EVALUATION AND APPLICATION OF BEST PRACTICE IN ANALYTICAL METHOD VALIDATION

GHULAM SHABIR

A thesis submitted in partial fulfilment of the
requirements of the University of Sunderland for the degree of
Doctor of Philosophy

April 2008

DEDICATION

I would like to dedicate this thesis in memory of my affectionate mother Nawab Bibi Arain (1919 – 2000) and in memory of my lovely wife Mumtaz (1968 – 2008). My mother was a very hard worker, very gracious, very strong and a great person.

Mumtaz was a loving wife and a caring mother to her five wonderful children, aged 2 – 15. She was a very courageous, brave and strong person and an inspiration to everyone who had met her.

ABSTRACT

The coherent body of research described in the existing published work is concerned with new assay method development and validation using novel systematic approaches for pharmaceutical and diagnostic compounds.

The first stage of the research was to study how analytical method development and validation are typically carried out at present and to formulate this into a simple step-by-step approach. Such a template and protocol was not only used as the foundation of this research programme but could also serve as a simple systematic guide for other practitioners and those new to the field. Furthermore, it was recognised that this protocol should satisfy the requirements of the most strategically important regulatory agencies.

The second stage of this research involved evaluation and application of the above validation approach to new methods that were developed for a diverse range of analytes and samples. A new purity assay for 1,10-phenanthroline-5,6-dione and 4,7-phenanthroline-5,6-dione using high-performance liquid chromatography (HPLC) was developed and validated. Impurities in these compounds were identified by liquid chromatography-mass spectrometry (LC-MS). Best practice in method development and validation is equally important in the analysis of both active components and excipients in formulated products. In the first case, a liquid chromatography assay method for determining the content of 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide in a gel formulation was developed and validated. In the second case, the individual contents of three *p*-hydroxy benzoic acid ester preservatives in a complex multi-component sample were determined following the development and validation of a liquid chromatography method.

Finally, the validation approach was evaluated as applied to another analytical technique. Here, gas chromatography (GC) successfully used to develop a novel assay for *p*-cymene in tea tree oil formulations presented different analytical problems because of the very complex nature of this natural product. Stability study information to increase the shelf life of the product and validation data for the analytical method for *p*-cymene content was critically evaluated.

In essence, the critical review of the requirements for method validation for various agencies and the subsequent preparation of guidelines on how to go about method validation have had a significant impact on how analytical practitioners worldwide go about method development and, more importantly, method validation. Further it was possible to apply these guidelines to conduct a series of effective, successful method validation for assays involving a range of typical pharmaceutical samples.

ACKNOWLEDGEMENTS

Firstly, I would like to thank Dr. W John Lough (School of Health, Natural and Social Sciences, University of Sunderland) for his support and advice throughout the preparation of this submission.

It is my great pleasure to thank Sunderland University Research Committee: namely Professor J R Brown (Pro-vice Chancellor), Professor Paul Groundwater and Professor Fridrun Podczeck for reviewing my initial application to confirm that there was a *prima facie* case for my publications, thus providing the basis for developing a justification for the degree of Doctor of Philosophy.

Thank you Dr. Nigel Forrow for always being there to help out when something was needed and for being such great person.

None of this would have been possible without the love and support of my family. A special thanks to my father, Abdul Aziz Arain and my mother Nawab Bibi Arain (1919-2000) for all the end-less love and support throughout my life. I thank you so much. Thanks to my sisters, Muneeran, Hamida and Nasreen for always giving me the ambition to do better and to try and keep up with you. Thanks to my brothers, Abdul Rashid and Abdul Hamid for their encouragement. Thanks to Saghir Ahmid for providing me with a laptop for writing this thesis.

Lastly, I wish to express my sincere gratitude to my loving wife Mumtaz (1968 – 2008) and my daughters, Sara, Fatima and Samra for their patience, moral support and encouragement during this work. To Eyssa and Zaid, my sons, for your unconditional love, playfulness and hugs and kisses when they are needed most.

TABLE OF CONTENTS

AUTHOR'S NOTE

Throughout this thesis, references to papers by other authors are presented by name and year, as are references to work published by the author which are not offered in specific support of this thesis.

References to publications by the author, offered in support of this thesis, are referred to as [1-9], in order of presentation and printouts are given in Appendix I.

The research described in the submitted papers was solely conducted by the author. Where others have made contributions, this is clearly indicated in Appendix II. I also certify that this work has not been accepted in substance for any degree and is not concurrently submitted for any degree other than that of Doctor of Philosophy at the University of Sunderland.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABREVIATIONS

APCI	atmospheric pressure chemical ionisation
API	atmospheric pressure ionisation
BP	British Pharmacopoeia
CGMP	Current Good Manufacturing Practice
cm	centimetres
°C	degree celsius
DAD	diode-array detector
ESI	electro spray ionisation
EU	European Union
EP	European Pharmacopoeia
FDA	Food and Drug Administration
FID	flame ionisation detector
GC	gas chromatography
GLP	Good Laboratory Practice
HPLC	high-performance liquid chromatography
ICH	International Conference on Harmonisation
ISO	International Organisation for Standardisation
LC	liquid chromatography
MHW	Ministry of Health & Welfare Japan
MS	mass spectrometry
m/z	mass-to-charge ratio
mg	milligram
mL	millilitre
λ_{max}	wavelength of band maxima
PDA	photodiode array detection
PDG	Pharmacopoeial Discussion Group
TLC	thin-layer chromatography
USP	United Estates Pharmacopoeia
UV	ultra-violet

1 Introduction

Analytical method development and validation is an important part of analytical chemistry and plays a major role in the discovery, development, and manufacture of pharmaceuticals. The official test methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency and performance of drug product 'quality' essential for drug safety and efficacy.

In the diagnostics and biotechnology industries and even more so in the pharmaceutical industry, a current major issue is the high cost of research in introduction of new drugs. In essence it takes several hundred million dollars to discover, develop and gain regulatory approval. One of the reasons research and development (R&D) is so costly in pharmaceuticals is that most new drug candidates fail to reach the market. Failure can result from toxicity, carcinogenicity, manufacturing difficulties, inadequate efficacy and analytical problems. Therefore there is a need for high throughput in order to maximise patent lifetime and consequently generate the profits to support the research and to increase the speed with which products can be delivered to the market. All the different stages of pharmaceutical R&D is underpinned by analysis so that high throughput is acutely dependent on effective and efficient analysis within which simple effective method development and comprehensive analytical method validation is of fundamental importance (Shabir *et al.,* 2007a).

A wide variety of materials are used in the pharmaceutical and diagnostic industries. All of these materials must be analysed in some way or other and, just as importantly, the method of analysis must be validated i.e. it must be shown that the method is fit for its intended purpose.

In the pharmaceutical field, method validation is very much a major issue as analysis is used primarily to control drug quality. This is important in its own right and also in that drug safety and efficacy are dependent on it. Different chemical entities with varying chemical and physical properties are used. These may include starting materials, intermediates, final drug substances and the final

formulated pharmaceutical products. The pharmaceutical analyst will be concerned with applying analytical methods to the determination of stability/shelf life, purity, side-product identity, dissolution etc. Here, the analyst is required to develop new methods of analysis appropriate to the information required. In many cases, the analyte may be known but is present in a new sample matrix such that a new sample preparation method is needed. The knowledge gained in the method R&D phase is important when it comes to validating the research data efficiently. This is an important coherent theme that runs throughout the publications on which this thesis is based.

Frequently, high-performance liquid chromatography (HPLC) is the analytical method of choice in pharmaceutical analysis [3, appendix I] because of its specificity (i.e. all the components of a sample are separated from one another before the measurement is made so that its results arise from the analyte and from nothing else). Although HPLC is a relatively mature technique, the analyst is continually required to innovate by adapting current methodology or indeed developing completely new protocols. For example, the coupling of HPLC with another technique such as mass spectrometry (MS) can be an especially powerful tool [5, appendix I].

In the diagnostic field, the variety of materials is further expanded due to the complexity of medical devices and their corresponding reagents. Such materials may include polymers, surfactants, enzymes, cofactors, mediators, stabilisers etc. The diagnostic analyst is therefore required to apply other techniques apart from HPLC in the analysis of key materials. An in-depth knowledge of the materials and their critical properties as applied to their use in the diagnostic device is necessary. Innovation is again needed if there is no directly applicable methodology reported in the literature. Once an analytical method is developed, validation is conducted in order to prove its results of the research are valid and its use for the intended application.

Method validation is a critical step for any product release for marketing authorisation. The literature contains diverse approaches to performing method validation (Crowther, 2001; Wilson, 1990; Clarke, 1994; Bressolle et *al.,* 1996;

Carr & Wahlich 1990; Green, 1996; Trullols *et al.,* 2004; Ermer, 2001; Daraghmeh, 2001; Badea *et al.,* 2004; Mendez *et al.,* 2003).

Many analytical methods appearing in the literature have not been through a thorough validation exercise and thus should be treated with caution until full validation has been carried out. Also there is no method validation reported with GMP/GLP considerations. Validation of a new method is a costly and very time-consuming exercise. However the result of not carrying out method validation could result in litigation, failure to get product approval, costly repeat analysis and loss of business and market share (Harvey *et al.,* 2002).

Currently, there is no completely worldwide single source or final guideline on method validation [4, appendix I] that helps analysts to perform validation in a systematic manner and most importantly under GMP/GLP considerations. Therefore industry depends on the analyst's knowledge and experience to develop simple and efficient methods of analysis.

The other major problem pharmaceutical industries are facing in today's world is that different validation data requirements are required for regulatory submissions for medicinal products' registration/approval depending upon the location of the regulatory body. For example the release of any medicinal product in USA, Europe and Japan would require the use of International Conference on Harmonisation (ICH) method validation criteria. (ICH, 2005). However, the release of the very same product, by the same industry, in any other part of the world would force the use of their local regulatory guidelines. This inevitably becomes a costly process due to issues of documentation and personnel training etc. Therefore, efforts are underway to streamline the method validation process through an idea commonly referred to as Harmonisation by ICH.

The birth of ICH took place at a meeting in April 1990, hosted by the European Federation of Pharmaceutical Industries and Associations (EFPIA) in Brussels. Representatives of the regulatory agencies and industry associations of Europe, Japan and the USA met, primarily, to plan an international conference but the meeting also discussed the wider implications and terms of reference of ICH.

The ICH Steering Committee, which was established at that meeting, has since met at least twice a year, with the location rotating between the three regions.

ICH is a joint initiative involving both regulators and industry as equal partners in the scientific and technical discussions of the testing procedures which are required to ensure and assess the safety, quality and efficacy of medicines. The focus of ICH has been on the technical requirements for medicinal products containing new drugs. The vast majority of those new drugs and medicines are developed in Western Europe, Japan and the United States of America and therefore, when ICH was established, it was agreed that its scope would be confined to registration in those three regions.

ICH is comprised of six parties that are directly involved, as well as three observers and the International Federation of Pharmaceutical Manufacturers & Associations (IFPMA). The six parties are the founder members of ICH that represent the regulatory bodies and the research-based industry in the European Union, Japan and the USA. These parties include the European Union (EU), European Federation of Pharmaceutical Industries and Associations (EFPIA), The Ministry of Health, Labour and Welfare, Japan (MHLW), Japan Pharmaceutical Manufacturers Association (JPMA), US Food and Drug Administration (FDA) and Pharmaceutical Research and Manufacturers of America (PhRMA).

The European Commission represents the 27 members of the EU. The Commission works through harmonisation of legislation and technical requirements and procedures, to achieve a single market in pharmaceuticals to allow free movement of products throughout the EU. The European Medicines Agency (EMEA) has been established by the Commission and is situated in London.

EFPIA is situated in Brussels and has, as its members, 29 national pharmaceutical industry associations and 45 leading pharmaceutical companies involved in the research, development and manufacturing of medicinal products in Europe for human use. Much of the Federation's work is concerned with the activities of the European Commission and the EMEA.

MHLW has responsibilities for approval and administration of drugs, medical devices and cosmetics in Japan. Technical and scientific support for ICH activities are provided by the Pharmaceuticals and Medical Devices Agency (PMDA) (which was established in April 2004 as a new administrative agency for scientific review for drug approval), and by the National Institute of Health Sciences (NIHS) and other experts from academia.

JPMA represents 75 members (including 20 foreign affiliates) and 14 committees. Membership includes all the major research-based pharmaceutical manufacturers in Japan. ICH work is coordinated through specialised committees of industry experts who also participate in the Expert Working Groups.

The FDA has a wide range of responsibilities for drugs, biological, medical devices, cosmetics and radiological products. The largest of the world's drug regulatory agencies FDA is responsible for the approval of all drug products used in the USA. The FDA consists of administrative, scientific and regulatory staffs organised under the Office of the Commissioner and have several Centres with responsibility for the various products that are regulated. Technical advice and experts for ICH work are drawn from the Centre for Drug Evaluation and Research (CDER) and the Centre for Biologics Evaluation and Research (CBER).

PhRMA represents the research-based industry in the USA. The Association has 67 companies in membership, which are involved in the discovery, development and manufacture of prescription medicines. PhRMA, which was previously known as the US Pharmaceutical Manufacturers Association (PMA), coordinates its technical input to ICH through its Scientific and Regulatory Section. Special committees have been set up, of experts from PhRMA companies, to deal with ICH topics.

Since ICH was initiated, in 1990, there have been observers to act as a link with non-ICH countries and regions. The ICH observers are: the World Health Organisation (WHO); the European Free Trade Association (EFTA), currently represented at ICH by Swissmedic Switzerland and Health Canada.

IFPMA is a non-profit, non-governmental organization representing national industry associations and companies from both developed and developing countries. Member companies of the IFPMA are research-based pharmaceutical, biotech and vaccine companies. IFPMA has been closely associated with ICH, since its inception to ensure contact with the research-based industry, outside the ICH Regions. IFPMA provides the ICH Secretariat.

ICH is administered by the ICH Steering Committee that is supported by the ICH Secretariat. The ICH Steering Committee was established in April 1990, when ICH was initiated. The Steering Committee, working with the ICH Terms of Reference, determines the policies and procedures for ICH, selects topics for harmonisation and monitors the progress of harmonisation initiatives. The Steering Committee meets at least twice a year with the location rotating between the three regions. The Secretariat operates from the IFPMA offices, in Geneva, and is primarily concerned with preparations for, and documentation of, meetings of the Steering Committee as well as coordination of preparations for Working Group and Discussion Group meetings.

The first guideline on method validation (Q2: Text on Validation of Analytical Procedures) was approved by the Steering Committee under ICH *Step 2* and release for public consultation in October 1993. This guideline was then finalised as Q2A and approved under ICH *Step 4* and recommended for adoption to the three ICH regulatory bodies in October 1994. The second guideline on method validation Q2B (Validation of Analytical Procedures: Methodology) was developed to complement the Parent Guideline and approved by the Steering Committee under *Step 2* and release for public consultation in November 1995. Q2B was approval by the Steering Committee under *Step 4* and recommended for adoption to the three ICH regulatory bodies in November 1996. In November 2005, the parent guidelines Q2A and Q2B were renamed Q2(R1). The new title is “Validation of Analytical Procedures: Text and Methodology”.

Method validation is the proof needed to ensure that an analytical method can produce results that are valid, reliable, reproducible and are fit for the purpose intended. Choosing the validation criteria depends on the method type [see paper 4]. In general, method validation parameters that should be studied are

linearity, range, accuracy, precision (repeatability and intermediate precision), specificity, limit of detection and limit of quantitation. The detailed explanation of these parameters is published in ICH Q2(R1) and in papers 2 and 4 (appendix I) including a step-by-step approach. Here only brief definitions are given.
Accuracy: Closeness of agreement between the value obtained by the method and the true value. Precision: Expresses the closeness of agreement between a series of measurements obtained from multiple sampling. Precision is often expressed as the relative standard deviation of replicate measurements. Specificity: The ability to measure the analyte in the presence of components, which we expect to be present in the sample matrix. Limit of detection: The lowest amount of analyte in a sample that can be detected, but not necessarily quantitated. Usually the lowest limit is evaluated as the signal-to-noise (s/n) ratio that is equivalent to 3 times the standard deviation of the noise (s/n = 3σ). Limit of Quantitation: The lowest amount of analyte in a sample that can be quantitated with suitable precision and accuracy. Usually the quantitation limit is evaluated as the s/n ratio that is equivalent to 10 times the standard deviation of the noise (s/n = 10σ). Linearity: The range of concentrations of analyte for which the procedure provides a test result that is in direct correlation to the amount of analyte in the sample.

The outcome of ICH efforts has been accepted by most regulatory agencies and pharmacopoeias such as U.S. Food and Drug Administration (FDA) (FDA, 1994, 1987), and USP (USP, 2007).

The USP established in 1820, contains legally recognised standards of identity, strength, quality, purity, packaging and labelling for drug substances, dosage forms and other therapeutic products including nutritional and dietary supplements. USP also contains monographs, which are recognised worldwide and may be enforceable by the US FDA and also by state agencies in the US.

The ICH guidelines achieved a great deal in harmonising the definitions of the required validation characteristics and their basic requirements. However, they provide only a basis for a general discussion of the validation parameters, their calculation and interpretation. However, this has not removed the confusion in industries because ICH, as yet, has not explained various other method types

such as response test (to detect a specific substance in a sample as indicated by test signal response), concentration test (for quantitation of a specific substance in a sample), physical test (for determination of the physical characteristics of a product or material) and cleaning test (for evaluating the cleanliness of equipment and areas used for manufacturing). Also ICH has not explained step-by-step approaches and most importantly GMP/GLP considerations that required during method validation to meet regulatory requirements. This impacts regulatory submissions (Shabir *et al.,* 2007a).

The strategy described in the papers on which this thesis is based, was to address some specific objectives i.e. (i) to critically evaluate current practices in method development and validation in order to identify best practices, (ii) to apply best practices with some improvements, in such a way as to ensure good quality and provide new knowledge on a wide range of pharmaceutical substances, products and compounds used in pharmaceuticals, diagnostics and, finally, (iii) to draw upon the outcomes of the programme to be able to recommend the way forward with respect to ensuring that the ever-evolving approaches to analytical method development and validation were enhanced, simple, systematic, efficient and effective while still being compliant with the requirements of regulatory agencies.

1.1 Critical evaluation of current best practice in analytical method development and validation

The first stage of the programme was to study how analytical method development and validation is typically carried out at present [1, 3, appendix I] and to formulate this into a simple step-by-step approach. Such a systematic protocol template [2, appendix I] was not only used as the foundation of this research programme but could also serve as a simple systematic guide for other practitioners and those new to the field. Furthermore, it was recognised that this protocol should satisfy the requirements of the most strategically important regulatory agencies. These requirements were critically evaluated, identifying the key similarities and, more importantly, differences between the validation requirements of the FDA, USP and ICH [4, appendix I]. The aim of the field was

to take forward to apply the identified best practices and to studies of a diverse range of analytes and complicated samples.

Everyday many analysts face the need and challenge to develop and validate HPLC, LC-MS and GC methods. Whereas individuals' approaches may exhibit considerable diversity, a best practice method development and validation follows the systematic approach (Figure 1.1) (Shabir *et al.,* 2007a). This is a highly successful approach to a method development and validation process. Before embarking on the development of a new method, the literature should always be searched to see if a suitable method already exists. If a suitable one is found, it will still be necessary to perform some method optimisation and validation to prove that the method can be successfully adapted for its intended use.

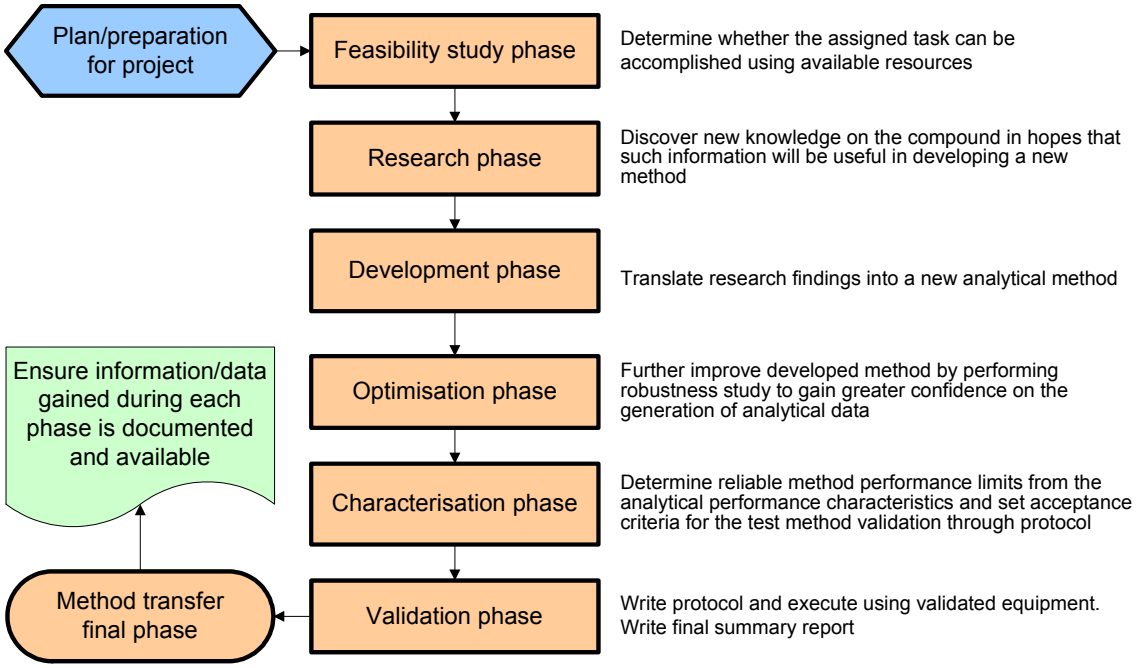


Figure 1.1 - Systematic approach in analytical method development and validation (Shabir *et al.,* 2007a).

In the feasibility study, the analyst will determine whether the assigned task can be successfully accomplished by using available resources. Research is defined as the activity aimed at discovering new knowledge on the compound in hopes that such information will be useful in developing a new method. Development is the translation of research findings into a new analytical method and the systematic use of knowledge or understanding gained from research directed toward the analytical methods, including the design and development of prototypes and processes. Robustness studies must be considered in this phase. Robustness: Measure of a method's capacity to remain unaffected by small but deliberate variations in method parameters [4, appendix I].

The development phase must also include system suitability testing and stability of analytical solutions that of the mobile phase. In the optimisation phase, the developed method can be further improved to gain greater confidence on the generation of analytical data. The search for the best solution amongst alternatives is the extreme value of variables. The current developed approach emphasises the allocation of greater resources during the development and optimisation phases. This allows the analyst to have more confidence on the quality of data generated and therefore considerably reduces the resources that are required for the process of validation (Shabir *et al.,* 2007a).

The purpose of the characterisation study is to determine reliable method performance limits from the analytical performance characteristics and set acceptance criteria for the test method validation. As a best practice, the characterisation protocol needs to be written and approved before execution. Prior to execution of the protocol, it is necessary that the analytical system itself is adequately designed, maintained, calibrated and validated. In all cases proper validation documentation should be archived to support the qualification process. All personnel involved in the characterisation protocol activities must be trained prior to performing their function. On completion of the characterisation study, the results/data should be critically assessed from a statistical point of view.

Validation is the last and critical step for the success of the whole method development project. If the validation fails, it can be seen as a wasted resource and inevitably can delay the product release date. Here, the validation protocol

needs to be written and approved by an appropriate cross-functional team. Upon successful completion of the validation, the data should be statistically analysed against its acceptance criteria by appropriate experts in order to test its validity. Timely implementation / method transfer plays an important role in expediting drug candidates through development stages. Method transfer is not a trivial task and requires careful planning and constant communication between the laboratory personnel involved in the transfer. Method transfer could occur within the same organisation or between pharmaceutical companies and analytical service providers. To have a successful transfer, the analytical method itself must be robust and the equipment differences between the delivering and receiving parties should be carefully evaluated. Unfortunately very limited information on method transfer can be found in the literature. Typically in any organisation, before the method transfer, scientists from both laboratories (R&D: method developer and quality control: end user) need to go through the method details very carefully. As a best practice in order to achieve successful transfer of the analytical method, a method transfer validation protocol should be prepared that is agreed by both sites. The approved method may then be validated (Shabir *et al.,* 2007a).

The second stage of the overall research programme involved evaluation and application of the above validation approach to methods that were developed for a diverse range of analytes and complex samples using analytical techniques such as HPLC, LC-MS and GC [5-9, appendix I]. The results / data generated from new validated assay methods were submitted to regulatory agencies for a pharmaceutical product licence for marketing authorisation. The results were critically reviewed by the regulatory agencies for its reliability, validity and approved for product licence. These validated new assay methods have been implemented in high compliance quality control laboratories for routine analysis of raw materials, bulks, intermediates and final products release.

2 Materials and Methods

The experimental techniques and materials details are published in the author's papers [5-9, appendix I]. Here only a brief general description of analytical techniques such as, reversed-phase HPLC, LC-MS and GC used in this research is given.

A modern HPLC system is shown systematically in Figure 2.1. The equipment consists of a high-pressure solvent delivery system, a sample auto injector, a separation column, a detector (often an UV or a DAD) a computer to control the system and display results. Many systems include an oven for temperature control of the column and a pre-column that protects the analytical column from impurities. The actual separation takes place in the column, which is packed with chemically modified 3.5-10 μm (often silica) particles. A mobile phase is pumped through the column with the high-pressure pump and the analytes in the injected sample are separated depending on their degree of interaction with the particles. A proper choice of stationary and mobile phase is essential to reach a desired separation.

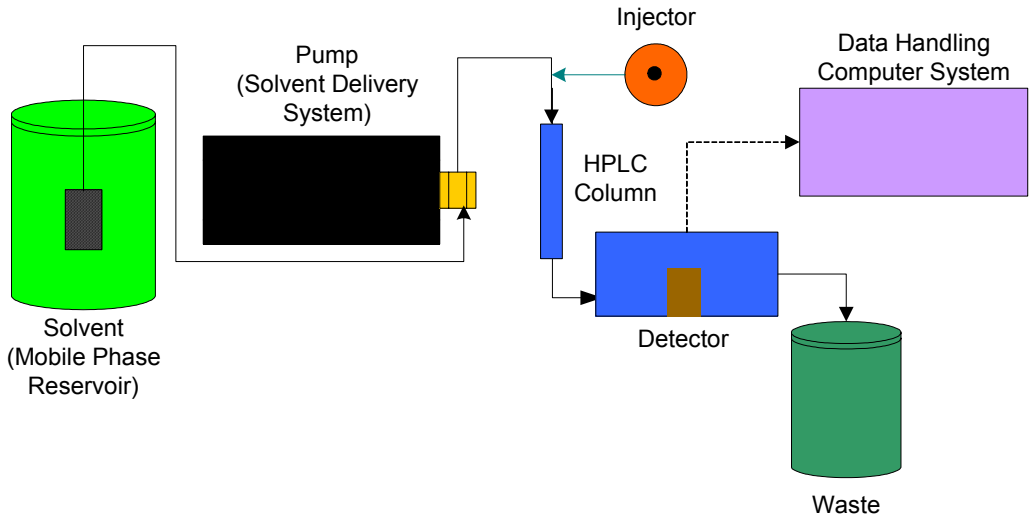


Figure 2.1 - Block diagram of a general LC system.

Reversed-phase chromatography is probably the most commonly used separation mechanism in liquid chromatography and consists of a non-polar stationary phase (normally octadecyl, C_{18} or octyl C_8 chains) bonded to a solid support that is generally micro particulate silica gel. Silica has a small pH range (3-8) where mixtures can be separated without degradation of the column performance. The mobile phase is polar and, therefore, the sample compounds are partitioned between the mobile and the stationary phases. The separation is normally performed using aqueous mobile phase containing different percentages of organic modifiers (e.g. methanol, ethanol, acetonitrile, or tetrahydrofuran) to increase the selectivity between species. Solute retention is also influenced by eluent pH, which affects the dissociation level of the analyte and therefore, its partition between the mobile and stationary phases (Shabir *et al.,* 2007a).

Mass spectrometry has progressed extremely rapidly during the last decade: production, separation and detection of ions, data acquisition, data reduction, etc. and this has led to the development of entirely new modern instruments and applications (Niessen, 2003, Shabir *et al.,* 2007a).

The combination of chromatographic separations with mass spectrometric detection is considered an indispensable tool for problem solving in analytical chemistry and increasingly for routine analytical methods. Mass spectrometric detection brings an added level of information, complementary to the chromatographic process that improves the certainty of identification and the specificity of detection. Mass spectral information can generally be obtained from sample sizes typical of common analytical methods. In the last ten years, research efforts in the field of LC-MS have changed considerably. LC-MS has rapidly matured to become a very powerful and useful analytical tool that is widely applied in many areas of chemistry, pharmaceutical sciences and biochemistry. Investigation into the coupling of HPLC and MS began in the early 1970s. In the first 20 years, most of the attention had to be given to solving interface problems and building new technology. However, most scientists with LC-MS today are only concerned with application of the commercially available techniques in their field of interest. Technological problems in interfacing appear to be solved, and from the wide variety of interfaces developed over the years basically only two dominate, i.e. electrospray ionisation (ESI) and atmospheric-

pressure chemical ionisation (APCI), which are both atmospheric-pressure ionisation (API) techniques (Shabir *et al.,* 2007a). With LC-MS, ESI and APCI has been implemented in analytical strategies in many application areas, e.g. environmental analysis, drug development within the pharmaceutical industry, characterisation of natural products and the characterisation of biomolecules like peptides, proteins, oligosaccharides etc.

The selection of the appropriate HPLC conditions, whether reversed-phase liquid chromatography, ion-pairing chromatography, capillary electrophoresis or ion chromatography, and ionisation mode, ESI or APCI, depends upon the polarity of the analyte. ESI is best applied to the highly polar nature of the analyte and APCI ionises most efficiently compounds with low to moderately high polarities and in this respect is complementary to electrospray, which gives the best sensitivity for ionic compounds. Both interfaces ESI and APCI can be operated in positive and negative ion mode. Often, an appropriate selection for a given analyte can be made by considering that ESI transfers ions from solution into the gas phase, whereas APCI ionizes in the gas phase. As a rule of thumb, analytes occurring, as ions in solution may be best analysed by ESI, while non-ionic analytes may be well suited for APCI.

As for the detection principles discussed above, all of these contribute significantly to the present-day success of hyphenation in HPLC. There is no doubt that, also today HPLC-photodiode-array (PDA) UV plays an important role (detection and peak-purity) in many research and development studies, and for a wide variety of routine analyses.

Since the first description of gas-liquid chromatography in 1952, (James & Martin, 1952), gas chromatography (GC), besides its own technical development and the development of separation methods as a scientific discipline has been used to solve a large number of significant problems in various branches of science.

GC has found an impressive number of industrial applications and the potential to be a powerful tool in routine analytical laboratories by increasing sample throughput and improving laboratory efficiency. Essential oil samples are

amongst the most complex samples known to analytical chemists. They contain a very large number of saturated and unsaturated alkanes, cyclic alkanes, aromatics and heteroatom-containing compounds. Therefore, GC technique/methods are very useful in the determination of essential oils, and offer a significant improvement in sensitivity over previous methods used for essential oils.

2.1 Chromatographic system suitability parameter

The quality of HPLC data collected begins with a well-behaved chromatographic system. System suitability specifications and tests are parameters that provide assistance in achieving this purpose. This section explains the terms used in HPLC assay development and method validation in this research.

Capacity factor (*k´*): The capacity factor (occasionally called retention factor) is a measure of where the peak of interest is located with respect to the void volume, i.e., elution time of the non-retained components. The peak should be well resolved from other peaks and the void volume. Generally the value of *k* is > 2 [4, appendix I], (Shabir & Arain, 2007b).

$$K' = (t_R - t_0) / t_0 \qquad (1)$$

where t_0 is elution time of the void volume of non-retained components and t_R is the retention time of the analyte.

Resolution (R_s): R_s is a measure of how well two peaks are separated. For reliable quantitation, well-separated peaks are essential for quantitation. This is a very useful parameter if potential interference peaks may be of concern. The potential peak eluting closest to the main analyte peak of interest should be selected. R_s is minimally influenced by the ratio of the two compounds being measured. R_s of > 2 between the peak of interest and the closest potentially interfering peak (impurity, excipient, degradation product, internal standard, etc.) is desirable (USP, 2007).

$$R_s = 2 (t_{R2} - t_{R1}) / (w_2 + w_1) \qquad (2)$$

where t_w is peak width measured at baseline of the extrapolated straight sides to baseline.

Relative retention or selectivity (α): Relative retention (also called selectivity or separation factor α value) is a measure of the relative location of two peaks. This is not an essential parameter as long as the resolution (R_S) is stated.

$$\alpha = k_2 / k_1 \qquad (3)$$

Tailing factor (T): The accuracy of quantitation decreases with increase in peak tailing because of the difficulties encountered by the integrator in determining where/when the peak ends and hence the calculation of the area under the peak. Integrator variables are pre-set by the analyst for optimum calculation of the area for the peak of interest. The acceptance criteria are that: T of ≤ 2 (USP, 2007).

$$T = W_x / 2f \qquad (4)$$

where W_x is width of the peak determined at either 5% (0.05) or 10% (0.10) from the baseline of the peak height. f is distance between peak maximum and peak front at W_x

Theoretical plate number (N): N is a measure of column efficiency, that is, how many peaks can be located per unit run-time of the chromatogram.

$$N = 16 (t_R / t_w)^2 = L / H \qquad (5)$$

N is fairly constant for each peak on a chromatogram with a fixed set of operating conditions. H, or the height equivalent of a theoretical plate (HETP), measures the column efficiency per unit length (L) of the column. Parameters, which can affect N or H, include peak position in the chromatogram particle size of stationary phase packing, flow-rate of mobile phase, column temperature, viscosity of mobile phase, and molecular weight of the analyte. The theoretical plate number depends on the elution time but in general should be > 2000 [USP, 2007, 4, appendix I].

3 Validation of a Reversed-phase HPLC Method for 1,10-Phenanthroline-5,6-Dione and Analysis of Its Impurities by HPLC–MS

Method development is not always a simple task since there are a substantial number of parameters in HPLC and LC-MS, which may influence the final results, that are obtained. This is especially the case, when a required method does not exist in the literature and it is then that the analyst needs advanced knowledge and experience on both the analytical equipment and drug substance, or drug product that needs to be analysed. In this situation, applying the systematic approach discussed previously (Chapter 1, Figure 1.1) can make the task simpler and reduce the resources that the company needs to apply to the tasks.

Using this philosophy, a new assay method was developed and validated for 1,10-phenanthroline-5,6-dione [5, appendix I] which is a useful class of heterocyclic o-quinone compounds (Figure 3.1).

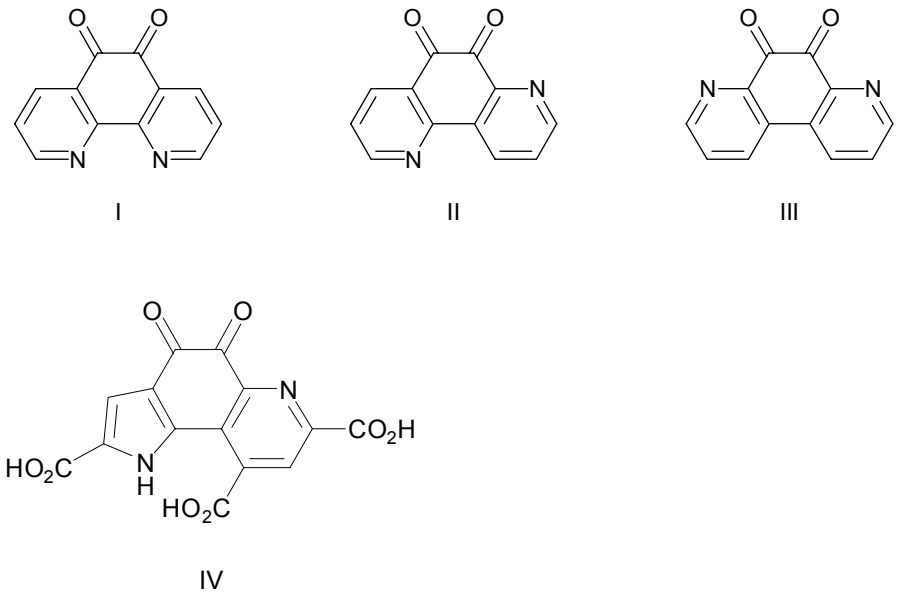
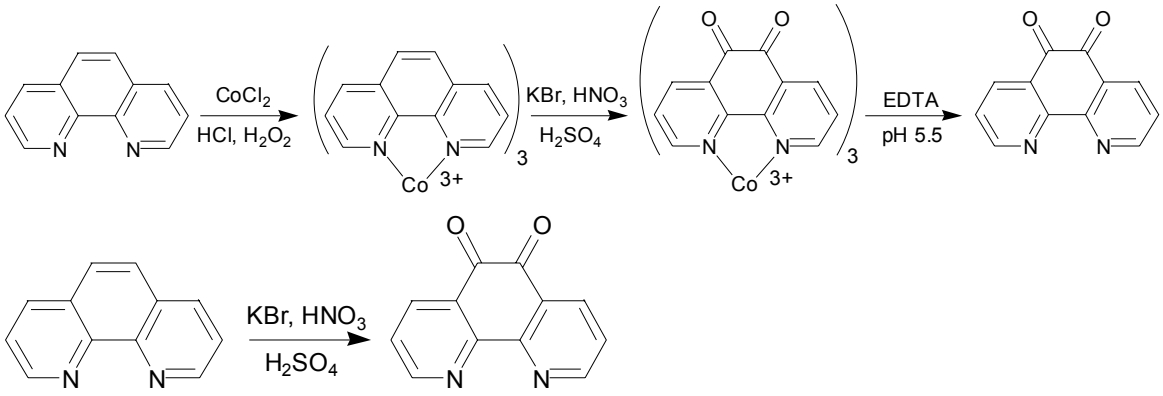


Figure 3.1 - Heterocyclic o-quinone compounds: (I) 1,10-phenanthroline-5,6-dione; (II) 1,7-phenanthroline-5,6-dione; (III) 4,7-phenanthroline-5,6-dione; (IV) pyrroloquinoline quinine.

The compound 1,10-phenanthroline-5,6-dione had been synthesized via two synthetic routes and its purity profile was determined. Impurity profiling is an important issue in pharmaceutical analysis, particularly during product development and quality control. The standard requirements of such an impurity method are that all likely synthetic and degradative impurities are resolved from each other and the main drugs and that the impurities can be monitored at the 0.1% (w/w) level or below.

Application of the method in LC-MS mode resulted in the identification of 4,5-diazafluoren-9-one (V) as the major impurity in 1,10-phenanthroline-5,6-dione prepared via the one-step oxidation of 1,10-phenanthroline. In contrast, the impurity (V) was absent from samples of 1,10-phenanthroline-5,6-dione produced using a three-step cobalt complexation route from 1,10-phenanthroline. As such, this latter method was preferred for the synthesis of high purity samples of 1,10-phenanthroline-5,6-dione. The comparison of two synthetic routes helped to identify the one yielding the best quality product. This approach of investigating the synthetic studies, analysis, impurity profiling and validating the method could also be applied to other similar compounds.

The main interest was to prepare 1,10-phenanthroline-5,6-dione by the two main synthetic routes and identifying any impurities, where possible, to evaluate its suitability for use as a mediator to nicotinamide adenine dinucleotide (NADH) in biosensors for diabetics. A number of potential impurities were expected from an examination of the literature concerning the reactivity of the phenanthroline-5,6-dione. Thus, compound (I) was known to undergo alkaline decarboxylation to afford 4,5-diazafluoren-9-one (V). Here, (V) may be obtained during the neutralisation of highly acidic reaction mixtures in the preparation of 1,10-phenanthroline-5,6-dione. The use of $KBr/HNO_3/H_2SO_4$ reaction medium may result in the formation of 5-bromo-1,10-phenanthroline (VI) and 5-nitro-1,10-phenanthroline (VII) as by-products. Under certain oxidation conditions, the 1,10-isomer (I) can be converted to 2,2'-bipyridine-3,3'-dicarboxylic acid (VIII) or 1*h*-cyclopenta[2,1-*b*:3,4-*b'*]dipyridine-2-5-dione (IX) as reported (Figure 3.2). The scheme 1 shows the synthesis and the structures are shown in Figure 3.2.



***Scheme* 1** - Synthesis of 1,10-phenanthroline-5,6-dione.

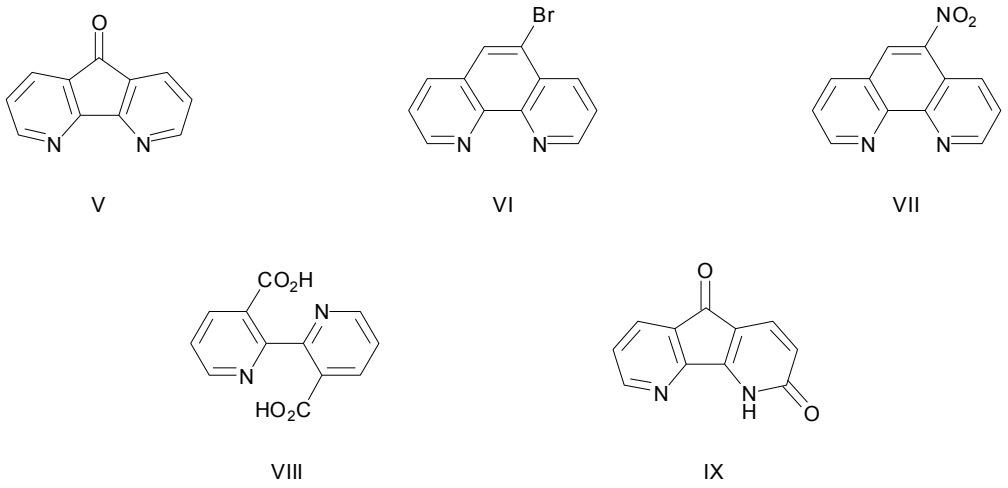


Figure 3.2 – Chemical structure of potential impurities in 1,10-phenanthroline-5,6-dione: (V) 4,5-diazafluoren-9-one; (VI) 5-bromo-1,10-phenanthroline; (VII) 5-nitro-1,10-phenanthroline; (VIII) 2,2'-bipyridine-3,3'-dicarboxylic acid; (IX) 1*h*-cyclopenta[2,1-*b*:3,4-*b'*]dipyridine-2-5-dione.

3.1 Step-by-step new method development by HPLC

A project charter/plan was written for the feasibility, research and development study. In the feasibility study, an assessment was made as to whether the assigned task could be successfully accomplished by using available resources. In the research phase, new knowledge was discovered on the compound in the hope that such information would be useful in developing a new method. The knowledge gained from the research phase was the physiochemical properties of the compound such as structure, solubility/stability in different solutions, pK_a values, spectra (UV, MS, NMR), the synthetic routes, synthetic impurities, and

literature review documenting the analysis of similar compounds if any. This knowledge was used to develop a new method for 1,10-phenanthroline-5,6-dione compound.

Good separation of 1,10-phenanthroline-5,6-dione from its synthetic impurities was achieved as demonstrated by the chromatogram displayed in Figure 3.3. The chromatographic run yielded four major peaks that are detailed as an area percent in Table 3.1. The first two peaks (at 2.07 and 2.37 min) are minor impurities and the third (3.74 min) is a major impurity. The peak at 2.75 min is the 1,10-phenanthroline-5,6-dione. The UV spectra of the potential impurities and 1,10-phenanthroline-5,6-dione are shown in Figure 3.4. Here, the data is from the analysis of a sample of crude (I) derived from the one-step oxidation of 1,10-phenanthroline.

Table 3.1 - Chromatographic results of 1,10-phenanthroline-5,6-dione and its impurities

Compound	Retention time (min)	Area (μV)	Area (%)
Impurity (1)	2.07	25404	0.64
Impurity (2)	2.37	23658	0.59
1,10-phenanthroline-5,6-dione	2.75	3725059	93.21
Impurity (3)	3.74	107954	2.70

The analytical conditions chosen during method development were then optimised to further improve to gain greater confidence on the generation of analytical data. After the optimisation phase, pre-validation characteristics such as robustness, stability of analytical solutions, linearity, precision, specificity and system suitability were studied [5, appendix I].

System suitability testing was performed to determine the accuracy and precision of the system by injecting six injections of a solution containing 0.6 mg 1,10-phenanthroline-5,6-dione/mL. All peaks were well resolved and the precision of injections for all peaks was acceptable. The percent relative standard deviation (%RSD) of the peak area responses was measured. The % RSD of peak areas was average 0.62 (n = 6) the tailing factor (T) for each peak of 1,10-phenanthroline-5,6-dione was 1.61 and theoretical plate number (N) was 3035. The resolution between each peak were > 1.2, and retention time (RT) variation RSD ≤ 2% for six injections.

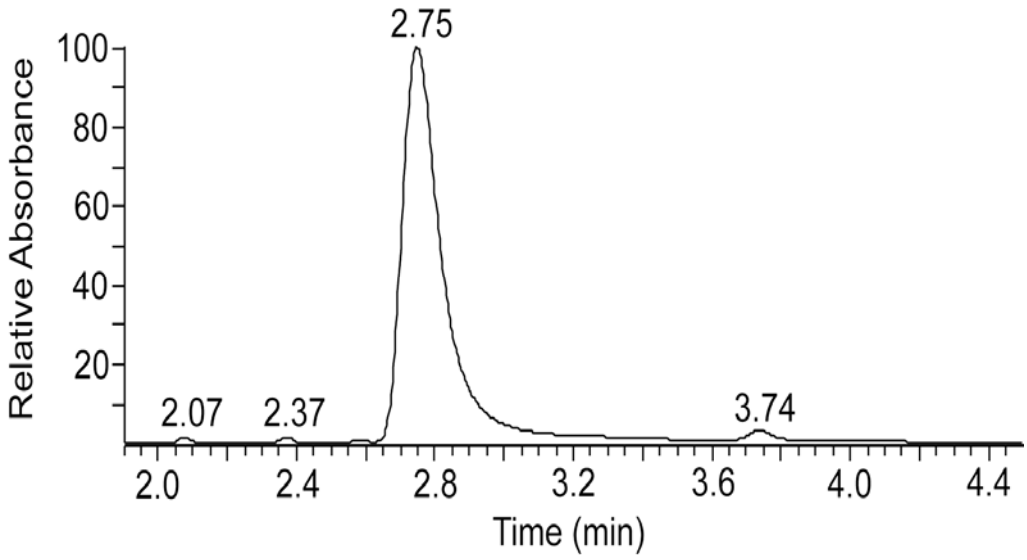


Figure 3.3 – Separation of crude 1,10-phenanthroline-5,6-dione displaying its impurities. This material was produced via the one-step oxidation method and was not crystallised. Conditions: isocratic elution with methanol/water (50/50, v/v) with 0.1% of triethylamine, flow rate 0.8 mL/min, Luna C_{18} column (150 x 4.60 mm, 5-µm particle size), temperature 40°C, injection volume 2µl, UV detection 254 nm.

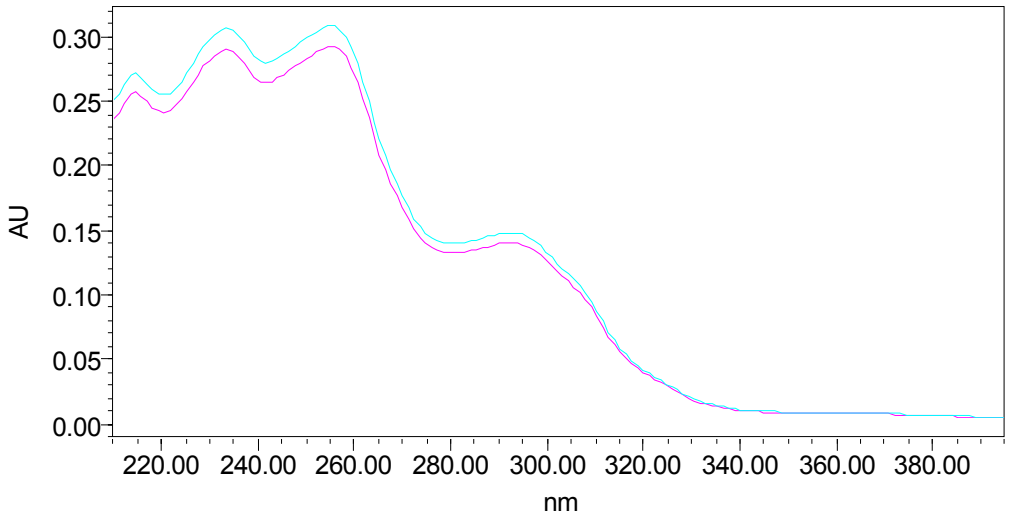


Figure 3.4 - PDA UV match spectra of the middle of the peak corresponding to the retention time of the main component of 1,10 phenanthroline-5,6-dione and a reference sample.

For the assessment of method robustness, a number of chromatographic parameters were investigated such as flow rate (± 0.2 mL/min), column temperature (± 5°C), mobile phase composition (± 5% organic) and wavelength (± 5 nm). In all cases, good separation were always achieved, indicated that the method remained selective for all components under the developed conditions.

3.2 Identification of impurities by LC-MS

It was not possible to identify the chemical structure of the impurities by HPLC/PDA UV alone. For this purpose, LC-MS was selected to obtain structural information. The full scan LC-MS spectra of 1,10-phenanthroline-5,6-dione and its impurities were measured in the mass range *m/z* 100-650. 1,10-Phenanthroline-5,6-dione displayed a single peak at 2.75 min with a corresponding protonated molecular ion of mass *m/z* 211.2. Impurity peak 3 at 3.74 min was assigned to 4,5-diazafluoren-9-one (V) on the basis of both HPLC (Figure 3.5) data and MS (Figure 3.6). Again, a protonated molecular ion of mass *m/z* 183.3 was observed in the mass spectrum.

The chemical structure of impurities 1 and 2 could not be identified from the HPLC and LC-MS data. However, the ions at *m/z* 183.3 and 241.2 for the two impurities (2.07 and 2.37 min) did not correspond with the other suspected impurities such as 1,10-phenanthroline, 5-bromo-1,0-phenanthroline (VI), 5-nitro-1,10-phenanthroline (VII), 2,2'-bipyridine-3,3'-dicarboxylic acid (VIII) or 1*h*-cyclopenta[2,1-*b*:3,4-*b'*]dipyridine-2-5-dione (IX). This was a first report for an HPLC purity assay on this important compound in the scientific literature.

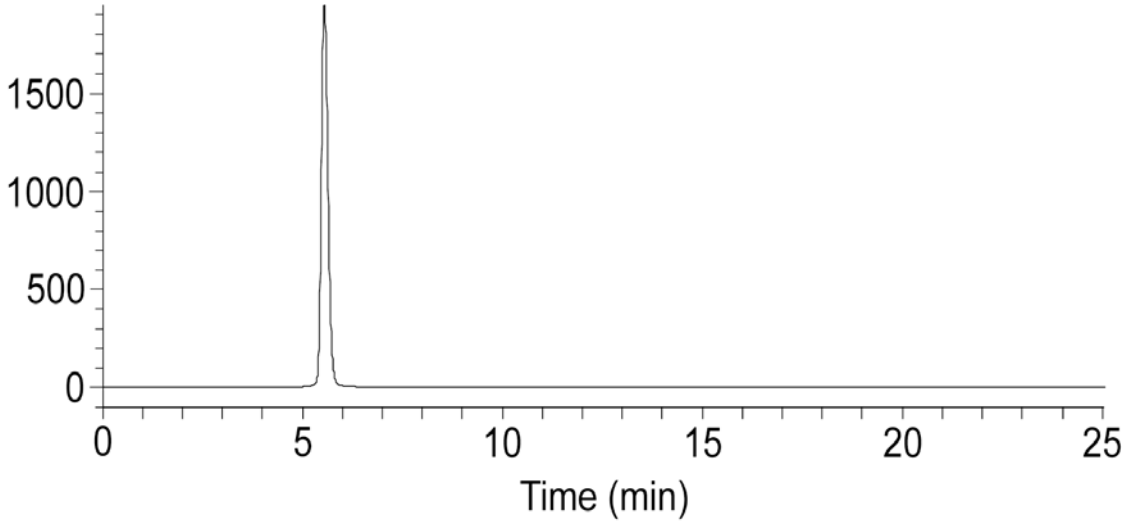


Figure 3.5 - Separation of 4,5-diazafluoren-9-one (V), (impurity 3). Conditions: isocratic elution with methanol/water (50/50, v/v) with 0.1% of triethylamine, flow rate 0.8 mL/min, Luna column C_{18} (150 x 4.60 mm, 5-μm particle size), temperature 40°C, injection volume 2μL, UV detection 254 nm.

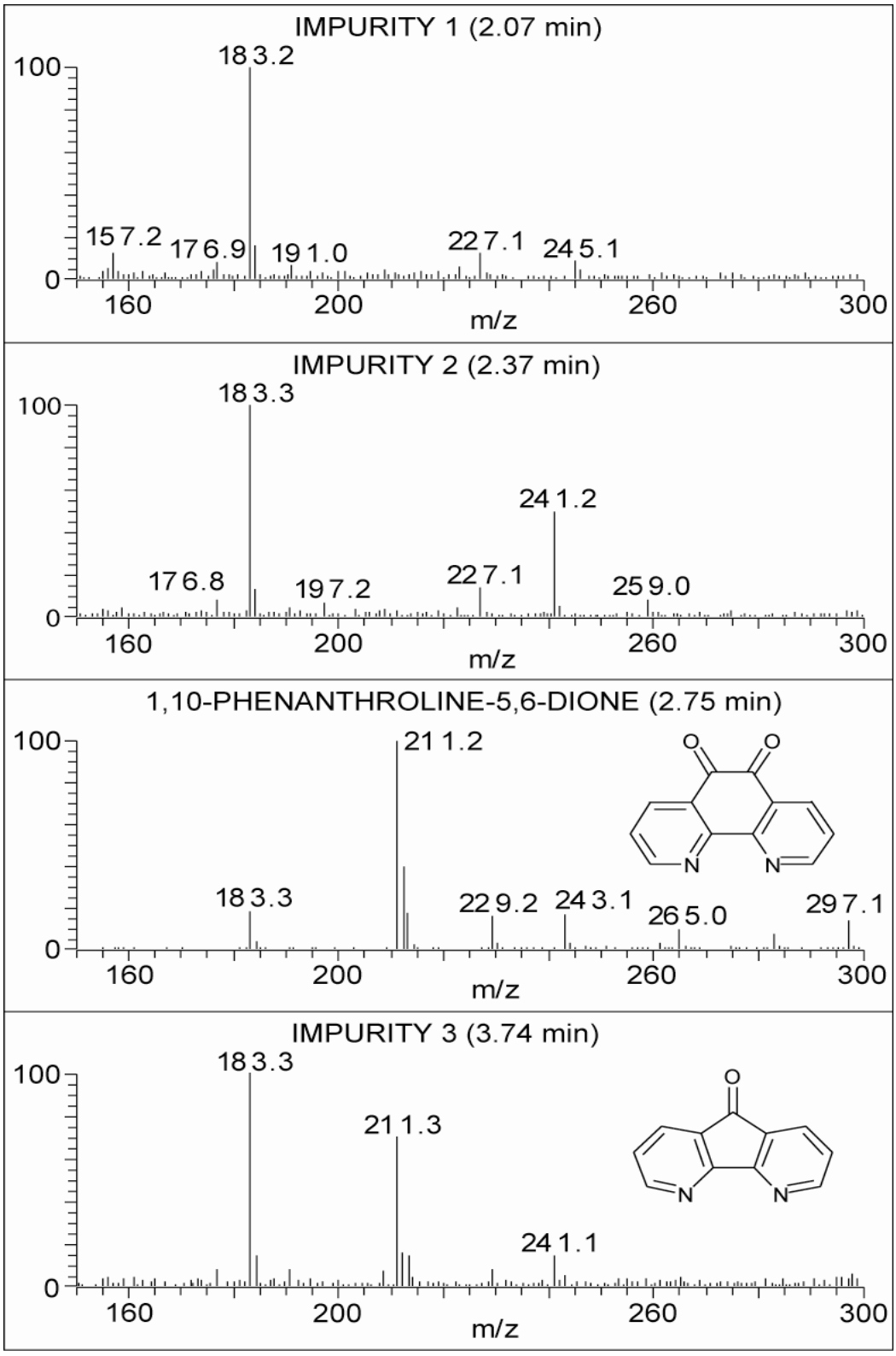


Figure 3.6 - Mass spectra (x-axis: relative abundance) of 1,10-phenanthroline-5,6-dione and its impurities 1-3. Conditions: ESI+, source temperature 100°C, capillary voltage 3.0 kV, cone voltage 30V.

3.3 Step-by-step method validation

The first step in test method validation was the characterisation phase. Initially a cross-functional team (CFT) was assembled. In this phase, the validation performance characteristics were chosen for the assay method type. The validation performance characteristics were linearity, range, intermediate precision, repeatability, accuracy and specificity studied.

For best practice, the characterisation protocol was written, approved and executed using validated equipment under highly GMP/GLP environment. Limits were set based on internal use purposes only and not for regulatory compliance of this analytical method. All personnel involved in the characterisation protocol activities were trained on characterisation protocol, equipment and test method prior to performing their functions. On completion of the characterisation study, the results/data were critically assessed from a statistical point of view. Finally, a validation step-by-step protocol was written and approved by the CFT and executed.

As has been indicated, great care was taken in the method development phase, which resulted in the subsequent validation being straightforward.

The linearity was studied over two days using six different amounts of 1,10-phenanthroline-5,6-dione in the range of 0.04 -1.60 mg/mL. The regression coefficient obtained (r^2: 0.9998 day 1, 0.9996 day 2), demonstrated the excellent relationship between peak area and the concentration of 1,10-phenanthroline-5,6-dione (Table 3.2).

Accuracy (percent recovery) study was investigated, five different solutions (10, 20, 40, 100 and 160% of target) were prepared with a known added amount of dione **I** and injected in triplicate. Percent recoveries of response factor (area/concentration) were calculated. Excellent recoveries were obtained averaged 99.9%.

Table 3.2 - Linearity assessment of the HPLC method for the assay of 1,10-phenanthroline-5.6-dione

Concentration (mg/mL)	Concentration (as %) of 0.6 mg/mL	Area (μV) as mean of 3 injections (day 1)	Area (μV) as mean of 2 injections (day 2)
0.0400	4.0	225761	241740
0.1000	10	577405	616281
0.2002	20	1126188	1401765
0.4004	40	2574365	3063222
1.0009	100	6561498	8541076
1.6016	160	10458276	13686533
Correlation coefficient:		0.9998	0.9996
Equation for regression line:		*y* = 7E+06x - 90335	*y* = 9E+06x - 263239

The PDA three-dimensional chromatogram demonstrated [5, appendix I] a good separation of the 1,10-phenanthroline-5,6-dione peak (c) (retention time 2.5 min) and from the impurities (retention time 1.4 and 2.0 min) and of the impurities from each other. A wavelength of 254 nm was found to be the most effective compromise to accomplish the detection and quantification of the two impurities and the main 1,10-phenanthroline-5,6-dione component in a single run. The impurities and the 1,10-phenanthroline-5,6-dione peaks were adequately resolved from each other; typical resolution values for the 1,10-phenanthroline-5,6-dione peak were greater than 1.5. This method possessed acceptable specificity.

Table 3.3 - Assay percent results of 1,10-phenanthroline-5,6-dione under stress conditions

Stress conditions	Sample treatment	RT (min)	Assay (%)	Area (μV)
Reference	Fresh solution	2.35	99.91	8582691
Acid	1N HCl for 24 hour	2.30	99.94	8753142
Base	1N NaOH for 4 hour	2.35	99.50	8697192
Heat	50°C for 1 hour	2.35	99.80	8630907
Light	UV Light for 24 hour	2.30	99.25	7189035

Forced degradation studies were also performed to evaluate the specificity with respect to 1,10-phenanthroline-5,6-dione and its impurities under four stress conditions (heat, UV light, acid, base). Solutions of 1,10-phenanthroline-5,6-dione were exposed to 50°C for 1h, UV light using a Mineralight UVGL-58 for 24h, acid (1M HCl) for 24h, and base (1M NaOH) for 4h. Summary data of the stress results is shown in Table 3.3. It is evident from Figure 3.3 that the method had been able to separate the peaks of the degraded products from that of the 1,10-phenanthroline-5,6-dione. This was further confirmed by peak purity analysis on a PDA UV detector.

The precision of the chromatographic method was investigated by measuring the repeatability on ten replicate injections at 100% test concentration (0.6 mg/mL) and inter day variation on injecting (n = 10) the same solution (day 2). The relative standard deviation (RSD) value for repeatability was 0.04% and inter day variation was 0.44%, and illustrated excellent precision for this LC assay method. The excellent results obtained due to the care taken during research phase.

Upon successfully completing the validation, the data was statistically analysed against its acceptance criteria by the statistician in order to test its validity. The validated method was then used routinely in the development laboratory for 1,10-phenanthroline-5,6-dione compound and its impurities.

3.4 Conclusion

A reversed-phase HPLC method for the assay and purity evaluation of 1,10-phenanthroline-5,6-dione obtained via two synthetic routes was developed using a systematic approach. Application of the method in LC-MS mode resulted in the identification of 4,5-diazafluoren-9-one (V) as the major impurity in 1,10-phenanthroline-5,6-dione prepared via the one-step oxidation of 1,10-phenanthroline. The research methodology was validated for its validity using validated equipment under a highly GLP/GMP regulated environment. The validation study showed excellent results of the careful step-by-step approach that had been followed. The method was precise with RSD ≤ 0.44% for intera and interday precision, and accurate with recoveries in between 99.76-100.00%. After the validation, the applicability of the method for determination and

identification of 1,10-phenanthroline-5,6-dione was demonstrated and adopted for routine use through Abbott.

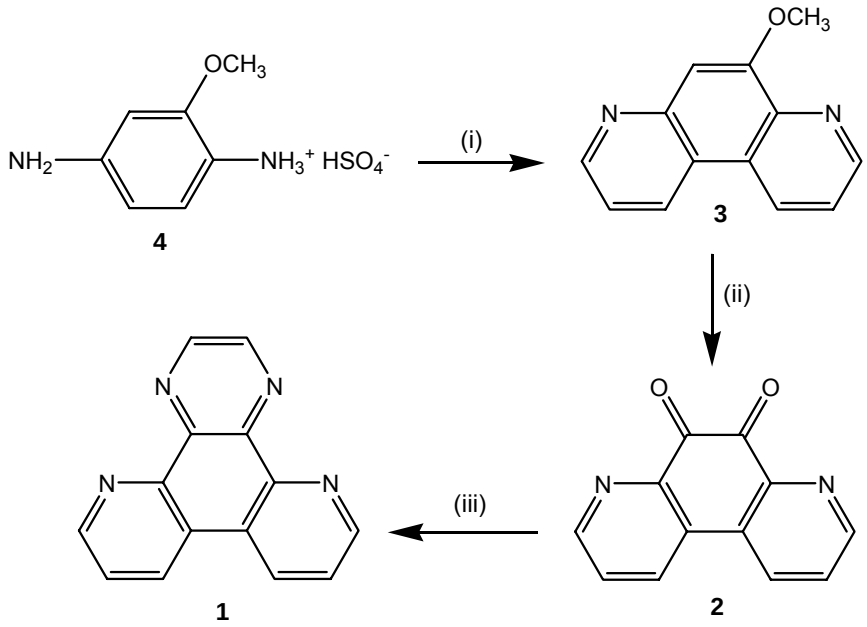
This research has made a significant and coherent contribution to new knowledge including new HPLC and LC-MS method; combined use of photodiode array UV and LC-MS to identify unknown impurities; newly identified information on 1,10-phenanthroline-5,6-dione impurities profile; newly identified detailed information on comparison of syntheses; a more detailed step-by-step approach to method development and validation and a robust validated method.

4 Development and Validation of a HPLC Method for 4,7-Phenanthroline-5,6-Dione and Identification of Its Major Impurity by HPLC–MS–APCI

In a similar to that described previously for 1,10-phenanthroline-5,6-dione manner (Chapter 3) a new purity assay for 4,7-phenanthroline-5,6-dione **2** was developed and validated [6, appendix I] by HPLC. Impurity was investigated using LC-MS with atmospheric-pressure chemical ionization and photodiode-array UV detection.

4,7-Phenanthroline-5,6-dione is an important heterocyclic quinone, which is known historically to be active against protozoa, amoebae and bacteria (Kradolfer, *et al.,* 1960). The compound has recently been suggested to be of use in the prevention and treatment of Alzheimer's (Xilinas & Gerolymatos, 2000), macular degenerative (Xilinas, 2002) and prion (Xilinas, & Hannoun, 2002) diseases. 4,7-Phenanthroline-5,6-dione is a starting material for phenanthroline ligands (Imor *et al.,*1996), which are useful for the preparation of complexes of transition metals, particularly ruthenium (D'Alessandro *et al.,* 2001). The quinone cofactor methoxatin can be replaced by in glucose dehydrogenase while maintaining enzyme activity. The interest in (**2**) stems from its possible use biosensor electrodes as a redox mediator for the cofactor NADH (Itoh *et al.,*1992).

A simple method was sought for the synthesis of (**2**), which was reportedly available via 5-methoxy-4,7-phenanthroline itself derived from a double Skraup reaction of 2-methoxy-1,4-phenylenediamine hydrosulphate or its diacetyl derivative (Figure 4.1).



(i) Glycerol ($C_3H_8O_3$), sulfuric acid, sodium m-nitrobenzene sulfonate ($C_6H_4NO_5SNa$)

(ii) Sulfuric acid (H_2SO_4), nitric acid (HNO_3)

(iii) Ethylene diamine ($H_2NCH_2CH_2NH_2$), methanol (MeOH)

Figure 4.1. Synthesis of phanquone: (1) 4,7-Phenanthrolino-5,6:5',6'-pyrazine; (2) 4,7-Phenanthroline-5,6-dione; (3) 5-Methoxy-4,7-phenanthroline; (4) 2-Methoxy-1 ,4-phenylenediamine hydrosulfate.

Avoiding the notorious Skraup synthesis, it was possible to the commercially available 4,7-phenanthroline to (**2**) using oxidation conditions previously applied to the 1,10-isomer (Hiort *et al.,* 1993; Calderazzo *et al.,* 1999; Mlochowski, 1974) (Figure 4.2). However, it had been suspected that the use of $KBr/HNO_3/H_2SO_4$ would result in the formation of brominated 4,7-phenanthrolines as by-products.

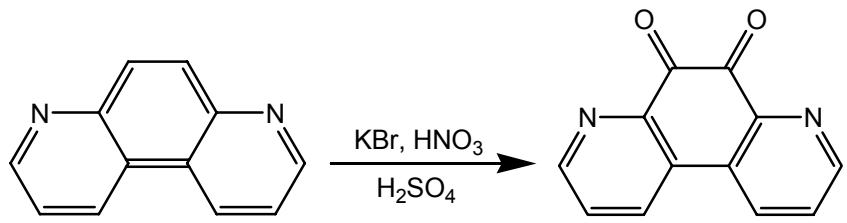


Figure 4.2 - Synthesis of 4,7-phenanthroline-5,6-dione.

4.1 Related substances

The quantity of each impurity peak (the known impurity designated as peak b plus any other impurity peak) is calculated as an area percent versus the total area of all peaks in the chromatogram. Each impurity peak, plus the total area percent of all impurity peaks, must fall within the requirements in the specification (Table 4.1).

Table 4.1 - Limits for chromatographic purity of 4,7-phenanthroline-5,6-dione and its impurity

Parameter	Assay	Related substances
Peak assignment	1(a)*	2(b)*
Retention time (min)	5.16	6.90
Limit as percent total area	c ≥ 96.0%	≤ 2.0% b + all other impurities. No other peak area can exceed 2.0%

* a, 4,7-Phenanthroline-5,6-dione (**1)**; b, impurity 1

4.2 Assay and purity calculations

The area percent of main 4,7-phenanthroline-5,6-dione peak (designated as peak a) is determined as for the impurities. The area percent must fall within the specifications (Table 4.1). The purity of the 4,7-phenanthroline-5,6-dione drug substance is calculated in relation to the reference standard using the area of the main peak a:

$$\text{Percent purity} = \left(\frac{\text{mg / 100 mL of standard x total area of peak a from sample}}{\text{area of peak a from standard x mg / 100 mL of sample}} \right)$$

4.3 Separation of 4,7-phenanthroline-5,6-dione and its impurity

The analytical method was developed using systematic step-by-step approach as previously described in Chapter 3. Good separation and high sensitivity was achieved by using acetonitrile containing 10 mM potassium dihydrogen phosphate containing 0.1% triethylamine as the mobile phase with varying detection wavelengths, based on the response of the main component. The main peak tailed badly on some C_{18} columns with these mobile phases but adding triethylamine minimised this. Amines such as triethylamine are often added in the mobile phase to reduce peak tailing caused by the strong intraction of basic analytes with the acidic surface silanols. The amount of organic modifier was adjusted so that the assay run time could be reduced for faster analysis of the (**2**) samples. A chromatogram illustrating the separation of (**2**) and the one potential impurity is illustrated in Figure 4.3 confirming specificity with respect to (**2**).

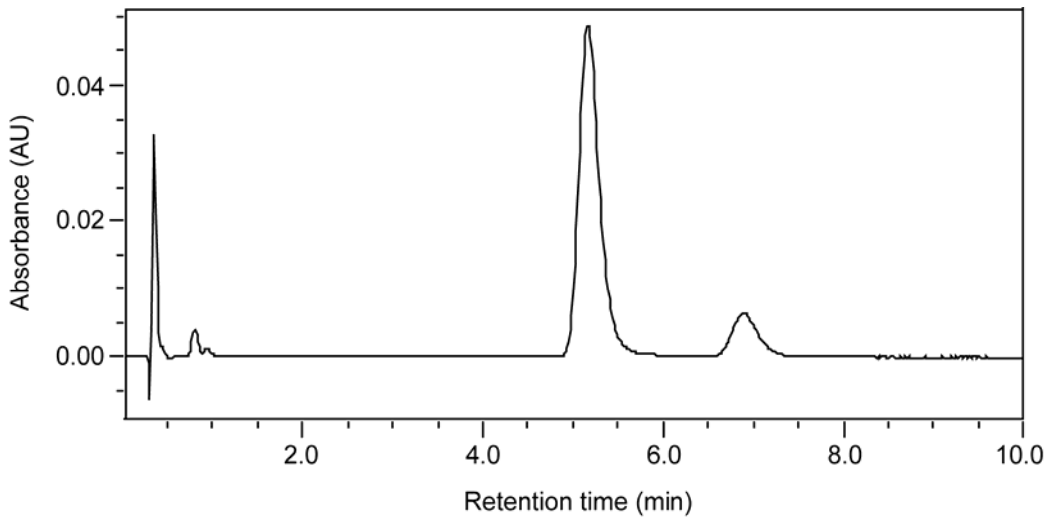


Figure 4.3 - Separation of 4,7-phenanthroline-5,6-dione (retention time 5.16 min) displaying its impurity (retention time 6.90 min). Conditions: isocratic elution with aqueous solution of 10 mM phosphate buffer (pH 3.05)/acetonitrile (85/15, v/v) containing 0.1% triethylamine, XTerra RP-18 column (150 x 4.6 mm, 5-μm particle size), flow rate 0.5 mL/min, injection volume 2 μL, column temperature 40°C, UV wavelength 254 nm.

The remaining chromatographic conditions listed in HPLC experimental section were chosen for the following reasons: the lower flow rate of 0.5 mL/min was chosen because of the potential problems associated with elevated back pressures. The photodiode-array detector (UV) was set at 254 nm.

During method development phase, great care was taken to develop a robust method. A number of chromatographic parameters were investigated. The column temperature was held at 40°C although separations at 30°C and 35°C indicated that slight variations in temperature did not have a significant effect on retention, resolution or peak shape. The injection volume of 2 μL and sample concentration of 0.40 mg 4,7-phenanthroline-5,6-dione per mL in the mobile phase were chosen to simplify sample preparation. This concentration of (**2**) allowed both assay (of the main component) and purity evaluation (of trace impurity). At this concentration, the (**2**) peak is well within the linear range for UV detection, and trace components were readily detectable.

System suitability testing was performed to determine the accuracy and precision of the system by making six injections of a solution containing 0.40 mg of 4,7-phenanthroline-5,6-dione per mL. All peaks were well resolved and the precision of injections for all peaks was acceptable. The RSD of peak areas averaged 0.30% (n = 6); the tailing factor (T) for each peak of (**2**) was 1.12 and theoretical plate number (N) was 3240. The resolution between each peak were > 2.40, and retention time variation RSD 0.3% for six replicate injections.

For the assessment of method robustness, a number of chromatographic parameters were investigated such as flow rate (± 0.2 mL/min), column temperature (± 5°C), mobile phase composition (± 5% organic, ± 5 mM buffer concentration), pH (0.2 unit) and wavelength (± 5 nm). In all cases, good separation were always achieved, indicated that the method remained selective for all components under the developed conditions.

4.4 Identification of impurities by LC-MS

Two major peaks are shown in the chromatogram in Figure 4.3. The first peak at 5.16 min (98.93 area %) was due to 4,7-phenanthroline-5,6-dione. The second

peak at 6.90 min (0.81 area %) was thought to be a brominated impurity. Elemental analysis of some (**2**) samples demonstrated the presence of *ca*. 0.3% Br. These samples had been produced via direct oxidation of 4,7-phenanthroline with $KBr/HNO_3/H_2SO_4$. In contrast, a sample of (**2**) prepared via 5-methoxy-4,7-phenanthroline was bromine-free. Consequently, a standard sample of 5-bromo-4,7-phenanthroline, the chromatogram in Figure 4.4, was prepared by a literature procedure (Mlochowski, 1974).

It was not possible to identify the chemical structure of this impurity by HPLC with PDA UV detection. For this purpose, LC-MS was selected. The full scan LC-MS spectra of 4,7-phenanthroline-5,6-dione and its impurity were measured in the mass range *m/z* 100-650. The compound (**2**) displayed a single peak at 5.16 min, which corresponded to the molecular mass at *m/z* 209.99 and an impurity peak (6.90 min) at *m/z* 258.88. The mass spectra of each peak ion chromatogram are shown in Figures 4.5 and 4.6 respectively. The impurity peak b at 6.90 min was identified as 5-bromo-4,7-phenanthroline, by both HPLC and MS data, with the $[M + H]^+$ ion pair at *m/z* 258.88 and 260.86 (Figure 4.6) being consistent with the presence of bromine due to the known 1:1 isotope cluster for ^{79}Br and ^{81}Br. It is interesting to note that the (**2**) displayed an M^+ ion rather than an $[M + H]^+$ ion as previously found for its 1,10-isomer and, indeed, the bromo compound above. The reason for this difference was unknown but the result was reproducible since external mass spectral analysis (EI, positive mode) also yielded an M^+ ion for the (**2**).

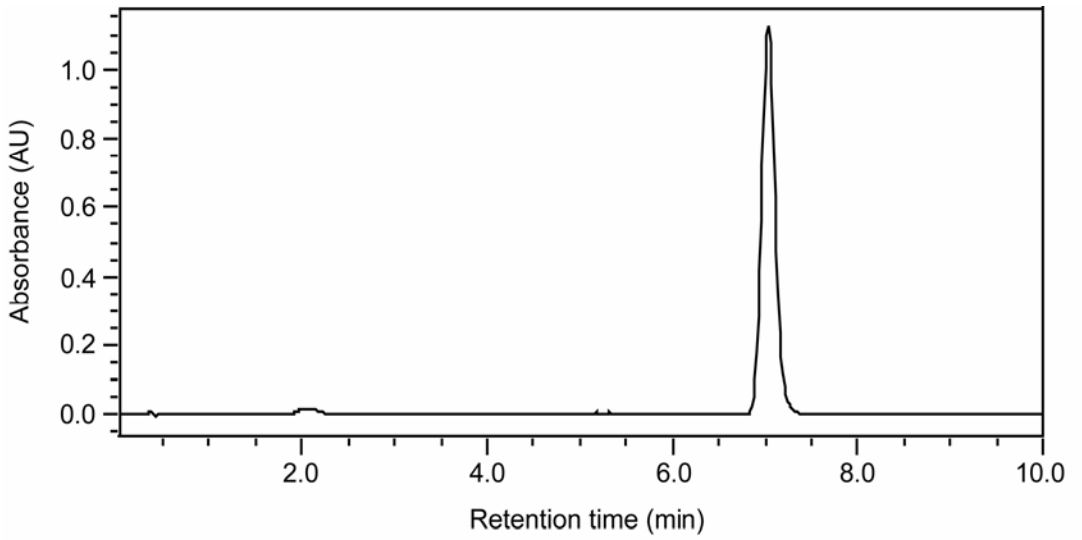


Figure 4.4 - Separation of 5-bromo-4,7-phenanthroline (impurity). Conditions: isocratic elution with aqueous solution of 10 mM phosphate buffer (pH 3.05)/acetonitrile (85/15, v/v) containing 0.1% triethylamine, XTerra RP-18 column (150 x 4.6 mm, 5-μm particle size), flow rate 0.5 mL/min, injection volume 2 μL, column temperature 40°C, UV wavelength 254 nm.

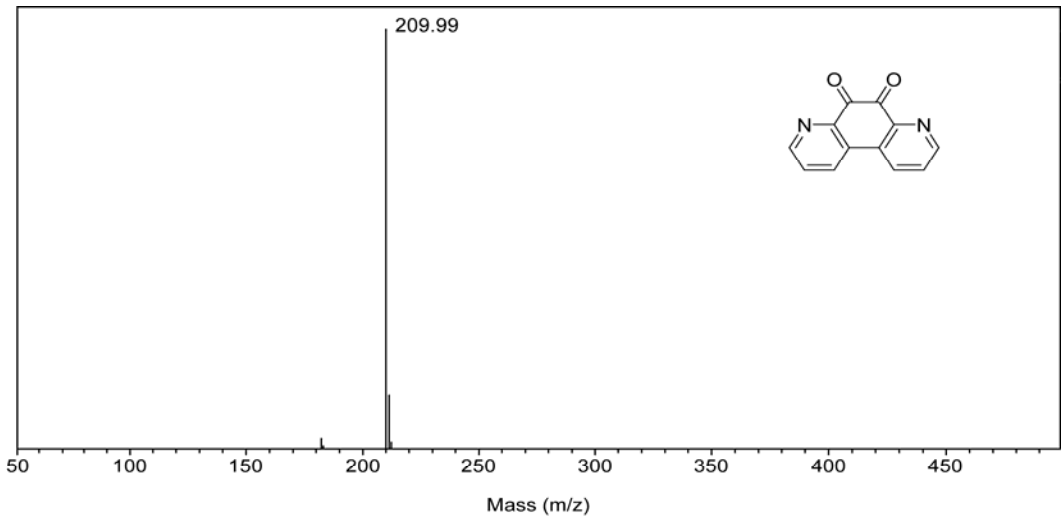


Figure 4.5 - Mass spectrum of 4,7-phenanthroline,5-6-dione. Conditions: XTerra MS C_{18} column (50 x 2.1 mm, 3.5-μm particle size), mobile phase 10 mM ammonium acetate/acetonitrile (90/10, v/v), injection volume 2 μL, APCI positive ionization mode, source temperature 120°C, capillary voltage 3.0 kV, Cone voltage 50V, cone gas flow, 100 l/h, APCI probe temperature 500°C, UV wavelength 210 to 400 nm.

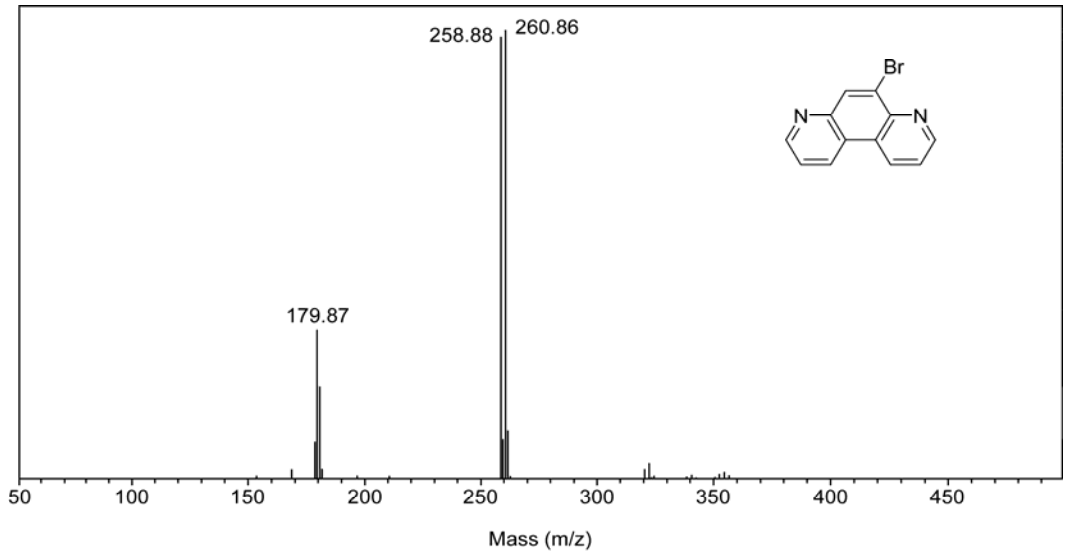


Figure 4.6 - Mass spectrum of main impurity of 5-bromo-4,7-phenanthroline. See Figure 4.5 for LC-MS conditions.

4.5 Step-by-step method validation

The analytical method validation was carried out using systematic step-by-step approach as previously described in Chapter 3.

The linearity was studied in the concentration range 0.05 to 1.50 mg/mL. Six solutions were prepared corresponding to 0.05, 0.25, 0.50, 0.75, 1.00 and 1.50 % of the nominal analytical concentration (0.40 mg/mL) and the following regression equation was found by plotting the peak area (*y*) versus the 4,7-phenanthroline-5,6-dione concentration (*x*) expressed in mg/mL: $y = 221070x + 116721$ ($r^2 = 0.9991$). The analyte response was linear across 80-120% of the target 4,7-phenanthroline-5,6-dione concentration.

The limit of detection (LOD) and limit of quantification (LOQ) tests for the procedure were performed on samples containing very low concentrations of analyte. For this method, LOD was 2.0 and the LOQ was 50 μg/mL and RSD 0.50% (*n* = 3).

The precision of the chromatographic method was investigated by measuring repeatability on six replicate injections at 100% test concentration (0.40 mg/mL)

and intermediate precision on three replicate injections at three different concentrations (0.05, 0.50 and 1.50 mg/mL). The RSD values were ≤ 0.30% in all cases and illustrated excellent precision for this method.

Accuracy was studied by preparing different solutions with a known added amount of (**2**) at three different concentrations (0.05, 0.50 and 1.50 mg/mL) and injected in triplicate. Mean recoveries for sample was found to be 99.0%.

The PDA three-dimensional chromatogram [6] demonstrated a good separation of the (**2**) peak a (RT = 5.16 min) and the impurity (RT = 6.90 min) from each other. A wavelength of 254 nm was found to be the most effective compromise to accomplish the detection and quantification of all impurities and the main component of (**2**) in a single run. The impurity and (**2**) peak area were adequately resolved from each other; typical resolution values for the (**2**) peak were greater than 2.39. This method demonstrated acceptable specificity.

Forced degradation studies were performed to evaluate the specificity of (**2**) and its impurity under four stress conditions (heat, UV light, acid, base). Solutions of (**2**) were exposed to 50°C for 1h, UV light using a Mineralight UVGL-58 light for 24h, acid (1M HCl) for 24h and base (1M NaOH) for 4h. In all cases, peak area was >99%. It is evident from Figure 4.3 that the method had been able to separate the peaks due to the degraded products from that of the (**2**). This was further confirmed by peak purity analysis on a PDA UV detector.

4.6 Conclusion

A new reversed-phase HPLC method for the identity, assay and purity evaluation of 4,7-phenanthroline-5,6-dione and its synthetic impurities had been successfully developed. 5-Bromo-4,7-phenanthroline was identified as main impurity by applying the method in LC-MS with photodiode-array UV detection. This impurity was only present in 4,7-phenanthroline-5,6-dione prepared via direct oxidation of 4,7-phenanthroline with $KBr/HNO_3/H_2SO_4$. 4,7-Phenanthroline-5,6-dione obtained via oxidation of 5-MeO-4,7-phenanthroline is bromine-free. Studies have demonstrated that the new assay method was rapid, simple, reliable and robust. It was extensively validated using validated LC-MS system.

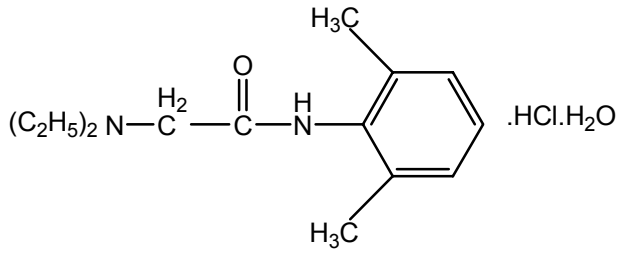
Very good validation data was obtained and this was in no small part due to the thoroughness of the initial ‘research’ and the careful step-by-step approach that had been followed.

This research has made a significant and coherent contribution to new knowledge including new validated HPLC and LC-MS method; combined use of PDA UV and LC-MS to identify unknown impurity; new information on 4,7-phenanthroline-5,6-dione compound; newly identified information on 4,7-phenanthroline-5,6-dione impurities profile; newly investigated information on comparison of synthesis and a more detailed approach to method development and validation.

5 Determination of 2-(Diethylamino)-*N*-(2,6-Diethylphenyl) Acetamide in a Gel Pharmaceutical Formulation by HPLC

Best practice in method development and validation is equally important in the analysis of active components in formulated products. In this study [7, appendix I], a rapid, simple, reliable and robust HPLC assay method for determining the content of 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide in a gel formulation was developed, validated and implemented to the quality control laboratory for bulk and final product release.

2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide (**1**) is widely used as a local anaesthetic that can be administered in a gel matrix (Stegman & Stoukides,1996). It has also achieved prominence as antiarrhythmic agent and is now in common use particularly as emergency treatment for ventricular arrhythmias that are encountered after cardiac surgery or acute myocardial infection.



2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide (**1**)

5.1 Step-by-step new method development

A project charter/plan was written for the feasibility, research and development study. In the feasibility study, an assessment was made as to whether the assigned task could be successfully accomplished by using available resources. In the research phase, new knowledge was discovered on the compound in the hope that such information would be useful in developing a new method. The

knowledge gained and received in the research phase was the physiochemical properties of the compound such as structure, solubility/stability in different solutions, pK_a values, spectra (UV, MS, NMR), any information regarding intended formulations, and literature review documenting the analysis of similar compounds. This information was used to develop a new method for 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide drug compound.

The chromatographic analysis of 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide (pK_a 7.86) was carried out in the isocratic mode using a mixture of 53% acetonitrile in aqueous buffer pH 7.0 (53:47, v/v) as mobile phase. Diluting the standard and sample in buffer pH 7.0 gave solutions that could be injected directly (without further dilution, filtration or centrifugation). Chromatograms of the 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide gave good peak shape (Figure 5.1) and co-elution of excipients was not observed (Figure 5.2) at the same retention time. Importantly, following the adapted step-by-step philosophy, robustness studies including system suitability and stability of analytical solutions were also conducted during the method development phase.

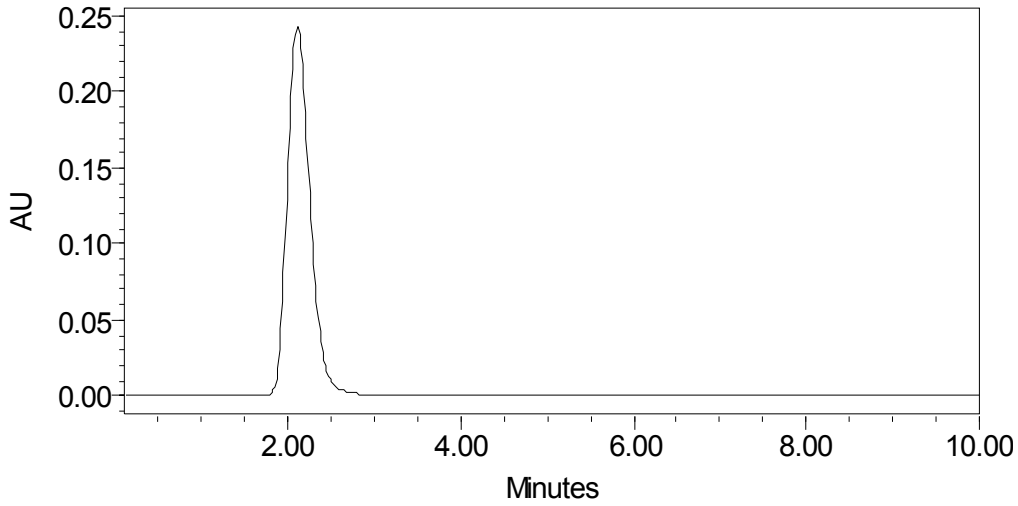


Figure 5.1 – Separation of 2-(diethylamino)-*N*-(2,6 dimethylphenyl) acetamide. Conditions: isocratic elution with potassium phosphate buffer (pH 7.0)/acetonitrile (47/53, v/v), flow rate 2 mL/min, injection volume 20 μL, UV wavelength 254 nm, C_{18} μ-Bondapak column 3.9 mm x 300 mm, 5-μm particle size.

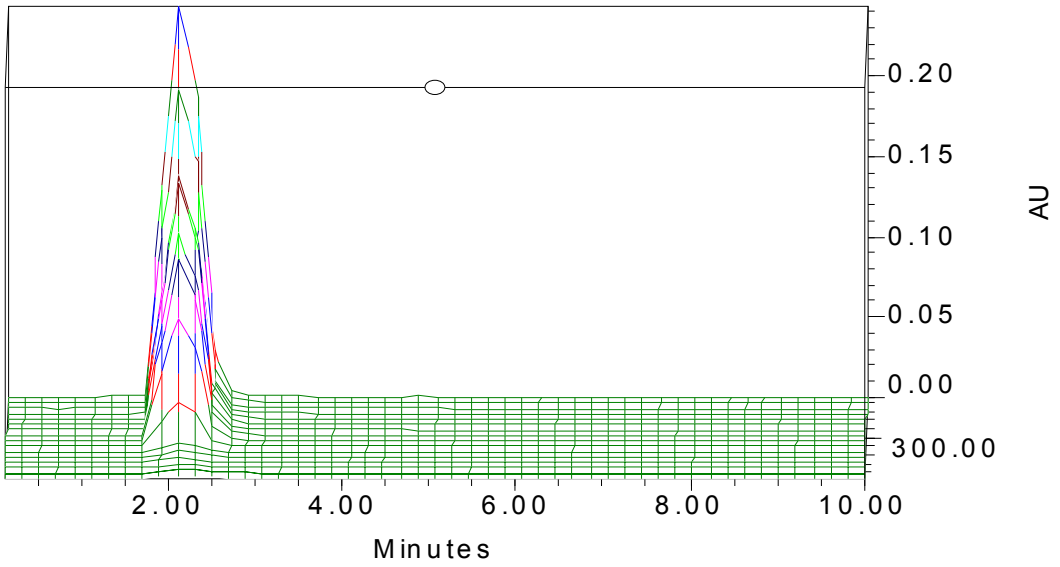


Figure 5.2 - Three-dimensional chromatogram of the mixture of 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide. For experimental conditions see Figure 5.1.

5.2 Step-by-step method validation

Initially a cross-functional team (CFT) was assembled. As a best practice, the characterisation protocol was written, approved and executed using validated equipment. In this phase, the validation performance characteristics were chosen for the assay method type. The validation performance characteristics were linearity, range, intermediate precision, repeatability, accuracy and specificity. All personnel involved in the characterisation protocol activities were trained on characterisation protocol, equipment and test method prior to performing their functions. On completion of the characterisation study, the results/data were critically assessed from a statistical point of view. Then a method validation protocol was prepared in compliance with GMP/GLP and approved prior to its initiation for this formulated product.

The linearity was studied using seven different amounts of 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide in the range of 20-140% around the theoretical values (1.40 μg/mL), and the following equation was found by plotting peak area (y) versus concentration (x) expressed in μg/mL: y = 1.30664e + 003x (r^2 = 1.000). The excellent linearity was seen for this chromatographic assay method.

The precision of the method was investigated with respect to repeatability and intermediate precision. Repeatability of the method was evaluated by assaying six replicate injections of the 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide at 100% of test concentration (1.4 μg/mL). The RSD of the retention time (min) was 0.01% and peak area was 0.18%. Intermediate precision was demonstrated by two analysts, using two HPLC systems and evaluating the relative peak area percent data across the two HPLC systems at three concentration levels (60, 100 and 120%) that covered the assay method range (0.2-14 mg/mL). The RSD values for both HPLC systems and analysts ware less than 0.13% (Table 5.1) and illustrated the excellent precision of the chromatographic method.

Table 5.1 - Intermediate precision results of 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide

Sample	HPLC System1			HPLC System 2		
	S1 (60%)	S2 (100%)	S3 (120%)	S1 (60%)	S2 (100%)	S3 (120%)
Analyst 1	99.85	99.88	99.82	99.95	99.98	99. 97
Analyst 2	99.89	99.85	99.80	99.92	99.96	99.98
Mean (HPLC)	99.87	99.86	99.81	99.94	99.97	99.98
Mean (Analyst)	99.90	99.93	99.90	99.91	99.90	99.89
RSD (criteria ≤ 2%) HPLC1 & 2	HPLC1 S1 + HPLC2 S1 = 0.05; HPLC1 S2 + HPLC2 S2 = 0.08; HPLC1 S3 + HPLC2 S3 = 0.12					
RSD (criteria ≤ 2%) Analysts	HPLC1 S1 + HPLC2 S1 = 0.07; HPLC1 S2 + HPLC2 S2 = 0.02; HPLC1 S3 + HPLC2 S3 = 0.07					

Injections of the extracted placebo were performed to demonstrate the absence of interference with the elution of the 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide. The results demonstrate that there was no interference (Figure 5.3) from the other materials in the gel formulation, and therefore confirm the specificity of the method.

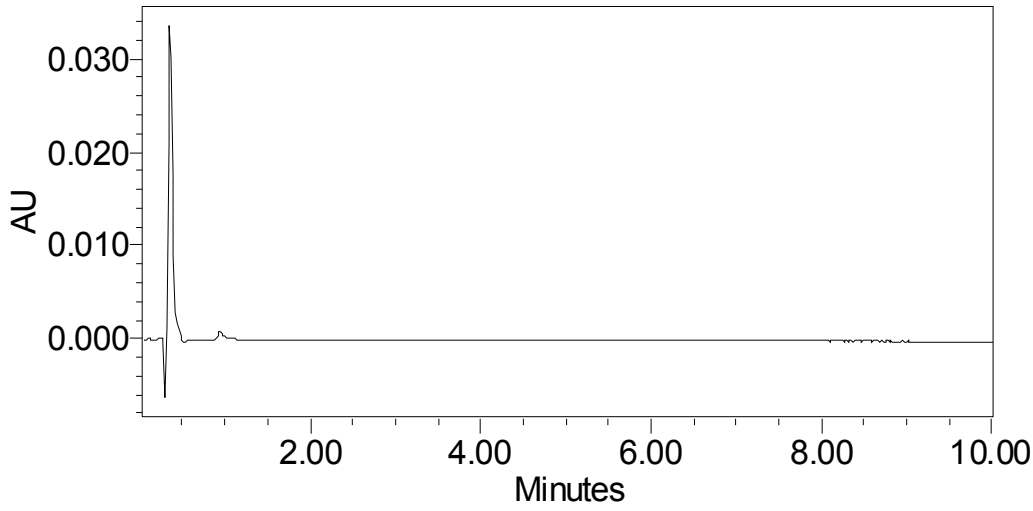


Figure 5.3 - Chromatogram of placebo demonstrating the absence of interference with the elution of 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide (retention time 2.0 min). Conditions: isocratic elution with potassium phosphate buffer (pH 7.0)/acetonitrile (47/53, v/v), flow rate 2 mL/min, injection volume 20 μL, UV wavelength 254 nm, C_{18} μ-Bondapak column (3.9 mm x 300 mm, 5-μm particle size).

The accuracy (percent recovery) of the method was evaluated by adding known quantities of 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide in the gel formulation samples to give a range of 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide concentration of 75-150% (*n* = 3) of that in a test preparation. These solutions were analysed and the amount of analyte recovered calculated. The recovery data expressed as an average percent of triplicate injections are presented in Table 5.2 and showed excellent recoveries of active compound from gel formulation.

Table 5.2 - Recovery results of 2-(diethylamino)-*N*-(2,6 dimethylphenyl) acetamide from samples with known concentration

Sample	Percent of nominal	Amount of analyte (mg)		Recovery (%, *n* = 3)	RSD (%, *n* = 3)
		Added	Recovered		
1	75	1.70	1.704	100.23	0.13
2	100	3.20	3.204	100.14	0.20
3	150	5.10	5.097	99.95	0.15

The LOD and LOQ tests for the procedure were performed on samples containing very low concentrations of analyte. The LOD was (s/n ratio 3.2) 100 ng/mL and the LOQ was (s/n ratio 10.2) 250 ng/mL and RSD 0.36% for three replicate injections.

Upon successfully completing the validation, the data was statistically analysed against its acceptance criteria by the statistician in order to test its validity. The validated method was then implemented and used routinely in the quality control laboratory for products containing 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide compound for release.

5.3 Conclusion

A new HPLC method for the assay of 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide was developed and validated successfully using systematic approach. The results showed that the method was very selective no significant interfering peak was detected; accurate with the percentage recoveries of 99.95-100.23 and reproducible with the RSD of ≤ 0.20% in each case.

The calibration curve was linear form 20-140% of the analytical concentration of 1.4 μg/mL with excellent correlation coefficient of r^2 = 1.0000. The method was sensitive as little as 100 ng/mL can be detected with the quantitation limit of 250 ng/mL. This excellent validation data did not arise by chance, but because validation was borne in mind during the method research and development phase. For example, then paraphrase the slot section “preliminary precision, linearity and robustness studies performed during the development phase that could be injected directly”. The method was transferred and used in the quality control laboratory for analysis of 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide in bulk, raw materials and final gel product for final release.

This research has made a significant and coherent contribution to new knowledge about the product, including more detailed step-by-step approach to method validation and also a very well validated (under GMP/GLP considerations) assay method.

6 Determination of Combined *p*-Hydroxy Benzoic Acid Preservatives in a Liquid Pharmaceutical Formulation by HPLC

The determination of excipients in a formulation can also be important and provide a difficult challenge to analytical scientists. For this case [8, appendix I], the individual contents of three *p*-hydroxy benzoic acid ester preservatives in a complex multi-component solution were determined following the development and validation of an HPLC method. The results / data generated from these new validated assay methods were submitted to the regulatory agency for product licence for marketing authorisation. The results were critically reviewed by the regulatory agency for its reliability, validity and approved for product licence. Thereafter, this assay method was implemented to the quality control laboratory and applied for the quantification and a stability study of all the compounds in the liquid pharmaceutical formulation for bulk, intermediate and final product release.

Combined *p*-hydroxy benzoic acid preservative is a combination of three esters including methyl hydroxybenzoate (methylparaben, MP), ethyl hydroxybenzoate (ethylparaben, EP) and propyl hydroxybenzoate (propylparaben, PP) (Figure 6.1). Its theoretical composition is given in Table 6.1. Each of these preservatives have been widely used as antimicrobial and anti-fungal agents in food, beverages, cosmetics and pharmaceuticals (Kang & Kim, 1997) because of their broad antimicrobial spectrum with good stability and non-volatility (Society of Japanese Pharmacopoeia, 1984). Hence, the simultaneous determination of these preservatives in commercial pharmaceutical products is particularly important both for quality assurance and consumer safety.

Table 6.1 - Theoretical composition of combined *p*-hydroxy benzoic acid

Component	Theoretical composition (%)
Methyl hydroxy benzoate	73.21
Ethyl hydroxy benzoate	16.07
Propyl hydroxy benzoate	10.71
Total	99.99

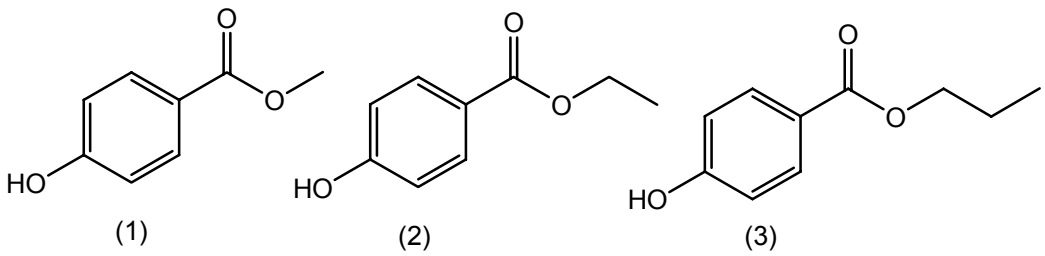


Figure 6.1 - Structures of the analytes, in order of elution: (1) methylparaben; (2) ethylparaben; (3) propylparaben.

6.1 Step-by-step new method development

A similar systematic strategy was used in developing a new method for combined *p*-hydroxy benzoic acid as discussed in Chapter 5. Initially, different chromatographic conditions were investigated to achieve best separation. Using optimised conditions the chromatographic analysis of combined *p*-hydroxy benzoic acid (pK_a ~8.4) was carried out in the isocratic mode using a mixture of 52.5% methanol in potassium phosphate buffer pH 7.05 ± 0.05 (52.5:47.5, v/v) as mobile phase. 20 μL of standard and sample solutions were injected automatically into the column. Subsequently, the liquid chromatographic behaviours of both drugs were monitored with a UV detector at 254 nm. Additionally, preliminary system suitability, precision, linearity and robustness studies performed during the development phase of the method showed that the 20 μL injection volume was reproducible and the peak response was significant at the analytical concentration chosen. Chromatograms of the resulting solutions gave excellent separation and resolution (Figure 6.2) and co-elution of excipients was not observed (Figure 6.3) at the same retention time as MP, EP and PP. The retention times are reported in Table 6.2.

Table 6.2 - Retention times (min) of methyl *p*-hydroxy benzoate, ethyl *p*-hydroxy benzoate and propyl *p*-hydroxy benzoate

Peak No.	Compound	Approximately RT (min)
1	Methyl *p*-hydroxy benzoate	2.90
2	Ethyl *p*-hydroxy benzoate	4.20
3	Propyl *p*-hydroxy benzoate	6.81

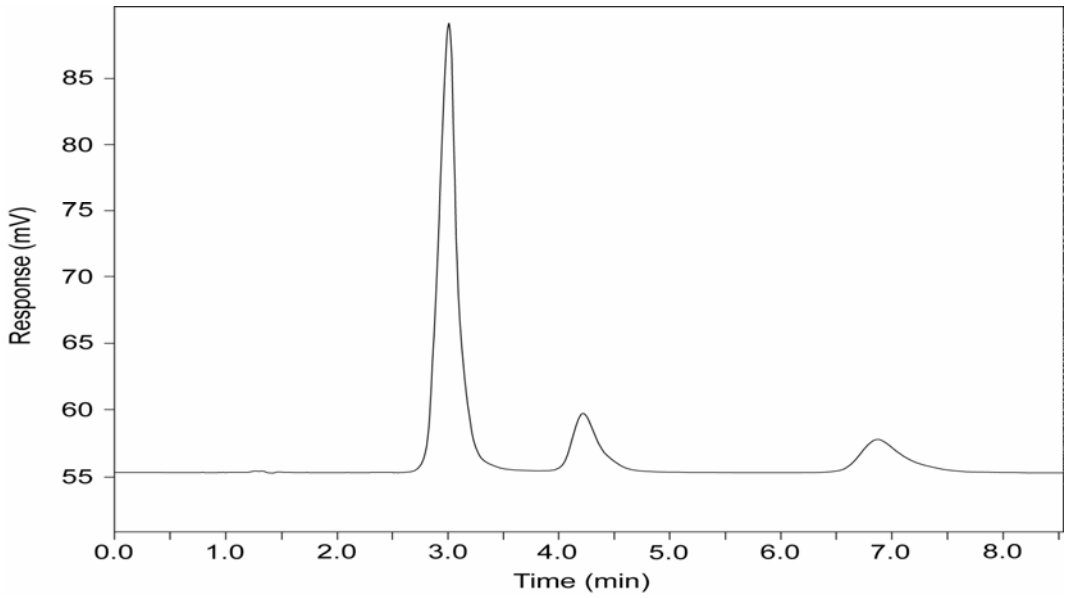


Figure 6.2 – Separation of combined *p*-hydroxy benzoic acid. Conditions: isocratic elution with aqueous solution of 52.5% (v/v) methanol containing 0.2 M potassium dihydrogen phosphate (pH 7.05), flow rate 2 mL/min, UV detection 254 nm, injection volume 20 μL, Waters μ-Bondapak C_{18} column (300 x 3.9 mm).

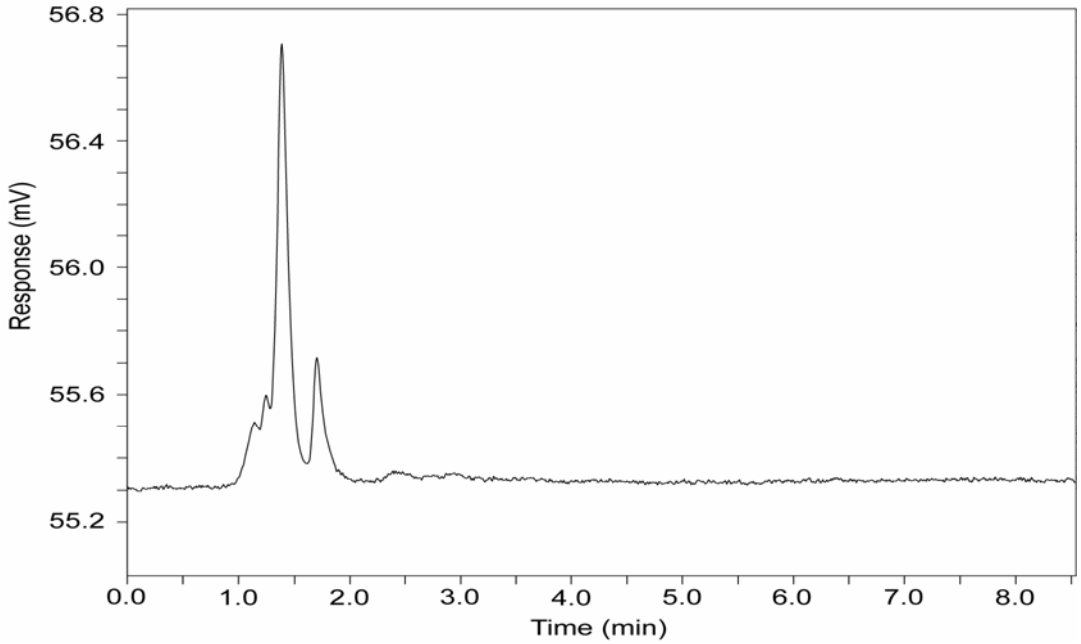


Figure 6.3 - Chromatogram of placebo without adding *p*-hydroxy benzoic acid. For experimental conditions see Figure 6.2.

6.2 Step-by-step method validation

For best practice, a similar approach was adopted in validation of this assay method as previously described in Chapter 5.

The linearity test was performed using seven different amounts of MP, EP and PP in the range of 20-140% around the theoretical values (MP 10 μg/mL, EP and PP 2 μg/mL). Solutions corresponding to each concentration level were injected and linear regression analysis of the MP, EP and PP peak areas (y) versus MP, EP and PP concentration ($\times$) were calculated (Table 6.3), MP (r^2 = 0.9999), EP (r^2 = 1.0000) and PP (r^2 = 0.9999).

Intermediate precision was studied by assaying five samples prepared by different analysts, using a different HPLC column, on a different day. The RSD across the system and analysts were calculated and found to be less than 0.6% (Table 6.3) for each of the multiple sample preparations, which demonstrated excellent precision and reproducibility for this developed new assay method.

The system precision was examined by analysing six determinations of the same batch of product at 100% of the test concentration. The samples were stored at 25°C for six months. The RSD of the areas of each paraben peak were found to be less than 0.9% (Table 6.3), which confirmed that the method was very reproducible and sufficiently precise.

Stability of sample and standard solutions were conducted and chromatographed immediately after preparation and then re-assayed after storage at room temperature for 24 hours. The results given in Table 6.3 showed that there was no significant change (< 0.14% response factor) in combined *p*-hydroxy benzoic acid concentration over this period.

A system suitability test was performed to determine the accuracy and precision of the system by injecting six replicate injections of combined *p*-hydroxy benzoic acid standard solutions. The RSD of the peak areas responses was measured. The RSD values were for MP (0.09%), EP (0.19%) and PP (0.24%) as can be seen in Table 6.3.

Table 6.3 - Method validation results of *p*-hydroxy benzoic acid

Validation step	Parameter	MP	EP	PP	Acceptance criteria
System precision	RSD (%)[a]	0.037	0.582	0.827	x < 2
Method precision: Analyst 1	RSD (%)[b]	0.163	0.421	0.356	x < 2
Analyst 2	RSD (%)	0.365	0.529	0.561	x < 2
Analyst 1 & 2	RSD (%)	0.300	0.480	0.444	x < 2
Linearity (n = 7)[c]	Correlation coefficient	0.9999	1.0000	0.9999	x > 0.9990
Standard stability[d]	% Change in response factors	0.140	0.140	0.140	x < 2
Sample stability	% Change in response factors	0.275	0.276	0.275	x < 2
System suitability	RSD (%) n = 6	0.09	0.19	0.24	x < 2

[a] Six injections

[b] Five preparations each, two injections of each preparation.

[c] At 20, 40, 60, 80, 100, 120, 140% levels.

[d] Two-day stability data.

A known quantity of pure combined *p*-hydroxy benzoic acid was added to the sample to give a concentration range of 75-125% (n = 3) of that in a test preparation. These solutions were chromatographed and the amount of combined *p*-hydroxy benzoic acid recovered calculated. Good recovery of combined *p*-hydroxy benzoic acid was observed as shown in Table 6.4.

Injections of the extracted placebo were performed to demonstrate the absence of interference (specificity) with the elution of the combined *p*-hydroxy benzoic acid. These results demonstrated (Figure 6.3) that there was no interference from the other materials (excipients) in the liquid pharmaceutical formulation, and therefore confirmed the specificity of the analytical method.

Table 6.4 - Accuracy /recovery of combined *p*-hydroxy benzoic acid from samples with known concentration

Sample	Percent of nominal	Amount of standard (mg)		Recovery (%, *n* = 3)	RSD (%, *n* = 3)
		Spiked	Found		
1	75	4.5	3.7	82.0	0.4
2	100	9.0	8.4	94.0	0.7
3	125	135	122	91.0	0.6

6.3 Conclusion

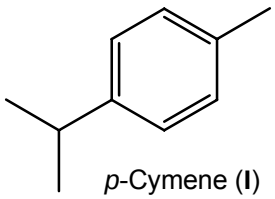
A new HPLC assay method with UV spectrophotometric detection was developed successfully for the determination of combined *p*-hydroxy benzoic acid preservatives. Preliminary studies during the method research and development phase illustrated the benefit of the step-by-step approach. The method was critically validated and the results obtained were accurate and precise with RSD $\leq$ 0.83% in each study and no significant interfering peaks were detected. The calibration curve were linear with correlation coefficient of >0.9999 for each preservative studied. The validated method was then submitted to the regulatory agency for product licence, which was successfully obtained. The method was then used for the routine quality control analysis (batch analysis and stability tests) of compounds in pharmaceutical products containing 0.3% of MP, EP and PP preservatives and the degradation products of the active compound. This method was successfully applied for the identification, quantitative analysis and stability tests of all compounds in the liquid pharmaceutical formulation for final product release.

This research has made a significant and coherent contribution to new knowledge about the product including a more detailed approach to method validation has resulted in an acceptable validated method by the regulatory agency. Also new information on shelf life extension of the product containing *p*-hydroxy benzoic acid preservatives has been obtained.

7 Method Development and Validation for the GC–FID Assay of *p*-Cymene in Tea Tree Oil Formulation

Finally in this research programme, the validation approach was evaluated as applied to another analytical technique. Here, GC was successfully used to develop a novel assay for *p*-cymene content in tea tree oil formulation [9, appendix I]. This presented a different analytical problem because of the very complex nature of this natural product, GC was the technique of choice for this method.

The use of essential oils in complementary medicine, particularly aromatherapy and also in the cosmetic and perfumery industry is becoming increasingly popular. The essential oil, which is distilled from the leaves of a tree *Melaleuca alternifolia*, commonly called tea tree is well known for its antimicrobial activity and has enjoyed increased medicinal uses in recent years. Oil of *Melaleuca alternifolia* (terpinen-4-ol type) is now clearly defined by the Draft ISO 4730. It sets a *p*-cymene content of not less than 0.5% and not more than 12%. Chemically *p*-cymene (**I**) is a 1-methyl-4-(1-methylethyl) benzene (Merck Index, 1996) occurs in a number of essential oils.



p-Cymene (**I**)

p-Cymene concentration can rise to levels approaching its upper limit (see research paper 9, appendix I). Two pathways are operating here (Figure 7.1), one involving hydrolysis of the pi bond at C-4 to produce terpinen-4-ol, the other involving oxidation of the *p*-menthane skeleton to its benzene analogue, *p*-cymene. The first pathway must involve water, naturally present in the oil through the steam distillation extraction process and possibly, trace volatile organic acids that may catalyse the reaction. The second pathway is well known in terpene

chemistry. However, the various oxidation agent and catalysts that are involved in tea tree oil degradation required further investigation.

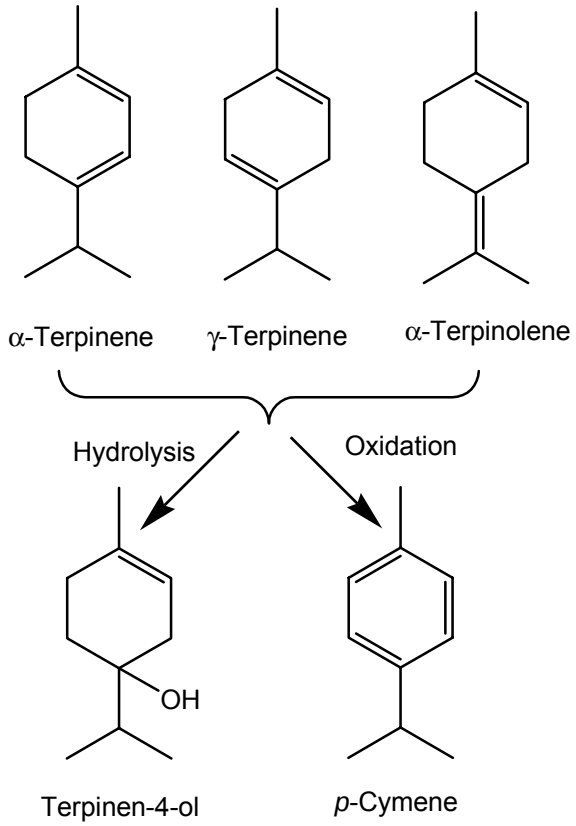


Figure 7.1 – End products of hydrolysis and oxidation of constituents of tea tree oil.

7.1 Step-by-step new method development

A similar systematic strategy was used in developing a new method for *p*-cymene as discussed in Chapter 5.

The method development for the assay of *p*-cymene was based on its chemical properties. *p*-Cymene ($CH_3C_6H_4CH(CH_3)_2$) is a non-polar molecule and, therefore, a non-polar solvent hexane (C_6H_{14}) was used as the diluent. The medium polarity five percent Carbowax was used for separation. The GC-FID parameters used in the method development were based on the boiling point (177.10 °C) and the flash point (47 °C) of *p*-cymene. The injection port and detector temperature were set to 220 °C and oven temperature was set to 100

°C. The oven programme was isothermal with a run time of 10 min. The head pressure was set to ensure a hydrogen flow of 36 mL/min. The split was then adjusted to 6:01. The solvent, column and acquisition parameters were chosen to be a starting point for the method development. However, the separations produced using these parameters were excellent. The retention time of *p*-cymene was approximately 8.45 min with good peak shape and USP tailing was approximately 1.0. Additionally, preliminary precision and linearity studies performed during the development of the method showed that the 1.5 µL injection volume was reproducible and the peak response was significant at the analytical concentration chosen. Diluting the standard and sample in hexane gave solutions that could be injected directly (without further dilution, filtration or centrifugation). Chromatograms of the resulting solutions gave very good peak shapes (Figure 7.2 and 7.3) and co-elution of excipients was not observed on the same retention time as *p*-cymene.

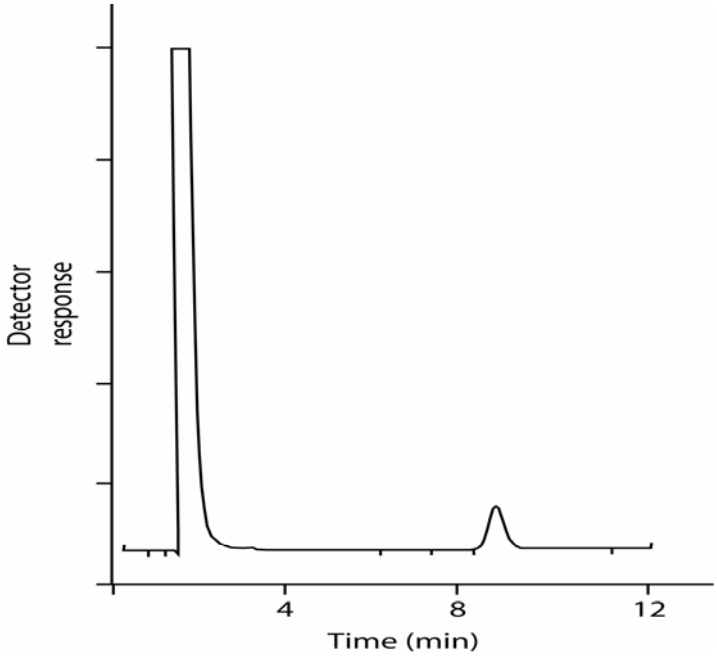


Figure 7.2 – Separation of *p*-cymene reference standard. Conditions: Phenomenex column packed 5% carbowax 20 m (6 x 0.25 mm) 80-100 mesh, hydrogen gas, FID, injector and detector temperature 220 °C, oven temperature 100 °C, flow rate 36 mL/min, injection volume 1.5 µL.

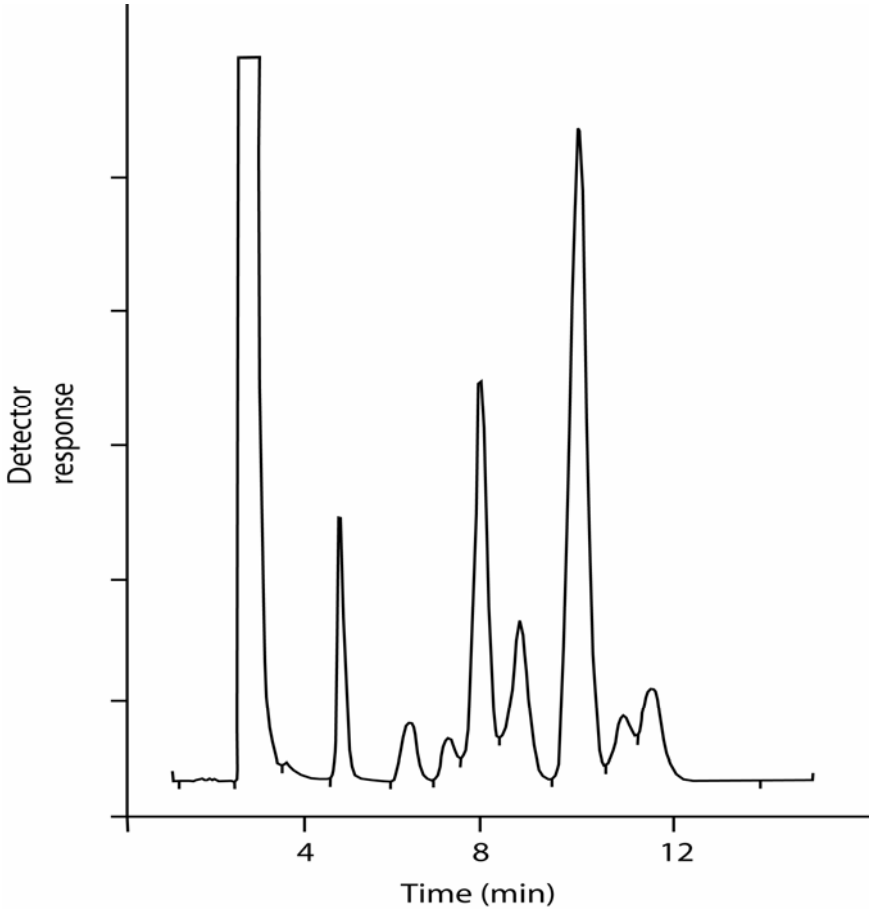


Figure 7.3 – Separation of 100% tea tree oil sample. *p*-Cymene eluted at 8.46 min. Conditions: Phenomenex column packed 5% carbowax 20 m (6 x 0.25 mm) 80-100 mesh, hydrogen gas, FID, injector and detector temperature 220 °C, oven temperature 100 °C, flow rate 36 mL/min, injection volume 1.5 μL.

7.2 Step-by-step method validation

For best practice, a similar step-by-step approach was adopted in validation of this assay method as used in Chapter 5.

Validation is a critical part of the development of a GC method. Although there is general agreement about what type of validation studies that should be done, there is great diversity in opinion as to how they are to be accomplished. The literature contains a variety of approaches to performing validation studies using gas chromatography (Robert & Eugene, 2004). This paper presents approaches

that serve as a basis to perform validation studies for most GC methods in compliance with the pharmaceutical and biotechnology fields. The validation characteristics performed were linearity, range, specificity, accuracy, precision (repeatability and intermediate precision), limit of detection and limit of quantitation.

The linearity was studied in the concentration range 20-120 µg/mL. Six solutions were prepared corresponding to 20, 40, 60, 80, 100, and 120% of the nominal analytical concentration (100 µg/mL) and the following regression equation was found by plotting the peak area (*y*) versus the *p*-cymene concentration (*x*) expressed in µg/mL: y = 354.05x + 0.218 (r^2 = 0.9995). The analyte response is linear across 80-120% of the target *p*-cymene concentration.

The precision was examined by analysing six different samples by only one operator. The repeatability (within-run precision) was evaluated by only one analyst within one day, whereas reproducibility (between-run precision) was evaluated for three different days. The RSD values for within-run precision was 0.13% and for between-run precision was 0.66% (Table 7.1), therefore meeting the acceptance criteria for the chromatographic *p*-cymene assay.

Table 7.1 - Within and between-run precision results for *p*-cymene in 100% tea tree oil formulation

Injection No.	Within-run precision		Mean between-run precision (*n* = 3 days)	
	Area (µV)	Retention time (min)	Area (µV)	Retention time (min)
1	29.2152	8.46	21.1505	8.45
2	29.0633	8.45	21.2953	8.45
3	29.6717	8.45	21.3440	8.45
4	28.3922	8.46	21.3291	8.46
5	28.5053	8.45	21.3453	8.45
6	28.2665	8.45	20.9958	8.45
Mean (6)	28.8523	8.45	21.2450	8.45
RSD (%)	0.13	0.06	0.66	0.05

The accuracy of the method was determined by fortifying sample with known amounts of the *p*-cymene reference substance at four concentration levels (0.5, 0.10, 0.05 and 0.01 mg/mL) and injected in triplicate. Mean recoveries for the samples analysed were found to be 95.3%.

The lower limit of detection for *p*-cymene was found to be (signal-to-noise = 3) 2.08 μg/mL. The LOQ values for *p*-cymene were found to be (signal to noise = 10) 10.39 μg/mL and RSD less than 2% for three replicate injections.

Assay interference (specificity) was investigated by injecting 12 month old stability solutions of tea tree oil. No interfering peaks were observed. Therefore, this method was specific for *p*-cymene.

7.3 Stability study

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light, and enables storage conditions to be recommended and re-test and shelf lives to be established.

Tea tree oil is susceptible to oxidation and this is highlighted by the analytical data (Table 7.2) presented for two batches of tea tree oil. Samples of tea tree oil containing *p*-cymene content were packed in sealed dark brown glass bottles (10 mL) and stored at room temperature. The samples were withdrawn periodically (0, 1, 2, 3, 6, 9, 12 and 24 months) and the validated method was utilized successfully for analysing these stability samples. The data obtained from stability batches is evident that the assay is suitable for the quantitative analysis and stability testing of *p*-cymene content in the tea tree oil formulation.

Table 7.2 - Stability data for *p*-cymene content in tea tree oil over 24 months

Batch #	Specification	Interval time in months							
		0	1	2	3	6	9	12	24
1	0.5-12%	2.2	2.8	2.7	2.4	3.1	3.0	3.5	10.1
2	0.5-12%	2.3	2.5	2.4	2.9	3.3	3.6	3.8	10.3

In this stability investigation, it could be seen that *p*-cymene concentration can rise to levels approaching its upper limit (maximum 12%). The key chemical indicator of oil treatment during processing and storage is *p*-cymene and elevated levels are usually an indication of poor storage conditions, old oil or harsh treatment during extraction.

Tea tree oil undergoes oxidation on storage via a number of different routes, some of which actually enhance oil, quality during the early stages. For long term storage for large quantities of neat oil stainless steel and aluminium, along with a nitrogen purge, provide the greatest stability (approximately two years). Dark glass with caps containing an impenetrable liner provides excellent long-term storage of small quantities. Stability of formulated products in glass is far superior to that in high-density polyethylene (HDPE) and polypropylene. However, laminates that incorporate aluminium or a fluorinated liner will enhance shelf life, although this may be dependent on the formulation matrix and tea tree oil concentration.

7.4 Conclusion

A new analytical method for the assay of *p*-cymene from a tea tree oil formulation was developed and validated using validated gas chromatography system. The validation study showed good linearity, (r^2 = 0.9995) sensitivity, accuracy and precession (RSD $\leq$ 0.66%). The proposed procedure was used in a quality control laboratory for analysis of formulations containing *p*-cymene products for final release.

This research has made a significant and coherent contribution to new knowledge including new validated GC method; new information on active *p*-cymene content; new information on tree tea oil product; a more detailed approach to method validation; a robust validated method and newly identified knowledge about product and shelf life extension.

8 Overall Conclusion and Suggestions for Further Work

Efficient analytical method validation is a critical element in the development of pharmaceuticals. Indeed, the principle of the validation of these methods is today widely spread in all the domains of activities where measurements are made. Nevertheless, the simple question of acceptability or not of an analytical method for a given application, remains incompletely determined in several cases despite the various regulations relating to the good practices (GLP, GMP) and other documents of normative character (ICH, USP, FDA). There are many official documents describing the criteria of validation to be tested, but they do not propose any simple step-by-step approaches to method development and validation activities and limit themselves most often to the general concepts.

For those reasons, critical analysis of the situation in the author's papers with respect to simple and step-by-step approach to analytical method development and validation has proved to be a very major contribution to new knowledge. A number of scientists and experts on method development and validation have used as guidelines and cited these papers as authoritative work. These papers have been cited in the field of advanced analytical chemistry books, high impact critical peer-reviewed journals and in regulatory compliance training courses / workshops around the world.

Application of identified good practice has led to a number of very sound, reliable, rapid and robust methods, each of which has performed well in practice and especially in some cases, made a major contribution to new knowledge and the development of the substance/product involved.

The concept of validation covered in the literature is mostly associated with development and validation of chromatographic methods. The description of equipment qualification is also discussed in the literature to a lesser extent (Grisanti & Zachowski, 2002; Zanetti & Huber, 1996; Hall & Dolan, 2002). However, the description of instrument qualification generally does not include

the need to validate the computer aspect of the instrument (software and computer hardware), which should be considered an important part of the qualification package prior to method validation.

Another one of the critical issues that have not been addressed by the consensus reports is when method validation is necessary. In the current highly cost-conscious environment, the balance of costs and benefits is an issue. The literature and regulatory agencies contain diverse approaches to performing method validation, as discussed above; there is a need for a single guideline worldwide on performing method validation. ICH should expand their effort on method validation globally for more input and set the minimum standard “one world – one standard” which ensures patient safety. Alternative guidelines to ICH are not preferred; the world should stay with ICH to achieve global harmonisation. This is because it is easier to revise and build upon existing guidelines that are already in operation. The guideline should cover step-by-step approaches from drug development to marketing authorisation. The guideline should also cover all the pre-validation requirement activities such as analytical instrument qualification (validation), which is another aspect of method validation. The benefits to the regulated industry of achieving the desired state (globally harmonised) will ensure, better quality, less recalls, less supplements, and facilitate new technology and continuous improvement. Focus will be on critical quality attributes and controls and will reduce regulatory burden of post approval changes.

USP and FDA have accepted ICH documents and have updated general chapters but old methods do not meet the criteria (e.g., TLC) and are currently not being updated. A major challenge to many pharmaceutical industries of today who still use old validated methods is that they need to upgrade/revalidate in order to meet current regulatory standards. The USP28-NF23 contains over 4000 monographs and over 180 general chapters. Approximately 200 drug substance, excipient, and drug product monographs are needed. Approximately 800-1200 current monographs need to be updated.

In considering the balance, the question of where things are going must be asked. Should a start be made with a blank piece of paper and a “desired state”

for pharmacopoeias and the public standards be developed. Does it make sense to retrofit for compendial standards from 1820 to the "21st century? The testing of compendial standards and the specification process has remained nearly unchanged. Adoption for the future is a must and re-assessment, re-evaluation and evolution is necessary. It is critical for the USP to be engaged with the changes and paradigm shift occurring at the FDA. Specifications must be based on FDA approved materials. The purpose of a monograph needs to be re-assessed. The role of monograph in release, stability and marketing surveillance needs to be re-evaluated.

The evolution and application of general chapters also needs to be considered: How will they be impacted by the changes and be applied (enforced) in the future? How will general chapters be developed to adapt to the new paradigm and how will existing chapters be modified? Will there be dual standards in the USP to accommodate the new approaches and how will content uniformity be addressed? Should it be addressed in the USP or should the current chapter remain and companies left with the option of different specifications / analytical methods based on agreements with the FDA? Chapters should not duplicate efforts underway in other areas (e.g. American Society for Testing and Materials (ASTM International Standards).

In Global Harmonisation: the path that the USP takes must be carefully considered. Since the USP has been engaged with the Pharmacopoeial Discussion Group (PDG) on harmonisation of general chapters and monographs it is important to move forward in collaboration with the other Pharmacopoeias and not in isolation. Work that has been done by the PDG must not be undone. The USP should work with the PDG to ensure that the current harmonised items are not negatively impacted and also work prospectively to harmonise new concepts.

Also input from the pharmaceutical industry and other users of their volumes is, therefore, essential in the provision of information as to what is most needed in the prioritisation and harmonisation of the work programmes. In a continually changing environment the PDG looks to industry to produce suggestions as to what issues need to be addressed in the formulation of its work programmes.

Industry professionals are, therefore, urged to take a keen and active interest in the work of the PDG, to monitor its progress by reading the appropriate forums and to let it know where major problems are occurring.

In overall conclusion, the status quo is no longer adequate and evolution is necessary. In order to prepare for the future the role of monographs and general chapters must be critically evaluated and what changes that must occur must be considered. These must be linked to the changes occurring at FDA and industry. The USP must engage the FDA, industry and PDG in the evolution process.
In final summary, the authors work as set out in the submitted papers provides a significant contribution to the knowledge base upon which then future development must take place and has included:

i) A critical review of the literature on analytical method development and validation – [1-4, appendix I];
ii) Application of sound experimental modern analytical chemistry techniques and development of rapid, simple, reliable and very robust new assay methods – [5-9, appendix I];
iii) Independent research that makes a significant and coherent contribution to new knowledge – [5-9, appendix I];
iv) The creation and interpretation of knowledge, which extends the forefront of discipline through original peer-reviewed and validated research – [5-9, appendix I];
v) Development of strategies to implement new processes at work place – [1-9, appendix I].

The author's contribution to the development of pharmaceutical standards for use in decision making and to achieve compliance with ICH, FDA and USP have been demonstrated, and the author's input to this development has been described. The author's development strategy for implementing analytical method development and validation systematic approaches with high degree of research standards has been demonstrated. The role of future of USP, FDA and ICH in global harmonisation in the field of analytical method validation has been discussed. The large number of citations already made to the submitted papers

verifies the fact that these combined constitute a major and coherent contribution to new knowledge.

References

Bressolle, F., Petit, M.B. & Audran, M. (1996). Validation of liquid chromatographic and gas chromatographic methods: application to pharmacokinetics. *J. Chromatogr. B*. **686**, 3-10.

Badea, D., Ciutaru, L., Lazar, D.N. & Tudose, A. (2004). Rapid HPLC method for the determination of paclitaxel in pharmaceutical forms without separation. *J. Pharm. Biomed. Anal.* **34**, 501–507.

Crowther, J.B. (2001). Validation of pharmaceutical test methods. *In: Handbook of Modern Pharmaceutical Analysis*, pp.415-543. California: Academic Press.

Clarke, G.S. (1994). The validation of analytical methods for drug substances and drug products in UK pharmaceutical laboratories. *J. Pharm. Biomed. Anal*. **12**, 643-652.

Carr, G.P. & Wahlich, J.C. (1990). A practical approach to method validation in pharmaceutical analysis. *J. Pharm. Biomed. Anal*. **8**, 613-618.

Calderazzo, F., Marchetti, F., Pampaloni, G. & Passarelli, V. (1999). Co-ordination properties of 1,10-phenanthroline-5,6-dione towards group 4 and 5 metals in low and high oxidation states. *Dalton Trans*. **24**, 4389-4396.

D'Alessandro, D.M., Kelso, L.S. & Keene, F.R. (2001). Stereochemical influences on intervalence charge transfer in homodinuclear complexes of ruthenium. *Inorg. Chem*. **40**, 6841-6844.

Daraghmeh, N., Al-Omari, M., Badwan, A.A. & Jaber, A.M.Y. (2001). Determinaton of sildenafil citrate and related substances in the commercial products and tablet dosage form using HPLC. *J. Pharm. Biomed. Anal.* **25**, 483–492.

Ermer, J. (2001). Validation in pharmaceutical analysis. Part 1: An integrated approach. *J. Pharm. Biomed. Anal*. **24**, 755-767.

FDA, Centre for Drug Evaluation and Research. (1994). Reviewer Guidance, validation of chromatographic methods, United States.

FDA, Centre for Drug Evaluation and Research. (1987). Guideline for submitting samples and analytical data for methods validation, United States.

Green, M.J. (1996). A practical guide to analytical method validation. *Anal. Chem.* **68**, 305A-309A.

Grisanti, V. & Zachowski, E.J. (2002). Operational and performance qualification.

LC/GC N.A. **20**(4), 356-362.

Harvey, I.M., Baker, R. & Woodget, B. (2002). Part 2, the analytical method. In: Series of tutorial lectures: Quality assurance in analytical sciences, http://new.filter.ac.uk/database/getinsight.php?id=47&seq=2, 25 January 2006.

Halperin, J.A. (1993). Proceedings of the second International Conference on Harmonisation, topic 3: pharmacopoeias, Orlando.

Hall, G. & Dolan, J.W. (2002). Performance qualification of LC systems. *LC/GC* N.A. **20**(9), 842-848.

Hiort, C., Lincoln, P. & Nordén, B. (1993). DNA binding of L- and L-[Ru(phen) $DPPZ_2]^{2+}$.*J. Am. Chem. Soc*. **115**, 3448-3454.

International Conference on Harmonisation (ICH) (2005). Q2(R1): Validation of analytical procedures: Text and Methodology, Geneva, Switzerland.

Imor, S., Morgan, R.J., Wang, S., Morgan, O. & Baker, A.D. (1996). An improved preparation of 4,7-phenanthrolino-5,6:5',6'-pyrazine. *Synth. Commun*. **26**, 2197-2203.

Itoh, S., Fukushima, H., Komatsu, M. & Ohshiro, Y. (1992). Heterocyclic o-quinones. Mediator for electrochemical oxidation of NADH. *Chem. Lett*. **8**, 1583-1586.

Kradolfer, F., Sachmann, W. & Bassil, G.T. (1960). Comparative microbiological studies of amebicides with special reference to the derivatives of phenanthroline quinine. *Ann. Biochem. Expt. Med*. **20**, 519-527.

James. A. T. & Martin, A. J. P. (1952). Gas-liquid partition chromatography: the separation and micro-estimation of volatile fatty acids from formic acid to dodecanoic acid. *Biochem. J.* **50,** 679-690.

Kang, S.H. & Kim, H. (1997). Simultaneous determination of methylparaben, propylparaben and thimerosal by high-performance liquid chromatography and electrochemical detection. *J. Pharm. Biomed. Anal*., **15**, 1359-1364.

Mlochowski, J. (1974). Investigations on reactivity of phenanthrolines. Part III. Bromination of phenanthrolines. *Rocz. Chem*. **48**, 2145-2155.

Merck index. (1996). AT-2832. **12**, pp. 467-467.

Mendez, A.S.L., Steppe, M. & Schapoval, E.E.S. (2003). Validation of HPLC and UV spectrophotometric methods for the determination of meropenem in pharmaceutical dosage form. *J. Pharm. Biomed. Anal.* **33**, 947–954.

Niessen, W.M.A. (2003). Progress in liquid chromatography-mass

spectrometry instrumentation and its impact on high-throughput screening. *J. Chromatogr. A* **1000**, 413-436.

Robert, L.G. & Eugene, F.B. (2004). Modern practice of gas chromatography, 4th ed. Wiley-Interscience, New Jersey.

Shabir, G.A., Lough, J.W., Arain, S.A. & Bradshaw, T.K. (2007a). Evaluation and application of best practice in analytical method validation. *J. Liq. Chromatogr. Rel. Technol.* **30**, 311-333.

Shabir, G.A. & Arain, S.A. (2007b). Method development and validation of 9α-fluoro-16β-methyl-prednisolone-17-valerate by HPLC. *J. Liq. Chromatogr. Rel. Technol.* **30,** 1923-1932.

Stegman, M.B. & Stoukides, C.A. (1996). Resolution of tumor pain with EMLA cream.*Pharmacotherapy*. **16**, 694-697.

Society of Japanese Pharmacopoeia. (1984). The Japanese Standards of Cosmetic Ingredients-with Commentary, 2nd ed. Yakuginippousha, Tokyo.

Trullols, E., Ruisanchez, I. & Rius, F.X. (2004). Validation of qualitative analytical methods. *Trends Anal. Chem.* **23**, 137-145.

U.S. Pharmacopeia 30. (2007). General chapters (1225) Validation of compendial procedures, United States Pharmacopeal Convention, Inc., Rockville Maryland. pp. 680.

U.S. Pharmacopoeia 30. (2007). General chapters (621) Chromatography, United States Pharmacopeal Convention, Rockville Maryland. pp. 243.

U.S. FDA HSS, 21 CFR Part 211. (1996). Current good manufacturing practice of certain requirements for finished pharmaceuticals, Proposed Rule.

Wilson, T.D. (1990). Liquid chromatographic methods validation for pharmaceutical products.*J. Pharm. Biomed. Anal*. **8**, 389-400.

Xilinas, M. & Gerolymatos, P.N. (2000). Use of phanquinone for the treatment or prevention of memory impairment. *Int. patent application WO 0040244.* March 20, 2000.

Xilinas, M. (2002). Use of chelators in the treatment of macular degenerative disease. *Int. patent application WO 0255081.* July 18, 2002.

Xilinas, M. & Hannoun, C. (2002). Use of sequestrants for treating prion diseases. *Int. patent application WO 0251415.* July 4, 2002.

Zanetti, M. & Huber, L. (1996). Validating automated analytical instruments to meet regulatory and quality standard regulations. *Mikrochim. Acta*. **123**(1-4), 23-31.

Appendix I

Re-Prints of Author's Published Papers

[1] Shabir, G.A. (2004). HPLC method development and validation for pharmaceutical analysis. *Pharm. Technol. Eur.* **16**(3), 37-49.

[2] Shabir, G.A. (2004). Step-by-step analytical methods validation and protocol in the quality system compliance industry, *J. Validation Technol.* **10**(4), 314-324.

[3] Shabir, G.A. (2004). A practical approach to validation of HPLC methods under current good manufacturing practices. *J. Validation Technol.* **10**(3), 210-218.

[4] Shabir, G.A. (2003). Validation of HPLC methods for pharmaceutical analysis: Understanding the differences and similarities between validation requirements of the U.S. Food and Drug Administration, the U.S. Pharmacopoeia and the International Conference on Harmonisation. *J. Chromatogr. A.* **987**(1-2), 57-66.

[5] Shabir, G.A. & Forrow, N.J. (2003). Validation of a reversed-phase HPLC method for 1,10-phenanthroline-5,6-dione and analysis of its impurities by HPLC-MS. *J. Pharm. Biomed. Anal.* **33**(2), 219-230.

[6] Shabir, G.A. & Forrow, N.J. (2005). Development and validation of a HPLC method for 4,7-phenanthroline-5,6-dione and identification of its impurities by HPLC-MS/APCI. *J. Chromatogr. Sci.* **43**(4), 207-212.

[7] Shabir, G.A. (2004). Determination of 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide in a gel pharmaceutical formulation by high-performance liquid chromatography. *J. Chromatogr. Sci.* **42**(5), 280-283.

[8] Shabir, G.A. (2004). Determination of combined *p*-hydroxy benzoic acid preservatives in a liquid pharmaceutical formulation and assay by HPLC. *J. Pharm. Biomed. Anal.* **34**(1), 207-213.

[9] Shabir, G.A. (2005). Method development and validation for the GC-FID assay of *p*-cymene in tea tree oil formulation. *J. Pharm. Biomed. Anal.* **39**(3-4), 681-684.

PAPER 1

HPLC method development and validation for pharmaceutical analysis. Pharmaceutical Technology Europe, 2004

PAPER 2

Step-by-step analytical methods validation and protocol in the quality system compliance industry.

Journal of Validation Technology, 2004

PAPER 3

A practical approach to validation of HPLC methods under current good manufacturing practices.

Journal of Validation Technology, 2004

PAPER 4

Validation of HPLC methods for pharmaceutical analysis: Understanding the differences and similarities between validation requirements of the U.S. Food and Drug Administration, the U.S. Pharmacopoeia and the International Conference on Harmonisation.

Journal of Chromatography A, 2003

PAPER 5

Validation of a reversed-phase HPLC method for 1,10-phenanthroline-5,6-dione and analysis of its impurities by HPLC-MS.

Journal of Pharmaceutical and Biomedical Analysis, 2003

PAPER 6

Development and validation of a HPLC method for 4,7-phenanthroline-5,6-dione and identification of its impurities by HPLC-MS/APCI.
Journal of Chromatographic Science, 2005

PAPER 7

Determination of 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide in a gel pharmaceutical formulation by high-performance liquid chromatography. Journal of Chromatographic Science, 2004

PAPER 8

Determination of combined *p*-hydroxy benzoic acid preservatives in a liquid pharmaceutical formulation and assay by HPLC.

Journal of Pharmaceutical and Biomedical Analysis, 2004

PAPER 9

Method development and validation for the GC-FID assay of *p*-cymene in tea tree oil formulation.

Journal of Pharmaceutical and Biomedical Analysis, 2005

Abbott
A Promise for Life

Appendix II

Statement of Authorship of Publications

On behalf of (*Ghulam Shabir)*

By (*Nigel Forrow, PhD, Abbott Diabetes Care, Abbott Laboratories, UK)*

I confirm that (Ghulam Shabir) contributed _85_ % to the following publications as shown below:

[1] Shabir, G.A. & Forrow, N.J. (2003). Validation of a reversed-phase HPLC method for 1,10-phenanthroline-5,6-dione and analysis of its impurities by HPLC-MS. *J. Pharm. Biomed. Anal.* **33**(2), 219-230.

[2] Shabir, G.A. & Forrow, N.J. (2005). Development and validation of a HPLC method for 4,7-phenanthroline-5,6-dione and identification of its impurities by HPLC-MS/APCI. *J. Chromatogr. Sci.* **43**(4), 207-212.

Signature _____

Date _____

Appendix III

Top Cited Article 2002 – 2007 Award