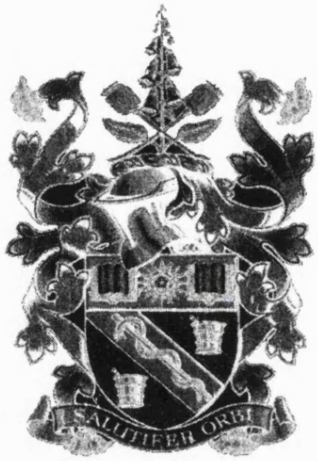
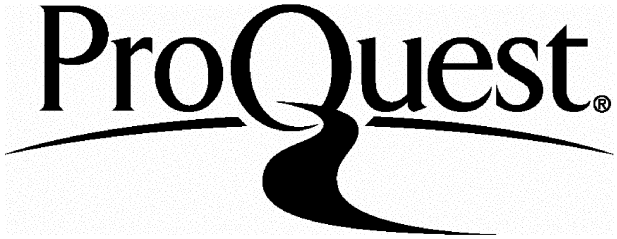
The development of high resolution techniques for the surveillance of medicines

Robert K. Waters

The School of Pharmacy
University of London
October 2000

A Thesis submitted to the University of London in partial fulfilment of the requirements for the degree of Doctor of Philosophy.





ABSTRACT

There is a continuing requirement for the development of rapid and accurate methods for the identification and quantification of drugs particularly in generic pharmaceutical formulations and forensic samples. The use of surveillance methods, (post-market), is of interest to the pharmaceutical industry and regulatory authorities alike. The rise of generic and parallel imported drug products means there are many more sources of the ever increasing range of drug formulations.

In the following work a non-buffered gradient HPLC system was considered as the basis of a rapid drug screening method. The system was originally developed by Gill *et al* for use in The Forensic Science Service, UK. Here the system has been developed further and tested as a surveillance technique on 'real' pharmaceutical samples.

Gradient and isocratic versions of the system were set up on a modular Gilson HPLC (column: Inertsil ODS-2), (System 1) and on a Waters Millennium system (column: Waters Symmetry), (System 2). A database of gradient retention indices for 178 and 237 was obtained on Systems 1 and 2 respectively.

System predictability and inter-laboratory transfer of data were investigated. A group of 28 barbiturates were run on System 1. The system demonstrated good repeatability with retention index values showing coefficients of variation of less than 0.46% for gradient and 0.23% for isocratic runs. Retention values were correlated with a range of structural and physical parameters to test the selective behaviour of the system. Correlation with molecular connectivity values was poor as was the correlation with GLC retention index values ($r = 0.45$). Correlation of the HPLC retention indices with molar volume ($r = 0.831$) and Log P values ($r = 0.891$) suggested these were factors affecting retention. Excellent correlation between the experimental retention indices and calculated indices, derived by the addition of substituent component values to the barbituric acid core, was obtained. This demonstrated the internal consistency and predictability of the system.

Using System 2, variations in eluent pH, column temperature and eluent composition were used to demonstrate the excellent robustness of the system. The repeatability of the 16 nitro-n-alkanes used to assign index values to the retention times of four acidic and

four basic drugs, were not affected by the applied conditions. The mean standard deviation for these retention times was less than 0.1 minute. The variation in retention indices for the acidic and basic drugs was monitored with changing conditions.

Gradient retention indices of 73 drugs run on System 1 common to those run on System 2 gave a correlation of r = 0.986. A data set of 237 drugs run on System 2 was used to generate a database of retention indices to demonstrate inter-laboratory transfer. These retention indices were obtained from the equations that described the correlations between System 2 retention indices and retention data given by nine other groups on various systems.

System 2 utilised a photodiode array detector so that ultraviolet spectra, as well as retention index data could be used to identify a drug. A modern polymeric column that could tolerate high pH was investigated as an orthoganol technique. It was demonstrated that for some drugs a change in ionisation state led to a different retention index and UV spectrum.

System 2 was used to test the surveillance procedure on sample supplied by a pharmaceutical exporter. Four powders for injection were considered. The active ingredient in each case was identified and quantified, the 95% confidence limits for the quantification fell within the BP limits for each drug. The decomposition of a new formulation of 5-aminolevulinic acid was monitored using the system demonstrating its flexibility for other analytical tasks.

The use of Near Infrared techniques for rapid, non-destructive analysis of drug samples was considered. In particular the role of glass in the transflectance of light was investigated by reflecting incident light on glass vials containing mercury and then correcting the spectra of the drug in glass with the result.

Acknowledgements

I would like to thank Professor A. T. Florence for awarding a School of Pharmacy Studentship for this work. Waters Chromatography are thanked for supplying HPLC equipment, service and training in support of this work. I would also like to thank FOSS NIR-Systems for the use of their NIR instrument.

I am grateful to Professor Tony Moffat for granting me the opportunity to return to The School of Pharmacy to study for this degree and for his guidance throughout my studies. I also acknowledge Professor Moffat's work in establishing of the Centre for Pharmaceutical Analysis and the great strides he has made in procuring equipment and finances for the laboratory.
I thank my immediate supervisor of Dr. Robert Watt for all his assistance with the following study. His help was supplied unstintingly and is gratefully acknowledged.

I must thank those who also assisted with the work; Dr. Roger Jee for his comments, Derek Marley and Andrea Gleich for their practical help.

Acknowledgements are also due to those people too numerous to mention, in the stores, departmental office and computer department who all helped me with these studies or matters associated with day to day college life.

Thanks to Pervaise Khan colleague and friend who made life in the lab interesting and humorous! I would also like to thank all those friends with whom I shared a varied sporting and social life - times that will be much missed.

I must thank my parents for their support especially through the undergraduate years.
Finally I owe deepest thanks to my wife Claire, who has been so very patient and supportive over the last few years.

TABLE OF CONTENTS

1. INTRODUCTION

1.1 BACKGROUND

The British Pharmacopoeia (BP) [1] has provided the backbone of drug analysis methods employed by the regulatory authorities for the past few centuries. Regulatory and forensic scientists use those methods and additional data provided by such texts as Clarke's Isolation and Identification of Drugs [2] as well as methods developed in-house. BP methods involve many wet chemistry techniques to identify and quantify drugs with a high degree of certainty. The BP methods are often expensive, time consuming and require highly trained staff. Further the BP is slow to produce methods for new drug substances and does not cover all substances that may need identifying and quantifying in the forensic area of analysis.

The post-market surveillance of medicinal products is carried out by agencies such as the Medicines Testing Laboratories (MTL) on behalf of the Royal Pharmaceutical Society of Great Britain (RPSGB) and the Medicines Control Agency (MCA). Recent changes towards a free market, mean that these services may be put out to competitive tender to any suitable applications laboratory. Although the following work is highly relevant to the forensic field it is the issues associated with post-market surveillance of drugs that it principally seeks to address.

The issue of post-market surveillance work is of growing importance. This is in part due to the sheer increase in volume of dispensed medicines. The Prescription Pricing Authority reported an increase of 110 million dispensed prescriptions, from 330 million to 440 million over the period 1986-1996 [3]. The problems in monitoring medicine stocks are compounded by exploding populations of new drug entities and the move towards novel drug delivery systems, leading to multiple formulations of both new and existing drugs. For the most part proprietary medicines that are within their expiry dates and stored correctly have only a small chance of offering quality problems. The main source of quality problems is expected to arise from the increased use of generic and Parallel Imported (PI) medicines. These represent

medicines that have come off patent and those that are made under license abroad and then imported into the UK respectively. These are both legitimate ways of producing medicines but they are also a difficult area for the regulatory authorities to police. Finally these two areas are the most likely target for the counterfeiter. Generics and PI medicines offer simple and multiple varieties of packaging for the counterfeiter to copy. Also the physical appearance of the medicine often varies making them easier to copy than the proprietary version. Additionally both of these medicine groups have a more complicated supply chain than proprietary drugs that is therefore more open to abuse. Counterfeiting can be defined as the supply of medicines which do not carry the full history of production and quality control of authentic material as produced by a properly licensed manufacturer. Counterfeiting may range from the supply of stolen or counterfeit drugs in forged packaging to the supply of medicines which contain no drug or the wrong drug. A counterfeit may contain the right drug in the right quantities with different excipients from the original. There has even been anecdotal evidence of a pharmaceutical company's staff producing their own stocks of a medicine out of hours for sale on the black-market. The potential for counterfeiters and the production of substandard medicines or the deterioration of good quality stocks during bad transport or storage conditions is now huge. The full extent of these problems has not yet been uncovered.

One step towards uncovering these problems is the use of a rapid screening system that determines whether each medicine contains what it says it does (the correct drug), in the right quantity and contains no additional drugs or dangerous excipients. Sterility testing would also be needed for relevant products but it is the former issues that will be considered in this thesis. Once a measure of the problems has been made, then attention can be focused on the less dramatic quality issues.

The logic for this surveillance method begins with the premise that if one takes a medicine off the shelf in a pharmacy there is a high expectation that it contains what it says it does and the rapid screening system is required to indicate serious faults.

Identification by rapid, suitably validated methods should be sufficient and need not proceed to full pharmacopoeial testing. Nor should a surveillance method need to

express the highest degree of accuracy possible in order to reveal dangerous errors in the medicines manufacture. The screening process could be used to monitor far higher numbers of samples than is presently the case. Once the screening system is revealing a certain level of seriously compromised drug products then resources might be adjusted to target a random population of the drug products that passed the initial screening with traditional Pharmacopoeial testing and specific product tests. These would ensure licensing, manufacturing and storage regulations are being adhered to in the day to day running of legitimate manufacturers, wholesalers and pharmacies. Such a surveillance method should be able to attain its required efficiency for the identification and quantification of samples by use of a single method and should be easily transferrable to a variety of different laboratories. The method must also be capable of handling a large throughput of samples, for example, by being capable of at least partial automation. High-performance liquid chromatography (HPLC) fits many of the criteria required in such a surveillance operation. HPLC has become the analytical method of choice for routine quality control in the pharmaceutical manufacturing industry and, suitably validated, is well accepted by the regulatory authorities as part of most product licence applications, although its introduction into the repertoire of BP methods has perhaps been slow. HPLC has also provided the backbone of forensic drug screening and many of the best examples of methods for individual drug identification in the literature have emanated from forensic analysis. Thus, the work presented in this thesis will discuss the development, validation and demonstration of the scope of a much more general or universal method of HPLC drug surveillance than has been common in forensic and pharmaceutical analysis. The use of HPLC as an alternative to other traditional methods and its use in conjunction to other modern techniques of chromatographic and spectroscopic analysis will also be considered.

1.2 ESTABLISHED AND NEW METHODS OF DRUG SURVEILLANCE.

A number of chromatographic techniques have been extensively investigated for the purpose of drug identification, often used in conjunction with a variety of spectroscopic methods to allow selectivity and sensitivity of detection. Thin layer Chromatography (TLC) has been used extensively for the identification and characterisation of drug substances [4,5,6,7]. TLC offers low cost analyses and good selectivity, with a variety of stationary phases and solvent systems available for separation and many reagents available for individual compound identification.

Owen *et al* [8] used discriminating power in order to choose a suitable TLC system for the analysis of a given basic or acidic drug. The reduction of systematic errors and hence improvement of the inter-laboratory transfer of TLC data (particularly for basic compounds), by the use of reference compounds has also been demonstrated by Flanagan *et al* [9]. However, while TLC is low cost and can be rapid, it has major drawbacks as a screening technique. Firstly if the compounds of interest are unknown or of an unknown class, then the selection of the initial solvent system is difficult. Resolution is usually poor, although modern plates coated in small uniform particles of, for example, ODS (octadecyl silica), are available and offer improved resolution leading to the term high performance – thin layer chromatography (HP-TLC). While quantification using TLC is possible using, for example, optical densiometers or radio-labelled samples it does not always provide the most precise results. High-performance TLC products have allowed quantification of specific compounds with improved precision [10].

Gas-liquid chromatography (GLC) has also been used extensively for drug identification. GLC is a reliable, selective and very sensitive method of drug screening with a long history of use among forensic scientists. As far back as 1975, Moffat [11] proposed that with the large number of stationary phases in use, retention indices would provide a more reliable method for drug identification than retention times and relative retention times for the inter-laboratory transfer data. The effect derivatisation had on the separation of barbiturates with GLC was also noted [12]. Methylation of the

barbiturates gave an improvement to peak shape but contracted the distribution of the retention index data. The issue of derivatisation is a major obstacle to GLC as a front-line drug surveillance technique in the context we are aiming for. While derivatisation may often improve or make possible the GLC determination of a drug by forming a less polar more volatile species, it does introduce extra handling stages into the determination. Also, if a drug to be examined is taken off the shelf in the pharmacy we do not know whether any adulterants present will be sufficiently volatile to be detected on a given GLC system. Even if we know that the drug supposed to be in the formulation requires derivatisation, that reaction would need to be carefully controlled and compared to a standard in order to give an accurate and precise quantification. This process may be further complicated by unlisted or adulterant excipient compounds present in the formulation.

Gill *et al* [12] explored the ability of TLC, GLC and HPLC to identify a series of barbiturates using a computer search system. For these compounds HPLC using an ODS column gave the highest spread of retention data, with TLC giving the lowest resolution. This work examined the correlation of results between these different systems. Further work [13] demonstrated a computer search system using multiple data sets of TLC, GLC and UV maxima which could achieve different discrimination indices for a particular drug by using various combinations of the data. The combination of different techniques, e.g. GLC with TLC, are described as orthogonal techniques when the results are obtained by different retention mechanisms, i.e. when there is a low correlation of data between the two systems. This usually leads to an increase in discrimination or identification power. This works well for a series of unrelated compounds, but if the series are related like the barbiturates then higher correlations between different techniques might be seen thus reducing discriminating power [12].

Advances in detection techniques have also improved the selectivity and sensitivity of chromatographic separation methods.

The so called hyphenated techniques such as gas chromatography with mass spectroscopy, (GC-MS) and HPLC-MS have been investigated. These hyphenated

methods have found specific use with drugs that are considered difficult to detect in forensic samples, such as high potency opiates.

The use of HPLC-MS is discussed in a review paper by Drummer [14]. This method offers great sensitivity when analysing particular classes of compounds. Developments in interfacing and ionization techniques mean that eluent may be diverted into an ionization chamber at atmospheric pressure. Thermo-spray devices atomize and superheat eluent flow before ionization. This method allows flow rates of up to 2mls/min therefore this is a suitable method to couple with standard HPLC systems. Electro-spray uses similar vaporization methods but much of the solvent is stripped away by a flow of nitrogen gas and the vapourised eluent passed over a highly charged probe producing a simple spectrum containing the molecular ion. Electro-spray offers great sensitivity however is limited to flow rates of up to 1 ml/min which is more suitable for coupling with micro-bore HPLC [15]. Electro-spray is most suited to polar molecules. Atomospheric pressure ionisation can be used to ionise less polar molecules at higher flow rates than Electro-spray.

These advances combined with improved ion separation technology such as ion trap, have allowed compact bench-top systems with greater practicality for the forensic or field analyst. The increased sensitivity is of great benefit to forensic scientists concerned with examining small samples after extraction procedures. Such systems might be usefully applied to the surveillance of pharmaceuticals, in that excipients not picked up by other detection methods could be analysed with great sensitivity. However although improving, chromatographic/MS interfaces still require a great deal of technical attention during routine use.

HPLC has also benefited from the advance in detection methods, particularly from the major development in UV detection in the form of the photo-diode array detector (PDA). The PDA allows absorption of UV/visible light by the eluent and analytes to be measured at multiple wavelengths simultaneously. The identification power of separation techniques coupled with such a detectors was increased by offering a UV spectra of an eluted peak, in addition to its retention properties. PDA detectors gave

analysts the ability to view the spectral make-up at time sections across a given chromatographic peak. This allowed the measurement of peak purity.

Fell *et al* [16] investigated the application of computer deconvolution techniques to chromatographic UV data in order to identify co-eluting peaks of closely related opiate drugs. Suppression techniques were used to allow more accurate quantification of the principal peak by removing the spectral contribution of the impurity. The use of a modern PDA detection system in a drug surveillance method was attractive since it allowed rapid identification as well as sensitive peak purity and quantification data.

There are new techniques receiving much development which might be of interest as pharmaceutical surveillance methods. Capillary Zone electrophoresis (CZE) is a method of separation attained by applying a large potential difference across a narrow capillary (usually made of fused silica) filled with a mobile phase containing an electrolyte. Analytes move with the electro-osmotic flow at a rate determined by their charge and ionic radius. This method offers resolving power many times greater than HPLC with shorter running times and lower all round costs. The method is not however as robust as HPLC and the sensitivity is lower. The high selectivity does have great surveillance potential in terms of enantiomer and excipient detection due to its very high resolving power. The use of CZE for systematic drug analysis is not wide spread although separation and UV detection of drugs of abuse has been reported [17,18].

It was noted that the resolution of particular compounds requires optimization of the different methods. This is because the factors that control migration are more complex than in HPLC, e.g. the effect of the capillary wall coating on electro-osmotic flow or localized heating due to the applied potential. Surfactants may be added to the mobile phase to increase the resolution this is called Micellar Electrokinetic Chromatography, (MEC) and further adds to the complexity of this method of separation. Although this method is cheap and has short analysis times, it does not yet offer ease of use or robustness for mass pharmaceutical surveillance.

Spectra in the Near-Infrared (NIR) region of light absorption have long been known to contain large amounts of chemical structure data, but it is only with aid of complex chemometrics and increases in computer power that useful information has begun to

become accessible to the analyst. The method can be non-destructive, use no additional consumables and can produce spectral information within a few minutes. NIR has the ability to identify drugs and excipients, detecting moisture content and physical properties, e.g. blend uniformity. Quantification of drugs using NIR is currently an active area of research. NIR is discussed extensively in this thesis and use of the technique for pharmaceutical analysis has been reviewed by Blanco *et al* [19].

NIR is plagued with problems of transferability but does offer the most promising new method for drug surveillance. Potentially, identification and quantification of a pharmaceutical's components could be achieved in a single rapid step, rather than the extraction, separation and detection steps required by the above methods.

1.3 THE DEVELOPMENT OF REVERSED-PHASE HPLC

High-performance liquid chromatography came about after Martin and Synge [20] realised the potential of classical adsorption chromatography. They showed that for adsorption chromatography to be efficient small particle size was required for the solid phase, and in turn the liquid phase must be placed under pressure to pass over the solid phase in a reasonable time.

Initially adsorption chromatography used polar solid phases such as silica and alumina eluting with non-polar eluents. The technique was improved by the development of reproducible high pressure pumps and different methods of detection, e.g. ultraviolet, fluorescence, refractive index. This method however required non-aqueous solvents and analytes to be extracted from their aqueous media. Further, slight changes of solvent water content, even those due to laboratory humidity, may cause changes to the chromatography.

Different separation techniques were developed using columns with different properties, including liquid-liquid partition, ion pair and ion exchange chromatography, size exclusion chromatography and chromatography with bonded

phases. Reversed-phase has probably become the most important chromatographic technique using bonded phases.

Reversed-phase chromatography [21] is a variation of partition chromatography utilising a non-polar hydrocarbon stationary phase and a polar alcohol or water based solvent as the mobile phase. Reversed-phase high performance chromatography (RP-HPLC), is a technique which uses non-polar microparticulate (5μm or smaller) stationary phases. The use of hydrocarbon bonded silicas have so far provided the majority of these stationary phases. RP-HPLC can efficiently separate a wide range of the chemicals which now make up the vast drug market. It is this technique that is most useful for the separation of modern drugs which are relatively low molecular weight, with significant lipophillic and more often than not, basic character, e.g. opiates, local anaesthetics, amphetamines, antihistamines etc.

The favoured method of producing RP silica bonded phases is given by reactions with substituted monochlorosilane [22] as shown in Figure 1.3.1

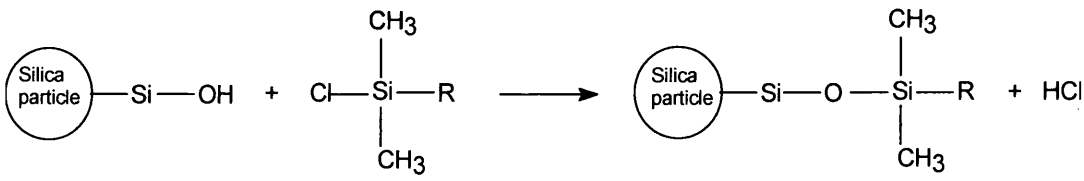


Figure 1.3.1 Formation of RP silica where R is the functional group e.g. -$(CH_2)_{17}CH_3$

Substituted dichlorosilane may be bonded to two silanol groups to give extended chains, leading to increased shielding and pH stability for the silica backbone. However, using second or third reactive functional groups can lead to such silanising agents reacting with each other in difficult to control reactions. Phenyl and butyl groups have been bonded to the silica but most use octyl or octadecyl as the functional groups.

After the functional layer is complete unreacted silanol groups are still present due to steric crowding. Additional octadecyl groups cannot fit between the existing chains

yet smaller molecules can fit in and give unwanted silanol interactions. These unreacted silanols need to be reacted with smaller molecules to prevent peak tailing especially of basic analytes. This process is known as end capping. Free silanols are reacted with trimethylchlorosilane to produce this blockade effect. Even after this reaction a large proportion of silanol groups remain unreacted.

The silanol surface in early bonded phases was not by any means uniform. Figure 1.3.2 shows silanol types; free silanols, geminal silanols and silanols associated by hydrogen bonding. Metals activating silanol groups by electron withdrawal are also shown, which causes considerable increases in the acidity of free silanols. Older silicas containing these heterogenous surfaces are currently classified as type A packings and the newer purer silicas are classified as type B packings.

Type B packings are engineered to give more geminal and associated silanols and fewer acidic silanols, i.e. free and metal activated silanols. These 'basic' silicas offer less tailing particularly with basic analytes [23].

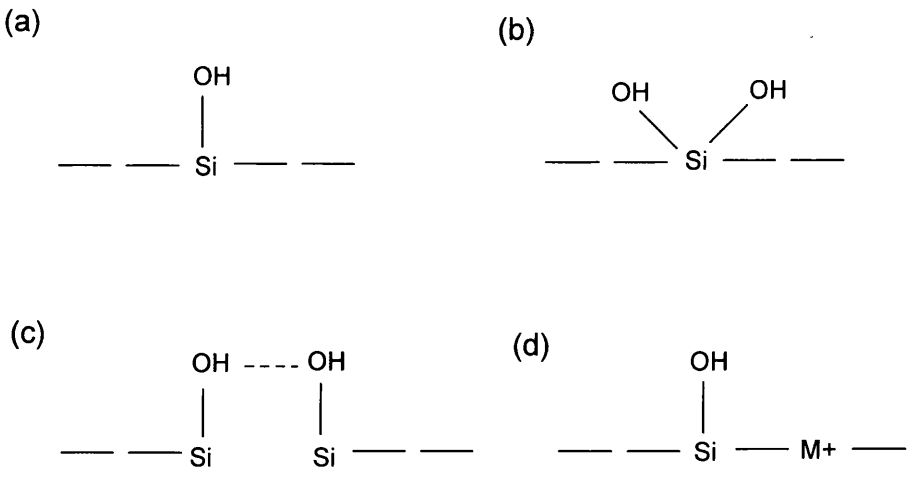


Figure 1.3.2 possible configurations at the silica surface: (a) free silanols (b) geminal silanols (c) Hydrogen bonded silanols (d) Metal activated silanols.

After reacting to form the bonded phase type B silicas still display a large number of free silanols, albeit less reactive ones. The interaction of remaining silanols with basic functions are sometimes reduced by the addition of amines to the mobile phase

in order to 'compete' with analyte for interaction with these sites, so reducing peak tailing. Alternatively, lowering the pH of the mobile phase to below the pKa of the silanols can achieve the same effect. The pH range of different silanol groups has been discussed [24,25] and the lowest pka is generally taken to be about 2.0. However this may lead to restrictions on the separation one is trying to achieve, therefore further modification of the bonded phase has been introduced.

The latest stage in the development of RP solid phases has been to shield these silanols. This may be achieved by steric protection given by bulky groups at the 'silanol end' of the bonded material. Alternatively the bonded phase may contain a functional group such as an amide or a carbamate – these are the so-called intrinsically base deactivated materials [26]. The mechanism by which these groups block the silanol groups is achieved is not clear. Possible mechanisms are shown in Figure 1.3.3. The analyte might interact with polar functions on the carbamate function in preference to the silica. The polar function of the carbamate might be interacting with the silica itself. Finally the carbamate function might be hydrogen bonding with water, increasing the water content at the surface layer and so reducing the analyte interaction with the silanol surface. These materials have been shown to further improve peak shape [26].

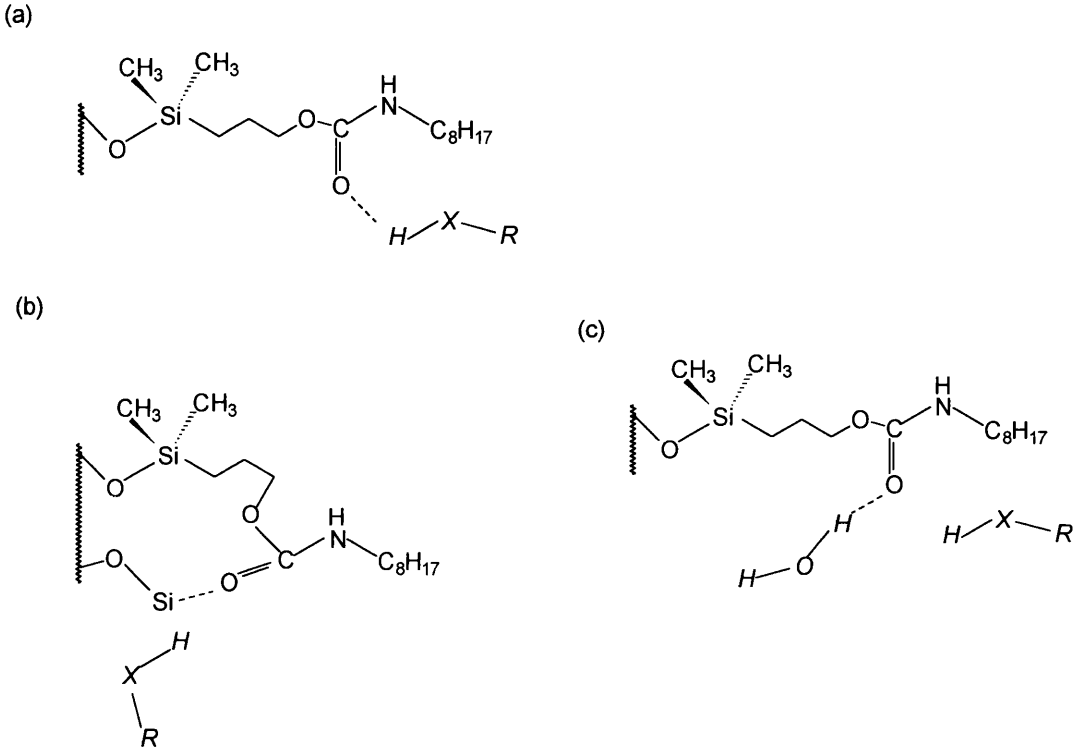


Figure 1.3.3 Showing the possible mechanisms by which base deactivation reduces analyte interaction with the silanol surface. (a) The analyte interacts with the carbamate functional group in preference to the silanol surface. (b) The analyte might compete with the polar function for silanol interaction. (c) The carbamate function raises water concentration at the silanol surface.

The silica based materials (Inertsil ODS-2, GL sciences, Japan and Waters Symmetry, Waters, USA), used in this work are both base deactivated columns based on low metal content type B silicas.

Reversed-phase packing materials may also be polymer based and these have the advantage of offering a wide tolerance to pH changes. The main aim of polymer based packing was however to eliminate the silanol interactions that plagued the chromatography of basic compounds.

Polymers of styrene and divinylbenzene provide a suitable RP material especially when structures are rigid, i.e. when they contain more than 8% divinylbenzene.

The formation of such a co-polymer is given in Figure 1.3.4. These materials may withstand high pressure and are stable in the pH range 1-13. They also have a macroporous structure and are therefore ideal for the elution of larger molecules.
Octa or octadecyl side chains may be added to the polymer, giving a more inert material than those based on silica.

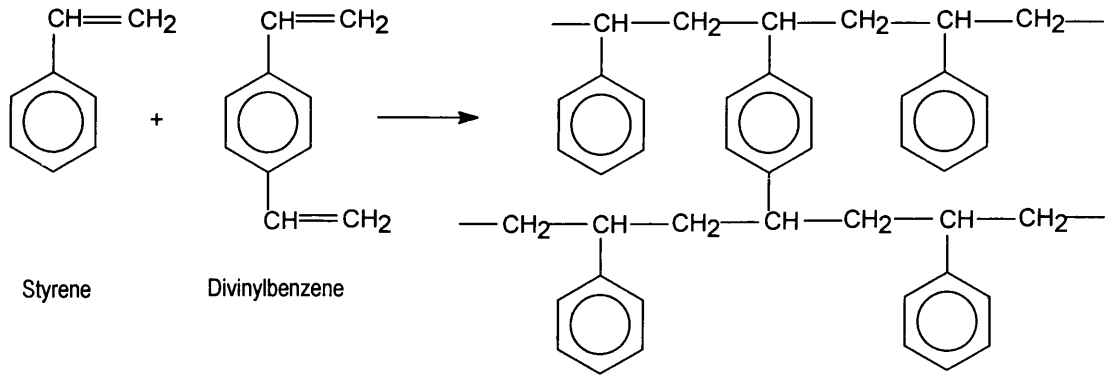


Figure 1.3.4 The formation of a Styrene-divinyl benzene polymer packing.

While silanol interactions have been eliminated it has so far been at the expense of good separations compared to a modern silica RP column.
RP columns are usually 4-6 mm in diameter and 50 to 500 mm in length; particle size is usually 5μm or below and eluent flow rates range from 0.5 - 3 ml/min. As in other types of HPLC the choice of mobile phase is governed by many factors including the viscosity of any mixtures of eluents, the effect of those eluents on detection, their cost and their toxicity.
In RP chromatography water as a polar material will have low eluting power, while non-polar eluents such as tetrahydrofuran will have great eluting power. Aqueous mixtures of methanol or acetonitrile are common eluents for the gradient analysis of low molecular weight drug substances. The use of methanol leads to high pressures as it has high viscosity when mixed with water. Acetonitrile is expensive and toxic but in a pure form gives a low UV cut off typically 190 nm.

Reversed-phase chromatography of drugs usually tends towards lower pH eluents as basic functional groups have significant interactions (particularly ion exchange), with free silanol groups on the silica surface. This is traditionally done using phosphate or acetate buffers. Inorganic acids have been avoided, as HPLC components have not always been so resistant to attack as they are today.

1.4 ADVANTAGES OF MODERN HPLC TECHNOLOGY

Modern HPLC hardware with PEEK and stainless steel fittings is resistant to deterioration by solvent and chemical attack. Such modern HPLC systems offer pumps with sophisticated mechanisms and electronic controls. These provide constant flow rates and accurate compositions even under gradient conditions. Such equipment lends itself to the goal of a reproducible and perhaps transferable HPLC system. However probably the most important technical advances have come in the form of the HPLC columns and packing materials.

Modern silica and polymer based packing materials are contained within precisely engineered and electro-polished stainless steel cartridges. The packing materials are found with a low spread of mean diameters whether they are irregular or spherically shaped particles leading to increased efficiency. The popular octadecyl silane (ODS) columns have been particularly highly developed and have shown dramatic improvements in performance. This is due to a series of developments that reduce the non-partition interactions of analytes with the bonded phase. The reversed-phase materials of today offer; high efficiency, excellent selectivity and most importantly for the purpose of this work, they give good batch to batch reproducibility.

Other methods of detection are used in RP-HPLC but ultra-violet (UV) detection is the most widely used for drug analysis. Today the chromatogram can be monitored at multiple wavelengths using high resolution diode array detectors. Linked with powerful computer software these detectors may provide UV spectra of separated

materials across the peak not only as a tool for identification but also giving a measure of purity.

The retention time of a compound is affected by the velocity of the mobile phase and the column length, therefore retention times are often expressed as retention factors or k as calculated in Equation 1.4.1.

$$k = \frac{t_r - t_0}{t_0}$$

Equation 1.4.1 Calculation of the retention factor *k*

Where t_r = the retention time of the compound and t_0 = the retention time of the void volume, a void volume marker or a standard related to the compound. The use of a standard related to the compound of interest will further protect the measurement against day to day changes in temperature and flow rate and slight variations in the nature of the packing material or mobile phase.

1.5 DEFINITIONS

Throughout this work the terms reproducibility and robustness are used regularly and are of the greatest importance, so their intended meanings are quoted at this point. The definition of reproducibility is taken from The Rules Governing Medicinal Products In The European Community [27]. 'Reproducibility expresses the precision under different conditions', e.g. different laboratories, different analysts or different days. In this work this will be described as inter-assay reproducibility. Where experiments are carried out by the same analyst on the same day using the same equipment, the precision is described as intra-assay reproducibility.

The United States Pharmacopoeia (U.S.P) [28] gives us a definition for robustness: 'the robustness of an analytical procedure is a measure of its capacity to remain unaffected

by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.'

1.6 HPLC SYSTEMS USED IN THIS WORK

This project work was carried out on two HPLC systems - a modular Gilson system (System 1) and a state of the art Waters Millennium (System 2). The latter was provided by the instrument manufacturer in order to develop a database of retention data and UV spectra. The Waters system offered greater precision in most aspects of its operation but the main advantage it offered was a diode array detector. In order to fully assess the application of these systems to rapid drug surveillance a number of key factors had to be examined in detail including optimisation of conditions and data handling. In order for the methods to have the greatest scope for application it is necessary to ensure they are reproducible, robust, have a defined capacity for transferability and behave in a predictable manner, e.g. when faced with sub-structural changes in the drugs under analysis. A comparison of the repeatability of data from these two systems and from literature data is made.

Gradient versus Isocratic performance

The elution profile in reversed-phase HPLC is the most common means of controlling separation. The advantages and disadvantages of isocratic and gradient runs are shown with respect to their possible use for rapid surveillance. The question of elution time and the number of drugs resolved are the obvious differences. The order of drug elution changes also provides a challenge to a transferable system. These questions are investigated using a series of acid and base probes which have a range of molecular weights and pKa values.

Predictability of the HPLC method

As well as proving robustness, reproducibility and repeatability, for an HPLC system to be transferable it must to some extent be predictable. Retention prediction studies

are not necessarily anything new but contribute to knowledge by adding to the understanding of the nature of retention in reversed-phase systems.

It is necessary for this project to demonstrate that the system behaves in a predictable manner if it is to form the basis of a transferable system. The retention selectivity must be a well defined relationship with molecular structure and be one that is comparable to other systems.

Retention prediction is investigated using physicochemical parameters and additive retention indices based on the contribution made by functional groups. The use of the former has been researched extensively on reversed-phase systems and is reviewed by Smith [29] and for example comparison of Log P values and other empirical information with retention indices gives us an understanding of the retention mechanisms at work in this particular system. The latter will highlight any anomalies that certain functional groups may cause in the predictability of the system.

Transferability of the HPLC method

There are many examples of HPLC transferability studies in the literature. The majority of these compare different systems directly or attempt to reproduce results from laboratory to laboratory. The way in which retention of a certain compound is recorded is as important to data reproducibility and consequently transferability as the physical characteristics of a system. A retention time is the simplest expression of a compound's retention on a given column. Slight changes in the elution conditions mean the retention time will be altered dramatically. In time (within the normal life of the column), there will be changes at the surface of the column such that repeatability will be affected. The use of retention factors was designed to overcome these problems. Here the retention time of a compound is reported relative to another. Often a standard is chosen that is hardly retained and therefore represents the time taken for the 'dead' or void volume to pass through the column to the detector. A retention factor of an analyte based on a void volume marker will be protected against changes in the void volume of the system and to a certain extent changes in elution conditions. However if a compound similar in structure to the analyte is used as the reference compound, then shifts in retention behaviour will be similar for both

compounds leading to a relatively constant retention factor. This type of standard will only lead to reproducible results if it is retained by the same mechanism as the compound of interest. This method is not therefore suitable for the analysis of unknown drugs.

The use of retention indices

In the following work retention indices (RI) are used to record retention information. RI methods use a series of standards, (which may or may not be related compounds), which were developed for use in gas chromatographic techniques notably the Kovat's retention indices [30]. Each standard is assigned an arbitrary value, before being run on the chromatographic system. The compound of interest is then run on the system. The retention times of the standards and the compound are then used to determine a value for the compound on the arbitrary scale. The way in which this calculation is made and the compounds chosen as standards, affect the repeatability and reproducibility of a given system; it is an issue as important as the chromatographic equipment employed. The many issues surrounding data handling and its importance to a 'universal' HPLC system are discussed fully.

Other workers attempting to transfer retention data from one system to another have done so largely by direct comparison. They generally found that using the same method for recording retention and a comparable HPLC system gave reasonably transferable results. Some workers tuned their results by using additional correction factors based on an additional set of standards [31]. The possibility of using retention data generated here as the basis of a 'universal' database is investigated.

Applications of the HPLC method

In the development of any research technique it is necessary to demonstrate the application to real problems in order to prove the practicality of the method, e.g. are any changes to the method required for a particular analysis problem? Here the method needed to be proved viable as a rapid surveillance technique for medicinal formulations as well as on pure drug compounds. An extensive study of the application of the method to real commercial powders for injection and for the

investigation of the stability kinetics of a relatively short shelf life pharmaceutical preparation, were used as examples of the viability of the method.

1.7 NIR AS A SURVEILLANCE TECHNIQUE

NIR is the most exciting new prospect for the mass surveillance of pharmaceuticals. The long term goals for NIR research, including quantification and transferability, once reached, will fulfil the philosophy of the surveillance method i.e. is a rapid low cost and non-destructive method that will give all the post-market quality control information required. The potential of this technique must be examined in the context of the philosophy of this work.

NIR is that region found between the visible part of the spectrum and the mid-infrared (mid-IR), i.e. 1100 - 2500 nm. Like the mid-IR, absorption of light in this region causes molecules to exhibit bending and stretching vibrations. However these are transitions at higher energy levels, where the oscillation is affected by surrounding electron densities thus the vibrations here are anharmonic. The NIR spectrum therefore consists of combinations of fundamentals and overtones, distinct peaks representing a specific absorption by a given bond type are not usually seen. However the whole spectrum is highly specific to the compound(s) being analysed, including its physical state, e.g. moisture content or polymorphism. Hence traditional NIR has been in routine use in the food and pharmaceutical industries, for example measuring moisture content. A non-destructive light source illuminates the sample through a variety of probes which then measures reflected/scattered or transmitted light. The absorption and scattering of light at these wavelengths is complex but in return the information given by NIR spectroscopy is highly specific.

There are two methods of sample presentation for the measurement of NIR absorption- transmission and reflectance.

When using transmission the sample must be reasonably transparent to NIR and the instrument is set up in much the same configuration as a conventional IR

spectrophotometer although commonly in single beam mode. A transflectance instrument doubles the path length by placing a reflector behind the sample and is configured with the light source and detector(s) on the same side of the sample.

Reflectance measures the reflected light from a sample as a ratio of the light reflected compared to a reference. NIR penetrates deeply into the sample (1-4 mm) where it may be absorbed. At the wavelengths where the reflected light is reduced by this absorption Beer's law may still hold. It is hoped this method will offer full quantitative capabilities.

Many factors may cause baseline shifts in the reflectance spectra of solids including particle size, polymorphic form and compaction. Derivitisation of spectra to reduce these effects is possible as the signal to noise ratio is large. A number of other chemometric methods of data handling may be used to extract useful data and eliminate unwanted interferences. These are discussed in detail where appropriate.

Qualitative identification information is most readily available and it is this which is investigated in this work. Thus those samples which have been used to demonstrate the application of HPLC surveillance to real pharmaceutical problems have also been investigated by NIR spectroscopy to demonstrate this complementary method. The low energy of the light samples used in NIR spectroscopy provides a non-invasive analytical measurement capable of monitoring real samples in a variety of forms and even through fibre optic probes.

1.8 AIMS OF THIS WORK

In the following work a non-buffered gradient HPLC method of qualitative and quantitative analysis is proposed as a primary, relatively rapid drug screening technique, that will be applicable to the majority of low molecular weight drugs on the market today. The method allows identification and quantification of drugs in a formulation, but not of excipients where present.

The system was originally developed by Gill *et al* [32] for use in the Forensic Science Service,UK. The system uses an inorganic acid as a modifier at a suitable concentration to maintain a low operating pH.

This system will be used to build a database for the purposes of checking the reliability and selectively of identification.

A complementary high pH system is investigated in order to provide increased identification power. The basic system would do this by providing different retention indices and UV spectra when the ionisation state of a compound changes with pH.

Within the industry, regulatory and forensic laboratories, HPLC systems are usually dedicated to the analysis of a particular drug or drug class. The column packing and eluent(s) used will be optimised for this analysis. An analysis of an unusual drug will require a system to be set up, or at worst a whole new method developed. In contrast our 'universal' system is designed to identify and quantify a large range of drugs. The development and practical use of this system is considered. The relationship of the application of this system to other modern analytical techniques will be explored.

2. HPLC MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 High Performance Liquid Chromatography (HPLC) instrumentation

The majority of the work for this thesis was carried out on two pieces of HPLC equipment whose technology differed by ten years. The initial work was carried out on the older system, henceforth known as **System 1**:

A modular Gilson system (Villiers-le-Bel, France) was used, controlled by Gilson 712 software on a IBM PS-2 personal computer via a Gilson 506B system interface. Detection was by a Gilson Holochrome single (variable) wavelength UV detector. A Gilson 231 auto-sampler and 401 dilutor (fitted with a 0.5ml syringe) were used to inject the sample, or sample mixtures, via a 7010 Rheodyne injection port fitted with a 20 µl loop. The auto-sampler was programmed from a separate key pad driven by custom programming language. It was also capable of making co-injections, i.e. withdrawing sample from more than one vial for a single injection. Two 302 Gilson pumps were used to give isocratic and programmed runs. These pumps could be controlled from the Gilson software. High pressure mixing of the eluents was achieved with a Gilson static mixer. The temperature of the column was maintained using a custom built column heater/cooler. The column was kept in a 35cm length of copper piping around which there was wrapped a spiral of copper tubing. Water was circulated around this copper tubing from a heated/cooled water bath. The thermocouple of a Phillips electronic thermometer was placed close to the column inside the copper pipe and the whole construction insulated with foam. Temperature could be maintained to within ± 0.5 °C using this device.

The newer HPLC system was supplied by Waters Chromatography, Milford, Massachusetts, USA, henceforth referred to as **System 2**:

System 2 consisted of a Digital 90 MHz, 24 MB personal computer with a dedicated Bus LAC/E, from which the entire system could be controlled using Waters

Millennium software via IEEE and RS 232 connections to the instrumentation. The Millennium software allowed live monitoring of chromatograms and spectral data. Detection was provided by a Waters photo-diode array detector with a simultaneous detection range of 190 to 600 nm with a resolution of down to 1.2 nm. The lamp of this detector was controlled from the Millennium software, diagnostic assessment of the lamp's condition were also available here. A Waters model 700 auto-sampler with a 96, 2ml vial capacity tray was used. This autosampler was fitted with a 200µl loop and a temperature control module with a range of 4 - 40°C. Precision for this autosampler was claimed to be <0.5 % relative standard deviation (RSD) for injections of 5-50µl. A Waters on-line vacuum degasser preceded the pump and was controllable from the Millennium software. The Waters 600 pump had two pump heads with lines for four separate eluents. Some low pressure mixing occurred before these pump heads, but most of the mixing took place in two high pressure mixing blocks after the pump heads. Below flow rates of 2ml an algorithm could be applied electronically to the pumps to provide a constant head of pressure. Column temperature was maintained by a Waters temperature control module with an accuracy of ± 0.5°C.

2.1.2 Drug samples and standards

Pure drug samples were gathered from libraries at the School of Pharmacy, London; The Forensic Science Service, Home Office Laboratories, Aldermaston, Berkshire, UK; The Medicines Testing Laboratories, Edinburgh, UK. Nitro-n-alkanes used as external standards, containing 1 - 6 carbon atoms were obtained from Aldrich Chem. Co. LTD, Poole, Dorset, UK. Nitro-n-alkanes containing 7 - 16 carbon atoms were custom synthesised by Cookson chemicals LTD, Southampton, UK and obtained from Dr. R. Gill.

2.1.3 HPLC eluent components

For System 1: Double de-ionised water (produced by a MilliQ system). Acetonitrile, Hypersolve grade, BDH, Poole, Dorset, UK Sulphuric Acid, Primar, Fisons, Loughborough , UK.

For System 2: As for System 1 except: Water, HPLC grade, BDH, Poole, Dorset, UK Acetonitrile, Far UV grade, BDH, Poole, Dorset, UK.

2.1.4 HPLC reversed-phase columns

For the Gilson system: Inertsil ODS-2, GI Sciences Inc., Japan (250 × 4.6mm).

For the Waters system: Waters Symmetry (C-18), Waters Chromatography, Milford, Massachusetts, USA. Two RS-paks D18-613 and ODP50 4 E, Shodex, from Waters Chromatography.

2.1.5 Other equipment

25-100µl Gilson (Anachem, Luton, Beds., UK), displacement pipettes. Heat resistant GC syringes, (50 µl). Chromacol (Anachem, Luton, Beds., UK), 2ml vials and caps. Waters 2ml vials and caps. Lambda 15 (Perkin Elmer, Norwalk, Connecticut, USA). 9410 Digital pH meter (Philips, Cambridge, UK).

2.2 METHODS

2.2.1 Sample preparation for HPLC (Systems 1 and 2)

Pure drug samples were assessed in order to determine a concentration that would give an absorbance of less than 2 absorbance units (AU) when using the HPLC with UV detection. The extinction coefficient [2,33], was used to estimate the appropriate weight of sample to be used. These weights of drug in injection solution were typically 0.5-1 mg/ml for injection volumes of 5-20 µl. The drug was weighed

directly into the vials and dissolved in a fixed amount of acetonitrile/water, (the Chromacol and Waters vials both had a volume of 2ml).

Drug samples were largely found to be soluble in a mixture of 50:50 acetonitrile/water, the proportions were changed or the solvent changed to dissolve poorly soluble drugs. Once in solution the drug samples were not filtered. The Gilson based system and the Waters based system both used capped vials, these vials were not re-used.

The C_1 - C_{16} nitro-n-alkanes standards were stored at 8°C. Each nitro-n-alkane (10µl) was diluted to 10ml with acetonitrile in a volumetric flask. Nitro-n-alkanes C_8 - C_{16} were warmed in a drying oven to make them fluid enough to draw up and inject with a warmed, heat resistant GC syringe. The resulting mixture of nitro-n-alkanes in acetonitrile was stored in the refrigerator at 8°C.

A saturated solution of glycine was used as a void volume marker and was co-injected with each sample, i.e. the glycine was drawn from a separate vial but the two were was co-injected with the sample; Glycine solution (5µl) was injected with each sample.

2.2.2 Preparation of HPLC eluents for System 1

Sulphuric acid (2.5 M) was prepared from sulphuric acid (135ml) added to double de-ionised water (700ml). This was allowed to cool and transferred to a 1l volumetric flask and made up to volume.

Eluent A: Sulphuric acid (0.5ml of 2.5M) was added to double deionised water (to 500ml) in a volumetric flask and inverted to mix.

Eluent B: Sulphuric acid (0.5ml of 2.5M) was added to acetonitrile (to 500ml) in a volumetric flask and inverted to mix.

These eluents were not filtered or degassed but transferred to conical flasks and covered with aluminium foil during use.

2.2.3 Preparation of HPLC eluents for System 2

As for System 1 except the grade of acetonitrile and water used, as specified in 2.1.3, additionally the eluents were vacuum degassed on line.

2.2.4 The standard gradient and isocratic runs on System 1

The 712 HPLC software and the autosampler controller had to be triggered simultaneously at the beginning of each run, after this point the software was a slave to the autosampler controller. The 712 software controlled the flow from the two pumps. The programmed gradient run began with the autosampler set to wait 10 minutes while the software set the pumps to 98% eluent A and 2% eluent B to equilibrate the column for 10 minutes. The autosampler then injected the sample(s). At this point the software began its integration, i.e. time 0 minutes on the chromatogram. These conditions were maintained by the software for a further 3 minutes as part of the gradient programmme. The percentage of eluent B was then raised linearly over 23 minutes to 98% and held at this composition for 5 minutes. Finally the eluent composition was returned to 98% eluent A over 2 minutes. For subsequent injections the software was triggered by the beginning of the auto-sampler's cycle at interval 34 minutes after injection. The gradient profile is given in Appendix I. For most experiments the column temperature was maintained at 25°C. The Holochrome UV detector was set to 215nm.

For isocratic elution 2 minutes of equilibration time was given at the desired eluent mixture. The integration and injection occurred simultaneously and the run continued for a further 20 minutes.

2.2.5 The standard gradient run on System 2

The vial positions in the autosampler tray, injection volumes and the run times were entered on the 717 auto-sampler's key-pad. The gradient was otherwise controlled from the Millennium software. For most gradient analyses the beginning of the gradient was synchronised with the time of injection and started at 98% eluent A and 2% eluent B which was maintained for 3 minutes. The proportion of eluent B was

then raised linearly to 98% over 23 minutes and stayed at this composition for 10 minutes. The composition was returned to 98% eluent A over 2 minutes and the system equilibrated to the end of the gradient with a total run time of 46 minutes. The gradient profile is given in Appendix I.

Integration began at 0 minutes and was inhibited after 36 minutes. The column temperature was maintained at 40°C. The chromatograms were monitored and reported at 215nm, although the Millennium software was set to gather spectral data from 190 to 450nm with a spectral resolution of 1.2nm. (Gradient elution on System 2 is shown in Appendix 1).

2.2.6 Presentation of retention data

Retention data were recorded as retention times, retention factors (k) or retention indices (RI). Retention times were expressed in minutes. k values were calculated as in Equation 1.4.1.

RI values for gradient and isocratic runs were calculated using Equations 2.2.1 and 2.2.2 respectively.

$$RI = 100\left[n + \left(\frac{(t_r - t_n)}{(t_{n+1} - t_n)}\right)\right]$$

(Equation 2.2.1 calculation of gradient RI)

$$RI = 100\left[n + \left(\frac{(Logt_r - Logt_n)}{(Logt_{n+1} - Logt_n)}\right)\right]$$

(Equation 2.2.1 calculation of isocratic RI)

Equations 2.2.1 & 2.2.2 used to calculate gradient and isocratic retention indices respectively. where: RI = retention index of a drug; n = number of carbons in the nitro-n-alkane eluting immediately before the drug; t_r = retention time of the drug; t_n = retention time of the nitro-n-alkane eluting immediately before the drug; t_{n+1} = retention time of the nitro-n-alkane eluting immediately after the drug.

2.2.7 Using UV spectra for identification using System 2

While retention indices usually provide for a rapid initial identification of a sample's component(s), examination of the UV spectrum can yield valuable confirmation and additional data. Identification of spectra can be carried out in one of two ways. Often spectra are compared visually, "the sample spectra has the same absorbance maxima and spectral shape as the standard spectra". Otherwise a standard may be run on a system utilising multi-wavelength detection, followed by the test sample. The two resulting spectra may then be compared mathematically. In the case of chromatography, especially gradient chromatography, it is important to obtain a standard spectrum under the same experimental conditions. This is because the eluent composition is unlikely to match any of the solvents used in standard texts. Further the degree of ionisation of an analyte at any given proportion of organic eluent will vary. Therefore the type of spectra obtained could depend on where in the gradient the solute elutes.

The Waters Millennium Chromatography software allows the storage of UV spectra in any number of libraries and a sample's UV spectrum may be compared to the library spectra as it elutes [34]. First the software performs a baseline correction on the spectrum by interpolation between liftoff and touchdown spectra. This baseline spectra is then subtracted from the sample spectra. This baseline corrected spectrum is then converted to a vector. This vector angle is then compared to the vector angle of the spectrum in the library.

The software also determines how much of this spectral difference is due to noise and solvent effects. Noise effects are determined from a section of baseline, which is at least five peak widths wide and free from any peaks. Solvent effects are user specified and are set at the highest level after which repeated injections of the same sample are not spectrally matched under a particular set of elution conditions.

If the angle between a sample and library vector representation is less than the vector angle contributed by noise and solvent effects then the two spectra are considered to match.

3. DEVELOPMENT OF THE RAPID HPLC ANALYSIS SYSTEM

Gradient or programmed elution HPLC describes a chromatographic run in which the eluting power is increased in a linear, staged or non-linear manner with respect to time. Such a technique is employed to separate two or more substances with a large difference in their retention properties in a single run of not significantly longer duration than an isocratic run might be. Ideally all compounds should be eluted between a *k* value of between 2-8 (below 2 and resolution is poorer and above 8 run times may become longer than necessary to achieve efficient resolution). Here, we are using a reversed-phase HPLC system so the increase in eluting power comes from an increase in the non-polar eluent B, with a corresponding reduction of the water based eluent A. This system was originally developed by Gill *et al* [32] at the Forensic Science Laboratories, Aldermaston, Berkshire, UK.

HPLC was in routine use at the Forensic Science Laboratories for the analysis of toxicological substances of interest, ranging from biological samples to powders believed to contain illicit drugs. HPLC systems were dedicated i.e. a given instrument was set up to identify and quantify a particular group of drugs e.g. opiates. This meant the careful design and monitoring of operating procedures and the expense of using different columns and eluents. More importantly it meant that HPLC could not be used as a rapid front line screening technique for samples presented to the laboratories awaiting analysis as evidence for court cases.

HPLC equipment development has provided more accurate pumping systems and sensitive detectors. But the biggest factor previously leading to irreproducibility of retention results has been the ever changing nature of solute-column interactions. In terms of the octadecasilane (ODS) column the use of type B silica supports have improved the batch to batch reproducibility of modern ODS materials. The use of external standards to assign retention indices has greatly increased the repeatability and reproducibility of retention data.

Gill *et al* [32] designed their 'universal' HPLC system to give the broadest range of reproducible compound identification possible while remaining simple to set up and operate. In our extension of this work we have examined the stability and transferability of the system and used a diode array detector to allow greater identification power and give accurate, corrected absorbance data that could improve peak area integration. The Inertsil ODS-2 column was chosen as it gave the best compound resolution and column-to-column reproducibility of the C-18 columns available at that time. The eluent system was required to be simple and of low pH in order to protonate any free silanol groups. Sulphuric acid diluted to about pH 2.2 was chosen as a simple modifier.

Buffered systems were thought to increase wear on the pumps and possibly introduce contaminants into the eluents. A water/acetonitrile based eluent was chosen as acetonitrile has a low UV cut-off, at about 190nm.

In all our work primary retention data was converted to retention indices using homologous nitro-n-alkanes as retention index standards. Nitro-n-alkanes were chosen as the external standards because the retention behaviour of these compounds was shown to be predictable under isocratic conditions and stable to pH changes in the mobile phase [35]. The nitro-n-alkanes have also been proposed as the best general retention scale for drug identification using gas chromatography [36].

Using HPLC the first nitro-n-alkane C_1 was found to elute before most polar compounds, which alleviated a problem found in previously used index sets such as the alkyl aryl ketones [37,38,39]. The importance of the retention index system is discussed fully in Chapter 4.

As part of the development of reliable surveillance methods the discrimination power should ideally be examined and if possible increased. The HPLC system employed here is based on a simple acidic eluent system. The use of an additional high pH system using the nitro-n-alkane retention index scale would lead to increased identification power since it is an orthogonal technique to the acidic system. That is to say many compounds will show different retention and UV properties once the pH of the eluent has shifted above that of a given compound's pKa. New column

technologies include C-18 columns bonded to polymer material which can be used across the full pH range, unlike silica based materials which dissolve at high pH. Some of the new polymer based columns are investigated as the basis for a high pH system.

Gradient elution provides rapid elution of a wide range of drugs, indeed all the drugs tested in these experiments. However identification and quantification have long been best served by isocratic elution. Isocratic elution can give better resolution of closely related drugs than gradient elution. This means that isocratic retention information has often been used in retention prediction studies. This is because up until this point pump technology has given more reproducible retention times and peak areas when the ratio of the eluents remains constant.

Reproducibility of gradient retention times are reduced by extra-column volumes and mixing inaccuracies. These effects can lead to alteration of retention times and in some cases loss of resolution [40]. Yet the advent of digitally controlled pumps and high pressure mixing systems has improved the potential of gradient elution by reducing mixing errors and extra-column volumes. The use of retention index markers will also help increase transferability from one system to another, although the behaviour of a given set of standards is not always reflected in the retention behaviour of the compound(s) of interest.

In order to develop such a screening method further it is important to have some understanding of the method's ability to discriminate between many different compounds. The discriminating power of a chromatographic system can be defined as the probability that two compounds chosen at random would be separated in that system [41]. The principle of discriminating power is applied to the acidic retention indices and UV spectra. However insufficient data was generated to show the increase in identification power with the corresponding high pH system.

Robustness of the gradient system is investigated to help demonstrate the suitability of the method for surveillance and as the basis of a transferable retention data set. The building of a structured isocratic database is also described and a comparison made with the gradient system.

3.1 SETTING UP THE GRADIENT RUN ON SYSTEM 1

The first requirement of this work was to reproduce and validate some of the results generated by Gill *et al* [32] at the Forensic Science Laboratories. The system was shown to be comparable to the original by showing the long term repeatability of the nitro-n-alkanes retention times and attaining RIs for a number of drugs that could be compared to the results given by Gill *et al* [32]. A correlation coefficient r = 0.998 was obtained for 44 retention indices of drugs run under gradient conditions.

The system was set up as described in the method although initially the 10 minute equilibration period was considered unduly long for a surveillance method and was replaced by one of only 3 minutes.

The retention times of the void volume marker and the nitro-n-alkanes C_1-C_{16} are given in Appendix II, 61 runs were recorded over a period of 5 months on a single column. Figure 3.1.1 shows a comparison of the coefficients of variation for the 16 nitro-n-alkanes and glycine for 30 runs on the following systems: System 1, Gill *et al* [32] and System 2 over 3 months.

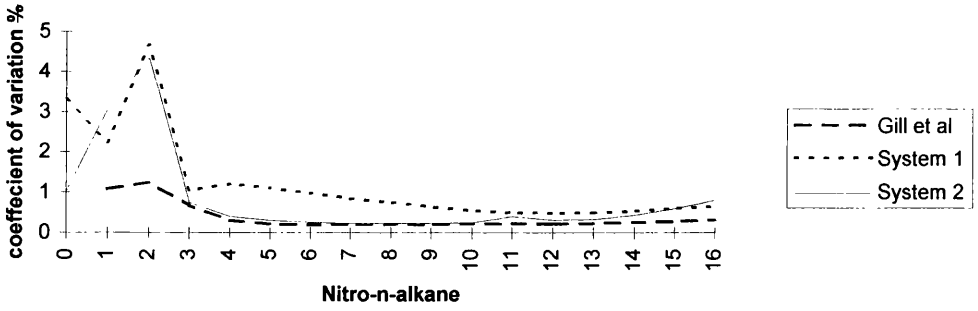


Figure 3.1.1 Coefficient of variation of retention times (%) for the nitro-n-alkanes, for System 1, 2 and Gill *et al* [32].

System 1 shows a coefficient of variation of up to 4.75 % for nitro-n-alkane C-2, but for Gill *et al* [32] this value is only 1.2 %. The retention times for C-2 and C-7 on System 1 correspond to the beginning and end of the gradient ramp respectively. This would suggest some eluent pumping or mixing errors were occurring with the older Gilson equipment especially with the pump handling the aqueous eluent component. In addition the Gill *et al* [32] system used a ten minute equilibration time at 98 % eluent B at the end of the run and ten minutes at 98 % eluent A at the beginning of the next. However the poorer coefficients of variation for retention times of these index standards did not translate into unreasonable variations in retention index of drugs from run to run. Appendix VIII contains a data set of gradient and isocratic retention indices for a set of barbiturates which were run four times under isocratic and gradient conditions. The highest coefficient of variation seen was 1.9 % for butalbarbitone under gradient conditions. This means that high repeatability of retention indices is seen on System 1 even using the older HPLC equipment.

3.2 SETTING UP THE GRADIENT RUN ON SYSTEM 2

The programmed run was set up on the Waters based system using a Waters Symmetry column. The Symmetry column was described as having similar chromatographic properties as the Inertsil ODS columns and Waters were keen to establish this column as an alternative. The use of this column was to be a requirement of the use of the Waters equipment.

Symmetry columns are examples of base deactivated columns and the general applicability of these columns to toxicological analyses has been demonstrated [42]. Basic drugs were found to be resolved under less specific elution conditions without the need to resort to the addition of specific modifiers when compared to the conditions required to achieve resolution on traditional silica based reversed phase materials. Further the Inertsil ODS-2 and the Symmetry columns were found to be very similar after characterisation of 30 reversed-phase columns using seven

physicochemical parameters and a variety of statistical representations [43]. The columns were closely matched for hydrophobicity, ion exchange and hydrogen bonding capacities.

The next step in the development of the system was the comparison with results obtained on this equipment and column with those given by Gill *et al* [32] and System 1. The retention times of the void volume marker and the 16 nitro-n-alkanes are given in Appendix II, and cover a period of nine months on two columns. Data from one column over three months is compared with similar data from System 1 and Gill *et al* [32] in Figure 3.1.1. Compared to System 1, a large coefficient of variation is seen for C-2. There is a lower coefficient of variation from C-3 to C-10, comparable to that of Gill *et al* [32] although towards the end of the run the variation is seen to rise again like System 1. The HPLC equipment used in System 2 is more modern than that used by Gill *et al* [32], therefore the better reproducibility obtained by Gill *et al* [32] for the nitro-n-alkanes must have been due to the longer equilibration. The general recommendation for column re-equilibration is 10-15 column volumes (about 28 minutes on System 2), after a full gradient elution, however in this work it been determined empirically.

Using System 2 a library of 237 drugs retention indices and UV spectra were obtained which was initially intended to be used as a powerful searchable database for various forensic and pharmaceutical screening purposes. These retention indices and UV spectral data are presented in Appendix III. The retention indices of 73 drugs common to System 1 and 2 gave a correlation coefficient of 0.990.

3.3 ROBUSTNESS TESTING OF SYSTEM 2

A robust set of retention data was required as the basis of a transferable or universal database. A 'robust' HPLC system is one that is stable to deliberate variations in the operating conditions. The system should not be sensitive to small changes in pH, pumping accuracy or temperature. The use of a retention index system may help to

overcome these problems. In a robustness study, Smith *et al* [44] used a buffered isocratic reversed-phase HPLC system with alkyl aryl ketones as external standards to determine the retention indices of a group of barbiturates. Temperature fluctuations and slight pH variations were found to cause small changes to retention indices, as the unionised standards and the ionisable barbiturates had different sensitivities to these factors. It was also shown, that in this buffered system, retention indices were virtually unchanged when the proportion of methanol was increased from 30% to 50%. These findings agreed with a similar study using the nitro-n-alkanes as standards [45].

The effect of temperature variations depends on the enthalpy of the solute-stationary phase interaction and a logarithmic relationship has been shown between retention factors and enthalpy [46]. Increases in temperature reduce the retention times of compounds run on isocratic systems to a greater extent than gradient elution [47]. However gradient elution is more likely to result in selectivity changes when the temperature changes. The nature of this selectivity depends on the compounds being eluted, this effect has been investigated as a tool for controlling selectivity [48]. Equilibration of temperature is important as plate numbers rise with an increase in temperature giving narrow peaks. If the mobile phase entering the column is not at the same temperature as that column then distortion of eluting peaks may occur. To avoid this, the eluent must either be pre-heated/cooled, or at least as much of the pre-column tubing must be placed in the column heater/cooler as is practicable. The operating temperature of 40°C was chosen for this system such that users in most countries might heat their columns rather than trying to cool them. We placed the column heater close to the pump outlets in order to enclose as much of the pre-column tubing as possible.

Statistical approaches to robustness testing have been investigated [49] as a means to examine the combined effects of experimental conditions upon retention and quantification. These regression methods can generate a standard error on for example peak area, that reflects day to day changes in experimental conditions.

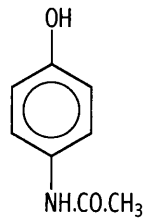
The robustness of System 2 to changes in temperature, pH and mobile phase composition were investigated. The effects were monitored using a set of acidic drugs and a set of basic drugs which were indexed against a series of nitro-n-alkanes. The acidic and basic drugs are typical of those that are routinely analysed on this system, they display a range of pKa values and are therefore ideally suited to this type of robustness testing. Structures and some physicochemical data of these compounds are presented in Figures 3.3.1 and 3.3.2.

A 50:50 acetonitrile/water mixture was prepared. Approximately 8 to 10mg of each of the acidic and basic drugs were dissolved in two separate aliquots (10ml) of the acetonitrile/water mixture.

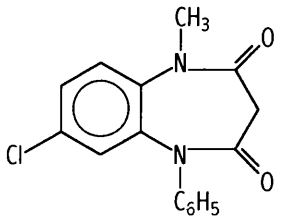
The nitro-n-alkane standard mixture and the acids and bases (5µl of each co-injected with 15µl of a saturated glycine solution), were run on the gradient three times under the standard conditions given above. All retention times and retention indices were recorded. These were re-recorded after each of the following changes to the method:

1. The percentage of eluent 'A' (the aqueous component), was reduced by 1% (of the total composition), over the entire gradient.
2. The percentage of eluent 'A' was raised by 1% over the entire gradient.
3. The standard sets were run at 5°C above and below the standard column temperature.
4. The amount of acid added was halved and doubled. The resulting pH of the 'normal', 'low acid' and 'high acid' aqueous eluents were measured using a Phillips 9410 digital pH meter.

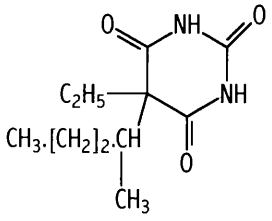
Acidic drug	Molecular weight	pKa	Log P [50]
Paracetamol	151.2	9.5	0.27
Pentobarbitone	226.3	8.0	2.00
Clobazam	300.7		1.82
Indomethacin	357.8	4.5	4.23



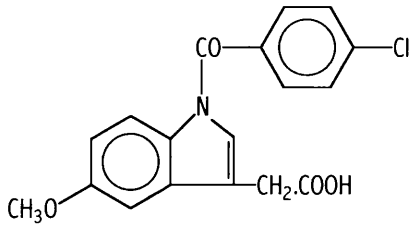
Paracetamol



Clobazam



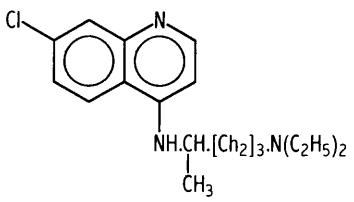
Pentobarbitone



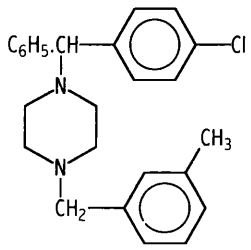
Indomethacin

Figure 3.3.1 Some properties of the acidic compounds used to investigate robustness and their structures. The Octanol/water partition coefficients were calculated according to the rules of Meylan & Howard [50].

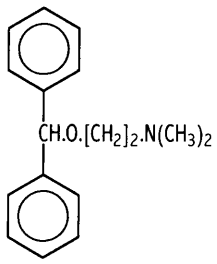
Basic drug	Molecular weight	pKa	Log P [50]
Chloroquine	319.9	8.4, 10.8	4.5
Diphenhydramine	255.4	9.0	3.11
Meclozine	391.0	3.1, 6.2	5.87
Amiodarone	681.8	5.6	8.81



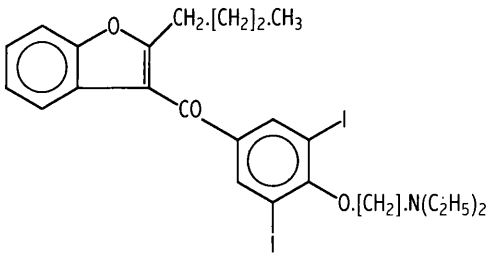
Chloroquine



Meclozine



Diphenhydramine



Amiodarone

Figure 3.3.2 Some properties of the basic compounds used to investigate robustness and their structures. The Octanol/water partition coefficients were calculated according to the rules of Meylan & Howard [50].

3.3.1 Effect of the applied changes on the nitro-n-alkanes

The retention times and indices of the robustness testing on the acidic and basic standards are given in Appendix IV.

The mean values of the retention time shifts from normal conditions for the 16 nitro-n-alkanes are given in Table 3.3.1. Using these mean figures, nitro-n-alkane retention appears to be most affected by changes in column temperature. However, when individual nitro-n-alkane retention time variations are considered, the latter part of the gradient shows greater variations due to changes in eluent composition. The resulting pH of the normal aqueous eluent (1ml/L 2.5M sulphuric acid), was 2.35, the low acid aqueous eluent (0.5ml/L 2.5M sulphuric acid), was 2.57 and the high acid aqueous eluent (1ml/L 2.5M sulphuric acid), was 2.15. The retention of the nitro-n-alkanes were unaffected by these changes in pH.

The mean standard deviation column (Table 3.3.1) represents the means of the standard deviations for the retention times of the nitro-n-alkanes after three runs. In each case this value is below 0.1 minutes and was not affected by the applied conditions, hence repeatability is excellent for the system.

Conditions	Retention time shift (mean of 16 nitro-n-alkanes)(mins).	Mean of standard deviations for each nitro-n-alkane given by 3 runs (mins).
Normal	0	0.044
Pump 'A' low (-1% of eluent A)	-0.26	0.029
Pump 'A' high (+1% of eluent A)	0.22	0.048
35°C	1.46	0.068
45°C	-0.58	0.032
High acid	-0.03	0.051
Low acid	-0.02	0.012

Table 3.3.1 Mean shifts of retention time of the 16 nitro-n-alkane standards and the mean of their standard deviations given by three runs.

3.3.2 Changes in eluent composition

The change in eluent composition of ± 1 % of eluent 'A' was selected as representing a reasonable difference one might expect between two given HPLC systems.

For the acidic and basic drugs the earliest eluting compounds (paracetamol and chloroquine) showed greater sensitivity to the eluent composition than later eluting compounds which were remarkably stable to these changes (Tables 3.3.2 and 3.3.3). However it has been shown that much larger changes in the proportion of acetonitrile may cause significant shifts in pKa of analytes [51]. Such large changes occur across the gradient and this complicates the effect of pH changes.

3.3.3 Changes in pH

The pH change was not great when the sulphuric acid concentration was halved and doubled as such changes (when using a strong acid), do not greatly affect pH (~ ± 0.22 pH units). However the changes in the amount of sulphuric acid added were intended to represent an obvious error an operator might make when preparing the eluents.

The alteration of acid concentration in the eluent showed no marked effect on the retention time of the nitro-n-alkanes. In our non-buffered system the acidic compounds showed slightly decreased RI values at lower pH while the bases showed an increase in retention indices, the opposite of the result expected from theory given later see Figure 3.3.3.

Among the acidic drugs indomethacin showed a variation of 3.96 RI units between the high and low acid concentrations as compared to variations of up to 7 RI units which are routinely recorded for repeat runs of a given drug under standard conditions. Apart from amiodarone the changes from normal conditions did not cause RI variations greater than 7 RI units. Amiodarone showed a large RI change, 25 units, between the high and low acid concentrations. The change in organic content of the eluent across the gradient means that the analytes will be subject to shifting pKa complicating the matter of relating the effect of pH on retention.

Schoenmakers *et al* [52] investigated the effects of variation in pH and organic modifier concentration on reversed-phase HPLC with a phosphate/citrate buffered isocratic system. From theoretical equilibrium considerations it was suggested that the retention factor for an acidic solute should decrease with increasing pH, a basic compound should show the opposite behaviour with an increase in retention factor with increasing pH, this relationship is shown in Figure 3.3.3.

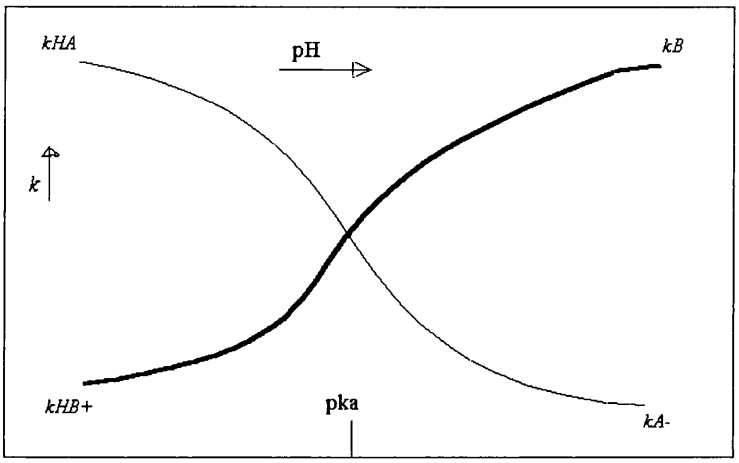


Figure 3.3.3 The expected relationship between pH and retention factor for acidic and basic compounds on a reversed-phase buffered isocratic system [52].

Schoenmakers *et al* [52] concluded that variation of pH may have a variable effect on retention shifts, dependent on the analyte, the column or the nature and proportion of the organic content. When optimising gradient RP-HPLC by varying pH, Goga *et al* [51] concluded that standardisation could be acheived in conjunction with commensurate changes in the proportion of organic modifier present in the eluent.
With our non buffered gradient system where the pH was well below the pKa of any of the compounds only the expected slight changes in retention indices were observed.

3.3.4 Changes in temperature

The potential effects of temperature changes on retention in HPLC are quite complex as the equilibrium of partition of a solute between mobile and stationary phase is altered through enthalpy and entropy factors as expressed by the van't Hoff

relationships as well as other extrothermodynamic quantities [46]. In addition changes in temperature can alter the equilibrium of ionisation of a drug and of the mobile phase. Indeed the pH of water itself is sensitive to temperature. In general retention times are seen to decrease with rising temperature [46].

The mean retention time of the nitro-n-alkanes was increased by 1.46 minutes (the largest shift), when the column temperature was reduced to 35°C.

The retention indices of the acidic drugs did not display significant changes at the lower or higher temperature. The retention indices of the bases were affected slightly more by temperature variations than the acidic drugs. The bases generally showed a decrease in RI with the reduction in temperature and an increase in RI at the higher temperature.

Acids: Conditions	Paracetamol	Pentobarbitone	Clobazam	Indomethacin	Mean standard deviation (RI)
Normal	240.6	382.6	454.9	587.8	1.20
Pump'A'low (-1% of eluent A)	236.9	383.6	455.6	587.8	0.57
Pump'A' high (+1% of eluent A)	243.0	382.8	454.9	588.0	1.49
35°C	240.5	382.8	454.9	588.0	1.06
45°C	240.0	383.9	456.1	587.7	0.42
High acid	238.4	382.3	452.1	584.1	2.55
Low acid	241.4	383.0	454.8	588.0	0.52

Table 3.3.2 The retention indices of the acidic drugs and the mean of all their standard deviations from three runs.

Bases: conditions	Chloroquine	Diphenhydramine	Meclozine	Amiodarone	Mean standard deviation (RI)
Normal	242.1	305.0	393.5	466.2	0.75
Pump'A' low (-1% of eluent A)	240.1	304.4	393.8	466.4	0.65
Pump'A' high (+1% of eluent A)	243.3	305.6	393.9	467.9	2.14
35°C	239.8	302.8	395.3	464.9	2.19
45°C	242.3	306.7	395.3	469.9	1.01
High acid	243.1	309.9	399.0	483.4	1.67
Low acid	240.1	301.9	391.1	458.8	0.55

Table 3.3.3 The retention indices of the basic drugs and the mean of all their standard deviations from three runs.

The results show good general robustness for the system. However individual compounds have shown unpredictable behaviour. These compounds highlight the importance of empirical robustness testing when the retention index, is influenced in a complex manner by the prevailing experimental conditions.

3.4 SETTING UP A HIGH pH GRADIENT RUN ON SYSTEM 2

Identification using the Waters system could be much improved by the use of UV diode array detection in addition to the use of RIs. Maier and Bogusz [53] compared the identification power of HPLC RIs linked to UV identification, with the combination of capillary GC RIs and UV identification. They found that HPLC/UV was slightly inferior.

The Millennium software integral with the Waters HPLC equipment utilised in System 2 allows the UV spectrum of a sample to be compared to a library of UV spectra of pure materials.

It was proposed that the identification power of our HPLC system could be greatly increased if an orthoganol method was used in parallel within a given analysis protocol. A high pH HPLC system was considered, as many individual compounds should show different retention indices and UV spectra due to a change in ionisation state at basic pH as compared to the strongly acidic conditions of the established method. The stable routine use of a high pH HPLC method depends on advances in modern column technology.

Any high pH HPLC system will require HPLC equipment and columns that are resistant to basic conditions. Previously, alkyl bonded silica columns have been used at high pH with eluents containing a high proportion of organic component [54], various buffers were tried [55,56,57,58] but only poor selectivity or peak shapes were obtained. Recently these methods were combined with a double end capped C-18 column protected by an untreated silica pre-column [59] in order to increase stability and column life. This work included the description of a new column later marketed as Zorbax Extend [60]. This is a densely packed bidentate C-18, double end capped materiai based on an ultrapure type B silica. Provided this column is used with organic buffers and methanol as the organic mobile phase this column gives good separations and is stable up to pH 11.

Divinylbenzene polymer columns are another alternative, however the properties of such columns were investigated by Gawdzik *et al* [61,62,63] and were found to suffer from low column efficiencies. Attempts to separate basic drugs at high pH gave poor peak shape [64]. Further, the retention index given by the alkyl aryl ketone scale were unpredictable due to the interaction of some bases with these columns which are weakly acidic.

Shodex now manufacture a column that is a C-18 supported by a polymethacrylate polymer. The manufacturer specifications state that the column is for routine reversed-phase use with a wide range of solvents and is able to tolerate pHs in the range 2 - 12. This column (RS-pak D18-613) was 150 x 6 mm, different dimensions to the other columns used in the acidic system.

Another polymeric column supplied by Shodex (ODP504e) 250 x 4.6 mm offered the same dimensions as the column used in the acidic system and a working pH range of 0 - 14. This material has been shown to be stable to long term use at high pH [65]. Both columns claimed to have better batch-to-batch reproducibility than silica supports.

3.4.1 Using Shodex RS-pak D18-613 column

The behaviour of the RS-pak D18-613 was examined first. In the first instance, the programme run remained the same as the acidic gradient except the sulphuric acid was substituted by a basic modifier. Sodium hydroxide was selected as a simple modifier that would give a high pH at a dosage of 1 ml/L of 1 M solution.

Figure 3.4.1 shows a chromatogram of glycine and the 16 nitro-n-alkanes run on the gradient without any modifier. Split and unresolved peaks can be seen, sodium hydroxide (1 ml of 1M) was added to each litre of water, (leading to a pH of 10.2) and acetonitrile to be used as eluents in the gradient run. Figure 3.4.2 shows glycine and the nitro-n-alkanes were resolved under these conditions, although there was some disruption at the front end of the chromatogram.

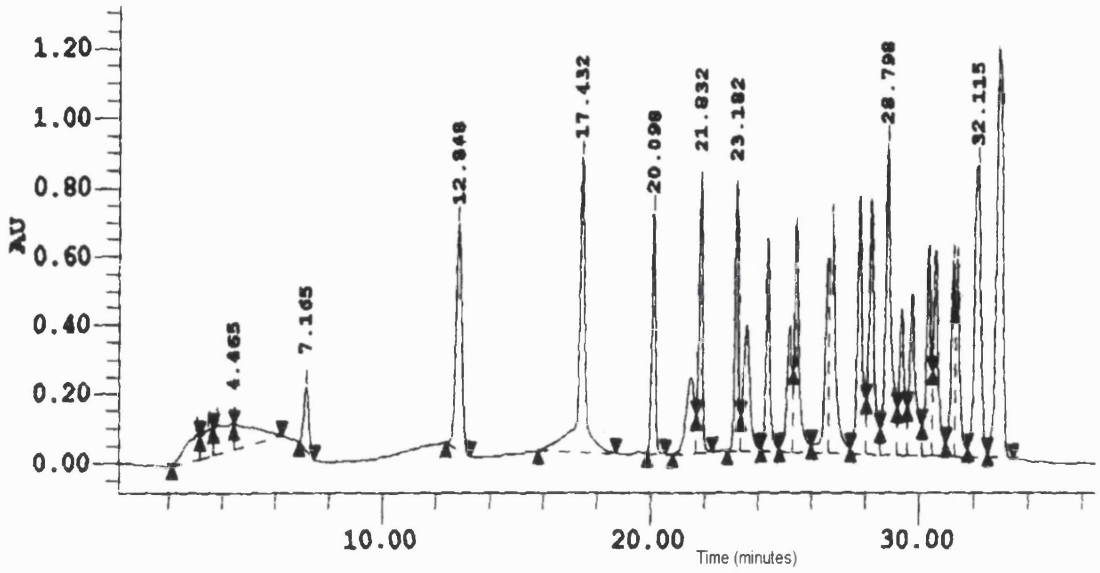


Figure 3.4.1 Gradient run of glycine and the nitro-n-alkanes on the RS-Pak D18-613 in the abscence of a modifier.

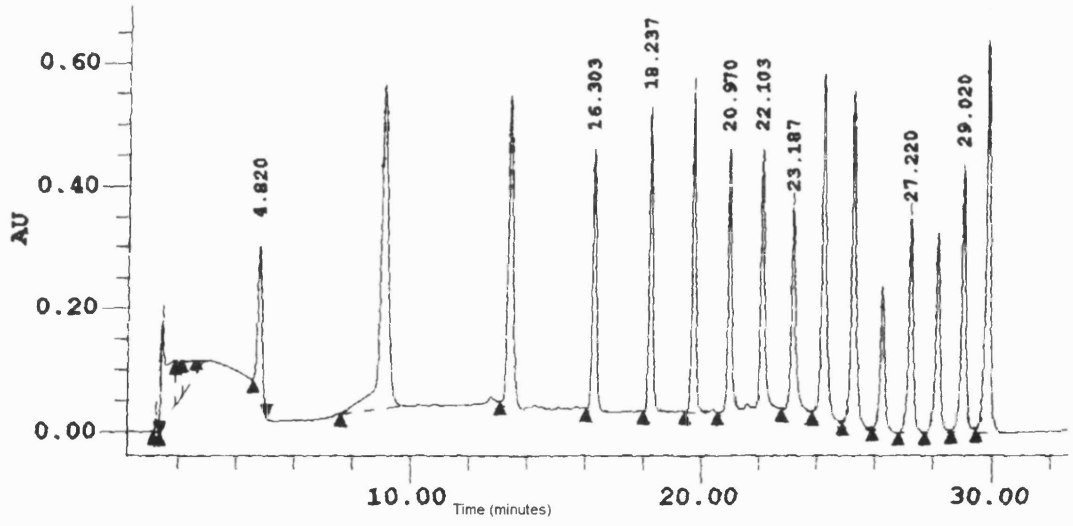


Figure 3.4.2 Gradient run of glycine and the nitro-n-alkanes on the RS-Pak D18-613 with 1ml of 1M NaOH added to each litre of eluent (pH 10.2).

The same acid and base standards as used in the non-buffered acidic system (Section 3.3), were run on the high pH system but it failed to satisfactorily resolve these

compounds. Further it was noted that precipitation occurred in the acetonitrile eluent containing the sodium hydroxide. This later lead to blockages in the system.

Potassium hydroxide (approximately 1M) was prepared and added to the water and acetonitrile resulting in a lower pH of 9.7. This resulted in a lesser precipitation problem and good resolution of the nitro-n-alkanes however again the acid or base standards were not resolved.

Tri-ethylamine (1M) was added to the water and acetonitrile eluents (1ml/L), but a sharp rise in the baseline was seen as the proportion of acetonitrile was increased. Therefore the maximum proportion of acetonitrile was reduced from 98% to 88% and the time taken to reach this concentration was reduced to fifteen minutes. It was hoped that this modified gradient would reduce baseline effects seen on the chromatogram when monitoring in the UV region between 215-230 nm while keeping the retention time spread of the nitroalkanes comparable to the established system. The reduction of the time taken to reach the maximum proportion of acetonitrile was supposed to counteract the increase in elution time given by the overall reduction in acetonitrile proportion of the eluent i.e. higher concentrations of acetonitrile were reached earlier in the gradient. This resulted in a minimal reduction in the rise in the baseline seen in the chromatogram. However the acid and basic standards were still not resolved.

Ammonium acetate was also investigated as a basic modifier with the same gradient profile as used as for the tri-ethylamine. Ammonium acetate (2M 100mls) was made up to volume (1000mls) water and acetonitrile to give the two eluents. A rising baseline was again seen on the chromatograms although the acid and base standards were resolved (see Figures 3.4.3 and 3.4.4 respectively). The first two acids were resolved poorly with broad peaks.

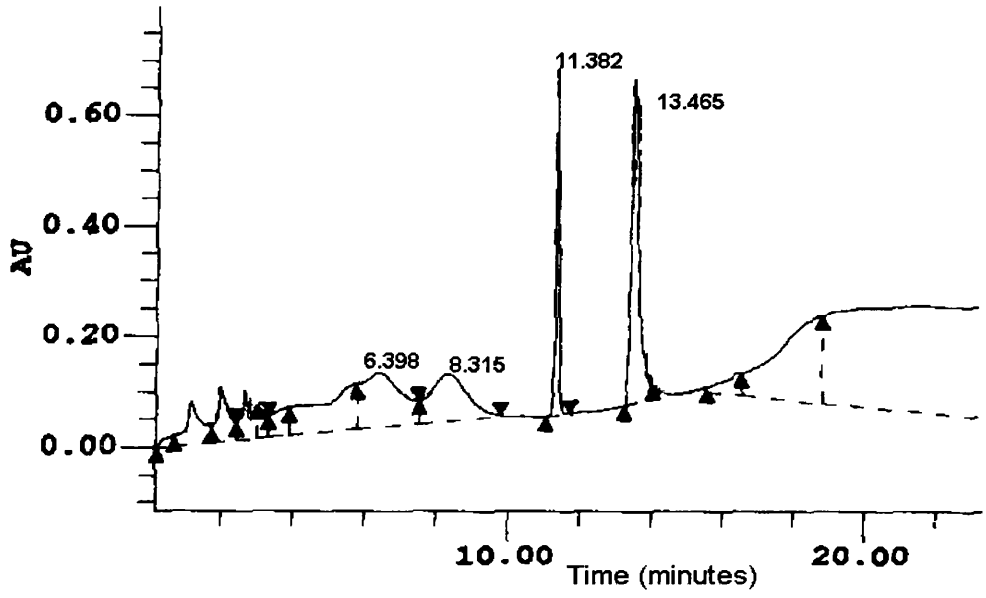


Figure 3.4.3 Gradient run of the acid standards on the RS-Pak D18-613 with 100mls of 2M of ammonium acetate added to each 900 mls of eluent.

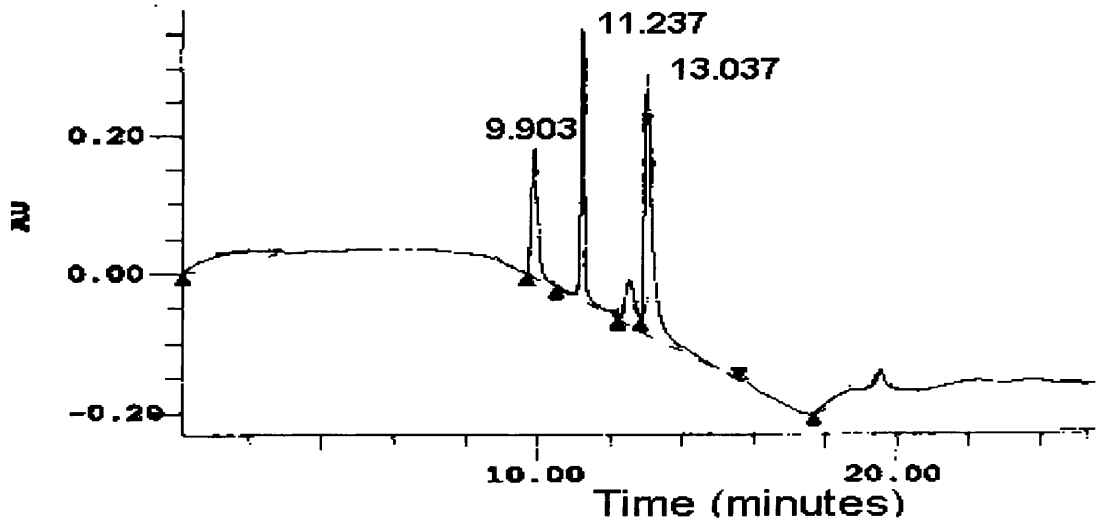


Figure 3.4.4 Gradient run of the basic standards on the RS-Pak D18-613 with 100mls of 2M of ammonium acetate added to each 900 mls of eluent.

3.4.2 Using Shodex RS-pak ODP504e

The alternative column, the Shodex RS-Pak ODP504e, was then investigated. The manufacturer claimed this column could operate across the full pH range. Initially

sodium hydroxide (1M 0.5ml/L) was used as the modifier, in this application it was bled in through a separate line at 2% of the total volume to give an aqueous pH of 10.2. The percentage of water and acetonitrile were each reduced by 1% to accommodate the sodium hydroxide solutions. At the end of the run a three minute washout period with water only from a fourth line was included, as it was hoped that this would prevent the build up of precipitates in the system. The gradient profile is given in Appendix I and was based on System 2 with the above changes.

Under these conditions the nitro-n-alkanes were resolved except for the first one and the glycine where there was still some front end disruption. This meant that retention indices under 200 RI units could not be recorded. The nitro-n-alkanes were run on the high pH system four times and their retention times are given in Appendix V. The coefficient of variation for each nitro-n-alkane was about ten-fold smaller than those given by the acidic System 2. This may only reflect the small sample size taken over a short period of time, however these results would suggest that polymer based columns like this are more inert than silica based materials and could offer much higher reproducibility.

The acidic and basic standards were run on the high pH system and their retention times are found in Appendix V (diphenhydramine was not included in the bases due to a supply problem at that time). The repeatability for these drugs are of the same order as those obtained on the acidic System 2. Figures 3.4.5 & 3.4.6 respectively show good resolution of the four acidic and three basic standards respectively.

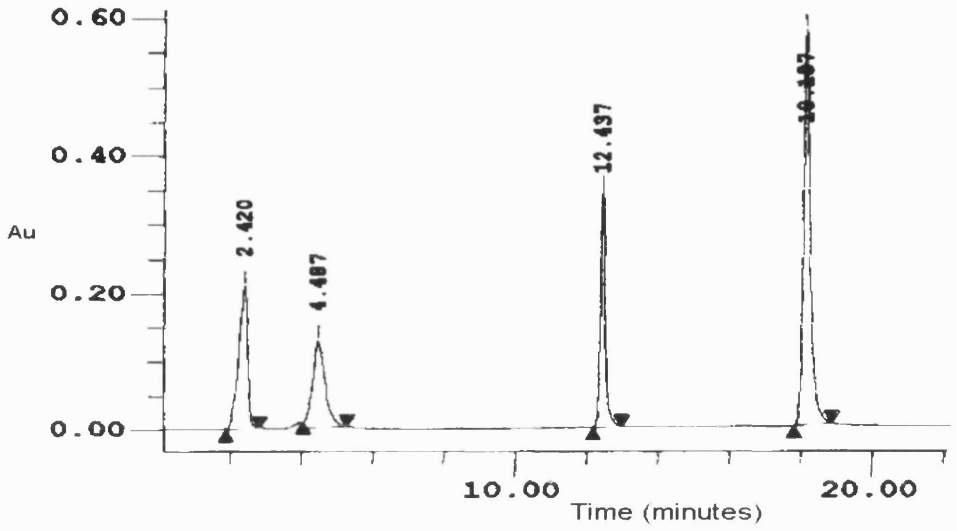


Figure 3.4.5 Gradient run of the acidic standards on the RS-Pak ODP504e with NaoH added (pH 10.2) to the acetonitrile and water via a separate line.

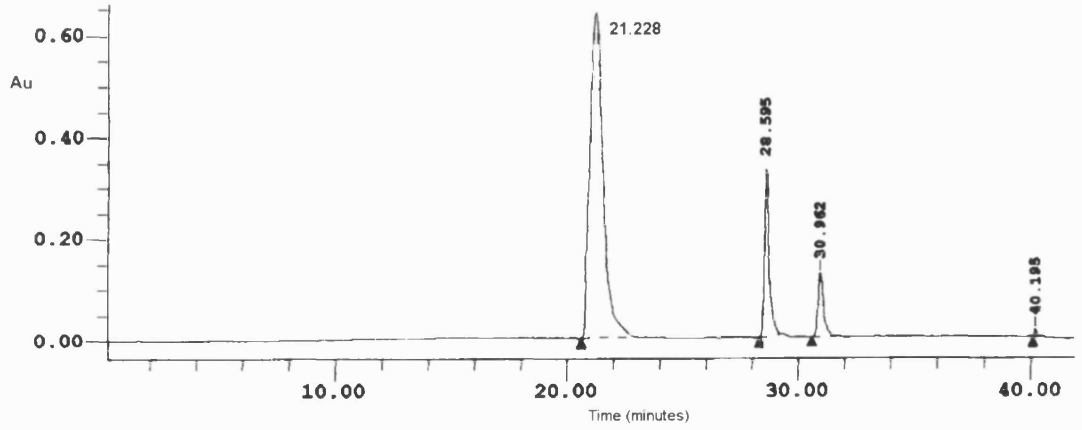


Figure 3.4.6 Gradient run of the basic standards on the RS-Pak ODP504e with NaoH added (pH 10.2) to the acetonitrile and water via a separate line.

Fifteen drugs that had previously been run on the acidic System 2 were selected at random for examination on the high pH system. Table 3.4.1 shows the RI and UV maxima obtained on each system for fourteen of these drugs. All show that different retention indices have been given by each system. This occurred due to differences in ionisation states, differences in the column and the gradient profiles. Most drugs also show a change in the UV spectrum when compared to the acidic system indicating the great potential of the parallel use of the high pH technique. One compound

amiodarone, did not show a basic shift in the UV spectra despite not having a particularly high pKa value (5.6). Clarke [2] shows a basic shift for this compound in aqueous alkali. These ionisation effects causing the basic shift in aqueous alkali, are not occurring in our eluent which has a significant organic content.

Drug		Basic conditions		Acidic Conditions	
	Pka	RI	UV Maxima (nm)	RI	UV Maxima (nm)
Amiodarone	5.6	589	202.5/240.1	476.4	203.6/240.1
Chloroquine	8.4/10.8	609.2	216.6/254.2/330.2	246.4	220.1/255.4/342.2
Cyclobarbitone	7.6	<200	204.8/237.7	352.2	196.6
Diazepam	3.3	489.1	197.8/228.3/312.4	429.1	201.3/233/280.3/361.6
Dichlorophen		326.2	202.5/305.2	590.6	204.8/283.8
Indomethacin	4.5	275.3	204.8/266.1/319.5	589.6	204.2/319.6
Meclozine	3.1/6.2	298.1	196.6	397.8	195.4/264.9
Mefenamic acid	4.2	252.9	194.3/286.2/333.8	685.2	196.6/218.9/277.9/350.5
Nalidixic acid	6.0	<200	202.5/255.4/333.8	379.9	216.6/256.6/317.1
Naproxen	4.2	212	261.3/330.2	468.3	270.8/330.2
Paracetamol	9.5	<200	203.6/255.4	241.3	200.2/241.1
Pentobarbitone	8.0	<200	203.6/238.9	383.7	199.8/208.5
Phenylbutazone	4.4	237.1	204.8/263.7	642.5	202.5/237.7
Trimethoprim	7.2	281.9	203.6/287.4	254.3	207.2/269.6

Table 3.4.1 Comparison of RI and UV maxima for 14 drugs run on System 2 and the high pH system.

The fifteenth drug run is not given in Table 3.4.1, this drug (thioridazine) gave two peaks the first eluting at 20.73 minutes and the second at 28.21 minutes. The UV spectra of these two peaks are shown in Figure 3.4.7. The first peak eluted gave a UV spectrum with the characteristic peak maxima at 277.9nm which corresponded to the thioridazine spectrum under basic conditions as given in Clarke [2] i.e. at a pH greater than 9.5 since this is its pKa value, (the aqueous portion of the eluent was at pH 10.4), while the second peak gave a UV spectrum corresponding to thioridazine under acidic conditions as shown in Clarke [2].

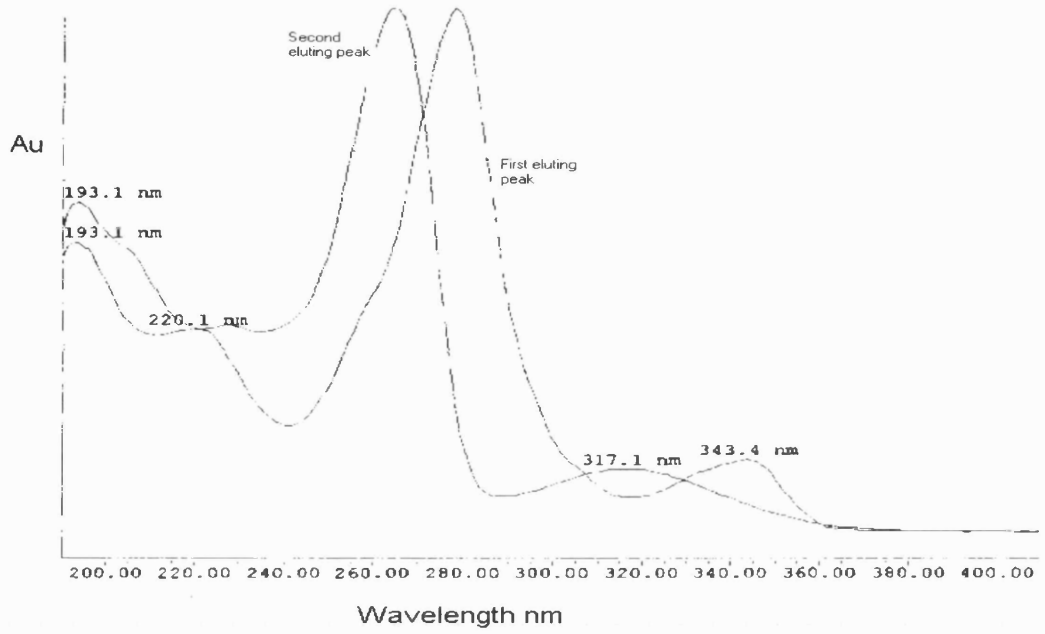
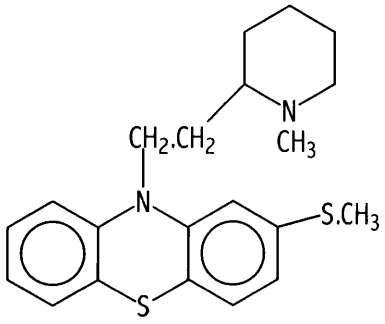


Figure 3.4.7 The UV spectra of the two peaks eluting when thioridazine was run on the high pH system the first peak gives a spectra consistent with thioridazine in basic conditions and the second peak acidic conditions shown in Clarke [2].

The initial explanation for this phenomena was that the unionised and ionised forms of the thioridazine were being eluted separately. However this seems unlikely given that the peaks eluted 7 minutes apart.

Additionally although a basic UV spectral shift is given in Clarke [2], ionisation of the nitrogen on the chromophore appears less likely than on the the piperidyl sidechain (Figure 3.4.8), which is remote from the chromophore and would not cause a basic shift in the UV spectrum. It is more likely that the sample used here suffered some oxidation at the sulphur on the heterocyclic ring.



Thioridazine

Figure 3.4.8 The stucture of thioridazine the sample was likely to have suffered oxidation at the heterocyclic ring sulphur of the phenothiazine.

3.5 SETTING UP ISOCRATIC RUNS ON SYSTEM 1

Gill *et al* [32] investigated the nature of isocratic elution extensively on this system. They found that the nitro-n-alkanes' interactions with the column were hydrophobic and that the elution order of these compounds did not change with alterations in isocratic conditions. Further, the changes in capacity factors became increasingly linear with the increase in the organic content of the eluent. In contrast the basic compounds showed changes in elution order with respect to each other on alteration of the isocratic mixtures. Acidic compounds did not show changes in retention order but did elute between different nitro-n-alkanes under different isocratic conditions. Acidic compounds are effectively neutral under the acidic elution conditions and therefore showed hydrophobic interactions more akin to the nitro-n-alkanes. These findings are supported by those of other workers [66].

For analytical purposes *k* values are considered acceptable when they fall between 2 and 8. Below *k* values of two analyte/column interactions are not efficient, beyond *k* values of 8 peak broadening often begins to occur. Here a series of isocratic data was required to provide a library for compound identification. To obtain a set elution order and a suitable *k*, a defined series of isocratic conditions must be used.

In order to establish the optimum parameters for the series, the nitro-n-alkanes were eluted at 10% increments of eluent B beginning at 30% B and ending at 90% B. The elution time in each case was set at twenty minutes.

Three isocratic strengths were selected for building an isocratic database, 30%, 60% and 80% B. The retention times of the nitro-n-alkanes are given in Appendix II. The 30% B eluted nitro-n-alkanes C_1 to C_4 with a *k* range of 0.93 - 8.5. The 60% B eluted nitro-n-alkanes C_1 to C_8 with a *k* range of 0.66 - 9.3. The 80% B eluted nitro-n-alkanes C_1 to C_{10} with a *k* range of 0.81 - 8.74. It was recognised that compounds eluting outside of the recommended *k* range (2-8) using any of the three isocratic strengths would need to be run under alternative conditions.

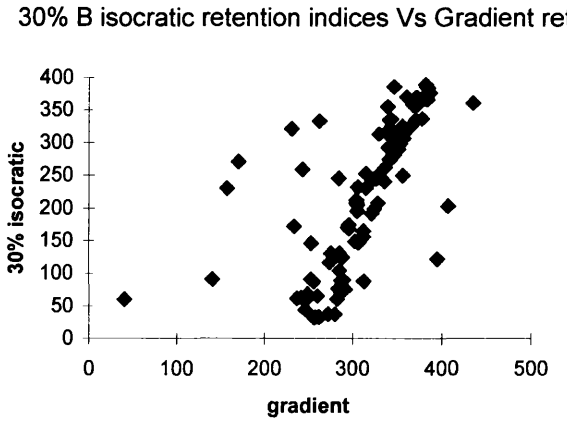
In total 152 drugs were examined under these established isocratic conditions. The gradient retention indices were used to determine which isocratic strength would be selected as most suitable for an individual drug. The retention indices that were obtained are given alongside the System 1 gradient data in Appendix VI.

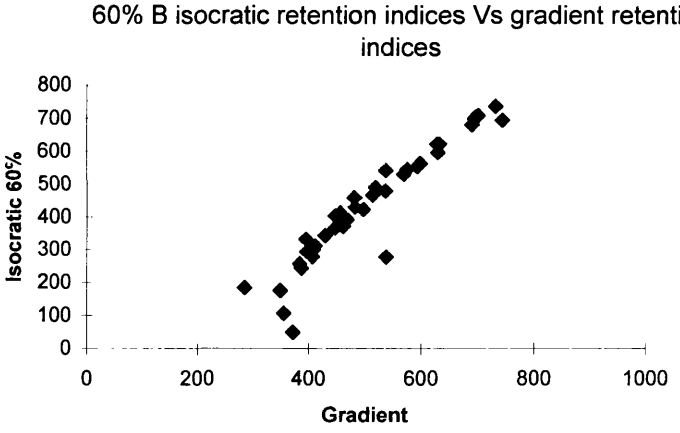
While direct correlations cannot be drawn between the two methods, if the gradient index suggested a compound would be eluted within reasonable *k* values at the 60% isocratic elution strength, then this was generally found to be the case. However when the gradient index suggested using 30 and 80% B often much higher or lower retention indices than expected were obtained. At the 30 and 80 % isocratic elution strengths some drugs are experiencing profound changes in their elution time relative to the nitro-n-alkane standards thus disrupting direct comparison of the index values. This is probably due to the extremes of ionisation or hydrophobicity interactions shown under these isocratic conditions.

A plot of isocratic retention indices against gradient indices (Figure 3.5.1), shows the reliability of the selection process.

Isocratic elution is favoured for analysis not only for its reproducibility and peak shape (less tailing), but also for the ability to resolve compounds. Figure 3.5.2 shows how the isocratic and gradient elutions compare in their ability to elute and separate compounds. The isocratic strength 30% B elutes 106 compounds over a range of ~ 40 - 400 RI units. The 80% B elutes 10 compounds over ~ 40 - 700 RI units. The

60% B elutes 45 compounds over ~ 40 - 750 RI units. The sigmoidal shape of the gradient separation profile shows that ~ 125 compounds of the 174 run are eluted between 200 and 400 RI units.





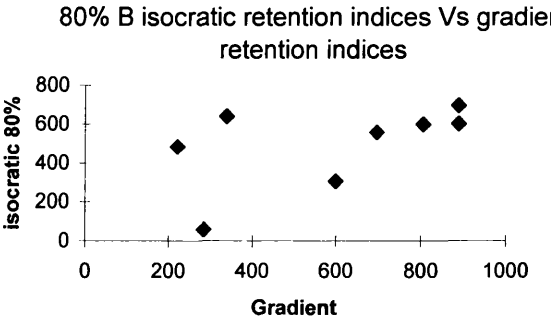


Figure 3.5.1 Plots of isocratic retention indices against gradient retention indices.

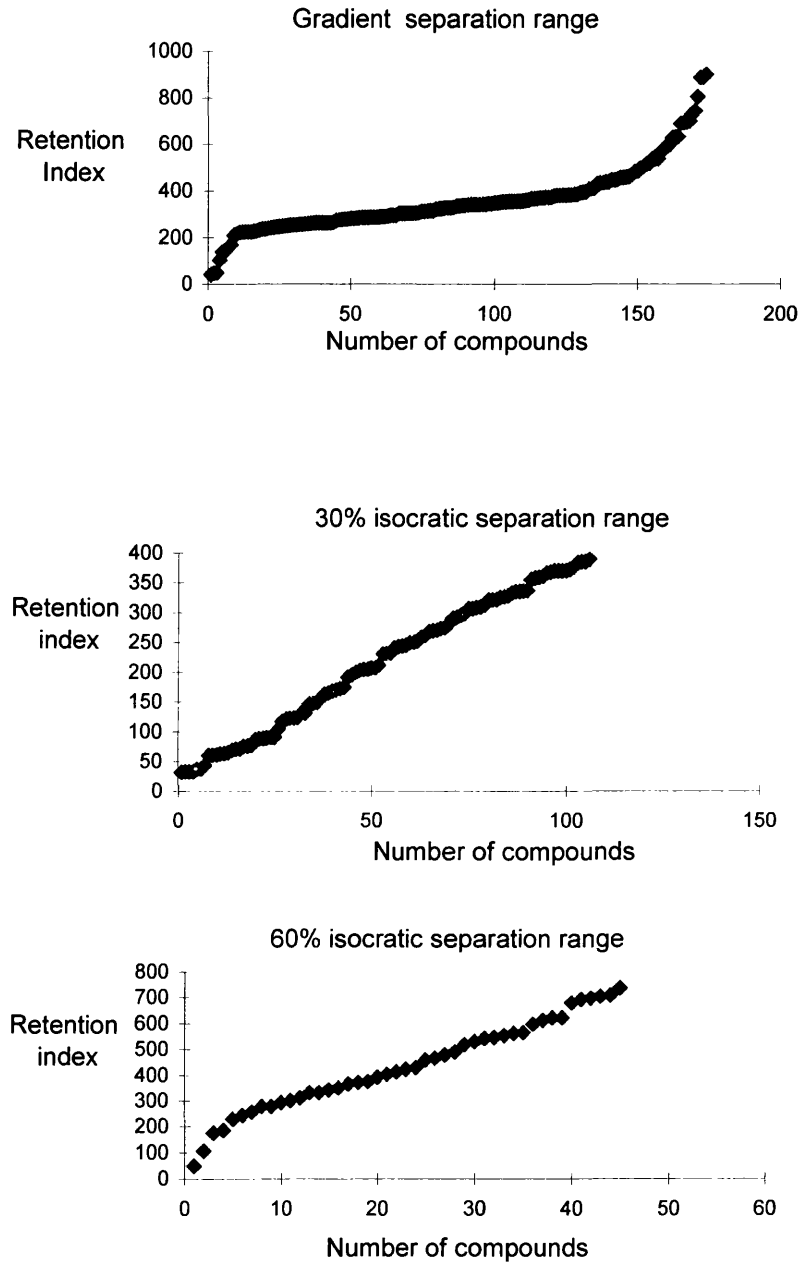


Figure 3.5.2 Separation slopes for gradient and isocratic systems.

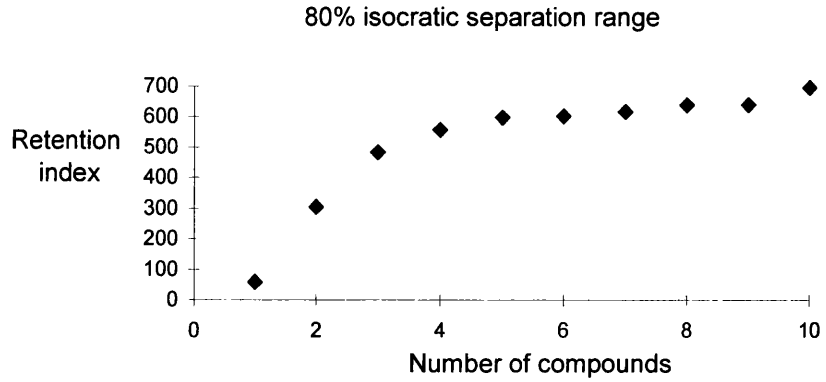


Figure 3.5.2 Separation slopes for gradient and isocratic systems (cont.)

This will inevitably lead to poor discrimination between compounds eluting in the mid-gradient region. By comparison the 30 and 60 % isocratic systems give a near linear profile indicating maximum discrimination power across the range of polarity.

3.6 DISCRIMINATION POWER OF SYSTEM 2

Moffat *et al* [41] were interested in the ability of various paper, thin-layer and gas chromatography methods to identify drugs of forensic interest.

Equation 3.6.1 was used to describe discriminating power.

$$DP_k = 1 - \frac{2M}{N(N-1)}$$

Equation 3.6.1 Where DP_k is the discrimination power of a series (k) chromatographic systems, M is the number of pairs that remain unresolved after running on each system and N is the total number of compounds.

Maier & Bogusz [53] applied this discrimination power to a database of 372 drugs using HPLC-RI data, HPLC-UV maxima data or the combination of HPLC-RI and UV data. These were calculated using a match window of +/- 10 RI units for the HPLC system and +/- 2 nm for the UV data maxima values.

In the present work a discrimination power for the acidic retention indices and UV absorption maxima were obtained and then the combined discrimination power was determined for 171 drugs.

A match window of +/- 7 RI units was applied to the retention indices and +/- 2 nm was applied to the UV absorption maxima. (These were chosen arbitrarily but were intended to be in excess of three standard deviations for samples repeatedly run on System 2.)

The discriminating powers obtained in this work are given with the results obtained by Maier & Bogusz [53] in Table 3.6.1

	HPLC- RI (DP)	UV maxima (DP)	HPLC-RI + UV (DP)
Maier & Bogusz [53] (372 drugs)	0.840	0.804	0.993
System 2 (171 drugs)	0.916	0.903	0.996

Table 3.6.1 The discriminating power of Maier & Bogusz's database [53] and the discriminating power of System 2.

The discriminating power of System 2 follows the same trend as Maier & Bogusz [53] but is higher in all cases. Maier & Bogusz [53] use an acetonitrile/water gradient HPLC with retention indices based on the nitro-n-alkane scale. Therefore this system will have a similar separation range to System 2.

The number of matching compounds that lead to the discrimination figures are interesting. For HPLC-RI only there were 1223 matches, for UV 1411 and for the HPLC-RI and UV combined there were only 64 matches. Although the combination leads to a dramatic reduction in the number of matches 64 is a large number when the database contains only 171 compounds. Improvements that could be made to the discrimination power of System 2 are discussed in the future work section of the conclusions.

Advantages and limitations of the non-buffered HPLC systems.

The use of consistent laboratory conditions and the use of the most up to date technology is important to attaining repeatable retention data. System 2 is shown to

be robust to experimental error and as such is suitable as the basis of a surveillance method and possibly a universal data set of retention indices.

Variability of the nitro-n-alkanes on System 1 was significantly higher than Gill *et al* [32] and System 2. High variability occurred at beginning and end of the gradient ramp to a larger extent than seen in the work of Gill *et al* [32]. It was concluded that long equilibration times were necessary to lower variability. Yet this long equilibration (twenty minutes) is costly analysis time and not suitable for a rapid surveillance method.

Despite greater variability of individual retention times on System 1 correlation of gradient retention indices with those obtained by Gill *et al* [32] ($r = 0.998$) is high. The correlation with System 2 is lower ($r = 0.990$). It was concluded that a large part of this difference was due to the slight difference in packing materials (ODS-2 in System 1 as opposed to Waters Symmetry in System 2). Therefore the large equilibration times can be eliminated if reasonably standardised elution conditions are used.

A comparable high pH system HPLC system appears possible and would add greatly to the power of the surveillance method. Further work is needed in order to raise the pH of the system to ensure the ionisation of more compounds while improving further the resolution of the chromatography especially at the front end.

Gradient retention indices may only be used as a general guide to the eluent composition to be used under isocratic conditions. The three isocratic eluent compositions provided suitable chromatographic conditions for resolution of all the drugs examined and should be fixed as standard conditions in order to build reproducible pharmaceutical, forensic and toxicological libraries.

The gradient elution resolves many compounds over a narrow range of retention indices. For this reason even if gradient elution on this system shows quantification comparable to isocratic elution it will not always be suitable for toxicological screening. The building of a standardised isocratic library will be useful for toxicological analysis of closely related compounds if this system is to be employed in this area.

4. THE INTER-LABORATORY TRANSFER OF DATA AND SYSTEM PREDICTABILITY

True transfer of data between different systems and laboratories is a common goal for many analytical techniques and it has been of special importance to high-performance liquid chromatographers for some time. Such transfer of data will be facilitated when the HPLC systems are robust and predictable and indeed the protocols required to ensure transferability are effectively identical to those necessary to validate robustness, repeatability and reproducibility for the analysis of medicines in HPLC. Retention of an analyte is affected by the chemistry of the stationary phase, temperature, composition of the mobile phase and of course the chemical structure of the analyte. It is these factors which must be allowed for in any study of predictability and transferability.

The author does not foresee the use of data gathered on other workers systems as being used exclusively in the rapid surveillance technique, although it would be of use to certain forensic scientists who have long been interested in this area. Thus, although a truly transferable system would allow the free exchange of retention data, an understanding of the limitations of transferability would at least allow for estimates to be made of interlaboratory variability for the provisional identification

In a review paper Kirschbaum [67] discussed the technical issues for the inter-laboratory transfer of HPLC data noting the importance of 'stable well defined columns' and the use of buffered eluents. Together with refinements to HPLC technology, the use of retention index methods have become the most important factor in improving the transferability of data.

The advantage of using external standards to provide an index scale was investigated by Baker & Ma [68], who concluded that the correct set of standards can provide a continuous scale that would be reproducible between laboratories. Baker *et al* [68,69] used the alkan-2-ones but discovered poor reproducibility using these standards. The mobile phase composition was found have a critical effect on retention indices, and isocratic and gradient retention indices were not found to be transferable.

Smith *et al* [44] investigated the use of the alkyl aryl ketones as retention indices. Here the brand of stationary phase and pH were critical factors if the compounds being identified were not neutral. However when acetonitrile was used as a modifier the retention indices obtained on different batches of the same packing material were less variable than before.

The order of elution of drugs was found to change when using different brands of ODS material [44]. Bogusz and Aderjan [70] proposed a method of correction for moving data between different columns using a series of secondary standards. The RI of the analyte was obtained using the alkyl aryl ketone scale on a particular column. The RIs of the secondary standards were also obtained on this column and also on the column the data was being corrected for. The difference between the secondary standards eluting before and after the analyte on the two columns was used to give a corrected retention factor for the analyte RI on each column. This provided an excellent improvement in the previous variability in retention index obtained on the two columns. This method was later successfully applied to the nitro-n-alkane retention scale [71].

It was later confirmed that this method of correction was more applicable to the nitro-n-alkanes indexed drugs, particularly basic compounds [72]. For applications in hospital toxicology, one group went further and used retention indices scales based on a series of acidic and basic primary standards to give a linear index scale [73,74]. Yet this scale would not prove to be linear if transferred to a different column type. At this time the nitro-n-alkanes provide the best homologous series of compounds that are easily obtained, encompass the lower molecular weight drugs and have a limited polarity that appropriately alters their retention in response to the prevailing chromatographic conditions.

The aim of this chapter is to demonstrate the possibility and requirements of the inter-laboratory transfer of HPLC and that HPLC System 2 is robust, predictable and therefore suitable as the basis of a transferable data set and as a rapid medicines surveillance method.

4.1 THE INTER-LABORATORY TRANSFER OF HPLC DATA

Comparisons of previous data sets [75] have concluded that good correlations are found between identical systems run in different laboratories but data obtained on similar systems may only be exchanged with caution. The need for standardisation of HPLC systems was emphasised.

In this work System 2 is presented as a robust HPLC method which is reproducible enough to be used as a rapid surveillance technique. This section investigates System 2 as the 'standard' in a compilation of HPLC retention data for the transfer of data between differing HPLC systems. The HPLC system employed in this work was considered to be robust and predictable enough to use as the basis of a transferable data set.

Nine significant HPLC databases were obtained from the literature. These were all derived from papers which expressed an interest in the concept of transferable databases, or were providing the data for use in toxicological analysis. The references are quoted in Table 4.1.1 along with the chromatographic systems employed by each set of workers.

The retention data given by each of these groups was plotted against the retention indices that were common to System 2. These plots are given in Figure 4.1.1.

A correlation was obtained by linear regression between the data, except in the case of Below & Burrman [83] where a power function was used to obtain the line of best fit. Using these best fit equations the primary data provided by the other workers was converted to the System 2 retention index scale.

Reference	Column dimensions (mm)	Stationary phase	Eluent	Analysis type	Retention recorded as:
Gill *et al* [32]	250 x 25	ODS-2 endcapped (Inertsil)	acetonitrile\water sulphuric acid pH 2.2	RP Gradient	Retention Indices
Logan *et al* [76]	250 x 4.6	C-8 endcapped Lichrospher	acetonitrile\water phosphate buffer pH 3.2	RP gradient	Retention time
Koves & Wells [77]	250 x 4.6	ODS Apex	acetonitrile\water phosphoric acid and triethylamine buffer to pH 3.4	RP isocratic	Retention time
Cosbey [78]	160 x 4.5	ODS endcapped Hypersil	methanol/nonylamine/ water with orthophosphoric acid and heptane sulphonic acid to pH 2.1	RP/Ion pairing isocratic	Relative retention times
Tracqui et al [79]	300 x 3.9	ODS endcapped Novapak	methanol/tetrahydrofuran phosphate buffer to pH 2.1	RP isocratic	Retention times
Hill & Kind [80]	250 x4.6	C- 18 Non-endcapped Zorbax RX	water/acetonitrile triethylamine phosphate buffer to pH 2.2.	RP gradient	Retention indices
Bogusz & Wu [81]	125 x 4	RP-18 endcapped Superspher	water/acetonitrile with triethylammonium phospate buffer to pH 3.0	RP gradient	Retention indices
Bogusz & Erkens [82]	125 x 4	RP-18 endcapped Superspher	water/acetonitrile with triethylammonium phospate buffer to pH 3.0	RP gradient	Retention indices
Below & Burrman [83]	150 x 3.2	ODS SI 100	water/acetonitrile with phosphate buffer to pH 2.3.	RP isocratic	Relative retention times

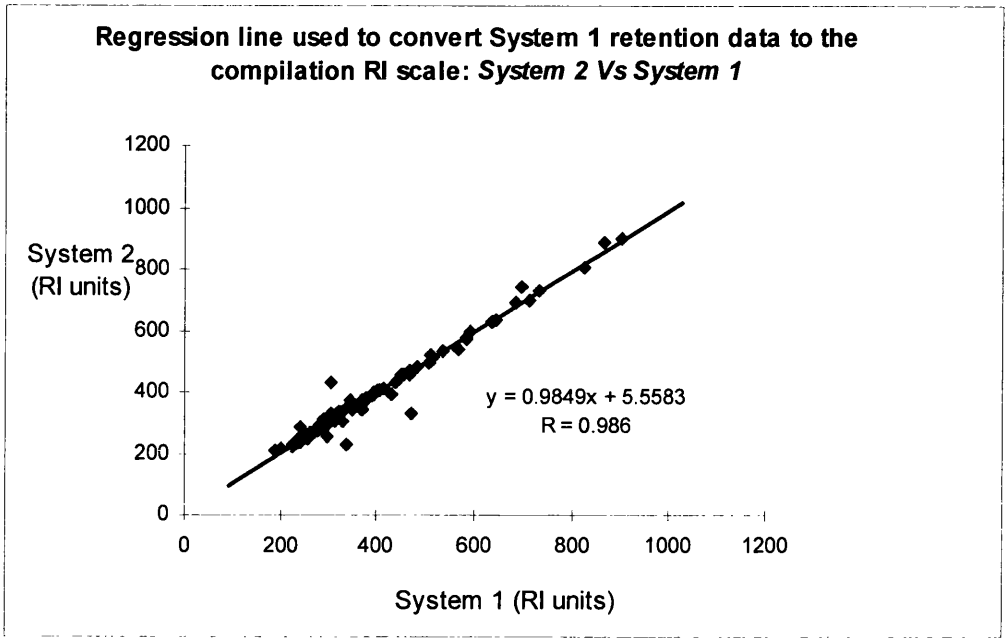
Table 4.1.1 References and chromatographic system descriptions of the retention database used to build a retention index database.

Where the correlation between the external data set and System 2 was poor i.e. $r < 0.8$ then the data were not included in the compilation of data. For example, Koves & Wells' [77] data gave a poor correlation $r = 0.435$ with System 2. This was more surprising as these workers used a C-18 column of the same dimensions as System 1 and the pH was also low at 3.4. However the use of an isocratic system using retention time measurements combined with a buffer system made this system perform differently to System 2. A similar problem occurred with data from

Cosbey [78], the use of an isocratic solvent system which used RP for acidic compounds but ion pairing for basic compounds, gave chromatography that correlated poorly with System 2, (r = 0.712). The data of Tracqui *et al* [79] led to a correlation of r = 0.38 with System 2. These workers used a methanol/tetrahydrofuran eluent that will have a different selectivity to acetonitrile/water eluents.

The valid retention indices as obtained were then placed in a table (Appendix VII). A compilation of retention index data based on System 2 was then produced. The rules for producing the compilation were somewhat arbitrary. The retention indices of those drugs run on System 2 were entered directly into the compilation. For other drugs the retention indices of the other workers data on the System 2 retention index scale were averaged and the standard deviation determined.

If there was a standard deviation greater than 50 retention index units then the data point causing that deviation was excluded. This was justified as in each correlation between System 2 and the other workers there were a few outlying compounds which did not behave as expected. These compounds are important and are discussed later.



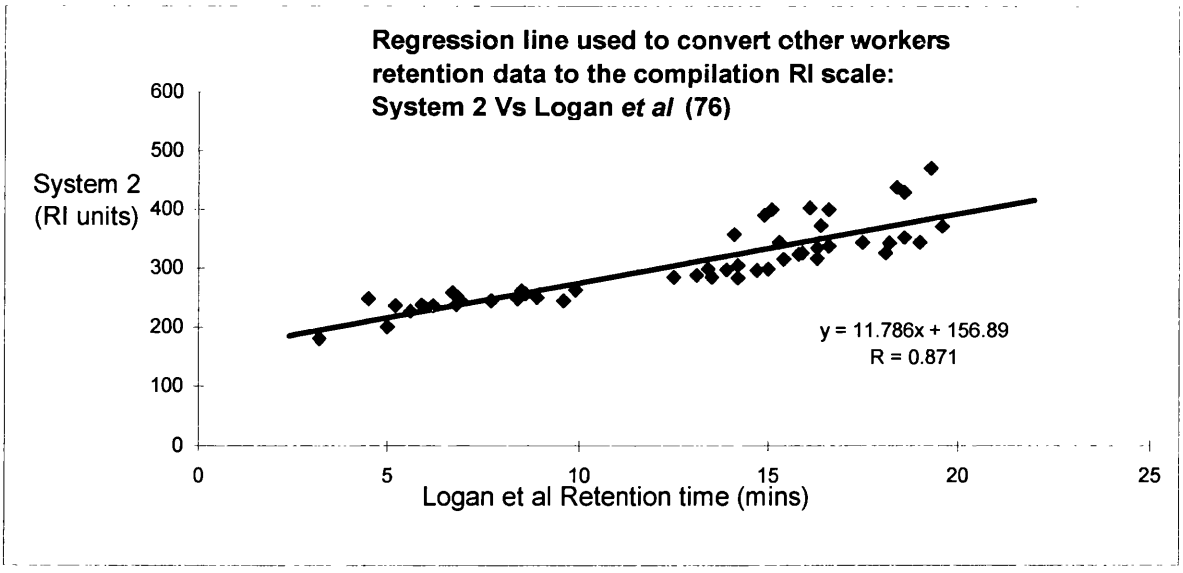
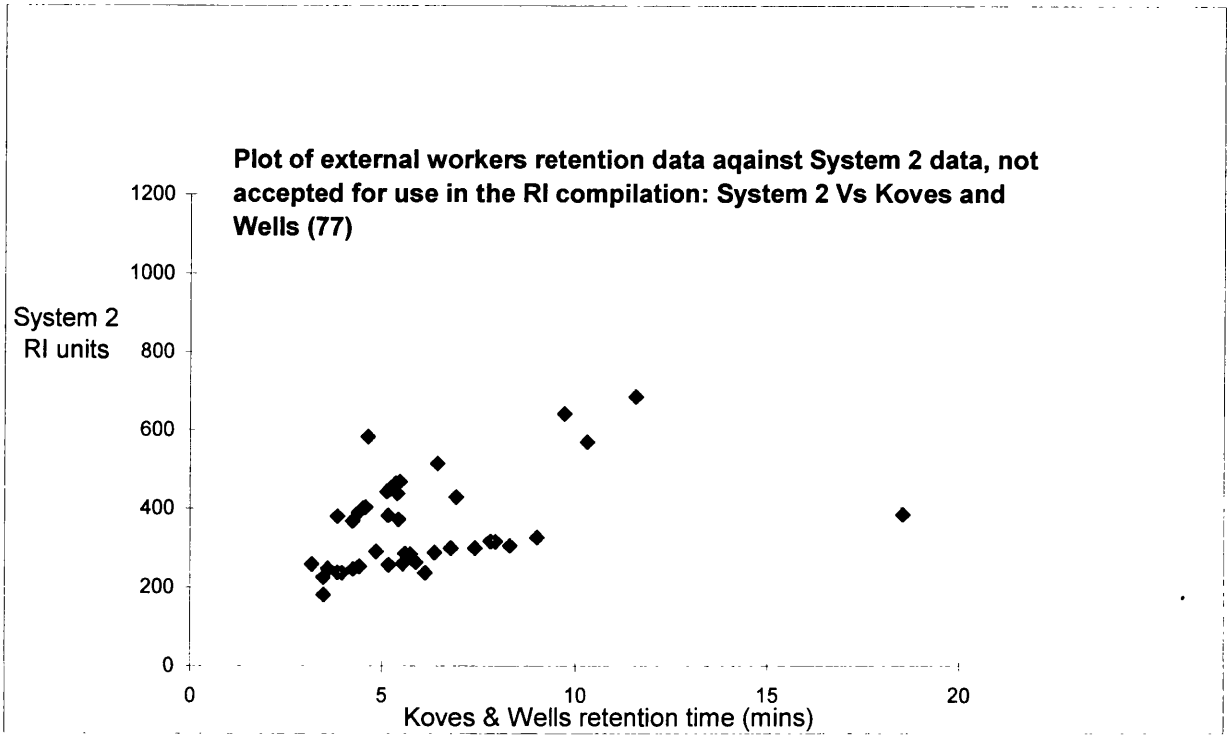


Figure 4.1.1 Plots of System 2 retention indices against System 1 and other worker's retention data used to generate the compilation of retention indices.



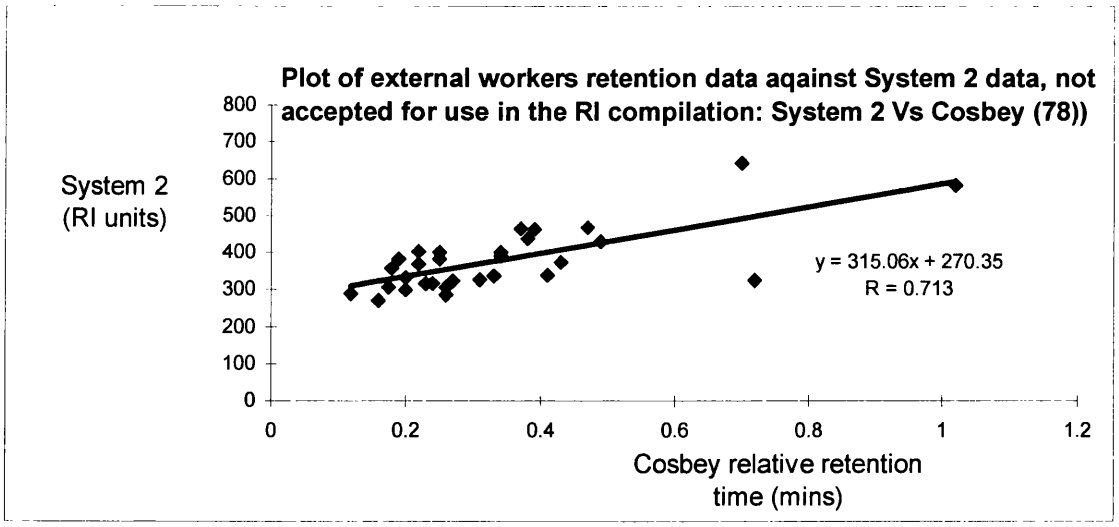
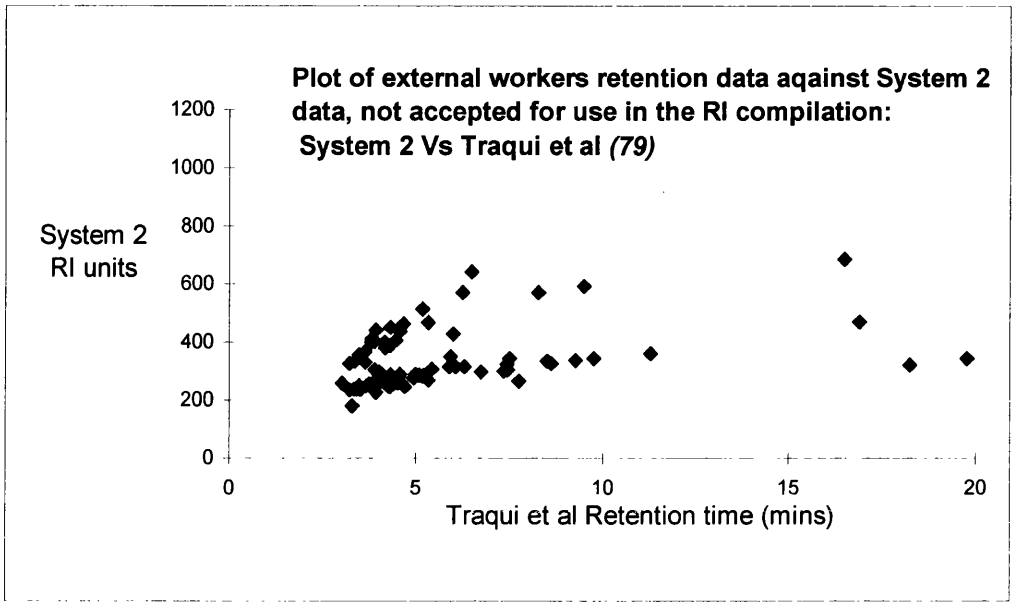


Figure 4.1.1 Plots of System 2 retention indices against System 1 and other worker's retention data used to generate the compilation of retention indices cont.



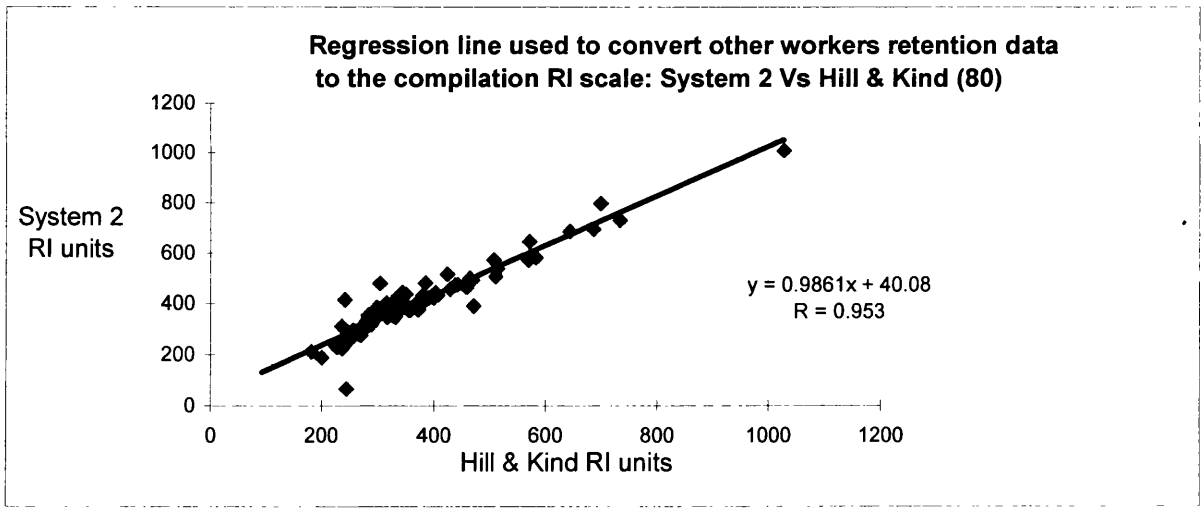
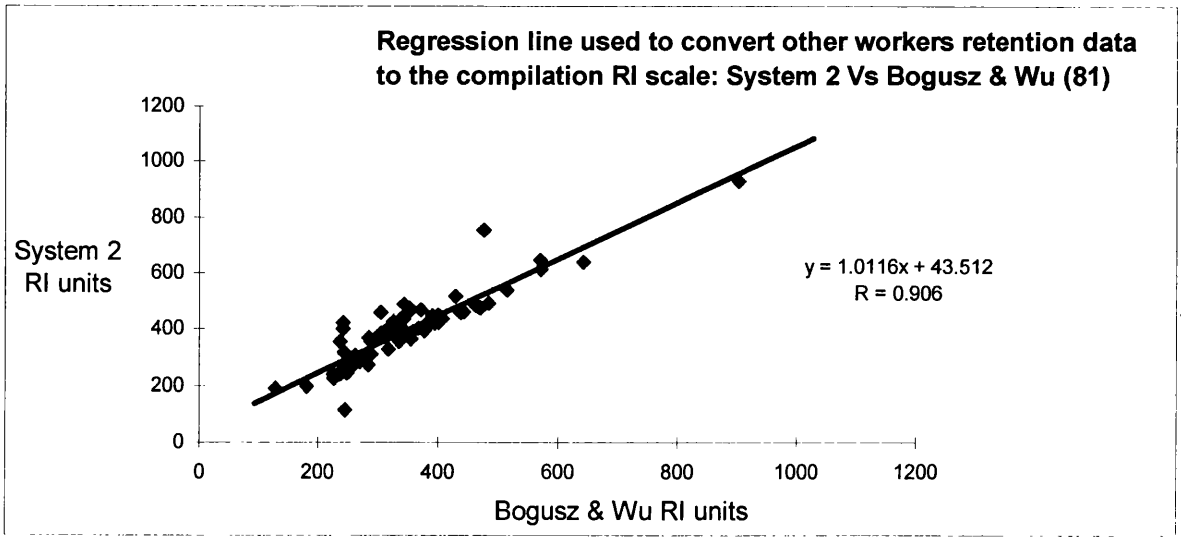


Figure 4.1.1 Plots of System 2 retention indices against System 1 and other worker's retention data used to generate the compilation of retention indices cont.



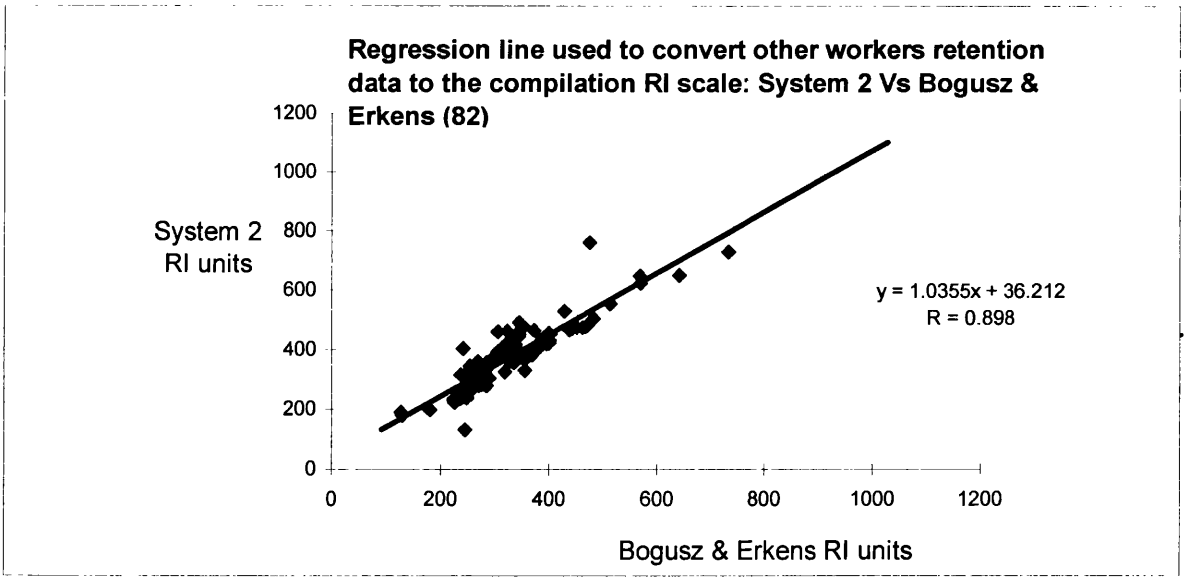


Figure 4.1.1 Plots of System 2 retention indices against System 1 and other worker's retention data used to generate the compilation of retention indices cont.

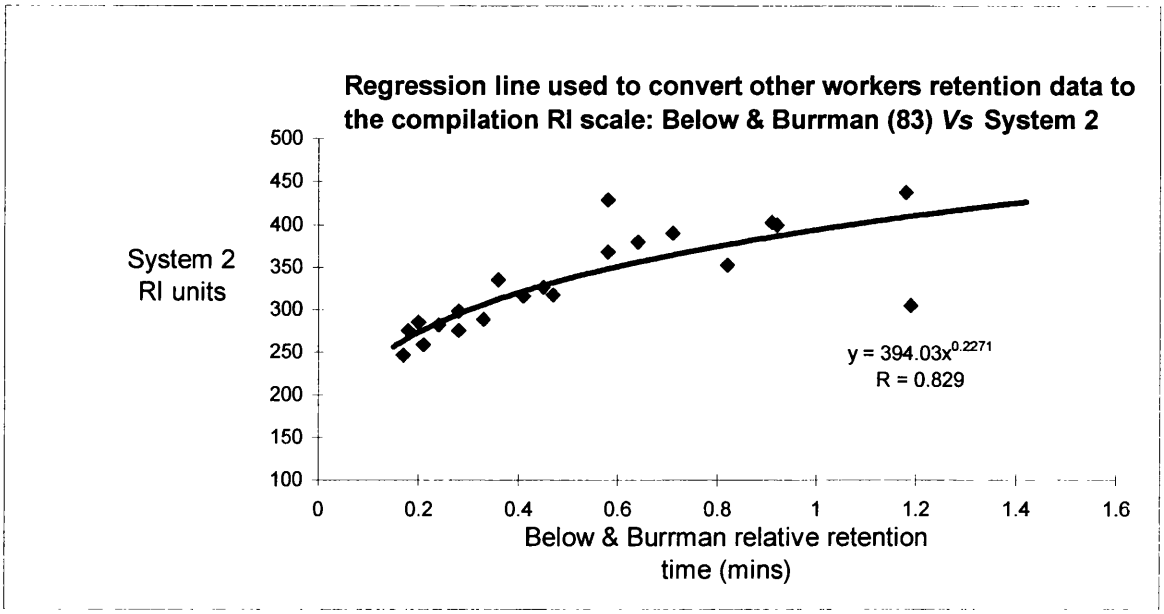


Figure 4.1.1 Plots of System 2 retention indices against System 1 and other worker's retention data used to generate the compilation of retention indices cont.

Table 4.1.2 show the correlation coefficients of those workers data that were accepted for the compilation with System 2 and also with the compilation itself. It can be seen that with the exception of Gill *et al* [32] the correlation of the data remains the same or is slightly improved after incorporation into the compilation.

Workers	Correlation of Original retention data with System 2 (r)	Correlation of Original retention data with Compilation data (r)
Gill *et al* [32]	0.998	0.998
Logan *et al* [76]	0.871	0.928
Hill & Kind [80]	0.953	0.991
Bogusz & Wu [81]	0.906	0.95
Bogusz & Erkens [82]	0.898	0.979
Below & Burrman [83]	0.828	0.869
System 1	0.986	0.985

Table 4.1.2 Correlation coefficients of other workers data with System 2 and the compiled data.

The Gill *et al* [32] data did not show an improvement because it was run on a system very similar to System 2 in the first instance. It would be expected that if the compilation were expanded to include additional data sets these correlations would improve further. Great improvements to the correlations might be achieved if the outlying compounds could be corrected in some way.

Examination of the correlations between the retention data converted to retention indices on the System 2 scale and the System 2 data itself revealed some of these 'outlying' compounds. The work of Bogusz & Wu [81] and Bogusz & Erkens [82] shared identical elution conditions and unsurprisingly shared the same outlying compounds, notably orphenadrine (pKa 8.4), chlorprothixene (pKa 8.8) and prazepam (pKa 2.7). Hill & Kind [80] used closely related elution conditions and shared diltiazem and ranitidine (pKa 2.3, 8.2) as outlying compounds with Bogusz & Erkens [82]. The identity of these two compounds were confirmed by their UV spectra. Therefore these compounds were discounted as degraded or mislabelled. Chlorprothixene was also identified as an outlying compound between the data of Below & Burrman [83] and System 2. Additionally for Logan *et al* [76] a notable outlying compound was temazepam.

Understanding the behaviour of the outlying compounds will improve the correlations with the compilation while using more external data sets will improve the correlation and provide more information about these and other outlying compounds. It may be possible to develop the compilation as a 'learning set'. This approach would be logical as the method used to build the compilation is an extended version of the corrective procedure used by Bogusz [70] and does not account for the anomalous behaviour of some compounds which also cannot be conclusively explained by their chemical structures.

As suggested although reasonable correlations have been made using this method of data transfer they are not good enough to allow the wholesale conversion of one set of retention data from one system to another for identification with a high level of certainty. However this method may be of use to analysts designing HPLC systems for

the general identification of drugs and it offers a contribution to the understanding of retention behaviour of drugs under varying chromatographic conditions.

4.2 RETENTION PREDICTION

4.2.1 Retention prediction using physicochemical parameters

The barbiturates were chosen as a model group of compounds to demonstrate system predictability as they possess a common polar core, functionalised with wide a range of alkyl or other non polar side chains which provide a subtle control over their retention characteristics, partition coefficients and biological properties. Other workers have used the barbiturates and other series of compounds to demonstrate HPLC as a method of determining physicochemical parameters [84-89]. At the pH used in this system (2.2), the barbiturates are unionised and on an inert support phase were expected to give predictable retention characteristics. Additionally, literature data are available for some of these barbiturates, not only their physiochemical properties but also data related to their gas liquid chromatographic behaviour [12,90]. The retention indices obtained for these barbiturates are then correlated with octanol/water partition coefficients (log P), partial molar volumes and molecular connectivities to demonstrate the system reliability and predictability.

For isocratic experiments the System 1 was run at 38 % B and 62% A. Approximately 10 mg of each barbiturate was dissolved in acetonitrile (2ml) and of double de-ionised water (1ml).

Three types of molecular connectivity were calculated according to the rules of Kier and Hall as modified by Stead *et al* [90]. ${}^{1}\chi^{O}$ reflects the number of hydrogen atoms attached to the carbon atoms substituted at the 5, 5 positions on the barbituric acid core. ${}^{1}\chi^{V}$ is based on the difference between carbon valency and the number of hydrogen atoms attached. ${}^{1}\chi^{V}_{N}$ includes the above but also accounts for the bond type between substituent carbons.

Additive partial molar volume substituent values were obtained from the literature [91]. A partial molar volume of the barbituric acid core was determined from its density [92]. Log P values were obtained using substituent values [93] and standardised by including the contribution of barbituric acid [94].

Eighteen values of the electronic parameter σ * for the barbituric acid substituents were obtained from the literature [93]. These values were then summed with the log P values of the corresponding barbiturates to examine the effect of the correlation with the HPLC retention indices. A typical chromatogram of glycine, (used as a void volume marker) and four barbiturates run on the isocratic system shows good resolution and peak symmetry (Figure 4.2.1).

Table 4.2.1 shows retention indices on the gradient and isocratic systems of the set of barbiturates, together with their gas chromatographic retention indices. The gradient and isocratic indices correlate well ($r = 0.976$) but they are not directly comparable since the order of elution varies slightly for these acidic compounds.

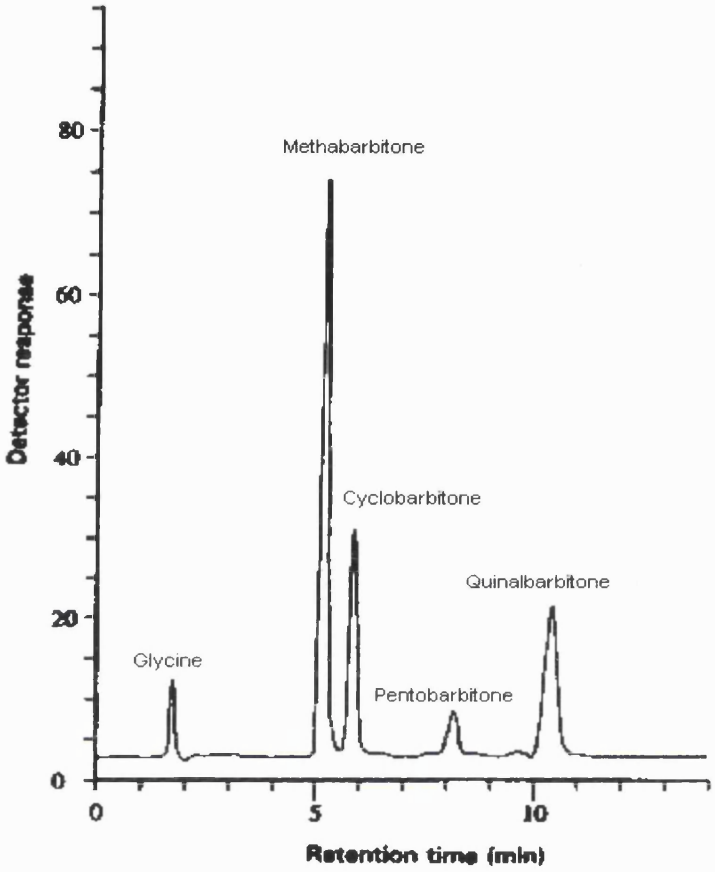


Figure 4.2.1 Four barbiturates resolved at 38% B on System 1

For the repeated runs of the barbiturates carried out on both gradient and isocratic systems, coefficients of variation of less than 0.46 % and 0.23 % were seen respectively, (retention data given in Appendix VIII). The isocratic data were selected for the correlation with the chosen physicochemical parameters because of their superior repeatability. This was rationalised as being due to fewer equilibration and eluent mixing problems than those given by the gradient system. These problems may have been reduced by the use of the Waters HPLC equipment but this was not available when this work was carried out.

Table 4.2.2 shows the correlation coefficients given by linear regression between the isocratic data and some physiochemical parameters. Poor correlations are seen with all three molecular connectivity values considered.

	Gradient index (mean of four runs)	Gradient index calculated from substituent values	Isocratic index (mean of four runs)	${}^{1}\chi^{V}_{N}$	GLC retention index	Partial molar volume	Log P
Allobarbitone	320	327	123.2	2.678	1586	161.85	0.9451
Amylobarbitone	388	387	338.3	3.477	1700	206.55	2.2563
Aprobarbitone	332.5	333	212.5	2.783	1600	183.35	1.2756
Barbitone	283.5	283	93.3	2.121	1482	141.855	0.7288
Brallobarbitone	343.5	350	179	3.203	1842	188.055	1.8359
Butalbital	373.5	371	282	3.256	1658	199.955	1.9902
Butobarbitone	357.5	359	263.7	3.121	1645	175.055	1.709
Cyclobarbitone	356.5	351	263.3	3.253	1945	187.655	2.42
Cyclopentobarbitone	356.5	357	262.1	2.693	1858	182.955	1.8945
Enallylpropymal	395.5	386	358.6	2.7825	NA	199.955	2.5937
Heptabarbitone	384.3	389	328.6	3.753	2035	NA	2.205
Hexethal	456.5	435	452.7	4.121	1835	208.255	2.6635
Hexobarbitone	389	366	344.5	1.947	NA	202.555	2.611
Ibomal	353	356	255.2	3.307	1866	209.555	2.4461
Idobutal	376.5	381	295.2	3.4	1698	168.45	2.2372
Metharbitone	334.5	336	232.3	2.121	NA	173.355	1.1385
Methohexitone	481.5	482	483.8	3.743	NA	263.855	2.8043
Methylphenobarbitone	398	397	365.9	5.55	NA	199.855	1.9997
Nealbarbitone	382.5	383	326.5	3.546	1720	230.855	2.4437
Pentobarbitone	385.5	387	332.6	3.542	1733	206.555	2.1266
Phenobarbitone	343.8	344	228.8	3.375	1934	168.355	1.4524
Phenylmethylbarbituric Acid	318.3	306	118.9	2.814	1875	151.755	0.9613
Probarbitone	311	311	115.9	2.504	1550	173.355	1.0506
Quinalbarbitone	408.3	409	378.9	3.821	1770	216.555	2.4813
Secbutobarbitone	350.8	349	236.8	3.042	1650	189.955	1.6355
Sigmodal	433.8	432	418.4	4.345	2031	242.755	2.881
Talbutal	370.5	371	296.5	3.321	1704	199.955	1.9902
Vinbarbitone	355.5	356	259	3.828	1755	214.855	2.0413

Table 4.2.1 Retention index data obtained on System 1 and the physicochemical parameters

Physicochemical parameter.	Correlation coefficient (r).
Partial molar volume.	0.831
Log P	0.891
Log P (for alkyl and allyl substituted barbiturates only)	0.949
${}^{1}\chi^{O}$	0.597
${}^{1}\chi^{V}$	0.759
${}^{1}\chi^{V}_{N}$	0.601

Table 4.2.2 The correlation coefficients and some physicochemical parameters obtained between the HPLC isocratic retention indices for the barbiturates.

Partial molar volume appears to have a limited role as a predictor of the retention of the barbiturates on the ODS support material.

Lipophilicity as reflected by octanol/water partition coefficients appears to be an important part of the retention mechanism even though calculated rather than observed log P values have been used. The alkyl electronic parameter σ * has previously been found to contribute to observed log P values by Lamb & Harris [95]. The subtraction of σ * gives some improvement to the correlation of log P with HPLC retention indices (from r = 0.868 to 0.878) for the 18 barbiturates with σ * data available. In agreement with the findings of Wong & McKeown [96] the σ * contribution was a small one.

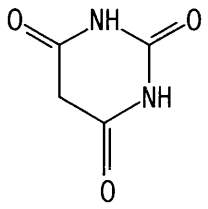
By removing barbiturates containing phenyl, cyclo- and polar functional groups (i.e. bromine containing) from the correlation between the isocratic data and log P, the correlation coefficient is significantly improved (r = 0.949). Barbiturates containing polar functional groups show lower log P values than expected from the linear regression with their retention indices. A similar trend between calculated and observed Log P values has been shown by Hansch *et al* [94].

Poor correlation of the HPLC retention data with the GLC data (r = 0.465) are seen. The GLC data however, do give excellent correlation with the molecular connectivity values, for example with ${}^{1}\chi^{V}_{N}$, where r = 0.997. This suggests that retention in the

HPLC condensed phase is more complex and cannot be simply related to bond arrangements of the analyte as in a simple gas/liquid partition. The poor correlation between these two techniques means that they may be used as independent screening methods.

4.2.2 Retention prediction using substituent retention indices

Novel additive substituent retention index values for the substituents on the 5, 5 position were calculated as follows. Barbituric acid (Figure 4.2.2) and its retention index was taken as the parent and the difference between the structure and the retention index of each analogue was taken in turn.



Barbituric Acid

Figure 4.2.2 the structure of barbituric acid on which the additive substituent retention indices for the barbiturate function groups was based.

From these data and using simultaneous algebraic equations, the individual substituent values were calculated. The table of barbiturate functional groups used for this iterative process is given in Appendix VIII.

The retention index substituent values calculated in this work (Table 4.2.3), give calculated gradient indices (Table 4.2.1) which compare very well with the observed gradient indices. The bromine functional group found in three of the barbiturates is not included as agreement could not be obtained for its retention index contribution.

The bromine function's electron withdrawing properties must alter the retention contribution of itself or other parts of the overall compound.

Substituent	Additive retention index value
$-CH_3$	81
$-NCH_3$	53
$-CH_2$	38
-CH	-15
C	-84
$=CH_2$	48
=CH	55
=C in a cyclo function	-25
=C	-63
≡C	29
Phenyl	180

(Barbituric acid has a retention index of 103.)

Table 4.2.3 The calculated additive substituent retention index values for the 5, 5 functions on the barbituric acid core.

The results of the novel gradient substituent investigation suggest the system is a predictable basis on which to build a surveillance method and a transferable data set. However the transferable data set needs further investigation of the anomalous behaviour of some compounds. Improvements to the general correlations between data sets are also required. The latter might be achieved by using a new series of standards that offer increased linearity but also respond to chromatographic conditions in a closer manner to the drug samples.

5. HPLC APPLIED TO SURVEILLANCE AND ANALYSIS PROBLEMS

It was considered essential to demonstrate the use of our stable non-buffered retention index based system for the screening of drugs in real pharmaceutical samples including both identification and quantification of the active principles.

Two short projects are presented in this chapter. The first considers the analysis of some pharmaceutical preparations. The pharmaceuticals were powders for injection. These were provided by a pharmaceutical importer/exporter concerned about the quality of some preparations produced by generic companies particularly those from certain overseas locations.

Many assays for drugs from pharmaceutical formulations have been reported, typically involving an extraction method for a specific formulation [97,98,99]. Other workers studying HPLC for the analysis of pharmaceutical dosage forms have proposed standardised extraction procedures [100,101,102] including an automated UV analysis method for drugs in suspension and solutions [103]. This investigation would help define the protocols and time scale required to characterise the pharmaceuticals compared to the existing conventional methods and to determine the precision with which this can be done.

The second application was considered in order to test the flexibility of the system for more unusual analytical problems. A new formulation of an unlicensed drug was proposed for clinical trials in a major teaching hospital. Its Pharmacy department had been asked to determine the rate of decomposition of the drug 5-aminolevulenic acid (5-ALA), in the formulation and to identify what breakdown products were formed.

5.1 ANALYSIS OF PHARMACEUTICALS POWDERS USING SYSTEM 2

The pharmaceutical powders for injection were provided by a non-profit making import/export company offering medical supplies to largely third world countries.

The charity is licensed by the Medicines Control Agency. All drugs offered were compliant with BP regulations on labelling. However the company was concerned about the quality of some of the pharmaceuticals in which they were dealing. The cost implications of setting up a Quality Control laboratory to carry out full BP testing would be considerable.

BP identification and assay of drugs would provide a reassuring level of confidence as these methods are robustness tested and proofed against mis-identification. However these tests are costly and laborious and do not lend themselves to a large throughput of samples. If a sample is presented with appropriate licensing documents from a foreign regulatory body recognised by the MCA then we could begin analysis on the premise that the pharmaceutical is more than likely to contain what its label says.

The rapid surveillance method was intended to examine a pharmaceutical, in order to identify the key component and quantify it within limits of accuracy that are well within the BP specified range. Such a method would detect any major flaw within the product. It would not however necessarily detect impurities, unsuitable excipients or stereo-isomers.

The method was intended to act as a filter in addition to the MCA testing scheme. In order to investigate the HPLC system in use here as a surveillance technique injectable formulations were selected as a simple presentation requiring little sample preparation and containing few excipients.

Three stages were required for the analysis: Calculation of equivalents between samples and standards and calculation of suitable dilution factors; preparing & running the samples; determining RI values and whether there is spectral match between sample and standard. Further the identification and quantification were confirmed using separate UV analyses.

5.1.1 Preparing and running pharmaceuticals on System 2

Calculation of equivalents between samples and standards

The standards were obtained from Sigma chemicals, UK. They were supplied as salts, free bases or hydrates. The formulations contained a salt or combinations of salts. Finally the expression used by the BP has to be considered, e.g. for Benzylpenicillin

injection BP " Content of penicillins, calculated as $C_{16}H_{18}N_2O_4S$ 95 to 105% of the content of benzylpenicillin stated on the label " .

Therefore to produce a calibration curve the equivalent weights of standard and formulation samples must be expressed in terms of the BP specification.

The equivalents were calculated either by using molecular weights or in the case of the penicillins by using the number of International Units (IU) expressed on the labels of standard and sample. These samples complied with the BP labelling requirements but were not supplied with any documentation. Therefore there was no information about exicipients.

Calculation of dilution factors

The minimum number of dilution steps were required to reduce errors. The resulting dilution of the samples and standards had to result in a suitable absorption at the UV detector.

Running the samples

The samples and standard were dissolved in the same diluent which was routinely a suitable water/acetonitrile mixture. Under normal circumstances the eluent mixture used at the beginning of the run would be used for the dilution. The standards were prepared using a single dilution while the whole drug required a two stage dilution.

The drug sample was prepared first and run on the HPLC to ensure the dilution gave suitable absorbance.

Two vials of the sample (so that substantial variability in the results would indicate any major dissolution or dilution problems) and one vial of each of the six standards were prepared.

The gradient HPLC System 2 was equilibrated (the samples were maintained at 8°C in the auto-injector). The System was programmed to run a vial containing the nitro-n-alkanes once. The sample was run a total of six times and each of the six concentration standards three times. This lead to a total run time of 19.2 hours for the generation of the calibration curve and identifying the first set of results.

Confirmation using a separate dedicated UV instrument

The identity of chloroquine and ampicillin were confirmed with UV spectrophotometry by comparing the standard and sample spectra in the range 190-450 nm on a dedicated UV instrument which had been calibrated.

Quantification was confirmed using absorption at the UV maxima for each drug. A six point calibration (50, 70, 90, 100, 120, 150% of to the BP specification for that drug) was used. Each of the standard concentrations for the calibration points were measured three times and the sample was run six times.

These samples were dissolved in the solvents specified in Clarke [2] for UV assays of these compounds.

5.1.2 Retention indices and UV data for identification and quantification

The retention indices of the standards and samples were obtained on the nitro-n-alkane scale as described for the gradient system (Section 2.2.6).

The peak areas of the standards were entered into a spreadsheet along with the concentration of sample as translated into the percentage of BP specification that each calibration point represents.

The peak area and concentration were plotted in order to give a calibration line. Each point on the calibration line was the mean of three measurements.

Each of the six sample readings were used to determine the percentage of the BP specification from the calibration line, these six percentages were then meaned.

Calibration for quantification using absorption in the separate UV analysis was set up in the same way as described for the peak area quantification.

The Millennium software was used to compare the spectra of the sample and standard of the drug in question using the spectral matching software.

Table 5.1.1 shows some results of the surveillance testing. Excellent agreement is seen for the RI's of the sample and the standard in each case. The highest discrepancy was seen for procaine in procaine penicillin formulation. This elution was probably disturbed by the presence of other penicillins in the formulation. All the spectra of the sample were matched to the standards using Millennium software (described in Chapter 1), Figure 5.1.1 shows a pair of closely matched chloroquine standard and

sample spectra. The standards and samples of chloroquine and ampicillin gave spectra matching in shape and absorbance maxima on the separate UV analyses. For the samples tested here chloroquine injection was found to be comfortably within BP limits using the peak area method and the separate UV analyses. Procaine in procaine penicillin was also found to be within BP limits using the peak area method. benzylpenicillin fell just outside of BP limits, while ampicillin was significantly outside of BP limits using the peak area method and separate UV analyses.

Accuracy and Precision of the quantification

The correlation coefficient given by the calibration gives an indication of the accuracy of the quantification (Table 5.1.1), yet this is not an indicator of error in the slope. It is known that with use of less than six calibration points the slope error increases significantly, however reducing the number of calibration points could lead to greatly reduced analysis time. Therefore three point HPLC analysis calibrations were considered. One of the three point calibration sets used calibration points close to the sample concentration, while the other used calibration points further from the sample concentration (see Table 5.1.1). The mean percentage of BP specification given by each of these three point calibrations lie 3 % either side of the value given by a six point calibration. Whereas the six point method for the example chosen gave a result in very close agreement with the standard UV method, the three point results were further out. Indeed the correlation coefficients for the three point calibration calibrations were generally lower than the six point ones indicating lower accuracy for this method.

Confidence intervals (95% limits) for the quantification are all low indicating excellent precision in the assay indeed they all fall well within the limits for the BP specification (Table 5.1.1). The three point calibrations show higher precision (lower standard deviations and confidence intervals), than the six point calibration.

Drug	Run information	Mean RI of standard	Mean RI of sample	Mean % BP specification given by method (standard deviation)	BP specification	Confidence interval for 95% limits	Correlation coefficient (r) given by peak area Vs Standard concentration	UV spectral matching achieved by Millennium
Chloroquine Phosphate injection BP	HPLC 6 point calibration	241.97	242.24	96.2 (0.26)	95-105% of the stated amount	0.2	0.999	Yes
	UV 6 point at 257nm			98.8 (0.45)		0.4	0.999	
Ampicillin injection BP	HPLC 3 point calibration (90, 110 & 120%)	257.1	257	106.9 (0.15)	95-105% of the stated amount	0.1	0.996	Yes
	HPLC 3 point calibration (50, 110 & 150%)			112.6 (0.17)		0.2	0.993	Yes
	HPLC 6 point calibration			109.8 (0.69)		0.8	0.993	Yes
	UV 6 point at 257nm			110.1 (0.48)		0.4	0.981	
	UV 6 point at 268 nm			111.1 (0.67)		0.54	0.936	
Fortified Procaine Penicillin injection B.P.	HPLC 6 point calibration considering the procaine part	269.15	271.9	43.8 (0.9)	36-44% of the stated amount of procaine penicillin	1.8	0.999	yes
Benzylpenicillin injection BP	HPLC 6 point calibration	377	377.1	105.6 (0.52)	95-105% of the stated amount	0.5	0.999	Yes

Table 5.1.1 surveillance testing results on some BP formulations.

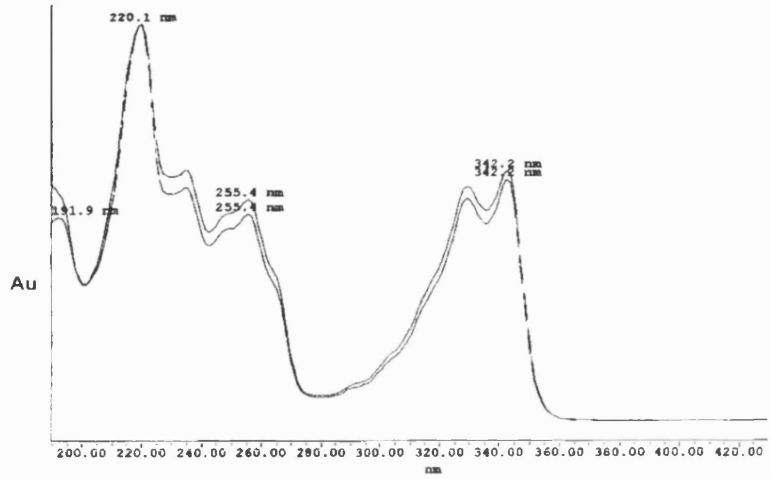


Figure 5.1.1 A pair of closely matched spectra, standard and sample of Chloroquine Phosphate injection BP.

Since all the confidence intervals fall well within BP limits then the case for using a six point calibration is its greater accuracy. Although the work up time and first analysis time is long (19.2 hours) subsequent analyses on the system could be configured to take only 4.6 hours of automated run time. This will be the case provided the system proves stable enough over a given period of time. Although a long BP test may approach this analysis time it involves the analyst's full attention, the automated screening process could ideally be quicker especially if carried out in the field. Near Infrared analysis, using whole sample scanning techniques and short analysis times promises many things as a screening method and is discussed in the next chapter.

5.2 The analysis of a new formulation of 5-aminolevulinic acid

5-Aminolevulinic acid, (5-ALA) is used in the photodynamic therapy of non-melanoma malignant tumours of the skin [104]. The 5-ALA is routinely applied topically and then the tumour irradiated with a laser. Increased effectiveness of the treatment has been shown in rats [105].

The aim of these experiments was to determine the rate of decomposition of 5-ALA (Figure 5.2.1) in aqueous formulation media and to identify the breakdown product(s). These experiments were carried out on samples at equivalent concentration and buffer pH to proposed formulations of an intravenous 5-ALA product.

The decomposition of 5-ALA under alkaline conditions is documented, notably by Franck and Stratmann [106]. A variety of reaction conditions do not affect the basic non-enzymatic formation of two main products a dihydropyrazine (Figure. 5.2.2) and porphobilinogen (Figure 5.2.3) in the ratio 10:1. The dihydropyrazine had previously proven difficult to isolate and identify.

Figure 5.2.1 5-Aminolevulinic acid.

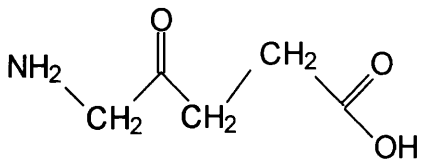
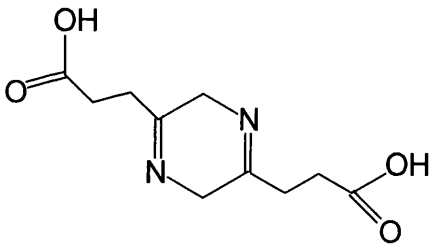


Figure 5.2.2 Dihydropyrazine decomposition product.



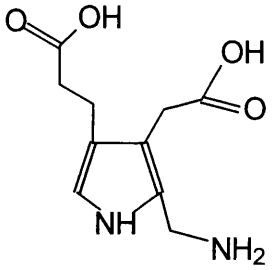


Figure 5.2.3 Porphobilinogen decomposition product.

Elfsson *et al* [107] investigated the degradation of 5-ALA using reversed-phase HPLC, at pH 4.81 and 7.42 at different temperatures and different initial concentrations of 5-ALA. Samples were incubated and sampled over a period of 37 days. They found the degradation to be second order overall and to be affected by temperature, pH and initial concentration of 5-ALA. With an initial concentration of 1% 5-ALA at pH 4.81 they determined the half-life to be 257 hours.

5.2.1 Preparation of 5-ALA formulations

The following suggested formulations were prepared in order to determine monitor the nature of 5-ALA decomposition:

In water:

1 g of 5-ALA to 50 ml with water for injections BP (WFI). (concentration 2%).

pH 5.0:

1 g of 5-ALA in WFI (25ml), add sodium bicarbonate (5.4ml of 8.4%) solution, then WFI to 50ml. (concentration of 5-ALA 2%).

pH 6.4:

1 g of 5-ALA in WFI (25ml), add sodium bicarbonate (7.8ml of 8.4%) solution, then WFI to 50ml.

5.2.2 HPLC analysis on System 2

A gradient system uses the two eluents A and B as previously described. The gradient consisted of four minutes at 1% B, then a rise to 40% B over six minutes and maintaince at 40% B for a further four minutes. The gradient was then returned to 99% A over two minutes in readiness for the next sample.

Samples of 5-ALA (20 µl) in water were run on the gradient HPLC system every hour for nine hours after its preparation.

The pH 5.0 and pH 6.4 formulations of 5-ALA (10 µl) were run on the gradient HPLC system every hour for fifty hours.

The chromatograms of 5-ALA in water act as a control and show no decomposition over 9 hours. The retention time (2.52 minutes) and UV spectrum of 5-ALA were obtained from these chromatograms.

Examples of chromatograms of 5-ALA formulated samples at pH 5.0 and 6.4 are shown in Figures 5.2.4 and 5.2.5.

Three peaks were identified in the chromatograms of the pH 5.0 and 6.4 samples.

Initially the 5-ALA peak is seen at 2.5 minutes. A secondary peak can be observed from 12.5 minutes and is due to the main decomposition product. Small secondary peaks may be equivalent to the porphobilinogen or related products.

The identity of the first peak as 5-ALA was confirmed by comparing its UV spectrum to one obtained from the 5-ALA on a Perkin-Elmer spectrophotometer in a separate experiment. The spectra of 5-ALA and the main decomposition product obtained from the chromatograms are given in Figure 5.2.6.

The peak at 2.49 minutes shows a shoulder, this is attributed to 5-ALA being a zwitterion resulting in an equilibrium disturbing the chromatography. Examination of the UV spectra at different points across the peak did not reveal any differences, indicating this to be an equilibrium effect rather than formation of a discrete product.

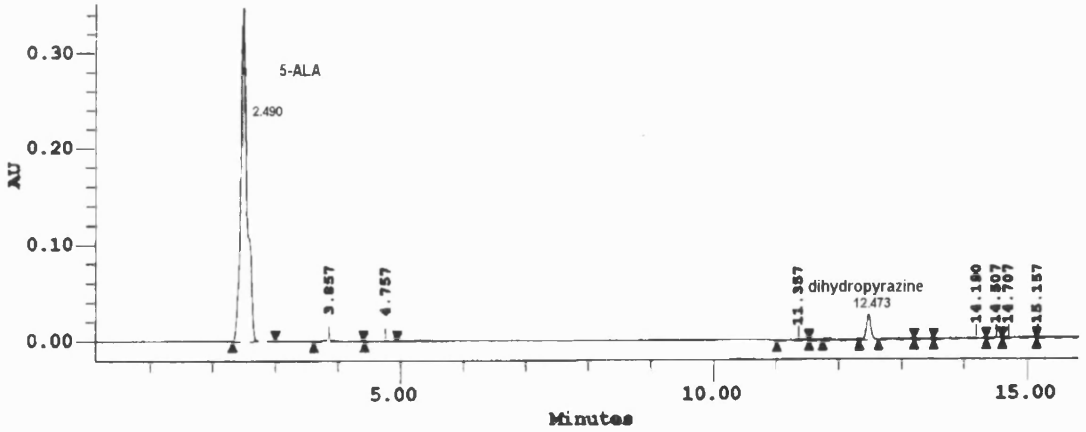


Figure 5.2.4 Chromatogram (263 nm) of 5-ALA in pH 5.0 formulation 17 hours after formulation.

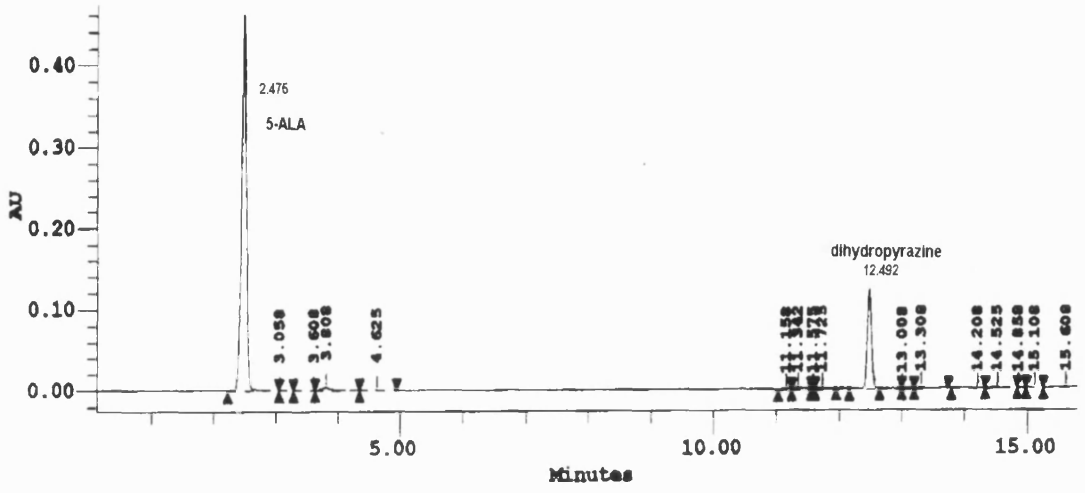


Figure 5.2.5 Chromatogram (263 nm) of 5-ALA in pH 6.4 formulation 9 hours after formulation.

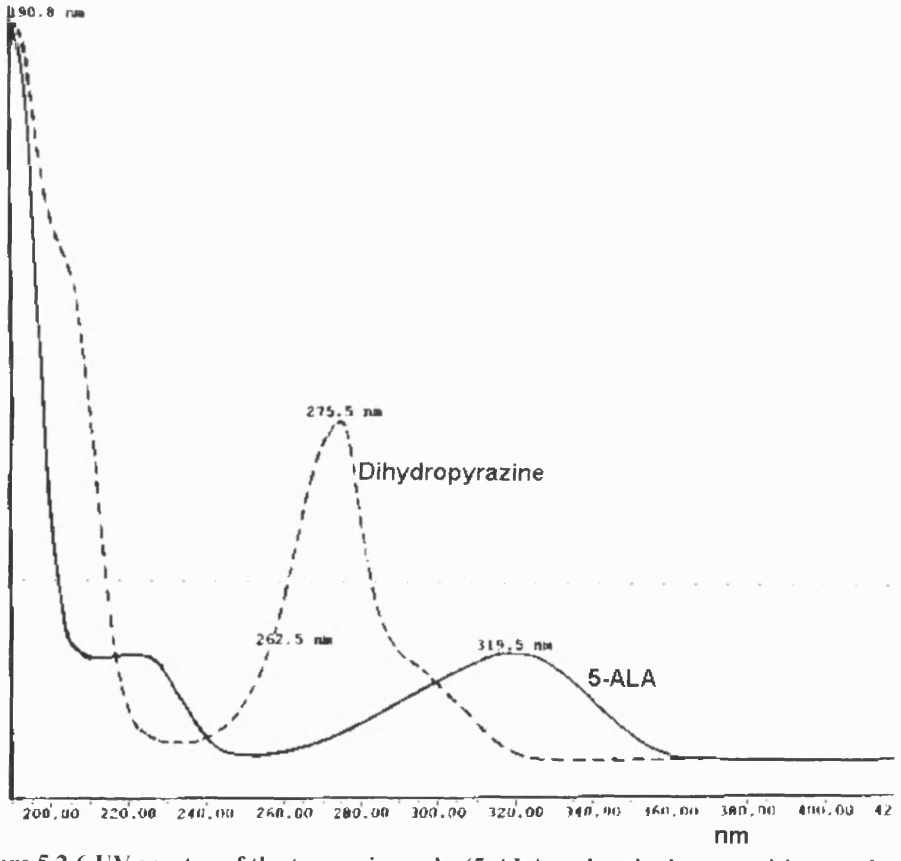


Figure 5.2.6 UV spectra of the two main peaks (5-ALA and main decomposition product) obtained from a chromatogram of the sample in pH 6.4 formulation.

Figures 5.2.7 and 5.2.8 shows the peak areas of the 5-ALA and its decomposition product. There is an overall increase in the peak area of 5-ALA although the correlation between time and peak area is low r = 0.87. This makes stability estimation impossible from direct monitoring of the amount of 5-ALA in the sample, although at pH 6.4 the peak shape of 5-ALA improves and the expected fall in 5-ALA and rise in decomposition product peak areas are seen (Figures 5.2.9. and 5.2.10).

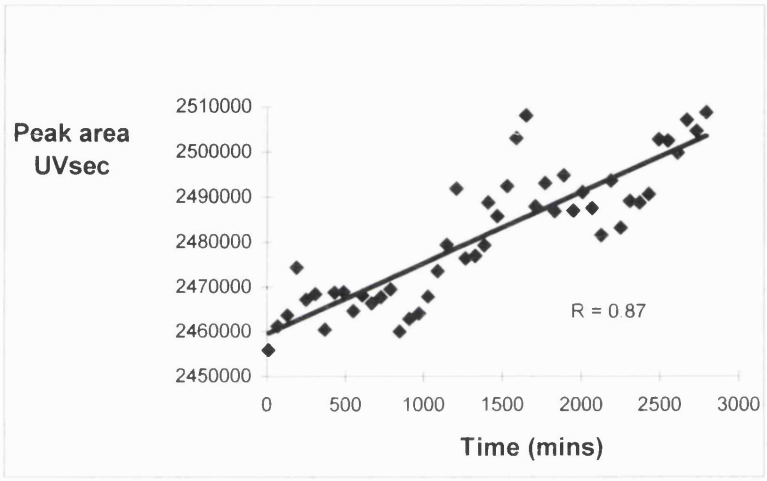


Figure 5.2.7 Peak areas Vs time for the 5-ALA peak at pH 5.0.

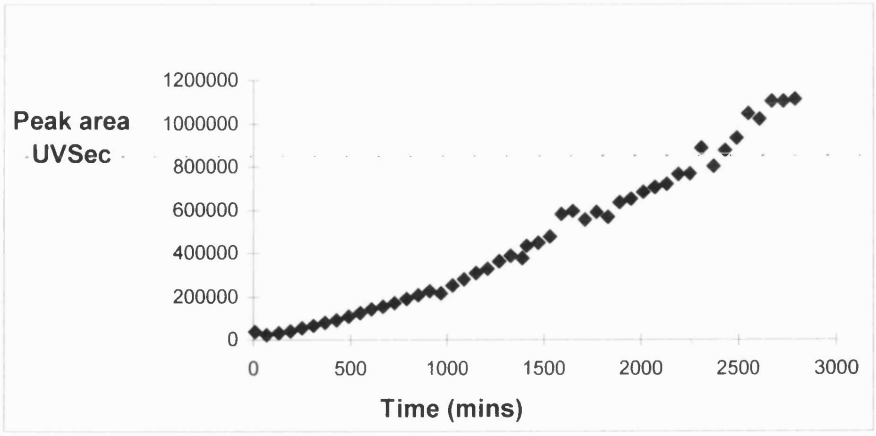


Figure 5.2.8 Peak areas (at 263 nm) Vs time for the main decomposition peak at pH 5.0.

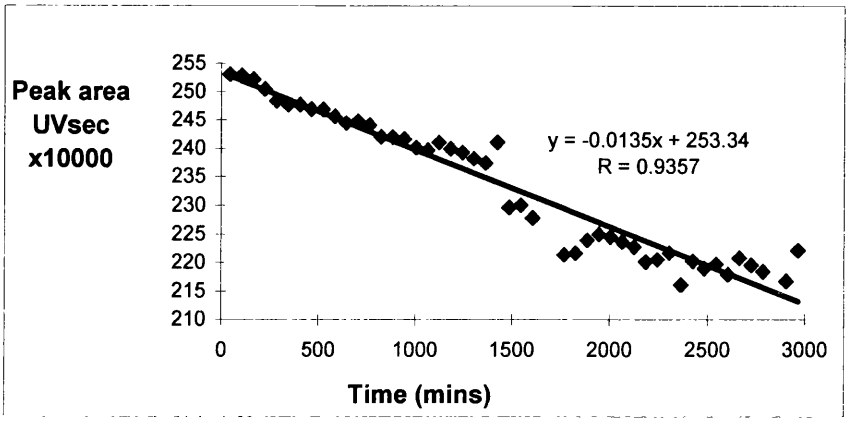


Figure 5.2.9 Plot of peak areas (at 263 nm) Vs time for the 5-ALA peak at pH 6.4.

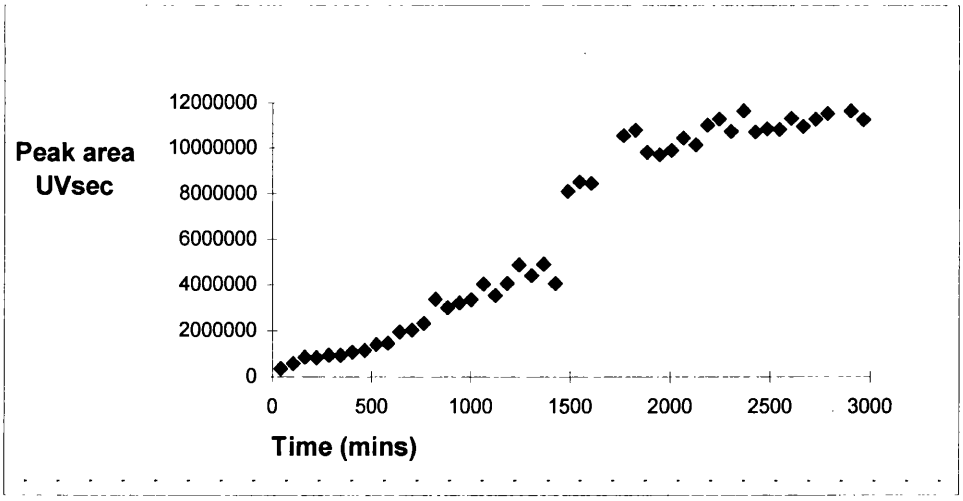


Figure 5.2.10 Peak areas (at 263 nm) Vs time for the main decomposition peak at pH 6.4.

The fall of the concentration of 5-ALA with over the investigated time period is basically linear. However the overall decomposition would be expected to be second order. A regression line is fitted giving a correlation coefficient R = 0.967. The estimated half-life obtained from this equation is 156.4 hours or 6.5 days. By this

reasoning the 5-ALA will be approximately 4.2 % degraded after 12 hours. This result is concurrent with that obtained by Elffson *et al* [107] however this method does not require long experimental times that would make the method impractical in a hospital quality control situation.

The overall change increase in peak area of the pH 6.4 formulation main decomposition product is approximately ten-fold of that formed at pH 5.0.

This value is only an estimate of 5-ALA's half-life under the formulation conditions. The limitations of this experimental method do not allow quantification of the amount of dihydropyrazine formed. Nor do the experiments make any account of the toxicity of the decomposition products of 5-ALA formed under the proposed conditions.

5.2.3 NMR analysis

NMR spectroscopy was used to confirm the nature of the principal decomposition product.

NMR spectra were recorded on a Bruker 500 MHz instrument at room temperature. A standard spectrum of 5-ALA in D_2O was recorded for confirmation. For kinetic studies, 5-ALA was prepared in D_2O with the pH adjusted to 6.8 by addition of NaOD in D_2O. Spectra were recorded immediately and after 96 hours.

The NMR spectra for 5-ALA in water and at pH 6.4 are given in Figures 5.2.11 and 5.2.12. For the 5-ALA in water sample a pair of triplets are seen at 2.989 and 2.718 PPM. These represent the protons of the methylene groups at positions 2 and 3. The methylene group adjacent to the carbonyl group is the further upfield of the triplets. A singlet at 4.13 PPM represents the protons on the methylene group at position 5.

The spectrum of the sample at pH 6.4 after 96 hours shows an extremely reduced singlet at 4.13 PPM as these protons are lost in the formation of the dimer. Triplets are seen at 2.5 and 2.76 PPM. These triplets still represent the two pairs of symmetrical methylene groups. This is due to the carbonyl being replaced by a nitrogen atom and a double bond. A singlet at 2.47 PPM represents the equivalent protons on the heterocyclic ring of the dimer. Additional weak signals are due to the porphorbolinogen product.

Dicarboxyethyldihydropyrazine has been clearly identified by NMR spectroscopy as the principal decomposition product of 5-ALA under the pH 6.4 formulation conditions. The rate of decomposition increases with increasing pH. At pH 6.4 the rate of formation of the dihydropyrazine is approximately ten times the rate at pH 5.0.

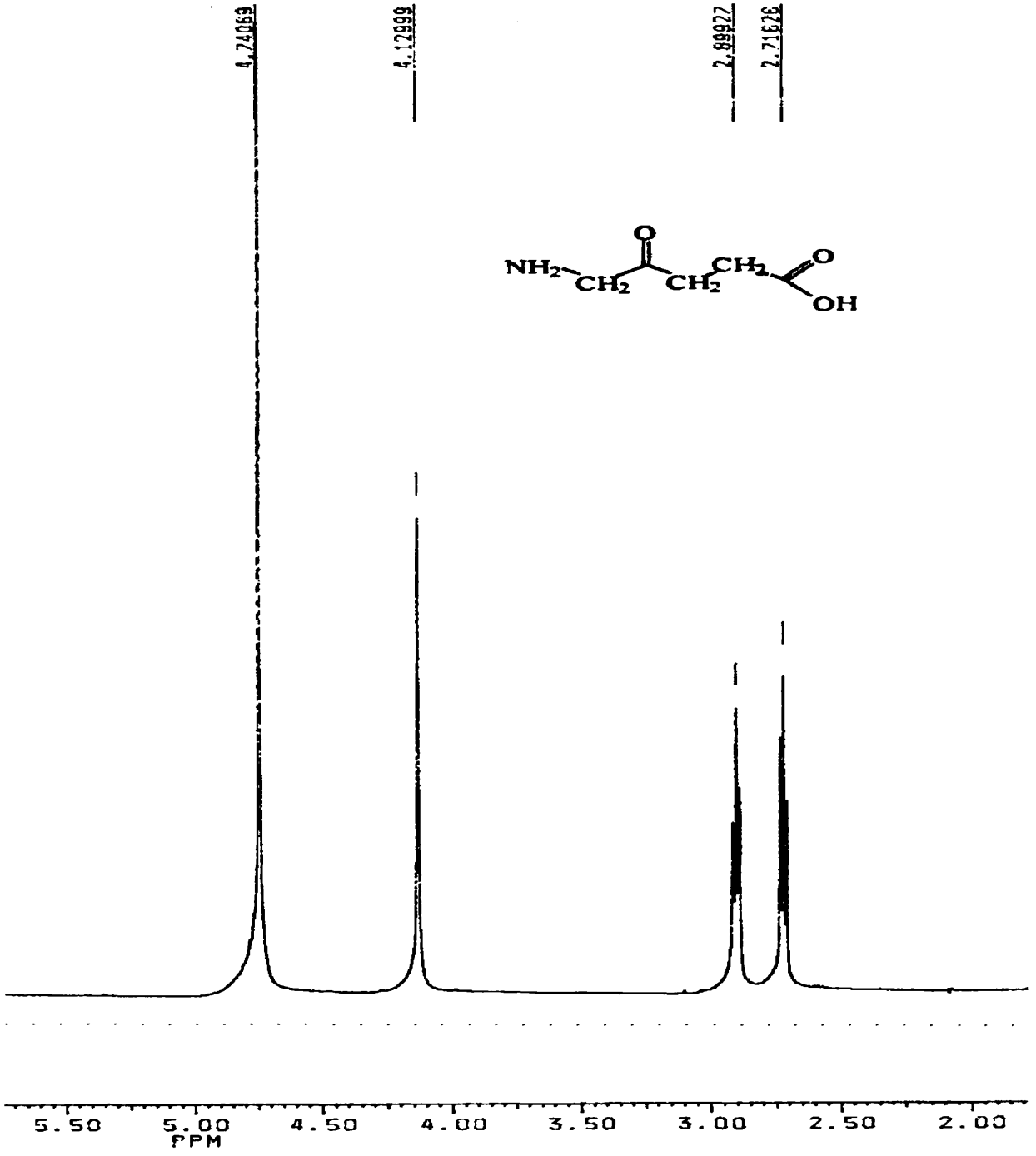


Figure 5.2.11 Proton NMR spectrum of 5 -ALA in D_2O.

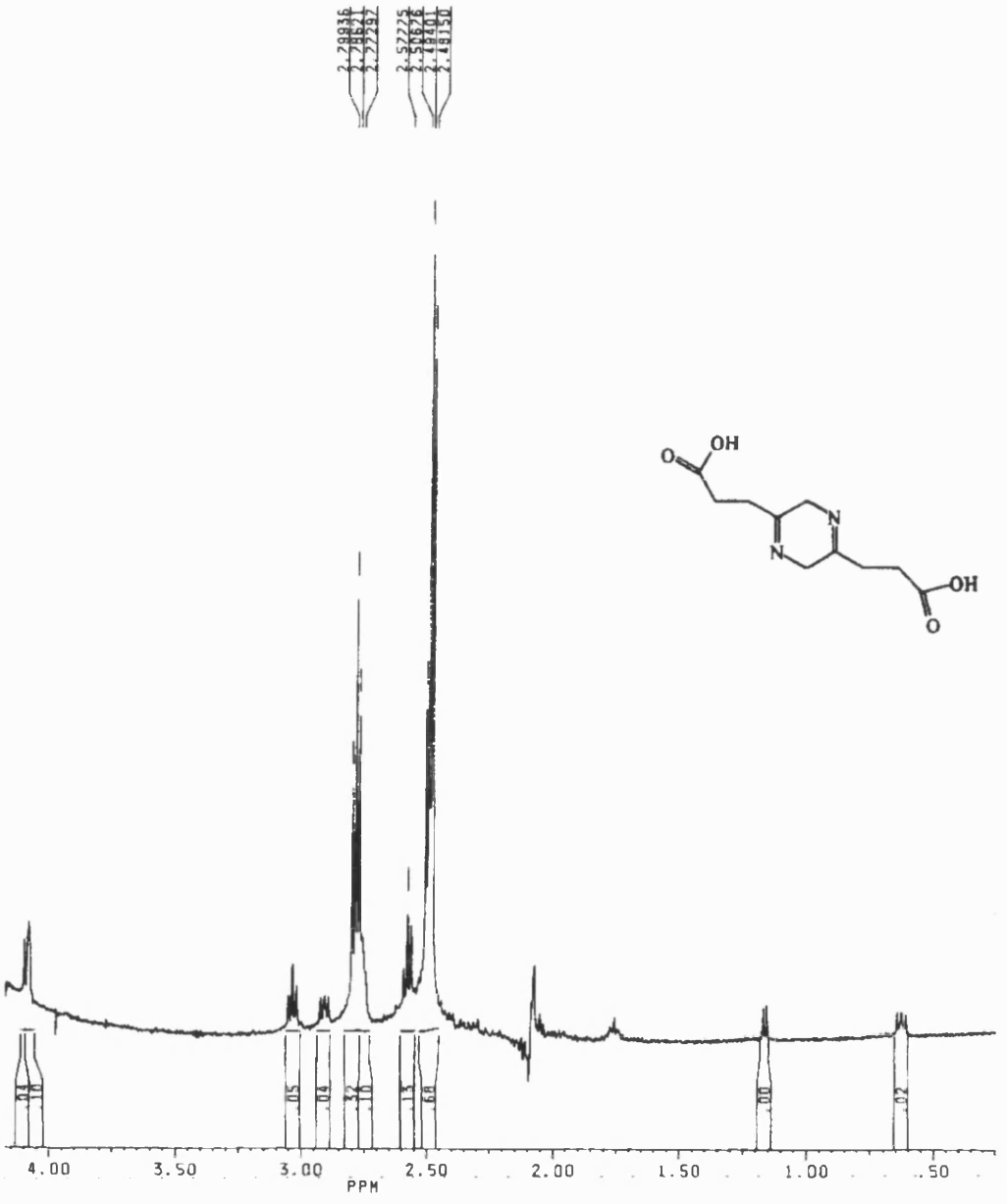


Figure 5.2.12 Proton NMR spectrum of 5-ALA in pH 6.4 formulation 4 days after preparation.

Most BP limits for the quantity of active drug in a dosage form are required to be within +/- 5% of the stated content by the specified method. The precision of the method proposed here is well within these limits. Further investigation of the stability of the system would be required in order to determine how much work is required for

each analysis. For example will dissolution of tablets and capsules in a acetonitrile\water mixture be sufficient extraction procedure to ensure repeatable results ? How often is it necessary to run the standards to produce the calibration line?

The 5-ALA, pH 6.4 formulation shows that an estimated decomposition of 4 % over 12 hours occurs. The product should remain within 95 % limits from the time of preparation to administration. Should routine quality control of the 5-ALA formulation be necessary, this HPLC method would be appropriate. However due to the relatively short shelf-life of the products calibrated apparatus would be required on site.

6. NEAR INFRARED SURVEILLANCE

An ideal surveillance method would be rapid, non-destructive, capable of detecting impurities and quantifying the material. Near Infrared (NIR), lays claim to a future that could fulfil all of these promises although there are many obstacles to be addressed before this point is reached.

The NIR region is generally accepted as being that between 780 and 2500 nm (European Pharmacopoeia [108]). This light is detected using lead sulphide detectors as opposed to the silicon ones used in mid-infrared (mid-IR) spectroscopy. Most instruments are single beam with long lasting halogen-tungsten lamps and the wavelength range is given by high precision holographic gratings. These instruments give high signal to noise ratios.

Spectra in the NIR region are characterised by overtones and additive combinations of organic functional groups (C-H, O-H, N-H and S-H) rather than the stronger fundamentals seen in the mid-IR region. These combinations may be up to a 1000 times weaker than the fundamentals and do not appear as well defined peaks. However the weak absorptivity in this region means that samples can be analysed undiluted and the light penetrates deeply into the sample. Because the absorption of light is weak, spectra can be measured by transmission or reflectance. Where the instrument is primarily used as a reflectance instrument (with detectors on the same side of the sample as the light source), then a reflector may be placed behind the sample to give a double path length variation of transmission called transflectance. Reflectance instruments offer a number of sample presentation options; cuvettes, spinning sample holders and fibre optic probes used for online process analysis.

In this work a single beam reflectance instrument was used, therefore a standard spectra must be recorded for baseline correction. (The standard must be robust such that its absorptive surface is not altered, ceramics are often the standards of choice.) When a sample is inserted in the spectrophotometer, the light reflected in this manner is called diffuse reflected light and contains chemical and physical information about the sample.

The Kubelka-Monk equation that describes absolute reflected light can be re-written [19] in terms of analyte concentration (Equation 6.1.).

$$f(R_\infty) = \frac{(1-R)^2}{2R} = \frac{c}{s/2.303\varepsilon}$$

Equation 6.1 The relationship between absolute reflected light at a given frequency (f (R_∞) and the concentration (c), of the analyte. Where R is the relative reflectance s is the dispersion coefficient and ε is the molar absorptivity of the sample.

A straight-line relationship between absolute reflectance and analyte concentration is only given if the absorption bands are weak, and the sample matrix does not show strong absorption. As this will often be the case, NIR reflectance spectra are recorded as an apparent absorbance (i.e. A= log 1/R), because for most powdered samples the Beer Lambert law is obeyed.

Variation in the spectra are caused by three factors: non-specific scatter of radiation, variable light path length through the sample and the chemical composition of the sample. The first two factors may be termed 'muliplicative scatter effects' and these are altered by the sample presentation. Multiplicative scatter effects lead to the unique identity of each NIR spectrum. The statistical methods NIR spectroscopists use to overcome these effects are discussed later.

NIR has been used for process analysis for some time in the agricultural, food and pharmaceutical industries. Routine uses include: moisture determinations as there is a strong absorption band at 1940nm [109-111]. Monitoring powder blending by demonstrating a reduction in the standard deviations of spectra as blend uniformity increases [112-115]. Physical properties are sometimes monitored using NIR. Polymorphism and the presence of different enantiomers can cause changes in the NIR spectral fingerprint [116,117].

The rapid growth of NIR use in the pharmaceutical industry has been reviewed by Blanco *et al* [19], who discuss the current state of the method for drug identification and quantification as well as the use of NIR in the applications mentioned above.

Interest in the use of NIR for drug identification has grown with the ability of computer based software to interpret the complex spectra [118-122]. The ability of NIR to analyse whole products non-destructively has been used to investigate products that have degraded, been tampered with [123-125] or are still enclosed in packaging material [126,127].

The technique suffers from two major drawbacks namely non-proven transferability [128,129] and sample presentation problems [130]. Sample presentation, affects transferability itself. However the geometry of detectors monochromators and connecting fibre-optics lead to large differences in the noise displayed in the spectra, even of two instruments of the same make and specification.

Sample presentation affects the spectra by scattering effects. These may be caused by such variables as particle size [131-134], markings on a tablet, the thickness of the sample, the nature of container [135], sample temperature, sample moisture content - although this is not an exhaustive list.

Methods used to interpret complex data using statistics or algorithms are described as chemometrics. It is largely the advances in such techniques and the availability of fast computers to calculate them, that has led to the current resurgence in interest in the NIR region. The basic techniques for achieving sample identification are outlined below.

Correlation spectral matching [135]

Spectra in a NIR library are converted to their second derivatives. A correlation coefficient is determined between the unknown spectrum and those in the library. This is based on a linear association between the absorbancies of the compared spectra at a number of given wavelengths. The equation (Equation 6.2) for this correlation coefficient is given below.

$$p_{jk} = \frac{\Sigma\left(x_{ij} - \overline{X_j}\right)\left(x_{ik} - \overline{X_k}\right)}{\sqrt{\Sigma\left(x_{ij} - \overline{X_j}\right)^2}\sqrt{\Sigma\left(x_{ik} - \overline{X_k}\right)^2}}$$

Equation. 6.2 Calculation of correlation coefficient.

Where p is the correlation coefficient; j and k are the two second derivative spectra; x is the second derivative of the absorbance at each wavelength (i) of each individual spectrum; $\overline{X}$ is the second derivative of the absorbance at each wavelength of the mean of each set of twelve spectra.

Wavelength distance match [135]

This algorithm calculates the maximum distance between an unknown derivatised spectrum and the mean of the standard deviation spectra for each of the drugs maintained in the library. The distance is measured in standard deviations at each wavelength. The most likely match between sample and library drug is given by the lowest overall distance.

Polar qualification system (PQS) [136]

Here the absorbance values are represented as distances (radii) and the associated wavelength is represented as an angle. Scanning probability windows are used to detect the wavelengths where the greatest variability lies between two spectra. The probabilities of variability are given and the wavelengths at which these occur is indicated.

Principal component analysis (PCA) [137]

Are matrix algebra algorithms which seek to express unrelated variations between spectra with the fewest possible vectors.

PCA has the advantage of removing much of the background noise after the first principal component. Both PCA and PQS require training sets for calibration. Wavelength distance, PCA and PQS are also useful for examining small or specific variability between spectra [138].

Rapid surveillance requires a comparison of samples in different forms; liquids, tablets and powders. These may be presented directly as raw or coated formulations or in a variety of glass and plastic packaging materials. Therefore in this initial work the correlation method was used to consider the possibility of identification on a 'macro-variability' scale. That is, can a drug be scanned on the instrument in its original presentation and be identified as the most likely product from a library of drugs.

The samples were limited to those powders for injection that were used in the HPLC surveillance work in order to compare the results given by each method. An investigation to the effect of sample presentation was carried out to examine the effect on this method on the identification.

6.1 IDENTIFICATION OF SAMPLES USING CORRELATION SPECTRAL MATCHING

The following powders for injection were used for the experiments:

Benzylpenicillin	Ampicillin
Cefuroxime (supplier 1)	Cefuroxime (supplier 2)
Thiopental	Procaine Penicillin

A library of 160 drugs (entered by other research workers in our laboratory) were held in the IQ^2 software accompanying the Foss 6500 NIR-VIS instrument, as standard spectra using a scan range of 1100-2500nm. These spectra were held on file as a mean of twelve scans the vial being rotated randomly on the instrument to give twelve different sample presentations.

The spectra of the powders for injection within their original vials and in Waters 4ml vials were scanned in the same fashion. The Waters 4ml vial and the vials containing the powders for injection were washed dried and scanned empty with their original caps on.

The spectra were averaged and converted to second derivative spectra. Using the IQ^2 software the powders for injection were compared with the derivatised spectra in the library using the correlation method. This was done for the powders both in their original vials and also in the Waters 4ml vials.

Using both the Waters vials and their original vials all the samples were identified correctly except for procaine penicillin which was not present in the library of pure samples. Table 6.1.1 shows the correlation coefficients for the first and second closest matches to the samples. The correlation coeffecients for the first match are 0.98 or above while the second match is of a much lower order. Although we can only make arbitrary comments about the certainty of the identification, this differentiation appears very promising. Although procaine penicillin was not present in the library, benzylpenicillin was given as the closest match to this sample with an appropriately low correlation coeffecient ($r = 0.554/0.565$ for original vial/Waters vial). This gives credibility to the correlation method's ability to differentiate but also to recognise chemical functions. The

method appears all the more robust since the identification is clear and only slightly improved by the use of Waters vials. Further the multiplicative scatter effects were not controlled in the samples or the pure drugs samples used to build the library. That is to say sample depth, particle size, water content were not controlled.

Sample	Correlation coefficient with drugs in IQ^2 library:	
	Sample in original container	**Sample in Waters vial**
Benzylpenicillin	0.995 = benzylpenicillin 0.388 = ampicillin	1 = benzylpenicillin 0.387 = ampicillin
Cefuroxime (supplier 1)	0.985 = cefuroxime 0.228 = amylobarbitone	0.991 = cefuroxime 0.236 = amylobarbitone
Cefuroxime (supplier 2)	0.996 =cefuroxime 0.226 = amylobarbitone	0.999 = cefuroxime 0.238 =amylobarbitone
Ampicillin	0.987 = ampicillin 0.523 = glutethimide	0.988 = ampicillin 0.522 = glutethimide
Thiopental	0.98 = thiopental 0.514 = progesterone	0.993 = thiopental 0.546 = progesterone
Procaine Penicillin	0.554 = benzylpenicillin 0.546 = ampicillin	0.565 = benzylpenicillin 0.557 = ampicillin

Table 6.1.1 Results of comparing drug samples in their original and in Waters vials, with the spectral library using the correlation method.

6.2 USING ONE DRUG TO CORRECT FOR VIAL DIFFERENCES

Light measured by the instrument in the diffuse reflectance mode travels through the glass of the container undergoes absorption and diffuse reflectance. The reflected light returns to the detector again via the glass of the container. The effect of the glass upon the identification was therefore of primary interest. The drugs in the library were all scanned in the Waters 4ml vial as the standard container. Ideally therefore it is necessary to correct the other types of container back to this one.

This was first attempted by filling a Waters vial and each of the suppliers original container with benzylpenicillin. These were each scanned twelve times and the second derivative of the mean spectrum recorded. The spectrum for benzylpenicillin in each of the others drug containers was subtracted from the spectrum of benzylpenicillin in the Waters vial. Each resulting spectrum was then added to the spectrum of each of the other drugs in its own container. The resulting spectrum from this addition was then scanned against the library. Benzylpenicillin was chosen as the drug to be used for the correction procedure because of its distinct peaks and even baseline. Correcting back to the Waters vial using benzylpenicillin did not improve the correlations with the pure drugs in the library (Table 6.2.1) in most cases they got worse. Except in the case of procaine penicillin where the correlation with benzylpenicillin jumped from r = 0.554 (for procaine penicillin in its own container), to r = 0.689 for procaine penicillin corrected back to the Waters vial.

Also ampicillin is seen to get significantly worse than the other compounds. This would suggest that the benzylpenicillin 'part' of the spectrum is still involved in the 'correction' as well as the contribution of the glass. Figure 6.2.1 shows the un-derivatised spectra of ampicillin in its original container. Figure 6.2.2 shows benylpenicillin in a Waters vial with large distinct peaks at 2060 and 2180 nm. The corrected spectra of ampicillin, (Figure 6.2.3), also displays increased relative absorption at these two wavelengths indicating that some residual absorption by benzylpenicillin has been retained after the correction subtraction and addition of spectra.

Sample	Correlation coefficient with drugs in IQ^2 library:	
	Sample, benzylpenicillin corrected	**Sample, mercury corrected**
Benzylpenicillin		1 = benzylpenicillin 0.4 = ampicillin
Cefuroxime (supplier 1)	0.982 = cefuroxime 0.219 = amylobarbitone	0.985 = cefuroxime 0.24 = amylobarbitone
Ampicillin	0.903 = ampicillin 0.713 = glutethimide	0.988 = ampicillin 0.529 = glutethimide
Thiopental	0.951 = thiopental 0.496 = progesterone	0.986 = thiopental 0.537 = progesterone
Procaine Penicillin	0.689 = benzylpenicillin 0.525 = ampicillin	0.561 = benzylpenicillin 0.523 = ampicillin

Table 6.2.1 Results of comparing drug samples in their original containers after benzylpenicillin and mercury correction, with the spectral library using the correlation method.

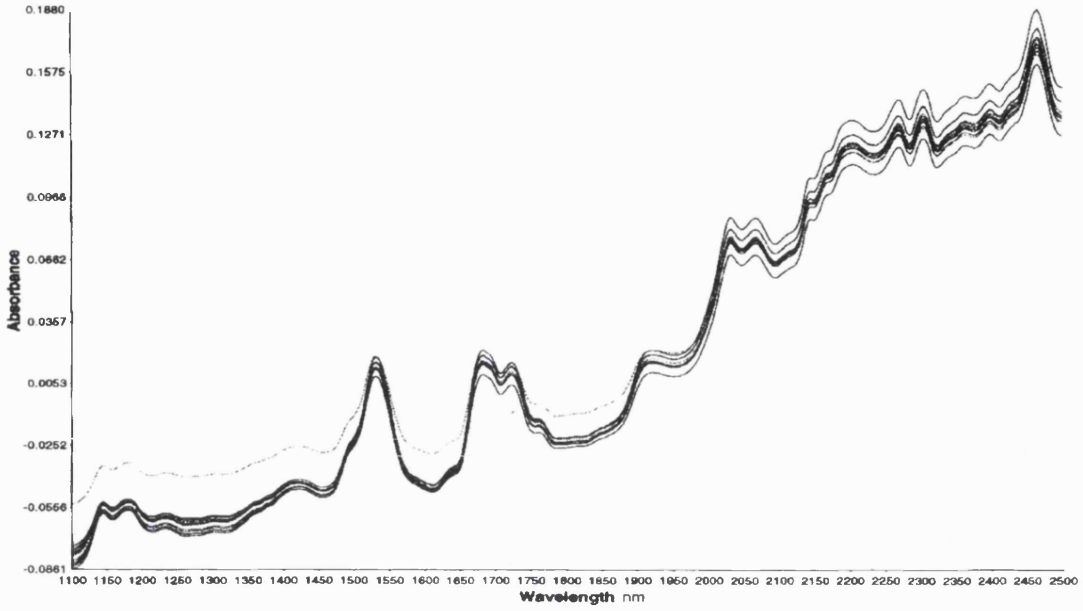


Figure 6.2.1 Un-derivatised NIR spectra of ampicillin in its original container.

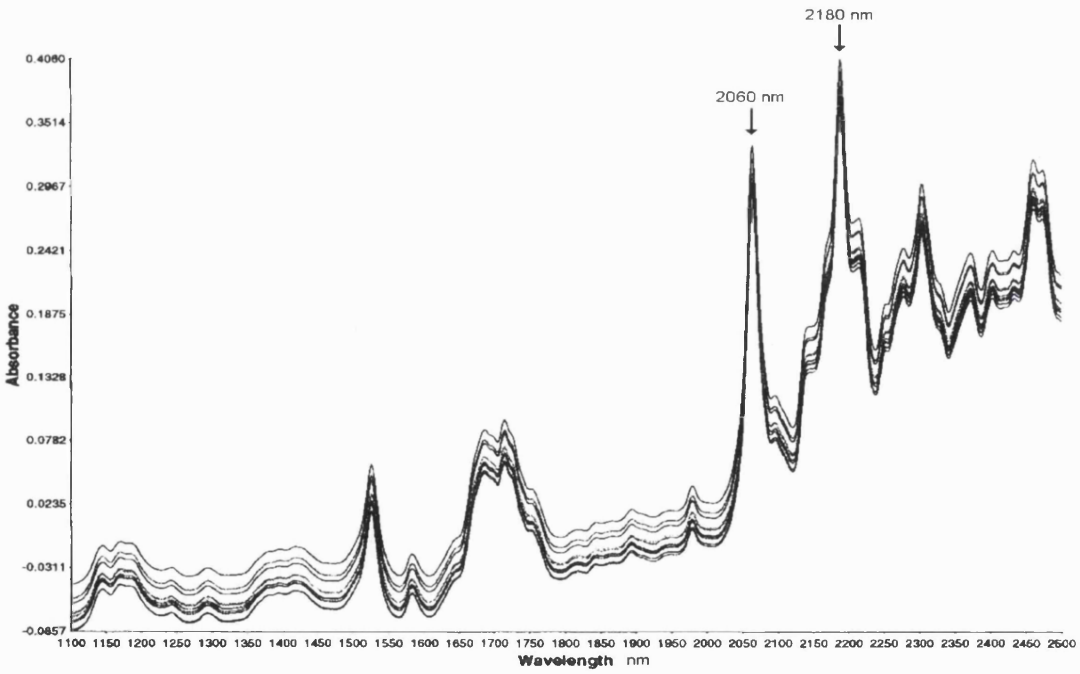


Figure 6.2.2 Un-derivatised NIR spectra of benzylpencillin in the Waters vial.

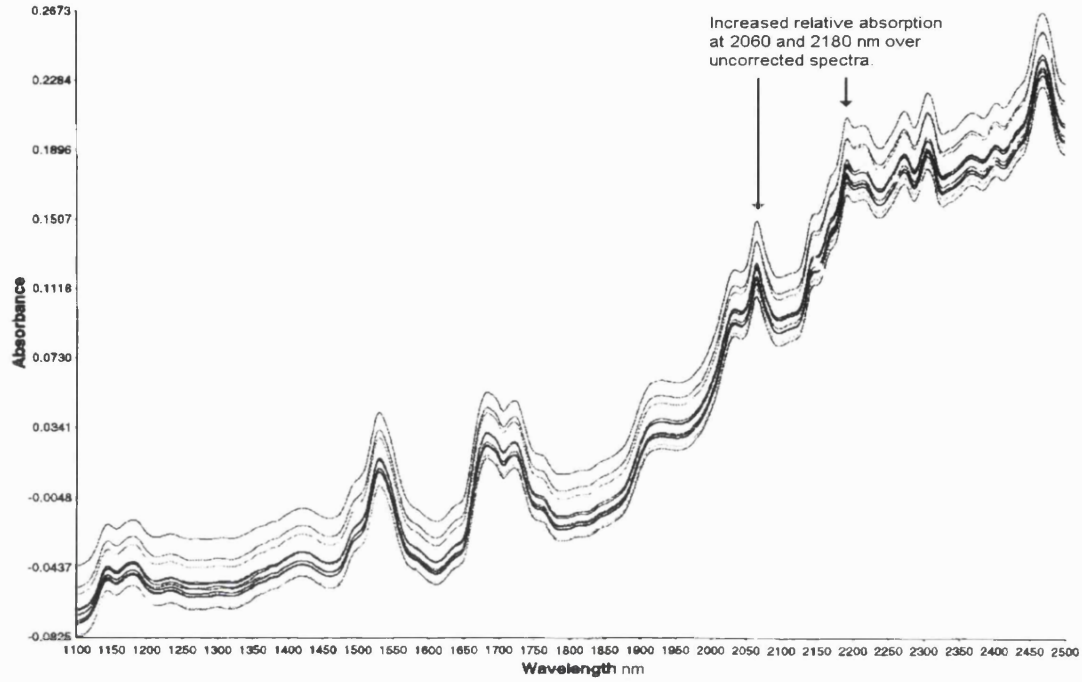


Figure 6.2.3 Un-derivatised spectra of ampicillin after correction back to the Waters vial using benzylpenicillin, showing residual effects on relative absorption caused by large peaks in the benzylpenicillin spectra.

The presence of residual benzylpenicillin effects could be due to other factors. The spectral effect of the glass, the difference in distance of the sample from the detector in different containers or the way the benzylpenicillin was packed in each container may have affected the final spectra in this way.

6.3 USING MERCURY TO CORRECT FOR VIAL DIFFERENCES

The previous experiment was repeated but the vials were filled with mercury to obtain a spectrum where the incident light was reflected back through the glass container by the mercury. Figure 6.3.1 shows a schematic of the light path within the Foss Nirsystem 6500 instrument.

For each drug and container; spectra of mercury in the drug's original container were subtracted from the spectra of a Waters vial containing mercury. The resultant spectra were added to the spectra of the drug in its original container. The spectra resulting from this addition were then scanned against the spectral library of drugs contained in Waters vials.

Mercury is a highly reflective material but will have absorption properties which would be further affected by its purity. It also had the advantage of filling the internal surfaces of the vial providing a good reflective surface.

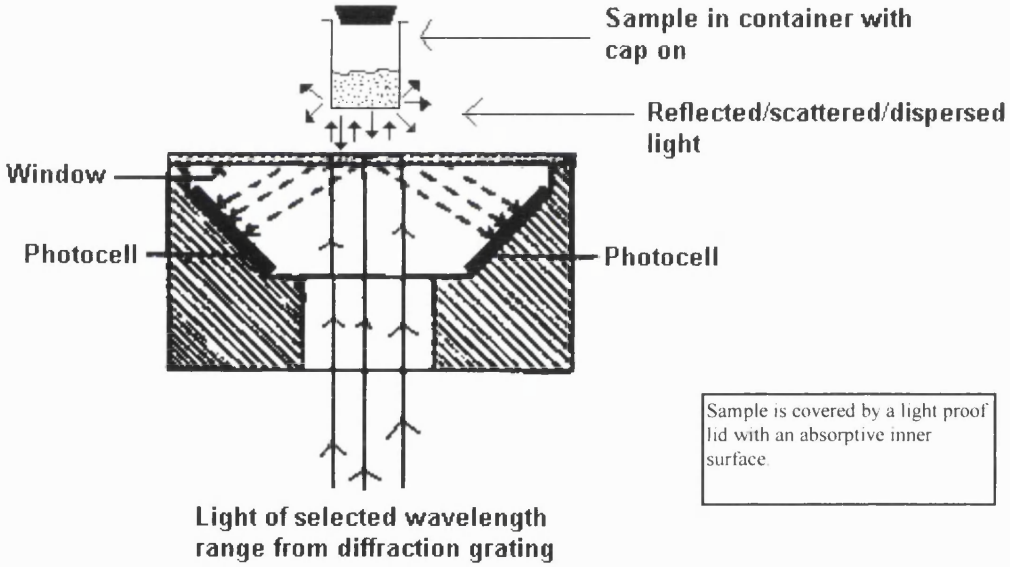
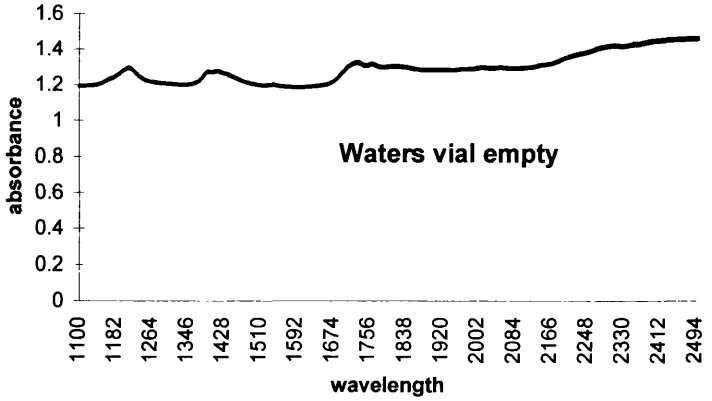


Figure 6.3.1 Schematic diagram of the arrangement of the Foss 6500 NIR spectrophotometer.

The un-derivatised spectra of an empty Waters 4ml vial and also filled with mercury are shown in Figure 6.3.2. Reduced absorption is seen with mercury in the vial but there is still significantly more absorption of any of the drugs presented in their vials. This massive loss of light is unlikely to be due to NIR absorptive properties of the mercury and or impurites as well as the glass. It is more likely that the mercury is working too well as a reflective surface sending light directly back to the light source instead of the detectors which are arranged at 45° (in order to avoid picking up specular light), to the sample.

This level of absorptivity will have some measure of effect on the correction of spectra, but not a large effect on the correlation of spectra since it is a relatively flat spectrum that will only have a dampening affect on the more distinct peaks after subtraction of spectra. Comparing the mercury corrected correlation results (Table 6.2.1) with the samples in their own vials and Waters vials correlation results (Table 6.1.1) little change was seen. The mercury results show a slight improvement over the samples presented in their own vials. They are slightly lower correlations than those obtained with the samples in uncorrected Waters vials.

Figures 6.3.3 - 6.3.12 show the spectra from individual empty and mercury filled drug vials and the corresponding effect of the mercury correction process to a Waters vial standard for the respective drug samples. The correction of the procaine penicillin vial shows the largest visible change to the resulting spectrum and this drug in this vial shows the most significant change in correlation.



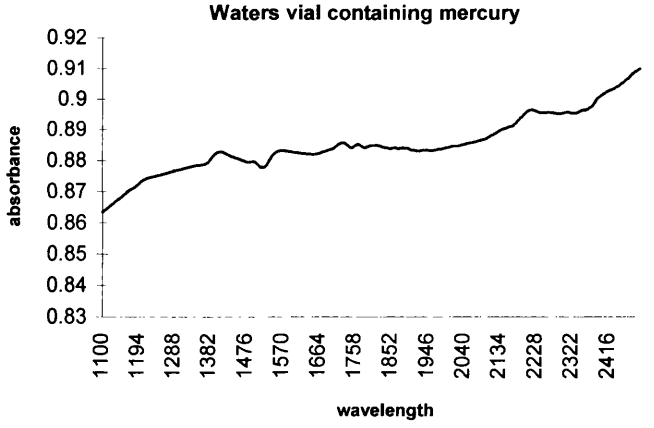


Figure 6.3.2 NIR spectra of empty and mercury filled Waters 4ml vials.

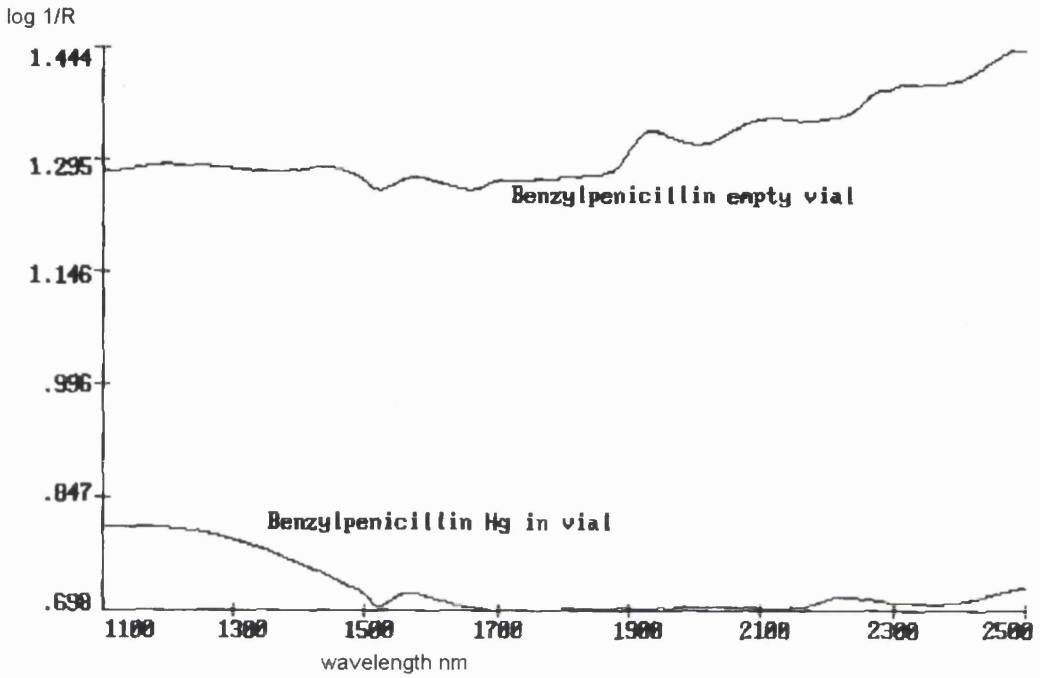


Fig. 6.3.3 NIR spectrum of an empty benzylpenicillin vial and also filled with mercury.

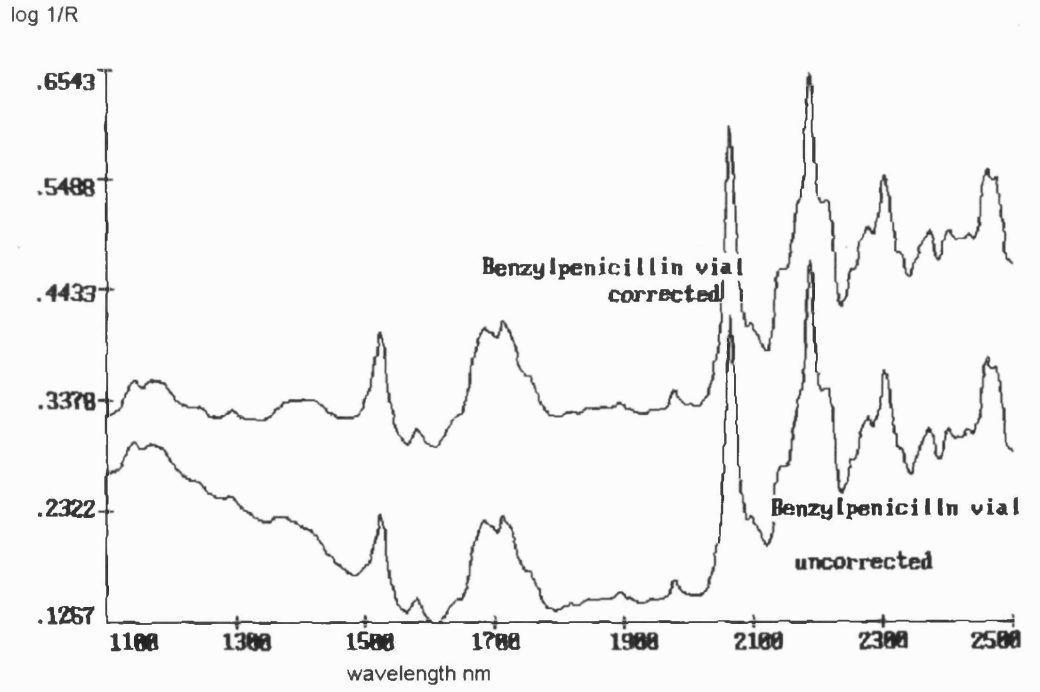


Figure 6.3.4 NIR spectrum of benzylpenicillin in its original vial corrected back to the Waters vial using mercury. Benzylpenicillin in its original vial uncorrected.

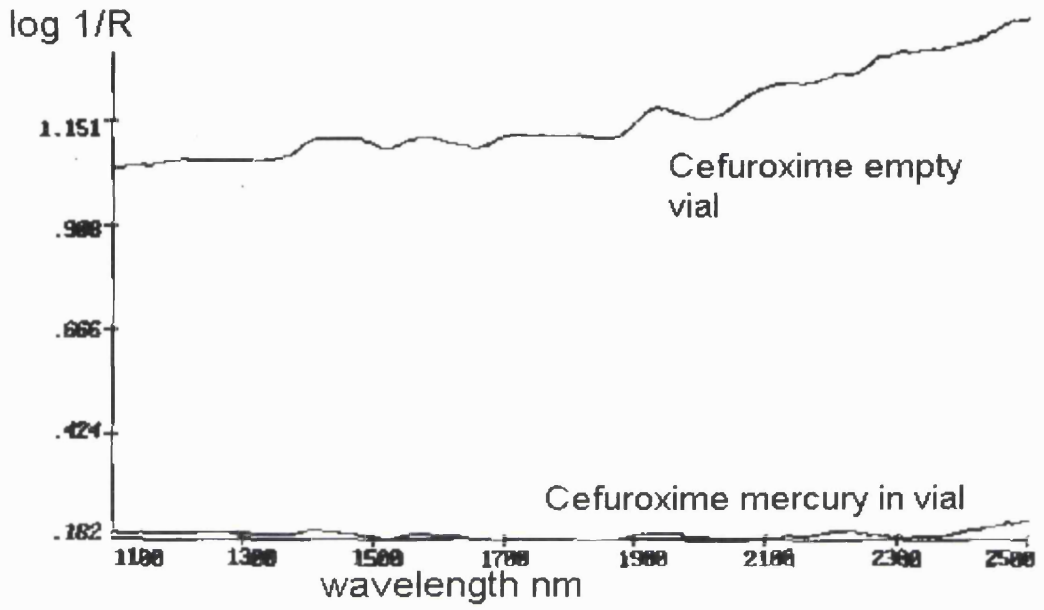


Figure 6.3.5 NIR spectrum of an empty cefuroxime (supplier 1) vial and also filled with mercury.

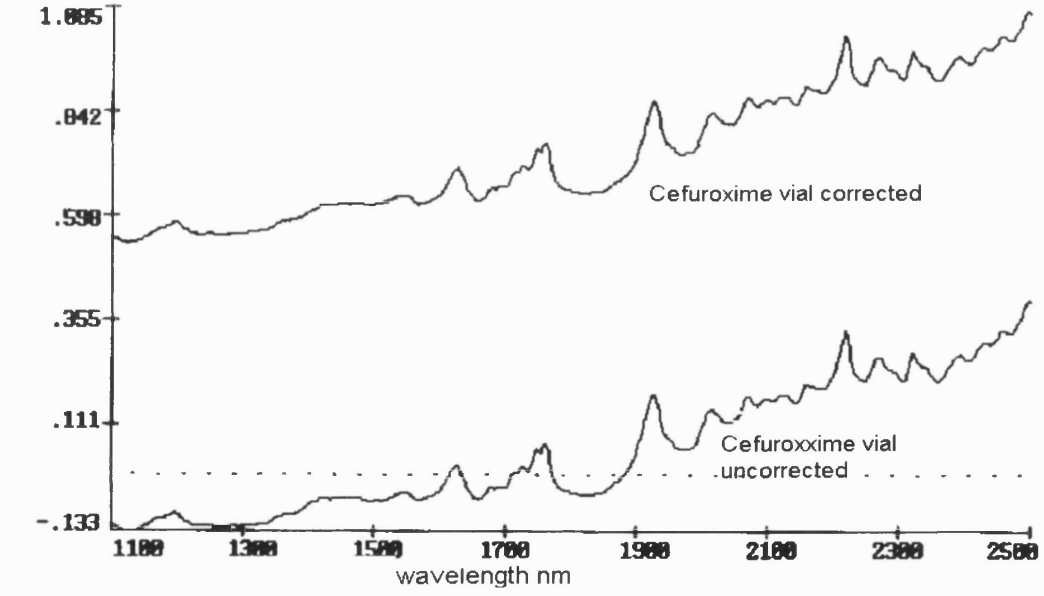


Figure 6.3.6 NIR spectrum of cefuroxime (supplier 1) in its original vial corrected back to the Waters vial using mercury. Cefuroxime (supplier 1) in its original vial uncorrected.

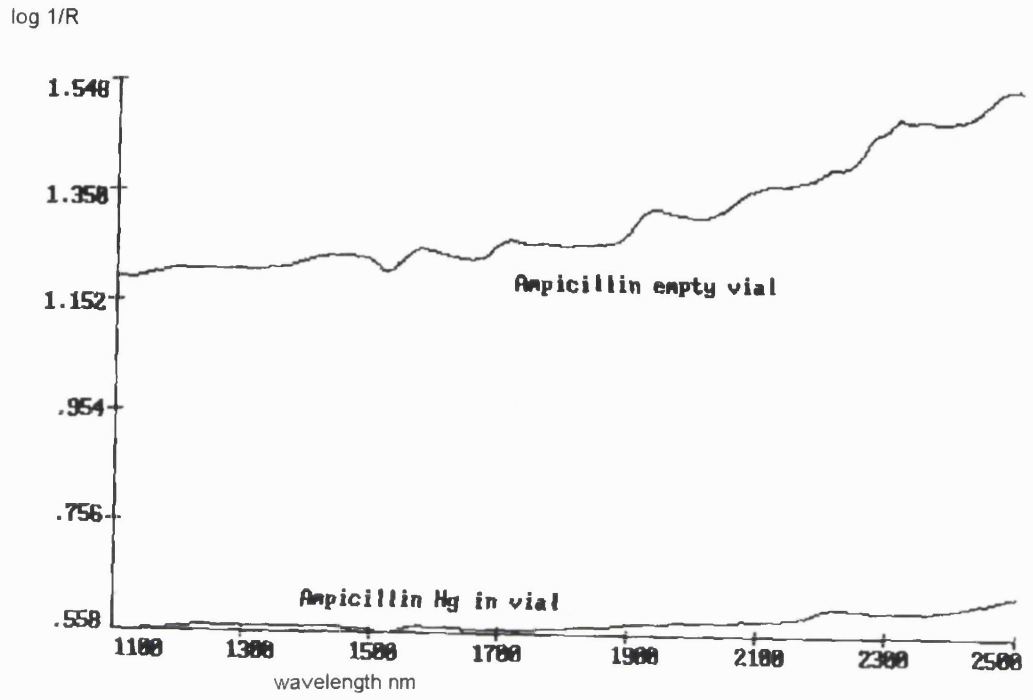


Figure 6.3.7 NIR spectrum of an empty ampicillin vial and also filled with mercury.

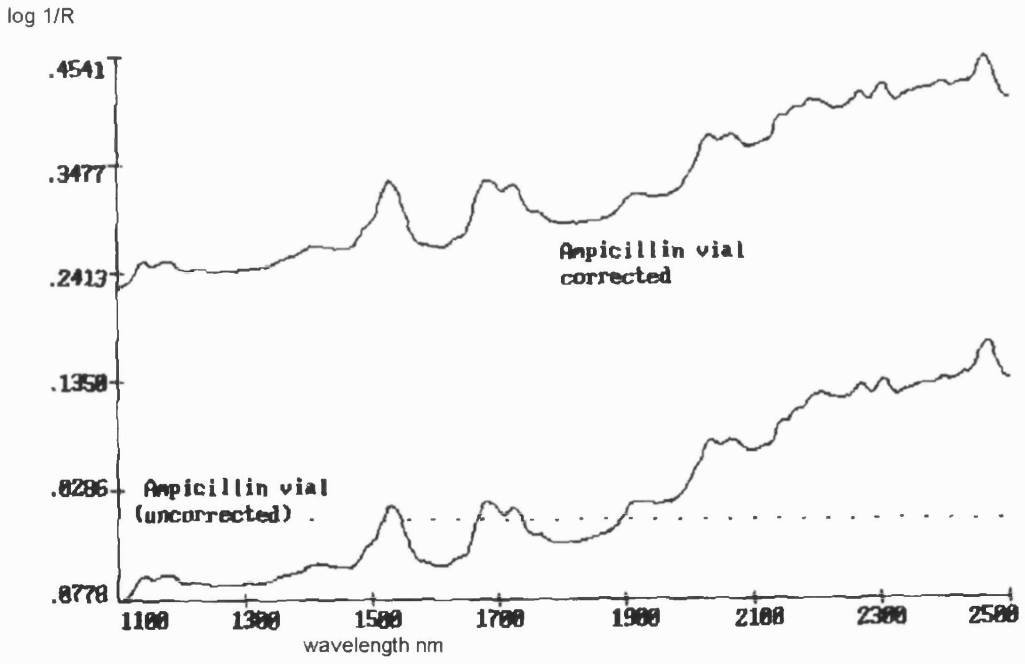


Figure 6.3.8 NIR spectrum of ampicillin in its original vial corrected back to the Waters vial using mercury. Ampicillin in its original vial uncorrected.

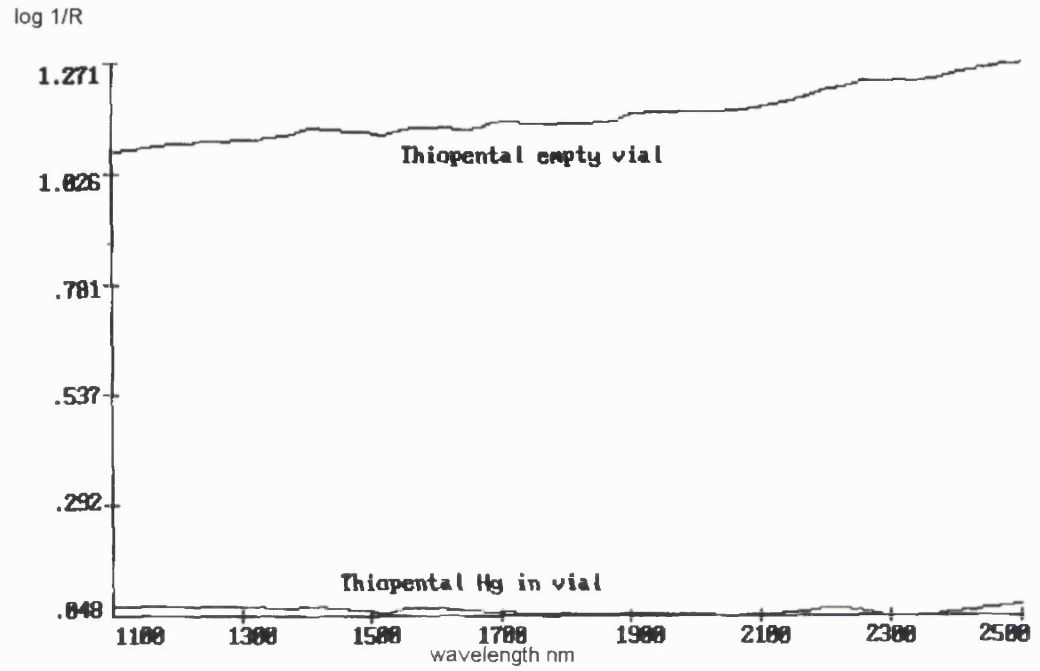


Figure 6.3.9 NIR spectrum of an empty thiopentone vial and also filled with mercury.

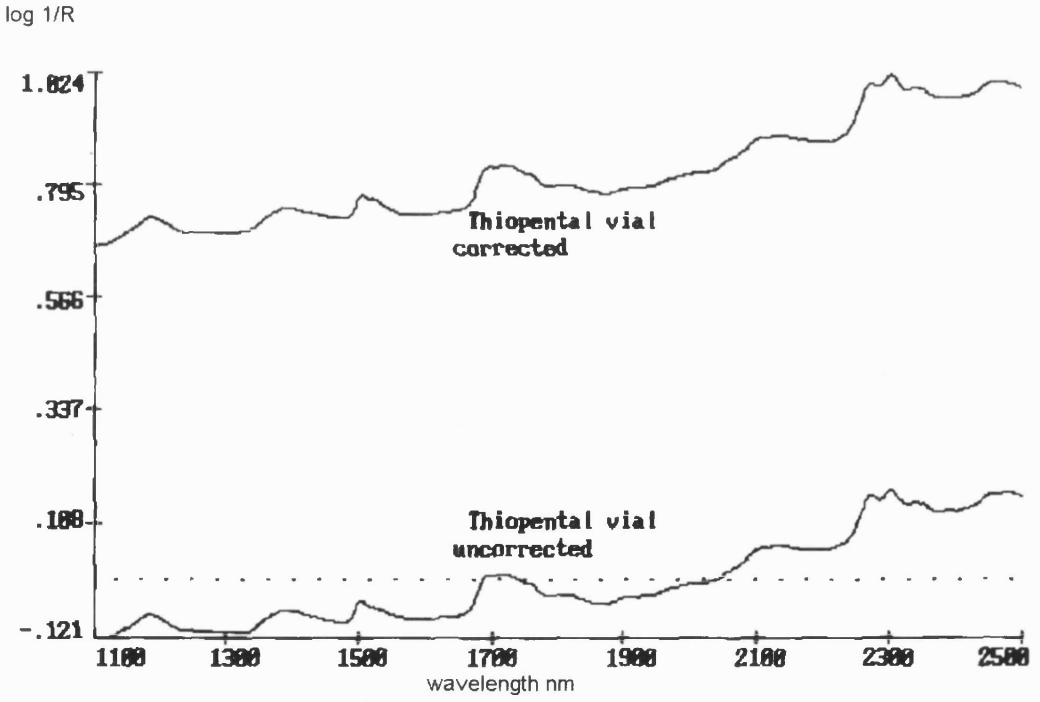


Figure 6.3.10 NIR spectrum of thiopentone in its original vial corrected back to the Waters vial using mercury. Thiopentone in its original vial uncorrected.

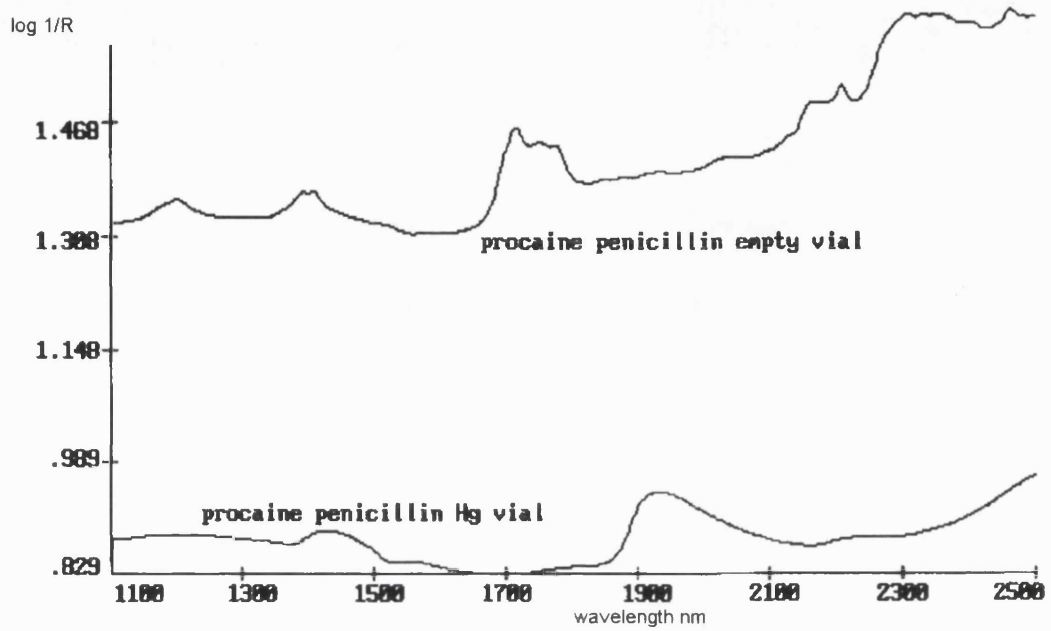


Figure 6.3.11 NIR spectrum of an empty procaine penicillin vial and also filled with mercury.

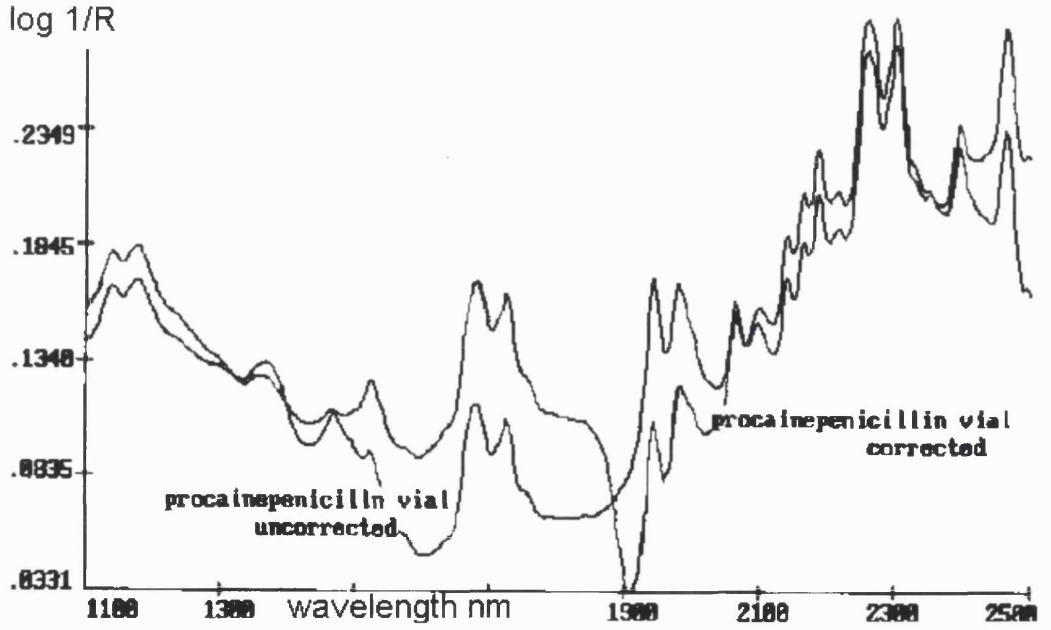


Figure 6.3.12 NIR spectrum of procaine penicillin in its original vial corrected back to the Waters vial using mercury. Procaine penicillin in its original vial uncorrected.

Diffuse reflectance NIR spectroscopy using the correlation method has potential as a rapid non-destructive screening technique. Correction using a drug substance is

unsuitable for correcting the spectral contributions of containers by this method as the spectral contribution of the drug is not eliminated here. Correction with mercury gives a small improvement to the identification of the drug substances. However correction procedures appear unnecessary with a library of this size. As the spectral library grows including more structurally related compounds then the differential between the correlations of the first and second matches will be reduced. A correction procedure would therefore be more essential with a larger sample size. For surveillance, particularly of generic and parallel imports a wide range of sample containers may be found. Therefore it is valuable to appreciate the effects of such containers when used in these external non-invasive methods. The corrective methods have had little impact on the identification of samples from this library. However a larger library would be expected for a surveillance method and plastic and coloured glass containers would be commonly encountered all of which would increase the need for a corrective procedure. NIR as used above demonstrates how the technique could be more efficient than HPLC. In the future NIR coupled with the correct chemometric technique could be used to monitor excipients or physical characteristics that may reveal counterfeit medicines as well as the active principles. This would offer substantial additional analytical information as well as increased efficiency.

7. CONCLUSIONS

The work presented has shown the development of HPLC utilising modern equipment, columns and data handling techniques for reliable pharmaceutical surveillance. A new basic HPLC method of identification has been investigated to increase the identification power of the method. NIR as an additional or alternative method of surveillance has been investigated and this work specifically included a study of the effect of different medicine containers on the reliability of identification.

The HPLC system provides a generally robust surveillance method capable of giving qualitative and quantitative information about a medicine's active principle. When real pharmaceutical samples were tested on System 2 the component drugs were successfully identified using the spectral software (selecting from a library of 237 drugs) and also on the retention index scale to within less than 3 RI units. They were also quantified with precision that fell within a fraction of the BP limits specified for a given drug.

The present surveillance system can achieve greater efficiency than BP testing provided the calibration curves for quantification are monitored and validated over time. The discrimination power of the standard gradient system in combination with diode array detection was found to be excellent and a limited study of the additional combination with the high pH system shows the potential for further enhancement of the discrimination power. In order to increase the discrimination power it is essential to progress further with the high pH system, additionally comparisons should be made with other hyphenated techniques such as HPLC-MS for use as pharmaceutical surveillance.

A rationalised method of transferring HPLC data from laboratory to laboratory has been proposed. A good level of agreement was seen for systems that had similar chromatographic properties. Correcting data back to one index using linear regression means that expanding the data set will increase its overall transferability. Building as many compilations from diverse systems into the data set as possible will mean that more workers will have access to the data set. As the data set becomes larger then other workers will be required to make fewer changes to their HPLC system in order to make the comparison with the data set a useful one. This interlaboratory transfer

method can be used by laboratories as a general guide to the requirements for an HPLC system in order to obtain similar elution results given by another laboratory e.g. when work in the field necessitates the use of a different column. The transfer method will contribute to the identification and understanding of the retention behaviour of outlying compounds.

The gradient HPLC system alone has not been shown to be entirely transferable to other systems to allow precise identification of all compounds. Many workers concerned with systematic toxicological analysis [76-83] require high levels of confidence in their identifications, which are not always guaranteed when using supposedly 'identical' HPLC systems in different laboratories. Bogusz & Rehorek [75] had concluded that even using a secondary standard correction process, interlaboratory standard deviations of up to 30 RI units may be obtained for the same nitro-n-alkane based HPLC system. Bogusz [35] later concluded that the composition of mobile phase is of critical importance in determining RI values in the nitro-n-alkane scale and should be strictly standardised for interlaboratory comparison. Additionally, data from isocratic databases cannot be compared to gradient data. This work does not reach any conclusions contrary to these views.

During the development of the acetonitrile/water non-buffered system a comparison of data has shown that the nitro-n-alkane retention index system can transcend differences in equipment and minor differences in the elution profile. During robustness testing any effects on the nitro-n-alkane retention times did not translate into unacceptable changes in retention indices when deliberate errors were applied to the system. However the robustness of the system when considering certain unusual compounds may not be guaranteed. It was noted that the greatest alterations were caused by sensitivity of the system to pH changes. Otherwise the combination of the nitro-n-alkane system combined with modern precise HPLC equipment led to a robust surveillance method.

The behaviour of compounds using this method shows good correlation with physicochemical parameters particularly molecular connectivity which is based on structural arrangement of functional groups. A novel retention index prediction based on the additive contributions from structural sub-units of the barbiturates gave excellent agreement with the retention index obtained when that barbiturate was run

on the system. This strong relationship between compound structure and retention might be investigated further using another series of compounds such as the steroids.

During the investigation of high pH columns, ODP50e provided the most suitable material of the two examined for a reversed phase basic system. Retention times given by this system were found to be highly repeatable. Using this column and sodium hydroxide as the modifier added to the eluent at the pumps led to more impressive peak shapes than those obtained by Gawdzik et al [62,63,64]. While peak shape did not match that given by Kirkland *et al* [60], there was no requirement to restrict the eluent in order to preserve the silica support. The basic system was originally devised in order to increase the identification power of the surveillance method which has been shown to be possible. However the development of a separate basic system is also necessary for improving the predictability and transferability of the method. Robustness and isocratic studies have shown that the greatest obstacles to predictability are caused by some basic compounds run on the acidic system.

NIR offers a method for the analysis of intact pharmaceutical products, that is fast and economical. The correlation method was shown to be highly selective in the identification of drugs from the library of 160 pure drugs when tested with real pharmaceutical powders for injection.

Novel investigations into the role of glass in identification using mercury as a reflective material by the correlation method have shown a negligible effect. This may be due to the physical arrangement of the instrument which is designed to avoid detecting specular light. The effect of glass does however have a visible effect on the raw spectra of these compounds and would be important if subtler differences between samples were being investigated. The effect of container or tablet coating materials will become more important when considering plastic or coloured glass medicine bottles or plastic strip packed tablets.

NIR offers rapid non-destructive identification of medicine but much work is required on transferability of results, quantification and detection of excipients before it offers real advantages over HPLC as a surveillance method.

Future work

The compilation of retention indices should be expanded by the addition of new workers data and by running more samples on System 2. The transferability of the data might be increased if the database were split into acidic and basic drugs run on

appropriate HPLC systems. The possibility of using correction factors for the remaining outlying compounds must also be investigated. Discriminating power of the UV spectra has in this work been limited to peak maxima. A computer programme to determine the number of matches given by whole spectral comparison, should be developed. Discrimination power should increase drastically when comparing the whole spectral range (190-450 nm) used by the UV in System 2.

It is necessary to develop the basic system further in order to resolve the first nitro-n-alkane and thus to obtain a retention index value for early eluting drugs. Also, an alternative mobile phase must be considered or the run time must be extended in order to reduce the initial baseline disruption. Once the high pH system is established and a database produced then the additional discriminating power this provides should be determined.

Pharmaceutical surveillance as described here is not the same as systematic toxicological analysis – unequivocal identification of each drug is not the intention. However the level of confidence in the screening process must be studied to decide what is required and what is actually obtained. As part of that larger numbers of 'real' pharmaceuticals need to be run and validated on the system.

There is much scope for development of NIR as a surveillance technique. Leading on from this work it would be logical to expand the drug database and investigate the discrimination power of the correlation library search method. Any restrictions that it might be necessary to apply to sample presentation could also be investigated. Major issues that are yet to be fully understood are identification and quantification of common excipients, these will provide major areas for new research.

REFERENCES

1. The British Pharmacopoeia, *The Stationery Office,* London, UK (1988)

2. Clarke's Isolation and Identification of Drugs. Second edition Ed. A. C. Moffat, *The Pharmaceutical Press*, London, UK (1986)

3. The Prescription Pricing Authority Annual Report. *PPA,* Newcastle Upon Tyne, UK (1996)

4. K. Sugden, G. B. Cox, C. R. Luscombe, *J. Chromatog.* 149 (1978) 377

5. B. B Wheals, *J. Chromatog.* 187 (1980) 65

6. A. H. Stead, R. Gill, T. Wright, J.P. Gibbs, A. C. Moffat, *Analyst*, 107 (1982) 1106

7. I. Ojanpera, R. L. Ojansivu, J. Nokua, E. Vuori, *J. Planar Chromatog. & Modern. TLC* 12 (1999) 38

8. P. Owen, A. Pendlebury, A. C. Moffat, *J. Chromatog.* 161 (1978) 195

9. R. J. Flanagan, G. C. A. Storey, D. W. Holt, *J. Chromatog.* 187 (1980) 391

10. B. Lin Ling, W. R. G. Baeyens, B. Del Castillo, K. Imai, P. De Moerloose, K. Stragier, *J. Pharm. Biomed. Anal.* 7 (1989) 1663

11. A. C. Moffat, *J. Chromatog.* 113 (1975) 69

12. R. Gill, A. H. Stead, A. C. Moffat, *J. Chromatog.* 204 (1981) 275

13. R. Gill, B. Law, C. Brown, A. C. Moffat, *Analyst* 110 (1985) 1059

14. O. H. Drummer, *j. Chromatog. B* 733 (1999) 27

15. M. E. Harrison *Ph.D Thesis* University of London 1991

16. A. F. Fell, H. P. Scott, R. Gill, A. C. Moffat, *J. Pharm. & Pharmacol.* 34 (1982) 99

17. F. Tagliaro, F. P. Smith, S. Turrina, V. Equisetto, M. Marigo, *J. Chromatog. A* 735 (1996) 227

18. B. Poksa, *J. Chromatog. A* 818 (1998) 251

19. M. Blanco, J. Coello, H. Iturriaga, S. Maspoch, C. De La Pezuela, *Analyst* 123 (1998) 35

20. A. J. P. Martin, R. L. M. Synge, *J. Biochem.* 35 (1941) 1358

21. G. A. Howard, A. J. P. Martin, *J. Biochem.* 46 (1950) 532

22. G. E. Berendsen, K. A. Pikaart, L. de Galan, *J. Liq. Chromatog* 3 (1980) 1437

23. J. Kohler, J. J. Kirkland, *J. Chromatog.* 385 (1997) 125

24. J. G. Dorsey, W. T. Cooper, *Anal. Chem.* 66 (1994) 857

25. G. B. Cox, *j. Chromatog.* 779 (1997) 29

26. K. J. Duff, *American Lab.* 30 (1998) 32AA

27. The Rules Governing Medicinal Products In the European Community. Volume III: Addendum. *Commission of the European Communities* (July 1990)

28. The United States Pharmacopoeia (USP-24) *United States Pharmacopoeial Commission* (1995)

29. Retention and Selectivity in Liquid Chromatography, Ed. R. M. Smith, *Journal of Liquid Chromatography Library*, Vol 57 (1995) Elsevier Science

30. E. Kovats, *Helv. chim. acta*, 42 (1958) 1915

31. M. Bogusz, R. Aderjan, *J. chromatog.* 435 (1988) 43

32. R. Gill, J. P. Davies, P. D. Upton, *Unpublished Communication* (1991)

33. The Merck index, Chapman & Hall, London UK (1996)

34. PDA Software User's Guide, Millipore, Milford USA (1994) 9.2

35. M. Bogusz, *J. Anal. Tox.* 15 (1991) 174

36. R. Aderjan, M. Bogusz, *J. Chromatog. A* 454 (1988) 345

37. R. M. Smith, *J. Chromatog.* 236 (1982) 313

38. R. M. Smith, T. G. Hurdley, R. Gill, A. C. Moffat, *Anal. Proc.* 22 (1985) 331

39. R. M. Smith, T. G. Hurdley, R. Gill, A. C. Moffat, *Chromatographia*, 19 (1984) 401

40. L. R. Snyder, J. W. Dolan, *LC-GC int.* 3 (1990) 28

41. A. C. Moffat, K. W. Smalldon C. Brown, *J. Chromatog.* 90 (1974) 1

42. M. Bogusz, M. Erkens, R. D. Maier, I. Schroder, *J. Liq. Chromatog.* 15 (1992) 127

43. E. Cruz, M. R. Euerby, C. M. Johnson, C. A. Hackett, *Chromatographia,* 44 (1997) 151

44. R. M. Smith, T.G. Hurdley, R. Gill, A. C. Moffat, *Chromatographia*, 19 (1984) 407

45. M. Wu, R. Aderjan, *J. Liq. Chromatog. & Rel. Technol.* 19 (1996) 1967

46. W. R. Melander, D. E. Campbell, Cs. J. Horváth, *J. Chromatog.* 158 (1978) 215

47. R. G. Woolcott, J. W. Dolan , *LC-GC int.* 12 (1999) 14

48. P. I. Zhu, J. W. Dolan L. R. Snyder, D. W. Hill, L. Van Heukelem, T. J. Waeghe, *J. Chromatog. A* 756 (1996) 51

49. R. Ragonese, M. Mulholland, J. Kalman, *J. Chromatog. A* 870 (2000) 45

50. W. M. Meylan, P. H. Howard, *J. Pharm. Sci.* 84 (1995) 83

51. S. Goga, S. Heinisch, J. L. Rocca, *Chromatographia* 48 (1998) 237

52. P. J. Schoenmakers, S. van Molle, C. M. G. Hayes, L. G. M. Uunk, *Anal. Chim. Acta.* 250 (1991) 1

53. R. D. Maier, M. Bogusz. *J. Anal. Tox.* 19 (1995) 79

54. S. O. Badriru, T. M. Jefferies, *J. Pharm. Biomed. Anal.* 6 (1988) 859

55. R. J. Flanagan, I. Jane, *J. Chromatog.* 323 (1985) 173

56. I. Jane, A. Mckinnon, R. J. Flanagan, J. Chrom*atog.* 323 (1985)191

57. D. J. Smith, *J. Chromatog. Sci.* 19 (1981) 253

58. R. M. Smith, J. P. Westlake, R. Gill, M. D. Osselton, *J. Chromatog.* 514 (1990) 97

59. J. J. Kirkland, M. A. Van Straten, H. A. Claessens, *J. Chromatog. A* 797 (1998) 111

60. J. J. Kirkland, J. D. Martosella, J. W. Henderson, C. H. Dilks, J. B. Adams, *American Lab.* 22 (1999) 22

61. J. Gawdzik, U. Czerwiska-Bil, *Chromatograpia.* 26 (1988) 399

62. B. Gawdzik, *Chromatograpia.* 31 (1991)21

63. B. Gawdzik, *J. Chromatog.* 549 (1991) 77

64. B. Gawdzik, *J. Chromatog.* 600 (1992) 115

65. E. Lamparter *J. Chromatog.* 635 (1993) 155

66. M. Bogusz, *J. Anal. Tox.* 15 (1991) 174

67. J. J. Kirschbaum, *J. Pharm. & Biomed. Analysis* 7 (1989) 813

68. J. K. Baker, C. Y. Ma, *J. Chromatog.* 169 (1979) 107

69. J. K. Baker, L. A. Cates, M. D. Corbett, J. W. Huber, D. L. Lattin, *J. Liq. Chromatog.* 5 (1982) 829

70. M. Bogusz, R. Aderjan, *J. Anal. Tox.* 12 (1988) 67

71. M. Bogusz, M. Erkens, J. P. Franke, J. Wijsbeek, R. A. De Zeeuw, *J. Liq. Chromatog* 16 (1993) 1341

72. M. Bogusz, G. Neidl-Fischer, R. Aderjan, *J. Anal. Tox.* 12 (1988) 325

73. S. P. Elliot, K. A. Hale. J. *Chromatog. B.* 694 (1997) 421

74. S. P. Elliot, K. A. Hale. *J. Anal. Tox.* 22 (1998) 279

75. M. Bogusz, D. W. Hill, A. Rehorek, *J. Liq. Chrom.& Rel. Technol.* 19 (1996) 1291

76. B. K. Logan, D. T. Stafford, I. R. Tebbett, C. M. Moore, *J. Anal. Tox.* 14 (1990) 154

77. E. M. Koves, J. Wells, *J. Forensic Sci.* 37 (1992) 42

78. S. H. Cosbey, *Forensic Sci. Int.* 32 (1986) 245

79. A. Tracqui, P. Kintz, P. Mangin, *J. Forensic. Sci.* 40 (1995) 254

80. D. Hill, A. J. Kind, *J. Anal. Tox.* 18 (1994) 233

81. M. Bogusz, M. Wu, *J. Anal Tox.* 15 (1991) 188

82. M. Bogusz, M. Erkens, *J. chromatog.* A 674 (1994) 97

83. E. Below, M. Burrmann, *J. Liq. Chromatog.* 17 (1994) 4131

84. S. Toon, J. Mayer, M. Rowland *J. Pharm. Sci.* 73 (1984) 625

85. R. J. E. Grouls, E. W. Ackerman, H. H. M. Korsten, L. J. Hellebrekers, D. Biemer, *J. Chromatog. B* 694 (1997) 42

86. K. Valko, C. Bevan, D. Reynolds, *Anal. Chem.* 69 (1997) 2022

87. W. J. Lambert, *J. Chromatog. A* 656 (1993) 469

88. E. Forgacs, T. Cserhati, *J. Liq. Chrom.& Rel. Technol.* 19 (1996) 1849

89. E. Forgacs, T. Cserhati, *J. Chromatog. A* 728 (1996) 75

90. A. H. Stead, R. Gill, A. T. Evans, A. C. Moffat, *J. Chromatog.* 234 (1982) 277

91. R. B. Barlow, Quantitative aspects of chemical pharmacology. *Croom Helm, London*, (1980) 80

92. B. Berking, B. M. Craven, *Acta. Crystallogr.* Sect. B 27 (1971) 1107

93. C. Hansch, A. Leo, Substituent constants for correlation analysis in chemistry and biology. *Wiley Interscience Publication*, New York, (1979) 65

94. C. Hansch, R. A. Steward, S. M. Anderson, D. J. Bentley, *J. Med. Chem.* 11 (1967) 1

95. D. J. Lamb, L. E. Harris, *J. Am. Pharm. Assoc.* 49 (1960) 583

96. O. Wong, R. H. McKeown, *J. Pharm. Sci.* 77 (1988) 926-932

97. O. Shakoor, R. B. Taylor, R. R. Moody, *Analyst* 120 (1995) 2191

98. M. B. Evans, P. A. Haywood, D. Johnson, M. Martin-Smith, G. Munro, J. C. Wahlich, *J. Pharm. Biomed. Anal* 7 (1998) 1

99. C. Ho, G. L. Chen, *J. Food Drug Anal.* 4 (1996) 271

100. A.S. Sidhu, J. M. Kennedy, S. Deeble, *J. Chromatog.* 391 (1987) 233

101. G. Hoogewigs, D. L. Massart, *J. Liq. Chromatog.* 6 (1983) 2521

102. L. Edno, F. Bressole, B. Combe, M. Gattier, *J. Pharm. Biomed. Anal.* 13 (1995) 785

103. B. Law, P. Hough, *J. Pharm. Biomed. Anal.* 15 (1997) 587

104. K. Svanberg, T. Andersson, D. Killander, I. Wang, U. Stenram, S. E. Andersson, R. Berg, J. Johansson, S. Svanberg, *Br. J. Dermatol.* 130 (1994) 743

105. J. Johansson, S. Svanberg, K. Svanberg, *Lasers Surg. Med.* 20 (1997) 272

106. B. Franck, H. Stratmann, *Heterocycles* 15 (1981) 919

107. B. Elfsson, I. Wallin, S. Eksborg, K. Rudaeus, A. M. Ros, H. Ehrsson, *Eur. J. Pharm. Sci.* 7 (1998) 87

108. European Pharmacopoeia, *The European Commission* (1997) 43

109. J. A. Jones, I. R. Last, B. F. MacDonald, K. A. Prebble, , *J. Pharm. Biomed. Anal.* 11 (1993) 1227

110. M. S. Kamat, R. A. Lodder, P. P. DeLuca, *Pharm. Research* 6 (1989) 961

111. J. Rantanen, S. Lehtola, P. Ramet, J. P. Mannermaa, J. Yliruusi, *Powder Technol.* 99 (1998) 163

112. E. W. Ciurczak, *Pharm. Technol.* 7 (1992) 12

113. F. Cuesta Sanchez, J. Toft, B. Van Den Bogaert, D. L. Massart, S. S. Dive, P. A. Hailey, *Fresenius J. Anal. Chem.* 352 (1995) 771

114. S. S. Sekulic, H. W. Ward, D. R. Brannegan, E. D Stanley, C. L. Evans, S. T. Sciavolino, P. A. Hailey, P. K. Aldridge, *Anal. Chem.* 68 (1996) 509

115. S. S. Sekulic, J. Wakeman, P. Doherty, P. A. Hailey, *J. Pharm. Biomed. Anal.* 17 (1998) 1285

116. R. Gimet, A. T. Luong, *J. Pharm. Biomed. Anal.* 5 (1987) 5

117. P. K. Aldridge, C. L. Evans, H. W. Ward, S. T. Colgan, N. Boyer, P. J. Gemperline, *Anal. Chem.* 68 (1996) 997

118. A. F. Zappala, A. Post, *J. Pharm. Sci.* 66 (1975) 292

119. E. W. Ciurczak, R. P. Torlini, *Spectroscopy* 2 (1987) 41

120. W. H. Kohn, A. N. Jeger, *J. Foren. Sci.* 37 (1991) 35

121. H. Wolfgang, W. H. Kohn, N. Alfons, A. N. Jeger, *J. Foren. Sci.* 37 (1992) 35

122. W. Plugge, C. Van Der Vlies, *J. Pharm. Biomed. Anal.* 6 (1993) 435

123. J. K. Drennen, R. A. Lodder, *J. Pharm. Sci.* 79 (1990) 622

124. R. A. Lodder, M. Selby, G. M. Hieftje, *Anal. Chem.* 59 (1987) 1921

125. P. R. Khan, R. D. Jee, R. A. Watt, A. C. Moffat, *Pharm. Sci.* 3 (1997) 447

126. B. F. MacDonald, K. A. Prebble, *J. Pharm. & Biomed. Anal.* 11 (1993) 1077

127. M. A. Dempster, B. F. MacDonald, P. J. Gemperline, N. Boyer, *Anal. Chim. Acta* 310 (1995) 43

128. Y. Wang B. R. Kowlaski, *Anal. Chem.* 65 (1992) 1301

129. E. Dreassi, G. Ceramelli, P. L. Perruccio, P. Corti, *Analyst* 123 (1998) 1259

130. M. W. Borer, X. J. Zhou, D. M. Hays. J. D. Hofer, K. C. White, *J. Pharm. Biomed. Anal.* 17 (1998) 641

131. R. J. Barnes, M. S. Dhanoa, S. J. Lister, *App. Spec.* 5 (1989) 772

132. E. W. Ciurczak, R. P. Torlini, M. P. Demkowicz, *Spectroscopy* 1 (1986) 36

133. M. Blanco, J. Coello, H. Iturriaga, S. Maspoch, F. Gonalez, R. Pous Near Infra-Red spectroscopy *Ellis Horwood Ltd* (1992) 401

134. P. Frake, I. Gill, C. N. Luscombe, D. R. Rudd, J. Waterhouse, U. A. Jayasorriya, *Analyst* 123 (1998) 2043

135. IQ^2 Version 3.52 Software manual, *NIR Systems, Inc.* Silver Spring, MD, USA (1995)

136. C. Van Der Vlies, K. J. Kaffka, P. Plugge, *Spectroscopy* 10 (1995) 46

137. D. A. Burns, E. W. Ciurczak, Handbook of NIR Analysis. *Marcel Dekker*, New York (1992) 128-155

138. W. Plugge, C. Van Der Vlies, *J. Pharm. Biomed. Anal.* 14 (1996) 898

APPENDICES

Appendix I

Gradient profiles

System 1

Time (min)	Flow ml/min	% H_2O + 1% 2.5M H_2SO_4	% CH_3CN + 1% 2.5M H_2SO_4
0	1.5	98	2
3	1.5	98	2
26	1.5	2	98
31	1.5	2	98
33	1.5	98	2
43	1.5	98	2

System 2

Time (min)	Flow ml/min	% H_2O + 1% 2.5M H_2SO_4	% CH_3CN + 1% 2.5M H_2SO_4
0	1.5	98	2
3	1.5	98	2
26	1.5	2	98
36	1.5	2	98
38	1.5	98	2
46	1.5	98	2

Basic System 2

Time (min)	Flow ml/min	% H_2O	% CH_3CN	% 0.5M NaOH
0	1.5	97	1	2
3	1.5	97	1	2
26	1.5	1	97	2
36	1.5	1	97	2
38	1.5	97	1	2
40	1.5	97	1	2
41	1.5	100	0	0
44	1.5	100	0	0
45	1.5	97	1	2
49	1.5	97	1	2

Appendix II

Retention times of the nitro-n-alkanes on System 1

DATE	GLYCINE	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16
03/01/95	1.6	3.32	7.17	15.7	20.42	22.7	24.4	25.79	27.08	28.29	29.37	30.43	31.52	32.64	33.95	35.44	37.2
06/01/95	1.68	3.51	7.67	15.4	19.64	22	23.71	25.88	26.56	27.82	28.47	30.03	31.07	32.18	33.43	34.89	36.7
06/02/95	1.61	3.34	7.18	15.4	19.75	22.1	23.9	25.38	26.73	27.98	29.13	30.21	31.27	32.39	33.66	35.15	37
06/02/95	1.7	3.55	7.97	15.4	19.49	21.8	23.59	25.11	26.5	27.79	28.97	30.05	31.1	32.24	33.52	35	36.9
07/02/95	1.61	3.32	7.11	15.4	19.89	22.3	23.98	25.43	26.76	27.98	29.11	30.17	31.22	32.35	33.61	35.09	36.9
08/02/95	1.62	3.36	7.22	15.3	19.67	22.1	23.84	25.39	26.77	28.05	29.22	30.3	31.37	32.52	33.8	35.52	37.2
09/02/95	1.59	3.31	7.13	15.4	19.85	22.2	23.99	25.48	26.8	28.06	29.21	30.26	31.31	32.44	33.69	35.33	36.9
13/02/95	1.58	3.32	7.04	15.5	20.08	22.5	24.24	25.68	27.02	28.24	29.37	30.42	31.47	32.63	33.96	35.6	37.4
20/02/95	1.56	3.31	7.09	15.7	20.25	22.5	24.16	25.58	26.88	28.1	29.21	30.24	31.28	32.68	33.68	35.16	37
21/02/95	1.6	3.31	7.16	15.6	20.1	22.4	24.02	25.45	26.74	27.99	29.12	30.17	31.21	32.32	33.61	35.08	36.9
22/02/95	1.6	3.31	7.13	15.6	20.27	22.6	24.32	25.77	27.07	28.33	29.45	30.46	31.52	32.63	33.94	35.41	37.2
23/02/95	1.74	3.61	8.08	15.9	20.2	22.4	24.1	25.55	26.88	28.11	29.26	30.31	31.37	32.53	33.84	35.36	37.2
23/02/95	1.54	3.27	6.97	15.5	20.14	22.5	24.27	25.73	27.03	28.21	29.31	30.33	31.37	32.46	33.71	35.22	37
24/02/95	1.57	3.3	6.91	15.5	20.15	22.5	24.23	25.65	26.94	28.13	29.23	30.25	31.29	32.44	33.73	35.21	37
25/02/95	1.59	3.3	7.17	15.7	20.33	22.7	24.4	25.81	27.1	28.31	29.44	30.49	31.56	32.71	34.05	35.58	37.4
26/02/95	1.61	3.27	7.15	15.7	20.23	22.6	24.22	25.64	26.95	28.19	29.33	30.41	31.52	32.71	34.07	35.65	37.5
27/02/95	1.61	3.38	7.25	15.7	20.21	22.5	24.06	25.46	26.76	27.99	29.14	30.21	31.29	32.46	33.8	35.38	37.2
27/02/95	1.63	3.5	7.37	15.8	20.17	22.4	24.09	25.52	26.84	28.09	29.25	30.35	31.43	32.63	33.96	35.51	37.4
02/03/95	1.55	3.32	7.44	15.8	20.28	22.6	24.25	25.66	26.96	28.18	29.29	30.32	31.38	32.5	33.78	35.3	37.1
02/03/95	1.54	3.31	6.92	15.6	20.28	22.6	24.33	25.76	27.08	28.3	29.44	30.49	31.56	32.7	34.01	35.49	37.2
03/03/95	1.6	3.37	7.52	15.8	20.17	22.5	24.15	25.55	26.84	28.06	29.19	30.24	31.28	32.43	33.76	35.26	37.1
12/03/95	1.54	3.26	6.86	15.5	20.24	22.7	24.41	25.84	27.14	28.34	29.44	30.48	31.54	32.71	34.03	35.61	37.6
12/03/95	1.64	3.25	6.78	15.4	20.25	22.7	24.42	25.87	27.18	28.38	29.45	30.48	31.52	32.64	33.92	35.43	37.2
13/03/95	1.71	3.39	7.87	15.9	20.33	22.6	24.26	25.66	26.98	28.19	29.33	30.4	31.46	32.62	33.93	35.45	37.3
13/03/95	1.52	3.25	6.77	15.5	20.24	22.7	24.42	25.87	27.17	28.38	29.46	30.5	31.53	32.63	33.94	35.38	37.1
14/03/95	1.71	3.39	7.81	15.7	20.19	22.5	24.19	25.64	26.93	28.12	29.22	30.28	31.34	32.45	33.73	35.22	37
15/03/95	1.58	3.29	6.92	15.7	20.55	22.9	24.57	25.99	27.29	28.47	29.59	30.68	31.74	32.89	34.24	35.97	37.7
16/03/95	1.64	3.4	7.8	15.9	20.59	23	24.64	26.03	27.3	28.45	29.53	30.54	31.58	32.78	34.17	35.72	37.6
17/03/95	1.5	3.24	6.8	15.6	20.68	23.2	24.86	26.27	27.55	28.7	29.8	30.81	31.85	32.96	34.29	35.89	37.7
07/04/95	1.71	3.55	7.9	15.4	19.49	21.8	23.57	25.09	26.47	27.77	28.95	30.04	31.11	32.24	33.5	34.95	36.8
21/04/95	1.56	3.31	6.98	15.1	19.52	21.9	23.59	25.07	26.43	27.69	28.86	29.93	30.98	32.11	33.39	34.9	36.7
21/04/95	1.57	3.28	6.81	14.9	19.41	21.8	23.54	25.71	26.4	27.68	28.85	29.93	30.99	32.12	33.4	34.86	36.7
22/04/95	1.56	3.27	6.84	14.9	19.34	21.7	24.2	25.89	26.33	27.6	28.77	29.83	30.87	31.97	33.19	34.61	36.3
04/05/95	1.6	3.28	7.9	15.6	20.61	23.1	24.83	26.26	27.56	28.75	29.81	30.84	31.89	33.08	34.44	36.05	37.9
23/05/95	1.68	3.55	7.7	15.5	19.62	21.9	23.64	25.12	26.49	27.77	28.94	30.01	31.07	32.19	33.46	34.94	*
23/05/95	1.71	3.56	7.86	15.4	19.55	21.9	23.61	25.1	26.47	27.75	28.92	29.99	31.04	32.15	33.41	34.87	*
23/05/95	1.93	3.55	7.76	15.3	19.52	21.9	23.58	25.07	26.43	27.7	28.87	29.93	30.97	32.07	33.31	34.75	*
23/05/95	1.71	3.55	7.82	15.3	19.5	21.8	24.31	25.08	26.45	27.72	28.9	29.97	31.01	32.12	33.36	34.81	*
23/05/95	1.73	3.57	7.81	15.3	19.49	21.8	23.58	25.07	26.45	27.72	28.89	29.96	31	32.1	33.34	34.7	*
24/05/95	1.73	3.54	7.73	15.3	19.46	21.8	23.53	25.7	26.37	27.64	28.8	29.86	30.9	32	33.23	34.66	36.4
31/05/95	1.7	3.52	7.66	15.2	19.42	21.8	23.52	25.01	26.38	27.65	28.82	29.89	30.93	32.02	33.25	34.68	36.4
31/05/95	1.69	3.49	7.56	15.1	19.35	21.7	23.45	24.94	26.31	27.58	29.26	29.83	30.86	31.95	33.17	34.57	36.2
07/06/95	1.79	3.56	7.92	15.4	19.41	21.7	23.51	25.04	26.43	27.73	28.92	30.01	31.08	32.2	33.44	34.86	36.6
13/06/95	1.71	3.55	7.97	15.5	19.53	21.9	23.6	25.82	26.51	27.8	28.99	30.08	31.15	32.32	33.65	35.21	37.3
14/06/95	1.72	3.57	8.02	15.5	19.53	21.9	23.6	25.83	26.52	27.82	29	30.09	31.18	32.34	33.69	35.25	37.5
20/06/95	1.7	3.55	7.44	15.4	19.52	21.8	23.58	25.08	26.47	27.75	28.93	30	31.06	32.19	33.48	35	36.9
21/06/95	1.71	3.54	7.98	15.4	19.48	21.8	23.57	25.08	26.46	27.75	28.43	30.01	31.07	32.2	33.47	34.46	36.8
27/06/95	1.71	3.55	7.93	15.4	19.52	21.9	23.61	25.04	26.51	27.8	28.97	30.05	31.11	32.25	33.55	35.08	37.1
29/06/95	1.78	3.54	7.9	15.3	19.38	21.7	23.5	25.02	26.41	27.69	28.85	30.62	30.95	32.85	33.29	34.73	36.5
30/06/95	1.71	3.54	7.86	15.3	19.38	21.7	23.5	25.02	26.4	28.51	28.84	30.61	30.94	32.04	33.27	34.69	36.4
03/07/95	1.57	3.36	7.55	15.5	19.71	22.1	23.8	25.27	26.63	27.89	29.06	30.14	31.22	32.37	33.67	35.17	37
13/07/95	1.69	3.54	7.87	15.3	19.35	21.7	23.45	24.97	26.36	27.65	28.84	29.92	30.96	32.06	33.28	34.68	36.3
20/07/95	1.71	3.51	7.76	15.2	19.26	21.6	23.39	24.91	26.3	27.6	28.75	29.86	30.82	31.92	33.05	34.37	35.9
21/07/95	1.7	3.51	7.77	15.3	19.36	21.7	23.49	25.01	26.44	27.67	28.84	29.91	30.96	32.03	33.22	34.6	36.2
25/07/95	1.71	3.63	8.08	15.6	19.59	21.9	23.63	25.21	26.64	27.88	29.04	30.14	31.16	32.33	33.56	35.07	36.9
06/08/95	1.71	3.63	8.15	15.6	19.54	21.9	23.63	25.14	26.51	27.81	29.04	30.14	31.23	32.4	33.77	35.34	37.6
03/09/95	1.63	3.37	7.48	15.4	19.56	21.9	23.58	25.07	26.45	27.73	28.91	30	31.07	32.21	33.51	35	36.8
03/10/95	1.52	3.26	6.83	15.3	20.31	22.7	24.44	25.89	27.25	28.44	29.56	30.59	31.66	32.8	34.1	35.42	37.5
03/11/95	1.57	3.3	7.33	15.6	20.18	22.6	24.37	25.81	27.17	28.38	29.45	30.47	31.53	32.69	33.99	35.51	37.4
07/11/95	1.72	3.55	7.84	15.3	19.39	21.7	23.5	25.07	26.4	27.68	28.86	30	31.03	32.07	33.32	34.78	36.6
06/12/95	1.71	3.44	7.91	16.1	19.71	21.9	23.62	25.12	26.52	27.82	29.01	30.1	31.19	32.37	33.72	35.31	37.6
mean	1.65	3.42	7.48	15.5	19.85	22.2	23.94	25.46	26.73	27.99	29.12	30.21	31.24	32.39	33.66	35.15	37
std.deviat	0.08	0.12	0.43	0.23	0.41	0.42	0.40	0.37	0.33	0.30	0.29	0.26	0.26	0.28	0.31	0.38	0.44
Coeff.varia	4.94	3.62	5.69	1.49	2.07	1.91	1.67	1.45	1.24	1.08	0.99	0.85	0.83	0.87	0.93	1.08	1.20
Count	61	61	61	61	61	61	61	61	61	61	61	61	61	61	61	61	56
Std.error	0.01	0.02	0.05	0.03	0.05	0.05	0.05	0.05	0.04	0.04	0.04	0.03	0.03	0.04	0.04	0.05	0.06

std.deviat: Standard deviation Std.error: standard error
Coeff.varia: Coefficient of variation * C-16 temporarily unavailable

Retention times of the nitro-n-alkanes on System 2

DATE	GLYCINE	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16
26/04/96	1.682	3.148	6.915	13.8	17.765	20.165	21.98	23.548	24.98	26.3	27.48	28.548	29.482	30.332	31.25	32.348	33.698
30/04/96	1.693	3.277	7.16	13.88	17.777	20.143	21.98	23.543	24.98	26.29	27.48	28.543	29.46	30.327	31.24	32.327	33.677
01/05/96	1.658	3.058	6.458	13.71	17.825	20.208	22.03	23.575	25.01	26.33	27.51	28.575	29.508	30.358	31.29	32.392	33.758
04/05/96	1.69	3.257	7.09	13.84	17.773	20.157	21.97	23.557	24.97	26.29	27.49	28.54	29.473	30.323	31.24	32.323	33.673
05/05/96	1.66	3.077	6.51	13.74	17.827	20.193	22.01	23.577	25.01	26.33	27.51	28.577	29.51	30.36	31.29	32.41	33.777
10/05/96	1.695	3.328	7.312	14.06	17.945	20.295	22.11	23.678	25.11	26.45	27.65	28.728	29.678	30.578	31.6	32.845	34.378
10/05/96	1.66	3.143	6.727	13.94	17.927	20.293	22.09	23.66	25.09	26.41	27.63	28.1	29.66	30.543	31.56	32.777	34.293
16/05/96	1.702	3.318	7.018	13.82	17.785	20.168	22	23.568	25	26.34	27.54	28.618	29.552	30.418	31.39	32.535	33.968
16/05/96	1.665	3.148	6.732	13.95	17.932	20.298	22.1	23.648	25.1	26.42	27.63	28.715	29.665	30.565	31.58	32.815	34.365
22/05/96	1.688	3.255	7.088	13.89	17.822	20.205	22.02	23.588	25.02	26.34	27.54	28.605	29.288	30.422	31.36	32.488	33.905
10/06/96	1.698	3.265	7.082	13.83	17.748	20.148	21.98	23.548	24.97	26.27	27.45	28.515	29.432	30.282	31.18	32.232	33.532
11/06/96	1.653	3.07	6.487	13.7	17.787	20.17	21.99	23.553	24.99	26.29	27.49	28.553	29.47	30.32	31.24	32.32	33.637
13/06/96	1.65	3.05	6.433	13.63	17.733	20.117	21.93	23.5	24.93	26.23	27.43	28.483	29.4	30.25	31.15	32.2	33.483
13/06/96	1.683	3.283	7.167	13.88	17.783	20.15	21.97	23.517	24.95	26.25	27.45	28.5	29.433	30.283	31.18	32.25	33.567
18/06/96	1.692	3.292	7.125	13.84	17.775	20.142	21.96	23.525	24.96	26.26	27.46	28.508	29.425	30.275	31.18	32.225	33.525
19/06/96	1.665	3.082	6.498	13.73	17.815	20.182	22	23.565	24.98	26.3	27.5	28.548	29.482	30.332	31.25	32.348	33.698
10/07/96	1.662	3.078	6.512	13.75	17.812	20.195	22	23.578	25	26.31	27.5	28.562	29.495	30.362	31.3	32.412	33.778
10/07/96	1.683	3.283	7.183	13.95	17.75	20.117	21.93	23.5	24.93	26.25	27.43	28.517	29.433	30.283	31.2	32.283	33.6
17/07/96	1.69	3.257	7.04	13.79	17.74	20.123	21.94	23.507	24.94	26.26	27.44	28.507	29.44	30.29	31.21	32.29	33.607
17/07/96	1.657	3.09	6.557	13.76	17.79	20.173	21.99	23.557	24.97	26.29	27.49	28.557	29.49	30.34	31.27	32.39	33.74
24/07/96	1.692	3.225	6.942	13.71	17.675	20.075	21.89	23.458	24.88	26.19	27.39	28.458	29.392	30.225	31.13	32.192	33.492
24/07/96	1.655	3.088	6.522	13.71	17.772	20.155	21.97	23.538	24.96	26.27	27.46	28.538	29.455	30.305	31.24	32.338	33.672
31/07/96	1.692	3.258	7.042	13.74	17.642	20.042	21.88	23.458	24.88	26.19	27.38	28.442	29.358	30.192	31.06	32.092	33.325
31/07/96	1.657	3.09	6.507	13.69	17.773	20.157	21.97	23.54	24.96	26.27	27.46	28.523	29.457	30.29	31.21	32.29	33.59
16/08/96		3.278	7.095	13.81	17.745	20.128	21.95	23.512	24.93	26.25	27.43	28.495	29.428	30.278	31.18	32.245	33.528
21/08/96	1.665	3.048	6.315	13.48	17.632	20.032	21.87	23.448	24.87	26.18	27.37	28.432	29.365	30.198	31.07	32.098	
21/08/96	1.673	3.107	6.557	13.74	17.79	20.173	21.99	23.54	24.97	26.29	27.49	28.573	29.507	30.373	31.31	32.423	
17/09/96	1.687	3.303	7.187	14.19	18.103	20.437	22.24	23.82	25.29	26.65	27.89	29.003	29.987	30.987	32.2	33.72	
18/09/96	1.693	3.26	7.077	14.18	18.143	20.477	22.28	23.843	25.31	26.68	27.93	29.043	30.027	31.06	32.29	33.877	
23/09/96	1.687	3.253	7.02	13.75	17.703	20.087	21.92	23.487	24.9	26.22	27.42	28.487	29.42	30.27	31.19	32.253	33.57
23/09/96	1.658	3.108	6.542	13.66	17.742	20.125	21.94	23.508	24.94	26.24	27.44	28.508	29.425	30.275	31.19	32.258	33.575
30/09/96	1.692	3.258	6.925	13.66	17.642	20.058	21.89	23.458	24.89	26.19	27.39	28.458	29.392	30.242	31.14	32.208	33.492
30/09/96	1.657	3.107	6.557	13.66	17.723	20.123	21.94	23.507	24.92	26.24	27.42	28.49	29.423	30.273	31.17	32.257	33.573
03/10/96	1.703	3.287	7.187	13.85	17.687	20.053	21.89	23.453	24.87	26.19	27.37	28.453	29.387	30.22	31.12	32.17	33.47
03/10/96	1.675	3.108	6.575	13.68	17.725	20.125	21.93	23.492	24.91	26.23	27.43	28.492	29.425	30.275	31.19	32.275	33.592
08/10/96	1.68	3.113	6.58	13.7	17.73	20.113	21.93	23.497	24.93	26.25	27.43	28.513	29.463	30.313	31.25	32.33	33.697
23/10/96	1.74	3.273	7.073	13.82	17.773	20.157	21.96	23.523	24.94	26.26	27.44	28.523	29.44	30.307	31.22	32.323	33.657
30/10/96	1.75	3.3	7.1	13.78	17.717	20.083	21.9	23.467	24.88	26.2	27.38	28.467	29.4	30.25	31.17	32.233	33.55
30/10/96	1.74	3.29	7.107	13.77	17.707	20.09	21.91	23.457	24.89	26.21	27.39	28.457	29.39	30.24	31.14	32.223	33.523
29/11/96	1.675	3.308	7.358	14.21	18.058	20.358	22.14	23.692	25.13	26.44	27.64	28.708	29.642	30.508	31.51	32.692	34.158
30/11/96	1.62	3.003	6.37	13.75	17.82	20.203	22.02	23.587	25.02	26.35	27.55	28.62	29.553	30.42	31.4	32.57	33.987
29/01/97	1.675	3.275	7.242	14.01	17.892	20.242	22.06	23.625	25.04	26.36	27.56	28.625	29.558	30.425	31.41	32.558	33.975
29/01/97	1.615	2.965	6.265	13.7	17.882	20.248	22.07	23.632	25.07	26.38	27.57	28.632	29.565	30.432	31.42	32.582	33.982
mean	**1.679**	**3.188**	**6.843**	**13.81**	**17.795**	**20.172**	**21.99**	**23.554**	**24.98**	**26.3**	**27.5**	**28.552**	**29.494**	**30.363**	**31.31**	**32.428**	**33.72**
std.deviat	**0.027**	**0.104**	**0.319**	**0.151**	**0.111**	**0.0949**	**0.088**	**0.087**	**0.096**	**0.106**	**0.117**	**0.143**	**0.142**	**0.174**	**0.247**	**0.355**	**0.251**
Coeff.varia	**1.596**	**3.255**	**4.658**	**1.095**	**0.626**	**0.470**	**0.399**	**0.370**	**0.385**	**0.404**	**0.427**	**0.501**	**0.482**	**0.575**	**0.788**	**1.093**	**0.746**
Count	**43**	**43**	**43**	**43**	**43**	**43**	**43**	**43**	**43**	**43**	**43**	**43**	**43**	**43**	**43**	**43**	**39**
Std.error	**0.004**	**0.016**	**0.049**	**0.023**	**0.017**	**0.0145**	**0.013**	**0.013**	**0.015**	**0.016**	**0.018**	**0.022**	**0.022**	**0.027**	**0.038**	**0.054**	**0.040**

Retention times of the nitro-n-alkanes on the Isocratic System

% B	Glycine	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
30%	1.68	3.24	4.7	8.2	16.5						
	1.68	3.17	4.6	8	16.1						
	1.68	3.24	4.66	8.1	16.2						
	1.68	3.15	4.6	8	15.8						
	1.67	3.21	4.62	8	16						
	1.68	3.17	4.58	8	15.8						
	1.67	3.18	4.61	8	15.9						
	1.67	3.15	4.56	7.9	15.8						
	1.68	3.17	4.57	7.9	15.8						
	1.63	3.21	4.61	8	16						
	1.67	3.31	4.82	8.5	17.1						
	1.65	3.2	4.62	8	16						
	1.71	3.4	4.94	8.6	17.1						
	1.59	3.18	4.65	8.1	16.3						
	1.65	3.17	4.55	7.9	15.6						
	1.62	3.15	4.51	7.8	15.4						
	1.67	3.14	4.55	7.9	15.6						
	1.54	3.12	4.52	7.8	15.5						
	1.67	3.26	4.7	8.1	16.3						
	1.68	3.14	4.64	8	15.9						
	1.69	3.25	4.69	8.2	16.5						
	1.68	3.19	4.63	8.1	16.1						
	1.65	3.14	4.55	7.9	15.6						
mean	**1.66**	**3.2**	**4.63**	**8**	**16**						
std.deviat	**0.04**	**0.06**	**0.1**	**0.2**	**0.44**						
Coeff.varia	**2.19**	**2.01**	**2.09**	**2.4**	**2.77**						
Count	**23**	**23**	**23**	**23**	**23**						
Std.error	**0.01**	**0.01**	**0.02**	**0**	**0.09**						
60% B	1.48	2.5	2.8	3.4	4.12	5.36	7.34	10.5	15.6		
	1.5	2.43	2.76	3.3	4.12	5.35	7.31	10.4	15.5		
	1.41	2.48	2.76	3.3	4.1	5.33	7.29	10.4	15.4		
	1.5	2.48	2.78	3.3	4.07	5.29	7.22	10.3	15.2		
	1.5	2.4	2.78	3.3	4.06	5.28	7.2	10.3	15.2		
	1.52	2.51	2.81	3.4	4.12	5.38	7.37	10.5	15.7		
	1.52	2.53	2.81	3.3	4.16	5.42	7.42	10.6	15.7		
	1.5	2.52	2.79	3.3	4.11	5.36	7.34	10.5	15.6		
	1.44	2.53	2.75	3.3	4.09	5.32	7.29	10.4	15.4		
	1.49	2.44	2.75	3.3	4.08	5.21	7.36	10.4	15.4		
	1.52	2.42	2.74	3.2	4	5.17	7.02	9.95	14.6		
	1.52	2.45	2.77	3.3	4.09	5.3	7.24	10.3	15.3		
mean	**1.49**	**2.47**	**2.78**	**3.3**	**4.09**	**5.31**	**7.28**	**10.4**	**15.4**		
std.deviat	**0.03**	**0.05**	**0.02**	**0**	**0.04**	**0.07**	**0.11**	**0.17**	**0.29**		
Coeff.varia	**2.30**	**1.83**	**0.86**	**1.1**	**0.97**	**1.33**	**1.44**	**1.63**	**1.88**		
Count	**12.00**	**12**	**12**	**12**	**12**	**12**	**12**	**12**	**12**		
Std.error	**0.01**	**0.01**	**0.01**	**0**	**0.01**	**0.02**	**0.03**	**0.05**	**0.08**		
80% B	1.7	2.48	2.76	3.1	3.57	4.25	5.23	6.66	8.74	11.76	16.2
	1.69	2.41	2.78	3.1	3.55	4.26	5.21	6.63	8.71	11.72	16.1
	1.66		2.77	3.1	3.55	4.24	5.22	6.65	8.74	11.77	16.2
	1.48	2.56	2.87	3.3	3.95	4.87	5.36	6.2	8.16	11	15.2
mean	**1.63**	**2.48**	**2.8**	**3.2**	**3.66**	**4.41**	**5.26**	**6.54**	**8.59**	**11.56**	**15.9**
std.deviat	**0.10**	**0.08**	**0.05**	**0.1**	**0.2**	**0.31**	**0.07**	**0.22**	**0.29**	**0.376**	**0.51**
Coeff.varia	**6.31**	**3.02**	**1.81**	**3.3**	**5.39**	**7.04**	**1.34**	**3.42**	**3.32**	**3.249**	**3.2**
Count	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**
Std.error	**0.05**	**0.04**	**0.03**	**0.1**	**0.1**	**0.16**	**0.04**	**0.11**	**0.14**	**0.188**	**0.25**

Appendix III

Gradient RI and UV spectral data, obtained from System 2			
DRUG	**mean RI**	**UV maxima (acid) nm**	**Clarke (UV maxima) nm**
1-napthol	444.5		
2-chlorophenazine	805.8		
acetazolamide	225.7		Aqacid 265 Aqalk 240/291
acetohexamide	438.3		Aqacid 247 Aqalk 249
acetylcysteine	175.8		nsa
allopurinol	128.1	204.8/249.5	Aqacid 250 Aqalk 257
amiodarone	476.4	203.6/240.1	Aqacid 241 Aqalk 251
amoxycillin	226.5	194.3/228.3/272	Aqacid 230/272 Aqalk 247/291
apomorphine	256.0	206/272	Aqacid 272 Aqalk 253
ascorbic acid	51.0		Aqacid 243
aspirin	317.7	203.6/226/275.5	
atenolol	194.3	195.4/223.6/273.2	Aqacid 274/280
beclomethasone	711.1		Alchol 239
benzamphetamine	297.0	193.1/256.6	Aqacid 251/257/263
benzocaine	358.1	200.1/220.1/288.6	Aqacid 272 Aqalk 285
benzoic acid	327.2		Aqacid 230/273
benztropine	344.4	202.5/257.8	Aqacid 253/259
benzylpenicillin	376.1		Water 257/264/325
betamethasone	583.7	194.3/238.9	Alchol 240
betaxolol	300.7		
brallobarbitone	335.7		NaOH 256
bromazepam	331.5	201.3/234.2	Aqacid 239/345 Aqalk 237/348
bromphenaramine	266.8		Aqacid 265 Aqalk 262/269
Buclizine Hcl	453.9		Alchol 255/260
butobarbitone	355.1		NaOH 254
caffeine	258.7	206/270.8	Aqacid 273
cannabidiol	902.0	207.2/270.8	Ethnol 278
cannabinol	1027.9		
carbamazapine	366.7	210.7/283.8	
carbenoloxone	823.9	195.4/248.4	
carbidopa	190.3		Aqalk 291
carbromal	377.5		nsa
cetrimide	229.4		
chloramphenicol	336.1	201.3/275.5	water 278
chlordiazepoxide	285.4	201.3/243.6/306.4	Aqacid 246/308 Aqalk 262
chlorhexidine	305.3	200.1/229.5/257.8	Aqacid 245 Aqalk 232/253
chlorocresol	454.6		Aqacid 279 Aqalk 244/299
chloroquine	246.3	220.1/255.4/342.2	Aqacid 257/329/343 Aqalk 254/330
chloroxylenol	509.7	202.5/279.1	Aqacid 279 Aqalk 244/299
chlorphenoxamine	345.7	257.8	Aqacid 279 Aqalk 242/296
chlorprocaine	250.3	207.2/289.8	
chlorpromazine	349.6	203.6/254.2/307.6	Aqacid 255
chlorpropamide	413.2	202.5/230.7	MethA 232
chlorprothixene	352.9	206/228.3/267.3/324.3	Aqacid 230/268/324
chlortetracycline	281.5	191.9/226/263.7/354.1	Aqacid 266/359 Aqalk 253/284/346
chlorthalidone	306.9	207.2/273.2	Alchol 275/284
cimetidine	226.2		nsa
ciprofloxacin	260.3		
cisapride	316.6	231/273.2/307.6	
clindamycin	290.8		nsa
clobetasol	635.9	196.6/236.6	Meth 239
clonazepam	403.5	198.9/307.6	Aqacid 273 meth 245/309
codeine	236.5	209.5/282.7	Aqacid 285

Appendix III

DRUG	mean RI	UV maxima (acid) nm	Clarke (UV maxima) nm
cyclobarbitone	352.2		NaOH 256
cyclobuthiazide	452.5	223.6/269.4/314.7	
cyclopentobarbitone	350.8		NaOH 254
cyclopentolate	286.5	195.4/255.4	Aqacid 252/258/264
D amphetamine	238.6		Aqacid 251/257/263
Dapsone	298.2	206/257.8/293.3	Aqacid 288 Aqalk 256/292
demeclocycline	273.4	196.6/226/266.1/363.6	Aqacid 237/305 Aqalk 243/255/310
dexamethasone	380.7	195.4/240.1	Meth 240
dextromethorphan	298.4	202.5/277.9	Aqacid 278
diamorphine	499.5	198.9/257.8	Aqacid 279 Aqalk 299
diazepam	429.1	201.3/233/280.3/361.6	Aqacid 242/284/366
dichlorophen	590.6	204.8/283.8	Aqacid 282 Aqalk 245/304
diclofenac	592.0	202.5/275.5	Aqacid 273 Aqalk 275
dicyclomine	575.4	204.8/280.3	nsa
dienoestrol	534.6	228.3	
diethylpropion	434.4	198.9/217.7/251.9/311.2	Aqa 253 Aqalk 246
diflunisal	582.6	206/227.1/314.7	Metha 251 Aqalk 277
digoxin	346.9	190.8/218.9	
diloxanide	499.7	202.5/257.8	Aqacid 262
diltiazem	315.9	207.2/236.6	
diphenoxylate	385.0	200.1/256.6	Aqacid 252/258/264
diphenhydramine	306.7	198.9/257.8	Aqacid 252/257
dipipanone	363.1	206/259/292.1	Aqacid 259/265/293
dipivefrin	347.3	277.9	
disulfiram	733.5	217.7	
dithranol	576.1	206/254.2/287.4/351.7	Aqalk 276 Meth 256/288/360
domiphen	506.4	216.6/268.4	meth 268/277
dopamine	347.8	197.8/262.5	Aqa 280
doxepin	315.9	206/293.3	Aqacid 292
doxycycline	290.8	195.4/268.4/347	Aqacid 269/346
econazole	384.8	201.3	Metha 265/271/280
embramine	353.5	202.5	
enallypropamal	394.2	198.9/220.1	NaOH 244
ephedrine	227.4	207.2/255.4	Aqacid 251/257/263
ergocalciferol	276.5		Alchol 265
erythrosine	683.8	202.5/242.4	
ethosuximide	276.1	203.6/243.6	Aqacid 244
fenoterol	243.7		Aqacid 275 Aqalk 295
fentanyl	298.7	204.8/256.6	Aqacid 251/257/263
flucloaxacillin	468.7		Aqacid 268/274/344 Aqalk 268/274/318
fluconazole	288.7	202.5/260.2	
flumazenil	327.4	203.6/243.6	
flunitrazepam	305.0	198.9/228.3/307.6	Meth 252/308
fluocinolone	490.5	217.7/251.9/310	Alchol 240
fluphenazine	426.3	203.6/255.4/306.4	Aqacid 256/306
flurazepam	305.1	198.9/228.3/307.6	Aqacid 236/284 Aqalk 231/312
frusemide	379.6	191.9/233/273.2/343.4	Aqacid 235/274/342 Aqalk 271/333
glibenclamide	573.2	201.4/226.3/297.8	Meth 275/300
glutethimide	401.4		Alchol 252/258/264
glyceryl trinitrate	499.0		
griseofulvin	487.7	211.9/235.4/292.1	Alchol 236/291
haloperidol	316.4	196.6/246	Metha 245
heptabarbitone	376.8		NaOH 255
hexethal	450.6		NaOH 253
hexobarbitone	242.4	202.5/275.5	NaOH 243

Appendix III

DRUG	mean RI	UV maxima (acid) nm	Clarke (UV maxima) nm
Hydralazine HCl	132.3	209.5/259/301.6	Water 240/260/304
hydrocortisone	349.1	193.1/244.8	Alchol 240
hydroxizine	326.1	204.8/230.7	Aqalk 232/258/263
hyoscine	234.4		Aqacid 251/257/263
Imipramine HCl	335.2	211.9/250.7	Aqacid 251 Aqalk 252
Indomethacin	589.6	204.2/319.6	Metha 318
isocarboxazid	352.7		Aqalk 274
isoniazid	245.5	206/256.6	Aqacid 266 Aqalk 298
ketamine	262.0	197.8/268.4	Aqacid 269/276
ketoprofen	464.3	206/254.2	Aqacid 260 Aqalk 262
labetalol	290.3	301.6	Aqacid 302 Aqalk 246/333
levamisole	238.1	190.8/213	
levodopa	45.4	206/279.1	Aqacid 280
lisinopril	250.3	193.1/256.6	
loratidine	361.9	197.8/272	
lorazepam	400.0	228.3/315.9	Alchol 230/316
lormetazepam	462.7		Aqacid 231/311
mazindol	284.1	197.8/268.4	Aqacid 271 Aqalk 269/275
MDA	248.0	203.6/233/283.8	
MDMA	252.4	204.8/233/285	
mebendazole	321.8	198.9/234.2/291	AcidIPA 234/288 AlkIPA 270/355
meclozine	397.8	195.4/264.9	Ethnol 230/266
medroxyprogesterone	689.5	240.1	Alchol 241
mefenamic acid	685.8	196.6/218.9/277.9/350.5	Metha 279/350 Aqalk 285
menapthone	467.9	200.1/249.5/333.8	
mepivacaine	260.2		Aqacid 263/271
mepyramine	257.1	200.1/237.7/313.5	Aqacid 239/316 Aqalk 248/312
mescaline	242.7	207.2/267.3	Aqa 268 no alk shift
methabitone	324.0	198.9/220.1	
methadone	343.4	201.3/257.8/292.1	Aqacid 253/259/264/292
methaqualone	399.6	203.6/226/264.9/304	Aqacid 234/269 Aqalk 265/306
methohexitone	483.5		NaOH 246
methotrimeprazine	343.1	250.7/304	Aqacid 250 Aqalk 259/323
methylparaben	331.9	208.3/255.4	
methylphenidate	237.0	194.3/256.6	Aqacid 251/257/264
methylphenobarbitone	395.2		NaOH 243
metoclopramide	263.0	213/273.2/308.8	Aqacid 273/309
metronidazole	226.2		Aqacid 277 Aqalk 319
mexiletin	277.6	198.9/261.3	Aqacid 260
midazolam	306.3	195.4/217.7	
minocycline	240.3		Aqacid 265/354 Aqalk 243
monoacetylmorphine	249.3		Aqacid 287
morphine	181.5	209.5/283.8	
nadolol	248.6	198.9/268.4	Aqacid 269/276
nalidixic acid	379.9	216.6/256.6/317.1	Aqacid 257/315 Aqalk 285/334
nalorphine	236.9		Aqacid 285 Aqalk 251/298
naloxone	237.8	206/280.3	Aqa 281
naltrexone	246.7	206/280.3	Aqacid 281 Aqalk 293
naproxen	468.3	230.7/270.8/330.2	Aqacid 262/315/328 Aqalk 261/271/330
nealbarbitone	382.3		NaOH 255
nicotinamide	18.8		Aqacid 261
nicotinic acid	46.2	193.1/260.2	
nitrofurantoin	287.2	190.8/264.9/364.6	DMF Water 266/367
norcodeine	235.3	210.7/283.8	Aqacid 284
nordiazepam	373.2	203.6/233/280.3/361.6	Aqacid 238/283/361 Aqalk 240/340

Appendix III

DRUG	mean RI	UV maxima (acid) nm	Clarke (UV maxima) nm
norethisterone	676.0		Alchol 240
nortryptiline	338.1	208.3/237.7	Aqacid 239
noscapine	288.9	211.9/312.4	Aqacid 290/312 Aqalk 281/315
oestriol	1005.6		Alchol 280
orciprenaline	130.5	275.5	Aqacid 276 Aqalk 297
orphenadrine	321.9		Aqacid 258/264
oxazepam	390.3	201.3/228.3/312.4	Aqacid 234/280 aqalk 233/344 Alchol 315
oxprenolol	283.7	203.6/273.2	Aqacid 273
oxycodone	246.4	206/280.3	Aqacid 280
oxytetracycline	258.9	194.3/216.6/268.4/354.1	Aqacid 268/352 Aqalk 246/269
p-aminobenzoic acid	211.7	196.6/222.4/280.3	
paracetamol	241.3	200.2/241.1	Aqacid 245
pemoline	270.6		Aqacid 256/262/268
penicillamine	51.0		
pentazocine	288.1		Aqacid 278 Aqalk 240/300
pentobarbitone	383.7	199.8/208.5	NaOH 255
Pethidine	280.5		Aqacid 251/257/263
phenacetin	335.0	202.5/247.2	Aqacid 244
phenazocine	299.1		Aqacid 278 Aqalk 238/298
phenazone	282.1	203.6/240.1	Aqacid 230/242/256
phencyclidine	284.5		Aqacid 252/258/263/270
phenidione	484.2	194.3/226	Aqalk 278/330
phenobarbitone	335.0		NaOH 254
phentermine	245.1		Aqacid 247/251/257/263
phenylbutazone	642.5	202.5/237.7	Aqacid 237 Aqalk 264
phenylephrine	52.2	196.6/213/272.0	Aqacid 273 Aqalk 238/291
phenylpropanolamine	198.2		Aqacid 251/257/262
phenytoin	381.4		Meth 258/264
pilocarpine	158.4		
pindolol	252.8	215.4/263.7	
piroxicam	381.8	200.1/242.4/343.4	
prazepam	569.6		Aqacid 240/285/360
predisone	340.2		Alchol 240
primadone	287.8	198.9/256.6	
primaquin	276.2	206/263.7/333.8	Aqacid 265/282/334
prochlorperazine	323.3	202.5/254.2/306.4	Aqacid 254/305
progesterone	698.4	194.3/241.3	Alchol 240
Promazine HCl	326.1	202.5/251.9/301.6	Aqacid 251/254/306
promethazine	323.8	201.3/249.5/299.3	Aqacid 249/298 Aqalk 254/305
propantheline HBr	533.9	207.2/280.3	Aqacid 243/281 Aqalk 249/282
propranolol	298.9	213/291	Aqacid 288/305/319 Meth 290/306/319
Propyl-paraben	458.3		
Pseudoephedrine	229.7		Aqacid 251/257/263
pyridoxine HCl	23.1	208.3/289.8	Aqacid 290
pyrimethamine	288.9	208.3/272	Aqacid 272/286
quinalbarbitone	407.4		NaOH 254
quinidine	245.1	207.2/249.5/315.9/345.8	Aqacid 250/317/345 Aqalk 280/330
Quinine	246.2		Aqacid 250/317/346 Aqalk 280/330
reserpine	350.7	216.6/267.3	Alchol 267/295
rifampicin	416.6	200.1/231.8/262.5/341	Alchol 315
salbutamol	237.5	202.5/233.6/275.5	Aqacid 276 Aqalk 245/295
salicylamide	289.3		Aqacid 235/298 Aqalk 241/328
salicylic acid	355.3	206/235.4/302.8	Aqacid 236/303 Aqalk 298
strychnine	257.2	207.2/253.1	Aqacid 254 Aqalk 255/278
sulphacetamide	240.5	196.6/269.6	Aqacid 271 Aqalk 256

Appendix III

DRUG	mean RI	UV maxima (acid) nm	Clarke (UV maxima) nm
sulphadimidine	277.1	201.3/241.3/263.7	Aqacid 243/301 Aqalk 242/258
sulphaguanidine	92.3	195.4/259	Aqacid 264 Aqalk 259
sulphamethoxazole	319.5	268.4	Aqacid 265 Aqalk 256
sulphinpyrazone	473.7		Aqacid 237 Aqalk 260
Sulpiride	235.4	211.9/291	Aqa 292 No alk shift
Tamoxifen	424.3	235.4	
temazepam	437.8		Aqacid 237/284/358 Aqalk 231/313
testosterone	508.0	191.9/242.4	Alchol 240
tetracycline	264.9	267.3/355.3	Aqacid 270/356
Thebaine	275.5		Aqacid 284
theophylline	248.9	207.2/270.8	Aqacid 270 Aqalk 275
thioproperazine	305.3	233/261.3/310	Aqacid 234/263/312
thioridazine	371.3	261.3/307.6	Aqacid 262/310 Aqalk 275
tiaprofenic acid	451.8	302.8	Ethnolacid 260/305
Tolbutamide	442.7		
tolnaftate	866.8	222.4/256.6	Meth 257
Triamcinolone	311.7		Meth 238
Trifluoperazine	344.2	256.6/307.6	Aqacid 256/305 Aqalk 258/308
trimeprazine	336.9	202.5/251.9/301.6	Aqacid 251/300 Aqalk 256/310
trimethoprim	254.3	207.2/269.6	Aqacid 271 Aqalk 287
Trimipramine	344.7	249.5	Aqacid 250
Tripolidine	270.2		Aqacid 290
tubocuraraine	257.0	279.1	Water 280
warfarin	514.4	204.8/281.5/304	Aqacid 270/280/303 Aqalk 293/308
Yohimbine	279.2	270.8	Aqa 271/277/287 Aqalk 280
	243		
Key			
nsa: no significant absorption		DMF water: dimethylformamide diluted with water	
Aqacid: aqueous acid		Ethnol: ethanol	
Aqalk: aqueous alkaline		Ethnolacid: ethanolic acid	
Alchol: dehydrated alcohol		AcidIPA: acidic isopropyl alcohol	
Water:water		AlkIPA: alkaline isopropyl alcohol	
NaOH: sodium hydroxide			
Meth: methanol			
Metha: methanolic acid			

Retention times and indices of acid standards obtained on system 2

	Retention times				Retention indices			
Date	paracetamol	pentobarbitone	indomethacin	conditions	paracetamol	pentobarbitone	clobazam	indomethacin
12/03/96	9.528	17.128	21.778	norm	238.8	383.7	455.9	588.1
12/03/96	9.56	17.16	21.81	norm	240.7	384.0	456.2	589.4
12/03/96	9.588	17.172	21.805	norm	240.6	383.2	454.6	586.9
13/03/96	9.607	17.173	21.823	norm	240.8	383.2	455.3	588.5
13/03/96	9.613	17.163	21.813	norm	240.8	382.9	454.6	587.1
13/03/96	9.64	17.173	21.823	norm	241.0	383.3	455.3	588.2
13/03/96	9.645	17.178	21.828	norm	240.7	382.6	454.7	587.3
13/03/96	9.633	17.2	21.833	norm	241.0	383.7	455.1	588.7
13/03/96	9.588	17.172	21.822	norm	240.3	371.4	454.6	588.1
13/03/96	9.565	17.132	21.782	norm	240.4	382.6	454.0	586.5
13/03/96	9.575	17.158	21.808	norm	240.9	383.3	455.1	587.9
14/03/96	9.562	17.162	21.795	norm	240.8	383.8	455.0	587.8
14/03/96	9.558	17.158	21.792	norm	240.7	383.6	454.8	587.5
14/03/96	9.567	17.133	21.783	norm	240.7	382.8	454.7	587.8
14/03/96	9.585	17.152	21.802	norm	241.0	383.4	454.6	587.8
14/03/96	9.618	17.152	21.802	norm	240.9	383.5	454.5	587.6
mean	**9.590**	**17.160**	**21.806**		**240.6**	**382.6**	**454.9**	**587.8**
sd	**0.034**	**0.019**	**0.017**		**0.5**	**3.0**	**0.5**	**0.7**
14/03/96	9.24	16.907	21.573	pump a low	235.3	383.0	455.1	587.7
14/03/96	9.187	16.903	21.553	pump a low	237.2	383.5	455.5	587.4
14/03/96	9.208	16.908	21.558	pump a low	237.5	383.8	456.1	588.3
14/03/96	9.217	16.917	21.55	pump a low	237.5	384.0	455.7	587.9
mean	**9.213**	**16.909**	**21.559**		**236.9**	**383.6**	**455.6**	**587.8**
sd	**0.022**	**0.006**	**0.010**		**1.1**	**0.4**	**0.4**	**0.4**

Key

norm: staandard gradient conditions

pump a low: The percentage of eluent A was decreased by 1% and B increased by 1%

pump a high: The percentage of eluent A was increased by 1% and B decreased by 1%

35 degrees: column temperature 35 degrees Celsius

45 degrees: column temperature 45 degrees Celsius

hi acid: 2ml of 1M sulphuric acid per litre of eluent

lo acid: 0.5ml of 1M sulphuric acid per litre of eluent

Retention times and indices of acid standards obtained on system 2

	Retention times				Retention indices			
Date	**paracetamol**	**pentobarbitone**	**indomethacin**	**conditions**	**paracetamol**	**pentobarbitone**	**clobazam**	**indomethacin**
15/03/96	9.775	17.375	22.025	pump a high	235.6	382.0	454.3	587.9
15/03/96	9.727	17.377	22.01	pump a high	242.9	383.1	454.7	587.7
15/03/96	9.718	17.385	22.035	pump a high	243.0	383.3	455.7	588.3
mean	**9.740**	**17.379**	**22.023**		**240.5**	**382.8**	**454.9**	**588.0**
s d	**0.031**	**0.005**	**0.013**		**4.2**	**0.7**	**0.7**	**0.3**
21/03/96	9.758	17.225	21.892	35 degrees	236.9	380.7	453.6	587.7
21/03/96	9.753	17.22	21.903	35 degrees	240.6	381.1	451.9	586.8
21/03/96	9.775	17.242	21.908	35 degrees	241.0	382.0	452.8	587.1
mean	**9.762**	**17.229**	**21.901**		**240.5**	**382.8**	**454.9**	**588.0**
sd	**0.012**	**0.012**	**0.008**		**2.3**	**0.7**	**0.9**	**0.5**
22/03/96	9.482	17.132	21.748	45 degrees	238.6	383.7	455.9	587.8
22/3/96	9.46	17.11	21.743	45 degrees	240.5	384.1	456.2	587.7
22/03/96	9.452	17.085	21.735	45 degrees	240.9	383.9	456.3	587.7
mean	**9.465**	**17.109**	**21.742**		**240.0**	**383.9**	**456.1**	**587.7**
sd	**0.016**	**0.024**	**0.007**		**1.2**	**0.2**	**0.2**	**0.1**
25/03/96	9.487	17.103	21.687	hi acid	234.9	381.8	451.9	583.7
25/03/96	9.45	17.05	21.667	hi acid	240.3	380.9	449.4	581.6
25/03/96	9.46	17.127	21.76	hi acid	240.1	384.2	454.9	586.9
mean	**9.466**	**17.093**	**21.705**		**238.4**	**382.3**	**452.1**	**584.1**
sd	**0.019**	**0.039**	**0.049**		**3.1**	**1.7**	**2.8**	**2.7**
26/03/96	9.523	17.157	21.807	lo acid	240.6	382.8	454.6	587.7
26/03/96	9.575	17.158	21.808	lo acid	242.2	383.4	455.3	588.5
26/03/96	9.513	17.13	21.797	lo acid	241.3	382.7	454.4	587.9
mean	**9.537**	**17.148**	**21.804**		**241.4**	**383.0**	**454.8**	**588.0**
sd	**0.033**	**0.016**	**0.006**		**0.8**	**0.4**	**0.5**	**0.4**

Retention times and indices of base standards obtained on system 2

	Retention times					Retention indices			
DATE	chloroquin	diphenhydramine	meclozine	Amiodarone	conditions	chloroquin	diphenhydramine	meclozine	Amiodarone
12/03/96	9.637	14.003	17.553	19.387	norm	240.4	305.6	394.3	467.4
12/03/96	9.668	14.018	17.568	19.418	norm	242.3	305.8	390.1	467.7
12/03/96	9.667	14.033	17.583	19.417	norm	241.7	305.1	393.5	466.4
13/03/96	9.69	14.007	17.557	19.407	norm	241.9	304.4	392.8	465.9
13/03/96	9.692	14.042	17.575	19.408	norm	241.9	304.9	393.2	465.5
13/03/96	9.782	14.018	17.585	19.418	norm	243	304.1	393.6	466.3
13/03/96	9.698	14.065	17.582	19.415	norm	241.4	304.7	392.7	465.1
13/03/96	9.695	14.062	17.562	19.395	norm	241.8	305.2	392.7	464.7
13/03/96	9.668	14.002	17.552	19.402	norm	241.8	304.4	393.1	466.1
13/03/96	9.665	14.015	17.565	19.415	norm	242.1	305.1	393.5	466.6
14/03/96	9.652	14.018	17.568	19.402	norm	242.1	305.8	393.8	466.5
14/03/96	9.652	13.985	17.535	19.385	norm	244	304.9	392.9	465.7
14/03/96	9.668	14.002	17.535	19.402	norm	242.1	304.9	392.9	466.8
14/03/96	9.675	14.008	17.558	19.392	norm	242.3	305.4	393.4	465.8
14/03/96	9.697	14.037	17.553	19.387	norm	242	305.3	393.5	465.7
mean	**9.68**	**14.021**	**17.562**	**19.403**		**242.1**	**305.0**	**393.1**	**466.1**
sd	**0.033**	**0.067**	**0.015**	**0.012**		**0.8**	**0.5**	**0.9**	**0.8**
14/03/96	9.42	13.763	17.313	19.147	pump a low	238.0	303.4	393.3	465.9
14/03/96	9.39	13.74	17.307	19.14	pump a low	240.1	304.4	393.6	466.1
14/03/96	9.417	13.75	17.3	19.15	pump a low	240.5	304.9	393.9	466.9
14/03/96	9.408	13.758	17.308	19.142	pump a low	240.2	304.7	393.8	466.6
15/03/96	9.497	13.757	17.323	19.14	pump a low	241.5	304.6	394.2	466.5
mean	**9.426**	**13.753**	**17.31**	**19.144**		**240.1**	**304.4**	**393.8**	**466.4**
sd	**0.041**	**0.009**	**0.009**	**0.004**		**1.3**	**0.6**	**0.3**	**0.4**

Retention times and indices of base standards obtained on system 2

	Retention times					Retention indices			
DATE	chloroquin	diphenhydramine	meclozine	Amiodarone	conditions	chloroquin	diphenhydramine	meclozine	Amiodarone
15/03/96	9.975	14.275	17.808	19.658	pump a high	238.5	302.0	393.1	467.5
15/03/96	9.957	14.257	17.823	19.673	pump a high	246.0	306.7	394.1	467.9
15/03/96	9.887	14.287	17.82	19.67	pump a high	245.5	308.2	394.4	468.4
mean	**9.94**	**14.273**	**17.817**	**19.667**		**243.3**	**305.6**	**393.9**	**467.9**
sd	**0.046**	**0.015**	**0.008**	**0.008**		**4.2**	**3.2**	**0.7**	**0.5**
21/03/96	9.777	14.127	17.66	19.477	35 degree	237.2	300.9	391.9	466.8
21/03/96	9.782	14.133	17.667	19.483	35 degree	241.0	303.6	386.9	463.8
21/03/96	9.782	14.125	17.658	19.475	35 degree	241.1	303.8	393.0	464.1
mean	**9.780**	**14.128**	**17.662**	**19.478**		**239.8**	**302.8**	**390.6**	**464.9**
sd	**0.003**	**0.004**	**0.005**	**0.004**		**2.2**	**1.6**	**3.3**	**1.7**
22/03/96	9.632	13.982	17.565	19.432	45 degree	240.7	305.0	394.6	469.2
22/03/96	9.627	13.993	17.577	19.427	45 degree	242.9	307.2	395.7	470.3
22/03/96	9.62	13.98	17.563	19.413	45 degree	243.2	307.8	395.6	470.1
mean	**9.626**	**13.985**	**17.568**	**19.424**		**242.3**	**306.7**	**395.3**	**469.9**
sd	**0.006**	**0.007**	**0.008**	**0.01**		**1.4**	**1.5**	**0.6**	**0.6**
25/03/96	9.823	14.19	17.723	19.79	hi acid	239.9	307.4	397.9	484.0
25/03/96	9.775	14.158	17.808	19.808	hi acid	244.8	310.4	399.4	483.5
26/03/96	9.787	14.197	17.747	19.763	hi acid	244.7	311.9	399.6	482.7
mean	**9.795**	**14.182**	**17.759**	**19.787**		**243.1**	**309.9**	**399.0**	**483.4**
sd	**0.025**	**0.021**	**0.0438**	**0.023**		**2.8**	**2.3**	**0.9**	**0.7**
26/03/96	9.428	13.862	17.478	19.228	lo acid	239.3	300.8	390.8	458.4
26/03/96	9.463	13.863	17.48	19.23	lo acid	240.6	302.4	391.3	459.0
26/03/96	9.445	13.862	17.478	19.228	lo acid	240.4	302.3	391.3	458.9
mean	**9.445**	**13.862**	**17.479**	**19.229**		**240.1**	**301.8**	**391.1**	**458.8**
sd	**0.018**	**0.001**	**0.001**	**0.001**		**0.7**	**0.9**	**0.3**	**0.3**

Basic Column results - obtained on the ODP504e column, pH 10.2 (NaOH)

Nitro-n-alkane	200	300	400	500	600	700	800	900	1000	1100	1200	1300	1400	1500	1600
Retention time	9.622	14.338	17.438	19.472	21.055	22.405	23.638	24.788	25.872	26.905	27.822	28.688	29.455	30.155	30.872
	9.613	14.33	17.413	19.48	21.063	22.43	23.647	24.797	25.897	26.913	27.83	28.68	29.463	30.163	30.88
	9.61	14.31	17.427	19.493	21.077	22.427	23.643	24.827	25.927	26.927	27.86	28.71	29.46	30.177	30.91
	9.603	14.287	17.387	19.453	21.07	22.437	23.67	24.82	25.903	26.92	27.853	28.687	29.437	30.137	30.82
Mean	**9.609**	**14.316**	**17.416**	**19.475**	**21.066**	**22.425**	**23.650**	**24.808**	**25.900**	**26.916**	**27.841**	**28.691**	**29.454**	**30.158**	**30.871**
St.dev	**0.005**	**0.023**	**0.022**	**0.017**	**0.009**	**0.014**	**0.014**	**0.018**	**0.023**	**0.009**	**0.018**	**0.013**	**0.012**	**0.017**	**0.037**
co-eff.var	**0.05**	**0.16**	**0.13**	**0.09**	**0.04**	**0.06**	**0.06**	**0.07**	**0.09**	**0.04**	**0.07**	**0.05**	**0.04**	**0.06**	**0.12**

Acidic compounds	Retention times				Mean	Std.dev	co-eff.var.	Retention indices				Mean	Std.dev	co-eff.var.
paracetamol	2.42	2.38	2.382	2.362	**2.386**	**0.021**	**0.89**							
pentobarbitone	4.487	4.472	4.465	4.478	**4.476**	**0.008**	**0.18**							
clobazam	12.437	12.422	12.465	12.445	**12.442**	**0.016**	**0.12**	259.7	260.7	259.6	260.7	**260.2**	**0.6**	**0.2**
indomethacin	18.137	18.122	18.132	18.112	**18.126**	**0.010**	**0.05**	434.4	434.1	434.3	435.1	**434.5**	**0.4**	**0.1**
Basic compounds														
chloroquin	21.14	21.122	21.228	21.335	**21.206**	**0.084**	**0.40**	606.3	611.2	604.3	619.4	**610.3**	**6.7**	**1.1**
meclozine	28.207	28.605	28.595	28.683	**28.523**	**0.185**	**0.65**	1244.5	1286.5	1291.2	1299.5	**1280.4**	**24.6**	**1.9**
amiodarone	31.207	30.955	30.962	31.05	**31.044**	**0.102**	**0.33**							

Appendix VI

Database of gradient and isocratic retention indices run on system 1

DRUG NAME	Gradient RI	30% B	60% B	80% B
2-chlorophenazine				616.7
2-ketotestosterone	368.0			
4-aminosalicylic acid		120.3		
5-androstone 3beta 17beta Diol	438.0			
acetazolamide	249.0	68.8		
acetohexamide			332.3	
acetomenapthone			518.5	
allobarbitone	314.5	231.1		
alprenolol	312.0	155.8		
amitriptyline	343.0	336.4		
amylobarbitone	383.9	384.2	257.4	
antazoline Sulphate	296.0	168.0		
apomorphine	262.5	22.7		
aprobarbitone	356.0	250.0		
aspirin	322.0	243.4		
astemizole	288.0	71.7		
b-oestradiol	447.0		365.7	
barbitone	278.2	122.0		
beclomethasone dipropinoate	701.0		708.7	
benadryl	305.0	205.2		
benzanilide	447.0		403.3	
benzil			613.5	
benzocaine	355.5	325.9		
benzoic acid		268.6		
benzotropine mesylate	339.0	355.4		
benzphetamine	306.5	146.9		
betamethasone valerate	576.0		545.5	
betaxolol		162.8		
brallobarbitone	341.0	275.1		
bromazepam	304.0	207.7		
brompheniramine	263.5			
buclizine			229.4	
buprenorphine	324.0	199.3		
butalbital	362.0	321.5		
butobarbitone	354.0	310.3		
caffeine	260.0	65.5		
cannabidiol	901.0			572.3
carbamazepine	371.0	370.2		
carbenoloxone	805.5			598.0
carbromal	372.5	358.8		
cetrimide	40.5	60.2		
chlophenoxamine	372.5	370.0		
chloramphenicol	346.0	281.7		
chlorcyclizine	341.5	322.1		
chlordiazepoxide	289.5	76.4		
chlorhexidine	305.0	195.4		
chlorhydroxyquinoline	221.5			484.0
chlorocresol	456.0		412.9	
chloroxylenol	518.5		489.5	
chlorpromazine	346.0	385.9		
chlorpropamide	412.0		312.1	
chlortetracycline	283.5	77.3		
chlorthalidone	328.0	207.8		
Cisapride	314.5	252.9		

DRUG NAME	Gradient RI	30% B	60% B	80% B
clobetasol propionate	628.5		621.9	
clomethiazole	284.5	246.0	185.6	59.1
clonazepam	407.0	202.9	278.7	
codeine	243.5	259.0		
cyclobarbitone	349.0	306.9	176.2	
dyclopentobarbitone	356.5	306.9		
dapsone	304.0	212.3		
demeclocycline	272.5	37.8		
desipramine	339.5	292.5		639.0
desogesterol				639.3
dextromethorphan	253.5	146.2		
diazepam	395.0	122.2	332.6	
dichlorophen	599.0		562.9	304.8
diclofenac			564.6	
dieoneostrol	536.5		478.9	
diethazine Hcl		295.9		
diflunisal	569.0		530.3	
diltiazem	157.5	230.6		
dimenhydrinate	305.0	205.0		
diphenoxylate			349.6	
diphenylpraline HCl		269.9		
dipipanone	355.5		107.0	
dipiverin HCl	360.0	370.6		
disulfiram	732.0		736.6	
dithranol	696.0		703.8	557.9
domiphen	496.5		423.1	
doxepin	306.0	232.5		
doxycycline	312.5	88.0		
empilan	888.0			695.9
erythrosine	694.0		698.6	
ethosuximide	276.0	130.9		
ethropropazine	339.0			
ethyl hydroxybenzoate		370.2		
ethyl paraamino benzoate		326.9		
fenoterol	242.0	63.9		
fentanyl	295.0	170.6		
fluconazole	285.5	131.0		
flumazenil	335.5	241.0		
flunitrazepam	429.5		343.6	
fluphenazine	329.5	313.2		
flurazepam	321.0	191.4		
frusemide	381.5	389.3		
haloperidol	328.0	249.9		
heptabarbitone	381.0	372.7		
hexethal	457.5		375.9	
hydrastine	282.5	61.0		
hydrochlorothiazide	274.5	117.1		
hyoscine	250.5	63.5		
ibomal	353.0	298.4		
ibuprofen	630.0		597.4	
idobutal	377.5	337.5		
indomethacin	594.0		553.8	
ketoprofen	461.5		371.6	
ledermycin	280.0	37.5		
lignocaine	262.0	33.3		

DRUG NAME	Gradient RI	30% B	60% B	80% B
mefenamic Acid	690.0		680.0	
mephentermine	257.0	32.3		
mepivacaine	258.0	33.3		
metharbarbitone	336.0	262.2		
methohexitone	482.5		429.7	
methoin	366.5	328.4		
methopromazine	343.0	308.6		
methylphenobarbitone	397.0		294.1	
metoclopramide	263.0	33.1		
midazolam	312.0	164.8		
minocycline	234.0	172.1		
n butylaminobenzoate	481.0		458.3	
nalidixic acid	381.5	367.7		
naproxen	469.0		392.0	
nealbarbitone	383.5	366.6		
neostigmine	237.0	61.7		
nitrazepam	367.5	358.1		
nitrofurantoin	296.0	174.5		
nordiazepam	341.0	335.4		
ofloxacin	262.5	333.2		
orphenadrine	170.5	271.1		
oxazepam	387.0		244.4	
oxprenolol	291.5	75.9		
papaverine	286.5	70.8		
pentobarbitone	386.5	376.7		
phenacetin	341.5	274.0		
phenazone	285.5	104.6		
phenylbutazone	633.0		622.3	
phenytoin	382.5	385.8		
prazepam	538.0		541.9	
primidone	288.5	124.0		
prochlorperazine	326.0	244.5		
progesterone	744.5		693.8	
propranolol	303.0	148.9		
pyrimethamine	290.0	90.1		
quinalbarbitone	408.5		300.6	
ranitidine	141.0	91.1		
secbutobarbitone	351.0	290.7		
sulphacetamide	287.0	89.0		
sulphadiazine	253.5	91.1		
sulphapyridine	256.0	87.5		
talbutal	370.5	335.7		
temazepam	434.5	361.1		
testosterone 17beta Hemisuccinate	538.0			278.0
theophylline	246.5	44.4		
thioridazine	372.0		48.8	
tolnaftate	889.0			601.8
trimeprazine	231.0	321.7		
warfarin	513.5		466.4	

Appendix VII

Compilation data based on System 2,
other workers data is shown converted to the compilation RI scale

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
acebutolol	263.9			321.1		317.0952		262.4	265.4		
acecarbromal	374.3							376.1	372.6		
acepromazine	350.1					350.22258	349.8		350.4		
acetanilide	281.1						292.0		270.2		
acetazolamide	**225.7**	245.9							205.5		
acetohexamide	**438.3**										
acetophenazine	309.2						309.2				
acetophenone	357.9						357.9				
acetylcysteine	**175.8**										
adrenaline	28.3						28.3				
alpha-acetyl-N,N-dinormethadol	363.0						363.0				
alpha-acetylmethadol	344.7						344.7				
n-acetylprocainamide	214.9						214.9				
ajmaline	277.5					323.45298		277.3	281.8		273.4
albuterol	184.5			307.6			184.5				
alimemazine	387.0					367.8459			387.0		
alimemazine Sulphoxide	287.9							288.1	287.6		
allobarbitone	300.6	312.8					307.2	289.1	293.4		
allopurinol	**128.1**							144.8	148.5		
allylcyclopentylbarbituric Acid	344.7						344.7				
allylisobarbitone	357.9						357.9				
alphenal	342.7						342.7				
alprazolam	378.9		346.6	327.4		321.22218		394.9	392.8	381.3	
alprenolol	322.6	310.2						324.7	333.0		
amethocaine	299.2									299.2	
amiloride	190.0								190.0		
4-aminoantipyrine	188.5						188.5				
4-aminobenzamide	37.4						37.4				
m-aminobenzoic acid	65.8						65.8				
o-aminobenzoic acid	235.2						235.2				
p-aminobenzoic acid	211.7										
2-aminobenzoylbenzamide	292.0						292.0				
2-amino-5-bromophenyl pyridin-2yl methanone 1st	410.2								410.2		
2 nd peak	443.1								443.1		
2-amino2',5'-dichlorobenzophenone	644.9					360.26118			644.9		
aminofenbendazole sulphone	279.8						279.8				
aminofenbendazole sulphoxide	254.5						254.5				
7-aminoflunitrazepam	289.5					309.95664			289.5		
2-amino-5-chlorobenzophenone	615.0					365.16894			615.0		
2-amino-2'-chloro-5-nitrobenzophenone	525.1								525.1		
2-amino-2'-fluoro-5-nitrobenzophenone	504.9								504.9		
2-amino-5-hydroxybenzylbenzimidazole	267.6						267.6				
2-amino-5-nitrobenzophenone	508.2					333.71466	521.2		495.2		
aminophenazone	204.4							209.1	199.7		
aminophylline	257.3						235.2				279.4
amiodarone	**476.4**							702.3	700.9		

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
amitriptyline	374.7	341.9	359.6092		390.1	377.66142	395.4	397.9	395.7	357.7	
amoxapine	317.8		329.0			359.14578	300.1		324.3		
amoxycillin	**226.5**	226.4									
amphetamine	**238.6**	241.8	237.0				204.8	196.2	197.8		
ampicillin	249.9	249.9									
amylobarbitone	373.6	383.7					375.1	365.3	365.8	378.2	
amylocaine	315.0					334.27236	303.1			326.9	
antazoline sulphate	293.9	293.9									
o-anisic acid	304.1						304.1				
antipyrene	266.5		266.5								
apomorphine	**256.0**	259.7					245.3				
astemizole	285.7	285.7				338.62242					
anthracine	730.1						730.1				
antropine	257.1		257.1								
aprobarbitone	319.2	355.2					319.4	302.0	300.1		
ascorbic Acid	**51.9**								51.9		
aspirin	**317.7**	320.5					312.3	283.2	279.9	318.7	331.9
atenolol	**194.3**	219.8					197.7	178.4	181.4		
atrazine	400.6								400.6		
atropine	250.8						260.5	249.6	242.2		
aureomycin	280.1	280.1									
azinphos methyl	512.5							512.5			
barbitone	258.3	275.7					261.6	240.7	242.2	271.5	
beclomethasone dipropinoate	**711.1**	707.8									
benadryl	303.1	303.1									
benperidol	324.0					326.24148		324.7	323.3		
benzanilide	448.2	448.2									
benzocaine	**358.1**	354.7	323.1			313.86054	338.6	344.5	332.0	351.5	
benzoctamine	322.3								322.3		
benzoic acid	**327.2**						318.3				
benzphetamine	**297.0**	304.6	330.1				319.4				
benzoylecgonine	236.3	131.4	250.0			309.2874	267.6	248.6	249.9	270.4	
benzthiazide	415.2									415.2	
benztropine	**344.4**		337.2				409.6		410.2		
benzylpenicillin	**376.1**										
berberine	327.5						327.5				
betamethasone valerate	**583.7**	580.0									
betaxolol	**300.7**					357.02652			323.3		
bezotropine mesylate	337.8	337.8									
bisacodyl	431.5					339.84936			431.5		
biphenyl	680.4						680.4				
brallobarbitone	**335.7**	339.9						311.9	311.7		
bretylium cation	274.5									274.5	
brodifacoum (a)	1070.3									1070.3	
brodifacoum (b)	1091.9									1091.9	
bromhexine	333.5										333.5

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
bromazepam	**331.5**	302.1			333.4	315.64518	312.3	334.6	330.1		
bromisoval	307.4							307.9	306.9		
bromophos - ethyl	737.6								737.6		
bromoprid	279.4										279.4
bromoxynil	413.1								413.1		
brompheniramine	**266.8**	260.7				361.48812	242.3		307.9		
brucine	266.6						266.6				
buclizine HCl	**453.9**										
buflomedil	277.9					343.64172			277.9		
bufotenine	181.4						181.4				
bupivacaine	310.5		313.6	379.5		335.27622		309.9	307.9		
bupranolol	325.2								325.2		
buprenorphine	338.6	322.5				334.49544	348.8	345.5	337.8		
buspirone	310.1					334.83006	314.3		305.9		
butacaine	330.5						330.5				
butalbital	342.3	361.3						333.6	332.0		
butanilicaine	279.7									279.7	
butaperazine	405.9							443.3	444.0		330.3
butobarbitone	**355.1**	353.2					344.7	318.8	317.5	347.4	
butorphanol	299.5		299.5								
butriptyline	369.0									369.0	
n-butylaminobenzoate	483.0	483.0									
butylparaben	494.8						494.8				
N,N-butyl-1-phenylcyclohexylamine	332.5						332.5				
caffeine	**258.7**	257.1	235.9	301.7		308.84124	258.5	224.9	220.9		276.4
camazepam	505.7							499.7	511.6		
cannabidiol	**902.0**	912.2						874.3			
cannabinol	**1027.9**						982.6			1040.5	
captopril	282.7									282.7	
carazolol	306.9								306.9		
carbamazepine	**368.0**	370.5		320.7	339.7	315.64518	364.0	355.4	332.0	373.1	348.2
carbamazepine-10,11-epoxide	336.1									336.1	
carbaryl	401.5								401.5		
carbenoloxone	**823.9**	814.6									
carbidopa	**190.3**	205.5									
2-carboxyibuprofen	346.7						346.7				
9-carboxy-11-nor-9-tetrahydrocannabinol	680.4						680.4				
carbromal	**377.5**	372.1					363.0	345.5	351.3	371.1	
carbutamide	321.1							320.8	321.4		
carpipramine	350.4					415.02732			350.4		
carvacrol	516.1						516.1				
chloramphenicol	**336.0**	345.0					332.5				
chlorcyclizine	340.4	340.4									
chlordiazepoxide	**285.4**	287.3	316.0	345.0	352.3	331.5954	302.1	321.8	309.8		273.4
chlorhexidine	**305.3**	303.1									
chlorhydroxyquinoline	217.8	217.8									

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
chloridazone	277.9								277.9		
chlormethiazole	291.7	282.1			333.4					301.2	
chlormezanone	333.9			318.6		312.6336			333.9		
chlorocresol	**454.6**	457.4									
5-chloro-2{(cyclopropylmethyl)-amino}benzophenor	929.8								929.8		
7,2-chloroethyltheophylline	306.2						306.2				
m-chlorophenol	385.3						385.3				
chlorprocaine	**250.3**		261.8				242.3				
chlophenoxamine	**345.7**	372.1									
chlorpromazine	**349.6**	345.0									
chlorpropamide	**413.2**	412.4				317.6529					
chloroquine	**246.4**	237.2				327.35688	236.2	218.9	220.9		
chlorotheophylline	241.7		241.7								
8-chlorotheophylline	256.7						263.6		249.9		
chlorthalidone	**306.8**	326.6									
chlorothiazide	239.3		232.3				246.3				
chloroxylenol	**511.0**	521.3					473.5				
chlorophacinone	1042.6					344.9802				1042.6	
chlorphenesin carbamate	347.8						347.8				
chlorpheniramine	263.9	262.7	301.9		323.9		227.1				
chlorpromazine	412.4		416.2		446.8	402.981	403.5	414.7	415.1		
chlorpropamide	410.6			329.7			410.6				
chlorprothixene	**352.9**	349.1	376.1					426.5	424.7		376.7
chlorpyrifos	684.5								684.5		
chlortetracycline	**281.5**	281.1					288.9				
chlorthalidone	308.5							298.0	311.7	315.6	
cimetidine	**226.2**	221.3		307.1			198.7		186.2		
cinchonidine	213.9						213.9				
cinchonine	208.8						208.8				
cinchocaine	371.1		362.0						380.3		
cinnamoylcocaine	318.7									318.7	
ciprofloxacin	**260.4**	260.7									
cisapride	**316.6**	312.8									
clenbuterol	282.4						279.8				285.0
clindamycin	**290.8**										
clobazam	**454.9**		347.8		358.6	319.326		435.4	432.4	453.2	
clobetasol propionate	**635.9**	633.7									
clomipramine	404.9			575.1	449.9	408.558		417.6	419.9	377.2	
clonazepam	**402.9**	407.3	346.6	326.6		318.43368	408.6	397.9	400.6		385.7
clonidine	193.6			336.2	349.1	318.87984		193.2	193.9		
clonixin	344.7						344.7				
clopamide	310.3							311.9	308.8		
clopenthixol	411.0				503.5			416.7	405.4		
clorazepate	388.2					332.59926	340.7	410.7	413.1		
clorexolone	390.6									390.6	
clotiapine	348.4								348.4		

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
clotiazepam	497.1								497.1		
clozapine	283.7					347.54562			283.7		
cocaine	289.3	284.7	293.6			327.6915	291.0	289.1	289.5	287.9	
codeine	**237.1**	240.2	229.9632	315.9		313.07976	221.0	201.2	199.7	242.7	
colchicine	326.7					308.95278	343.7		309.8		
cortisone	372.1						372.1				
creatine	21.2						21.2				
coumachlor	596.9									596.9	
coumarin	334.1						347.8		320.4		
coumatetralyl	598.0									598.0	
crotylbarbitone	302.0							302.0			
1-cyano-1,2-dihydrohydroxyquinoline	346.7						346.7				
cyclazocine	288.9						288.9				
cyclobarbitone	**352.2**	348.1						324.7	326.2		
cyclobenzaprine	378.2						378.2				
cyclobuthiazide	**452.5**										
1-cyclohexenyl-5-chloro-2-methyl-amino ketone	834.2								834.2		
cyclomethycaine	413.2									413.2	
cyclopenthiazide	453.3	451.3								455.3	
cyclopentobarbitone	**352.2**	355.7						327.7	329.1		
cyclopentolate	**286.5**										
cyclothiazide	433.2	439.5					426.9				
cyheptamide	410.6						410.6				
cymarin	373.1						373.1				
cyproheptadine	353.7		353.7			360.37272					
danazol	710.8						710.8				
danthron	603.3						603.3				
dapsone	**298.2**	302.1					308.2				
DCFB	407.3								407.3		
DDT	523.0									523.0	
debrisoquine	245.3					320.10678	245.3				
demeton -S-methyl	353.2								353.2		
demeton-S-methylsulphone	602.4								602.4		
demeclocycline	**273.4**	269.9									
demoxepam	340.6			316.0	339.7			341.5	339.7		
desalkylflurazepam	408.3			335.3	374.3				408.3		
desipramine	361.1	338.4	344.3			370.41132	379.2	372.2	371.6		
detajmium	256.1										256.1
dexamethasone	**380.7**						398.5				
dexchlorpheniramine	295.3					337.1724			295.3		
dextromethorphan	**298.4**	250.5	320.7			319.66062	331.5		322.3	314.6	
dextromoramide	389.9					353.3457			389.9		
dextropropoxyphene	374.0				352.3	355.46496	374.1	380.1	388.0	353.6	
1,4-diaminobutane	42.3								42.3		
1,5-diaminopentane	51.9								51.9		
diamorphine	281.9						292.0	277.3	280.8	277.6	

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
diazepam	**429.1**	395.1	376.1	368.7	424.7	341.74554	423.8	468.1	475.9	412.1	348.2
diazinon	628.5								628.5		
diazoxide	299.1					309.62202		300.0	298.2		
dibenzepin	299.7							303.0	302.1	294.0	
2,4-dibromoacetophenone	545.5						545.5				
dibucaine	372.1						372.1				
dichlorophen	**590.6**	603.6									
diclofenac	**592.0**					380.78454					
didesmethylchlorpheniramine	210.9						210.9				
2-{2-(diethylamino)ethylamino}-5-chloro-2'-fluorober	407.3								407.3		
2',5dicloro-2-(methylamino)benzophenone	689.3								689.3		
7-diethylamino-4-methylcoumarin	560.7						560.7				
N,N-diethyl-1-phenylcyclohexylamine	304.1						304.1				
dicyclomine	**575.4**										
diethylcarbamazine	191.6						191.6				
diethylpropion	230.1						230.1				
diethylstilbestrol	558.7						558.7				
diethyltryptamine	275.8						275.8				
dienoestrol	**534.6**	539.7									
diethylpropion	**434.4**										
difenacoum (a)	934.8									934.8	
difenacoum (b)	962.5									962.5	
diflunisal	**582.6**	572.9		327.9	591.7		549.6			564.1	
digitoxin	468.4						468.4				
digoxin	**346.9**						351.8	342.5	340.7		
10,11-dihydrocarbamazepine	377.2						377.2				
dihydrocodeine	207.6						207.8	195.2	193.9	233.5	
dihydromorphine	156.5									156.5	
3,4-dihydroxymandelic acid	102.3						102.3				
3,4-dihydroxyphenethyl alcohol	211.9						211.9				
3,4-dihydroxyphenylacetic acid	222.0						222.0				
3,4-dihydroxyphenylglycol	108.4						108.4				
diloxanide	**499.7**										
diltiazem	**315.9**			386.7		340.5186	357.9		343.6		
dimenhydrinate	182.3	303.1							61.6		
dimethindene	287.6								287.6		
dimethoate	289.3							289.1	289.5		
2,5-dimethoxy-4-ethylamphetamine	317.3						317.3				
2,5-dimethyl-1-methylphenyl-3-pyrazoline-5-one	325.4						325.4				
4-dimethylaminopyridine	165.2						165.2				
1,3-dimethylbarbituric Acid	206.8						206.8				
N,N-dimethyl-1-phenylcyclohexylamine	299.1						299.1				
N,N,-dimethyltryptamine	228.1						228.1				
1,7-dimethylxanthine	232.1						232.1				
3,5-dinitrobenzoyl chloride	363.0						363.0				
diphenhydramine	**335.5**		332.5		333.4	345.98406	336.6	338.6	336.8	324.8	

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
diphenoxylate	**385.0**			577.5			448.1				
5,5-diphenyl-2-thiohydantoin	434.0						434.0				
dipipanone	**363.1**	354.7									
dipiverin HCl	**347.3**	359.3									
diprenorphine	301.1						301.1				
diprophylline	227.1						227.1				
dipropyltryptamine	322.4						322.4				
dipyridamol	334.7					330.14538		330.7	338.8		
dipyrone	193.6						193.6				
disopyramide	280.8			353.1		319.43754			280.8		
disulfiram	**733.5**	739.5					700.7		670.0		
disulfoton	691.2								691.2		
5,5`-dithiobis-(2-nitrobenzoic acid)	440.0						440.0				
6,6`-dithiodinicotinic acid	359.9						359.9				
dithranol	702.7	702.7									
diuron	417.0								417.0		
domiphen	**506.4**	498.8									
dothiepin	367.4				358.6			372.2	378.4	351.5	
doxapram	312.3				311.3		312.3				
doxepin	**315.9**	304.1	338.4		342.8	345.09174	343.7	348.4	352.3	337.2	
doxycycline	**290.8**	310.8									
doxylamine	258.6					325.68378			229.6		287.6
droperidol	323.1			344.3		326.5761		324.7	321.4		
dyclonine	347.4									347.4	
econazole	**384.8**										
edifenphos	602.4								602.4		
embramine	**353.5**										
empilan	898.9	898.9									
enallypropymal	**394.2**	393.5									
ephedrine	**227.4**	222.4	222.9			318.7683	193.6	181.4	181.4	225.2	
epinine	59.8						59.8				
17a-epitestosterone	535.4						535.4				
ergocalciferol	**276.5**										
erythrosine	**683.8**	700.6									
estazolam	374.5					318.65676			374.5		
ethenzamide	303.5							304.0	303.0		
ethacrynic Acid	497.3						531.3		463.3		
ethosuximide	**276.1**	273.5					260.5	246.6	239.3		295.1
ethropropazine	337.8	337.8									
ethylmorphine	244.4		250.0	322.9			252.4	236.7	238.3		
2-ethyl-2-phenylmalonamide	255.5						255.5				
2-ethyl-2-phenylmalonate	252.4						252.4				
N-ethyl-thienylcyclohexylpiperidine	305.2						305.2				
2-ethyl-2-(p-tolyl)malonamide	292.0						292.0				
N-ethylnornicotine	33.4						33.4				
ethylparaben	373.1						373.1				

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
etilefrine	141.8								141.8		
etomidate	417.3							419.6	415.1		
etonitazene	325.4						325.4				
etorphine	292.0						292.0				
eugenol	429.9						429.9				
euphylline	225.8								225.8		
famotidine	190.6						190.6				
fenbendazole	370.1						370.1				
fenbendazole sulphone	337.6						337.6				
fenbendazole sulphoxide	284.9						284.9				
fenbufen	461.2				440.5	335.38776		453.2	469.1		
fencamfamin	309.2						309.2				
fenethylline	277.1							275.3	278.9		
fenfluramine	315.1		313.6			333.60312			316.6		
fenfuram	416.7							416.7			
fenoprofen	524.3			367.8		355.0188	532.3	508.6	531.9		
fenoterol	**245.8**	238.7		309.4							
fenproporex	226.1						226.1				
fentanyl	**298.7**	292.9	333.7	377.4			341.7		329.1		
flencainide	355.2					332.93388		349.4	361.0		
fluanisone	349.4					333.15696			349.4		
flubendazole	356.9						356.9				
flucloxacillin	**468.7**	460.5									
fluconazole	**288.7**	283.2									
flufenamic acid	667.1						656.0			678.1	
flumazenil	**327.4**	334.3				311.07204			314.6		
flunarizine	523.9							531.3	516.5		
flunitrazepam	**305.0**	430.3			352.3	318.43368	446.1	410.7	408.3		409.9
flunixin	413.7						413.7				
fluocinolone	**490.5**										
5-fluorouracil	96.3						96.3				
fluoxetine	399.6			430.0		376.65756			399.6		
fluoxymesterone	426.9						426.9				
flupenthixol	435.3								435.3		
fluphenazine	**471.0**	328.1	384.4			462.98952	355.9	428.5	428.6		
flurazepam	**305.1**	319.4	324.3	393.4	325.5			338.6	343.6	320.7	
fluvoxamine	362.9					371.86134			362.9		
frusemide	**379.6**	381.3		313.7			387.3	357.3	364.8		356.1
genotinine aminopropazine	296.0						296.0				
gentisic Acid	263.6						263.6				
glafenine	276.0					337.95318			276.0		
glibenclamide	**571.1**					344.4225	614.5	563.0	566.7		
gliclazide	482.7					317.76444		480.9	484.6		
glipizide	423.4						459.3	397.9	413.1		
gliquidone	667.5							671.7	663.2		
glisoxepide	399.7							396.9	402.5		

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
glutethimide	**401.4**				339.7	317.42982	402.5	376.1	380.3		
glyceryl trinitrate	**499.0**										
griseofulvin	**487.7**										
guaiacol	306.2						306.2				
guaifenesine	261.5								261.5		
halazepam	625.6						625.6				
haloperidol	**316.4**	326.6	349.0	384.5	346.0	342.52632	368.0	352.4	360.0		321.8
halostachine	115.5						115.5				
harmaline dinitrohydrate	272.7						272.7				
harmalol	236.2						236.2				
harmine	270.8						285.9		255.7		
heptabarbitone	**376.8**	380.8						353.4	355.2		
hexahydrocannabinol	1115.4						1115.4				
hexethal	**450.6**	458.9									
hexobarbitone	**242.4**						381.2	353.4	355.2		
4-hexylresorcinol	528.3						528.3				
hippuric acid	252.4						252.4				
homatropine	223.0						223.0				
homocaine	327.8		327.8								
homovanillic Acid	262.6						262.6				
hydralazine	**132.3**										
hydrastine	280.1	280.1									
hydrochlorothiazide	254.9	271.9					252.4	229.8	230.6	265.3	279.4
hydrocodone	231.1		244.1	329.0			231.1		218.0		
hydrocortisone	**349.1**						364.0				
hydromorphone	186.9		175.8	311.2			196.7		188.1		
hydroxyamphetamine	166.2						166.2				
2-hydroxethylpromazine	335.6						335.6				
hydroxyethyltheophylline	233.5		233.5								
b-hydroxyethyltheophylline	238.2						238.2				
p-hydroxyfenbendazole	324.4						324.4				
3-hydroxyflunitrazepam	368.7			327.0					368.7		
o-hydroxyhippuric acid	285.9						285.9				
2-hydroxyibuprofen	358.9						358.9				
5-hydroxy-3-acetic Acid	249.4						249.4				
6-hydroxy-7-methoxycoumarin	303.1						303.1				
11-a-hydroxy-a-methyltestosterone	371.1						371.1				
5-hydroxyisoquinoline	182.5						182.5				
6-hydroxynicotinic acid	195.6						195.6				
p-hydroxynorephedrine	37.4						37.4				
3`-hydroxypentobarbitone	252.4						252.4				
5-(p-hydroxyphenyl)-5-(p-tolyl)-hydantoin	345.7						345.7				
5-(m-hydroxyphenyl)-5-phenylhydantoin	331.5						331.5				
5-(p-hydroxyphenyl)-5-phenylhydantoin	321.4						321.4				
p-hydroxyphenylpyruvic Acid	294.0						294.0				
2-hydroxyquinoline	306.2						306.2				

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
4-hydroxyquinoline	241.3						241.3				
5-hydroxyquinoline	176.4						176.4				
hydroxyzine	**326.1**		370.2	405.9		370.85748		380.1	378.4		
hyoscine	**253.1**	247.4		319.3			219.0	226.9			
hyoscine butyl bromide	293.7							296.1	291.4		
ibogaine	321.4						321.4				
ibomal	352.1	352.1									
ibuprofen	598.5	635.2		407.9		392.71932	588.1	563.0	592.7	613.4	
idobutal	357.0	377.2						347.5	346.5		
iminostilbene	590.1						590.1				
imipramine	**335.2**		349.0		374.3	369.85362	389.3	382.1	384.2		
indole	397.7								397.7		
3-indoleacetic acid	325.4						325.4				
indole-3-carboxyaldehyde	328.5						328.5				
indomethacin	**589.6**	598.4		383.6			580.0	559.0	554.1		
indoprofen	406.0				355.4					406.0	
3-isobutyl-1-methylxanthine	307.2						307.2				
isocarbostyril	309.2						309.2				
isocarboxazid	**352.7**						348.8				
isoniazid	**245.5**						27.3	70.7	92.5		
N-(isopropyl)-1-phenylcyclohexylamine	306.2						306.2				
isoproterenol	108.4						108.4				
isoquinoline-N-oxide	280.8						280.8				
isosorbide dinitrate	395.8							396.9	394.8		
isoxsupine	301.1						301.1				
ivermectin	1109.3						1109.3				
ketamine	**262.0**		257.1			321.44526	256.5	250.6	249.0		
ketazolam	521.8			368.7	424.7			515.5	528.0		
ketobemidone	245.3							240.7	249.9		
ketoprofen	**464.3**	463.0		340.7	386.9	327.02226	466.4			462.4	
ketotifen	315.2					323.7876		312.9	317.5		
kynurenic acid	260.5						260.5				
labetalol	**290.3**			331.7		325.79532			303.0		
lamotrigine	272.5									272.5	
ledermycin	277.5	277.5									
levallorphan	291.1							291.1			
levamisole	**238.1**										
levomepromazine	381.1					370.52286		393.9	395.7		353.5
levorphanol	264.5			337.1			266.6		262.5		
lidocaine	258.2		258.2	345.7		324.3453					
lignocaine	248.7	259.1					245.3	239.7	240.3	259.1	
linuron	506.2							507.6	504.9		
lisinopril	**250.3**					313.749					
loprazolam	325.9				330.2	336.39162			331.0	320.7	
loratadine	**361.9**					400.63866					
lorazepam	**400.0**		334.9	325.4	377.5	321.44526	388.3	377.1	372.6	398.8	

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
lormetazepam	**462.7**				393.2		450.2	434.4	422.8		
loxapine	336.0		338.4			356.91498	319.4		350.4		
lysergide	306.6						306.2	303.0	310.8		
lysergic Acid	235.6						248.4	222.9			
lysergic acid hydroxyethylamide	226.1						226.1				
maleate anion	126.7									126.7	
maprotiline	389.0				396.4	374.31522		388.0	389.9		
mazindol	**285.6**		304.2				311.2				
mebendazole	**321.8**						346.7				
meclofenamic acid	689.5						689.5				
meclozine	**397.8**										
medazepam	333.6					348.54948	319.4	341.5	346.5	326.9	
medroxyprogesterone	**689.5**										
mefenamic Acid	**685.8**	696.6		452.3		458.52792	664.2			686.3	
mefruside	417.3									417.3	
megosterol acetate	778.7						778.7				
meperidine	286.5		286.5								
melperone	280.8								280.8		
menapthone	**467.9**										
mepacrine	299.1						299.1				
mephenesin	322.4					314.08362	322.4				
mephentermine	246.1	254.0				322.6722	238.2				
mephenytoin	343.2						359.9	335.6	333.9		
mepivacaine	**260.2**	255.1		343.9		325.34916	251.4	242.7	254.7		
mepyramine	**257.1**	245.4									
meptazinol	269.4									269.4	
mescaline	**242.7**						221.0	376.1	211.3		
mesoridazine	336.8		327.8				345.7				
metamizol	251.2							242.7	244.1		266.9
metapramine	310.8					331.37232			310.8		
metaproterenol	315.6			306.3					315.6		
methadol	365.0						365.0				
methadone	**343.4**		371.4			358.69962	400.5	389.0	392.8	355.7	
methamphetamine	245.3		245.3								
methapyrilene	196.7						196.7				
methaqualone	**399.6**		352.5		349.1	321.44526	400.5	402.8	404.4	392.6	386.6
methabitone	**324.0**	334.8								325.9	
methazolamide	264.6						264.6				
methocarbamol	296.9			304.4		308.28354	301.1				292.7
methohexitone	**483.5**	484.5						443.3	450.8	474.8	
methoin	365.9	365.9									
methomyl	225.8								225.8		
methopromazine	341.9	341.9									
methotrimeprazine	**343.1**										
methoxamine	228.1			323.6			228.1				
4-methoxyamphetamine	226.1						226.1				

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
2-methoxy-4,5-methylenedioxyamphetamine	246.3						246.3				
methsuximide	387.3						387.3				
methychlorthiazide	363.9									363.9	
2-methylamino-5-chlorobenzophenone	762.7								762.7		
a-methylaminomethylbenzyl alcohol	116.5						116.5				
methylaminorex	255.0									255.0	
methylamphetamine	215.8						222.0	214.0	211.3		
2-methyl-5H-dibenzapine-5-carboxamide	411.6						411.6				
methyldopamine	191.8		255.9				127.7				
3,4-methylenedioxyamphetamine (MDA)	**248.0**						215.9		217.1		
5-amino-2'-fluruor-2-(methylamino)-benzophenone	275.9		259.4282						292.4		
3,4-methylenedioxyethylamphetamine (MDEA)	227.6						254.5		200.7		
3,4-methylenedioxymethylamphetamine (MDMA)	**252.4**						237.2		235.4		
methylparaben	**332.6**						332.5				
methyl phenidate	**237.0**			354.3			274.7		270.2		
methylphenobarbitone	**395.2**						393.4	373.2	370.6		
N-methyl-1-phenylcyclohexylamine	279.8						279.8				
5-methyl-5-phenylhydantoin	286.9						286.9				
methylprednisolone	390.3						390.3				
4-methylprimidone	312.3						312.3				
methylphenidate	277.1		277.1								
methylsalicylate	449.2						449.2				
delta-9(11)-methyltestosterone	535.4						535.4				
methyltestosterone	587.1						587.1				
1-methyluric acid	189.6						189.6				
3-methyluric acid	172.3						172.3				
3-methylxanthine	199.7						199.7				
7-methylxanthine	187.5						187.5				
methyprylon	302.1						302.1				
metoclopramide	**263.0**	260.2	273.6	350.0		318.43368		259.5	262.5		
metolachlor	561.8								561.8		
metolazone	371.1			317.7						371.1	
metoprolol	272.4		272.4	332.6		321.5568	276.8	269.4	271.2		
metronidazole	**226.2**							194.2	192.9		
mexiletine	**277.6**					329.9223					
mianserin	342.1				336.5	341.85708		342.5	341.7		
mibolerone	554.6						554.6				
midazolam	**306.3**	310.2				335.27622		334.6	337.8		
minaprine	233.5								233.5		
minocycline	**240.3**	230.5									
molsidomine	227.7					308.39508			227.7		
6-monoacetylmorphine	**249.3**					313.52592	237.2	222.9	231.6	245.8	
monolinuron	410.5							416.7	404.4		
monopropionyldapsone	356.9						356.9				
moperon	338.8					337.39548			338.8		
morazone	294.2							291.1	297.2		

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
morphine	**181.5**		194.6	307.2		311.74128	174.3	152.7	156.2	172.9	
morphine glucuronide	168.3						168.3				
morphine-3-glucuronide	113.3									113.3	
nadolol	**248.6**			309.2		315.86826	237.2	226.9	226.7		
naftidrofuryl	409.3								409.3		
nalidixic acid	**380.0**	381.3									
nalorphine	**236.9**		218.2			313.97208	205.8	198.2	193.9	232.4	
naloxone	**237.8**	233.6	226.4	316.6		314.41824	221.0	309.9	207.4	235.5	
naltrexol	224.0						224.0				
naltrexone	**246.7**					313.63746	228.1				
naphazoline	262.6			313.7			262.6				
naphthalene	557.7						557.7				
a-naphthol	438.0						438.0				
2-naphthoxyacetic acid	415.7						415.7				
naproxen	**468.1**	470.7		342.7	418.4	334.27236	458.3	429.5	424.7		
nealbarbitone	**382.3**	383.3									
nefopam	312.6						319.4	305.9			
neostigmine	233.6	233.6									
niacinamide	52.7						52.7				
nifedipine	464.3			345.9		321.66834	497.8	444.3	450.8		
niflumic acid	529.6					370.96902	528.3		530.9		
nikethamide	238.6							241.7	235.4		
nimodipine	584.1					345.64944			584.1		
nitrazepam	369.9	367.0		324.7		319.66062	369.0	373.2	380.3	368.0	362.2
nitrendipine	554.1					341.634			554.1		
m-nitrobenzaldehyde	353.8						353.8				
nitrofurantoin	**287.5**	293.9						264.4	261.5		
nitroglycerine	469.5							461.1	477.8		
m-nitrophenol	344.7						344.7				
p-nitrophenol	345.4										345.4
nizatidine	178.5					313.97208			178.5		
nomifensin	296.1					329.02998		293.1	299.2		
norcodeine	**235.3**						212.9				
nordiazepam	**372.3**	339.9	350.2	341.9	405.8		341.7	419.6	413.1	356.7	
norephedrine	148.5								148.5		
norepinephrine	21.2						21.2				
norethisterone	**676.0**										
norfenefrine	42.3								42.3		
norflurazepam	374.5								374.5		
a-N-normethadol	337.6						337.6				
normethadone	366.0							364.3	367.7		
normeperidine	279.5		279.5								
N-normorphine	132.8						132.8				
norpropoxyphene	374.9		374.9								
noroxymorphone	178.4						178.4				
norpethidine	291.2						293.0	288.1	292.4		

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
norpseudoephedrine	168.3						168.3				
19-nortestosterone	437.8									437.8	
nortriptyline	**338.1**		352.5		399.5	378.21912	384.3	377.1	368.7	355.7	
noscapine	**288.9**						303.1	308.9	306.9		
noxiptyline	330.0									330.0	
nylidrin	305.2						305.2				
b-oestradiol	455.8	448.2					463.4				
oestrone	521.2						521.2				
ofloxacin	259.7	259.7									
opipramol	339.6					359.70348		340.5	338.8		
orciprenaline	**130.5**								138.9		
orphenadrine	**323.2**				355.4			363.3	366.8		
oxazepam	**390.3**	386.9	332.5	323.6	377.5	323.11836	387.3	399.8	390.9	382.3	364.5
oxazolam	293.4								293.4		
oxprenolol	**283.7**	289.3		347.5		332.48772			285.6		
oxycodone	**246.4**		238.2	321.1			225.0	216.0	216.1	242.7	
oxydemeton -S- methyl	224.8								224.8		
oxymorphone	184.2		166.5				184.5			201.6	
oxyphenbutazone	458.6				361.7		482.6	442.4	450.8		
oxytetracycline	**259.5**						241.3				
papaverine	295.0	284.2	301.9				299.1	302.0	299.2	283.8	
paracetamol	**240.6**					309.2874	220.0	188.3	191.0	237.6	
paraoxon	427.6							427.5	427.6		
paraxanthine	232.1						232.1				
pargyline	202.7						202.7				
paroxetin	336.8								336.8		
pecazine	381.6							373.2	389.9		
pemoline	**270.6**						255.5	238.7	236.4		
penfluridol	656.5					478.2705			656.5		
pentazocine	**288.1**		311.3	358.7		323.00682	322.4	308.9	309.8	296.1	
pentobarbitone	**382.6**	386.4					374.1	358.3	356.1		
pentoxifylline	274.1			304.4					274.1		
perazine	370.9							373.2	368.7		
pericyazine	355.8			372.0				355.4	356.1		
perphenazine	394.9							401.8	388.0		
persantine	327.5						327.5				
pethidine	**280.5**					325.4607	296.0		287.6		
phenacemide	266.3								266.3		
phenacetin	**335.0**	340.4					335.6	309.9	308.8	335.1	312.4
phenazocine	**299.1**						350.8				
phenazone	**282.1**	283.2					292.0	259.5	257.6		285.0
phenazopyridine	314.3						314.3				
phencyclidine	**284.5**		324.3			332.93388	319.4	227.8	236.4		
phendimetrazine	217.9						217.9				
p-phenetidin	179.4						179.4				
pheniramine	206.4						178.4		234.5		

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
phenobarbitone	**335.0**					312.41052	330.5	312.9	309.8	333.1	
phenothiazine	636.8						636.8				
phemetrazine	240.6		240.6								
phensuximide	347.1						345.7			348.5	
phentermine	**245.1**		247.6				232.1	228.8	232.5		
phenylbutazone	**642.5**	638.3		418.9	490.9	347.32254	656.0	589.6	593.7		
1-phenylcyclohexamine	293.0						293.0				
1-(1-phenylcyclohexyl)morpholine	298.1						298.1				
phenylcyclopentylamine	248.4						248.4				
phenylephrine	78.7	95.6					61.8				
1-phenylethylamine	157.2								157.2		
2-phenylethylamine	156.2								156.2		
m-phenylphenol	471.5						471.5				
phenylpropanolamine	**200.7**	210.1	215.8				151.0				
phenytoin	**381.4**	382.3			330.2		378.2	359.3	365.8	379.3	
pholcodine	91.8									91.8	
physostigmine	240.3						240.3				
pilocarpine	**158.4**										
pindolol	**252.8**			323.8		319.10292		232.8	232.5		
pipamperone	241.2					330.25692			241.2		
piperocaine	312.3						312.3				
piracetam	129.0							121.1	136.9		
piribedil	255.7								255.7		
piritramide	343.0							339.5	346.5		
piroxicam	**381.8**			337.3	349.1	321.5568		375.1	375.5		
prajmalin	340.1							338.6	341.7		
prazepam	**569.6**	541.2		429.7		367.17666	539.4	597.6	590.8	563.1	
prednisone	**340.2**										
prednisolone	360.9						360.9				
primaquin	**276.2**										
primadone	**287.8**	286.2					283.9	264.4	262.5		
probenecid	506.9						508.0		505.8		
procainamide	160.4			316.9		315.4221		160.6	160.1		
procaine	225.2	221.3	234.7					197.2	192.9	231.4	273.4
prochlorperazine	**323.3**	324.6			497.2	478.2705			411.2	326.9	
a-prodine	317.3						317.3				
progesterone	**698.4**	752.2					767.6				
promazine	**326.1**		344.3		368.0			367.2	368.7	342.3	328.7
promethazine	**323.8**		343.1			357.91884		359.3	361.9		
propafenone	349.3					357.02652			359.0		339.7
probanthelline	**533.9**										
propiomazine	358.9						358.9				
N-propionylprocainamide	231.1						231.1				
propoxur 1	486.5								486.5		
propoxur 2	567.6								567.6		
propoxphene	359.6		359.6								

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
propranolol	**298.9**	301.0	314.8	366.2	333.4	350.11104	315.3	324.7	322.3	312.5	295.1
N-propylamphetamine	274.7						274.7				
n-propylparaben	**458.3**						429.9				
N-(n-propyl)-1-phenylcyclohexylamine	311.2						311.2				
propyphenazone	370.4							368.2	372.6		
protriptyline	362.0		347.8		374.3	363.60738		374.1	374.5	351.5	
proxibarbal	211.3								211.3		
proxymetacaine	269.4									269.4	
pseudoephedrine	**229.7**						193.6			229.4	
psilocin	226.4		226.4								
psilocybin	185.2		185.1764								
puromycin	226.1						226.1				
pyrilamine	221.0						221.0				
pyrimethamine	**288.9**	287.8				330.48					
pyrithyldione	290.0						284.9				295.1
quazepam	765.6			497.1			765.6				
quinalbarbitone	**407.4**	408.9				324.67992	399.5	389.0		403.9	
quinaldic acid	234.2						234.2				
quinidine	**245.1**		270.0			323.11836	224.0	270.4	270.2		
quinine	**246.2**					322.44912	239.2	267.4	262.5		263.5
ranitidine	174.6	135.5				312.41052	191.6		196.8		
recinnamine	406.6						406.6				
reserpine	**350.7**					340.96476	402.5	410.7	421.8		
resorcinol	210.9						210.9				
rifampicin	**416.6**										
saccharin	234.4						258.5		210.3		
salbutamol	**237.5**					312.29898	187.5			221.1	
salicyl alcohol	234.2						234.2				
salicylamide	**289.3**				308.2		282.9	264.4	259.6	288.9	306.3
salicylic Acid	**355.3**				327.1		339.6		284.7	347.4	
scopoletin	307.2						307.2				
secbutobarbitone	331.3	350.1					337.6	319.8	317.5		
sigmodal	431.0	434.4								427.5	
SKF-525A	395.7									395.7	
spironolactone	538.7								538.7		
strychnine	**257.2**		258.2	337.4		316.76058	259.5	243.7	247.0	261.2	
sulphacetamide	**240.5**	284.7					225.0				
sulphadiazine	233.6	250.5					234.2		216.1		
sulphadimethoxine	351.8						351.8				
sulphadimidine	**277.1**										
sulphaethidole	314.3						314.3				
sulphaguanidine	**92.3**										
sulphamerazine	247.4						247.4				
sulphamethazine	257.5						257.5				
sulphmethoxizole	**319.5**						322.4				
sulphanilamide	86.1						86.1				

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
sulphapyridine	242.1	253.0					231.1				
sulphasoxizole	332.5						332.5				
sulphinpyrazole	**473.7**										
sulindac	462.3						462.3				
sulpiride	**235.4**					311.07204		193.2	196.8		
sulthiam	275.0								275.0		
suprofen	95.2							74.6	115.7		
talinolol	290.2										290.2
talbutal	370.0	370.0									
tamoxifen	**424.3**						482.6				
tebuconazole	551.2								551.2		
temazepam	**437.6**	435.4	373.8	341.6	390.1	325.79532	439.0	410.7	415.1	436.8	409.1
terbutryn	442.1								442.1		
terfenadine	512.6					479.16282			512.6		
testosterone	**508.0**						540.4				
testosterone acetate	894.4						894.4				
testosterone propionate	1002.9						1002.9				
tetrabromophenolphthalein	823.4						823.4				
tetracaine	321.4		327.8	423.6		343.41864	318.3		333.0		306.3
tetracycline	**264.9**	260.2					255.5				
tetrazepam	482.7							480.9	484.6		
tetroxoprim	241.2								241.2		
d8-tetrahydrocannabinol	1062.7						1062.7				
tetramisole	222.0						222.0				
thebaine	**275.5**						279.8		277.9		266.9
theobromine	200.9		217.0	244.3			222.0	183.4	181.4		
theophylline	**248.9**	243.3	209.9				233.2	199.2	195.8	245.8	
thiabendazole	233.7						229.1		238.3		
thiamine	21.2						21.2				
thiamylal	475.5						475.5				
thioproperazine	**305.3**					358.03038					
thioridazine	**371.3**	371.6	387.9								
thienylcyclohexypiperidine morpholine	279.8						279.8				
thiobarbituric Acid	163.2						163.2				
thiopentone	433.0			345.3				426.5	429.5	449.1	426.7
thioridazine	427.0				503.5	424.50822	419.8	455.2	451.8	381.3	
thiosalicylic Acid	345.7						345.7				
thiothixene	373.8		373.8								
cis-thiothixene	340.7						340.7				
thymol	544.5			389.9			544.5				
tiaprofenic acid	**451.8**					323.11836			423.7		
tilidine	289.8							289.1	290.5		
timolol	256.2			325.7		327.80304		260.5	251.8		
tocainide	207.7			323.6				208.1	207.4		
tolazamide	445.1						445.1				
tolazoline	179.4						179.4				

DRUG NAME	compilation/System 2	System 1	Logan et al comp	Koves & Wells	Cosbey	Traqui et al	Hill & Kind	Bogusz & WU	Bogusz & Erkens	Gill et al	Below & Burrman
tolbutamide	424.2			336.7		318.7683	440.0	413.7	418.9		
tolmetin	434.0			333.1			434.0				
tolnaftate	**866.8**	899.9									
o-toluenesulphonamide	295.0						295.0				
p-toluenesuphonamide	297.0						297.0				
tosylate anion	246.8									246.8	
tramadol	267.3							266.4	268.3		
tranylcypromine	196.2						194.6	196.2	197.8		
trazodone	305.5			348.4	320.8	330.25692			310.8	300.2	
triamcinolone	**311.7**						330.5				
triamterene	262.7			323.4					246.1		279.4
triazolam	390.5		374.931	328.8		317.98752		394.9	401.5		
tribenzylamine	360.9				364.9		360.9				
trichlorfon	737.6								737.6		
trichlormethiazide	340.5						349.8	330.7	329.1	352.6	
trifluoperazine	**344.2**		380.8			495.33612	360.9	439.4	439.2		
m-trifluoromethylbenzoic acid	419.8						419.8				
trifluperidol	306.0					348.43794		308.9	303.0		
triflupromazine	454.5							456.2	452.7		
trifluralin	699.9								699.9		
trihexyphenidyl	381.2						381.2				
trimeprazine	**336.9**	227.5									
trimethoprim	**254.3**	256.6					249.4		298.2		
2,4,6-trimethoxyamphetamine	298.1						298.1				
3,4,5-trimethoxyamphetamine	232.1						232.1				
trimipramine	**344.7**		363.1			383.79612		402.8	400.6		
tripelennamine	264.8		304.2				199.7		290.5		
triprolidine	**270.2**				320.8	334.27236	239.2		312.7		
tropacocaine	282.9						282.9				
tryptamine	190.0				308.2				190.0		
tubocurarine	**257.0**									259.1	
tyramine	77.3						69.9		84.8		
d-valerolactam	195.6						195.6				
vanillylmandelic Acid	192.6						192.6				
verapamil	386.1			404.9		334.71852	402.5	392.9	403.5		345.4
vincamine	312.3						312.3				
viloxazine	273.2					324.79146		271.3	275.0		
vinbarbitone	363.1							365.3	361.0		
vinblastine	354.7	354.7				338.8455					
warfarin	**514.4**	516.2		359.9		332.48772	504.9	490.8	501.0	523.0	
yohimbine	**279.2**					323.7876	281.8		286.6		
zimeldine	270.4									270.4	
zolpidem	290.6					326.68764			296.3		285.0
zomepirac	495.3			346.9658						495.3	
zopiclone	269.2					319.8837			268.3		270.2
zoxazolamine	283.9						283.9				

Appendix VIII

The retention indices of the Barbiturates run on gradient System 1

Barbiturate	Run 1	2	3	4	Mean	Std.dev.	coeff.var.
Barbitone	282.60	282.80	284.20	284.20	283.45	0.9	0.3
Probarbital	310.50	310.50	310.70	310.70	310.60	0.1	0.0
Phenylmethylbarbituric acid	318.50	318.50	316.10	319.00	318.03	1.3	0.4
Allobarbitone	317.90	319.90	320.10	322.00	319.98	1.7	0.5
Aprobarbitone	331.40	331.40	334.00	334.00	332.70	1.5	0.5
Metharbarbitone	332.80	332.80	336.20	336.20	334.50	2.0	0.6
Brallobarbitone	342.30	342.00	345.00	345.00	343.58	1.7	0.5
Phenobarbitone	343.30	343.50	344.10	344.10	343.75	0.4	0.1
Secobutobarbitone	350.70	350.20	351.20	351.20	350.83	0.5	0.1
Ibomal	351.60	351.60	354.40	354.40	353.00	1.6	0.5
Vinbarbitone	354.70	354.70	355.90	355.90	355.30	0.7	0.2
Cyclobarbitone	355.40	355.40	358.20	357.90	356.73	1.5	0.4
Cyclopentobarbitone	354.50	354.50	357.90	355.90	355.70	1.6	0.5
Butobarbitone	356.90	356.90	358.20	358.20	357.55	0.8	0.2
Talbutal	370.30	370.10	371.10	371.10	370.65	0.5	0.1
Butalbarbitone	375.30	363.00	378.30	378.30	373.73	7.3	2.0
Idobutal	374.90	374.90	377.90	377.60	376.33	1.7	0.4
Nealbarbitone	381.00	381.00	383.70	383.90	382.40	1.6	0.4
Heptabarbitone	383.00	383.00	385.70	385.20	384.23	1.4	0.4
Pentobarbitone	383.90	383.90	387.00	387.00	385.45	1.8	0.5
Amylobarbitone	387.10	386.90	389.00	389.30	388.08	1.3	0.3
Hexobarbitone	387.60	387.80	390.40	390.40	389.05	1.6	0.4
Enallypropymal	394.30	394.30	396.60	396.60	395.45	1.3	0.3
Methylphenobarbitone	396.60	396.10	399.10	399.60	397.85	1.8	0.4
Quinalbarbitone	407.50	407.50	407.80	409.20	408.00	0.8	0.2
Sigmodal	433.10	432.60	434.20	435.10	433.75	1.1	0.3
Hexethal	454.50	454.50	458.20	458.20	456.35	2.1	0.5
Methohexitone	480.00	480.40	482.80	482.80	481.50	1.5	0.3

The barbiturate retention indices run on the isocratic system 1 (38% 'B')

Barbiturate	Run 1	2	3	4	Mean	Std.dev.	coeff.var.
Barbitone	63.74	64.29	63.19	63.74	63.74	0.45	0.71
Probarbital	100.72	100.48	100.48	100.72	100.6	0.14	0.14
Phenylmethylbarbituric acid	103.62	103.38	103.62	103.38	103.5	0.14	0.13
Allobarbitone	107.28	107.48	107.48	107	107.31	0.23	0.21
Aprobarbitone	107.28	107.48	107.48	107.48	107.43	0.1	0.09
Metharbarbitone	125.12	125.84	124.88	125.12	125.24	0.42	0.33
Brallobarbitone	105.56	105.31	105.56	106.1	105.63	0.33	0.32
Phenobarbitone	123.91	123.43	123.91	123.43	123.67	0.28	0.22
Secobutobarbitone	127.29	127.05	127.05	127.78	127.29	0.34	0.27
Ibomal	137.44	135.99	135.75	135.5	136.17	0.87	0.64
Vinbarbitone	138.41	137.92	138.41	137.92	138.17	0.28	0.21
Cyclobarbitone	140.58	140.1	140.1	140.82	140.4	0.36	0.26
Cyclopentobarbitone	139.61	140.57	139.13	139.86	139.79	0.6	0.43
Butobarbitone	140.82	140.82	140.58	140.34	140.64	0.23	0.16
Talbutal	159.18	158.94	158.7	159.18	159	0.23	0.15
Butalbarbitone	150.24	151.45	150.24	150.24	150.54	0.6	0.40
Idobutal	158.7	157.73	158.45	157.97	158.21	0.44	0.28
Nealbarbitone	183.09	182.85	181.88	183.82	182.91	0.8	0.44
Heptabarbitone	185.5	185.5	185.26	185.5	185.44	0.12	0.07
Pentobarbitone	188.16	187.43	187.92	188.64	188.04	0.5	0.27
Amylobarbitone	193.71	193.96	193.48	193.24	193.6	0.31	0.16
Hexobarbitone	199.75	199.03	199.03	200.13	199.49	0.55	0.28
Enallypropymal	208.36	207.44	207.17	207.17	207.54	0.56	0.27
Methylphenobarbitone	211.95	211.82	211.69	211.82	211.82	0.11	0.05
Quinalbarbitone	219.65	220.58	219.12	219.65	219.75	0.61	0.28
Sigmodal	250.07	250.73	249.67	249.4	249.97	0.58	0.23
Hexethal	283.8	285.65	284.06	283.67	284.3	0.92	0.32
Methohexitone	340.66	339.95	338.33	338.53	339.37	1.12	0.33

Table used to determine the novel barbiturate substituent retention index values.

Barbiturate ⇓		Substituent value ⇒	81	38	180	-15	53	48	55	-63	29	84	78		
	RI	Δ RI	$-CH_3$	phenyl	$-CH_2$	-CH	$-NH_3$	$=CH_2$	=CH	=C	≡C	C	=CBr	Calc.RI	Observed RI
Barbituric acid	45														45
Barbitone	283.5	237	2	2										283	283.5
Phenobarbitone	344	299	1	1	1									344	344
Probarbitone	311	266	3	1	1									311	311
Butobarbitone	357.5	313	2	4										359	357.5
Secobarbitone	351	306	3	2		1								349	351
Phenylmethylbarbitone	318	273	1		1									306	318
Pentobarbitone	385.5	341	3	3		1								387	385.5
Amylobarbitone	388	343	3	3		1								387	388
Hexethal	456.5	412	2	6										435	456.5
Metharbabarbitone	334.5	290	2	2			1							334	334.5
Methylphenobarbitone	398	353	1	1	1		1							397	398
Nealbarbitone	382.5	338	3	2				1	1			1		383	382.5
Methohexitone	481.5	437	2	2		1	1	1	1		2			482	481.5
Quinalbarbitone	408	363	2	3		1		1	1					409	408
Enallypropamal	395.5	351	2	1		1	1	1	1					386	395.5
Aprobarbitone	332.5	288	2	1		1		1	1					333	332.5
Allobarbitone	320	275		2				2	2					327	320
Iodobutal	376.5	332	1	4				1	1					381	376.5
Vinbarbitone	353	311	3	2					1	1				356	353
Talbutal	370.5	326	2	2		1		1	1					371	370.5
Butalbarbitone	373.5	329	2	2		1		1	1					371	373.5
Sigmodal	434	389	2	3		1		1	1				1	432	434
Brallobarbitone	343.5	309		2				2	1				1	350	343.5
Ibomal	350	308	2	1		1		1					1	346	350
Cyclopentobarbitone	356.5	312		3		1		1	3					357	356.5
Cyclobarbitone	355.5	312	1	5					1	1				351	355.5
Heptabarbitone	384	339	1	6					1	1				389	384
Hexabarbitone	389	344	1	4			1		1	1				366	389

Appendix IX

Published papers

Pharmaceutical Sciences 1995, 1: 593–596
Received November 13, 1995
Accepted December 19, 1995

Correlation of Physicochemical Parameters of 28 Barbiturates to their Retention Properties on an ODS Reversed-phase HPLC System*

R. K. WATERS, R. D. JEE, R. A. WATT AND A. C. MOFFAT

Centre for Pharmaceutical Analysis, The School of Pharmacy, 29–39 Brunswick Square, London WC1N 1AX, UK

Abstract

A group of 28 barbiturates has been run on a novel non-buffered reversed-phase HPLC system under both gradient and isocratic conditions.

The system demonstrated good repeatability with retention values showing coefficients of variation of less than 0·46% for gradient and 0·23% for isocratic runs. Retention values were correlated with a range of structural and physical parameters to test the selective behaviour of the system. Correlation with molecular connectivity values was generally poor as was the correlation with a standard set of GLC retention indices ($r = 0·465$), suggesting different mechanisms of retention. Significant and improved correlations with the barbiturate retention indices were obtained with molar volume ($r = 0·831$) and log P ($r = 0·891$) values, respectively. Excellent correlation between the experimental retention indices and calculated indices, derived by the addition of substituent component values to the barbituric acid core, was obtained.

This excellent correlation demonstrates the internal consistency and regular, predictable behaviour of these compounds, on the HPLC system.

There is a continuing requirement for the rapid and accurate identification of drug substances particularly in generic pharmaceutical formulations and forensic samples. A robust and reproducible automated HPLC system that can identify each of a wide range of drugs is therefore ideally required.

The advantage of using external standards to provide an index scale was investigated by Baker & Ma (1979), who concluded that the correct set of standards can provide a continuous scale that would be reproducible between laboratories. Further work by Bogusz (1991) and Bogusz et al (1988), using the n-nitroalkanes as a set of external standards examined the effect of elution conditions upon this scale. The mobile phase composition was found to have a critical effect on retention indices, and since isocratic and gradient retention indices were not found to be transferable, a one-step gradient elution was recommended for reproducible interlaboratory transfer of HPLC data.

A reversed-phase HPLC system as devised by Gill et al (personal communication), using a modern inert C-18 support, acetonitrile/water as eluents and with an inorganic modifier is described in this paper. An homologous series of nitroalkanes is used to calculate a retention index for each drug, using linear interpolation. Under gradient conditions this system will rapidly elute a range of drugs from ascorbic acid to highly lipophilic compounds such as tetrahydrocannabinol and is therefore potentially useful as a time-efficient universal drug screening technique.

In this paper we report the reproducibility of the retention indices of 28 barbiturates. The extent of the correlations of these indices with given physicochemical parameters is then considered, to give an indication of system predictability.

The barbiturates have been chosen as a model group of compounds to investigate, as they possess a common polar core, functionalized with a wide range of alkyl or other nonpolar side chains which provide a subtle control over their retention characteristics, partition coefficients and biological properties. At the pH used in this system (2·2), the barbiturates are un-ionized and on an inert support phase should give predictable retention characteristics. Additionally, literature data are available for some of these barbiturates, not only for their physicochemical properties but also for their gas liquid-chromatographic behaviour (Stead et al 1982). The retention indices obtained for these barbiturates are then correlated with octanol/water partition coefficients (log P), partial molar volumes and molecular connectivities to demonstrate the system reliability.

Materials and Methods

Materials and the HPLC system

An Inertsil ODS-2 (GI Sciences Inc., Japan) column (250 × 4·6 mm) and eluents A and B were used to run gradient and isocratic systems (eluent A: double deionized water (produced by a MilliQ system), with 0·1% 2·5 M sulphuric acid (Hopkins and Williams, Ultrapure); eluent B: acetonitrile (Hypersolve grade, BDH), with 0·1% 2·5 M sulphuric acid).

The eluents were not filtered nor degassed. The barbiturates and the n-nitroalkanes were supplied by the Forensic Science Service, Aldermaston, UK.

A modular Gilson system controlled by Gilson 712

*Presented at the meeting of the Joint Pharmaceutical Analysis Group on October 19, 1995.

Correspondence: A. C. Moffat, Centre for Pharmaceutical Analysis, The School of Pharmacy, 29–39 Brunswick Square, London WC1N 1AX, UK.

software on an IBM PS-2 personal computer was used. Detection was by a Gilson Holochrome UV detector set at 215 nm.

High pressure mixing of the eluents was used. The programmed gradient used a 10-min equilibration at 2% B continuing for a further 3 min after injection. The percentage of B was then raised linearly to 98% over 23 min and held at 98% for 5 min. At the end of the run the solvent mixture was rapidly altered to the 2% b mixture. For isocratic experiments the system was run at 38% B and 62% A. Approximately 10 mg of each barbiturate was dissolved in 2 mL acetonitrile and 1 mL double deionized water.

A Gilson 401 autosampler and dilutor were used to inject 10 µL sample mixture, then 3 µL double deionized water, followed by 7 µL saturated glycine solution, into a 7010 Rheodyne injection port fitted with a 20-µL loop.

A mixture containing 5 µL of each n-nitroalkane (C_1 to C_{16} warming up where necessary to liquify), made up to 10 mL with acetonitrile was used for the external retention standards. Before running the series of barbiturates, the mixture of nitroalkanes was run and their retention times measured. These were later used to calculate a retention index for the barbiturates using equations 1 and 2 for the gradient and isocratic retention times, respectively:

$$RI = 100[n + ((t_r - t_n)/(t_{n+1} - t_n))] \quad (1)$$

$$RI = 100[n + ((\log t_r - \log t_n)/(\log t_{n+1} - \log t_n))] \quad (2)$$

where RI is the retention index of a barbiturate; n is the number of carbons in the nitroalkane eluting immediately before the barbiturate; t_r is the retention time of the barbiturate; t_n is the retention time of the nitroalkane eluting immediately before the barbiturate; and t_{n+1} is the retention time of the nitroalkane eluting immediately after the barbiturate.

Physicochemical parameters

Three types of molecular connectivity were calculated according to the rules of Kier and Hall as modified by Stead et al (1982). $^1\chi^0$ reflects the number of hydrogen atoms attached to the carbon atoms substituted at the 5, 5 positions on the barbituric acid core. $^1\chi^v$ is based on the difference between carbon valency and the number of hydrogen atoms attached. $^1\chi^v$ includes the above but also accounts for the bond type between substituent carbons.

Additive partial molar volume substituent values were obtained from the literature (Barlow 1980). A partial molar volume of the barbituric acid core was determined from its density (Berking & Craven 1971).

Log P values were obtained using substituent values (Hansch & Leo 1979) and standardized by including the contribution of barbituric acid (Hansch et al 1967). Eighteen values of the electronic parameter σ* for the barbituric acid substituents were obtained from the literature (Hansch & Leo 1979). These values were then summed with the log P values of the corresponding barbiturates to examine the effect of the correlation with the HPLC retention indices.

Novel additive substituent retention index values for the substituents on the 5, 5 position were calculated as follows. Barbituric acid and its retention index was taken as the parent and the differences between the structures and the retention indices of each analogue were taken in turn. From these data and using simultaneous algebraic equations, the individual substituent values were calculated.

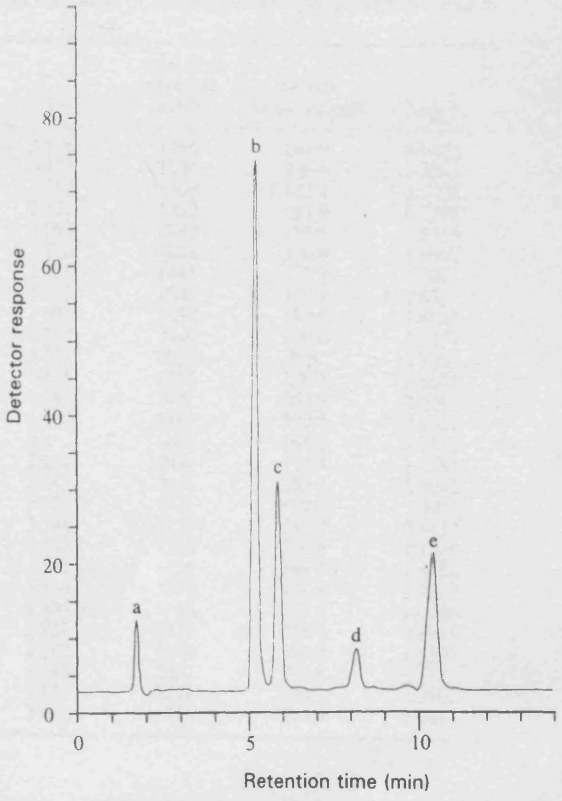


FIG. 1. A typical isocratic chromatogram. a, Glycine; b, methabarbitone; c, cyclobarbitone; d, pentobarbitone; and e, quinalbarbitone.

Results and Discussion

A typical chromatogram of glycine (used as a void volume marker), and four barbiturates run on the isocratic system shows good resolution and peak symmetry (Fig. 1).

Table 1 shows retention indices on the gradient and isocratic systems of the set of barbiturates, together with their gas chromatographic retention indices. The gradient and isocratic indices correlate well ($r = 0{\cdot}976$) but they are not directly comparable since the order of elution and index ranges are different; for example, allobarbitone gives a retention index of 320 retention index units on the gradient system and 123 on the isocratic system.

For the repeated runs of the barbiturates carried out on both gradient and isocratic systems, coefficients of variation of less than 0·46% and 0·23% are seen, respectively. The isocratic data were selected for the correlation with the chosen physicochemical parameters because of their superior repeatability. This was obtained because of fewer equilibration and eluent mixing problems than those given by the gradient system.

Table 2 shows the correlation coefficients given by linear regression between the isocratic data and some physiochemical parameters. Poor correlations are seen with all three molecular connectivity values considered. Partial

Table 1. Observed and calculated HPLC retention indices, GLC retention indices and some of the physicochemical parameters used in the correlations.

Barbiturate	Gradient index (observed)	Gradient (calculated)	Isocratic index (observed)	$^1\chi^v$	GLC retention index	Partial molar volume	Log P
Allobarbitone	320	327	123·2	2·678	1586	161·9	0·9451
Amylobarbitone	388	387	338·3	3·477	1700	206·6	2·2563
Aprobarbitone	332·5	333	212·5	2·783	1600	183·4	1·2756
Barbitone	283·5	283	93·3	2·121	1482	141·9	0·7288
Brallobarbitone	343·5	350	179	3·203	1842	188·1	1·8359
Butalbital	373·5	371	282	3·256	1658	200·0	1·9902
Butobarbitone	357·5	359	263·7	3·121	1645	175·1	1·709
Cyclobarbitone	356·5	351	263·3	3·253	1945	187·7	2·42
Cyclopentobarbitone	356·5	357	262·1	2·693	1858	183·0	1·8945
Enallylpropymal	395·5	386	358·6	2·783	NA	200·0	2·5937
Heptabarbitone	384·3	389	328·6	3·753	2035	NA	2·205
Hexethal	456·5	435	452·7	4·121	1835	208·3	2·6635
Hexobarbitone	389	366	344·5	1·947	NA	202·6	2·611
Ibomal	353	356	255·2	3·307	1866	209·6	2·4461
Idobutal	376·5	381	295·2	3·4	1698	168·5	2·2372
Methabarbitone	334·5	336	232·3	2·121	NA	173·4	1·1385
Methohexitone	481·5	482	483·8	3·743	NA	263·9	2·8043
Methylphenobarbitone	398	397	365·9	5·55	NA	199·9	1·9997
Nealbarbitone	382·5	383	326·5	3·546	1720	230·9	2·4437
Pentobarbitone	385·5	387	332·6	3·542	1733	206·6	2·1266
Phenobarbitone	343·8	344	228·8	3·375	1934	168·4	1·4524
Phenylmethylbarbituric acid	318·3	306	118·9	2·814	1875	151·8	0·9613
Probarbitone	311	311	115·9	2·504	1550	173·4	1·0506
Quinalbarbitone	408·3	409	378·9	3·821	1770	216·6	2·4813
Secbutobarbitone	350·8	349	236·8	3·042	1650	190·0	1·6355
Sigmodal	433·8	432	418·4	4·345	2031	242·8	2·881
Talbutal	370·5	371	296·5	3·321	1704	200	1·9902
Vinbarbitone	355·5	356	259	3·828	1755	214·9	2·0413

NA, Not applicable (data unavailable).

Table 2. The correlation coefficients obtained between the HPLC isocratic retention indices for the barbiturates and some physicochemical parameters.

Physicochemical parameter	Correlation coefficient (r)
Partial molar volume	0·831
Log P	0·891
Log P (for alkyl and allyl substituted barbiturates)	0·949
$^1\chi^0$	0·597
$^1\chi^v$	0·759
$^1\chi^s$	0·601

molar volume appears to have a limited role as a marker in the retention of the barbiturates on the ODS support material.

Lipophilicity as reflected by octanol/water partition coefficients appears to be an important part of the retention mechanism even though calculated rather than observed log P values have been used. The alkyl electronic parameter σ^* has previously been found to contribute to observed log P values (Lamb & Harris 1960). The subtraction of σ^* gives some improvement to the correlation of log P with HPLC retention indices (from $r = 0{\cdot}868$ to 0·878), however in agreement with the findings of Wong & McKeown (1988) its contribution was a small one.

By removing barbiturates containing phenyl, cyclo- and polar functional groups (i.e. bromine-containing) from the correlation between the isocratic data and log P, the correlation coefficient is significantly improved ($r = 0{\cdot}949$). Barbiturates containing polar functional groups show lower log P values than expected from the linear regression with their retention indices. A similar trend between calculated and observed log P values has been shown by Hansch et al (1967).

The retention index substituent values calculated in this work (Table 3), give calculated gradient indices (Table 1)

Table 3. The calculated additive substituent retention index values for the 5, 5 functions on the barbituric acid core.

Substituent	Additive retention index value
$-CH_3$	81
$-NCH_3$ (in the 1 position)	53
$-CH_2$	38
$-CH$	−15
C	−84
$=CH_2$	48
$=CH$	55
=C in a cyclo function	−25
=C	−63
$\equiv C$	29
Phenyl	180

Barbituric acid has a retention index of 103.

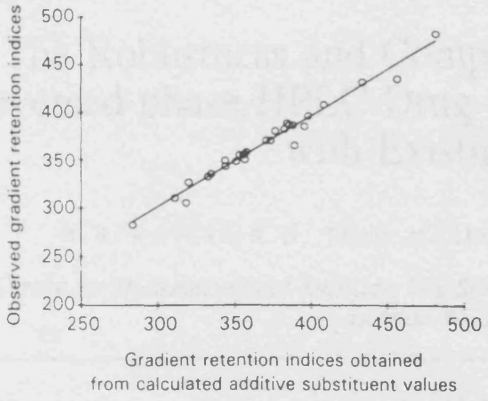


FIG. 2. Linear regression between observed gradient retention indices and indices given by the calculated additive substituent retention index values. $y = 0{\cdot}9513x + 16{\cdot}354$, $r = 0{\cdot}987$.

which compare very well with the observed gradient indices. The linear regression plot ($r = 0{\cdot}987$) between these two indices is shown in Fig. 2. These substituent values demonstrate that the system is behaving very reliably with this group of compounds.

Poor correlation of the HPLC retention data with the GLC data ($r = 0{\cdot}465$) is seen. The GLC data however, do give excellent correlation with the molecular connectivity values, for example with $^{1}\chi^{v}$, where $r = 0{\cdot}997$. This suggests that retention in the HPLC condensed phase is more complex and cannot be simply related to bond arrangements of the analyte as in a simple gas-liquid partition. The poor correlation between these two techniques means that they may be used as independent screening methods.

Acknowledgements

R. Waters thanks the School of Pharmacy for financial support. The authors are grateful to Dr Richard Gill for helpful discussions and to the Forensic Science Service, for supplying them with n-nitroalkanes and barbiturate samples.

References

Baker, J. K., Ma, C. Y. (1979) Retention index scale for liquid-liquid chromatography. J. Chromatogr. 169: 107–115

Barlow, R. B. (1980) Quantitative Aspects of Chemical Pharmacology. Croom Helm, London, pp 80

Berking, B., Craven, B. M. (1971) Crystal structure of sodium 5,5 diethylbarbiturate. Acta. Crystallogr. B 27: 1107–1115

Bogusz, M. (1991) Influence of elution conditions on HPLC retention index values of selected acidic and basic drugs measured in the 1-nitroalkane scale. J. Anal. Toxicol. 15: 174–178

Bogusz, M., Neidi-Fischer, G., Aderjan, R. (1988) Use of corrected retention indices based on 1-nitroalkane and alkyl arylketone scales for HPLC identification of basic drugs. J. Anal. Toxicol. 12: 325–329

Hansch, C., Leo, A. (1979) Substituent Constants for Correlation Analysis in Chemistry and Biology. Wiley Interscience Publication, New York, pp 65–339

Hansch, C., Steward, R. A., Anderson, S. M., Bentley, D. (1967) The parabolic dependence of drug action on lipophilic character as revealed by a study of hypnotics. J. Med. Chem. 11: 1–12

Lamb, D. J., Harris, L. E. (1960) Correlation of distribution coefficients of various barbituric acids. J. Am. Pharm. Assoc. 49: 583–585

Stead, A. H., Gill, R., Evans, A. T., Moffat, A. C. (1982) Predictions of gas chromatographic retention characteristics of barbiturates from molecular structure. J. Chromatogr. 234: 277–283

Wong, O., McKeown, R. H. (1988) Substituent effects on partition coefficients of barbituric acids. J. Pharm. Sci. 77: 926–932

Pharmaceutical Sciences 1997, 3: 419–423
Received April 30, 1997
Accepted May 24, 1997

The Robustness and Comparability of a Novel Rapid Reversed-phase HPLC Drug-screening Method Compared with Existing Systems

R. K. WATERS, R. D. JEE, D. MARLEY, R. A. WATT AND A. C. MOFFAT

Centre for Pharmaceutical Analysis, The School of Pharmacy, 29–39 Brunswick Square, London WC1N 1AX, UK

Abstract

Acidic and basic drugs have been run on a novel non-buffered reversed-phase HPLC system.

Variations in eluent pH, column temperature and eluent composition were used to demonstrate the excellent robustness of the system. The repeatability of the 16 nitro-*n*-alkanes used to assign index values to the retention times of acidic and basic drugs were not affected by the varied applied conditions (mean standard deviation less than 0·1 min). Due to the low operating pH of the system (2·2) the largest variation in retention index was seen to occur with the basic standard drug set, 7 retention index units between the two extremes of pH.

Retention indices of 73 drugs run on different HPLC equipment using an ODS column from a different manufacturer, gave a correlation $r = 0.990$ with the retention indices of the same drugs run on the system used here. A correlation $r = 0.977$ was obtained for 48 common retention indices given by a buffered system published by other workers.

A data set of 168 drugs run on the previous ODS system was used to generate a database of retention indices. These retention indices were obtained from the equations that described the correlations between this data set and that of other workers. For 115 drugs run on the system described here that are also present in this compilation, a correlation $r = 0.945$ was obtained from linear regression between them.

A robust and reproducible automated HPLC system that can identify each of a wide range of drugs with a single gradient run is required for the rapid identification of drug substances in forensic samples and pharmaceutical formulations. In a previous paper such a system was demonstrated using retention predictability studies (Waters et al 1995a, b) and this has now been further developed to give a more reproducible and robust system.

A robust set of retention data is required as the basis of a transferable or universal database. A robust HPLC system is one that is stable to deliberate variations in the operating conditions. The system should not be sensitive to small changes in pH, pumping accuracy or temperature. The use of a retention index system may help to overcome these problems. In a robustness study, Smith et al (1984) used a buffered isocratic reversed-phase HPLC system with alkylaryl ketones as external standards to determine the retention indices of a group of barbiturates. Temperature fluctuations and slight pH variations were found to cause small changes to retention indices (RI) as the un-ionized standards and the ionizable barbiturates had different sensitivities to these factors. It was also shown that, in this buffered system, the retention indices were virtually unchanged when the proportion of methanol was increased from 30 to 50%.

The effect of temperature variations depends on the enthalpy of the solute–stationary phase interaction and a logarithmic relationship has been shown between retention factors and enthalpy (Melander et al 1978). Anomalous temperature effects on retention may sometimes be seen, which are usually associated with poor column efficiency. Variations of retention indices due to temperature are determined by the relative changes in ionization of the standards and the samples.

Correspondence: A. C. Moffat, Centre for Pharmaceutical Analysis, The School of Pharmacy, 29–39 Brunswick Square, London WC1N 1AX, UK.

In this study, the robustness of a non-buffered gradient system to changes in temperature, pH and mobile phase composition is investigated. The effects are monitored using a set of acidic drugs and a set of basic drugs which are indexed against a series of nitro-*n*-alkanes.

In the previous paper (Waters et al 1995a), this non-buffered system was described using a different reversed-phase column and different HPLC equipment. Results generated on that system and those of other workers (Hill & Kind 1994) are compared directly with those given here to give an indication of the comparability of the systems. The data generated using this system are further investigated by comparing them with a compilation of retention indices calculated from literature data (Cosbey 1986; Gill 1986; Logan & Stafford 1990; Bogusz & Wu 1991; Koves & Wells 1992; Bogusz et al 1993; Below & Burrman 1994; Hill & Kind 1994; Gill et al unpublished data) as previously described (Waters et al 1995b).

Materials and Methods

Materials and the HPLC system

Nitro-*n*-alkanes C_1 to C_{16}, were supplied by the Forensic Science Service, Aldermaston, UK. A Waters Symmetry column (250 × 4·6 mm) held at 40 ± 0·5°C was used. The HPLC system consisted of a Waters 616 pump and a Waters 717 autosampler (temperature module set at 25°C). A Waters on-line degasser and a Waters column temperature control module were also used. Detection was by a Waters 996 photodiode array detector, monitoring at 215 nm. The system was controlled by a Waters Millenium Chromatography manager running on a Digital personal computer. Eluents A and B were used to run the gradient: eluent A: water for HPLC, (BDH), with 0·1% of 2·5 M sulphuric acid (Hopkins and Williams, Ultrapure); eluent B: acetonitrile, (Far UV grade, BDH), with 0·1% of 2·5 M sulphuric acid.

Chromatography

The programed gradient began at 2% B continuing for a further 3 min after injection. The percentage of B was then raised linearly to 98% over 23 min and held at 98% for 10 min. At the end of the run the solvent mixture was rapidly altered to 2% B over 2 min and maintained for 8 min to re-equilibrate the column.

Methods

A 50 : 50 acetonitrile/water mixture was prepared. Approximately 8–10 mg of each of the acidic (paracetamol, pentobarbitone, clobazam, indomethacin) and basic (chloroquine, diphenhydramine, meclozine, amiodarone) drugs was dissolved in two separate 10-mL samples of the acetonitrile/water mixture.

A mixture containing 5 μL of each nitroalkane, (C_1 to C_{16}), made up to 10 mL with acetonitrile, was used for the external retention standards (the void volume retention marker glycine was co-injected to give the value of C_0 on the nitroalkane scale). Equation 1 was used to calculate the retention indices of the acidic and basic drugs.

$$RI = 100(n + (t_r - t_n)/(t_{n+1} - t_n)) \qquad (1)$$

where RI = retention index of an acidic/basic drug; n = number of carbons in the nitroalkane eluting immediately before the acidic/basic drug; t_r = retention time of the acidic/basic drug; t_n = retention time of the nitroalkane eluting immediately before the acidic/basic drug; t_{n+1} = retention time of the nitroalkane eluting immediately after the acidic/basic drug. Nitroalkane standards (5 μL) and the acids and bases (each co-injected with 15 μL saturated glycine solution), were run on the gradient three times under the standard conditions given above. All retention times and retention indices were recorded. These were re-recorded after each of the following changes to the method:

The percentage of eluent A (the aqueous component), was reduced by 1% (of the total composition), over the entire gradient.

The percentage of eluent A was raised by 1% over the entire gradient.

The standard sets were run at 5°C above and below the standard column temperature.

The amount of acid added was halved and doubled. The resulting pH of the normal, low-acid and high-acid aqueous eluents was measured using a Phillips 9410 digital pH meter. A total of 115 drugs (acidic, basic and neutral) was run on the above system under normal conditions and RI values measured.

Results and Discussion

Robustness

The means of the retention time shifts from normal conditions for the 16 nitroalkanes are given in Table 1. Using these mean figures, nitroalkane retention appears to be most affected by changes in column temperature. However when individual nitroalkane retention time variations are considered the latter part of the gradient shows greater variations due to changes in eluent composition. The resulting pH of the normal aqueous eluent (1 mL per L 2·5 M sulphuric acid), was 2·35, the low-acid aqueous eluent (0·5 mL per L 2·5 M sulphuric

Table 1. Mean shifts of retention time of the 16 nitroalkane standards and the mean of their standard deviations given by three runs.

Conditions	Retention time shift[a] (min)	Mean of s.d.[b] (min)
Normal	0	0·044
Pump A low (− 1% eluent A)	− 0·26	0·029
Pump A high (+ 1% eluent A)	0·22	0·048
35°C	1·46	0·068
45°C	− 0·58	0·032
High-acid	− 0·03	0·051
Low-acid	− 0·02	0·012

[a]Mean of 16 nitro-*n*-alkanes, [b]for each nitroalkane (n = 3).

acid), was 2·57 and the high-acid aqueous eluent (1 mL per L 2·5 M sulphuric acid), was 2·15. The retention of the nitroalkanes was unaffected by these changes in pH.

The mean standard deviation column represents the means of the standard deviations for the retention times of the nitroalkanes after three runs. In each case this value is below 0·1 min and was not affected by the applied conditions.

The acidic and basic drugs are typical of those that are routinely analysed on this system; they display a range of pK_a values and are therefore ideally suited to this type of robustness testing.

Changes in eluent composition

For the acidic and basic drugs the earliest eluting compounds (paracetamol and chloroquine) showed greater sensitivity to the eluent composition than later eluting compounds (Tables 2 and 3). The change in eluent composition of ± 1% of eluent A was selected as representing a reasonable difference one might expect between two given HPLC systems. Larger variations in eluent composition would be expected to cause greater changes within the basic drugs set as this would alter the enthalpy of the analyte interaction with the stationary phase, thereby altering the extent of ionization.

Changes in temperature

The mean retention time of the nitroalkanes was increased by 1·46 min (the largest shift), when the column temperature was reduced to 35°C. The RI of the bases were affected more by temperature variations than the acidic drugs. This is expected as the extent of ionization will be altered by temperature changes.

Changes in pH

The pH change was not great when the sulphuric acid concentration was halved and doubled as such changes (when using a strong acid) will not greatly affect pH. However the changes in the amount of sulphuric acid added were intended to represent an obvious error an operator might make when preparing the eluents.

The alteration of acid concentration in the eluent showed no marked effect on the retention time of the nitroalkanes. Among the acidic drugs, indomethacin showed a variation of 3·96 RI units between the high and low acid concentrations, (variations of up to 7 RI units are routinely recorded for repeat runs of a given drug under standard conditions). The bases however have ionization states that are susceptible to these small pH changes.

Apart from amiodarone, the changes from normal conditions did not cause RI variations greater than 7 RI units. Amiodarone shows a large RI change, 25 units, between the high and low acid concentrations. The reason for this extreme variation is probably due to it having a pK_a of 5·6 rather than the much higher values of the other bases.

Comparability

Of the 115 drugs run on the present system, 73 had previously been run in this laboratory using a similar system (Waters et al 1995b). The previously used HPLC equipment was a modular Gilson system and the column was an Inertsil ODS-2 (GI Sciences Inc., Japan), with the same dimensions as the Symmetry column. A correlation coefficient of 0·990 (r) was obtained using linear regression between these two data sets. Hill & Kind (1994) determined 469 retention indices on a buffered system pH 2·2; 48 of these drugs were common to those used here and their RI values gave a correlation r = 0·977, following linear regression. These correlations indicate good comparability of the chromatographic system.

The above good correlations indicate that it may be possible to transfer data from one system to another and therefore data on the present system were compared with a previously compiled database. For the 115 drugs run on the system, a correlation coefficient of 0·945 was obtained from the

Table 2. The retention indices of the acidic drugs and the mean of all their standard deviations from three runs.

Conditions	Paracetamol	Pentobarbitone	Clobazam	Indomethacin	Mean s.d.
Normal	240·6	382·6	454·9	587·8	1·20
Pump A low (− 1% eluent A)	236·9	383·6	455·6	587·8	0·57
Pump A high (+ 1% eluent A)	243·0	382·8	454·9	588·0	1·49
35°C	240·5	382·8	454·9	588·0	1·06
45°C	240·0	383·9	456·1	587·7	0·42
High-acid	238·4	382·3	452·1	584·1	2·55
Low-acid	241·4	383·0	454·8	588·0	0·52

Table 3. The retention indices of the basic drugs and the mean of all their standard deviations from three runs.

Conditions	Chloroquine	Diphenhydramine	Meclozine	Amiodarone	Mean s.d.
Normal	242·1	305·0	393·5	466·2	0·75
Pump A low (− 1% eluent A)	240·1	304·4	393·8	466·4	0·65
Pump A high (+ 1% eluent A)	243·3	305·6	393·9	467·9	2·14
35°C	239·8	302·8	395·3	464·9	2·19
45°C	242·3	306·7	395·3	469·9	1·01
High-acid	243·1	309·9	399·0	483·4	1·67
Low-acid	240·1	301·9	391·1	458·8	0·55

linear regression between the compilation (Waters et al 1995b) and these RI values (Figure 1). From Figure 1, there are a number of outliers which do not fall into any particular chemical group and the reason for their different chromatographic behaviour needs further investigation. It is by understanding the mechanisms which cause these drugs to be outliers that will enable true interlaboratory ruggedness to be achieved. However, it does show that good correlation between varying chromatographic systems exists and further opens the way for interlaboratory transferability of systems and data.

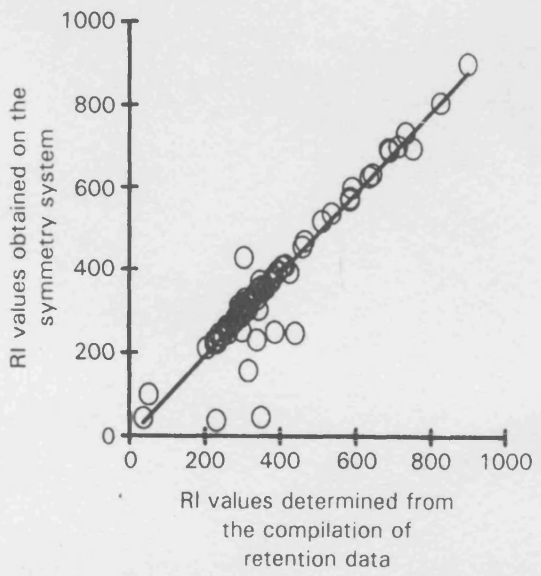


Figure 1. Linear regression between symmetry gradient retention indices and retention indices from the compiled data set. r = 0·945 (n = 115).

Acknowledgements

R. Waters thanks the School of Pharmacy for financial support. The authors are grateful to Dr Richard Gill and the Forensic Science Service, UK for supplying them with the standards and to Waters Chromatography for supporting this work.

References

Below, E., Burrman, J. (1994) Application of HPLC equipment with rapid scan detection for the identification of drugs in toxicological analysis. Liq. Chrom. 17: 4134–4144

Bogusz, M., Wu, M. (1991) Standardised HPLC/DAD system based on retention indices and spectral library, applicable for systematic toxicological screening. J. Anal. Tox. 12: 325–329

Bogusz, M., Erkens, M., Maier, R. D., Franke, J. P., Wijsbeek, J., Zeeuw, R. A. D. (1993) Interlaboratory applicability of a retention index library of drugs for screening by reversed-phase HPLC in systematic toxicological analysis. J. Liq. Chrom. 16: 1341–1354

Cosbey, S. H. (1986) Drugs and the impaired driver in northern Ireland: an analytical survey. Forensic Sci. Int. 32: 245–258

Gill, R. (1986) High pressure liquid chromatography. In: Moffat, A. C. (ed.) Clarke's Isolation and Identification of Drugs. 2nd edn, Pharmaceutical Press, London, pp 201–220

Hill, D., Kind, A. J. (1994) Reversed-phase solvent-gradient HPLC retention indexes of drugs. J. Anal. Tox. 18: 233–242

Koves, E. M., Wells, J. (1992) Evaluation of a photodiode array/HPLC-based system for the detection and quantitation of basic drugs in postmortem blood. J. Forensic Sci. 37: 42–60

Logan, B. K., Stafford, D. T. (1990) Rapid screening for 100 basic drugs and metabolites in urine using cation exchange solid-phase extraction and high performance liquid chromatography with diode array detection J. Anal. Tox. 14: 154–159

Melander, W. R., Campbell, D. E., Horváth, C. (1978) Enthalpy-entropy compensation in reversed-phase chromatography J. Chromatogr. 158: 215–225

Smith, R. M., Hurdley, T. G., Gill, R., Moffat, A. C. (1984) The application of retention indices using the alkylarylketone scale to the separation of the barbiturates by HPLC. I. The effect of eluent. Chromatographia 19: 401–406

Waters, R. K., Jee, R. D., Watt, R. A., Moffat, A. C. (1995a) Correlation of physicochemical parameters of 28 barbiturates to their retention properties on an ODS reversed-phase HPLC system. Pharm. Sci. 1: 593–596

Waters, R. K., Jee, R. D., Watt, R. A., Moffat, A. C. (1995b) The interlaboratory transfer of HPLC data. Proceedings of the 55th International Congress of FIP Stockholm pp 195