr. = syringe**

Table 2.2 Stability of a 10 ml extemporaneously prepared injection containing clonidine (150 µg), bupivacaine (45 mg) and diamorphine (50 mg) stored at 37 °C.

	Clonidine % remaining			**Diamorphine % remaining**			**Bupivacaine % remaining**		
Time (h)	Syr.1	Syr.2	RSD%	Syr.1	Syr.2	RSD%	Syr. 1	Syr. 2	RSD%
0	100.0	100.0	0%	100.0	100.0	0%	100.0	100.0	0%
4	99.4	99.8	± 0.3%	81.8	80.4	± 1.2%	100.3	100.1	± 0.1%
24	99.2	99.5	± 0.2%	81.5	78.8	± 2.4%	100.1	102.2	± 1.5%
48	99.6	99.7	± 0.1%	80.7	77.8	± 2.6%	99.9	101.4	± 1.1%
96	99.4	99.9	± 0.4%	79.2	77.4	± 1.6%	100.4	102.1	± 0.5%
192	99.0	99.6	± 0.4%	75.5	70.1	± 5.2%	100.0	102.0	± 1.4%

***syr. = syringe**

Table 2.3 Change in the pH values of the clonidine (150 μg), bupivacaine (45 mg) and diamorphine (50 mg) combination at 22°C with time.

Time (h)	pH
0 minute	4.55
4 hours	4.54
24 hours	4.51
48 hours	4.45
96 hours	4.40
192 hours	4.33

Table 2.4 Change in the pH values of the clonidine (150 µg), bupivacaine (45 mg) and diamorphine (50 mg) combination at 37°C with time.

Time (h)	pH
0 minute	4.55
4 hours	4.52
24 hours	4.44
48 hours	4.34
96 hours	4.21
192 hours	4.10

Table 2.5 Change in the pH values of the clonidine (150 µg), bupivacaine (45 mg) and diamorphine (50 mg) combination at 4 °C with time.

Time (h)	pH
0 minute	4.55
4 hours	4.55
24 hours	4.52
48 hours	4.48
96 hours	4.44
192 hours	4.40

Table 2.6 Stability of a 10 ml extemporaneously prepared injection containing clonidine (150 µg), bupivacaine (45 mg) and diamorphine (50 mg) stored at 4 °C.

	Clonidine % remaining			**Diamorphine % remaining**			**Bupivacaine % remaining**		
Time (h)	Syr.1	Syr.2	RSD%	Syr.1	Syr.2	RSD%	Syr. 1	Syr. 2	RSD%
0	100.0	100.0	0%	100.0	100.0	0%	100.0	100.0	0%
4	100.2	99.7	± 0.4%	88.5	90.4	± 1.5%	100.0	99.9	± 0.1%
24	100.1	99.8	± 0.2%	87.6	86.3	± 1.1%	99.4	98.3	± 0.8%
48	99.4	99.4	0%	86.1	85.9	± 0.2%	97.6	95.9	± 1.2%
96	99.5	99.2	± 0.2%	85.5	85.5	0%	97.6	97.8	± 0.1%
192	100.0	99.7	± 0.2%	84.6	84.6	0%	96.4	96.3	± 0.1%

***syr. = syringe**

Tables 2.7-2.9 show the data obtained for the stability of the clonidine (150 μg), bupivacaine (80 mg) and diamorphine (100 mg) combination at room temperature, 37°C and 4°C respectively. In all cases the bupivacaine and clonidine were stable over the course of the experiments but as was observed for the other combination slow degradation of diamorphine through to monoacetyl morphine occurred. This was not as extensive for the formulation containing 50 mg of diamorphine/10 ml this is possibly again consistent with a buffering effect due to the acetic acid released upon hydrolysis. With a higher concentration of diamorphine present a lower % of hydrolysis would be required to release the same amount of acetic acid when compared to the less concentrated solution.

Table 2.7 Stability of a 17 ml extemporaneously prepared injection containing clonidine (150 µg), bupivacaine (80 mg) and diamorphine (100 mg) stored at room temperature (*ca* 22°C).

	Clonidine % remaining			**Diamorphine % remaining**			**Bupivacaine % remaining**		
Time (h)	Syr.1	Syr.2	RSD%	Syr.1	Syr.2	RSD%	Syr. 1	Syr. 2	RSD%
0	100.0	100.0	0%	100.0	100.0	0%	100.0	100.0	0%
4	101.5	98.4	± 2.2%	97.7	98.1	± 0.3%	99.2	99.2	0%
24	101.4	99.4	± 1.4%	96.7	96.9	± 0.1%	100.4	98.9	± 1.1%
48	101.4	99.3	± 1.5%	95.6	95.3	± 0.2%	99.6	98.6	± 0.7%
96	101.4	98.9	± 1.8%	94.5	92.1	± 1.8%	99.0	99.2	± 0.1%
192	101.5	98.7	± 2%	91.1	90.5	± 0.5%	101.9	98.5	± 2.4%

***syr. = syringe**

Table 2.8 Stability of a 17 ml extemporaneously prepared injection containing clonidine (150 µg), bupivacaine (80 mg) and diamorphine (100 mg) stored at 37°C.

	Clonidine % remaining			**Diamorphine % remaining**			**Bupivacaine % remaining**		
Time (h)	Syr.1	Syr.2	RSD%	Syr.1	Syr.2	RSD%	Syr. 1	Syr. 2	RSD%
0	100.0	100.0	0%	100.0	100.0	0%	100.0	100.0	0%
4	99.5	100.2	± 0.5%	97.5	96.7	± 0.6%	100.8	98.1	± 2%
24	100.0	103.3	± 2.3%	97.0	96.6	± 0.3%	99.4	98.6	± 0.6%
48	100.6	100.5	± 0.7%	96.8	96.5	± 0.2%	99.8	100.9	± 0.8%
96	98.6	100.3	± 1.2%	90.2	89.2	± 0.8%	101.1	100.0	± 0.8%
192	100.5	100.3	± 0.1%	89.5	88.9	± 0.5%	100.7	98.9	± 1.3%

Table 2.9 Stability of a 17 ml extemporaneously prepared injection containing clonidine (150 µg), bupivacaine (80 mg) and diamorphine (100 mg) stored at 4 °C.

	Clonidine % remaining			**Diamorphine % remaining**			**Bupivacaine % remaining**		
Time (h)	Syr.1	Syr.2	RSD%	Syr.1	Syr.2	RSD%	Syr. 1	Syr. 2	RSD%
0	100.0	100.0	0%	100.0	100.0	0%	100.0	100.0	0%
4	100.7	100.4	± 0.2%	98.5	98.8	± 0.2%	100.1	100.3	± 0.1%
24	101.0	101.2	± 0.1%	97.0	97.2	± 0.1%	99.1	100.2	± 0.8%
48	101.3	101.0	± 0.2%	96.2	96.8	± 0.4%	99.9	98.4	± 1.1%
96	100.0	100.3	± 0.2%	95.6	96.0	± 0.3%	100.0	99.4	± 0.4%
192	100.7	100.2	± 0.4%	93.4	93.6	± 0.2%	98.5	98.3	± 0.1%

***syr. = syringe**

Tables 2.10-2.12 show the stability data for the combination containing clonidine (300 µg), levobupivacaine (110 mg) and diamorphine (650 mg). Again the clonidine and bupivacaine were stable under all the storage conditions. The diamorphine again degraded up to 10% over the time period of the study apart from at 4°C where it appeared to be more stable in this more concentrated formulation. Tables 2.13-2.15 show the change in pH in the 650 mg diamorphine formulation with time.

Table 2.10 Stability of a 17 ml extemporaneously prepared injection containing clonidine (300 μg), levobupivacaine (110 mg) and diamorphine (650 mg) stored at room temperature (22 °C).

	Clonidine % remaining			**Diamorphine % remaining**			**Bupivacaine % remaining**		
Time (h)	Syr.1	Syr.2	RSD%	Syr.1	Syr.2	RSD%	Syr. 1	Syr. 2	RSD%
0	100.0	100.0	0%	100.0	100.0	0%	100.0	100.0	0%
4	101.5	98.48	± 2.1%	97.73	98.08	± 0.3%	99.22	99.23	± 0.01%
24	101.4	99.40	± 1.4%	96.70	96.92	± 0.2%	100.4	98.91	± 1.1%
48	101.4	99.30	± 1.5%	95.56	95.30	± 0.2%	99.62	98.56	± 0.8%
96	101.4	98.99	± 1.7%	94.50	92.07	± 1.8%	98.97	99.18	± 0.2%
192	101.5	98.65	± 2%	91.06	90.51	± 0.4%	101.9	98.49	± 2.4%

***syr. = syringe**

Table 2.11 Stability of a 17 ml extemporaneously prepared injection containing clonidine (300 µg), levobupivacaine (110 mg) and diamorphine (650 mg) stored at 37 °C.

	Clonidine % remaining			**Diamorphine % remaining**			**Bupivacaine % remaining**		
Time (h)	Syr.1	Syr.2	RSD%	Syr.1	Syr.2	RSD%	Syr. 1	Syr. 2	RSD%
0	100.0	100.0	0%	100.0	100.0	0%	100.0	100.0	0%
4	100.6	100.4	± 0.1%	95.7	95.5	± 0.2%	100.1	102.9	± 2%
24	100.8	100.4	± 0.3%	95.0	94.3	± 0.5%	101.3	100.8	± 0.4%
48	100.2	100.1	± 0.1%	94.0	93.4	± 0.5%	99.2	100.8	± 1.1%
96	99.8	98.7	± 0.9%	92.7	91.9	± 0.6%	101.3	101.5	± 0.1%
192	100.5	99.7	± 0.6%	90.5	90.9	± 0.3%	100.0	100.7	± 0.5%

***Syr. = syringe**

Table 2.12 Stability of a 17 ml extemporaneously prepared injection containing clonidine (300 µg), levobupivacaine (110 mg) and diamorphine (650 mg) stored at 4 °C.

	Clonidine % remaining			**Diamorphine % remaining**			**Bupivacaine % remaining**		
Time (h)	Syr.1	Syr.2	RSD%	Syr.1	Syr.2	RSD%	Syr. 1	Syr. 2	RSD%
0	100.0	100.0	0%	100.0	100.0	0%	100.0	100.0	0%
4	100.2	100.0	± 0.1%	99.1	99.5	± 0.3%	100.4	101.8	± 1%
24	100.0	100.4	± 0.3%	98.6	99.0	± 0.3%	102.2	100.0	± 1.5%
48	100.6	99.7	± 0.6%	98.4	98.7	± 0.2%	100.9	100.7	± 0.1%
96	99.8	100.4	± 0.4%	97.8	97.7	± 0.1%	101.0	102.2	± 0.1%
192	100.0	99.7	± 0.2%	97.8	96.7	± 0.8%	102.3	103.1	± 0.6%

***syr. = syringe**

Table 2.13 Change in pH of the clonidine (300 µg), levobupivacaine (110 mg) and diamorphine (650 mg) combination at room temperature (22°C) with time.

Time (h)	PH
0 minute	4.75
4 hours	4.75
24 hours	4.71
48 hours	4.69
96 hours	4.62
192 hours	4.59

Table 2.14 Change in pH of the clonidine (300 µg), levobupivacaine (110 mg) and diamorphine (650 mg) combination at room temperature (37°C) with time.

Time (h)	PH
0 minute	4.75
4 hours	4.73
24 hours	4.69
48 hours	4.64
96 hours	4.60
192 hours	4.53

Table 2.15 Change in pH of the clonidine (300 µg), levobupivacaine (110 mg) and diamorphine (650 mg) combination at room temperature (4°C) with time.

Time (h)	pH
0 minute	4.75
4 hours	4.75
24 hours	4.73
48 hours	4.71
96 hours	4.68
192 hours	4.66

2.6 Conclusion

A method was developed for the determination of the stability of various combinations of diamorphine, bupivacaine and clonidine. After some initial development work the best mobile phase conditions were found to be buffering at pH 8.0 since buffering at a lower pH did not produce sufficient resolution between the diamorphine hydrolysis product monoacetyl morphine and the very small amount of clonidine in the samples. Acetonitrile was found to be a more suitable for analysis since use of methanol in the gradient method produced too much baseline drift. In addition it was found necessary to analyse the combinations in two stages, first for the very small clonidine peak without dilution and then by diluting x 50 or x 100 to bring the bupivacaine and diamorphine within the range of the UV detector and to avoid overloading the HPLC column. It was found the performance of an ACE C18 column declined quite rapidly in the case of bupivacaine (bupivacaine is only 50% ionised at pH 8.0) where the peak started to tail after perhaps 100 runs. The Varian Pursuit column has ultra-high purity silica, extensive capping, high bonded phase density, minimal metal and silanol activity and wide pH range (1.5-10), which make it ideal for the majority of reverse phase applications and basic compounds analysis [38]. Thus aVarian Pursuit column was purchased with guard column and this column was found to give better peak shapes for the bases and also maintained its performance over a longer period. Both column types are claimed to be base deactivated but the Varian column proved to be more robust.

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Chapter 3

A stability Indicating Assay for a Combination of Morphine Sulphate with Levomepromazine Hydrochloride Used in Palliative Care

3.1 Introduction

Drug treatment plays a major role in symptom control in palliative care. High numbers of patients develop nausea, vomiting or dysphagia or become too weak to take drugs orally. In this situation the required medicines are commonly delivered as a subcutaneous infusion with a number of drugs mixed together in a small volume and delivered by a syringe driver.

Potential problems of mixing injections include degradation of the drug(s) and therefore reduced efficacy, and precipitation/crystallisation; the greater the number of injections mixed together, the greater the possibility that drug interactions and physical changes will occur. Data from chemical stability and compatibility studies are only available for a few of the subcutaneous injection combinations used in palliative care; injection combinations are often used in practice without any prior assessment of the chemical or even physical stability of the admixtures. HPLC methods can be used to determine drug stability, for example giving the percentage of original concentration drug present after 24h. Simple visual inspection of an admixture before and during administration can detect many problems of crystallisation/precipitation, although other techniques are usually necessary to detect fine particles that are not visible to the naked eye.

In July 2009, the MHRA published the outcome of its consultation regarding the legal position of practitioners mixing and administering medicines in palliative care [1] together with the Commission on Human Medicines' recommendations for changes to medicines legislation. The changes proposed apply not only to palliative care but to all

clinical areas where the mixing of medicines is accepted practice. The Commission's recommendations have been accepted by Ministers and will a) allow doctors and dentists to direct other to mix, b) allow non-medical prescribers to mix medicines themselves and direct others to mix, and c) allow nurse and pharmacist, independent prescribers, to prescribe unlicensed medicines for their patients on the same basis as doctors and supplementary prescribers. As the MHRA intends to extend the types of practitioners authorised to be involved in the mixing of medicines and the situations where mixing of medicines is acceptable, there could well be an increase in the number of medicines that are mixed. This will certainly require an assessment of the physical and chemical stability of drugs when medicines are mixed. While many medicines are stable in this situation, without investigation of this, the stability of such combinations cannot be assumed. Previous studies at Strathclyde have investigated the stability of a number of injection admixtures [2-5] and found in all cases that there were no major stability problems. Several studies have been published where the stability of combinations of morphine injections with other drug injections are reported [6-17], and the stability of a number of other analgesic combinations have also been investigated [18-27]. However, there are examples where mixing of morphine sulphate injection with other injections has resulted in issues with either the physical stability [28-31] or the chemical stability [32,33] of the drugs.

In palliative care, morphine and levomepromazine are considered essential medicines. Morphine is used routinely in clinical practice to manage moderate to severe pain, while levomepromazine is commonly used at low doses to manage

intractable nausea and vomiting. Levomepromazine is also a vital treatment option in the management of severe delirium/agitation in a dying patient when it is administered at higher doses. Combinations of morphine sulphate and levomepromazine injections for subcutaneous infusion via syringe drivers are recommended in several published palliative care guidelines used within the NHS in Scotland. While it has been reported that an injection combination of morphine sulphate (0.5 mg/ml) and levomepromazine (0.1 mg/ml) was physically compatible, showing no evidence of precipitation or changes in turbidity over 48 hours [33], data on the chemical stability of combinations of these drugs has not been reported. In the absence of published chemical stability data on morphine and levomepromazine admixtures suitable for use in syringe drivers, hospital pharmacy aseptic units may be unwilling to prepare these injection combinations. The following combinations of morphine sulphate and levomepromazine hydrochloride are used in palliative care in Scotland, UK, and were considered by the Scottish Palliative Care Pharmacists Association to be representative the concentrations used in palliative care across Scotland : a) morphine sulphate 100 mg, levomepromazine hydrochloride 12.5 mg water for injections to 17 ml b) morphine sulphate 200 mg , levomepromazine hydrochloride 25 mg, water for injections to 17 ml c) morphine sulphate 360 mg, levomepromazine hydrochloride 50 mg, water for injections to 17 ml.

3.2 Aims

In the current study the stability of injection combinations of morphine sulphate and levomepromazine hydrochloride will be investigated at 4˚C, room temperature (about

22˚C) and 37°C for up to 8 days in order to cover the storage conditions likely to be encountered in clinical practice. In order monitor the stability of the combination a stability indicating HPLC will be developed.

3.3 Methods

3.3.1 Chemicals and Materials

Orthophosphoric acid was obtained from Sigma-Aldrich (Dorset UK). HPLC grade methanol was obtained from ThermoFisher (Reading UK). HPLC grade water was prepared "in house" with a MilliQ filter (Millipore, Watford, UK).

Morphine sulphate was available as 10mg/ml and 30mg/ml ampoules(Martindale Pharmaceuticals, Romford, UK) and levomepromazine hydrochloride injection was available as 25 mg/ml ampoules from Link Pharmaceuticals Ltd (Horsham, UK).

Three part, polypropylene Luer-LokTM Syringes (30 mL) were obtained from Becton Dickinson (Oxford, UK), and HelapetCombi- Caps from B.Braun (Melsungen, Germany).

3.3.2 Sample Preparation

A method was established to analyse 100 mg of morphine sulphate and 12.5 mg of levomepromazine hydrochloride by using HPLC. Two syringes each containing 100 mg morphine sulphate and 12.5mg of levomepromazine hydrochloride were prepared. The combination was prepared by mixing together enough morphine sulphate to make a total of 200mg (8 ml), and then adding the content of one ampoule of levomepromazine hydrochloride (25 mg/ml). To this 9 ml was added 25 ml of water for injection to make a volume of 34 ml, rinsing out the ampoules with the sample solution in order to make

sure the correct final concentration was achieved. Each syringe was filled with 16 ml of the sample and just over 1 ml of the sample was reserved to perform a time zero analysis. The syringes were stored at room temperature (*ca* 22˚C). At each time point, 2ml of sample was withdrawn from each syringe, and 1 ml aliquots were transferred with a Gilson pipette to separate 25 ml volumetric flasks and diluted to volume with water. Aliquots were then transferred to auto-sampler vials. This sampling procedure was carried out at each time point (4, 24, 48, 96 and 192 hours). The above procedure was repeated with two syringes stored at 4˚C and 37˚C.

The other combinations were prepared as follows:

For the 200 mg/25 mg combination enough morphine sulphate injection was used to give a total of 400 mg (14 ml), and mixed with 2 ml of levomepromazine hydrochloride injection (25mg/ml). To this mixture was added 18 ml of water for injection to make a final volume of 34 ml. The procedure was then carried out as described above for the 100 mg/12.5 mg combination. For the 360 mg/50 mg combination enough morphine sulphate injection was mixed together to give a total of 720 mg (24 ml), and 4 ml of levomepramazine hydrochloride injection (25 mg/ml) and 6 ml of water for injection was added to make a total volume of 34 ml. The procedure was then carried out as described above for the 100 mg/12.5 mg combination.

3.3.3 pH Measurement

The combinations were diluted X5 with HPLC grade water and the pH of the diluted solution was measured with a pH meter which was calibrated with buffers at pH 4.0 and pH 7.0.

3.3.4 HPLC Analysis

HPLC analysis was carried using a ThermoFisher P2000 HPLC system with an AS3000 autosampler and a UV 2000 variable wavelength detector. The column used was a Varian Pursuit C18 column (150 x 4.6 mm i.d.)(5 µm) fitted with a guard column. The solvent system used was: A) orthophosphoric acid (0.05% w/v); B) methanol. The solvent gradient programme shown in Table 3.1 was used, with a flow rate of 1 ml/min, a column oven temperature of 40°C, and a detection wavelength of 237 nm. The data was processed using Chromquest software.

Table 3.1 Solvent gradient programme used to elute morphine and levomepromazine.

Time (min)	% A	% B
0	98	2
5	75	25
7	55	45
10	30	70
15	20	80

3.3.5 Calibration Curve

The linearity of response around the nominal content (80-120%) in the injections was estimated by using freshly injection preparation itself and diluting aliquots of 0.8 ml, 1 ml and 1.2 ml of the three preparations to 25 ml with water. As shown in figures below (3.1-3.6) , Duplicate injections for each preparation were carried out to make sure that the method was well calibrated.

3.3.6 Degradation

1ml of the injection admixture containing morphine sulphate (50 mg) and levomepromazine hydrochloride (12.5 mg) in 17 ml was placed in a 4ml vial, to which 1ml of 0.0001M HCl was added. The sample was heated to 90°C for 90 minutes, allowed to cool down for 15 minutes, and then analysed by liquid chromatography mass spectrometry (LC-MS).

3.3.7 Liquid Chromatography Mass Spectrometry (LC-MS)

Tandem MS was carried out on a TSQ 7000 instrument using the same columns as was used in the HPLC analyses but with methanol - 0.1% formic acid as the mobile phase. Analysis was carried out in electrospray ionization (ESI) mode with a needle voltage of 4.5 kV. The sheath and auxiliary gas pressures were 60 and 20 psi respectively. MS/MS was carried out with argon as the collision gas at a pressure of 3.0 torr and the fragmentation energy was 25 V.

3.4 Results

The calibration curves (figures3.1-3.6) for morphine sulphate and levomepromazine hydrochloride, prepared in duplicate, were linear over the range between 80% and 120% of stated content for the 3 combinations studied. The correlation coefficients and equations for the calibration lines were as follows: morphine sulphate 100 mg/levomepromazine hydrochloride 12.5 mg in 17 ml ($y = 4699830x – 25758$ R^2=0.999 and $y = 30702583x + 18497$ R^2=0.999, respectively); morphine sulphate 200mg/levomepromazine hydrochloride 25 mg in 17 ml ($y = 4079377x + 103222$ R^2 = 1.000 and $y = 33744190x - 122597$ R^2 = 1.000, respectively); morphine 360 mg / levomepromazine 50 mg in 17 ml ($y= 3912869x + 189192$, R^2 = 1.000 and $y = 30702583x + 18497$ R^2 = 1.000, respectively).

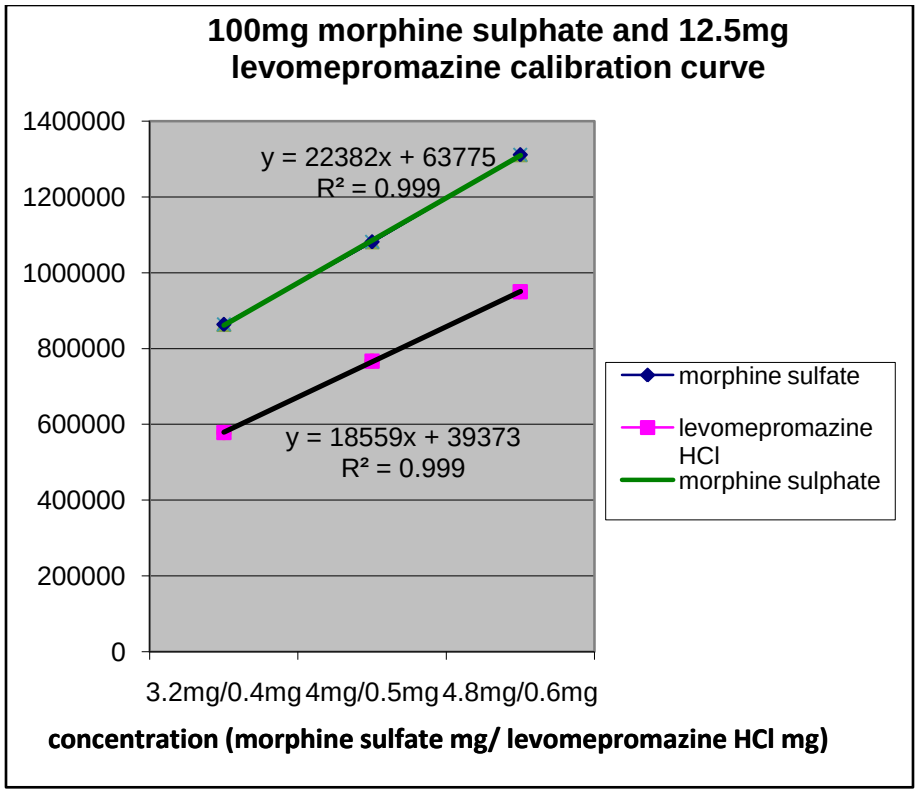


Figure 3.1 Calibration curves for morphine sulphate over the ranges 80-120% of stated content.

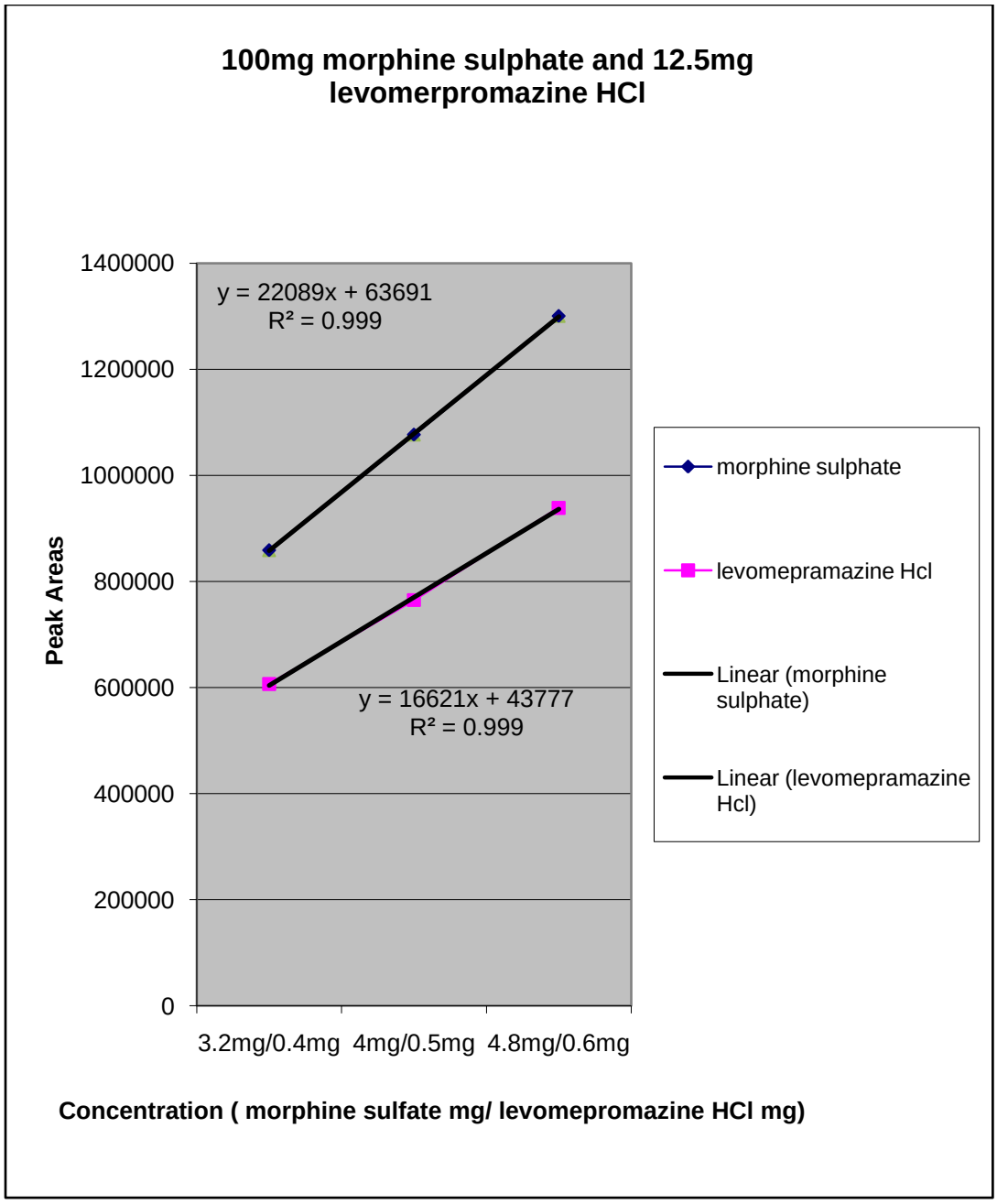


Figure 3. 2 Calibration curves for morphine sulphate over the ranges 80-120% of stated content.

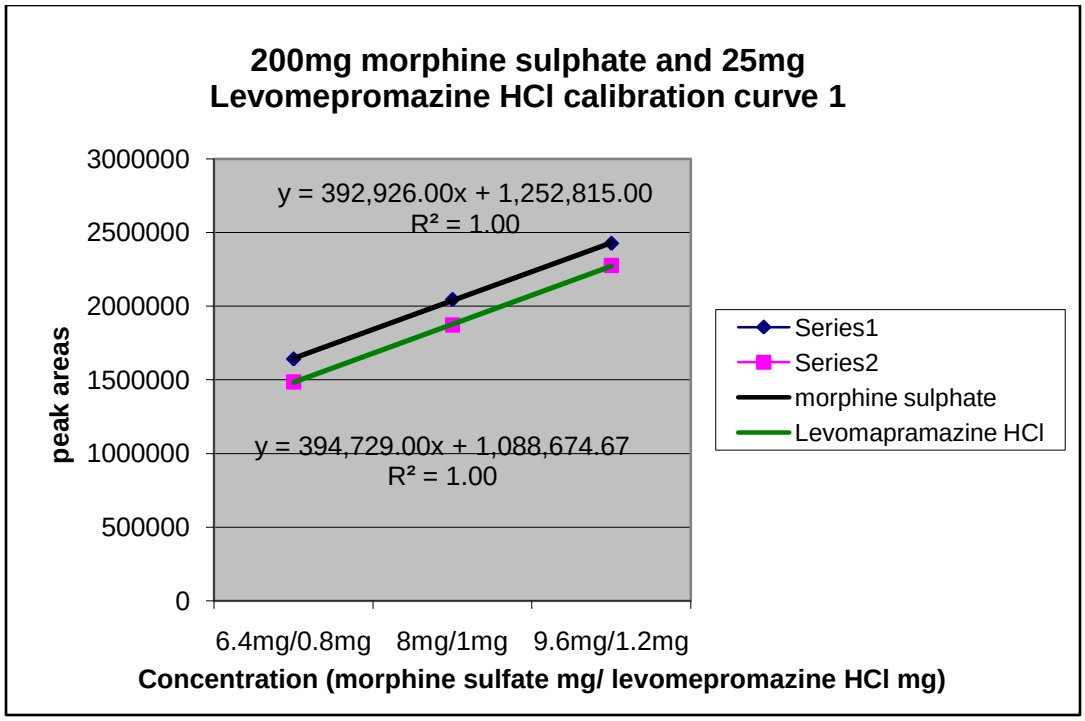


Figure 3.3 Calibration curves for morphine sulphate over the ranges 80-120% of stated content.

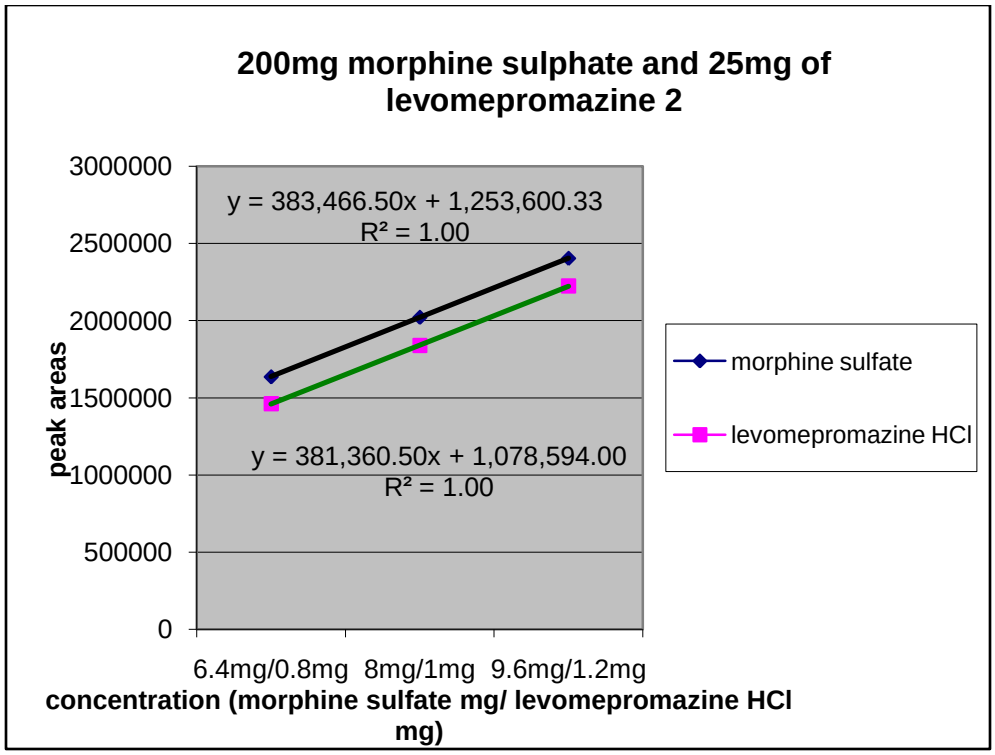


Figure 3.4 Calibration curves for morphine sulphate over the ranges 80-120% of stated content.

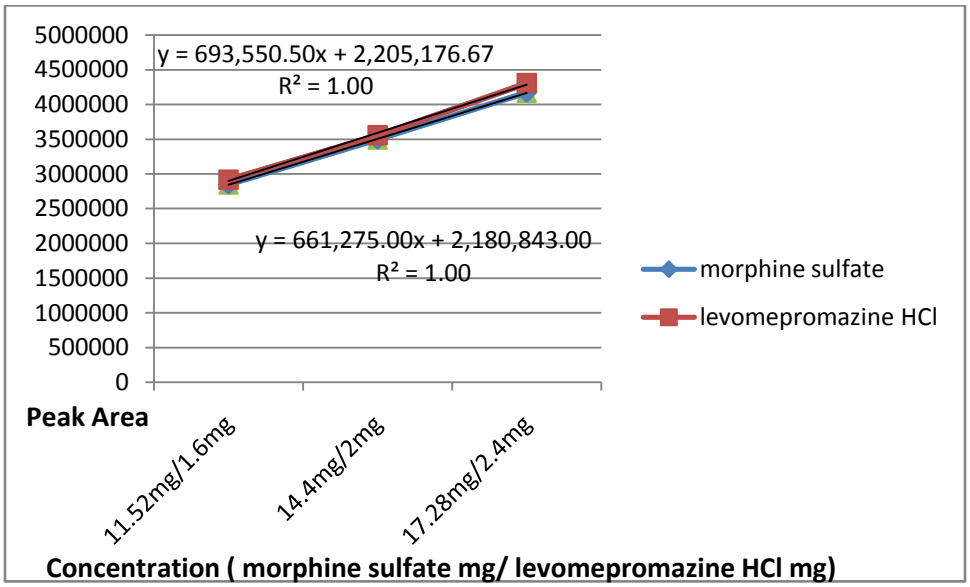


Figure 3.5 Calibration curves for morphine sulphate over the ranges 80-120% of stated content.

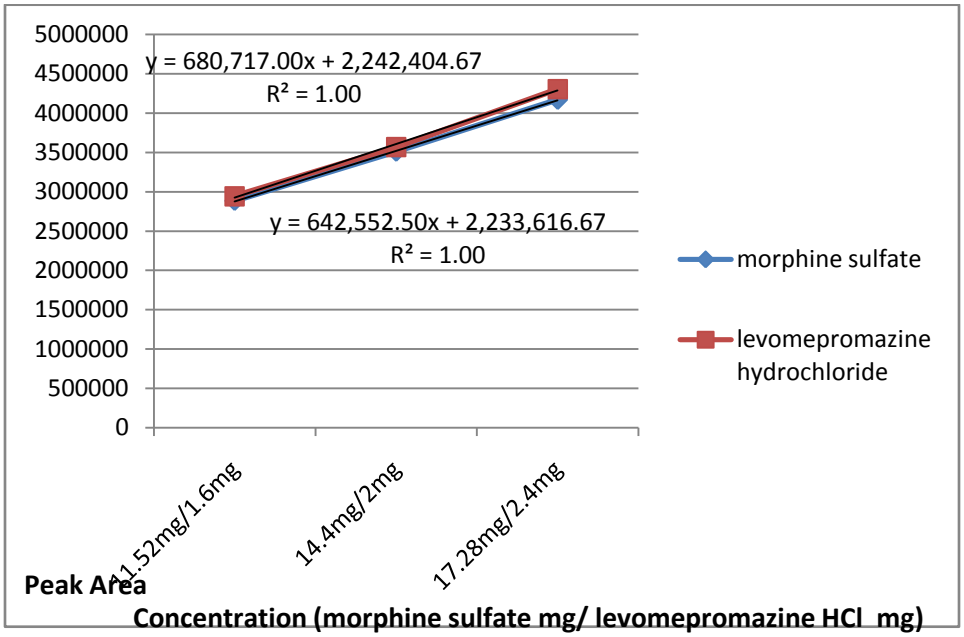


Figure 3.6 Calibration curves for morphine sulphate over the ranges 80-120% of stated content.

The chromatogram obtained for admixtures containing morphine sulphate 100 mg and levomepromazine hydrochloride 12.5 mg in a 17 ml volume after diluting 25 times with water prior to injection into the HPLC is shown in Figure 3.7.

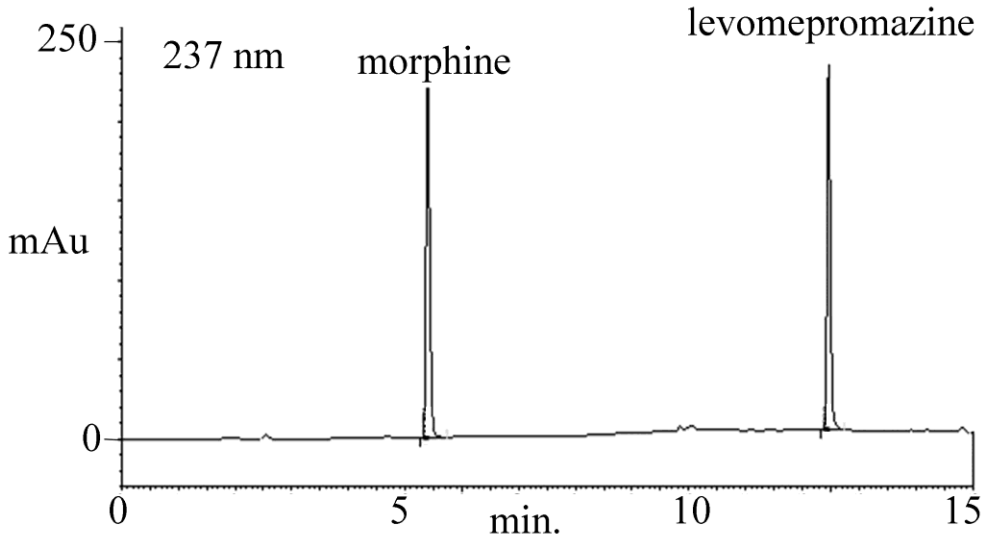


Figure 3.7 HPLC analysis of an injection combination containing morphine sulphate (100 mg) and levomepromazine hydrochloride (12.5 mg) in 17 ml at zero minutes.

In Figure 3.8 the chromatogram obtained for the same admixture after storage a 37˚C for 4 days is shown. There was a loss of levomepromazine hydrochloride in the sample with a corresponding increase in the peak of a degradant. For a good analytical results, wavelength selection for a UV detection should provide acceptable absorbance by morphine sulphate and levomepromazine hydrochloride. Although the chosen wavelength was where levomepramazine exhibited maximum absorbance the morphine sulphate absorbance was also acceptable for quantitative analysis. As shown in table 3.2, the method precision for sample preparation was tested by diluting a sample

containing morphine sulphate (200 mg) and levomepromazine hydrochloride (25 mg) in 17 ml of water (X5) and analysing them using the HPLC method. The relative standard deviation (RSD) for the area obtained for the morphine sulphate peak was ± 1.2% and for the levomepromazine hydrochloride peak was ± 0.16 %. The precisions for the retention times were ± 0.13 min. and ± 0.1 min. respectively. Tables 3.2-3.10 show the data obtained for the duplicate analysis of the contents of syringes containing the different combinations at room temperature, 37˚C and 4˚C.

Table 3.2method precision for sample preparation was tested by diluting 5 samples containing morphine sulphate (200 mg) and levomepromazine hydrochloride (25 mg) in 17 ml

component	Morphine sulphate		Levomepramazine HCL	
time	Retention time	Peak area	Retention time	Peak area
First sample	5.165	2827223	12.167	1583349
Second sample	5.153	2894200	12.185	1589376
Third sample	5.170	2841092	12.182	1583830
Forth sample	5.167	2801530	12.172	1584219
Fifth sample	5.162	2841123	12.157	1587278
RSD%	0.13%	1.2%	0.1%	0.16%

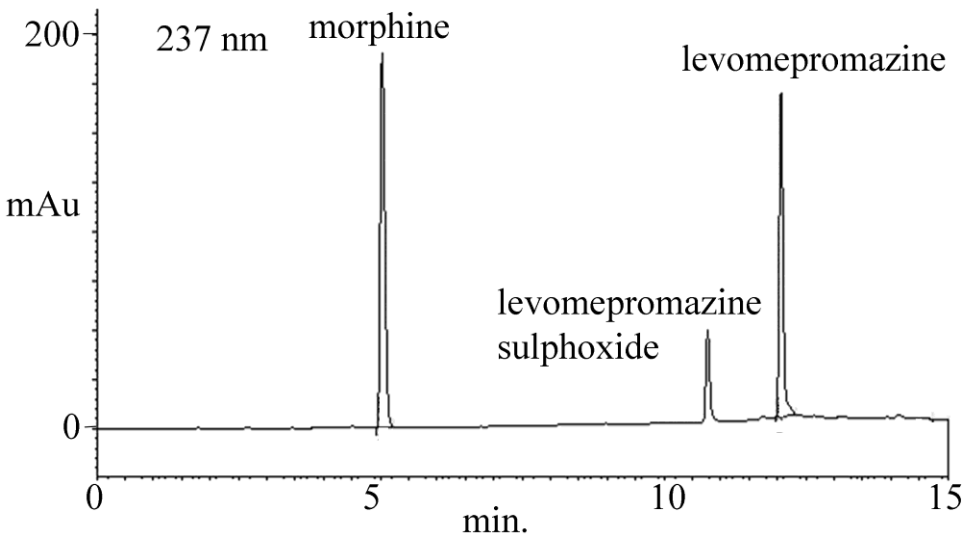


Figure 3.8 HPLC analysis of an injection combination containing morphine sulphate (100 mg) and levomepromazine hydrochloride (12.5 mg) in 17 ml after 4 days storage at 37˚C.

Table 3.3 Stability data for morphine sulphate (100 mg) and levomepromazine hydrochloride (12.5 mg) in a 17 ml volume at room temperature

	Morphine sulphate			Levomepromazine hydrochloride		
Time (hours)	% remaining Syringe 1	% remaining Syringe 2	%RSD	% remaining Syringe 1	% remaining Syringe 2	%RSD
0	100.0%	100.0%	0%	100.0%	100.0%	0%
4	101.1%	100.4%	± 0.5%	100.0%	99.5%	± 0.4%
24	100.3%	99.0%	± 0.9%	98.2%	95.7%	± 1.8%
48	100.3%	100.2%	± 0.1%	95.9%	93.2%	± 2%
96	102.6%	101.2%	± 1%	93.3%	92.1%	± 0.9%
192	103.4%	100.1%	± 2.3%	88.2%	87.6%	± 0.5%

Table 3.4 Stability data for morphine sulphate (100 mg) and levomepromazine hydrochloride (12.5 mg) in a 17 ml volume at 37˚C.

	Morphine sulphate			Levomepromazine hydrochloride		
Time (hours)	% remaining Syringe 1	% remaining Syringe 2	%RSD	% remaining Syringe 1	% remaining Syringe 2	%RSD
0	100.0%	100.0%	0%	100.0%	100.0%	0%
4	100.2%	100.4%	± 0.1%	96.8%	98.9%	± 1.5%
24	99.5%	103.0%	± 2.4%	89.2%	94.6%	± 4.1%
48	99.6%	102.4%	± 2%	87.8%	94.5%	± 5.1%
96	102.1%	103.8%	± 1.1%	83.5%	90.7%	± 6%
192	94.6%	95.9%	± 1%	82.6%	88.5%	± 4.8%

Table 3.5 Stability data for morphine sulphate (100 mg) and levomepromazine hydrochloride (12.5 mg) in a 17 ml volume at 4˚C.

	Morphine sulphate			Levomepromazine hydrochloride		
Time (hours)	% remaining Syringe 1	% remaining Syringe 2	%RSD	% remaining Syringe 1	% remaining Syringe 2	%RSD
0	100.0%	100.0%	0%	100.0%	100.0%	0%
4	99.3%	101.2%	± 1.3%	99.6%	99.9%	± 0.2%
24	95.6%	96.1%	± 0.4%	99.3%	98.5%	± 0.6%
48	95.5%	94.8%	± 0.5%	98.2%	98.1%	± 0.1%
96	95.3%	96.3%	± 0.7%	96.9%	97.7%	± 0.6%
192	101.1%	100.5%	± 0.4%	96.1%	95.6%	± 0.4%

Table 3.6 Stability data for morphine sulphate (200 mg) and levomepromazine hydrochloride (25 mg) in a 17 ml volume at room temperature

	Morphine sulphate			Levomepromazine hydrochloride		
Time (hours)	% remaining Syringe 1	% remaining Syringe 2	%RSD	% remaining Syringe 1	% remaining Syringe 2	%RSD
0	100.0%	100.0%	0%	100.0%	100.0%	0%
4	98.3%	98.7%	± 0.3%	99.6%	99.0%	± 0.4%
24	100.5%	100.8%	± 0.2%	98.9%	98.2%	± 0.5%
48	99.9%	100.5%	± 0.4%	98.2%	97.4%	± 0.6%
96	97.9%	98.5%	± 0.4%	97.5%	96.5%	± 0.7%
192	99.4%	99.9%	± 0.4%	92.4%	91.0%	± 1.1%

Table 3.7 Stability data for morphine sulphate (200 mg) and levomepromazine hydrochloride (25 mg) in a 17 ml volume at 37˚C.

	Morphine sulphate			Levomepromazine hydrochloride		
Time (hours)	% remaining Syringe 1	% remaining Syringe 2	%RSD	% remaining Syringe 1	% remaining Syringe 2	%RSD
0	100.0%	100.0%	0%	100.0%	100.0%	0%
4	99.6%	101.6%	± 1.4%	99.6%	98.1%	± 1.1%
24	99.2%	98.4%	± 0.6%	86.8%	84.3%	± 2.1%
48	95.9%	95.8%	± 0.1%	81.5%	80.8%	± 0.6%
96	96.4%	95.6%	± 0.6%	74.4%	72.4%	± 2%
192	98.1%	98.0%	± 0.1%	67.5%	66.8%	± 0.7%

Table 3.8 Stability data for morphine sulphate (200 mg) and levomepromazine hydrochloride (25 mg) in a 17 ml volume at 4˚C.

	Morphine sulphate			Levomepromazine hydrochloride		
Time (hours)	% remaining Syringe 1	% remaining Syringe 2	%RSD	% remaining Syringe 1	% remaining Syringe 2	%RSD
0	100.0%	100.0%	0%	100.0%	100.0%	0%
4	100.7%	95.6%	± 3.7%	99.8%	99.6%	± 0.1%
24	99.77%	101.5%	± 1.2%	98.7%	98.7%	0%
48	101.4%	100.2%	± 0.8%	97.0%	95.4%	± 1.2%
96	102.2%	96.2%	± 4.2%	94.3%	93.8%	± 0.4%
192	100.8%	96.9%	± 2.8%	94.1%	93.2%	± 0.7%

Table 3.9 Stability data for morphine sulphate (360 mg) and levomepromazine hydrochloride (50 mg) in a 17 ml volume at room temperature

	Morphine sulphate			Levomepromazine hydrochloride		
Time (hours)	% remaining Syringe 1	% remaining Syringe 2	%RSD	% remaining Syringe 1	% remaining Syringe 2	%RSD
0	100.0%	100.0%	0%	100.0%	100.0%	0%
4	98.3%	98.7%	± 0.3%	99.6%	99.0%	± 0.4%
24	100.5%	100.8%	± 0.2%	98.9%	98.2%	± 0.5%
48	99.9%	100.5%	± 0.4%	98.2%	97.4%	± 0.6%
96	97.9%	98.5%	± 0.4%	97.5%	96.5%	± 0.7%
192	99.4%	99.9%	± 0.4%	92.4%	91.0%	± 1.1%

Table 3.10 Stability data for morphine sulphate (360 mg) and levomepromazine hydrochloride (50 mg) in a 17 ml volume at 37˚C

	Morphine sulphate			Levomepromazine hydrochloride		
Time (hours)	% remaining Syringe 1	% remaining Syringe 2	%RSD	% remaining Syringe 1	% remaining Syringe 2	%RSD
0	100.0%	100.0%	0%	100.0%	100.0%	0%
4	100.2%	99.5%	± 0.5%	99.6%	97.5%	± 1.5%
24	98.4%	99.8%	± 1%	97.2%	96.1%	± 0.8%
48	100.3%	99.7%	± 0.4%	96.5%	94.3%	± 1.6%
96	93.5%	94.9%	± 1%	82.1%	77.7%	± 3.9%
192	98.6%	97.6%	± 0.7%	72.0%	68.7%	± 3.3%

Table 3.11 Stability data for morphine sulphate (360 mg) and levomepromazine hydrochloride (50 mg) in a 17 ml volume at 4˚C

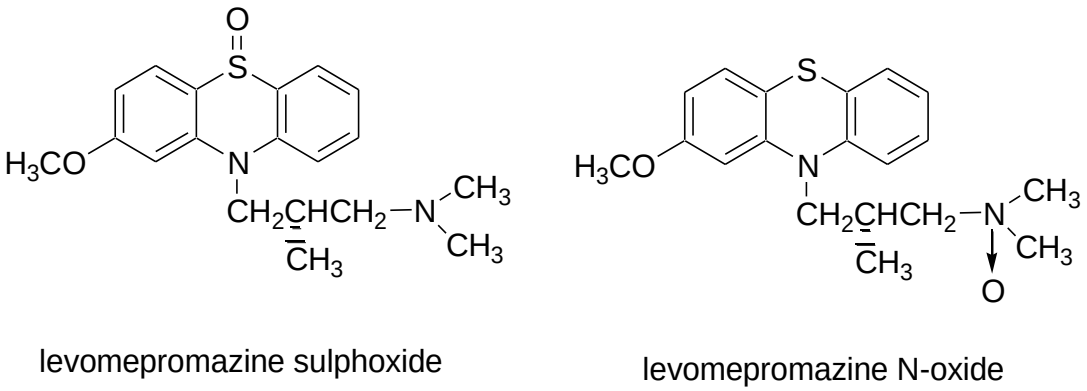
	Morphine sulphate			Levomepromazine hydrochloride		
Time (hours)	% remaining Syringe 1	% remaining Syringe 2	%RSD	% remaining Syringe 1	% remaining Syringe 2	%RSD
0	100.0%	100.0%	0%	100.0%	100.0%	0%
4	100.1%	99.3%	± 0.5%	99.3%	97.9%	± 1%
24	99.9%	99.5%	± 1%	99.0%	97.8%	± 0.9%
48	99.7%	100.2%	± 0.4%	97.9%	97.2%	± 0.5%
96	96.8%	94.9%	± 1%	97.5%	95.5%	± 1.4%
192	99.3%	100.1%	± 0.7%	90.1%	91.5%	± 1.1%

There was little degradation of morphine sulphate under any of the storage conditions, and in all syringes the amount of morphine sulphate remained above 94% of the initial concentration. After 192 hours storage, degradation of morphine sulphate appeared to be slightly greater at 37˚C than at the other two temperatures studied. Yeh and Lach [34] have previously reported that there is an increase in the degradation rate of

morphine as the temperature increases, although this is less important than the effects of pH or the presence of oxygen; however, degradation was independent of molarity or the ionic strength of buffers. However, this was not the case for the levomepromazine hydrochloride in the admixtures where a steady decline in concentration was observed, with the rate of decline being greatest at 37˚C. In some admixtures stored at 37˚C the concentration of levomepromazine hydrochloride fell to below 70% after 192 hours; (this appeared to be more of a problem with the more concentrated admixtures). As the level of levomepromazine hydrochloride declined, the level of the major degradant peak, seen at 10.9 min, as shown in Figure 2, increased.

The mixture of morphine sulphate (50 mg) and levomepromazine hydrochloride (12.5 mg) in 17 ml was diluted with weak acid and heated at 90˚C for 90 minutes in order to promote degradation. The relatively mild degradation conditions are more relevant to likely degradations under the conditions of storage being studied. The only degradant peak formed was the additional peak attributed to levomepromazine hydrochloride which was observed during storage of the injections when mixed. There was no evidence for the presence of pseudomorphine which has been reported to form under more strongly acidic degradation conditions (2). The identity of the major degradant peak was confirmed by LC-MS to be most probably due to the sulphoxide of levomepromazine (Figure 3.3). Levomepromazine produced a protonated molecular ion at m/z 329 and the impurity was shifted by a mass increment of 16 corresponding to the addition of an oxygen atom to produce an ion at m/z 345. It is possible that modification could occur at the nitrogen group giving an N-oxide (Figure 3.3) but this reaction occurs

less readily than S-oxidation and, from previous experience, these compounds are very early eluting from reversed phase columns and readily lose their oxygen under ESI conditions [35]. The tandem MS spectrum of the degradant contained a base peak ion at m/z 58 due to alpha cleavage next to the dimethylamine group in the side chain. This was consistent with oxidation being on the sulphur rather than the nitrogen since, in theory, the alpha cleavage next to an oxidized nitrogen would have yielded a peak at m/z 74. The oxidation of the sulphur atom phenothiazines is thought to be catalysed by UV radiation and has been known for many years [36].



levomepromazine sulphoxide

levomepromazine N-oxide

Figure 3.9 Structures of levomepromazinesulphoxide and N-oxide

Visual inspection of the injection admixtures showed that their appearance changed with time going from a clear, colourless solution when prepared to a brown coloured solution, this became noticeable after 48 hours. It is suggested that this brown discoloration is probably associated with the oxidation of the levomepromazine molecule since oxidatively catalysed colorometric assays have been used to determine

phenothiazines [37]. Apart from the discolouration of the injection from visual inspection there was no evidence of particulate formation and the pH of the injection solution remained over 192 hours (Table 3.11).

Table 3.12 pH profile of three injection combinations at room temperature over 192 hours.

Time (h)	pH 100mg/12.5mg	pH 200mg/25mg	pH 360mg/50mg
0	5.61	5.53	5.32
4	5.62	5.55	5.39
24	5.64	5.58	5.40
48	5.65	5.62	5.41
96	5.68	5.63	5.42
192	5.72	5.66	5.47

3.5 Conclusion

In conclusion, the stability of three different combinations of morphine sulfate and levomepromazine hydrochloride prepared by the Pharmacy Department in Western General Infirmary in Edinburgh was tested at 4°C, 22°C and 37°C. A HPLC method was established to indicate the stability of morphine sulfate and levomepromazine

hydrochloride at different temperatures. The HPLC method was able to separate morphine sulfate and levomepromazine hydrochloride with a high resolution and with a reasonable retention time. Although levomepromazine hydrochloride was oxidized to a sulphoxide degradant at all temperatures, the combinations showed a reasonable stability at room temperature and a high stability at 4°C with levomepromazine remaining above 90% of the original content. Levomepromazine hydrochloride underwent extensive degradation in all combinations at 37°C, while morphine sulfate showed a high stability at all temperatures. Combinations showed a colour change to yellowish brown but no precipitate was observed while a slight change of pH was noticed during the 8 days of the experiment. The established method for degradation studies was able to separate morphine sulfate, levomepromazine hydrochloride and its sulphoxide degradant with a high resolution, reasonable retention time and the components were identified easily. Although the sample was placed at 90°C for 90 minute during stress testing morphine sulphate was stable and no change was noticed. On the other hand, levomepromazine hydrochloride was rapidly oxidized to the sulphoxide degradant. As a result, the three combinations were regarded safe to be used if stored in a cool (4°C) and dry temperature, while unsafe if stored at a warm temperature (37°C).

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Chapter 4

The Hydrophilic Interaction-like Properties of Some Reversed Phase High Pressure Liquid Chromatography Columns in the Analysis of Basic Drugs and Applications in Impurity Profiling.

4.3 Introduction

4.1.1 Applications of Hydrophilic Interaction Chromatograph (HILIC)

The majority of drugs are basic compounds and their chromatographic analysis can still pose problems, particularly where the bases are very polar an do not retain strongly on reversed phase columns. Thus continuing the theme of the chromatography it was of interest to investigate the relatively new chromatographic technique of hydrophilic interaction chromatography (HILIC). The term hydrophilic interaction chromatography was coined by Alpert in 1990 [1]. There are a number of HILIC phases, the simplest kinds being based on the use of bare silica, but one of the most popular is the ZIC-HILIC phase the structure of which is shown in figure 4.1. The mobile phase used is generally acetonitrile mixed with water or aqueous buffer. In this mode of chromatography the water associated with the surface of the stationary phase is regarded as acting as a pseudostationary phase. The phase thus works in the opposite way to a reversed phase: 1. The more polar or the lower the partition coefficient of a compound the more strongly it is retained by the aqueous pseudostationary phase. 2. The higher the water content in the mobile phase the more quickly compounds elute. However, is also the possibility of ion exchange interactions which can occur in the case of strongly acidic and basic compounds.

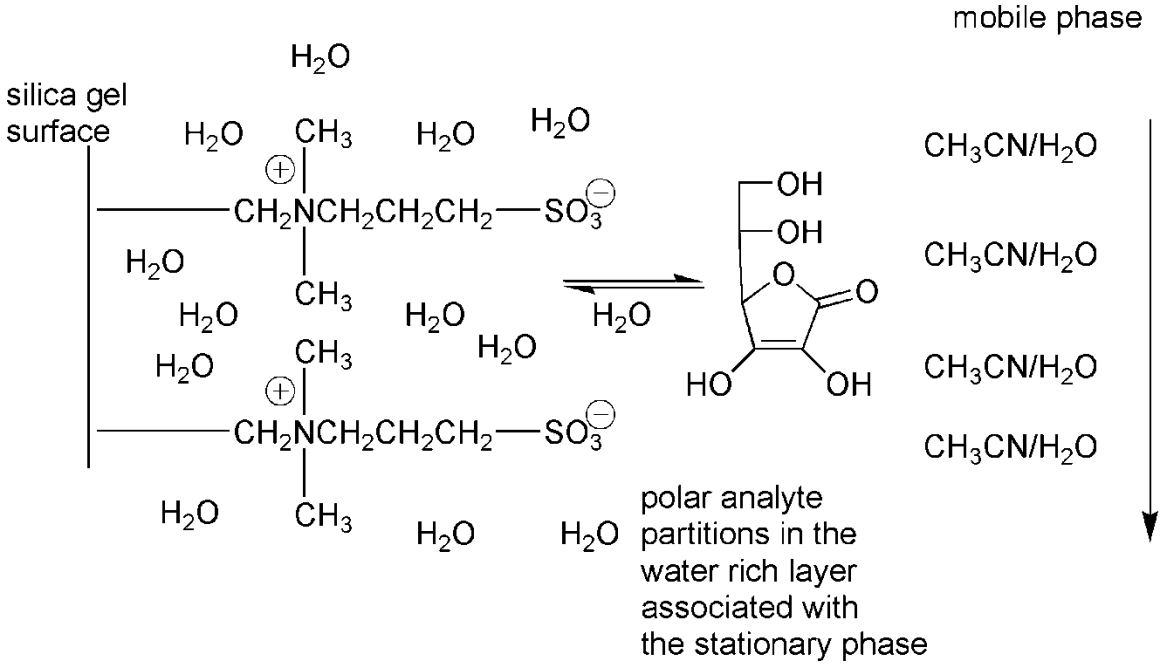


Figure 4.1 The structure and proposed mode of action of the ZIC-HILIC stationary phase illustrated by its interaction with ascorbic acid. [Watson, 2012, MSc Course Notes]

In figure 4.2 the chromatograms obtained on a ZIC-HILIC phase for two important physiological antioxidants ascorbic acid and glutathione (GSH) and for oxidised glutathione (GSSG) which forms as a result of oxidative stress are shown. The chromatographic peaks were detected using mass spectrometry. None of these molecules would be retained by a reversed phase column even in 100% water.

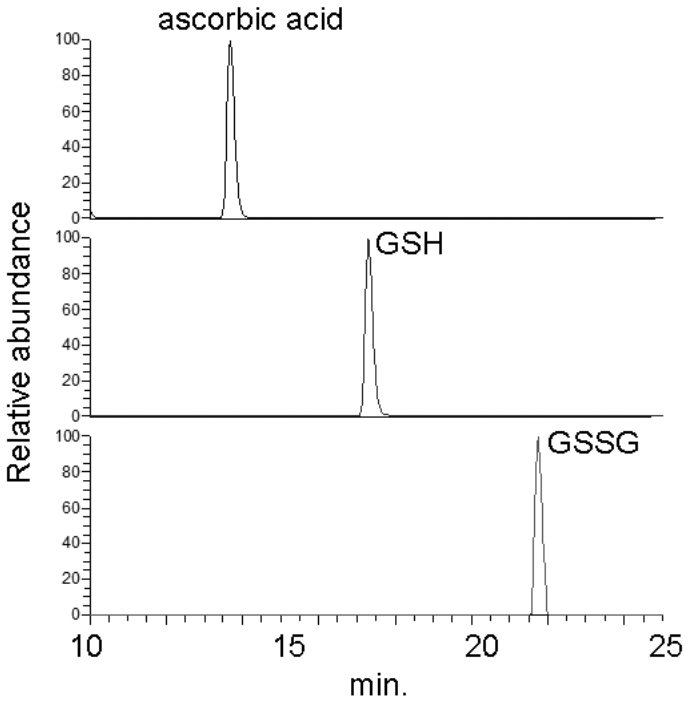


Figure 4.2 Chromatograms for ascorbic acid, GSH and GSSG analysed on a ZIC-HILIC column (150 x 4.6 mm i.d.) with a gradient between 0.1% formic acid/acetonitrile (10:90, v/v) and 0.1% formic acid/acetonitrile (80:20, v/v). [Watson, 2012, personal communication]

In the pharmaceutical industry the analysis of basic compounds can still present problems with regard to peak shape and column overloading [3,4]. The requirement for good peak symmetry in the analysis of basic compounds has driven the development of high purity silica gel phases free from metallic impurities for use in the preparation of reversed phases. In addition processes such as end-capping have been developed which are designed to reduce the interaction of basic compounds with silanol groups. Another problem which has arisen in the development of reversed phases is the need to

adequately retain very polar basic compounds which have limited affinity for reversed phases and hence polar embedded phases have been developed which can be used with high water content in the mobile phase without the alkyl stationary phase coating collapsing. However, another method for the HPLC analysis of basic drugs is hydrophilic interaction chromatography (HILIC) and there have been a number of studies in this area. Bare silica gel and modified silica phases have been used for the separation of basic drugs using conditions with high organic modifier content in the mobile phase and a low level of aqueous content [5-11]. Much work has been carried out on the analysis of basic drugs since many pharmaceutical compounds fall into this category. In a recent paper the retention of some model basic compounds on bare silica gel, a zwitterionic phase and a silica bonded diol phase was studied [3]. Of the phases studied silica was the most retentive and where the silanol groups on the silica gel surface were blocked, for instance with diol groups, the retentivity of the phase was reduced. The studies were carried out at pH 3.0 and increased ionic strength of the mobile phase and increased water content of the mobile phase reduced the retention times of the test probes [3]. The overall conclusion of the study was that retention mechanisms in HILIC mode are complex and it is not possible to fully explain them. Since it was discovered that HILIC can provided a solution to the chromatographic retention of polar analytes, the applications of HILIC chromatography have increased rapidly during the past 5 years. Some recent examples are discussed below.

A rapid LC-MS assay with a nine minute run time was developed for cocaine and its metabolites in hair [4]. The stationary phase was bare silica gel and the eluent was

aqueous ammonium acetate buffer pH4.5 and acetonitrile which was varied between 82% and 40% in a gradient elution. The method was fully validated and LOQs of 0.001 ng/mg were obtained for cocaine and its metabolites. The method was able to produce strong retention of the metabolite ecogonine methyl ester which is only weakly retained on reverse phase columns.

A stability indicating method was developed for the analysis of brimonidine tartrate and its degradants. A bare silica gel column was used with an isocratic mobile phase consisting of acetonitrile - 0.5% w/v ammonium acetate (92:8, v/v) adjusted to pH 7.0 with acetic acid. Increasing the % of acetonitrile caused an increase in retention of brimonidine and increasing the ionic strength of the mobile phase or its pH caused a decrease in its retention time [5].

Impurities in streptomycin and dihydrostreptomycin were investigated by using LC-MS in conjunction with a bare silica column in HILIC mode with acetonitrile/ammonium formate buffer (75:25, v/v). Using a fused core silica column with 2.7 µm particle size peaks with high efficiency were obtained and 18 impurities could be characterised in the samples [6].

Six impurities in mildronate were separated using a variety of HILIC columns. Principally a ZIC-HILIC column and a bare silica gel column were compared. The effect of acetonitrile and buffer content in the mobile phase on retention times were investigated. Overall the ZIC-HILIC column was deemed most fit for purpose. The method was fully validated for quantitative determination of the impurities [7].

A number of different HILIC columns were tested for their ability to separate 4-aminomethylpyridine and its related substances. Mobile phase pH, buffer salts and organic modifier were all investigated for their effect on retention times and silica gel columns from several manufacturers were compared [8]

A HILIC method was developed on bare silica gel for the analysis of dextromethorphan, pseudoephedrine and diphenhydramine in a cough formulation. The mobile phase consisted of methanol and a buffer consisting of ammonium and triethylamine. All three components were separated and the method was validated for quantitative determination [9].

In addition to silica based columns the sulphobetaine column ZIC-HILIC has been used for analysis of bases. For example the determination of dimethindine maleate in a topical gel [10]. In previous work in at Strathclyde extensive use of the ZIC-HILIC stationary phase for the retention of polar compounds in metabolomics studies has been made [11-13]. This phase produces useful retention of acidic, basic and neutral compounds. However, it is too strongly retentive for compounds such as polyamines such as spermine and spermidine which have very long elution times. In the course of developing a lipidomics method it was found that bare silica gel offered useful retention of a range of phospholipids when eluted under HILIC like conditions [14]. In the course of trying to improve a method for the analysis of a small di-amine cross-linking agent used in pharmaceutical polymer preparation an ACE CN (cyanopropyl column) was found to produce suitable retention of these compounds. This column was previously

studied extensively with regard to its ability to produce dipole-dipole interactions and it was also noted to have useful HILIC properties at this time, however, it was also noted there was a tendency for the ligand coating of the column to be unstable [16].

The use of CN columns in HILIC mode has not been extensive and as recently as 2008 the CN column was presented as being suitable for normal phase separations where a mixture of hydrocarbon based solvent and a polar modifier was used in the chromatography of basic drugs [17]. In addition, the CN column has been used as an alternative reversed phase column [18-20]. However, it has also been used successfully in HILIC mode with the authors of the papers perhaps not appreciating that was the mechanism they were exploiting [21,22]. In addition, there are some examples where a CN column was used explicitly in HILIC mode. In a recent paper a number of HILIC columns were tested with regard to their ability to retain catecholamines and their acidic metabolites [23]. The methodology exploited the ion exchange interactions of the amines with ionised silanol groups in the phase as well as the a purely HILIC mechanism to retain acid compounds. A CN column was among the columns tested and it was found that retention of the amines increased as the pH of the mobile phase was increased which was attributed to increased ionisation of the silanol groups in the stationary phase promoting increased interaction with the amine functionalities of the analytes [23]. A cyanoethyl column was investigated for its ability to retain strongly basic alcohol denaturants in HILIC mode using acetonitrile as the organic modifier [24]. Successful chromatography was only achieved when perchlorate or trifluoroacetate were used to form strong ion pairs with the analytes in order to reduce their interaction with the

silanol groups of the stationary phase. By varying the % of acetonitrile it was possible to resolve all three analytes of interest and the optimum resolution was achieved at 60% acetonitrile. A sensitive and quantitative method was developed for the analysis of piperazine in HILIC mode using a cyanopropyl column [25]. The method worked best when strongly acidic conditions were used with either nitric acid or trifluoroacetic acid being used to suppress the ionisation of the silanol groups or form ion-pairs with the analytes. Several phases were tested for their ability to retain folates in order to develop a tandem MS method for the analysis of these compounds in plasma [26]. Most of the columns tested were used in reversed phase mode but among the columns tested was a cyanopropyl column which was eluted with acetonitrile-methanol (60:40 v/v) containing ammonium acetate or ammonium formate, thus under HILIC conditions. The CN column gave broad tailing peaks and in the end a bare silica column was used for the analysis in HILIC mode. A CN column was tested for its ability to retain a series of peptides under normal phase conditions (HILIC conditions), however the peptides were found elute at the void volume of the column [27]. The applications of CN columns and other columns in HILIC mode has been recently reviewed [28].

From the review above it can be seen that although CN columns have been used in HILIC mode there has been no detailed evaluation their performance in this mode.

4.1.2 Applications of Mass Spectrometry to Impurity Profiling

In addition to using HILIC chromatography it was proposed that its uses in drug impurity profiling would be investigated by combining it with high resolution mass spectrometry.

4.1.2.1 Electrospray Ionisation (ESI)

ESI began to be popularized around 20 years ago and has revolutionized the applicability of MS as an analytical tool. Thus ESI is now the most widely applied method of ionization because of its ready compatibility with high performance liquid chromatography (HPLC). The ionization takes place under atmospheric pressure. The basis of the technique is shown in figure 4.3.

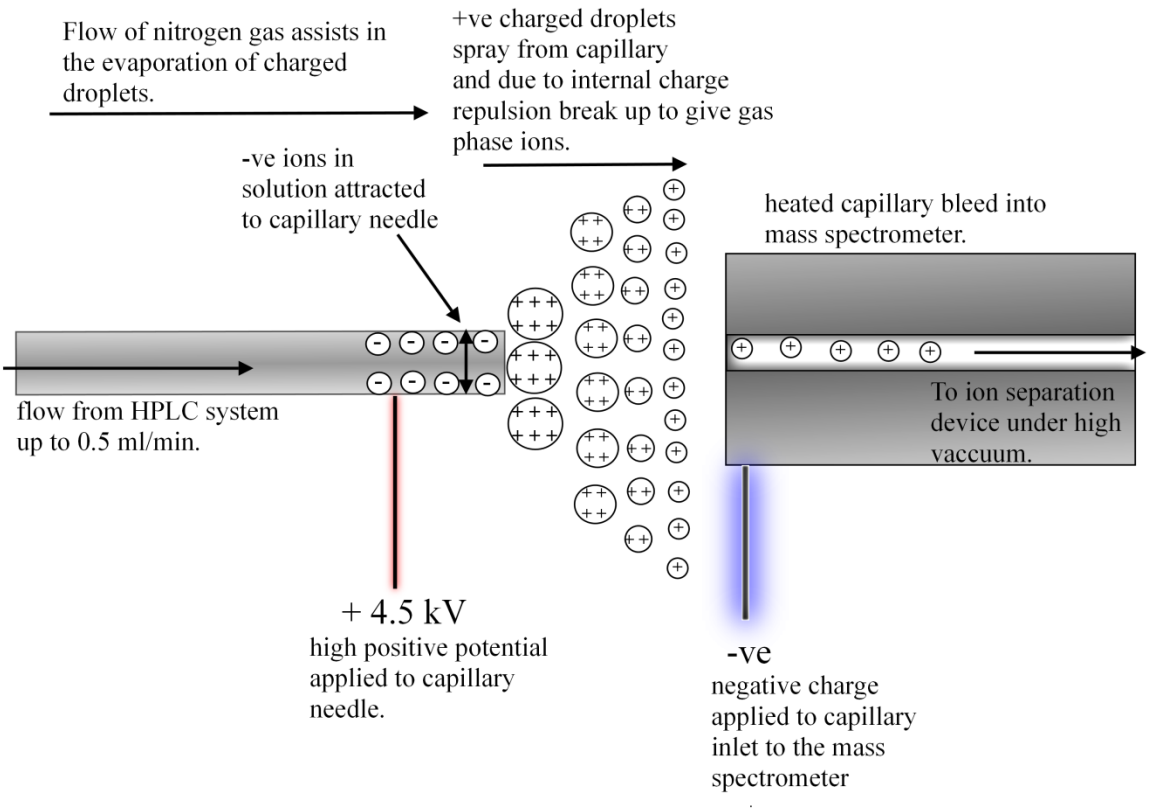


Figure 4.3 The electrospray ionization process.

The eluent from a HPLC system passes through a quartz or metal needle to which a high electrical potential e.g. 4.5 kV is applied. If a positive potential is applied, then the negative ions in the eluent are stripped away by being attracted to the needle thus leaving positively charged solvent droplets which spray out of the capillary. Under the influence of a coaxial flow of nitrogen gas the droplets evaporate and as they do so break up due to internal charge-charge repulsion. The end stage of the process is that gas phase ions are produced, which are then attracted into the mass spectrometer by an opposite charge applied to a heated capillary which allows a slow bleed from the

atmosphere into the mass spectrometer, which has to operate under high vacuum. In order to maintain high vacuum in the instrument two pumping stages are used, an intermediate stage immediately after the heated capillary and a high vacuum stage in the ion separation stage.

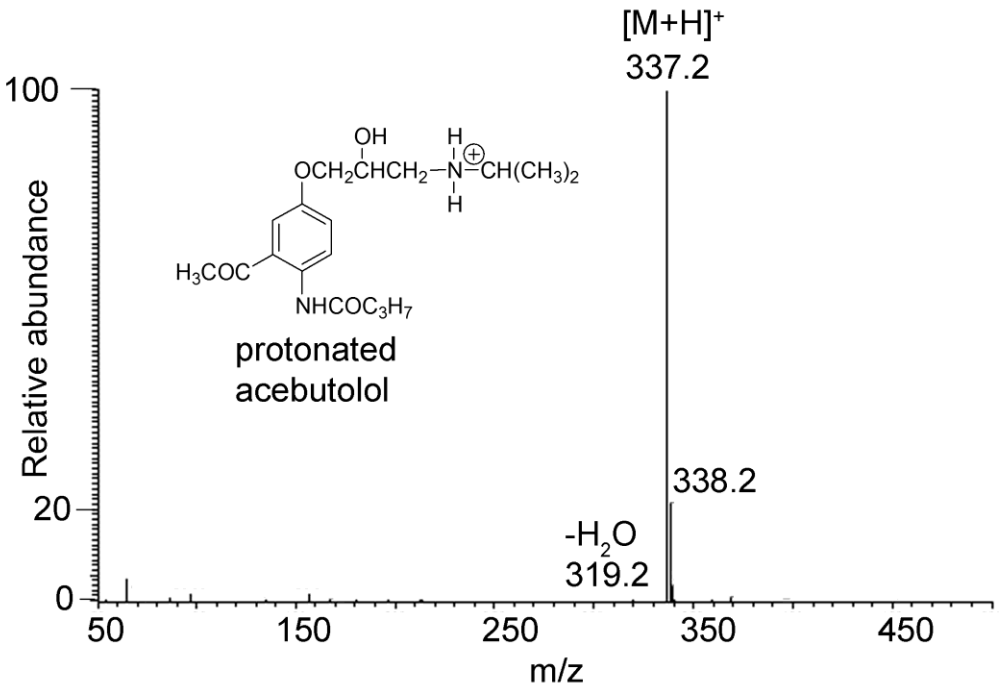


Figure 4.4 A positive ESI spectrum of acebutolol

In Figure 4.4 an electrospray spectrum of the basic drug acebutolol is shown, the positive charge on the molecule is generated by its basic centre being protonated, this occurs even in a weakly acidic HPLC mobile phase. The main ion seen at m/z 337.2 (m/z stands for mass over charge ratio) is due to the molecular weight of the drug plus a

proton. ESI is known as a soft ionization technique and in Figure 4.4 it can be seen that there is little fragmentation of the molecule. A very minor fragment can be observed at m/z 319.2 which is due to the loss of water (18 amu).

4.1.2.2 High Resolution Ion Separation Techniques

- **Time of Flight (TOF) Ion Separation**

The patent for separating ions by TOF was registered in 1952 but the first commercial instruments using the technology did not become available until around 1990. In TOF, there will be no external force to separate ions of different m/v values, however, pulses of ions are accelerated into an evacuated field free region called a drift tube. The velocity of an ion will depend on its mass, thus if all ions have the same kinetic energy and charge, on this basis the ions will be separated by TOF. Smaller ions will travel faster along the drift tube than larger ions and are detected first. Pulses of ions can be produced from the sample using matrix assisted laser desorption ionization (MALDI) method, which is using short pulses of laser energy focused on a sample dissolved in a UV light absorbing matrix. Pulses ions arev produced with frequencies of 10-50 KHz. The ions are accelerated into the drift tube by a pulse electric field called the ion extraction field. Accelerating voltages up to 30 KV and extraction pulse frequencies of 5-20 KHz are used. An ion mirror, called reflectron, is used to improve the resolution of a TOF analyzer. The role of reflectron is to reverse the direction in which the ions are travelling and to energy-focus the ions to improve resolution. The electrostatic field of the

reflectron allows faster ions to penetrate more deeply than slower ions of the same m/z value. Therefore, faster ions follow a longer path before they are turned around, which allows all ions with the same m/z value to end up travelling exactly the same distance and arrive at the detector together.TOF resolution can be further improved by using a W-configuration where the ions move back and forth twice passing into two reflectrons. TOF separation is often used with MALDI as described above but it is also used in conjunction with ESI, in this case a hybrid instrument is produced where the ions can be initially filtered through a quadrupole prior to entering the TOF separation stage. In Figure 4.5 the TOF separation process is shown in combination with electrospray and a quadrupole ion filter. This type of combination is used to produce ion fragmentation in ESI mode and will be discussed further below. This type of configuration is found in instruments such as the QTOF. TOF instruments are capable of making high resolution mass measurements to four decimal places.

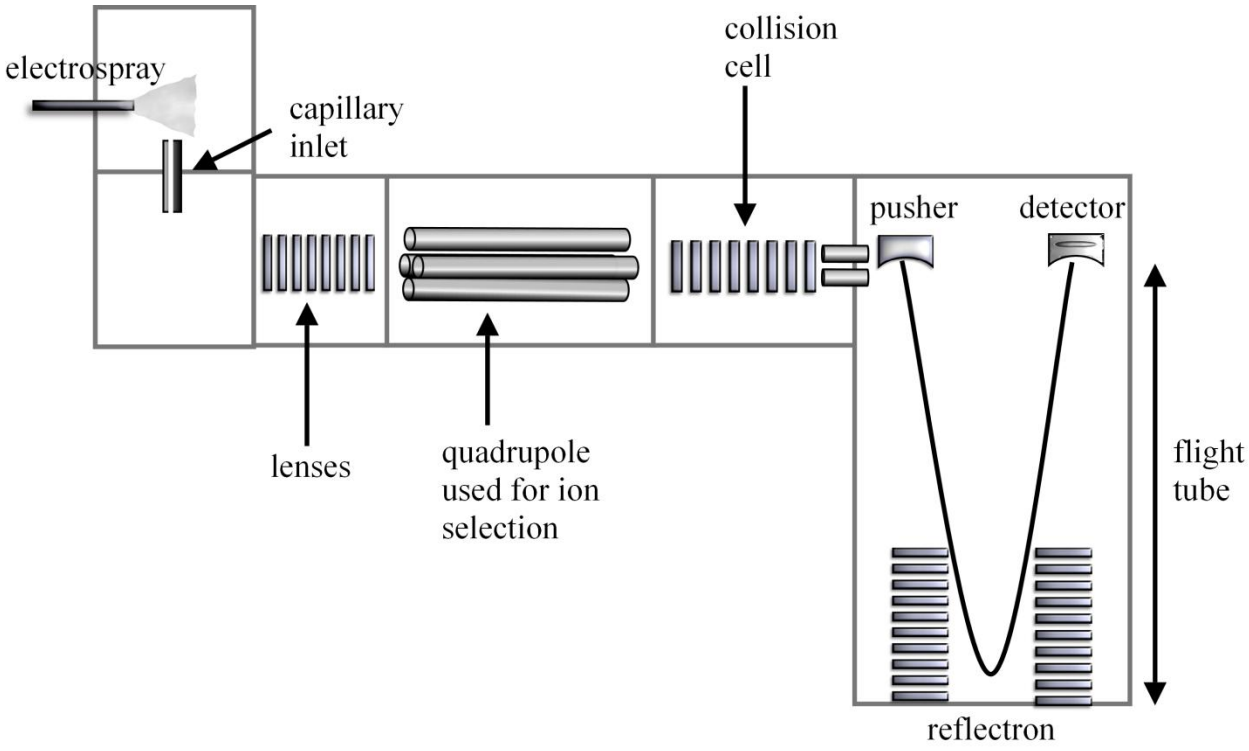


Figure 4.5 Schematic diagram of a quadrupole time of flight (QTOF) mass spectrometer.

- **Ion Trap Separation**

Ion trap technology first became commercialized in the late 1980s. In Figure 4.6 one version of an ion trap is shown. Ions are injected into the trap and are then trapped in an rf field which is applied to a circular electrode. The trap is filled with helium which quenches the kinetic energy of the ions. The ions can then be ejected from the trap in order of mass by applying a DC electric field to an end-cap electrode. If it is of interest to fragment a particular ion the trap can be set to empty itself of all the ions apart from the ion of interest. The rf voltage can then be altered so that the ion becomes excited and collides with helium atoms in the trap thus producing fragments and the fragments derived from the selected ion can then be ejected from the trap

and detected. Ion trapping technology can be used to create high resolution mass spectrometry separations.

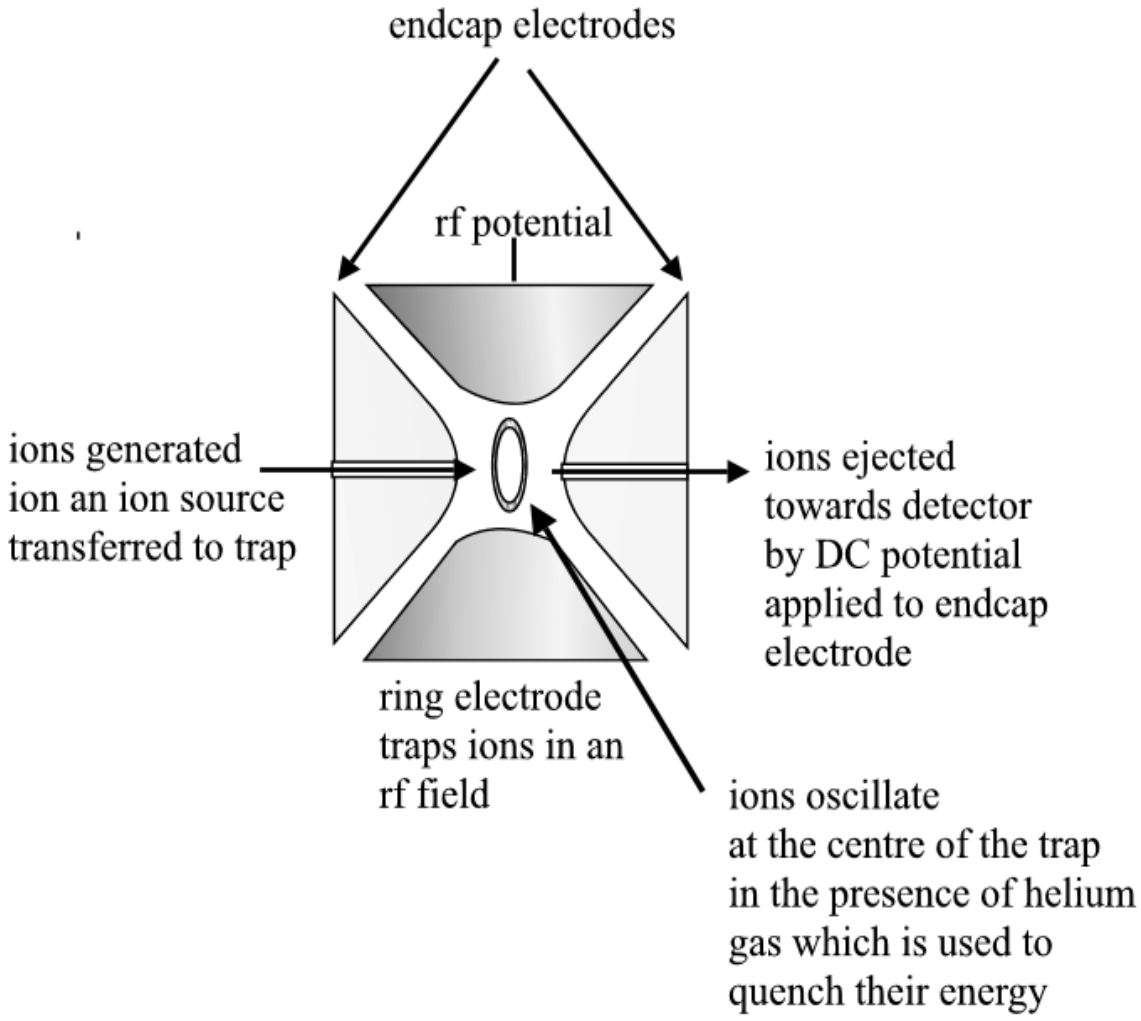


Figure 4.6 Schematic diagram of an ion trap.

- **Fourier Transform Mass Spectrometry**

Fourier transform mass spectrometers produce very accurate mass measurements using ion trapping technology. The original cyclotron resonance instruments have been used for many years and are very expensive instruments. These instruments trap ions in a magnetic field in order to measure their masses. However, in 2005 the Orbitrap Fourier transform mass spectrometer was launched and is in the same price range and TOF instruments making the technique much more routine. The Orbitrap uses an electrostatic field to trap ions so that they orbit around a central spindle electrode (figure 4.7). The ion oscillations are detected as an image current which can be converted into highly accurate mass and the Orbitrap can measure masses to five decimal places.

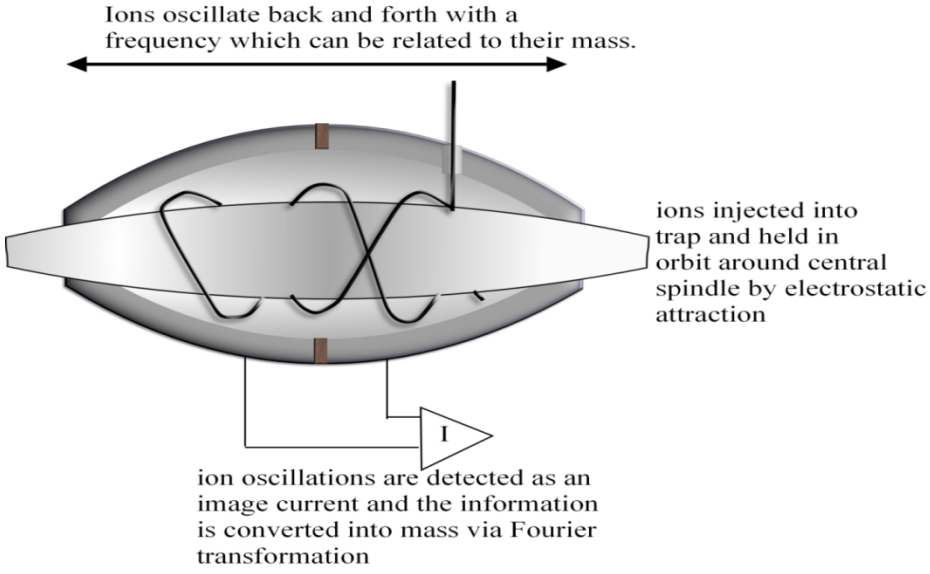


Figure 4.7 The Orbitrap high resolution ion trap.

4.2 Aims

The aim of the current study will be to study the mechanism of retention of basic compounds on an ACE CN column in HILIC mode and compare the retention of a base test mixture under the same conditions on two other reversed phases prepared from the same base silica gel.

4.3 Materials and Methods

4.3.1 Chemicals

Ammonium acetate, tris free base, HPLC grade acetic acid, HPLC grade methanol and HPLC grade acetonitrile were obtained from Fisher Scientific, Loughborough, UK. HPLC water was prepared in house using a Milli Q purification system. The bases used in the running tests were from either Sigma Aldrich, Dorset UK, were European Pharmacopoeia Standards or were part of an in house stock donated by companies over the years. Anisole and 1,2 dinitrobenzene (1,2 DNB) were obtained from Sigma Aldrich, Dorset UK.

4.3.2 HPLC Columns

ACE CN, ACE Phenyl (Ph), ACE Butyl (C4), ACE silica gel (Si), Zorbax Stable Bond CN and Hypersil BDS CN columns (150 x 3 mm i.d.)(3 µM or 3.5 µM) were obtained from HiChrom Ltd., Reading U.K.

4.3.3 HPLC Instrumentation

HPLC analysis was carried out on a ThermoFinnegan HPLC system consisting of a P2000 pump, P200 UV dual wavelength detector and an AS2000 autosampler. The mobile phase compositions were mixed off-line in the proportions required and the HPLC system was used in isocratic mode, 10 µl of sample was injected. The flow rate was 0.8 ml/min and the column was run at room temperature. The UV detector was set to monitor 220 and 280 nm. The continued stability of the CN column was monitored by injecting a mixture of anisole and 1,2 DNB with a mobile phase composition consisting of methanol/water (30:70, v/v).

4.3.4 LC-MS Instrumentation

LC-MS was carried out on an Orbitrap Exactive instrument (ThermoFisher, Hemel Hempstead U.K.). The instrument was interfaced with a Dionex HPLC system (Dionex, Camberly, U.K.). The flow rate was 0.4 ml/min. the gas flows in the ESI source were sheath gas 60 (arbitrary units) and auxiliary gas 25 (arbitrary units), the capillary temperature was 275ºC and the needle voltage was 4.5kV. The instrument was freshly tuned with the manufacturer's standard tuning solution containing pyridine to calibrate the lower mass range. The residual caffeine peak from the tuning solution was used as a lock mass.

4.3.5 Sample Preparation

Samples were prepared as stock solutions of 1 mg/ml in methanol and diluted to a concentration of 0.1 mg/ml in mobile phase. Samples for impurity profiling were dissolved at a concentration of 2 mg/ml in mobile phase.

4.4 Results

4.4.1 Chromatography of Basic Drugs on an ACE CN Column

In Figure 4.8Aa chromatogram of a test mixture containing five basic compounds: propranolol, chlorpromazine, salbutamol, nortriptyline and benzalkonium chloride (figure 4.9) run on an ACE CN column with 0.025% w/v ammonium acetate in the mobile phase is shown. The peak shapes obtained for the bases are excellent with peak efficiencies >60000 plates/m. There is no immediately clear mechanism for the retention of the compounds except that the quaternary ammonium compound is most strongly retained. The three secondary bases propranolol, salbutamol and nortriptyline, which have similar pK_a values, are retained to a differing extent. In Figure 4.1B the effect of increasing the ammonium acetate strength in the mobile phase to 0.05 % w/v which reduces the retention of the bases by nearly 50% is shown. This supports the proposal that much of the retention mechanism is due to ion exchange with increased ionic strength reducing the interaction of the bases with the stationary phase. In Figure 4.1 C the effect of decreasing the water content of the mobile phase from 5% to 3% which produces a marked increase in retention times is shown. The effect of changing the aqueous content of the mobile phase is complex since there several interacting effects.

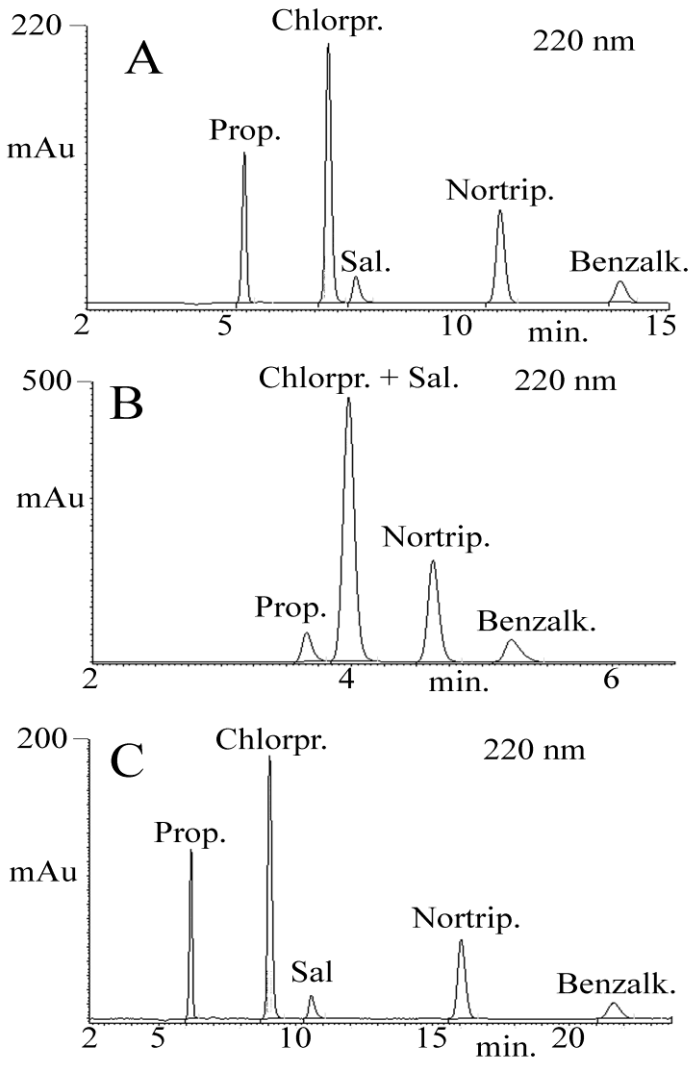


Figure 4.8 A-Separation of test mixture of basic compounds (propranolol, chlorpromazine, salbutamol, nortriptyline, benzalkonium chloride)on an ACE CN column (150 x 3 mm i.d.) in acetonitrile - water (95:5, v/v) containing 0.025% w/v ammonium acetate, flow rate 0.8 ml/min. **B-** Separation of test mixture of basic compounds on an ACE CN column (150 x 3 mm i.d.) in acetonitrile - water (95:5, v/v) containing 0.05% w/v ammonium acetate, flow rate 0.8 ml/min. **C-** Separation of test mixture of basic compounds on an ACE CN column (150 x 3 mm i.d.) in acetonitrile - water (97:3, v/v) containing 0.025% w/v ammonium acetate, flow rate 0.8 ml/min.

Figure 4.9 Structures of the bases included on the test mixture and manufacturing impurities in salbutamol.

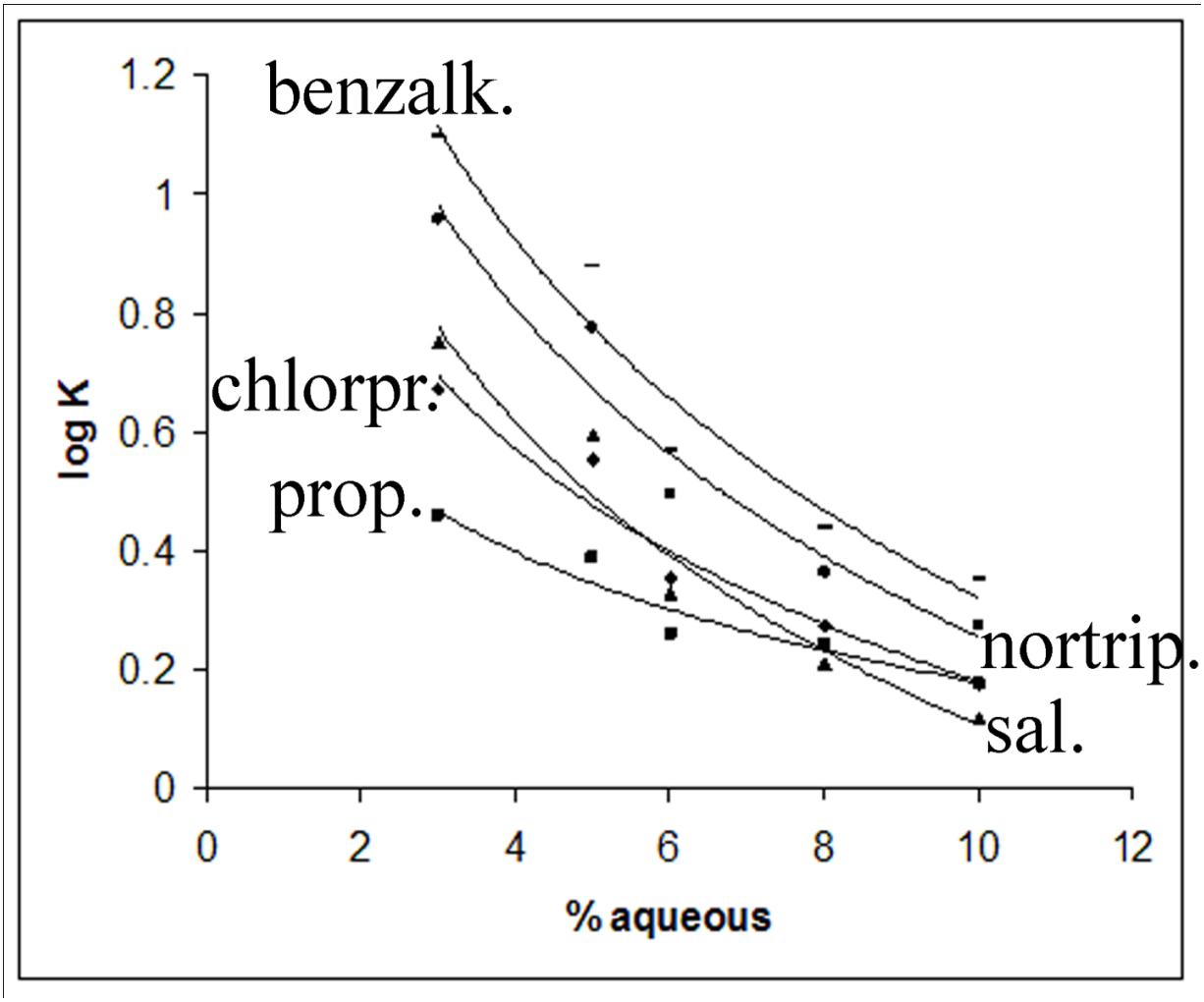


Figure 4.10 Log *k* plots for the bases in the test mix against % of aqueous component in the mobile phase.

In Figure 4.10 the log *k* plots for the bases in the test mixture can be seen, rather than being linear they approximate to a logarithmic fit, however, the correlation coefficients are not high. A general rule, which can be demonstrated by potentiometric titration, is that the pK_a of acids becomes higher as the organic content of the titration medium increases and the pK_a of bases becomes lower. In the current example there are three sets of pK_a values to consider the pK_a values of the bases in the test mixture, the pK_a value of the ammonium ion and the pK_a value of the silanol groups in the stationary phase. As the water content in the mobile phase decreases all three species will become less ionised but at different rates i.e. weaker bases will be affected to a greater extent. The exception is the benzalkonium chloride which will remain at a constant level of charge. Thus there are only two factors governing the retention of benzalkonium chloride which are the concentration of ammonium ions and the degree of ionisation of silanol groups in the stationary phase. The shift from 5% to 3% aqueous content will cause less ionisation of both the ammonium ion and the silanol groups. The former would favour greater retention of the benzalkonium chloride the latter lower retention and it would appear that the former effect predominates since moving from 5% to 3% aqueous content causes marked increase in retention time. The effect of very high concentrations of acetonitrile on base pK_a values has not been studied. However, in the range between 0 and 60% acetonitrile a study which used capillary electrophoresis to estimate pK_a found that a series basic compounds experienced a pK_a shift of between -0.22 and -0.77 [29]. This might explain why the log *k* plots obtained in the current study are approximately logarithmic since they reflect the Henderson Hasselbalch equation

where at a fixed pH % ionisation varies logarithmically with pK_a. However, neither pH nor pK_a are well defined in organic solvents so any model is only approximate [30]. Thus if the concentration of the ammonium ion is primarily responsible for the elution of the bases from the CN column and its concentration varies logarithmically with % acetonitrile then, in contrast to the linear log *k* plot, obtained when % organic modifier is varied linearly, one might expect the log *k* plot for a base to approximate to a log plot. The steepness of the log plot would depend on how far the pK_a value of the base was above the pK_a of ammonia and retention times of weaker bases should not be as affected by % acetonitrile because their % ionisation will more closely reflect the change in the level of the competing counter ion ammonium. This is roughly what is observed in figure 4.10 where the log *k* plots are steeper for the stronger bases. The rate of change of pK_a with % organic modifier is difficult to predict and varies from compound to compound [29]. However, applying the model discussed above, bases which are much weaker than ammonia would not be strongly retained since they would not compete very well with the counter ion. Table 4.1 gives the retention times for a series of bases on the ACE CN column. In Table 4.1 it can be seen that the very weak bases itraconozole, papaverine, lidocaine and bupivacaine elute at the void volume of the column. Considering that lidocaine, which has low pK_a value of 7.9 (the pK_a is lowered by the proximity of an amide group to the basic centre), has the same dimethyl tertiary amine group as chlorpromazine (pK_a 9.0) then this suggests that pK_a is more important than steric effects in governing retention. Thus the important factor governing retention

might be the pK_a of the analyte in comparison with the pK_a of the basic counter ion present in the mobile phase.

Table 4.1 Retention times of basic compounds on the ACE CN column (150 x 3 mm i.d.) (3 µm) in 0.025% w/v ammonium acetate in acetonitrile - water (95:5, v/v), flow rate 0.8 ml/min. p = primary amine, s= secondary amine, t=tertiary amine, q=quaternary amine. pK_a values taken from Clark's Analysis of Drugs and Poisons Third Edition. NA =not available.

Base	**t_r (min)**	**pK_a**	**Base**	**t_r (min)**	**pK_a**
Itraconozole(t)	1.2	3.7	Chlorpromazine (t)	7.3	9.0
Lidocaine(t)	1.2	7.9	Salbutamol (s)	7.9	9.5
Bupivacaine(t)	1.5	8.1	Acebutolol (s)	7.9	9.4
Papaverine(t)	1.0	6.4	Atenonol (s)	8.5	9.6
Ketamine(s)	1.0	7.5	Methoxytryptamine (p)	9.0	NA
Cyclizine(t)	3.0	7.7	Chlorpheniramine (t)	9.2	9.2
Propranolol(s)	5.4	9.7	Synephrine (s)	9.9	8.9
Dosulepin(t)	5.4	NA	Nortriptyline (s)	11.0	9.7
Diphenhydramine(t)	6.0	9.0	Pseudoephedrine (s)	10.1	9.8
Oxprenolol(s)	6.6	9.5	Homoatropine (t)	11.1	9.9
Dopamine(p)	6.9	8.8	Benzalkonium Cl (q)	13.8	
Benzylamine (p)	7.1	9.7			

Ammonia has a pK_a value of 9.2 and will be much more charged than weak bases such as lidocaine or bupivacaine. In order to test the hypothesis that pK_a of the basic counter ion governs elution tris acetate buffer pH 6.0 was prepared. The pK_a of tris is 7.0 and in comparison it will be less charged than most of the bases listed in table 1 at any pH.

Table 4.2 Retention times for bases run on an ACE CN column (150 x 3 mm i.d.) in acetonitrile - water (95:5, v/v) containing 0.025 % tris acetate buffer pH 6.0, flow rate 0.8 ml/min.

Base	Rt (min)	Base	Rt (min)
papaverine	2.0	salbutamol	8.4
lidocaine	2.7	Chlorpromazine (t)	14.5
bupivacaine	3.7	Nortriptyline	24.9
propranolol	5.3	Benzalkonium Cl	28.7

In Table 4.2 the retention times obtained for some of the bases listed in table 4.1 when tris acetate buffer pH 6.0 was used in the mobile phase in combination with acetonitrile in a (5:95, v/v) at a concentration of 0.025 M can be seen. The weak bases lidocaine, bupivacaine and papaverine are now retained and the retention times of the stronger bases are longer in comparison with those obtained under the same conditions with ammonium acetate buffer. Thus it would appear that the retention times of bases can manipulated in accord with the pK_a of base used to buffer the mobile phase.

4.5 Applications of the ACE CN Column to Impurity Profiling

Regardless of the exact retention mechanism the CN column performs well for the separation of basic compounds.

4.5.1 Separation of Salbutamol from three impurities listed in the European Pharmacopoeia

In Figure 4.4 the separation of salbutamol (1 mg/ml) from three of its manufacturing impurities (figure 4.2) spiked at 1 μg/ml can be seen and although the differences in structure of the impurities from salbutamol are quite minor the column exhibits good selectivity for the mixture. The impurities are well resolved from the salbutamol peak and would be quantifiable at this level.

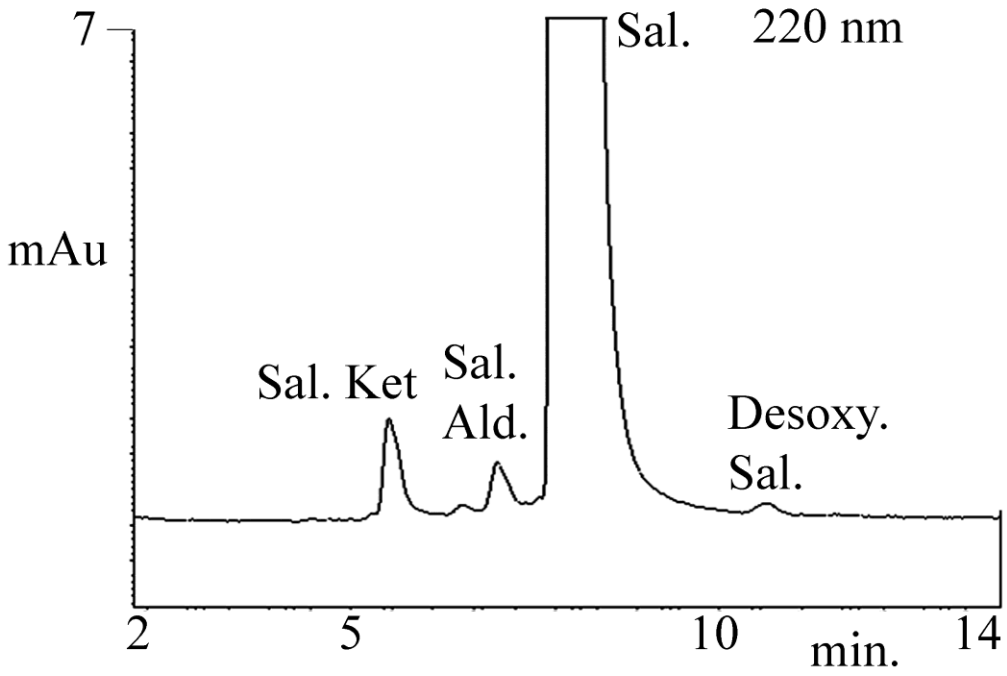


Figure 4.11 Separation of salbutamol and its manufacturing impurities on an ACE CN column (150 x 3 mm i.d.) in acetonitrile - water (95:5, v/v) containing 0.025 % w/v ammonium acetate, flow rate 0.8 ml/min.

4.5.2 Impurity Profiling of Oxprenolol Using an ACE CN Column with high resolution mass spectrometry

Another example is provided by oxprenolol. In Figure 4.5 the analysis of a 2 mg/ml solution of oxprenolol where impurities at a level <0.05% of the height of the main peak can be observed. Although there are no impurities listed in the European Pharmacopoeia for oxprenololit is possible to propose identities for the impurities

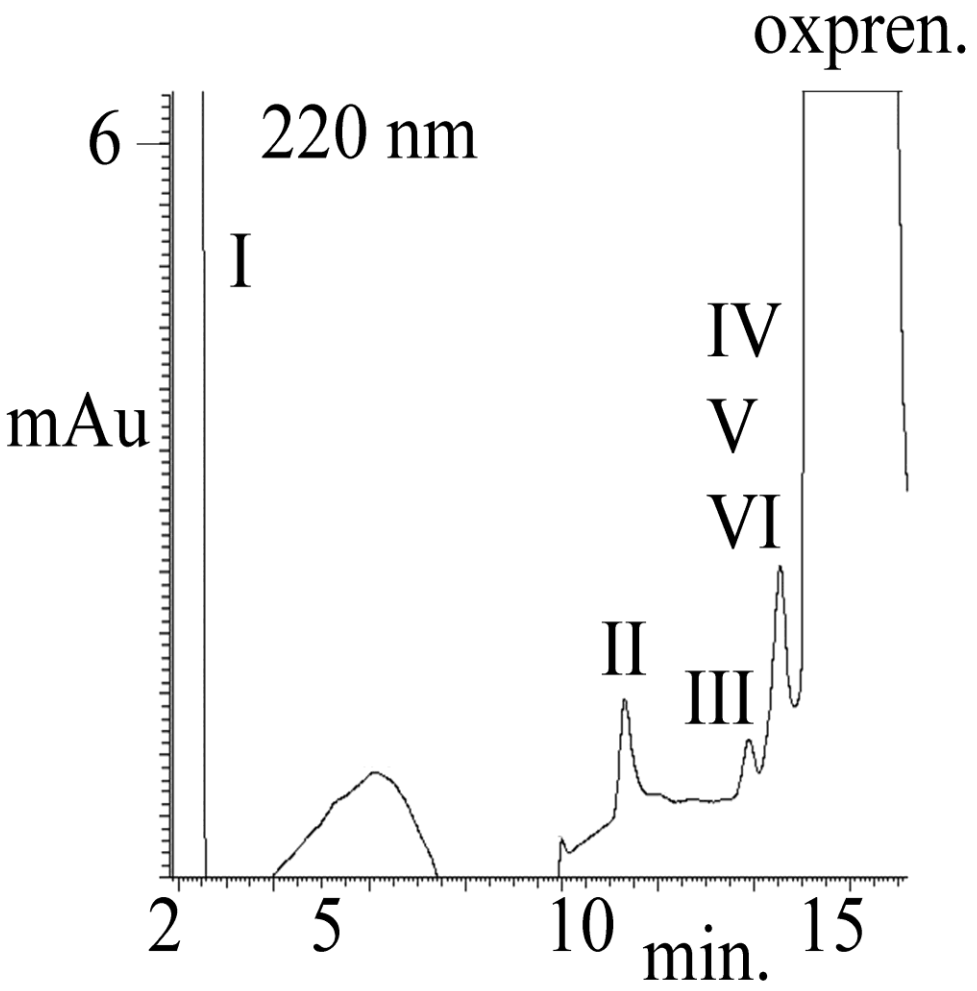
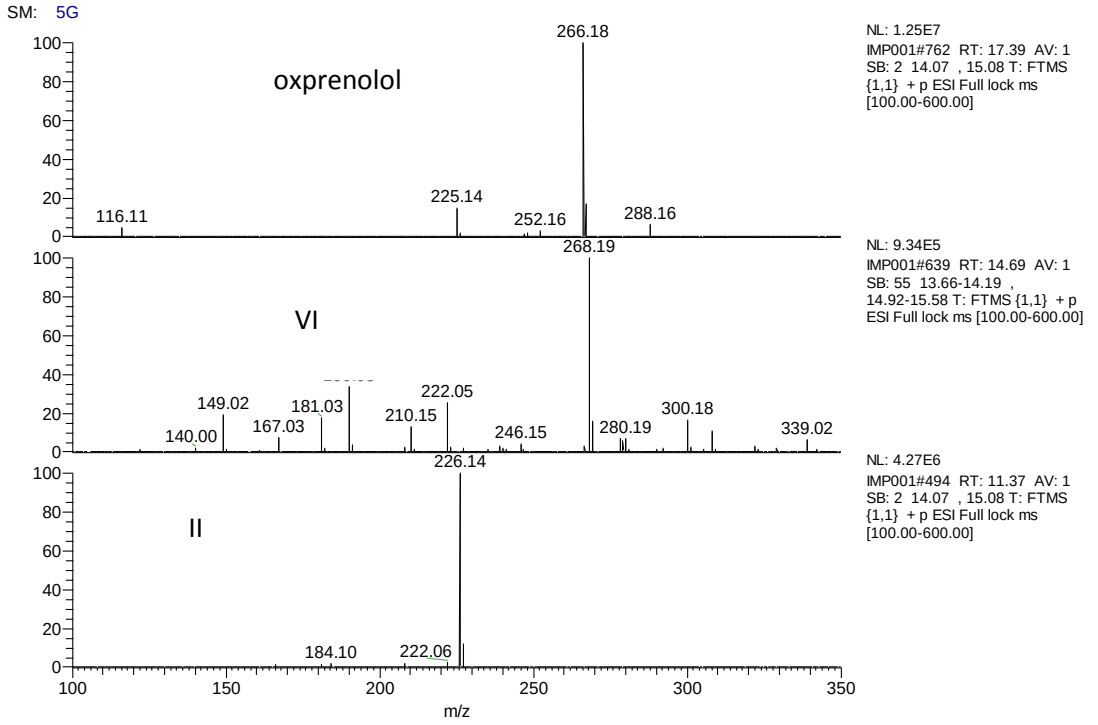
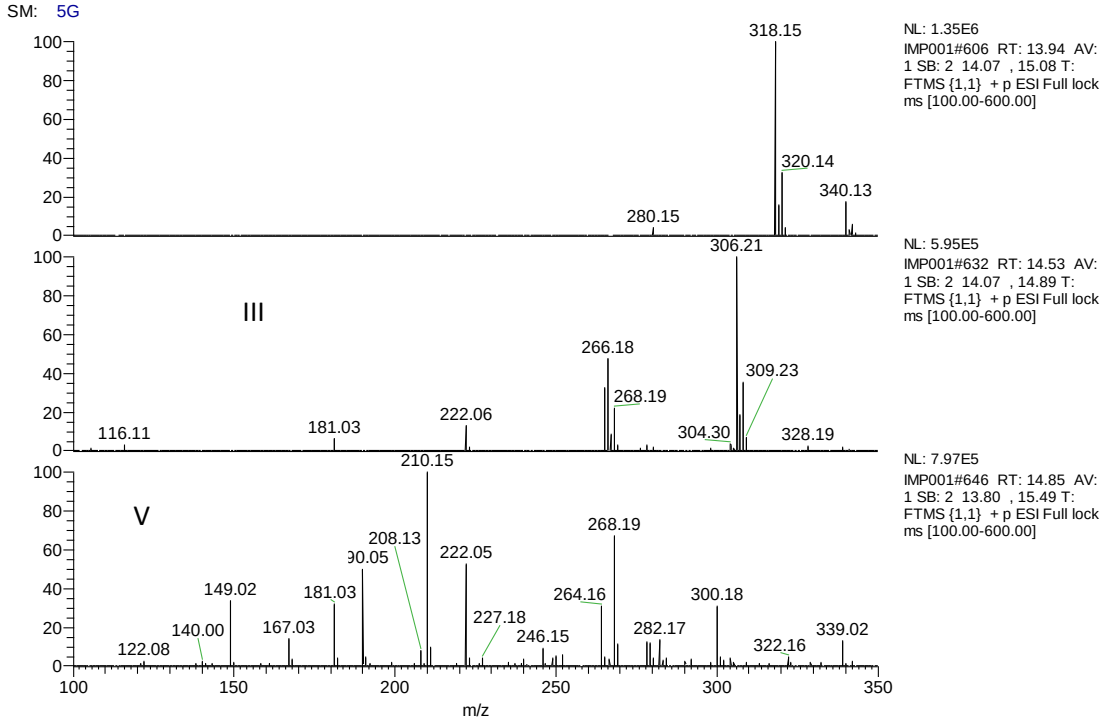


Figure 4.12 Separation of oxprenolol and its manufacturing impurities on an ACE CN column (150 x 3 mm i.d.) in acetonitrile - water (95:5, v/v) containing 0.025 % w/v ammonium acetate, flow rate 0.4 ml/min.

on the basis of the synthetic route to the compound and by analogy with impurities listed for other β-adrenergic blocking agents such as atenolol or propranolol in the EP. In Figure 4.13 the mass spectra of oxyprenolol and some of its impurities can be seen.


SM: 5G
oxprenolol
266.18
225.14
116.11
252.16
288.16
NL: 1.25E7
IMP001#762 RT: 17.39 AV: 1 SB: 2 14.07 , 15.08 T: FTMS {1,1} + p ESI Full lock ms [100.00-600.00]
VI
268.19
149.02
181.03
222.05
210.15
140.00
167.03
246.15
280.19
300.18
339.02
NL: 9.34E5
IMP001#639 RT: 14.69 AV: 1 SB: 55 13.66-14.19 , 14.92-15.58 T: FTMS {1,1} + p ESI Full lock ms [100.00-600.00]
II
226.14
184.10
222.06
NL: 4.27E6
IMP001#494 RT: 11.37 AV: 1 SB: 2 14.07 , 15.08 T: FTMS {1,1} + p ESI Full lock ms [100.00-600.00]
m/z


SM: 5G
318.15
320.14
340.13
280.15
NL: 1.35E6
IMP001#606 RT: 13.94 AV: 1 SB: 2 14.07 , 15.08 T: FTMS {1,1} + p ESI Full lock ms [100.00-600.00]
III
306.21
266.18
309.23
268.19
222.06
116.11
181.03
304.30
328.19
NL: 5.95E5
IMP001#632 RT: 14.53 AV: 1 SB: 2 14.07 , 14.89 T: FTMS {1,1} + p ESI Full lock ms [100.00-600.00]
V
210.15
208.13
268.19
222.05
149.02
181.03
264.16
300.18
227.18
140.00
167.03
246.15
282.17
339.02
122.08
322.16
NL: 7.97E5
IMP001#646 RT: 14.85 AV: 1 SB: 2 13.80 , 15.49 T: FTMS {1,1} + p ESI Full lock ms [100.00-600.00]
m/z

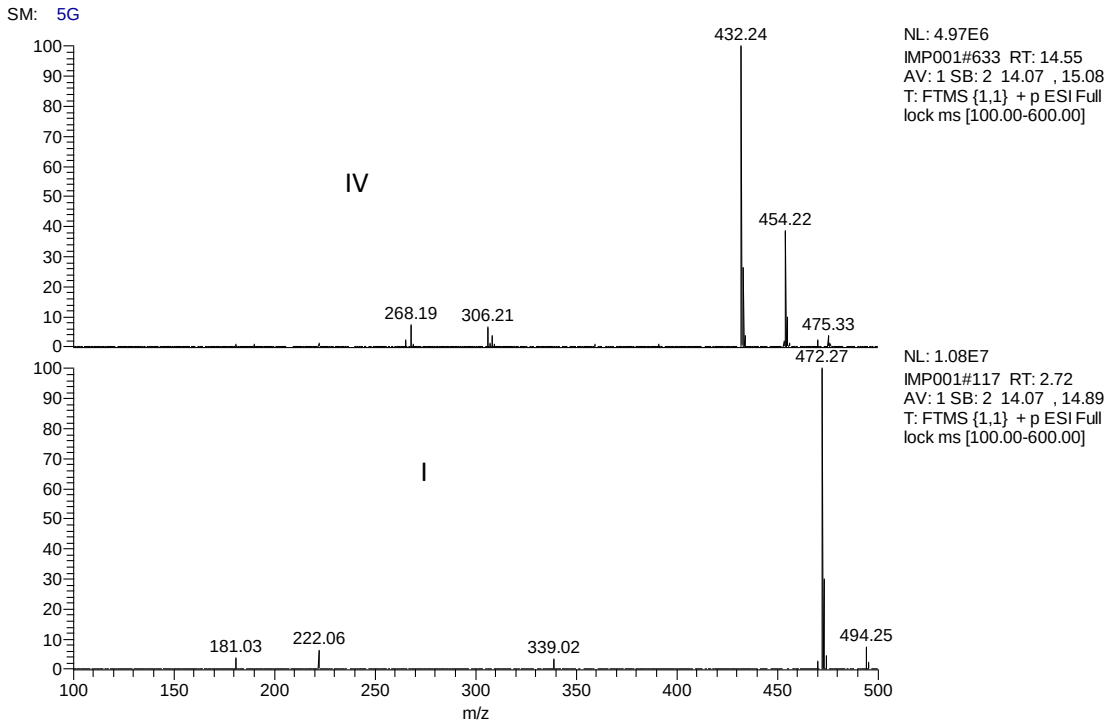


Figure 4.13 Mass spectra of oxprenolol and its major impurities in a sample of oxprenolol hydrochloride.

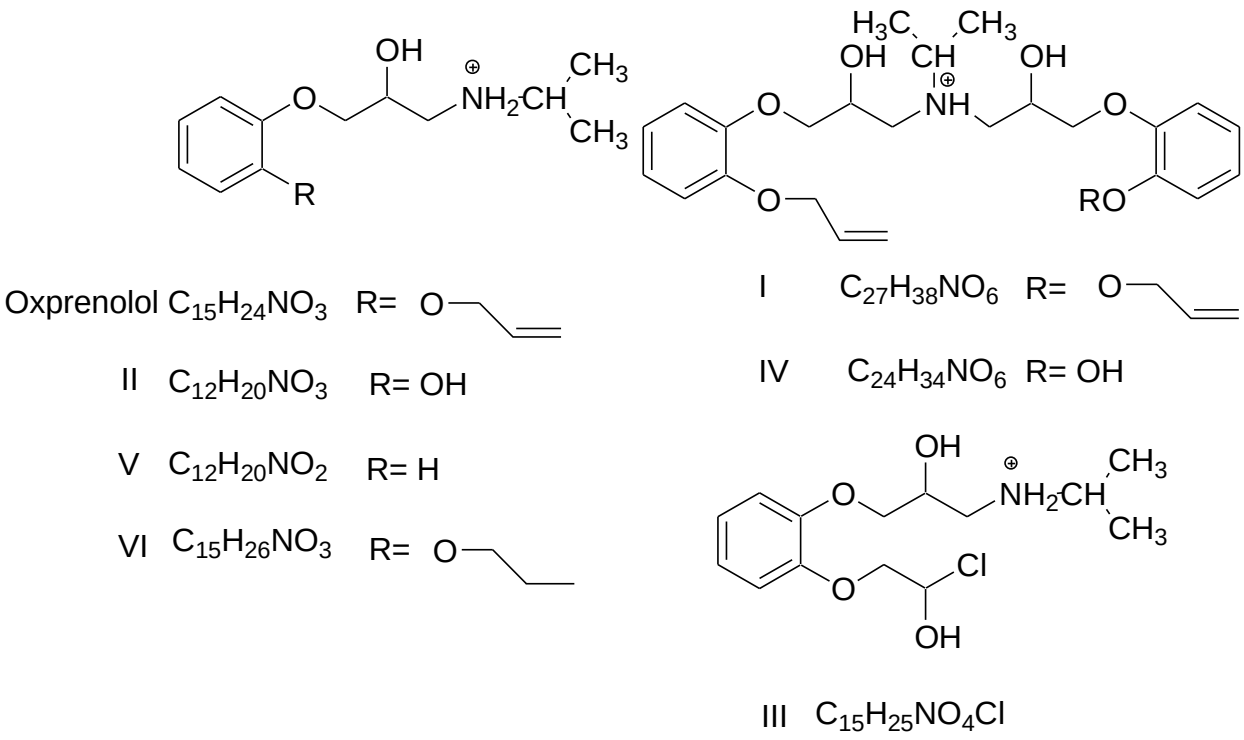


Figure 4.14 Manufacturing impurities (as the protonated bases) in a sample of oxprenolol proposed from elemental composition data.

(Table 4.3). This analysis indicates that the retention mechanism on the CN column is also influenced by molecular shape for instance absence of the propenyl side chain causes impurity II to elute 4 minutes earlier than oxprenolol and the bulky product I formed from reaction of oxprenolol with an epoxide intermediate is very early eluting.

Table 4.3 Impurities in oxprenolol determined by LC-MS on an Orbitrap Exactive instrument using an ACE CN column in 0.025% w/v ammonium acetate in acetonitrile/water (95:5, v/v) flow rate 0.4 ml/min.

Elemental comp.	Deviation ppm	t_r min
I $C_{27}H_{38}NO_6$	+0.46	2.8
II $C_{12}H_{20}NO_3$	-0.22	11.4
III $C_{15}H_{25}NClO_4$	+0.24	13.9
IV $C_{24}H_{34}NO_6$	+0.50	14.6
V $C_{15}H_{26}NO_3$	-0.11	14.7
VI $C_{12}H_{20}NO_2$	0.21	14.8
Oxprenolol $C_{15}H_{24}NO_3$	-0.11	15.2

4.5.3 Impurity profiling of dosulepin using an ACE CN Column with high resolution mass spectrometry

In Figure 4.8 an impurity profile for dosulepin can be seen, the impurity peaks are at <0.05% of the drug concentration. The elemental compositions for the impurities found in dosulepin are listed in table 4.5. Three of the impurities correspond to impurities listed in the EP, a sulphone, a sulphoxide and traces of an intermediate where water has not been eliminated to produce a double bond.

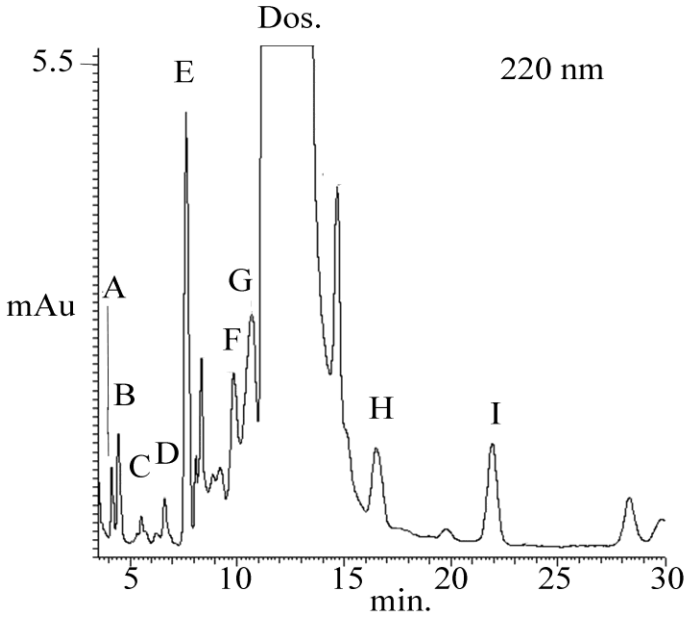


Figure 4.15 Separation of dosulepin and its manufacturing impurities on an ACE CN column (150 x 3 mm i.d.) in acetonitrile - water (95:5, v/v) containing 0.025 % w/v ammonium acetate, flow rate 0.4 ml/min.

In Figure 4.16 mass spectra of dosulepin and its major impurities can be seen and in figure 4.17 the structures proposed for the impurities are shown. In addition there is an

impurity which is clearly due to nordosulepin which, since it is a secondary amine, is characteristically later eluting from the column.

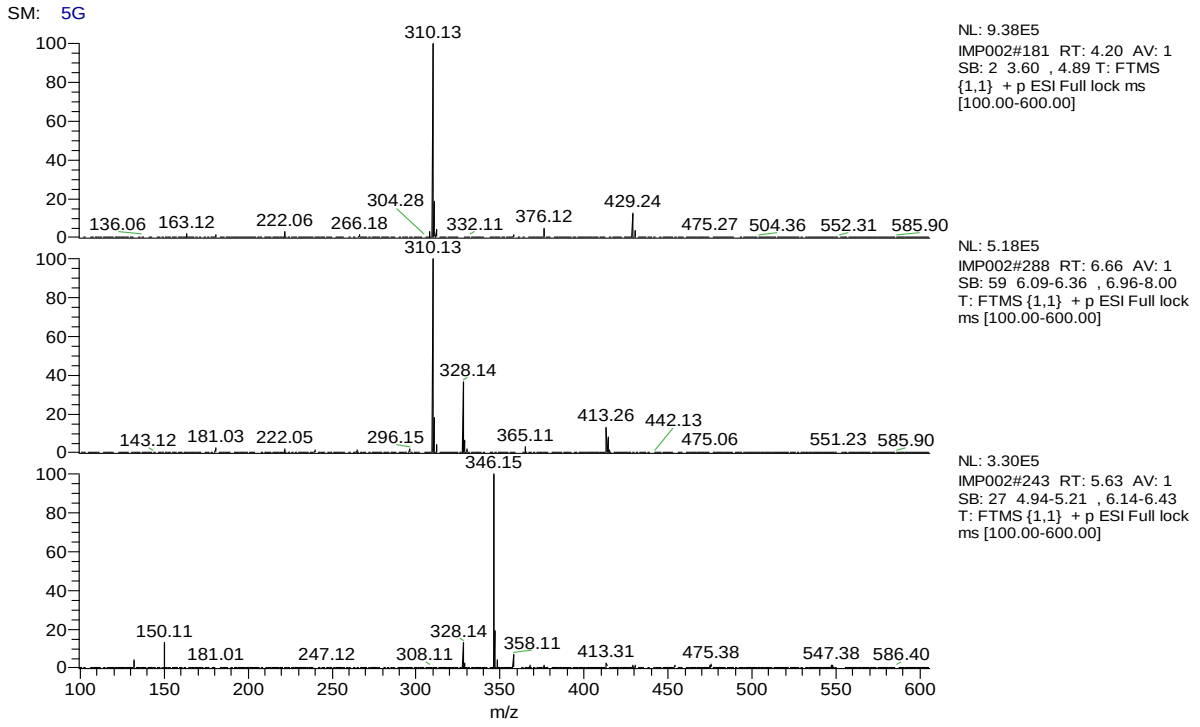
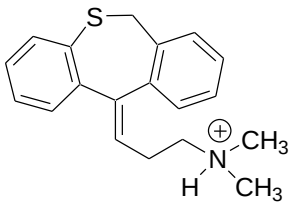
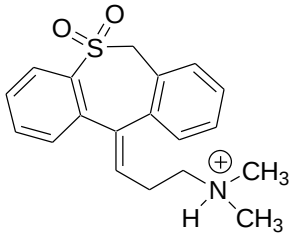


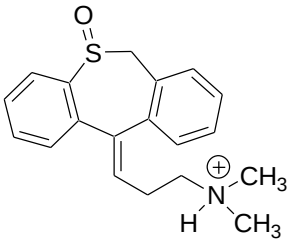
Figure 4.16 Mass spectra of major impurities in dosulepin



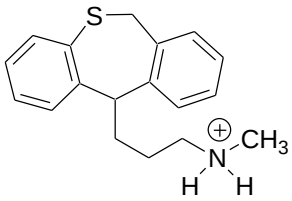
dosulepin $C_{19}H_{22}NS$



A $C_{19}H_{22}NSO_2$



B $C_{19}H_{22}NSO$



C $C_{18}H_{20}NS$

Figure 4.17 Manufacturing impurities in dosulepin (in the form of their protonated bases) proposed from elemental composition data.

The other impurities are variations on a theme where up to three oxygens are added to the structure and in some cases two hydrogen atoms are lost. The impurities which are missing two hydrogens are earlier running which would suggest the additional double bond is alpha to the amine (the only possible position in the dosulpin structure) which would cause its pK_a to be lower.

Table 4.4 Impurities in dosulepin determined by LC-MS on an Orbitrap Exactive instrument using an ACE CN column in 0.025% w/v ammonium acetate in acetonitrile/water (95:5, v/v) flow rate 0.4 ml/min.

Elemental comp.	Deviation ppm	Rt min
$C_{19}H_{22}NS$ Dosulepin	-0.23	12.8
A $C_{19}H_{20}NSO$	+0.03	4.2
B $C_{19}H_{20}NSO_2$	+0.26	4.4
C $C_{19}H_{24}NSO_3$	-0.12	5.5
D $C_{19}H_{24}NSO_2$	-0.23	6.6
E $C_{21}H_{24}NSO_2$	+0.07	7.6
F $C_{19}H_{22}NSO$	-0.23	9.9
G $C_{19}H_{22}NSO$	-0.23	10.7
H $C_{19}H_{22}NSO$	-0.23	16.9
I $C_{18}H_{20}NS$	-0.02	21.9

4.6 The Stability of the ACE CN Column to Hydrolysis

In general, cyano columns know to be a popular choice for both reverse-phase and normal phase separations, despite their history of poor chemical and mechanical stability [31].

In the literature, CN silica-based columns have been reported to have a poor retention time stability in low pH mobile phases due to acid induced ligand hydrolysis. Moreover, high pH mobile phases can cause silica skeleton dissolution, especially for short chain ligand CN columns [31, 32]. In addition, the CN column stability can be improved by using a longer ligand chain length, which can protect the silica skeleton from dissolution when used in high pH, and large steric side group, such as isopropyl, to protect the ligand from hydrolysis in low pH [33-35]. During practical work, ACE CN column was used and the stability of cyanopropyl ligand was evaluated. The ACE CN column was found to produce good peak shape and the retention times for the base test mix were stable over 200 hours of running time. A small amount of hydrolysis of the ligand might have no bearing on the retention characteristics of the column since the interaction of the analytes is with silanol groups rather than with the bonded phase. The stability of the column was monitored with two test probes anisole and 1,2 dinitrobenzene (DNB) both run in methanol - water (30:70, v/v). The lipophilicity of the ACE CN column is quite low and the anisole was not strongly retained under the test conditions, however, it does give an indication of any changes in the lipophilicity of the column. The 1,2 DNB undergoes dipole-dipole interaction [16] with the CN group in the stationary phase and is a sensitive indicator of the loading of the cyanopropyl ligand. The retention times of the anisole (2.7 min) and the 1,2 DNB (5.5 min) had not changed after 6000 column volumes of mobile phase has passed through the column. The stability of the column may be greater than was found under highly aqueous conditions because of the low

activity of the species such as H^+ and HO^- in a largely acetonitrile mobile phase which has low dielectric constant in comparison to mobile phases with a higher water content.

4.7 Preliminary Investigation of the HILIC Properties of other Alkyl Columns

The ion exchange effect of course can occur on other silica based columns in Figure 4.18 the chromatogram obtained for the basic test mixture run on an ACE phenyl in acetonitrile - water (95:5 v/v) containing 0.025% w/v ammonium acetate can be seen. It should possible to optimise separations of bases on this column and get different selectivity, however, the initial impression under the test conditions was that the CN column had better selectivity and better mass transfer characteristics.

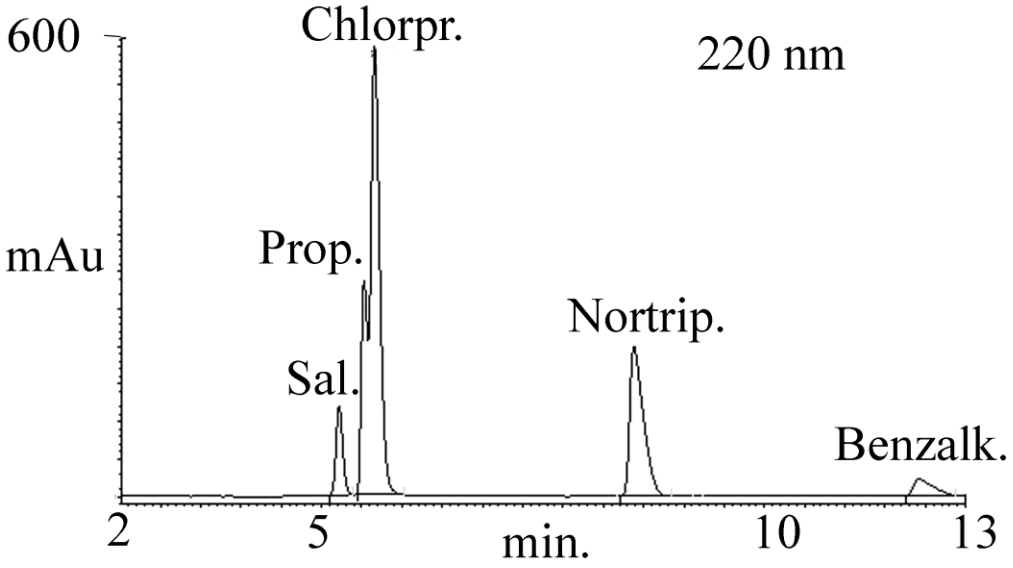


Figure 4.18 Separation of the basic test mixture on an ACE Ph column (150 x 3 mm i.d.) in acetonitrile - water (95:5, v/v) containing 0.025 % w/v ammonium acetate, flow rate 0.8 ml/min.

Table 4.5 compares the retention times of the bases in the test mixture on ACE CN, C4, Ph and Si columns. The selectivity of the columns is rather different and on both the C4 and the Ph columns salbutamol elutes earliest suggesting that the ligand attached the surface of the column may affect the ability of different analytes to interact with the silanol groups. Comparison with the silica gel column is most informative where the retention times of the bases in the test mix are very different from those obtained on the CN column. It would appear that steric effects are more marked on the silica gel column than on the CN column with salbutamol, which has a bulky tertiary butyl group attached to its basic centre eluting relatively early on the silica column and nortriptyline, which is a relatively unhindered base eluting later that the quaternary base benzalkoniumchloride which is more hindered. The pK_a of the silanol group varies according to its environment and the surfaces modified silica gels have a different population of silanol groups from a bare silica gel column and this might account for their different selectivity. However, further experiments are required to determine the exact basis of their selectivity.

Table 4.5 Comparison of retention times of basic compounds on the ACE CN, ACE Ph, ACE C4 and ACE Si columns (all 150 x 3mm i.d.) (3µm) in 0.025% w/v ammonium acetate in acetonitrile - water (95:5, v/v) flow rate 0.8 ml/min.

Base	Rt (min) CN	Rt (min) Ph	Rt (min) C4	Rt (min) Si
Chlorpromazine	5.4	5.1	4.8	9.9
Propranolol	7.3	5.2	5.4	14.9
Salbutamol	7.9	4.8	4.7	5.7*
Nortriptyline	11.0	8.5	8.2	18.2*
Benzalkonium Cl	13.8	12.1	11.5	13.7,14.3,14.8**

* wide peak ** Compound has a range of alkyl chain lengths.

4.8 Comparison of CN columns with different base silica gel and bonding chemistry

In order to compare the effects of changing the base silica gel on the performance of a CN column a 5 base test mix was used to evaluate a BDS Hypersil CN column and it was found that although the elution order of the bases (Figure 4.19) was the same but the retention times of the bases were not identical to those on the ACE CN columns with retention times tending to be lower. This would suggest that the number of accessible silanol groups on this column is lower than on the ACE CN column. Otherwise the selectivity of the column looked very similar to that of the ACE CN column.

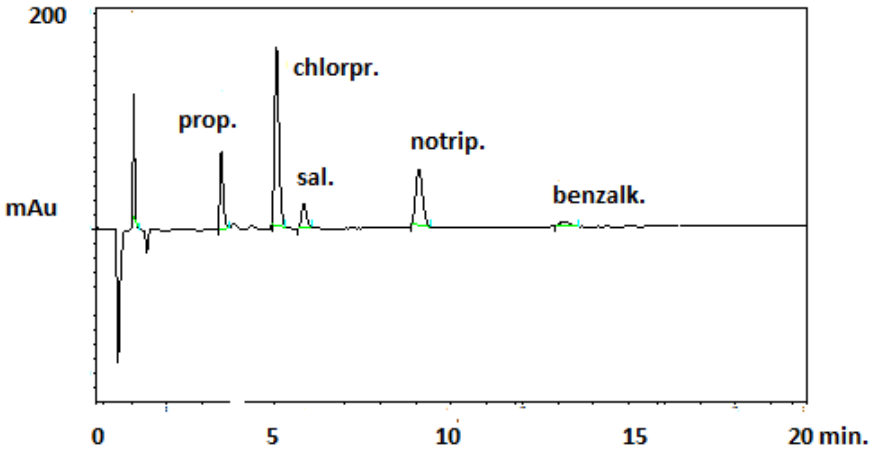


Figure 4.19 A chromatogram of the test mixture of bases on a BDS Hypersil CN column (150 x 3mm i.d.) (3 µm) with ACN - H_2O (95:5, v/v) containing 0.025% w/v ammonium acetate. Flow rate 0.8 ml/min.

In addition the test mixture of bases was run on a Zorbax Stable Bond CN column. This column has a different bonding chemistry to the standard dimethyl cyanopropyl ligand with having isopropyl groups in place of the methyl groups. This gives the phase greater stability towards hydrolysis. The chromatogram obtained for the test mixture is shown in Figure 4.20. The retention times of the bases are much shorter on this phase possibly indicating that the silanol groups are more shielded from interaction with the bases and the phase is closer to an alkyl column in character.

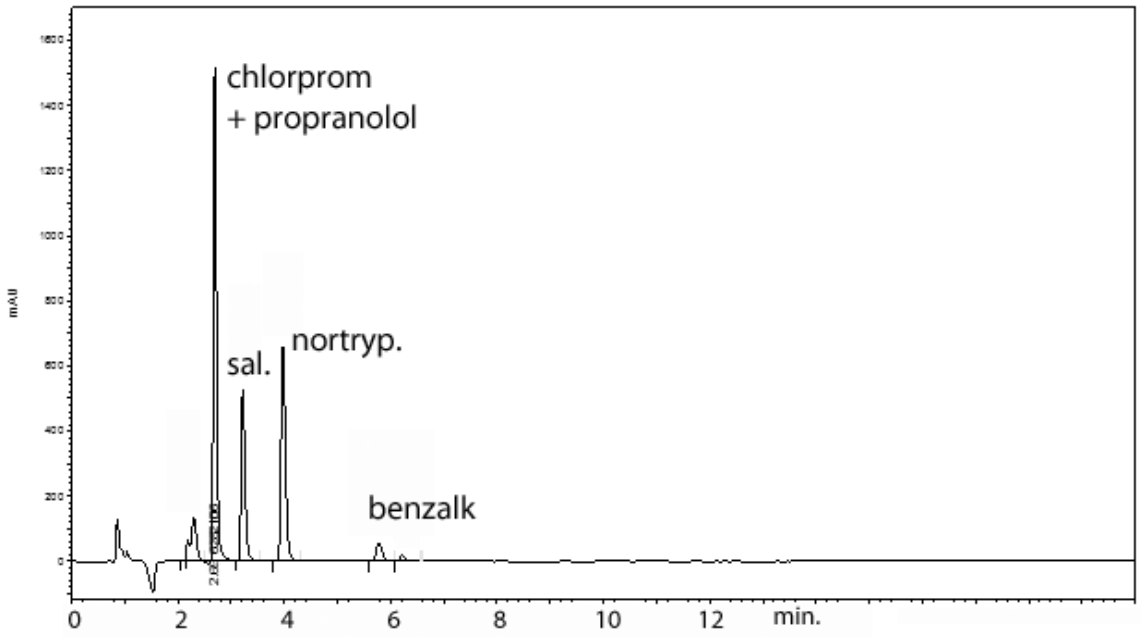


Figure 4.20 A chromatogram of test mixture of bases on a BDS Hypersil CN column (150 x 3mm i.d.) (3.5 µm) with ACN - H_2O (95:5, v/v) containing 0.025% w/v ammonium acetate. Flow rate 0.8 ml/min.

4.9 Conclusion

The observation that different types of reversed phase silica based columns exhibit ion exchange properties opens up a wide field of research. Thus there is potentially a wide range of chromatographic selectivities to be exploited since there are many columns which could be studied from different manufacturers with a variety of ligands bound to them. In this studythe behaviour of neutral and acidic molecules was not evaluated although there is evidence that silica based columns provide purely HILIC properties

which will retain these compounds [1] as opposed to cation exchange properties which only apply to basic compounds. Working with high organic content in the mobile phase offers improved mass transfer properties and the efficiencies obtained on the CN column studied in this paper are very high. The low backpressures obtained with high organic content mean that longer 3µm columns could be used and it there were a need to economise on acetonitrile long narrower bore columns should work well with the high acetonitrile mobile phases which generate low backpressures.

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Chapter 5

Examination of the HILIC like properties of some alkyl columns.

5.1 Introduction

Following on from the work described in Chapter 4 it was decided that the HILIC like properties of some alkyl columns should be examined. This was because there was still some doubt about the long term stability of the stationary phase ligand on the CN stationary phase. Although the CN columns appeared stable to normal use with ammonium acetate in the mobile phase it was noticed that they were for some reason less stable to tris buffer. The hydrolysis of the stationary phase was indicated by a gradual increase in the retention time of the test mixture which indicated that more silanol groups were becoming exposed due to loss of the coating ligand.Despite the fact that HILIC chromatography is a rapidly expanding area [1-4] there are no literature references to the use of alkyl silica based columns in hydrophilic interaction mode and thus the introduction to this section is brief and follows on from the observations made in Chapter 4. Thus this study was entirely speculative.

5.2 Materials and Methods

5.2.1 Chemicals

Ammonium acetate, HPLC grade acetic acid, HPLC grade methanol and HPLC grade acetonitrile were obtained from Fisher Scientific, Loughborough, UK. HPLC water was prepared in house using a Milli Q purification system. The bases used in the running tests were from either Sigma Aldrich, Dorset UK.

5.2.2 HPLC Columns

ACE CN, ACE Phenyl (Ph), ACE Butyl (C4) (150 x 3mm i.d.)(3 µm) were obtained from Hichrom Ltd., Reading U.K.

5.2.3 HPLC Instrumentation

HPLC analysis was carried out on a ThermoFinnegan HPLC system consisting of a P2000 pump, P200 UV dual wavelength detector and an AS2000 autosampler. The mobile phase compositions were mixed off-line in the proportions required and the HPLC system was used in isocratic mode, 10µl of sample was injected. The flow rate was 0.8 ml/min and the column was run at room temperature. The UV detector was set to monitor 220 and 280 nm. The continued stability of the CN column was monitored by injecting a mixture of anisole and 1,2 DNB with a mobile phase composition consisting of methanol - water (30:70, v/v).

5.3 Results

5.3.1 Butyl (C4) Column

In Figure 5.1 a separation of the standard text mixture of basic drugs, used to assess the ACE CN column in the work described in Chapter 4, on an ACE C4 column in Acetonitrile - water (95:5, v/v) containing 0.025 % w/v ammonium acetate at flow rate of 0.8 ml/min is shown. It can be seen that the retention of the test compounds was much lower than was observed on the ACE CN column. However, despite the short retention times the test mixture was almost completely resolved and the benzalkonium chloride, which is a mixture of different chain lengths was separated into three peaks. This would

suggest that the free silanol groups within the C4 phase are less readily accessible to basic analytes thus the cation exchange interactions are much smaller than was observed on the CN column (the levels of free silanol groups in these phases are similar [5]. The CN column has a polar ligand attached to the surface and the stationary phase ligands probably stand up from the surface to a greater extent thus allowing better access to bulky analytes. This is entirely logical since the CN group will interact with water in the mobile phase although currently there is no evidence for this. The benzalkonium chloride mixture of chain lengths does not separate on the CN column and this suggests that on the more lipophilic C4 column that there may also be lipophilic interactions with the analytes although this has yet to be proved.

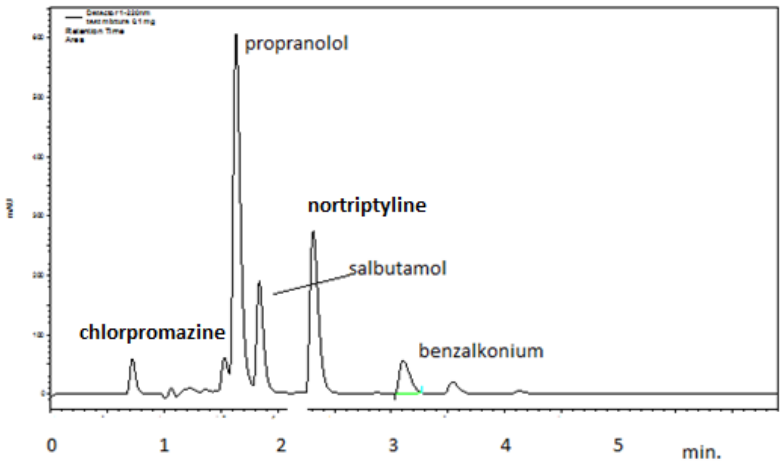

Figure 5.1 Separation of basic test mixture in on an ACE C4 column using Acetonitrile - water (95:5, v/v) containing 0.025 % w/v ammonium acetate at flow rate of 0.8 ml/min.

5.3.2 Xterra Octadecyl (C18) Column

In Figure 5.2 the separation achieved for the basic test mixture on a Xterra C18 column is shown. The separation is similar to that obtained on the ACE C4 column indicating that interaction with silanol groups is still possible for a column with bulky stationary phase ligands attached. In fact the retention on the Xterra column is slightly greater than on the ACE C4 column which may be due to the different type of base silica gel used to prepare the column. The separation between the different chain length benzalkonium was even more marked than on the ACE C4 column again supporting the proposal that there are secondary lipophilic interactions contributing to the separation as well as the interactions with free silanolol groups. Further work is required to support this hypothesis.

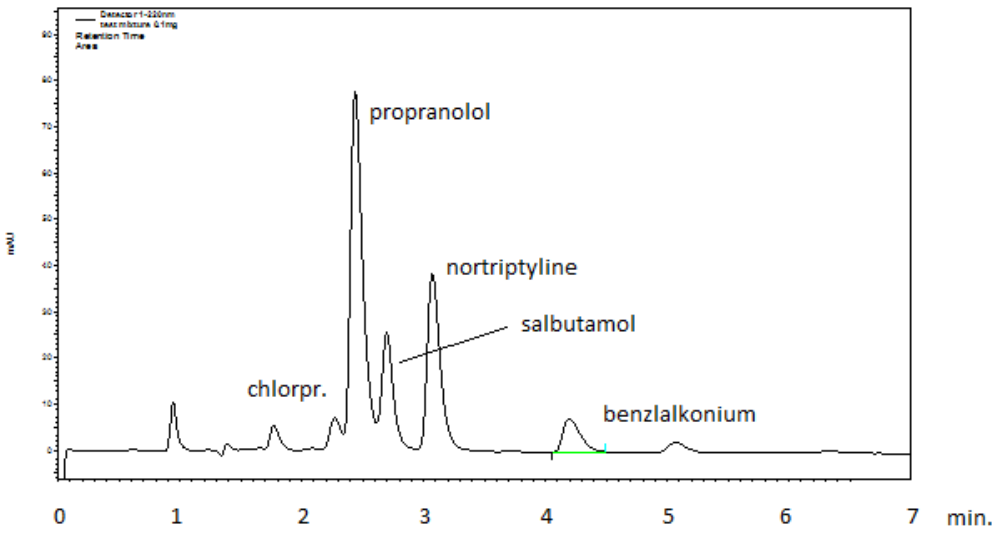

Figure 5.2 Separation of basic test mixture in on an Xterra C18 column (150 x 4.6 mm i.d.) (5 µm) using acetonitrile - water (95:5, v/v) containing 0.025 % w/v ammonium acetate at flow rate of 0.8 ml/min.

5.3.3 Hypersil C1 Column

In order to further test the effect of stationary phase ligand chain length on retention a Hypersil C1 column was tested. In Figure 5.3 a chromatogram obtained for benzalkonium on the C1 column is shown. The retention of benzalkonium was much stronger on the C1 column indicating that the shorter chains on the surface of the stationary phase allowed easier access by the analyte to the uncapped silanol groups. The Hypersil column is based on a low purity silica gel and thus the peak shapes of obtained on the Hypersil C1 column were not as good and tailing tended to occur. In Figure 5.4 a chromatogram for nortriptyline analysed on this column which tailed to a greater extent than on the Xterra and ACE C4 columns which are based on modern high purity silica gels is shown.

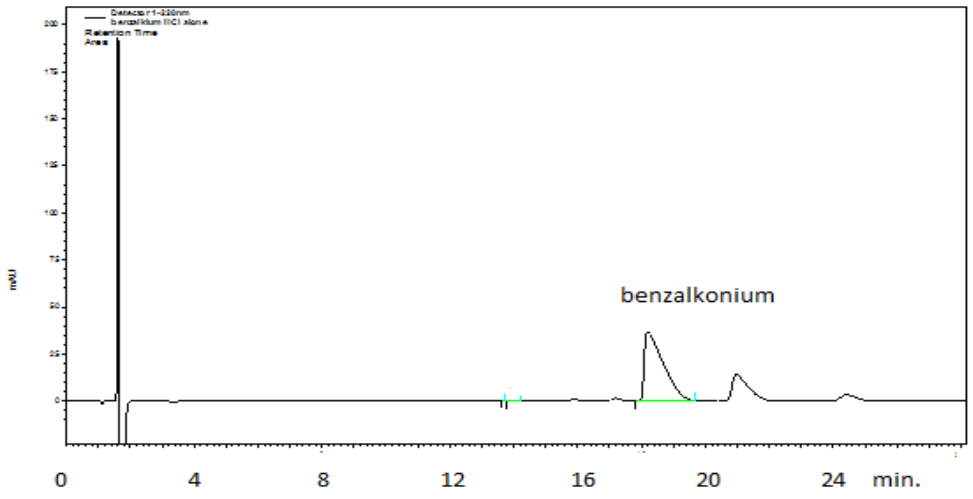

Figure 5.3 Separation of benzalkonium on a Hypersil C1 column (150 x 4.6 mm i.d.)(5µm) using acetonitrile - water (95:5, v/v) containing 0.025 % w/v ammonium acetate at flow rate of 0.8 ml/min.

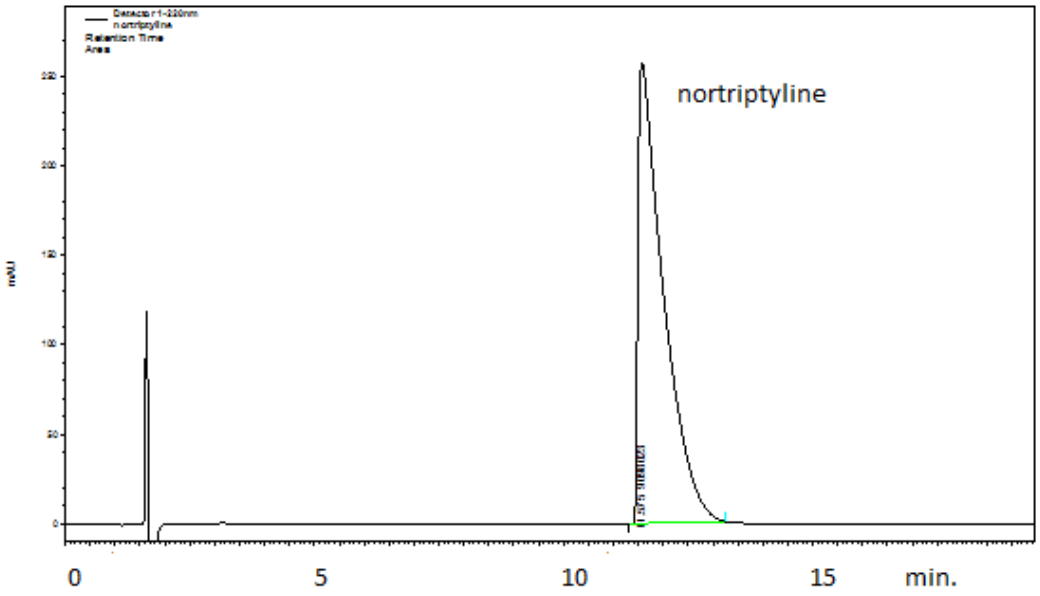

Figure 5.4 Separation of nortriptyline on a Hypersil C1 column (150 x 4.6 mm i.d.)(5 µm) using acetonitrile - water (95:5, v/v) containing 0.025 % w/v ammonium acetate at flow rate of 0.8 ml/min.

Future work will test the hypothesis developed above further by using the other test-bases

5.3.4 A detailed examination of the hydrophilic interaction properties of an ACE Phenyl (Ph) Column

A preliminary test was carried out on an ACE Ph column in Chapter 4 and it was found to have HILIC properties when used with a mobile phase with a high content of acetonitrile. The column was tested with a series of basic drugs (Figure 5.5) with different percentages of acetonitrile. Tables 5.1 – 5.4

Table 5.1 Retention times for secondary amines retention analysed on an ACE Ph column (150 mm x 3 mm i.d.) (3 µm) water - acetonitrile (60:40, v/v) containing 0.025% w/v ammonium acetate pH 6.0. Flow rate 0.8 ml/min.

Compound	Retention time (minutes)
Acebutolol	1.9
Atenolol	1.4
Despiramine	6.1
Doxylamine	2.8
Isoxsuprine	3.0
Ketamine	4.0
Metanephrine	1.4
Nortriptyline	6.8
Propranolol	3.4
Salbutamol	1.3
Chloromeprazine HCl	10.7
Diphenhydramine HCl	4.9
Promethazine HCl	6.8
Dosulepin	7.6

The drugs showed U shaped plots (Figures 5.6 and 5.7) which are typical for HILIC type interactions [6,7]. At 40% acetonitrile the reversed phase retention mechanism dominated with lipophilic compounds like chlorpromazine, dosulepin nortriptyline and desipramine eluting latest and polar compounds like salbutamol eluting early. At 80% acetonitrile all compounds elute quickly from and column and at 95% the retention times, particularly for lipophilic secondary amines like nortriptyline and desipramine start to increase again due to the increase in ion exchange interactions with the uncapped silanol groups in the stationary phase. The mechanism involved is complex but part of the mechanism must involve the high level of organic solvent in the mobile

phase containing 95% acetonitrile causing the stationary phase ligands to move away from the surface of the phase thus exposing the silanol groups for interaction with the basic drugs. Although further work would be required to fully support this hypothesis. In addition the nature of the basic centre on the drug is important with basic centres with smaller ionic radius such as the secondary amine centres in nortriptyline and desipramine interacting more strongly with the silanol groups than tertiary amines such as propranolol and salbutamol which have a bulky isopropyl group substituted on their basic centre. The exception is ketamine which has the methyl substituted amine centre like desipramine but the basic centre is very hindered by the groups around it. In order to assess the effect of the type of basic centre on retention on the phenyl column a series of simpler test compounds (figure 5.8) were investigated. These compounds only have an amine as their functional group thus eliminating any effects from additional substituents.

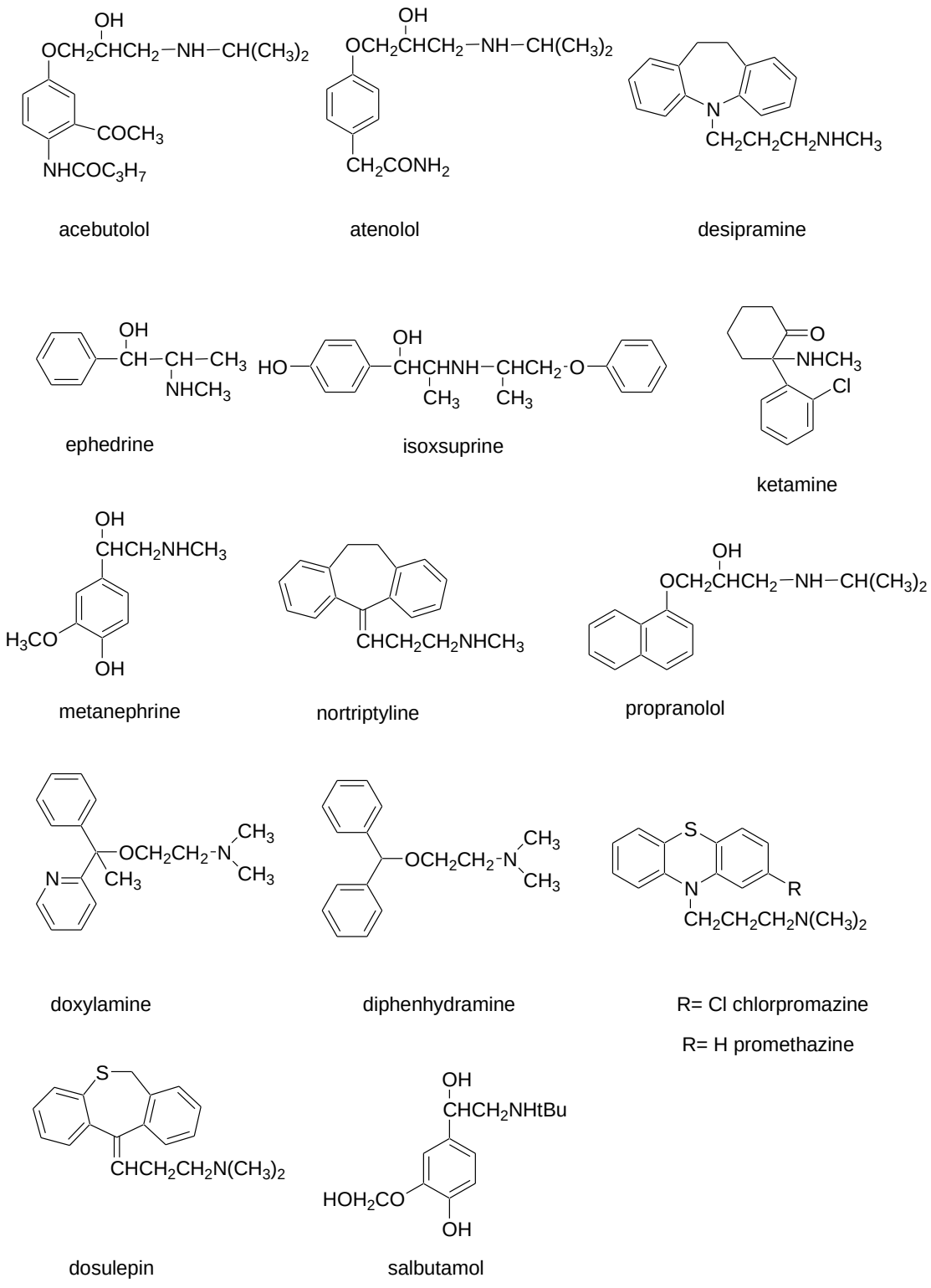

Figure 5.5 Secondary and tertiary amine drugs used to test the ACE Ph column in HILIC mode

Table 5.2 Retention times for secondary amines retention analysed on an ACE Ph column (150 x 3 mm i.d.)(3 µm) water - acetonitrile (40:60, v/v) containing 0.025% w/v ammonium acetate pH 6.0. Flow rate 0.8 ml/min.

Compound	Retention time (minutes)
Acebutolol	1.6
Atenolol	1.4
Despiramine	2.6
Doxylamine	2.1
Ephedrine	1.4
Isox suprine	2.1
Ketamine	2.7
Metanephrine	1.4
Nortriptyline	2.8
Propranolol	2.1
Pseudo ephedrine	1.6
Salbutamol	1.4
Chloromeprazine HCl	3.7
Diphenhydramine HCl	2.6
Promethazine HCl	3.2
Dosulepin	3.1

Table 5.3 Retention times for secondary amines retention analysed on an ACE Ph column (150 x 3 mm i.d.)(3 µm) water - acetonitrile (20:80, v/v) containing 0.025% w/v ammonium acetate pH 6.0. Flow rate 0.8 ml/min.

Compound	Retention time (minutes)
Acebutolol	1.7
Atenolol	1.5
Despiramine	2.3
Doxylamine	2.1
Ephedrine	1.5
Isox suprine	1.8
Ketamine	2.1
Metanephrine	1.6
Nortriptyline	2.3
Propranolol	1.9
Pseudo ephedrine	1.7
Salbutamol	1.6
Chloromepromazine HCl	2.9
Diphenhydramine HCl	2.3
Promethazine HCl	2.6
Dosulepin	2.6

Table 5.4 Retention times for secondary amines retention analysed on an ACE Ph column (150 x 3 mm i.d.)(3 µm) water - acetonitrile (5:95, v/v) containing 0.025% w/v ammonium acetate pH 6.0. Flow rate 0.8 ml/min.

Compound	Retention time (minutes)
Acebutolol	2.5
Atenolol	2.2
Despiramine	4.2
Doxylamine	3.5
Isox suprine	1.3
Ketamine	1.2
Metanephrine	2.6
Nortriptyline	4.0
Propranolol	2.6
Salbutamol	2.4
Diphenhydramine	2.7
Dosulepin	3.0
Promethazine	2.0
Chlropromazine	2.7

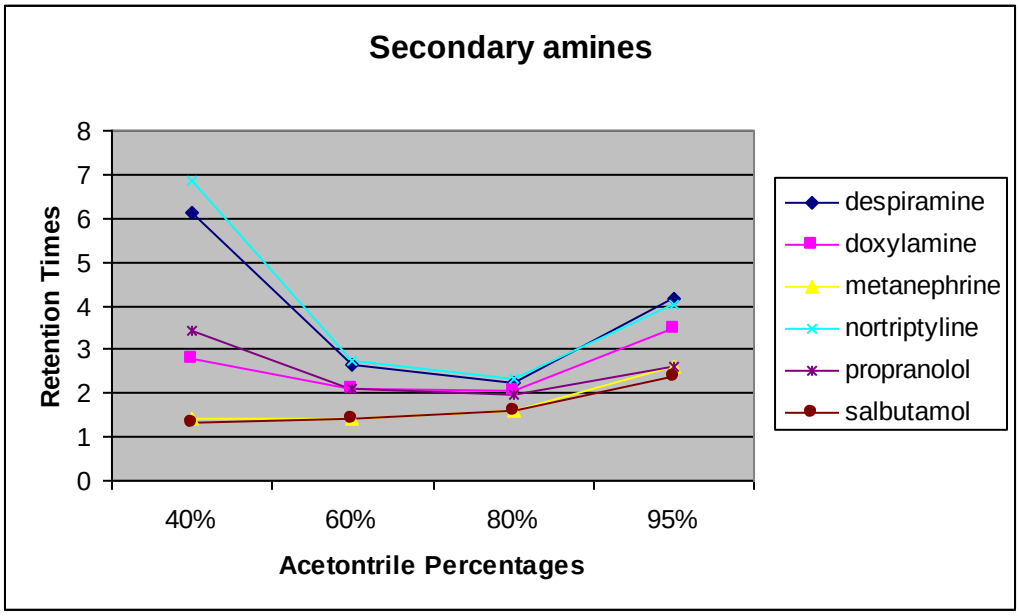

Figure 5.6 Plots of the retention times of secondary amines against % of acetonitrile showing that the retention times pass through a minimum in the course of moving from a lipophilic retention mechanism to a HILIC retention mechanism [6,7].

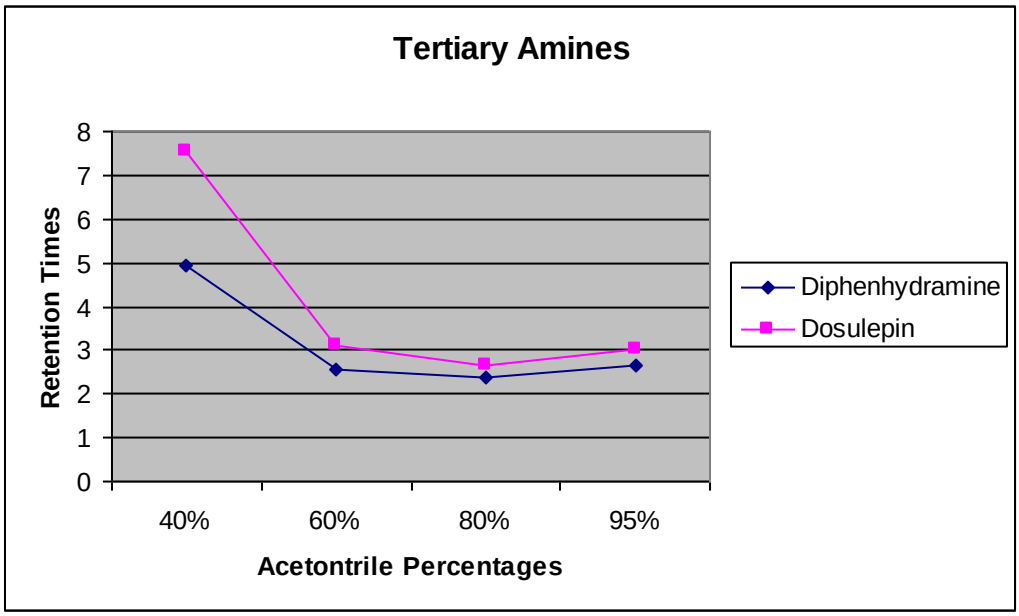

Figure 5.7 Plots of the retention times of tertiary amines against % of acetonitrile showing that the retention times pass through a minimum in the course of moving from a lipophilic retention mechanism to a HILIC type retention mechanism. The HILIC retention mechanism is less marked in this case because of weaker interaction of the tertiary amine centre with the uncapped silanol groups.

The simple test probes 2-methylbenzylamine, N-tert-butylbenzylamine (TBBA) ,α-methylbenzylamine (AMBA), benzylamine (BA), dimethylbenzylamine (DMBA), benzyltriethylamine (BTEA), N-methyl-, N-ethyl and N-isopropylbenzylamine (MBA, EBA and PBA) (Figure 5.8) were used to try to better understand the retention mechanism on the Ph column. The retention times of the probes are shown in table 5.5. Under the conditions used the retention times are shorter than the drug molecules shown in table 5.4 and this indicates that the lipophilicity of the probe may be important as well as it capacity to undergo ion exchange interactions with the uncapped silanol groups. The quaternary amine BTEA, due to its pH independent charge interacted most strongly with the Ph column, the secondary amines eluted in order of the size of their alkly substituent with MBA being the most strongly retained and TBBA being the least retained of the secondary amines. The tertiary and primary amines were least retained. The greater retention of the secondary amines compared with the primary amine may be due to their pK_a values being higher than those of the primary amines. Thus this reflects the fact that they were more charged under the conditions of analysis than the primary amines. The effect of the structural elements within the simple amine test probes can be seen more clearly when the mobile phase was changed from containing ammonium as the counter ion to containing tris buffer as the counter ion. As seen in Chapter 4, since tris is a weaker amine than ammonium it is less charged and thus it competes less effectively with the analytes for the silanol groups in the stationary phase leading to longer elution times. In Table 5.6 the data obtained for the amine probes when 0.04% w/v tris acetate pH 6.0 was used as the ionic modifier in the mobile phase

is shown. The ionic strength of the buffer was equivalent to 0.025% w/v ammonium acetate. In figure 5.10 it can be clearly seen that the order of elution of the secondary amine test probes is governed by the size of the alkyl substituent on the secondary amine centre with the smallest methyl substituted amine eluting the latest. The primary amine and tertiary amine probes still elute much earlier than the secondary amines and the quaternary amine BTEA elutes later than the secondary amines reflecting is pH independent charge.

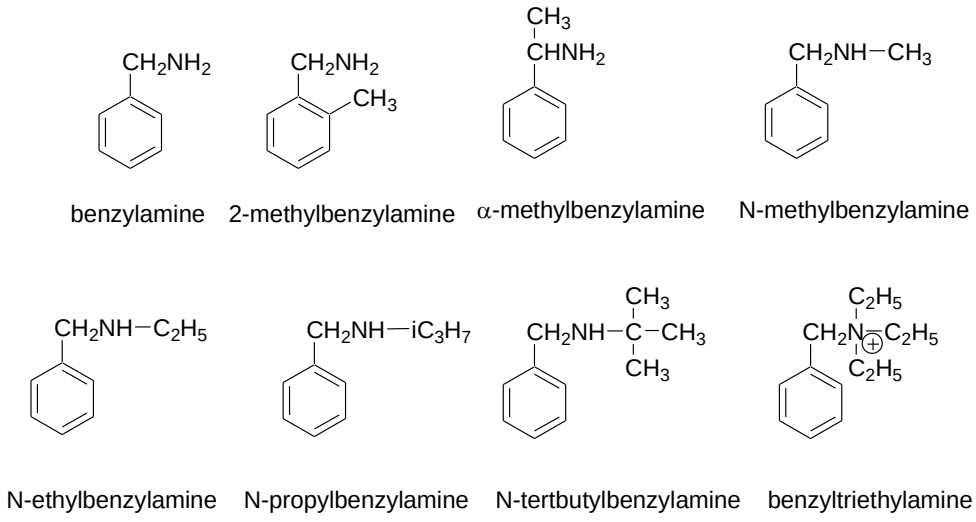

Figure 5.8 Structures of simple amine test probes used to test the ACE Ph column.

Table 5.5 Retention times of the simple amine probes on an ACE Ph column (150 x 3 mm i.d.)(3 µm) water - acetonitrile (5:95, v/v) containing 0.025% w/v ammonium acetate pH 6.0. Flow rate 0.8 ml/min.

Compound	**Retention time (minutes)**
Benzylamine	1.6
2-methylbenzylamine	1.6
α-methylbenzylamine	1.6
N-methylbenzylamine	2.1
N-ethylbenzylamine	2.0
N-isopropylbenzylamine	1.9
N-tertbutylbenzylamine	1.8
benzyltriethylamine	2.8

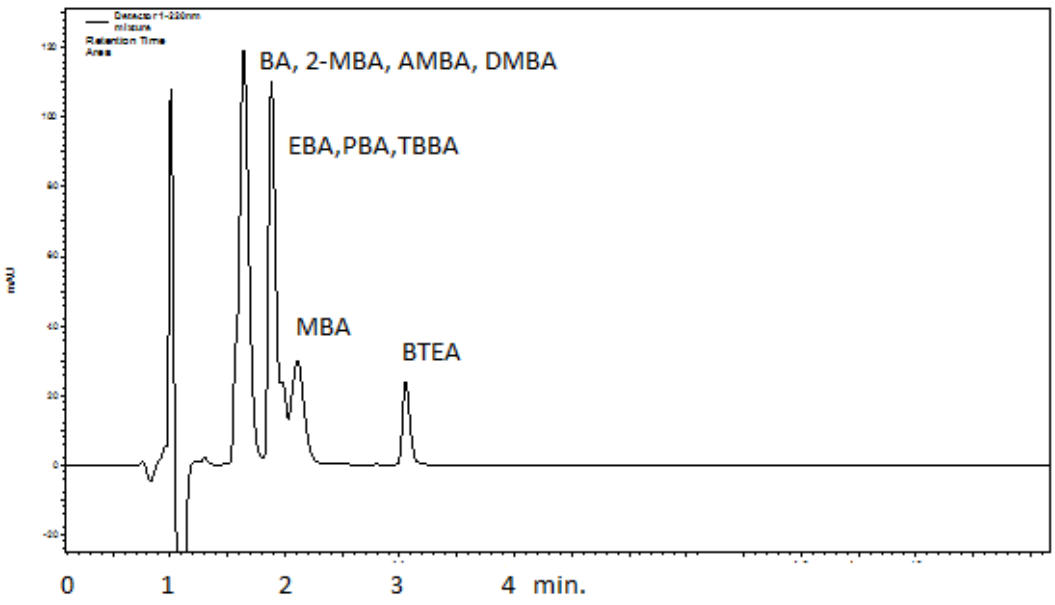

Figure 5.9 A Mixture of simple test probes 2-methylbenzylamine (2 MBA), N-tert-butylbenzylamine (TBA),α-methylbenzylamine (AMBA), benzylamine (BA), dimethylbenzylamine DMBA, benzyltriethylamine (BTEA), N-methyl-, N-ethyl and N-isopropylbenzylamine (MBA,EBA and PBA) run on an ACE Ph column (150 x 3 mm i.d.)(3 µm) water - acetonitrile (5:95, v/v) containing 0.025% w/v ammonium acetate pH 6.0. Flow rate 0.8 ml/min.

Table 5.5 Retention times of the simple amine probes on on an ACE Ph column (150 x 3 mm i.d.)(3 µm) water - acetonitrile (5:95, v/v) containing 0.04%* w/v tris acetate pH 6.0. Flow rate 0.8 ml/min.

Compound	**Retention time (minutes)**
Benzylamine	2.0
2-methylbenzylamine	2.0
α-methylbenzylamine	1.9
N-methylbenzylamine	2.7
N-ethylbenzylamine	2.6
N-isopropylbenzylamine	2.5
N-tertbutylbenzylamine	2.3
benzyltriethylamine	4.1

*****Equivalent ionic strength to 0.025% w/v ammonium acetate since these concentrations are the same molar strength.

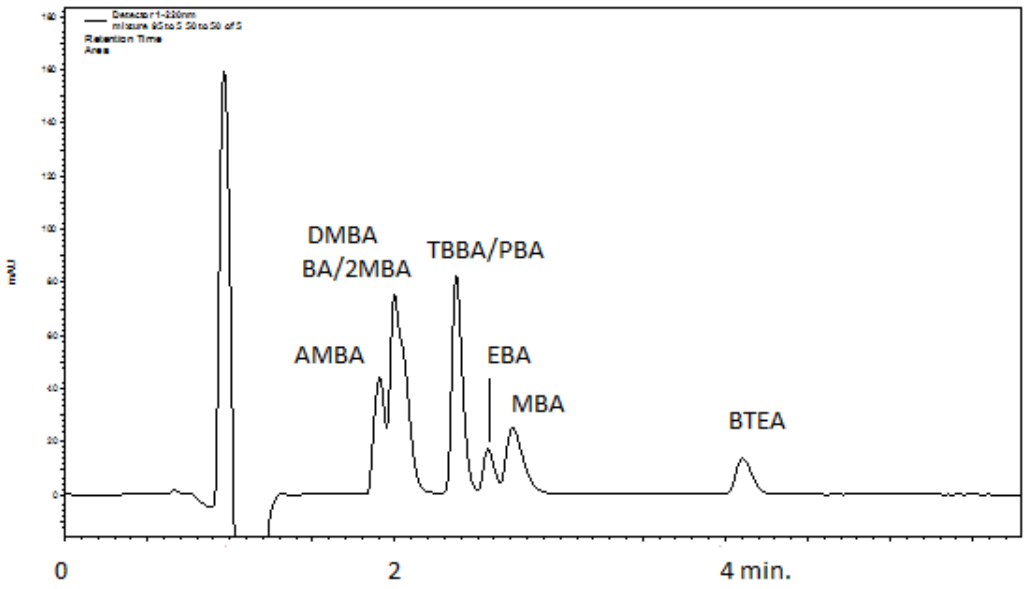

Figure 5.10 A Mixture of simple test probes 2-methylbenzylamine (2MBA), N-tert-butylbenzylamine (TBBA), α-methylbenzylamine (AMBA), benzylamine (BA), dimethylbenzylamine (DMBA), benzyltriethylamine (BTEA), N-methyl-, N-ethyl and N-isopropylbenzylamine (MBA, EBA and PBA) run on an ACE Ph column (150 x 3 mm i.d.) (3 µm) water - acetonitrile (5:95, v/v) containing 0.04% w/v tris acetate pH 6.0. Flow rate 0.8 ml/min.

The standard test mixture of basic drugs was also run with tris acetate pH 6.0 as the mobile phase ionic modifier. These conditions gave very good performance with all the drugs in the test mixture being well retained. The secondary amine nortriptyline was retained to a much greater extent than the simple secondary amines shown in figure 5.10 indicating that there are probably two factors at work, the nature of the basic

centre and the overall lipophilicity of the molecule. Even chlorpromazine which is a tertiary amine is quite strongly retained. Overall this supports the idea that the analyte must have strong partitioning into the stationary phase in order to make contact with the silanol groups which to some extent may be buried under layers of stationary phase modifier, in this case the dimethylphenyl silane group.

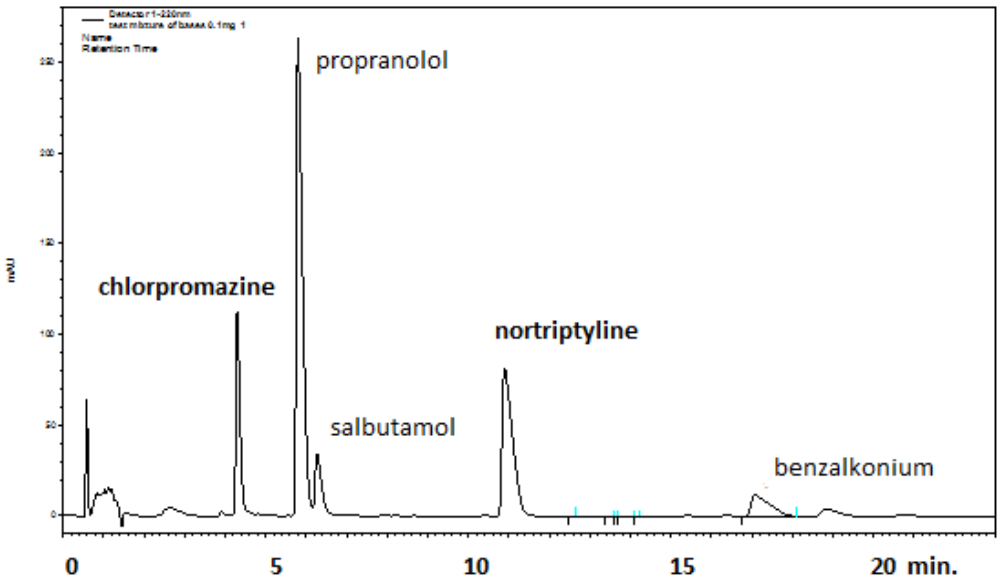

Figure 5.11 Test mixture of basic drugs run on an ACE Ph column (150 x 3 mm i.d.) (3 µm) water - acetonitrile (5:95, v/v) containing 0.04% w/v tris acetate pH 6.0. Flow rate 0.8 ml/min.

5.4 Conclusion

The use of alkyl phases in hydrophilic interaction mode for the separation of bases has great potential and there is still much research to do in order to completely understand the type of interactions observed. The advantages of this mode of chromatography are: 1. The high efficiencies obtained due to the favourable mass transfer characteristics of high organic solvent mobile phases 2. There is good evidence from this study that the mode of separation is sensitive to small differences in molecular structure thus having potential in the separation of isomers.

5.5 References

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Chapter 6

Conclusions and Future Work.

6.1 Conclusions

Two separate studies were carried out in this thesis linked by the common theme of the chromatographic separation of basic drug compounds.

Stability studies are critical part of the drug development process and are essential for drug product marketing approval. Several factors affect drug shelf- life including chemical and physical stability during pre-clinical formulation stages, process development, drug packing development and post marketing life. The stability of a drug substance and a drug product can be assessed by stability indicating method, which can evaluate chemical and physical stability and identify drug degradation products and pathways. There are many extemporaneous formulations used within hospitals for which there is no stability information. In the work described in this thesis the stability of two such extemporaneously prepared formulations were evaluated.

In chapter 2, the stability of a formulation containing clonidine.HCl, diamorphine .HCl and bupivacaine.HCl administered via epidural infusion to manage severe pain in palliative care patients was evaluated. The injection is freshly prepared by the Pharmacy Department at the Western General Hospital in Edinburgh. In the laboratory, the extemporaneous formulations were prepared in a manner suitable for laboratory testing and a stability indicating HPLC method was developed to evaluate the formulation stability during storage. The formulation was tested under three sets of storage conditions (4, 22, and 37 ^{0}C) for 8 days. Clonidine and bupivacaine were stable under all examined conditions, while diamorphine underwent slow degradation into

monodactyl morphine at room temperature and degraded more rapidly when stored at 37^0C for 8 days. This type of study is important since it can have a direct impact on pharmacy practise.

In chapter 3, the stability of a formulation consisting of morphine sulphate and levomepramazine.HCl was evaluated by developing a stability indicating HPLC method. The formulation is recommended by the Scottish Palliative Care Pharmacists Association to be effective in pain control, if administered via subcutaneous infusion. In the laboratory, the extemporaneous formulations were prepared in a manner suitable for laboratory testing and a suitable stability indicating HPLC method was developed to evaluate the formulation stability. The formulation stability was examined under three different sets of storage conditions (4, 22. 37 °C). Morphine sulphate was found to have very little degradation under any of the storage conditions and the amount of morphine sulphate remained above 94% of the initial concentration in all syringe. In contrast, levomepramazine.HClwas observed to undergo a steady decline in concentration with time with a greatest rate of decline at 37°C. In some admixtures stored at 37°C the concentration of levoumepramazine.HCl fell to below 70% over 8 days and as the level of levoumepramazine.HCl decline, the level of the major degradant peak increased. The identity of the major degradant peak was confirmed by LC-MS to be due to the sulphoxide of levomepramazine.

Both formulations studied in Chapters 2 and 3 should be stored in a fridge at 4 $^{\circ}$C or below to ensure that the components involved in the formulations are stable.

Since stability indicating methods are to some extent routine although requiring careful development it was decided that some more speculative research should be pursued. In chapter 4, the challenges of analysing basic compounds by HPLC were discussed. For instance some peak shape problems were experienced when analyzing bupivacaine.HClin Chapter 2 on a C18 column using reversed phase liquid chromatography. Hydrophilic interaction chromatography (HILIC) was discussed as an alternative technique to reversed phase liquid chromatography. HILIC is particularly effective in to retaining and separating basic components that are not strongly retained using reversed phase liquid chromatography. It was demonstrated comprehensively for the first time that a CN column was very effective at retaining basic compound under HILIC conditions using water - acetonitrile (5:95, v/v) containing 0.025% w/v ammonium as the mobile phase. The method was very sensitive to the type of bases with the order of retention of basic compounds being quaternary> secondary> tertiary (there are very few drug molecules with primary amines in their structures). The mechanism of retention was proved to be ion exchange since higher concentrations of ammonium ion in the mobile phase reduced retention times and using the weaker, less ionised, base tris as the counter ion increased retention times. The usefulness of the HILIC method developed on the CN column was demonstrated in three impurity profiling studies.

The stability was assessed by 1,2 dinitrobenzene and anisole. The retention times of the anisole and 1,2 dinitrobenzene did not change after 6000 column volumes of mobile phase had passed through the column, which indicated adequate stability of cyano column under HILIC conditions. The higher organic content in the mobile phase offers

improved mass transfer properties and the efficiencies obtained on the CN column were very high. In addition, the low back pressures obtained by HILIC mode could allow the use of smaller particle sizes and longer columns. During the course of the work in Chapter 4 it was observed that other alkyl bonded stationary phases exhibited HILIC like properties and these were explored further in Chapter 5.

In chapter 5, the HILIC like properties of some alkyl columns: An ACE butyl (C4) column, an Xterra octadecyl (C18) column, a Hypersil (C1) column and an ACE phenyl column, were examined. There was a positive relationship between stationary phase ligand chain length and basic compounds retention time. The stationary phase with the shortest C1 ligand produced the greatest retention of the basic test probes. It was demonstrated that the alkyl columns produced a typical U shaped plot for retention times of basic test probes against % of organic modifier showing a switch from lipophilic interaction to a HILIC like mechanism. The lipophilic interactions may also remain a subsidiary component in the interactions under HILIC like conditions. The ACE phenyl column was examined in more depth. The retention of the simple test probes: 2-methylbenzylamine,N-tert-butylbenzylamine,α-methylbenzylamine, dimethylbenzylamine, benzylamine, benzyltriethylamine, N-methyl, N-ethyl and N-isopropylbenzylamine was studied in order to try to better understand the retention mechanisms on these phases. The quaternary amine benzyltriethylamine was found to have longer retention time than the other amines due to its pH independent charge interacting most strongly with the silanol groups of phenyl stationary phase. The secondary amines were found to have greater retention times than the primary and

tertiary amines due to their higher pK_a valueswhich meant they were more fully charged than primary and tertiary amines. The pK_a of counter ion used as a modifierin the HPLC mobile phase was found to have a big effect on the retention times of the amines. This can be explained since from switching ammonium acetate buffer (ammonia pK_a = 9.2) to tris buffer (pK_a = 8), provided the pH is kept constant, produces a counter ion in the latter case which is less charged. Where the counter ion in the mobile phase was less charged than the ammonium ion used in the initial investigations, then the retention of the analytes increased since the counter ion was less effective at displacing the amines from their interactions with silanol groups in the stationary phase. When alkyl columns were used in separating basic compounds they were shown to have a good sensitivity to small differences in molecular structure.

6.2 Future Work

- Since it is common for combinations of two or more medicinal products to be mixed in managing palliative care, further stability studies should be done to evaluate compatibility and stability of drugs in such combinations. In fact there were a number of other examples which could have been worked on during the course of the current study but time did not allow this.
- Acidic and neutral molecules would be examined on silica based stationary phase under HILIC like interaction mode.
- A wider comparison could be made between different alkyl phases used in HILIC like mode. The effect of different base silica gels on the retention properties of the phase could be investigated.
- A more thorough investigation could be carried out in order to determine the exact mechanisms at work during chromatography of bases on alkyl phases in HILIC like mode. This would require using an extended range of phases and test probes under HILIC conditions.
- Silica based stationary phases under HILIC mode showed a great sensitivity towards small differences in molecular structure, therefore the potential in the separation of isomers could be studied.

Publications Arising from the Thesis

Al-Tannak N.F., Cable C.G., McArthur D.A. , Watson D.G. A stability indicating assay for a combination of morphine sulphate with levomepromazine hydrochloride used in palliative care (2012). J. Clin. Pharm. Therap. 37, 71-73.

Al-Tannak N.F., Bawazeer, S., Siddiqui T.H. and Watson D.G. (2011) The hydrophilic interaction like properties of some reversed phase high performance liquid chromatography columns in the analysis of basic compounds. J. Chromatography A. 1218, 1486-91.