DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF DIPHENHYDRAMINE, PROMETHAZINE, CHLORPHENIRAMINE AND EPHEDRINE IN COLD-COUGH SYRUPS

A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Pharmacy in Pharmaceutical Analysis

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DECLARATION

This research thesis is my original work and has not been presented elsewhere for examination.

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DEDICATION

This work is dedicated to my family.

My parents, Mr. and Mrs. Njuguna for their unwavering faith and belief that I can achieve all I dream of.

To Kim, Maureen, Joe and Edith for trying to understand the meaning of it all.

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ABBREVIATIONS

°C	Centigrade (degrees)
µm	Micrometer
Å	Angstrom unit
B.P.	British Pharmacopoeia
C18	Octadecyl silane reversed phase liquid chromatography stationary phase
cm	Centimeter(s)
CNS	Central Nervous System
CPM	Chlorpheniramine
DPH	Diphenhydramine
EPD	Ephedrine
FTIR	Fourier Transform Infra-Red
g	Gram
GC	Gas Chromatography
GIT	Gastro-Intestinal Tract
h	Hour(s)
HCl	Hydrochloride
HPLC	High Performance Liquid Chromatography
I.C.H.	International Committee on Harmonization
I.P.	International Pharmacopoeia
IR	Infra-Red
k'	Capacity factor

ABBREVIATIONS

kg	Kilogram
KShs.	Kenyan Shillings
LC	Liquid Chromatography
LC-MS	Liquid Chromatography coupled to Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantitation
M	Molar (concentration)
MAL	Maleic acid
MeOH	Methanol
mg	Milligram(s)
min	Minute(s)
mL	Milliliter(s)
mm	Millimeter
Mw	Molecular Weight
nm	Nanometer(s)
OTC	Over-The-Counter
pK_a	Acid dissociation constant
PRM	Promethazine
QA	Quality Assurance
QC	Quality Control
R^2	Square of Pearson correlation regression coefficient

ABBREVIATIONS

RSD	Relative Standard Deviation
S/N	Signal to Noise (Ratio)
TBAH	Tetrabutyl ammonium hydroxide
TBAHS	Tetrabutyl ammonium hydrogen sulphate
TEA	Triethylamine
TLC	Thin Layer Chromatography
U.S.P.	United States Pharmacopoeia
UV	Ultra-violet
Ver.	Version
λ_{max}	Wavelength of maximum absorbance

ABSTRACT

The quality of essential drugs used to treat diseases such as HIV/AIDS, tuberculosis and malaria is often monitored locally through market surveillance initiatives driven by different Ministry of Health departments in Kenya. The same scrutiny is not applied to drugs used in the treatment of less serious ailments. This is the case especially with drug mixtures used to relieve the symptoms associated with conditions such as the common cold. One of the reasons for the absence of quality monitoring is the lack of reliable, accurate or cost-effective analytical methods capable of simultaneously analyzing the multiple active ingredients in such products.

In this study, a simple, rapid, precise and sensitive isocratic elution reversed-phase liquid chromatographic method was developed for the simultaneous determination of the antihistamine drugs diphenhydramine, promethazine and chlorpheniramine as well as the decongestant ephedrine contained in commercially available cold-cough syrups in the Kenyan market. The effect of chromatographic parameters including use of both inorganic and organic mobile phase buffers, pH, column temperature, organic modifier concentration and ion pairing agents were studied during method development.

Combinations of these drugs were separated by the liquid chromatography method comprising a mobile phase consisting of methanol-water-triethylamine-0.2 M ammonium acetate pH 5.0 (50:9.85:0.15:40, % v/v/v/v) delivered at a flow rate of 1.0 mL per minute with ultraviolet detection at 254 nm. The stationary phase used was a silica based octyldecyl silane Gemini-NX column of dimensions 250 mm length and 4.6 mm internal diameter with particle size 5 µm maintained at a temperature of 40 °C.

Validation of the method showed that it exhibited good linearity over the 25% to 150% range of the analytical concentration with linear regression coefficient R^2 values of 0.9999 for ephedrine, 0.9997 for both chlorpheniramine and diphenhydramine and 0.9979 for promethazine. Limits of detection were 234 ng, 0.2 ng, 13 ng and 0.04 ng while the limits of quantitation were 1125 ng, 1.1 ng, 32.6 ng and 0.7 ng for ephedrine, chlorpheniramine, diphenhydramine and promethazine respectively. The method was found to exhibit good precision with the intra-day analysis coefficients of variation ranging from 0.9% to 1.7% and inter-day coefficients of variation being 1.7% to 2.1% for the four drug compounds.

The developed method was used to analyze twelve batches of commercially available samples of cold-cough syrups from the Kenyan market. Results obtained indicated low levels of ephedrine in all the samples tested with assay values ranging from 71.8% to 89.7% of the labeled amount. Promethazine was also noted to exhibit inter-batch variation with assay values ranging from 69.1% to 103%. Chlorpheniramine and diphenhydramine were found to exhibit the least degree of inter-product and inter-batch variation with assay values ranging from 93.4% to 100% and 86.1% to 96.0% respectively.

From the findings obtained in the evaluation of commercial samples, it can be concluded that the developed method can be adopted for the routine quality analysis of these cold-cough medicine ingredients by the pharmaceutical industry and drug regulatory authority quality control laboratories.

days. The annual cumulative economic burden of the common cold worldwide is considerable in terms of cost of remedies and hours of work lost.

The common cold is caused by different viruses, most common of which are the rhinoviruses, respiratory syncytial virus, coronaviruses and adenoviruses. The type of virus causing the infection and the symptoms that occur is determined by such factors as the season of the year, age of the patient and history of prior exposure. Infections resulting from respiratory syncytial virus and parainfluenza viruses are most common and severe in children younger than 3 years of age. Parainfluenza viruses often produce lower respiratory symptoms with first infections, but less severe upper respiratory symptoms on re-infections.

Rhinoviruses, of which there are more than 100 serotypes, are the most common cause of colds in persons aged between 5 and 40 years old. Although individuals acquire lifelong immunity to an individual serotype, it would take an impossibly long time to attain immunity against all serotypes [3].

1.2 Symptoms, complications and diagnosis of the common cold

Infection by any of the causative viruses is followed by an incubation period of 24 to 72 h. Initial symptoms of the common cold often include a "scratchy" or sore throat, followed by sneezing, rhinorrhea, nasal obstruction and malaise sometimes accompanied by fatigue, headaches, muscle weakness and loss of appetite. Temperature is usually normal, particularly when the causative pathogen is a rhinovirus or coronavirus. The nasal secretions produced are watery and profuse during the first days but then become more mucoid and purulent. Cough is usually mild but often lasts up to two weeks. Most

symptoms due to uncomplicated colds resolve within 10 days but are often more severe in infants and young children [4].

Complications of the common cold are mostly seen in children, with acute bacterial otitis media being most common. In children under three years of age, 90% of acute otitis media cases are often preceded or accompanied by common cold. In adults, acute sinusitis is a more frequent complication of the common cold than otitis media [5]. Bacterial super-infections of the sinuses, middle ear or both are also potential complications. Viral pneumonia or worsening of bronchospastic airway disease (such as asthma) is seen particularly in children and immunocompromised hosts [6]. In some cases, severe complications of the common cold, particularly involving the middle ear may result in hearing loss or pneumonia in the case of lower respiratory tract involvement [7].

Diagnosis of the common cold is generally made clinically and presumptively, without diagnostic tests. Allergic rhinitis is the most important consideration in differential diagnosis [4].

1.3 Prevalence and incidence of the common cold

No statistics are available on the global prevalence and incidence of the common cold, however, the impact of this illness in countries such as the United States of America (USA) has been published. In the USA, upper respiratory tract infections, including the common cold, are the most common types of infectious diseases among adults. It is estimated that each adult in the USA experiences two to four respiratory infections annually. The morbidity of these infections is measured by an estimated 75 million physician visits per year, approximately 150 million days lost from work, and more than

$10 billion in costs for medical care. Serotypes of the rhinoviruses account for 20 to 30 percent of episodes of the common cold. However, the specific causes of most upper respiratory infections are undefined [8].

1.4 Treatment of common colds

There is no specific treatment for the common cold. Management of the condition mainly involves amelioration of the symptoms using a variety of drug compounds of different therapeutic classes.

Herbal remedies that have been used traditionally to relieve symptoms of the common cold and research even carried out to determine the effectiveness of some. Plants such as eucalyptus, basil, hyssop, pine, rosemary and thyme all contain volatile oils that possess antimicrobial properties, stimulate mucous membranes and together with their expectorant properties, help to loosen and clear phlegm. Garlic has been demonstrated to be effective in non-specific prevention of acute respiratory infections [9].

Conventional antipyretics and analgesics such as paracetamol and ibuprofen may relieve fever or pain and inflammation associated with sore throat. Nasal obstruction may improve with topical or systemic decongestants that act by decreasing oedema through vasoconstriction. Rhinorrhea may be decreased by first generation antihistamines or anticholinergic compounds such as intranasal ipratropium bromide. First-generation antihistamines such as triprolidine, chlorpheniramine and promethazine frequently produce sedation, but second generation (non-sedating) antihistamines such as cetirizine, ebastine and loratadine are ineffective for treating the condition [4]. Anti-tussives such as codeine and dextromethorphan act centrally to suppress the cough reflex associated with common colds at the medullary cough center.

Other non-conventional remedies such as zinc and vitamin C have all been evaluated as common cold therapies but none has been clearly demonstrated to be beneficial.

There are no vaccines available for prevention of the illness because of the numerous causative viral serotypes. Polyvalent bacterial vaccines, citrus fruits, vitamins, ultraviolet light, glycol aerosols and other folk remedies do not prevent the common cold. However, hand washing and use of surface disinfectant in a contaminated environment may reduce spread of infection. Experimental therapies with interferon-2α and interleukocyte A interferon show some role in the prevention of colds. Active vaccination can be used for influenza types A and B along with the prophylactic use of amantadine or rimantadine.

Use of antibiotics in management of common colds is not recommended unless there is evidence of secondary bacterial infection. In patients with chronic lung disease, antibiotics may be administered with less restriction [4,6].

1.5 Antihistamines and decongestants used in common cold

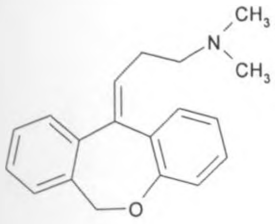
Although incorporated in numerous medications that are used for the relief of symptoms associated with the common cold, the use of antihistamines for this purpose remains controversial. In many sources, these compounds are not recommended especially since studies have shown that histamine levels are not significantly elevated in colds caused by rhinoviruses [10]. However, when antihistamines are incorporated into cold remedies, it is their anticholinergic properties rather than their antihistamine activity that are targeted to provide symptomatic relief [11]. Thus, those compounds devoid of anticholinergic activity are generally not incorporated in cold relief regimens.

The term "antihistamines" is generally used to refer to compounds that act as antagonists at H_1 histamine receptors in the body. These compounds are used primarily in the symptomatic relief of hypersensitivity reactions such as urticaria, angioedema, rhinitis and conjunctivitis as well as in controlling pruritus associated with skin disorders. Some H_1-receptor antagonists have anti-emetic properties and are used to control nausea and vomiting. Many antihistamines cause some degree of sedation although this generally becomes less troublesome after a few days of continued use [12]. Other adverse effects may include antimuscarinic effects, extra-pyramidal symptoms, gastro-intestinal disturbances and blood disorders. Newer antihistamines are less likely to cause sedation or antimuscarinic effects.

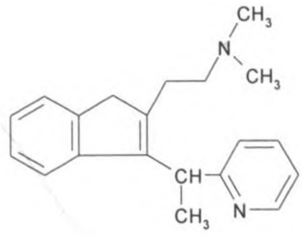
Antihistamines are classified as being either first or second generation compounds. First generation antihistamines are characterized by their central nervous system (CNS) effects particularly sedation due to their ability to cross the blood-brain barrier. On the other hand, the newer second generation or non-sedating antihistamines do not cross the blood-brain barrier to enter the CNS in appreciable amounts when administered at therapeutic doses. H_1-receptor antagonists can be classified into seven groups (Table 1.1 and Figure 1.1) based upon their chemical structure [11].

Table 1.1. Chemical classification of histamine H_1-receptor antagonists

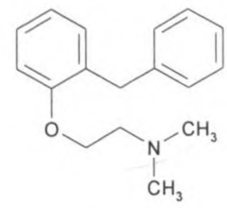
Chemical Class	Drugs
Tricyclic dibenzoxepins	Doxepin
Alkylamines	Dimethindene, chlorpheniramine, brompheniramine
Ethanolamines	Phenyltoloxamine, diphenhydramine, dimenhydrinate,
Ethylenediamines	Antazoline, halopyramine, histapyrrodine, mepyramine
Phenothiazines	Dimethothiazine, mequitazine, promethazine
Piperazines	Cetirizine, buclizine, cinnarizine, cyclizine, flunarizine
Piperidines	Cyproheptadine, phenindamine



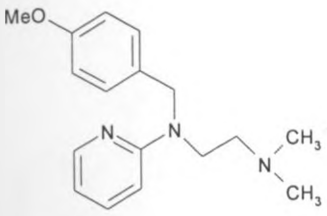
Doxepin



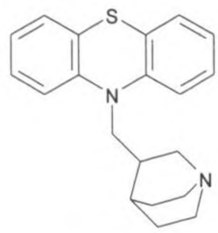
Dimethindene



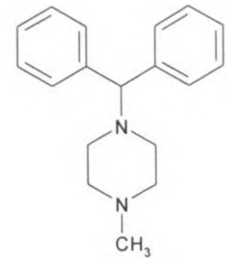
Phenyltoloxamine



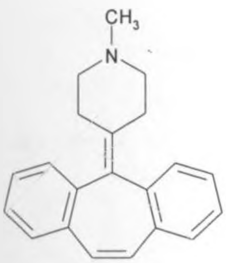
Mepyramine



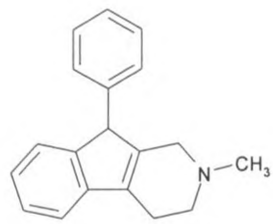
Mequitazine



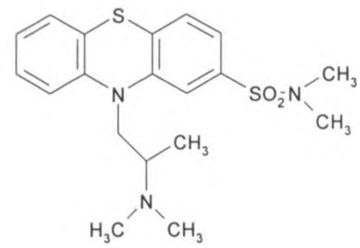
Cyclizine



Cyproheptadine



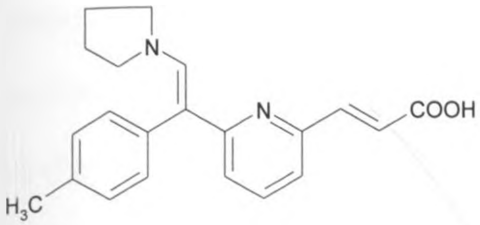
Phenindamine



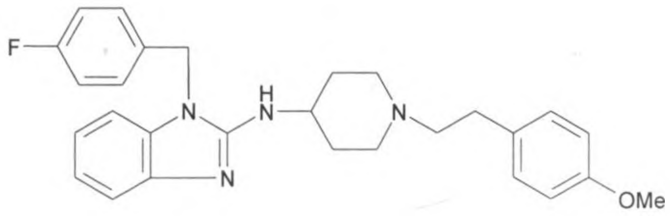
Dimethothiazine

Figure 1.1. Chemical structures of selected structurally classifiable antihistamines

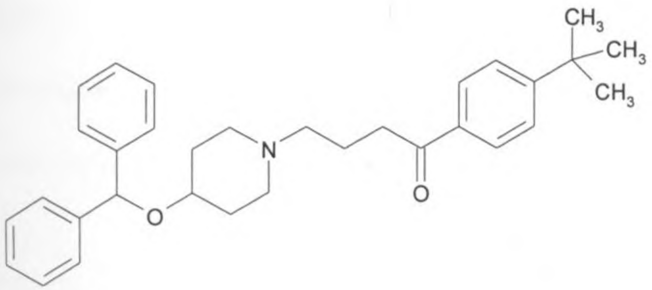
Other antihistamines exist that have entirely diverse and unrelated chemical structures that cannot be systematically organized into any specific chemical groupings. These include such drugs as acrivastine, astemizole, ebastine, terfenadine, triprolidine and clemastine (Figure 1.2) [11,13].



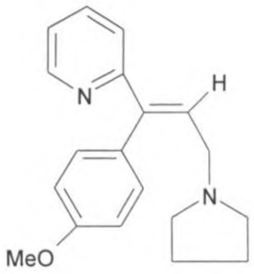
Acrivastine



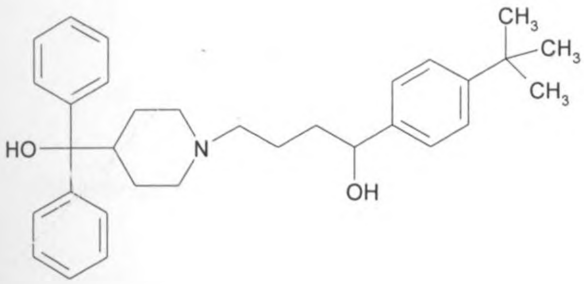
Astemizole



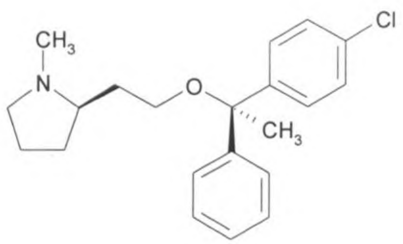
Ebastine



Triprolidine



Terfenadine



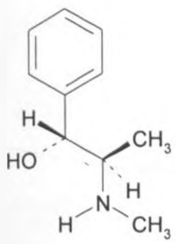
Clemastine

Figure 1.2. Chemical structures of selected chemically unclassified antihistamines

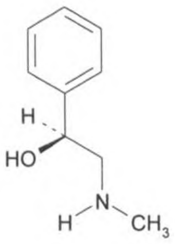
Decongestants are drugs that reduce the inflammation of the nasal passages, which in turn opens clogged nasal passages and enhances drainage of the sinuses. These drugs are used for the temporary relief of nasal congestion caused by the common cold as well as other conditions such as hay fever, sinusitis and other respiratory allergies.

Most decongestant drugs are α-adrenergic agonists that act by activating receptors in venous capacitance vessels in nasal tissues that have erectile characteristics. Such activation leads to decrease in resistance to airflow by decreasing the volume of the nasal

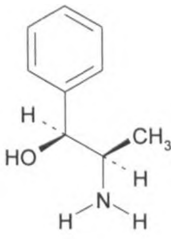
mucosa. Decongestants may be administered topically as nasal sprays or orally as tablets, capsules or syrups. They are sympathomimetic compounds that act by producing localized vasoconstriction of the small blood vessels of the nasal membranes. Vasoconstriction reduces swelling in the nasal passages. Topical nasal decongestants such as phenylephrine are more effective than oral decongestants, but the use of such topical drugs for more than 3 to 5 days may result in rebound congestion [14]. Systemic decongestants include such drugs as pseudoephedrine, ephedrine, phenylephrine, phenylpropanolamine, xylometazoline and oxymetazoline (Figure 1.3).



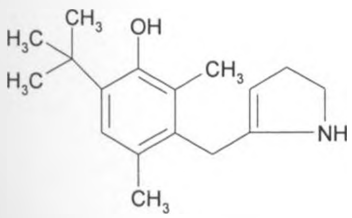
Pseudoephedrine



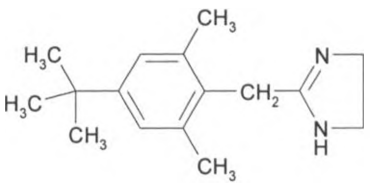
Phenylephrine



Phenylpropanolamine



Oxymetazoline



Xylometazoline

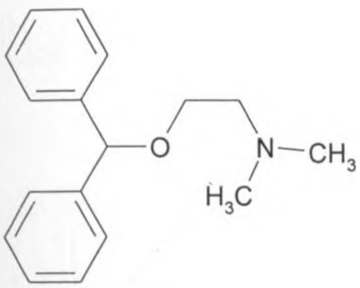
Figure 1.3. Chemical structures of selected sympathomimetic decongestants

When administered at prescribed doses, topical decongestants usually have minimal side effects. The most significant adverse effect associated with their use is rebound congestion or '*rhinitis medicamentosus*'. This phenomemon often occurs following chronic use or sudden cessation of these drugs and is thought to be possibly due to receptor desensitization and damage to the nasal mucosa. Less severe effects may include

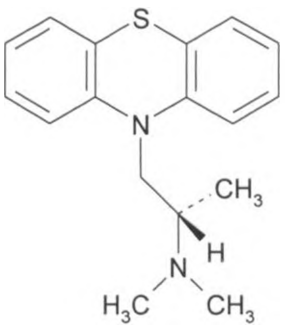
nasal burning, stinging or dryness. Orally administered decongestants may result in more serious side effects including tachycardia and other cardiac arrhythmias, blurred vision, nausea and vomiting, nervousness, restlessness and insomnia [14].

1.6 Chemistry and pharmacology of diphenhydramine, promethazine, chlorpheniramine and ephedrine

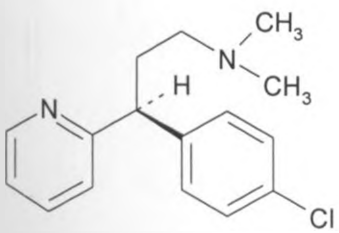
Figure 1.4 illustrates the chemical structures of the four compounds evaluated in this study. All four compounds are weak organic bases incorporated into pharmaceutical formulations in their more stable salt forms in most cases.



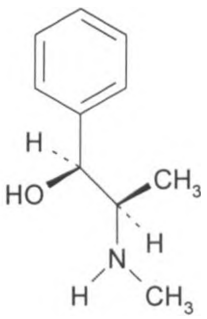
Diphenhydramine



Promethazine



Chlorpheniramine



Ephedrine

Figure 1.4. Chemical structures of the compounds under study

1.6.1 Diphenhydramine

Diphenhydramine, also known as benzhydramine, is a synthetic antihistamine compound that was first synthesized in 1943 by George Rieveschl Jr. while studying muscle relaxants at the University of Cincinnati. The pharmaceutical company Parke-Davis, which began marketing it in 1946, patented the drug in 1947. Since the 1980s, diphenhydramine has been marketed commercially as an over-the-counter medication [15,16].

Diphenhydramine [2-(benzhydryloxy)-N,N-dimethylethylamine], molecular formula $C_{17}H_{21}NO$ and molecular weight 255.35 [14] is an ethanolamine antihistamine with a molecular structure comprising two phenyl rings bonded to a common methoxy carbon to whose oxygen atom is attached a dimethyl substituted ethyl amino group. Most pharmaceutical formulations contain the hydrochloride salt of diphenhydramine, which occurs as a white crystalline powder that slowly darkens upon exposure to light and has a bitter taste. Diphenhydramine hydrochloride has a melting point of 167° to 172 °C, is very soluble in water, ethanol and chloroform but practically insoluble in diethyl ether. Diphenhydramine has pK_a of 9.0 at 25 °C [17].

Diphenhydramine hydrochloride is well absorbed from the gastro-intestinal tract though high first pass metabolism appears to affect systemic availability. Peak plasma concentrations are achieved within 1 to 4 h after oral administration. The drug is widely distributed in the body, including the CNS. It crosses the placenta and has been detected in breast milk as well. Diphenhydramine is highly bound to plasma proteins. It undergoes extensive metabolism and is excreted mainly in urine as the metabolites with little being

excreted as the unchanged drug in urine. Excretion is almost complete within 24 h after administration [12].

Clinically, diphenhydramine is used for the symptomatic relief of hypersensitivity reactions and for its anti-emetic properties, particularly in the prevention and treatment of motion sickness. It is also used for its antimuscarinic properties in the control of Parkinsonism and drug-induced extrapyramidal disorders. Because of its pronounced central sedative properties, diphenhydramine can be used as a hypnotic in the short-term management of insomnia [12].

Commercial pharmaceutical products containing diphenhydramine hydrochloride are formulated as tablets containing 25 or 50 mg of the drug, flavoured syrups for paediatric use containing 5 or 10 mg diphenhydramine hydrochloride per 5 mL. Intramuscular and intravenous injections of diphenhydramine hydrochloride at a concentration of 1% w/v or 5% w/v are also marketed. In addition, topical applications containing 1-2% w/v diphenhydramine hydrochloride are available in addition to numerous cold preparations containing mixtures of several active ingredients that include the drug [18].

1.6.2 Promethazine

Promethazine is a phenothiazine derivative synthetic anti-histamine compound chemically related to anti-psychotic drugs such as chlorpromazine and trifluoperazine. The compound was first synthesized by Paul Charpentier in 1946 and subsequently patented in 1950 by the French pharmaceutical company Rhône-Poulenc [16,19].

The molecular structure of promethazine consists of a substituted tricyclic phenothiazine ring in which the ring nitrogen is bonded to a propyl chain on which a hydrogen atom on C_2 is substituted with a dimethyl amino group.

Chemically, promethazine is 10-(2-dimethylaminopropyl)phenothiazine with molecular weight 284.41. It occurs as a crystalline solid with a melting point of about 60 °C. In most pharmaceutical preparations, the drug is incorporated in its hydrochloride salt form. Promethazine hydrochloride occurs as a white or faintly yellow crystalline powder that is soluble in water, ethanol and chloroform but practically insoluble in ether. It is slowly oxidized on prolonged exposure to air and becomes blue in colour. It has a melting point of about 222 °C accompanied by decomposition. The pK_a at 25 °C is 9.1 [13,17].

Promethazine is well absorbed after oral or intramuscular administration. Peak plasma concentrations are observed 2 to 3 h after administration using these routes although there is low systemic bioavailability after oral administration due to high first-pass metabolism in the liver. The drug is widely distributed and crosses both the blood-brain-barrier and the placenta. Plasma protein binding values have been reported to range from 76 to 93%. Promethazine undergoes extensive metabolism, predominantly to promethazine sulphoxide and also to N-desmethylpromethazine. It is excreted slowly via urine and bile chiefly as metabolites. Elimination half-lives of 5 to 14 h have been reported [12].

Clinically, promethazine is used much like diphenhydramine, chiefly in the symptomatic relief of hypersensitivity reactions as well as in preventing and treating nausea and vomiting in conditions such as motion sickness. Promethazine hydrochloride is also employed pre- and post-operatively in surgery and obstetrics for its sedative effects and the relief of apprehension [12].

Formulations containing promethazine in the market include tablets containing 10 or 25 mg of the hydrochloride, elixirs and syrups containing 5 mg/ 5mL promethazine hydrochloride for pediatric use and ampoules containing 25 mg/mL of the drug for

intravenous or deep intramuscular injection. Suppositories for rectal administration are also available as are creams containing 2% w/v of the active ingredient for topical use. Promethazine HCl is also used in combination with many other compounds as an ingredient in numerous cough and cold preparations, particularly those formulated as syrups [18].

1.6.3 Chlorpheniramine

Chlorpheniramine, also referred to as chlorphenamine, is a synthetic first generation alkylamine anti-histamine compound that was first synthesized by Simon Sperber in 1951 and patented by the USA pharmaceutical company Schering.

Chlorpheniramine, a propylamine derivative antihistamine derivative, is chemically 1-(*p*-chlorophenyl)-1-(2-pyridyl)-3-dimethylaminopropane with molecular weight 274.80 that occurs as an oily liquid with a boiling point of about 142 °C. Its molecular structure consists of two ring systems bonded to a common methylene carbon to which a dimethyl amino ethyl chain is also attached. The aromatic systems in the molecule are a pyridine ring and a phenyl ring that is chloro-substituted at the para position.

In most pharmaceutical preparations, the drug is incorporated as its maleate salt. Chlorpheniramine maleate ($C_{16}H_{19}ClN_2 \cdot C_4H_4O_4$, molecular weight 390.9) occurs as a white, odorless, crystalline powder that is soluble in methanol, water, chloroform and ethanol but only slightly soluble in ether and benzene. This salt has a melting point of between 130 °C and 135 °C and the base has a dissociation constant of 9.2 at 25 °C [13, 17].

Because the methylene carbon to which both ring systems are bonded is a chiral center, chlorpheniramine exhibits optical isomerism. In most preparations, both the

dextrorotatory and levorotatory isomers are present in equal quantities as a racemic mixture. In a few pharmaceutical preparations, only the more physiologically active *d*-isomer is included as dexchlorpheniramine or *d*-chlorpheniramine maleate. On a weight-to-weight basis, the dextro isomer is approximately twice as active as racemic chlorpheniramine [18].

Chlorpheniramine maleate is well absorbed after oral administration but undergoes substantial metabolism in the gastro-intestinal mucosa during absorption and on first pass through the liver. Limited data indicates that about 25-45% of a single oral dose of the drug in conventional tablet form reaches systemic circulation as the unchanged drug. Peak plasma concentrations are observed within 2 to 6 h of oral administration. Following intravenous administration in humans, the drug undergoes rapid and extensive distribution and is present in saliva as well as in bile in small amounts. *In vitro,* chlorpheniramine is approximately 69-72% bound to plasma proteins. Chlorpheniramine is rapidly and extensively metabolized in the body with initial and substantial metabolism occurring in the gastro-intestinal mucosa during absorption and on first pass through the liver after oral administration. The drug undergoes *N*-dealkylation to form monodesmethylchlorpheniramine and didesmethylchlorpheniramine but is principally metabolized to at least two other as yet unidentified metabolites. The unchanged drug and its metabolites are apparently excreted almost completely in urine. Urinary excretion of chlorpheniramine and its *N*-dealkylated metabolites varies with urinary pH and urine flow - decreasing substantially as urinary pH increases and urine flow decreases. Less than 1% of the orally administered dose of the drug is eliminated in faeces [18].

Clinically, chlorpheniramine acts as a potent antihistamine that causes a moderate degree of sedation and also exhibits antimuscarinic activity. Both racemic chlorpheniramine and dexchlorpheniramine maleate are used for the symptomatic relief of hypersensitivity reactions including urticaria and angioedema, rhinitis and conjunctivitis as well as skin disorders. Intravenous chlorpheniramine may be administered as an adjunct in the emergency treatment of anaphylactic shock [12,18].

Formulations containing chlorpheniramine in the market include tablets and extended release capsules containing 2 to 8 mg of the maleate salt, oral syrups containing 1-2 mg/5mL chlorpheniramine for pediatric use and ampoules containing 10 mg/mL of the drug for subcutaneous, intravenous or deep intramuscular injection. Chlorpheniramine is also used in combination with many other compounds as an ingredient in numerous cough and cold preparations, particularly those formulated as syrups [18].

1.6.4 Ephedrine

Ephedrine is a naturally occurring alkaloid that has been used in China for over 2000 years and which was introduced into conventional Western medicine in 1924 as the first orally active sympathomimetic drug. Ephedrine was originally isolated from the Chinese herbal medication "Ma-huang", and is present in many species of plants belonging to the genus *Ephedra* such as *E. sinica, E. equisetina* and *E. nevadensis* [20].

Ephedra sinica, used as the original Chinese Ma-huang remedy, is considered one of the world's oldest medicines. It is used as a stimulant and for the management of bronchial disorders. Ancient Aryans from India discovered that *ephedra* or the *Soma* plant could be used as an energizer cum euphoriant. Historically, *ephedra* has been recommended for

colds and flu, coughing, wheezing, nasal congestion, fever, chills, headaches, hyperhydrosis and bone pains.

Ephedra is an evergreen perennial herb native to Central Asia and is now widely distributed and cultivated throughout the temperate and subtropical zones of Asia, Europe and the Americas. The *Ephedra* genus includes more than 40 species, the majority of which contain ephedrine. Ephedrine was first isolated from these plants in 1885. The alkaloid yield from ephedra plants ranges from 0.5 to 2.5%, of which 30 to 90% is ephedrine. In its natural form, ephedrine exists as the L-isomer whereas synthetic ephedrine is generally a racemic mixture of both L and D isomers [21].

Ephedrine (1-phenyl-1-hydroxy-2-methylaminopropane), molecular formula $C_{10}H_{15}NO$ has molecular weight 165.23 [14]. Most pharmaceutical formulations contain the hydrochloride salt form ($C_{10}H_{15}NO.HCl$), which occurs as a colourless or white crystalline powder with a melting point of 217° to 220 °C. The hydrochloride is soluble in water and ethanol but only slightly soluble in chloroform and practically insoluble in diethyl ether. Ephedrine has a dissociation constant of 9.6 at 25 °C.

Ephedrine is a sympathomimetic agent that exerts both direct and indirect effects on adrenergic receptors. It has both alpha- and beta- adrenergic activity and has pronounced stimulating effects on the central nervous system. Ephedrine acts primarily through the release of stored catecholamines in addition to some direct action on adrenoreceptors.

On oral administration, ephedrine is readily and completely absorbed from the gastro-intestinal tract. It is resistant to monoamine oxidase and is excreted largely unchanged in urine, together with small amounts of metabolites produced by hepatic metabolism. The

drug has been reported to have a plasma half life of 3 to 6 h depending on urinary pH with elimination being enhanced in acidic urine.

Clinically, ephedrine is used to prevent bronchial spasm in asthmatic patients when given orally. Parenterally administered ephedrine salts are used to combat fall in blood pressure during spinal anaesthesia. Ephedrine salts have also been used either alone or in combination with other agents in the symptomatic relief of nasal congestion associated the common cold, hay-fever, rhinitis and sinusitis.

Pharmaceutical products containing ephedrine hydrochloride are formulated as injections containing 3 mg/mL in 10 mL ampoules, tablets containing 15 mg of the salt as well as syrups, nasal drops and mixtures containing between 5 and 15 mg of the drug per 5 mL of the preparation [12].

1.7 Multi-component cold-cough syrups

Multiple active ingredients have been incorporated into mixtures used to treat coughs and colds. The rationale of this being that the ingredients required to treat the various symptoms of the condition may be incorporated into a single formulation thus simplifying dosage regimens and reducing the cost of treatment.

Routine prolonged administration of fixed-dose combinations containing anti-histamines, nasal decongestants, anti-cholinergics, analgesic-antipyretics, caffeine, anti-tussives and expectorants has been questioned. Single-ingredient products generally are safer than combination products while also facilitating dosage adjustment. There is no evidence that combinations containing 2 or more anti-histamines are more effective than one anti-histamine or that combinations of sub-therapeutic doses of 2 or more anti-histamines are

more effective than therapeutic doses of one anti-histamine. Oral anti-histamine combinations containing an analgesic-antipyretic, nasal decongestant and anti-tussive may be rational if each ingredient has demonstrated clinical effectiveness and is present at therapeutic dose. Combinations containing an anti-histamine and an expectorant, anticholinergic agent or bronchodilator are not considered rational [18].

In theory, it would appear that incorporating such diverse ingredients into a single formulation would greatly ease treatment of cold and cough symptoms. However, the administration of many different drug compounds with widely differing pharmacological and toxicity profiles carries with it the inherent risk of increasing the possibility of adverse drug reactions in patients receiving such medications. The dilemma in this case is further compounded by the fact that by their very nature, colds, despite being discomforting to most people, are generally self limiting and usually resolve by themselves within a matter of days.

In Kenya, the controversy regarding the use of such multi-component preparations was highlighted by both print and broadcast media in March 2009 when a number of privately owned hospitals publicly announced the withdrawal of several cold-cough preparations for use in children from their hospital formularies [22].

1.8 Analytical challenges in the quality control of cold-cough syrups

Quality control (QC) of syrup formulations through laboratory testing is often complicated by presence of the numerous inactive excipients incorporated into these products. Unlike the case of pharmaceutical formulations such as capsules and tablets where relatively few additives are included, it is often necessary to add agents that improve the palatability and stability of syrups. Flavouring agents, stabilizers, coloring

Alternatively, a simpler approach in quality control testing of multi-component syrup might involve using a technique that inherently separates the different compounds in the product, thereby making them available to subsequent quantification. Liquid chromatography provides this possibility and coupled with a suitable detection technique, allows very accurate determination of the separated sample components. In order to enhance the separation further, sample pretreatment prior to chromatography aimed at eliminating interfering excipients may be carried out as well. The use of chromatography in this case would greatly reduce both the duration and cost of quality control analysis of cold-cough syrups by greatly simplifying or even totally eliminating the need for sample pretreatment, as well as allowing the simultaneous determination of all sample components in a single run. Another advantage of liquid chromatography in quality control testing of pharmaceutical products is the possibility of automating the testing process, greatly minimizing the amount of human input required once sample preparation has been concluded.

1.9 Quality control of cold-cough syrups in Kenya

Currently, there are no published pre-registration or post-market surveillance studies conducted to determine the quality of multi-component cold-cough syrup formulations in circulation in Kenya or the East African region. In Kenya, these products, though controlled by the Pharmacy and Poisons Board, like any other pharmaceutical products remain largely untested since they are not classified as essential medicines. Indeed, public hospitals do not routinely include multi-component cold-cough syrups as part of their institutional formularies. Even the process of granting market authorization for such products is not as rigorous as that undertaken for drugs considered to be essential medicines. This is emphasized by the fact that few, if any, of these products are ever

subjected to mandatory laboratory testing to determine their quality prior to granting the marketing authorization. The lack of comprehensive scrutiny of these products might explain the presence of syrups in the Kenyan market that contain multiple anti-histamine ingredients, all incorporated at sub-therapeutic doses, despite the fact that current medical opinion largely discourages the use of anti-histamines in treatment of colds and coughs.

Another possible reason why no comprehensive studies have been carried out to determine the quality of these products in Kenya might be the lack of reliable and accurate test methods that could allow such a study be conducted on a large scale and within a reasonable timeframe and cost.

1.10 Study Justification

The quality of pharmaceutical products is of critical importance if they are to elicit the desired clinical outcomes. To ensure that pharmaceutical preparations are of the appropriate quality, products are subjected to tests specified in official compendia or in-house monographs. Such tests include those for identity, assay, microbial contamination and various physico-chemical properties. World Health Organization guidelines on current Good Manufacturing Practices (cGMP) specify that the role of ensuring the quality of pharmaceutical products is the responsibility of the Quality Assurance (QA) department of any pharmaceutical manufacturing setup [24]. In order to test with the requisite accuracy and efficacy the quality of pharmaceutical products, it is necessary that the QA department develop methods that are capable of meeting this requirement and which are subsequently validated to confirm and ascertain their suitability. The process of method development can therefore only be effectively conducted in an environment where the method developer is involved in the design of the product formulation. This

involvement is important since it allows the method development process to take into account the different ingredients incorporated into the finished pharmaceutical product and therefore include test techniques or steps designed to eliminate any possible interference by such ingredients in product analysis. Methods that are used during the development of a drug product formulation should be able to assess reacting or catalyzing excipients and any undesired reactions leading to degradation products. Methods should be able to separate the active pharmaceutical ingredient (API) from the drug product degradation products, excipients, excipient degradation products and any synthetic impurities that are present in the API. These development steps are known as excipient compatibility studies [25].

Advances in technology have resulted in new pharmaceutical formulations by drug manufacturers as well as more sophisticated methods of analysis. These methods are characterized by their relatively high level of selectivity and accuracy, allowing them to be used in the assay of pharmaceutical products as well as in detection and quantification of impurities especially related substances. Perhaps the most commonly employed technique for routine quality control testing of pharmaceutical products is liquid chromatography which offers the advantages of high selectivity and sensitivity. It is also relatively more robust and affordable especially when compared to such techniques as capillary electrophoresis.

Typically, multi-component cold-cough syrups contain 5 mg diphenhydramine HCl, 2.5 mg promethazine HCl, 1 mg chlorpheniramine maleate and 7.5mg of ephedrine HCl in every 5 mL. Other ingredients commonly incorporated include sodium citrate (45 mg per 5 mL) and ammonium chloride (90 mg in 5 mL) as well as various flavouring and

colouring agents and sweeteners. The use of such combinations, especially containing anti-histamines in combating the symptoms of the common cold remains a controversial issue. These products, however, continue to be manufactured and marketed in Kenya. The fact that most of them are readily available as over-the-counter (OTC) or pharmacy only medications available to patients without the need for a physician's prescription further raises concerns over the need to have them properly tested and evaluated to ensure that they are of good quality.

1.11 Study objectives

The general objective of this project was development and validation of a liquid chromatography method capable of the simultaneous determination of diphenhydramine hydrochloride, promethazine hydrochloride, chlorpheniramine maleate and ephedrine hydrochloride in cold-cough syrup formulations.

Specific objectives of the study were:

a. To develop a liquid chromatography (LC) method for the optimum separation and determination of diphenhydramine hydrochloride, promethazine hydrochloride, chlorpheniramine maleate and ephedrine hydrochloride

b. To optimize the developed LC method through systematic adjustment and examination of chromatographic variables including temperature, pH, mobile phase composition and concentration.

c. To validate the developed LC method.

d. To apply the developed method in the assay of some available samples of cold-cough syrups containing the four ingredients obtained from the Kenyan market.

CHAPTER TWO

METHOD DEVELOPMENT

2.1 Introduction

The need to continually develop and refine analytical test methods to determine the quality of pharmaceutical products is dictated by the fact that new formulations are constantly being produced by pharmaceutical manufacturers. In addition, advances in analytical chemistry often provide the opportunity to develop newer, faster, more accurate and reliable means for testing pharmaceutical products through instrumental or other test methods. In many cases, the development of new test methods relies heavily on data obtained from pre-existing techniques and is often geared towards overcoming shortcomings identified in such. The ultimate goal when developing methods to be used in testing the quality of pharmaceutical products is to come up with techniques that are accurate, reproducible, efficient, robust, cost-effective and reliable.

2.2 Literature Review

Diverse official and non-official test methods have been published for analysis of preparations containing the four compounds under study in numerous literature sources, especially as individual ingredients in pharmaceutical and biological samples. However, few test methods capable of simultaneously quantifying various mixtures of the four compounds have been reported in literature.

Official pharmacopeias contain monographs in which diphenhydramine HCl as raw material and in finished products may be assayed through direct potentiometric titration or reversed-phase liquid chromatography respectively [26-29]. Methods reported in non-

pharmacopoeial sources include capillary electrophoresis, isothermal gas chromatography (GC), Fourier Transform Raman spectroscopy and high performance thin layer chromatography which have been used to quantify the drug in biological samples as well as in cold-cough syrups containing other active ingredients as well [30-37].

Both aqueous and non-aqueous potentiometric and colour indicator titrations can be carried out to quantify promethazine HCl raw material according to official methods [26-28]. Single component finished pharmaceutical formulations can be suitably tested using ultraviolet (UV) spectroscopy after appropriate sample preparation typically involving extraction or alternatively employing ion-paired reverse phase LC [29]. Non-pharmacopoeial methods have been reported for the determination of promethazine including differential pulse stripping voltammetry for drug combinations with chlorpromazine in synthetic mixtures and in blood as well as capillary zone electrophoresis [38,39]. An extractive-spectrophotometric technique for determination of phenothiazines in pure solutions and pharmaceuticals involving reaction of the samples with dipicrylamine and picric acid in neutral media is described by Regulska *et al.* [40]. By far the most common technique employed in determination of promethazine in different formulations has been reversed-phase LC, sometimes involving the use of ion-pair reagents in the mobile phase with UV detection [41-44].

The British and European Pharmacopoeia recommend potentiometric non-aqueous titration as the method of choice for the assay of chlorpheniramine maleate raw material while UV spectroscopy is used for single active ingredient injection and tablet formulations either directly or after extraction. Gas chromatography is recommended for

the assay of oral solutions containing the chlorpheniramine maleate [26,27]. Reversed-phase liquid chromatography is indicated as the assay method for extended release chlorpheniramine maleate capsules in the United States Pharmacopoeia [28]. Non-official methods have been reported that employ chemiluminescence, isocratic high performance liquid chromatography (HPLC) using either reversed-phase C18, poly(ethyleneglycol) or propylcyano stationary phase columns as well as capillary electrophoresis for the determination of chlorpheniramine and other ingredients of cold-cough preparations [45-52]. Less common techniques reported include near infrared spectroscopy and atomic emission spectroscopy [53,54].

The official methods for analysis of ephedrine raw material prescribe use of either direct potentiometric aqueous or non-aqueous titration of the sample while finished products are analysed using a reversed-phase LC with UV detection at 263 nm or alternatively using UV spectrophotometry after extraction of the drug from the formulations being tested. Liquid and gas chromatography have both been used to determine ephedrine in cough and cold syrups [37,55]. Gas chromatography has also been used to determine ephedrine and its related alkaloids in supplements containing the Chinese *Ma-huang* herbal remedy [56]. Less frequently used techniques include capillary zone electrophoresis preceded by derivatization of the samples with fluorescein to increase the sensitivity of the method which uses laser induced fluorescence as the detection method [57]. A method involving in-capillary derivatization of the sample using 4-fluoro-7-nitro-2,1,3-benzoxadiazole as the fluorescent reagent has also been reported in quantifying both ephedrine and pseudo ephedrine [58]. Circular dichroism and LC with dual optical rotation/UV absorbance detection have both been employed in determining the enantiometric purity of ephedrine [59,60].

Despite the numerous and diverse techniques present in literature for the quantification of the four analyte compounds of interest in pharmaceutical products and biological samples, whether singly or in combination with other compounds, none could be found that allows for the simultaneous determination of all four compounds. Capillary electrophoresis methods could in theory be developed for the simultaneous determination of the components under study in cold-cough syrups but the main impediment to their use in Kenya is their high application cost compared to HPLC. Circular dichroism would present a similar financial challenge to most pharmaceutical testing facilities while simpler techniques involving analyte derivatization prior to determination using spectroscopy or titration would be too time-consuming and laborious for routine application.

2.3 Experimental

2.3.1 Reagents and solvents

Analytical grade potassium dihydrogen phosphate, dipotassium hydrogen phosphate (Loba Chemie PVT Ltd., Mumbai, India), orthophosphoric acid (Merck Chemicals PTY Ltd., Gauteng, South Africa), tetrabutylammonium hydroxide (Fischer Scientific U.K. Ltd, Loughborough, U.K.), tetrabutylammonium hydrogen sulphate, triethanolamine (BDH Laboratory Supplies, Poole, England), anhydrous sodium acetate (Fluka Chemie GmbH, Buchs, Switzerland), ammonium acetate (Loba Chemie PVT. Ltd) and glacial acetic acid (Sigma-Aldrich Laborchemikalein GmbH, Seelze, Germany) were all used during method development.

Methanol (Fischer Scientific U.K. Ltd.) and acetonitrile (VWR International Ltd., Poole, England) used in preparation of all chromatography mobile phases were of HPLC grade.

All aqueous solutions used in the study were prepared using purified water obtained through reverse osmosis treatment and ultra filtration through successive 0.45 µm and 0.2 µm membrane filters using a combined Arium 61316 RO and Arium 611 VF water system (Sartorius AG, Göttingen, Germany).

2.3.2 Instrumentation

2.3.2.1 Liquid chromatography apparatus

The LC apparatus consisted of a Merck Hitachi LaChrom HPLC System (Hitachi Ltd, Tokyo, Japan) incorporating the following components: a quaternary low pressure gradient pump model L7100, a variable wavelength UV detector model L7400, a variable injection volume autosampler model L7200 supported by Merck-Hitachi Model D-7000 Chromatography data station software - HSM Manager Version 4.1 (Merck KGaA, Darmstadt, Germany and Hitachi Instruments Inc., San Jose, USA). Mobile phase preparations were degassed by using a Sonorex Super RK103H ultrasonic bath (Bandelin Electronic, Berlin, Germany) for 15 min. The variable injection volume autosampler allowed sample solutions to be injected the chromatograph without the need for a sample loop of fixed volume. The advantage of this setup was that it allowed virtually any sample volume to be injected without having to physically replace sample loops.

The chromatography column temperature was controlled using a thermostatically controlled Clifton unstirred water bath (Nickel-Electro Ltd., Weston-S-Mare, Somerset, England). Mobile phases were pumped at a constant flow rate of 1.0 mL/min throughout method development. Column back pressures were maintained below 200 bar which was set as the upper working limit.

2.3.2.2 Infra-Red spectrophotometer

The infrared spectra of the analyte reference compounds were recorded using a Shimadzu IRPrestige 21 Fourier Transform Infra-Red (FTIR) spectrophotometer (Shimadzu Corp., Kyoto, Japan) supported by IRSolution ver. 1.3 software (Shimadzu Corp). Sample discs for determination of the FTIR spectra were prepared using spectroscopic grade potassium bromide (E. Merck, Darmstadt, Germany) and a manually operated hydraulic pellet press (Perkin Elmer GmbH, Uberlingen, Germany).

2.3.2.3 Ultra-Violet spectrophotometer

Ultra-violet absorption spectra of the analyte reference materials were measured using a double beam Perkin Elmer UV/Vis Lambda 12 Spectrometer (Perkin Elmer GmbH) over a 10 mm path length using quartz cuvettes.

2.3.2.4 Melting point apparatus

Determination of the melting points of the working standards was done using a Buchi melting point apparatus Model B-540 (Buchi Labortechnik AG, Flawil, Swizerland). The apparatus was calibrated using certified primary chemical reference melting point standards caffeine, vanillin and phenacetin obtained from the U.S.P. (Rockville, Maryland, USA).

2.3.3 Working Standards

Working standards of chlorpheniramine (CPM) maleate, diphenhydramine (DPH) hydrochloride, ephedrine (EPD) hydrochloride and promethazine (PRM) hydrochloride were a kind donation from Dawa Limited, Nairobi, Kenya. These powders were stored in well sealed amber colored bottles kept in a sealed dessicator at room temperature to protect them from direct light and excessive moisture. The working standards were

subjected to preliminary tests aimed at confirming their identities against primary chemical reference standards. These tests included: determination of melting points, measurement of both IR and UV absorption as well as confirmation of purity.

2.3.3.1 Determination of melting point

The melting point of each of the four working standards (Table 2.1) was determined as a simple identity test. For this purpose, melting points were compared against those of certified primary standards tested simultaneously and values published in literature.

Table 2.1. Melting points of working standards

Compound	Determined Melting Point (°C)	Reported in Literature (°C)
Chlorpheniramine maleate	132.7 - 133.9	130 - 135
Diphenhydramine HCl	168.4 - 169.5	167 - 172
Ephedrine HCl	218.6 - 219.0	217 - 220
Promethazine HCl	219.7 - 221.1	Approx. 222

2.3.3.2 Infra-Red spectroscopy

Running of the infra-red absorption spectra of all four analyte compounds was carried out as a more reliable technique for confirming their identities. Fourier Transform Infra-Red spectra were measured for both the working standards as well as primary chemical reference compounds using the same apparatus under identical test conditions. Spectra were run as 1 mm thick potassium bromide pellet disks containing approximately 1% w/w of the analyte compounds over the range of frequencies 400 to 4000 cm^{-1} cumulatively with each sample disk being scanned a total of 45 cycles to obtain more accurate absorption spectra (Appendices 1 - 4).

The frequencies of principal IR absorption bands were compared against those observed from primary chemical reference substances and those published in literature sources for all compounds. Key observations of these are summarized below (Table 2.2).

Table 2.2. Principal absorption bands observed in FTIR spectra of working standards

Compound	Principal IR absorption bands at wave number (cm^{-1})
Chlorpheniramine maleate	578, 652, 762, 872, 1005, 1094, 1200, 1356, 1479, 1587, 2453, 3019
Diphenhydramine hydrochloride	528, 710, 756, 1020, 1107, 1177, 1381, 1462, 2450, 2565, 2887, 3026
Ephedrine hydrochloride	525, 673, 700, 752, 991, 1049, 1115, 1240, 1354, 1393, 1456, 1589, 2461
Promethazine hydrochloride	517, 760, 858, 930, 1040, 1128, 1227, 1333, 1454, 1568, 2380, 2926

2.3.3.3 Ultra-Violet spectroscopy

Ultra-violet absorption spectra of the four working standards separately dissolved in methanol to yield concentrations of EPD 0.4 mg/mL, DPH 0.3 mg/mL, CPM 0.024 mg/mL and PRM 0.008 mg/mL were scanned over the wavelength range 220-300 nm using 10 mm quartz sample cells (Appendix 5-8). The λ_{max} determined from the scans were to be used in selecting the detection wavelength for use during the chromatographic method development. Additionally, the test provided further confirmation of the identity of the raw materials.

From the UV spectra, the wavelength of maximum absorbance (λ_{max}) for each of the four raw materials was determined as shown in Table 2.3.

Table 2.3. Determined λ_{max} values for working standards dissolved in methanol

Compound	Measured λ_{max} (nm)	Reported λ_{max} in Literature (nm)
Chlorpheniramine maleate	262.0	262.0
Diphenhydramine hydrochloride	258.8	257.0
Ephedrine hydrochloride	258.0	257.0
Promethazine hydrochloride	252.0	254.0

The differences noted in the determined λ_{max} values for DPH (+1.8 nm), EPD (+1.0 nm) and PRM (-2.0 nm) from those reported in literature were most probably due to the difference in solvent used for preparation of the test solutions, in this case methanol, from that used to establish the values reported in literature, in most cases reported as being aqueous acid.

2.3.3.4 Confirmation of purity

Assay of each of the working standards against WHO and USP chemical reference substances was carried out to confirm their purities prior to use. The values obtained are summarized in Table 2.4 below:

Table 2.4. Percentage purity of working standards

Compound	% Purity
Chlorpheniramine maleate	100.1
Diphenhydramine hydrochloride	99.6
Ephedrine hydrochloride	99.7
Promethazine hydrochloride	100.1

2.3.4 Liquid chromatography method development

2.3.4.1 Column selection

The chromatographic column selected for use in the development of the method was a reversed-phase Phenomenex Gemini-NX® C18 column of dimensions 250 mm length and

4.6 mm internal diameter with particle size 5μm and pore size 110Å (Phenomenex, Torrance, California, USA).

The choice of this column was based on the wide pH stability range claimed by its manufacturer (pH 1-12). This claim was anchored on the fact that in this column brand, the silica based stationary phase is modified by the chemical insertion of ethyl bridges that connect neighbouring free silanol groups resulting in a marked improvement in the pH stability of the column compared to conventional C18 phases. Wide pH stability allowed for greater flexibility in the method development process and offered a significant advantage over most conventional silica based reversed phase C18 columns that are designed to operate within a much narrower pH range (2.0-8.0). Another consideration in the choice of this column was the fact that being a silica based column, it was expected to exhibit greater efficiency and superior peak shapes compared to synthetic polymer based columns.

2.3.4.2 Selection of detection wavelength

The UV absorption spectra of the working standards had revealed that all four exhibited definitive and appreciable absorbance maxima at wavelengths ranging from 252 to 262 nm. Additionally, CPM, DPH and EPD all exhibited even more significant absorbance at wavelengths below 240 nm. Promethazine did not, however, show similar behaviour, with its UV absorbance decreasing at wavelengths below 250 nm. Consequently, the detection wavelength used in the development process was fixed at 254 nm since at this wavelength, all the four compounds showed an absorption intensity comparable to that at their λ_{max} values. Another consideration made in selecting this wavelength was the fact that it would allow the method developed to be successfully applied using HPLC

instrumentation equipped with fixed wavelength UV detectors, typically set at 254 nm, without any complications, hence enhancing the method's versatility.

2.3.4.3 Fixed chromatographic conditions

At the onset of the method development process, some chromatographic variables were fixed based on preliminary observations. These parameters included mobile phase flow rate, organic modifier and sample injection technique.

Mobile phase flow rate was fixed at 1.0 mL/min. Flow rate influences column back pressures, analysis time as well as mobile phase consumption, all of which influence the cost of analysis. Most analytical column stationary phase packings are designed to work within explicitly defined pressure limits specified by the manufacturer. Excessive backpressure resulting from among other factors, high mobile phase flow rate can cause damage to the column. For the column used in method development process, the upper pressure limit had been set at 200 bar. Consequently, a flow rate of 1.0 mL/min was considered ideal in ensuring pressures did not exceed this limit.

From the very beginning of the method development process, methanol was chosen to be the organic modifier solvent to be used in mobile phase preparation.

2.3.4.4 Preparation of the working standard solution

Working standard solution was prepared by accurately weighing and dissolving the working standards in a mixture of methanol and water (50:50) as diluent. The concentrations of the analyte compounds in this solution were: Ephedrine hydrochloride 1.5 mg/mL, chlorpheniramine maleate 0.2 mg/mL, diphenhydramine hydrochloride 1.0 mg/mL and promethazine hydrochloride 0.05 mg/mL.

2.3.5 Mobile phase composition

The different mobile phases prepared during method development comprised variable proportions of methanol, organic and inorganic buffer solutions (0.2 M) at different pH values and water. In some cases, a solution of ion-pairing agent was also incorporated into the mobile phase. Mobile phases were prepared by mixing appropriate volumes of the stock buffer solution with water before adjusting pH to the desired value using the molar equivalent solution of the parent acid or buffer salt. Thereafter, the volume of methanol required to yield the desired proportions of the different mobile phase components was measured separately and then added to the pH adjusted buffer solution before degassing the resultant mixture in an ultra-sonic water bath.

Initial chromatographic analysis of the working standard solution was carried out using unbuffered mobile phase containing only a mixture of methanol and water (50:50, % v/v). Working standard solution (20 µL) was injected into the LC system. Under these conditions, very poor separation of the analyte compounds was achieved (Figure 2.1), with only chlorpheniramine exhibiting a distinct peak from the other compounds whereas promethazine did not even yield any peak in the chromatogram.

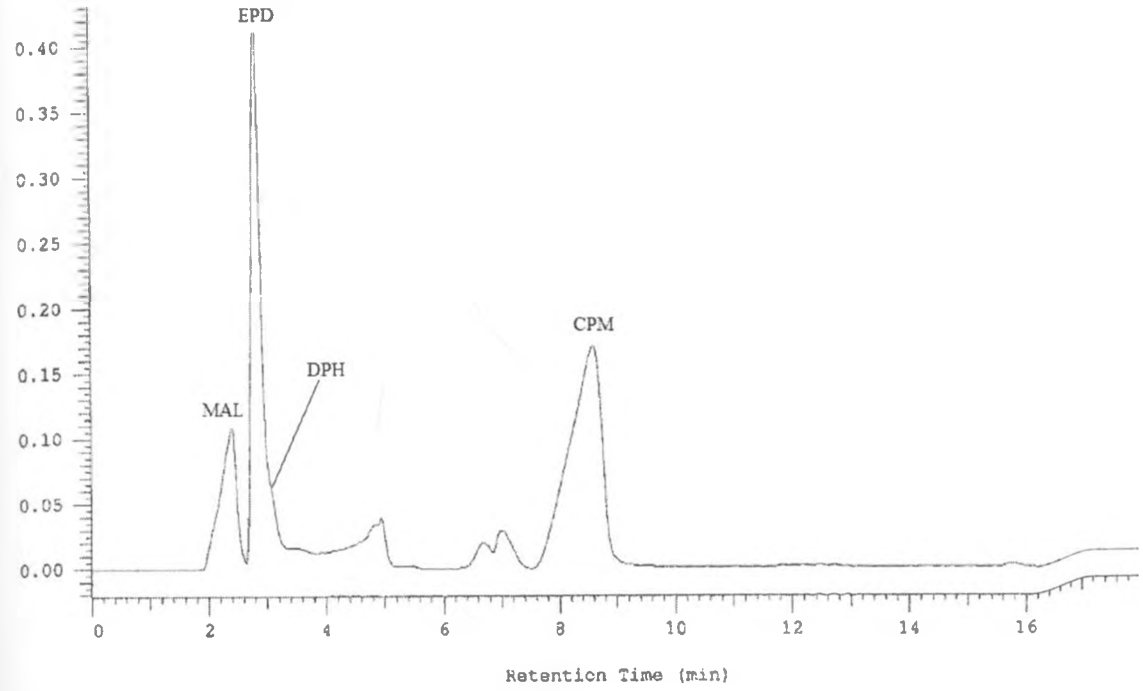


Figure 2.1. Chromatogram of a mixture of maleic acid (MAL), ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine using methanol-water mobile phase
Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-water (50:50, % v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 20 µL.

2.3.5.1 Effect of inorganic aqueous buffer and pH

The consequence of incorporating an inorganic buffer in the mobile phase was tested to determine its effect on separation compared to that observed using unbuffered mobile phase. Monobasic potassium phosphate (KH_2PO_4) was selected as buffer because it is commonly used in reverse phase LC, readily available and has particularly wide buffering capacity that could allow preparation of solutions with pH values ranging over almost the entire pH range (pH 3-13). Because the proportion of organic modifier (methanol) in the mobile phase was high at 50%, to avoid precipitation of KH_2PO_4, the effective concentration of the buffer was restricted to 0.02 M. The mobile phase was prepared by mixing a stock solution of 0.2 M KH_2PO_4 with water and methanol in the ratio 10:40:50 (% v/v) then degassing using an ultrasonic water bath. At this stage, pH of

the buffer solution was not adjusted. The chromatogram obtained (Figure 2.2) revealed a noticeable improvement in separation of the component peaks.

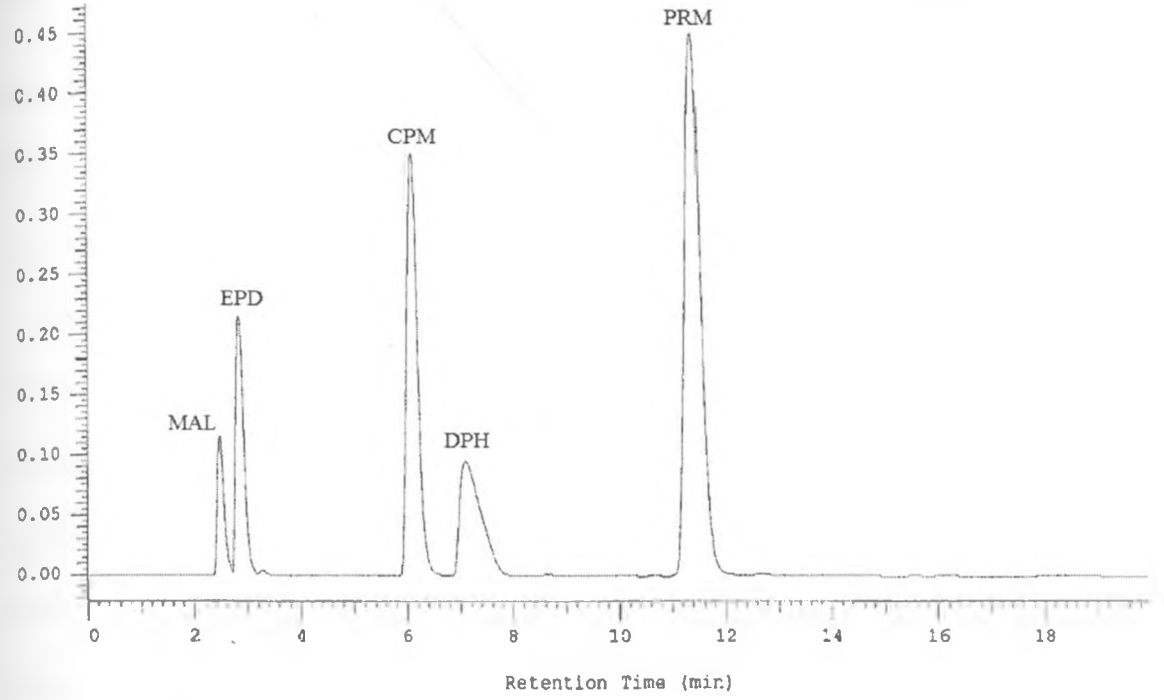


Figure 2.2. Chromatogram of a mixture of maleic acid (MAL), ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM) using phosphate buffer in mobile phase
Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-0.2 M potassium dihydrogen phosphate-water (50:10:40, % v/v/v). Flow rate 1.00 mL/min. Detection: 254 nm. Injection volume: 20 µL.

From the chromatogram obtained, 5 distinct peaks were recorded that included the 4 analyte compounds EPD, CPM, DPH, PRM and maleic acid (MAL) component of chlorpheniramine maleate.

Dissociation of chlorpheniramine maleate in solution yielded both the free chlorpheniramine base as well as the maleic acid moiety. Both species possess chromophores capable of absorbing UV radiation at 254 nm. Therefore, unlike the case with EPD, DPH and PRM which in solution dissociated to yield only inorganic and non-UV absorbing hydrochloride ions apart from the detected free bases, the maleic acid from chlorpheniramine was clearly observed as a distinct peak in the chromatogram in addition

to the parent base CPM. The identity of the maleic acid was confirmed by injecting a solution of maleic acid working standard into the LC system under identical conditions and comparing the retention time.

The elution sequence of the peaks, in order of increasing retention time was MAL, EPD. CPM, DPH and PRM. The overall run time for elution of all 4 component peaks was approximately 15 min with PRM being the last peak to elute from the column at about 11.2 min. Although the peaks were all distinctly separated from each other, the selectivity between MAL and EPD was poor. The resolution between these two peaks was 1.58, indicating that baseline separation had not been achieved under these conditions. In addition, MAL co-eluted with the solvent peak. Another characteristic noted under these conditions was the poor symmetry of the DPH peak which exhibited significant tailing (peak symmetry factor > 2.4). However, separation between the other critical peak pair of DPH and CPM, though not perfect, was achieved at a resolution of 1.94 with distinct baseline separation between the two peaks.

The effect of adjusting pH of the phosphate buffer on the separation of the component peaks and improving the symmetry factors of all the observed signals was then investigated. For this purpose, mobile phases were prepared at pH 5.0 and 6.0. The unadjusted pH of the 0.2 M KH_2PO_4 solution used in the preceding step had been determined as 4.3. Adjustment of the buffer solution to the higher pH 5.0 and 6.0 values was therefore achieved by adding equimolar solutions of 0.2 M dipotassium hydrogen phosphate (K_2HPO_4). The effect of pH of the phosphate buffer mobile phase on the retention, resolution and symmetry of the different component peaks are summarized in Table 2.5.

Table 2.5. Effect of mobile phase inorganic buffer pH on chromatographic parameters of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM)

Mobile phase composition	Drug	Retention time (min)	Resolution*	k'	Peak symmetry
MeOH-0.2 M KH_2PO_4 pH 4.3-H_2O (50:10:40, % v/v/v)	EPD	2.83	1.58	0.14	1.84
	CPM	6.01	11.07	1.42	1.69
	DPH	7.02	1.94	1.83	2.41
	PRM	11.19	7.00	3.51	1.53
MeOH-0.2 M KH_2PO_4 pH 5.0 -H_2O (50:10:50, % v/v/v)	EPD	2.84	1.80	0.15	1.81
	CPM	6.48	12.75	1.61	1.52
	DPH	7.91	3.88	2.19	2.26
	PRM	15.35	15.50	5.19	1.20
MeOH-0.2 M KH_2PO_4 pH 6.0 -H_2O (50:10:40, % v/v/v)	EPD	2.95	2.55	0.19	1.82
	CPM	10.39	23.64	3.19	1.21
	DPH	15.87	10.98	5.40	0.86
	PRM	49.03	32.24	18.77	1.21

*Resolution in each case calculated with reference to the peak eluting immediately before the component whose value is indicated.
Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 20 µL.

Raising the pH of the mobile phase buffer had the effect of progressively increasing the retention of CPM, DPH and PRM whereas EPD was not affected significantly. At pH 5.0 there was increased retention of both CPM and DPH by up to 1 min while the retention time of PRM was increased by more than 4 min to give an overall run time of about 16 min. The symmetry of all the four component peaks was improved at pH 5.0 although DPH still gave an asymmetry factor of 2.2. At this pH also, the EPD peak was still not completely baseline resolved from the MAL peak which still co-eluted with the solvent front.

The four analytes under study are all basic compounds with dissociation constants ranging from 9.0 to 9.6. At pH less than 7.0, which is two units below the pKa values of these compounds, they would all be expected to exist as virtually completely ionized

species in solution. Therefore, during the LC analysis of the working standard mixture, the use of buffered mobile phase at the pH ranges investigated (4.0-6.0) would be expected to cause the analytes to exist in predominantly ionic and hydrophilic form in the chromatographic column. In reversed-phase LC, more polar hydrophilic compounds are eluted from the column earlier than less hydrophilic compounds. In the case of EPD, DPH, CPM and PRM this mechanism would not be very significant in determining retention times since at the pH range studied, all four compounds would exhibit almost identical hydrophilic character by virtue of all being completely ionized at pH 4.0, 5.0 and 6.0. Therefore, another factor apart from ionization of the analytes must have been the cause of the observed changes in retention time observed as mobile phase buffer pH was adjusted. This behavior may have been attributed to the degree of analyte solvation resulting from changes in mobile phase pH. Weak organic bases such as EPD, DPH, CPM and PRM when ionized at low pH exhibit increased solvation as the solution pH is lowered. This phenomenon is especially pronounced when the bases are in a solution containing a protic solvent such as methanol [25]. This characteristic of the analyte compounds might explain the trend of consistently decreasing retention times as mobile phase pH was lowered, since the subsequent increased degree of solvation of the drugs would result in their increased interaction with the mobile phase as opposed to the hydrophobic stationary phase and hence faster elution from the column.

At pH 6.0, there was improved separation of all the component peaks with baseline separation. However, the retention times of CPM, EPD and PRM were all significantly increased with PRM exhibiting the most dramatic change to 49.0 min. Because of this change in retention times, the analysis time for the working mixture was impractically

long at approximately 55 min. The peak symmetry factors for CPM and DPH were further reduced at pH 6.0 but DPH now showed significant peak fronting (As 0.86).

Introduction of an inorganic buffer into the mobile phase was thus observed to significantly improve the separation of EPD, CPM, DPH and PRM. In addition, the pH of the buffer was noted to influence both the retention times and symmetry factors of most of the component peaks.

2.3.5.2 Effect of organic sodium acetate buffer and pH

To further improve the separation achieved using inorganic phosphate buffer, an alternative organic buffer was investigated as a possible replacement. For this, sodium acetate (CH_3COONa) was selected. Sodium acetate (0.2 M) was introduced into the mobile phase to replace inorganic potassium dihydrogen phosphate as buffer at the same concentration (10% v/v). The effect of pH using this buffer was investigated at values of 4.0, 5.0 and 6.0. The elution profile (Figure 2.3) observed using this buffer was similar to that seen using potassium phosphate with the 5 peaks being eluted in the same order.

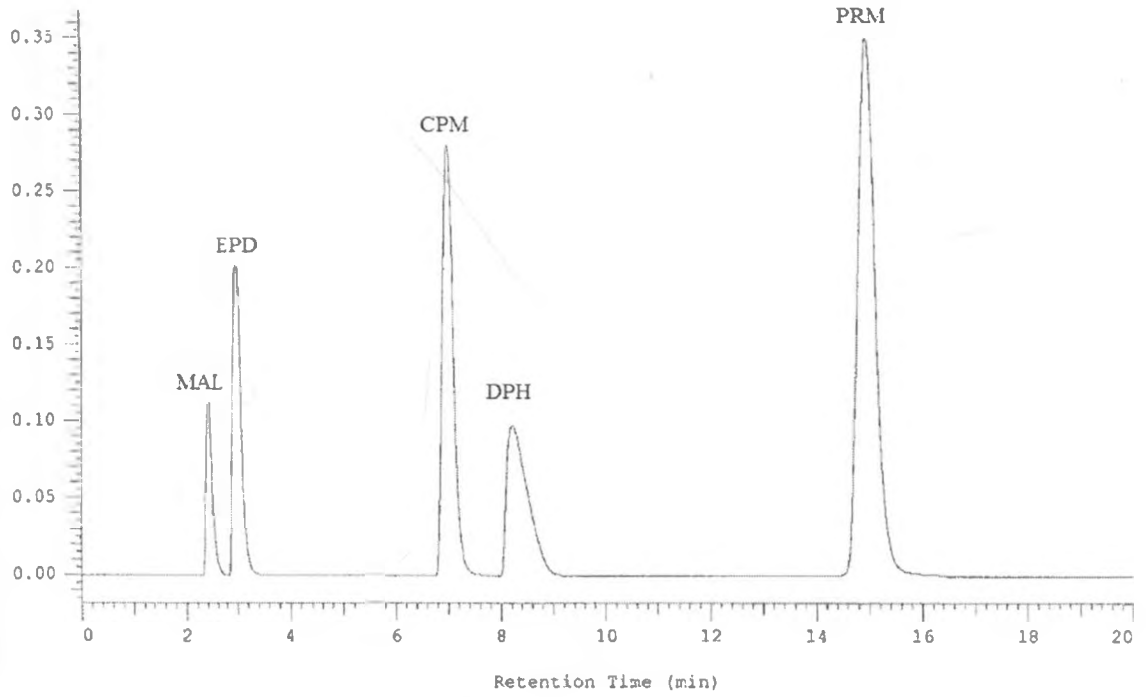


Figure 2.3. Chromatogram of a mixture of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM) using sodium acetate in mobile phase
Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-0.2M sodium acetate pH 5.0-water (50:10:40, % v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 20 µL.

Under these chromatographic conditions, the MAL peak was not retained and eluted first from the column at the same time as the solvent front. At the three pH values, good base line separation of the component peaks was observed and the resolution between neighbouring peak pairs was greater than 2.0 in all cases. The retention times and symmetry factors of the component peaks observed are summarized in Table 2.6.

Table 2.6. Effect of mobile phase sodium acetate buffer pH on chromatographic parameters of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM)

Mobile phase composition	Drug	Retention time (min)	Resolution	k'	Peak symmetry
MeOH-0.2 M CH_3COONa pH 4.0-H_2O (50:10:40, % v/v/v)	EPD	2.94	2.44	0.20	1.80
	CPM	6.42	12.36	1.63	1.58
	DPH	7.40	2.00	2.03	2.31
	PRM	11.95	7.95	3.90	1.41
MeOH-0.2 M CH_3COONa pH 5.0-H_2O (50:10:40, % v/v/v)	EPD	2.99	2.64	0.23	1.82
	CPM	6.98	13.53	1.87	1.59
	DPH	8.23	2.34	2.39	2.40
	PRM	14.93	10.40	5.14	1.42
MeOH-0.2 M CH_3COONa pH 6.0-H_2O (50:10:40, % v/v/v)	EPD	3.03	2.93	0.26	1.86
	CPM	8.83	18.47	2.66	1.36
	DPH	12.18	7.38	4.05	0.91
	PRM	31.60	25.52	12.11	1.17

Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 20 µL.

Increasing pH from 4.0 to 6.0 gradually increased the retention times as illustrated by the change in capacity factors of CPM, DPH and PRM while EPD remained largely unaffected (Figure 2.4). At both pH 4.0 and 5.0, the retention times of the component peaks were almost identical to those observed using KH_2PO_4. The main difference noted between the two mobile phase systems was the reduced peak retention at pH 6.0 using the acetate buffer. At this pH, increase in retention from that observed at pH 5.0 was noted for CPM, DPH and PRM, but the change was not as significant as that observed while using KH_2PO_4 especially regarding PRM whose retention increased from 11.95 min to 31.60 min. The reduced PRM retention allowed for a better analytical run time of about 35 min compared to the 55 min that was the case using phosphate buffer at the same pH. Another advantage observed by the substitution of sodium acetate for potassium

dihydrogen phosphate as buffer was the better resolution achieved between the critical peak pairs MAL/EPD (2.44) and CPM/DPH (2.00) even at the lowest pH value 4.0. This was a marked improvement over the resolutions obtained when using the inorganic buffer. Although the acetate buffer appeared to offer an advantage over the phosphate at pH 6.0 in terms of reducing analysis run time, no noticeable improvement on peak symmetries was observed. The DPH peak still exhibited asymmetry factors of 2.31 at pH 4.0 and at pH 6.0, the peak showed fronting with an asymmetry factor of 0.91.

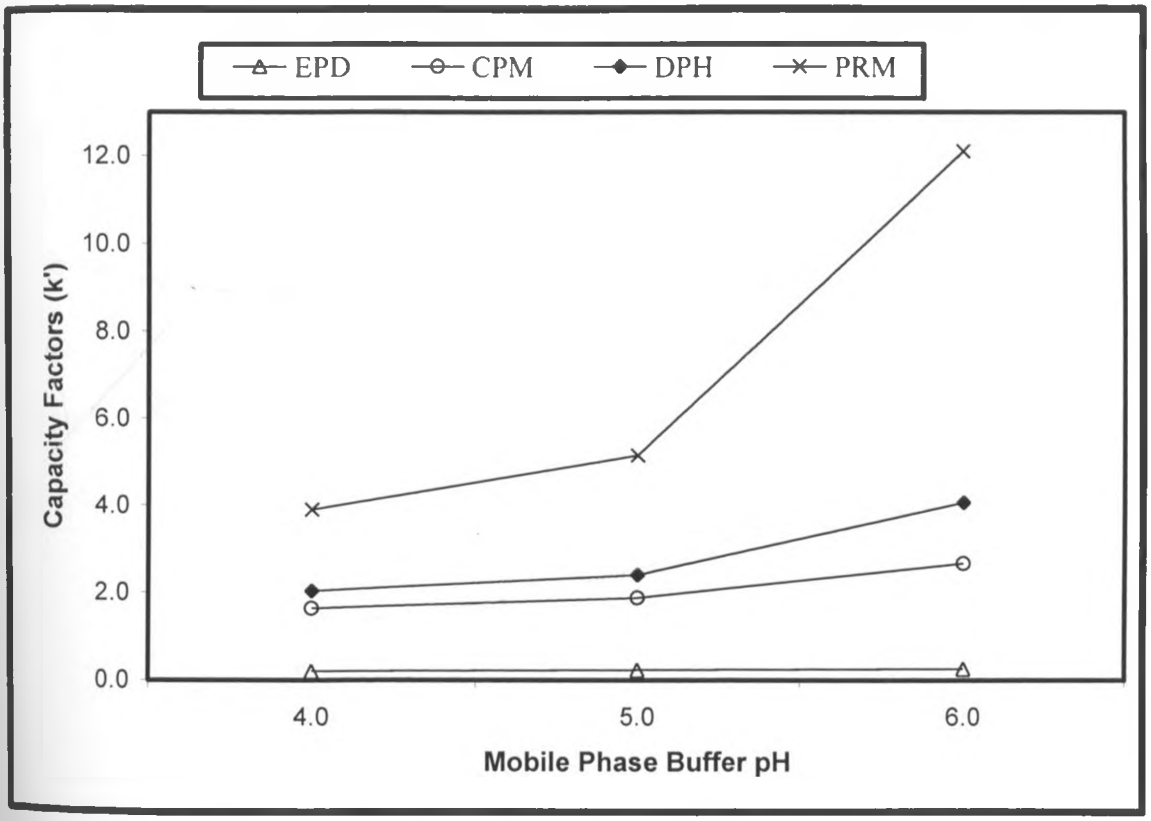


Figure 2.4. Effect of mobile phase 0.2 M sodium acetate buffer pH on capacity factors (k') of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM)
Column: Phenomenex Gemini-NX 5 µm. Mobile phase: Methanol-0.2 M Sodium acetate-Water (50:40:10, % v/v/v). Column temperature: 40 °C. Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 20 µL.

Another advantage of this buffer system was its higher miscibility with methanol compared to that of the inorganic phosphate buffer. Consequently, its incorporation into

the mobile phase would allow for higher concentrations of buffer to be used without the risk of precipitation of salts in the mobile phase during the course of analysis.

2.3.5.3 Effect of volatile organic ammonium acetate buffer and pH

In an attempt to improve on overall analysis run time as well as the peak symmetry particularly of DPH peak, the effect of employing a more volatile acetate buffer in place of sodium acetate was investigated. For this, ammonium acetate (CH_3COONH_4) was selected. Volatile organic buffers such as ammonium acetate are commonly used especially in LC-MS applications in which inorganic buffers cannot be used.

The effect of using ammonium acetate as the mobile phase buffer at pH 4.0, 5.0 and 6.0 was studied as a possible alternative to both sodium acetate and potassium phosphate. Stock 0.2 M ammonium acetate buffer solution was incorporated at a concentration of 10% v/v into the mobile phase. The pH was adjusted by use of 0.2 M glacial acetic acid. Figure 2.5 illustrates a typical chromatogram of the working standard solution analyzed using ammonium acetate buffer at pH 5.0.

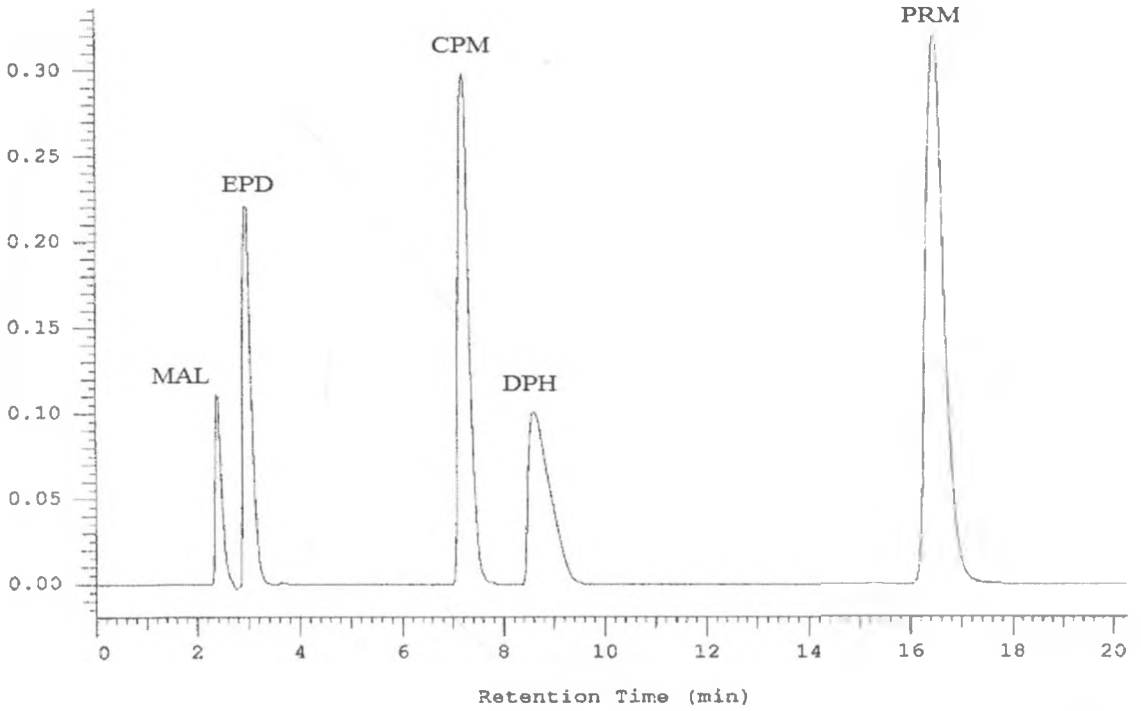


Figure 2.5. Chromatogram of a mixture of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM) using 0.02 M ammonium acetate in mobile phase
Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-0.2 M ammonium acetate pH 5.0-water (50:10:40, % v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 20 µL.

Table 2.7 is a summary of the effect of different pH values on the chromatographic parameters of component peaks in the working standard preparation. From the chromatograms obtained at different pH values using ammonium acetate as mobile phase buffer, it was evident that no significant change in peak symmetries or resolution between the critical peak pairs of MAL and EPD was achieved. Ammonium acetate would however be a better buffer to use in method development than the sodium salt because of its greater volatility and ideal application in LC-MS analysis. This buffer was therefore chosen as the one to use in the subsequent method development steps.

Table 2.7. Effect of mobile phase ammonium acetate buffer pH on chromatographic parameters of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM)

Mobile phase composition	Drug	Retention time (min)	Resolution	k'	Peak symmetry
MeOH-0.2 M CH_3COONH_4 pH 4.0-H_2O (50:10:40, % v/v/v)	EPD	2.96	2.46	0.21	1.80
	CPM	6.72	12.83	1.75	1.57
	DPH	7.79	2.01	2.19	2.49
	PRM	12.92	8.25	4.29	1.41
MeOH-0.2 M CH_3COONH_4 pH 5.0-H_2O (50:10:40, % v/v/v)	EPD	2.99	2.64	0.23	1.90
	CPM	7.23	14.03	1.98	1.54
	DPH	8.63	2.52	2.55	2.52
	PRM	16.50	11.60	5.79	1.35
MeOH-0.2 M CH_3COONH_4 pH 6.0-H_2O (50:10:40, % v/v/v)	EPD	3.12	3.09	0.29	1.72
	CPM	10.33	20.23	3.29	1.46
	DPH	13.94	6.46	4.78	2.46
	PRM	37.73	25.78	14.65	1.20

Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-0.2 M ammonium acetate pH (4.0-6.0)-water (50:10:40, % v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 20 µL.

2.3.5.4 Effect of ammonium acetate buffer concentration

Introduction of CH_3COONH_4 as the buffer in mobile phase at 0.02 M concentration had not resulted in improvement of the peak symmetry factors, especially that of DPH. The effect of increasing buffer concentration was investigated as the next step since this usually has the effect of improving peak shape and asymmetry [25]. Effect of ammonium acetate buffer concentration was studied while maintaining mobile phase pH at 5.0. This pH was selected as the optimum value since it allowed for a better analysis run time of 20 min compared to pH 6.0 at which analysis time was more than 40 min. Also, at pH 5.0, better resolution between the critical peak pairs of MAL/EPD and CPM/DPH was obtained than was the case at the lower pH 4.0 which, though it allowed for shorter run times, did not offer the same degree of selectivity. The range of concentrations

investigated were 10% to 40% v/v of 0.2 M ammonium acetate. The results obtained were as recorded in Table 2.8.

Table 2.8. Effect of mobile phase ammonium acetate buffer concentration on component peak asymmetry factors

0.2 M CH_3COONH_4 Concentration (% v/v)	Asymmetry Factors			
	EPD	CPM	DPH	PRM
10	1.90	1.54	2.52	1.35
20	1.80	1.48	2.30	1.27
25	1.84	1.45	2.11	1.34
30	1.80	1.43	2.13	1.25
35	1.79	1.43	2.00	1.32
40	1.80	1.39	2.01	1.24

Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-0.2 M ammonium acetate pH 5.0-water (50:x:50-x, % v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 20 µL.

Increasing ammonium acetate concentration improved the symmetry of all four component peaks with DPH exhibiting the most significant change from 2.52 at 10% v/v concentration to 2.01 at 40% v/v. The EPD peak showed no change in its tailing factor at concentrations from 20% to 40% while both PRM and CPM were only slightly affected over the entire concentration range investigated.

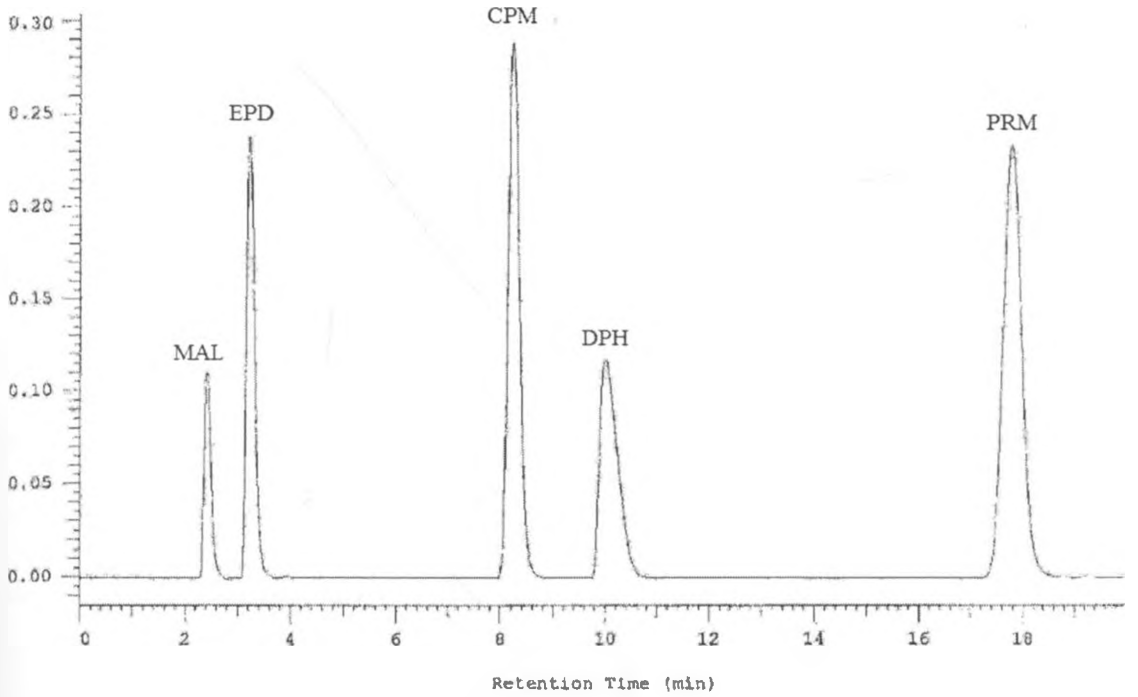


Figure 2.6. Chromatogram of a mixture of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM) using 0.08 M ammonium acetate in mobile phase
Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-0.2 M ammonium acetate pH 5.0-water (50:40:10, % v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 20 µL.

Increasing mobile phase CH_3COONH_4 concentration to 40% v/v improved the peak symmetry of DPH but it was not still possible to obtain the desired symmetry factor of < 2.0 for all the component peaks. Although solubility of even higher buffer concentrations in mobile phase containing methanol was not a concern in this case, increasing ammonium acetate concentration would limit the application of the proposed method in LC-MS applications where buffer concentrations are generally restricted to below 0.1 M [25]. For this reason, alternative methods were sought to improve peak shapes especially through the introduction of ion-pairing agents into the mobile phase.

2.3.5.5 Effect of ion-pairing agents

Incorporation of ion-pairing agents in LC mobile phases is often employed as a technique to improve the separation and symmetry of component peaks. The most commonly

employed ion-pairing agents in reversed-phase LC are salts of alkyl sulfonates, as well as tertiary and quartenary ammonium compounds. These reagents are typically added to the mobile phase at very low concentrations (usually ≤ 10 mM) and interact with basic and acidic analyte molecules as well as the stationary phase to improve separation of components in the LC column.

Four different ion-pairing agents, namely, triethylamine, triethanolamine, tetrabutyl ammonium hydrogen sulphate (TBAHS) and tetrabutyl ammonium hydroxide (TBAH) were incorporated into the mobile phase in order to improve the symmetry of the DPH peak and enhance the separation between MAL and EPD. Stock solutions containing 0.2 M of each ion-pairing agent were prepared and added to the 0.2 M CH_3COONH_4 buffer solution before adjusting pH to 5.0. The volume of stock ion pair reagent solution added to the mobile phase was intended to give a final effective concentration of 10 mM of the agent upon addition of the methanol organic modifier solvent.

Table 2.9 summarizes the effect of incorporation of the different ion-pairing agents in mobile phase on chromatographic parameters of component peaks.

Table 2.9. Effect of ion-pairing agents in mobile containing 10% v/v, 0.2 M ammonium acetate on chromatographic parameters of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM)

Ion-pairing agent (10 mM)	Asymmetry Factors				k'			
	EPD	CPM	DPH	PRM	EPD	CPM	DPH	PRM
None	1.90	1.54	2.52	1.35	0.23	1.98	2.55	5.79
Triethylamine	1.86	1.47	2.17	1.35	0.24	1.93	2.51	5.22
Triethanolamine	1.81	1.49	2.16	1.42	0.25	1.90	2.44	5.08
TBAH	1.64	1.42	1.55	1.23	0.00	0.98	1.33	3.17
TBAHS	1.76	1.32	1.21	1.14	0.04	1.13	1.49	3.50

Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-0.2 M ammonium acetate pH 5.0-water (50:40:10, % v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 20 µL.

The tertiary amine ion-pairing agents triethylamine and triethanolamine yielded comparable results in reducing the tailing factors of all component peaks while at the same time causing a noticeable decrease in PRM peak retention time. The capacity factors of all other peaks were not significantly altered. It was also evident that both reagents appeared to have an appreciable effect on especially the tailing factor of the DPH peak, reducing this significantly from 2.52 to about 2.2. The effect on other peaks was less pronounced with triethylamine having only a slightly greater impact in reducing CPM asymmetry compared to triethanolamine with the converse being the case with EPD. The PRM asymmetry was unchanged by addition of triethylamine while triethanolamine actually resulted in a slight increase in tailing.

The hydroxide and hydrogen sulphate salts of the quaternary ammonium ion-pairing agent tetrabutylammonium resulted in almost identical but even more significant alterations in both peak symmetry and peak retention compared to triethylamine and triethanolamine (Figure 2.7).

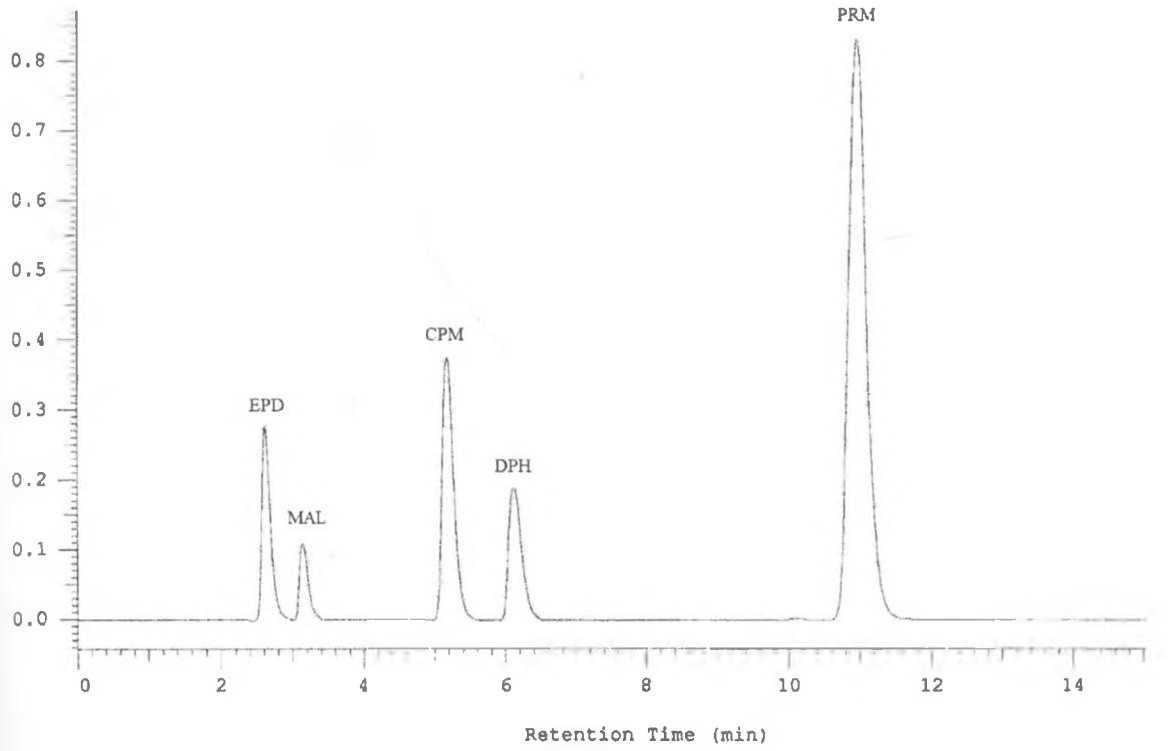


Figure 2.7. Chromatogram of a mixture of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM) using 0.01 M tetrabutyl ammonium hydroxide in mobile phase

Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-0.2 M tetrabutyl ammonium hydroxide- 0.2 M ammonium acetate pH 5.0-water (50:5:40:5, % v/v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 20 µL.

Both these ion-pairing agents caused a reduction in the peak tailing for all component peaks, including DPH with resultant asymmetry factors of less than 2.0 for all peaks. Additionally, they caused a significant reduction in retention times for all the peaks with CPM, DPH and PRM being most affected. From an initial retention time of approximately 17 min for PRM, addition of tetrabutylammonium ion-pairing agents into the mobile phase resulted in a decrease to about 10-12 min. Incorporation of either of these reagents also resulted in the interchange of the elution sequence of MAL and EPD with EPD eluting first but in the process, also merging with the solvent front. In principle, it was more appropriate for MAL to elute before EPD and even though the tetrabutylammonium reagents appeared to provide the desired effect of reducing peak

symmetry of all components to within the desired limits, the main impediment to their use was their reversal of the elution sequence of these two peaks. This was a significant drawback because co-elution of EPD with the solvent front would greatly undermine the ability of the method developed to accurately quantify this component and consequently its use in calculating the content of ephedrine in test samples.

Further investigations were performed in full using triethylamine with this compound being selected in favour over triethanolamine mainly due to its greater volatility and hence potential compatibility for application in LC-MS analysis in addition to its more common application in numerous diverse officially recognized LC methods.

2.3.5.6 Effect of triethylamine and increased buffer concentration

From the studies carried out on the effect of mobile phase buffer concentration and incorporation of different ion-pairing agents in the mobile phase, ways in which both these factors could be combined to try improve peak symmetry were investigated. Triethylamine (TEA) at concentration 10 mM was added to different mobile phase preparations containing varying concentrations of 0.2 M ammonium acetate ranging from 10% to 40%.

The results obtained from these experiments (Table 2.10) indicated a gradual improvement of component peak symmetries with increasing ammonium acetate concentration. In the case of DPH, which had previously exhibited the highest asymmetry of the analyte peaks, tailing reduced from 2.17 at 10% CH_3COONH_4 concentration to 1.92 at 40% buffer concentration.

Table 2.10. Effect of ammonium acetate buffer concentration in mobile phase containing 10 mM triethylamine on chromatographic parameters of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM)

0.2M CH_3COONH_4 Concentration (% v/v)	Asymmetry Factors				k'			
	EPD	CPM	DPH	PRM	EPD	CPM	DPH	PRM
10	1.86	1.47	2.17	1.35	0.24	1.93	2.51	5.22
20	1.83	1.44	2.09	1.31	0.27	2.13	2.78	5.72
25	1.80	1.42	2.09	1.28	0.30	2.29	2.99	6.13
30	1.80	1.39	1.96	1.28	0.30	2.22	2.90	5.88
40	1.73	1.35	1.92	1.18	0.31	2.19	2.86	5.77

Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:x:49.85-x, % v/v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 20 µL.

To further improve the symmetry of the peaks, the effect of varying concentration of triethylamine while maintaining the ammonium acetate buffer concentration at 40% was investigated at pH 5.0 (Table 2.11).

Table 2.11. Effect of triethylamine concentration in mobile phase containing 40% v/v, 0.2 M ammonium acetate pH 5.0 on chromatographic parameters of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM)

Triethylamine Concentration (mM)	Asymmetry Factors				k'			
	EPD	CPM	DPH	PRM	EPD	CPM	DPH	PRM
0	1.80	1.39	2.01	1.24	0.35	2.58	3.37	6.85
5	1.77	1.36	1.98	1.18	0.32	2.25	2.94	5.94
10	1.73	1.35	1.92	1.18	0.31	2.19	2.86	5.77
15	1.74	1.33	1.89	1.18	0.32	2.21	2.89	5.81

Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:x:40:10-x, % v/v/v/v). Flow rate: 1.00 mL/ min. Detection: 254 nm. Injection volume: 20 µL.

The most conspicuous effect of incorporating TEA into the mobile phase was its impact in reducing the retention times of all 4 analyte peaks. This was most dramatic with PRM resulting in a shortening of the run time for this compound by more than 2 min at 5 mM. The other effect of TEA was the improvement of all four peak asymmetry factors. In the

case of DPH, asymmetry reduced from 2.01 to 1.98. Further increase of TEA concentration to 10 mM improved DPH asymmetry even more, reducing it to 1.92 while reducing retention times of CPM, DPH and PRM only slightly. At 15 mM TEA concentration, no further significant improvement on component peak asymmetries was achieved while the retention times of CPM, DPH and PRM increased slightly compared to 10 mM TEA.

On the basis of the observations made, 10 mM TEA was chosen as the optimum level at which its effect on both peak asymmetry and retention was most advantageous.

2.3.5.7 Effect of organic modifier concentration

The influence on retention times as well as capacity and retention factors of the methanol concentration in the mobile phase was studied while fixing 0.2M ammonium acetate buffer concentration at 40% and pH 5.0 with triethylamine content at 10 mM. The effect of methanol concentration ranging from 40% to 60% is summarized in Table 2.12 and illustrated in Figure 2.8.

Table 2.12: Effect of mobile phase methanol concentration on chromatographic parameters of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM)

% v/v Methanol content in Mobile Phase	Drug	Retention time (min)	Resolution	k'	Peak symmetry
40	EPD	3.93	5.95	0.57	1.77
	CPM	17.73	29.84	6.06	1.51
	DPH	22.93	5.54	8.13	2.79
	PRM	45.67	16.98	17.19	1.25
45	EPD	3.52	4.78	0.42	1.78
	CPM	11.21	22.11	3.52	1.47
	DPH	14.05	4.69	4.66	2.24
	PRM	25.96	14.59	9.47	1.25
50	EPD	3.23	3.77	0.31	1.73
	CPM	7.70	15.70	2.19	1.35
	DPH	9.30	3.72	2.86	1.92
	PRM	15.97	12.12	5.77	1.18
55	EPD	3.03	2.94	0.24	1.80
	CPM	5.72	10.92	1.35	1.50
	DPH	6.66	2.83	1.73	1.75
	PRM	10.63	9.85	3.36	1.30
60	EPD	2.88	2.29	0.19	1.83
	CPM	4.57	7.57	0.89	1.57
	DPH	5.14	2.07	1.12	1.68
	PRM	7.66	7.85	2.17	1.41

Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:40:9.85, % v/v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 20 µL.

Increasing the concentration of methanol in the mobile phase was observed, as expected, to systematically reduce the retention times of all the component peaks due to the increase in the eluting power of the mobile phase. Reduction in peak retention was most noticeable in the case of PRM, with CPM and DPH exhibiting almost identical trends in

this behavior. Ephedrine was the component peak least affected by increase in methanol concentration, with retention only reducing marginally compared to the other peaks.

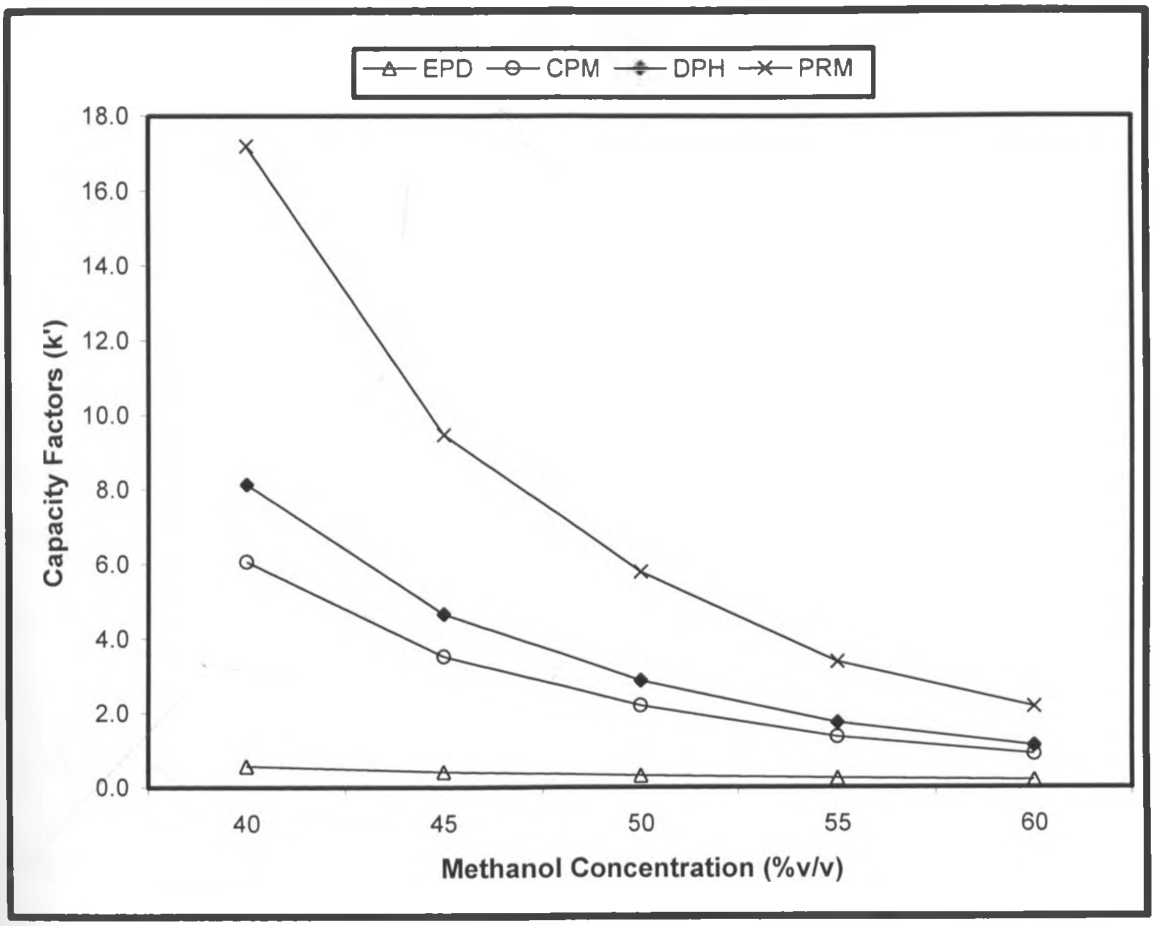


Figure 2.8. Effect of mobile phase methanol concentration on capacity factors of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM)

Another phenomenon noted with increasing methanol was the change in component peak symmetries. Increasing methanol systematically resulted in reduction of DPH tailing - with this peak having been noted to be consistently problematic with regard to this parameter. The effect on methanol on the other component peaks was however not as clear-cut. Increasing concentration from 40% to 50% resulted in the general reduction in peak symmetry for virtually all peaks, the optimum being observed at 50%. However, further increase in the amount of methanol beyond 50% in the mobile phase was

observed to have the opposite effect on the peak symmetries of EPD, CPM and PRM leading to an increase in the peak tailing factors of all 3 compounds. As noted before, only DPH appeared to consistently exhibit reducing peak tailing with increasing methanol concentration in the mobile phase.

Although increasing methanol had the advantage of reducing peak retentions and therefore offered an opportunity to decrease the analytical run-time, at concentrations of 55% and 60%, it was observed that the resolution between CPM and DPH was adversely affected. From a resolution value for this peak pair of 4.7 at 50% methanol concentration, there was a decrease to 3.4 at 55% methanol concentration and further decrease to 2.1 at 60% methanol. In addition, these higher methanol concentrations were also noted to have the negative influence of increasing peak symmetry factors for EPD, CPM and PRM. Consequently, methanol concentration of 50% was settled upon as the optimum level at which to incorporate the organic modifier in the mobile phase.

2.3.5.8 Effect of column temperature

The temperature at which LC is carried out has a significant bearing in the ability of the technique to separate components in a mixture. Temperature affects the density and viscosity of the mobile phase and consequently column back pressures. Most liquid mobile phases exhibit lower viscosity and density at higher temperatures, resulting in reduced column back pressures. Higher temperatures also increase the mass transfer of analyte components separated in the column resulting in reduced retention and thus shorter analysis time in most cases. There is, however, a limit to the temperatures under which silica based columns can be optimally utilized. Beyond 60 °C, silica based packings become unstable especially when used with mobile phases at pH above 7.0.

Additionally, many compounds may become unstable at elevated temperatures, resulting in their hydrolysis and degradation while under analysis.

At the beginning of the method development process, column temperature had been fixed at 40 ˚C with this value being maintained in all subsequent development steps. Having studied the effects of other chromatographic factors especially mobile phase composition, the effect of temperature on separation of the analyte components was investigated using mobile phase of composition methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:40:9.85, % v/v/v/v). The temperature range investigated was 30 ˚C to 50 ˚C at 5 ˚C intervals (Table 2.13).

Increase in column temperature from 30 ˚C to 50 ˚C was observed to systematically reduce the retention times of all components with the exception of EPD which exhibited only slight variation with rise in temperature. From an overall analysis run-time of approximately 25 min at 30 ˚C, increasing temperature to 50 ˚C reduced the analysis time to about 18 min. which represented only 2 min improvement on the 20 min run-time achieved at 40 ˚C. Increasing the temperature was also observed to reduce the resolution between component peaks. However, this change, even in the case of the critical peak pairs of MAL/EPD and CPM/DPH was not considered significant since even at the highest temperature (50 ˚C), resolution values for these 2 sets of peaks was still > 3.0 indicating complete baseline separation. The effect of raising column temperature on improving peak symmetries was observed to be negligible for most peaks, while in the case of EPD, temperatures higher than 40 ˚C actually resulted in slightly increased tailing.

Table 2.13. Effect of column temperature on chromatographic parameters of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM)

Column Temperature (° C)	Drug	Retention time (min)	Resolution	k'	Peak symmetry
30	EPD	3.38	4.25	0.36	1.73
	CPM	9.10	18.39	2.65	1.29
	DPH	11.23	4.24	3.51	1.95
	PRM	20.61	13.75	7.28	1.15
35	EPD	3.30	4.08	0.33	1.76
	CPM	8.45	17.75	2.42	1.33
	DPH	10.35	4.09	3.19	1.95
	PRM	18.55	13.42	6.51	1.16
40	EPD	3.23	3.93	0.32	1.77
	CPM	7.93	17.11	2.25	1.34
	DPH	9.62	3.95	2.94	1.94
	PRM	16.90	13.03	5.92	1.14
45	EPD	3.16	3.75	0.31	1.85
	CPM	7.46	16.36	2.08	1.33
	DPH	8.97	3.79	2.71	1.93
	PRM	15.49	12.59	5.40	1.11
50	EPD	3.10	3.59	0.29	1.86
	CPM	7.05	15.61	1.93	1.35
	DPH	8.42	3.64	2.49	1.91
	PRM	14.31	12.14	4.94	1.11

Column: Phenomenex Gemini-NX 5 µm. Mobile phase: methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:40:9.85, % v/v/v/v). Flow rate: 1.00 mL/ min. Detection: 254 nm. Injection volume: 20 µL.

On the basis of the observations made on the influence of column temperatures on chromatographic parameters, 40 °C was retained as the optimum setting at which to carry out analysis using the method. The only advantage of using higher temperature than this was a slight reduction in analysis run-time which when weighed against the potential damage to the stationary phase on prolonged operation at such elevated temperatures was

considered injudicious. Lower temperatures on the other hand would unnecessarily delay the analysis run-time resulting in a more time-consuming method not ideal for routine application, while at the same time leading to use of higher volumes of mobile phase, thereby increasing the cost of analysis.

2.3.6 Optimized chromatographic conditions

From the data collected, optimum chromatographic separation parameters were established as: mobile phase consisting of methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:40:9.85, % v/v/v/v) delivered at a flow rate of 1.00 mL/min. The stationary phase comprising a reverse phase Phenomenex Gemini-NX C18 chromatography column of dimensions 250 mm length and 4.6 mm internal diameter with particle size 5 µm maintained at a temperature of 40 ˚C with detection wavelength set at 254 nm.

Figure 2.9 is a representative chromatogram showing the separation of components in the working standard solution tested under the established optimum conditions.

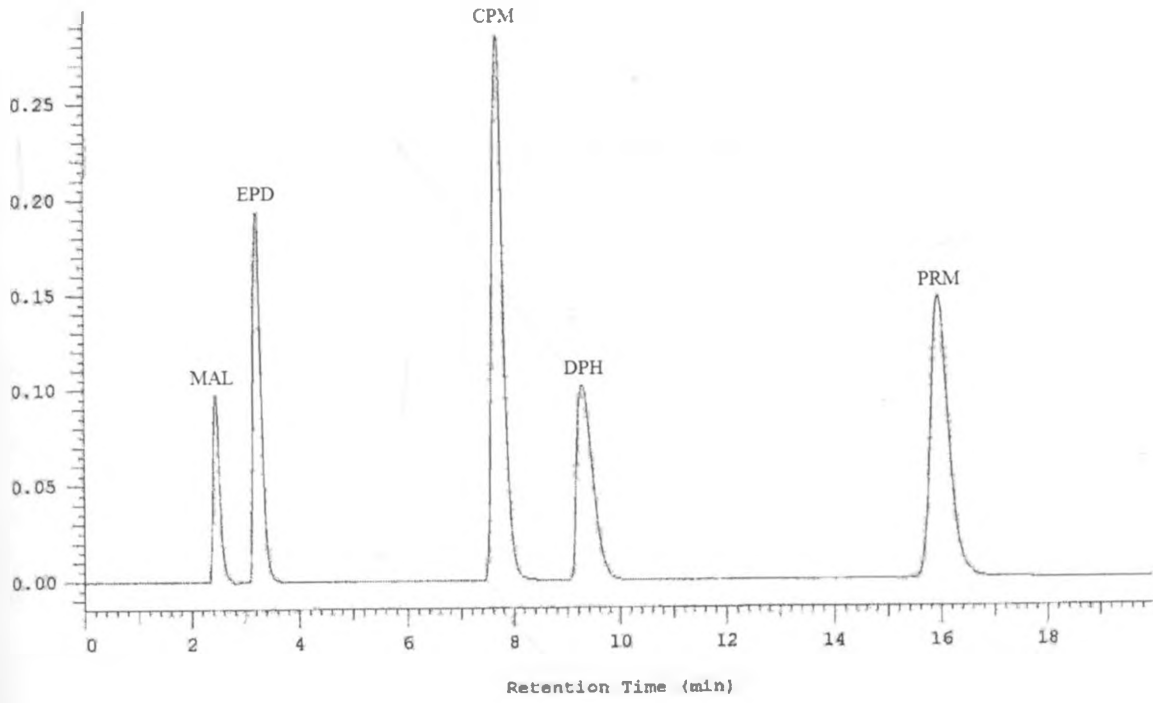


Figure 2.9. Chromatogram of a mixture of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM) at optimized chromatographic conditions Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:40:9.85, % v/v/v/v). Flow rate: 1.00 mL/ min. Detection: 254 nm. Injection volume: 20 µL. Concentrations: EPD 1.5 mg/mL, CPM 0.2 mg/mL, DPH 1.0 mg/mL, PRM 0.05 mg/mL.

CHAPTER THREE

METHOD VALIDATION

3.1 Introduction

Validation of an analytical method is the process by which it is determined, through laboratory studies, that the performance characteristics of the method meet the requirements for its intended analytical applications. Typically, the characteristics that are studied during method validation include: sensitivity, accuracy, precision, specificity, linearity of detector response, range of analyte concentration and robustness. Accuracy of a method is usually determined by comparing the assay results obtained using the method under validation against those obtained using an established and well validated pre-existing method. In the case of the test method under development, accuracy could not be determined using such a comparison since no prior validated method has been documented. Consequently, as proposed in the International Committee on Harmonisation (ICH) guidelines for analytical method validation, accuracy under such circumstances may be inferred from the results obtained from the other validation parameters namely sensitivity, precision and linearity [28,61].

3.2 Determination of sensitivity

The sensitivity of a liquid chromatography technique may be defined as its ability to detect and respond to the presence of analytes separated in the analytical column. Sensitivity depends on the magnitude of detector response per unit amount or concentration of separated component eluting from the column. Estimation of the sensitivity of a LC method is done quantitatively through the limit of detection and limit

of quantitation. High sensitivity is an ideal characteristic since it indicates that the analytical method is capable of detecting minute quantities of analytes.

3.2.1 Limit of Detection

The limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected but not necessarily quantified using an analytical technique under specified experimental conditions. The LOD was determined by preparing serial dilutions of each analyte compound from stock solutions containing EPD 0.6 mg/mL, CPM 0.2 mg/mL, DPH 0.4 mg/mL and PRM 0.05 mg/mL. The solutions were injected in triplicate and the signal to noise ratio values of the component peaks determined with reference to a blank injection of the diluent solution run under the same LC conditions. The LOD was determined from the lowest concentration of the analyte that yielded a definitive peak at S/N ratio of between 2 and 3 [28,61]. The results obtained are summarized in Table 3.1.

3.2.2 Limit of Quantitation

The limit of quantitation (LOQ) is defined as the lowest amount of analyte in a sample that can de determined with acceptable precision and accuracy under specified experimental conditions. The degree of precision considered to be acceptable for purposes of LOQ determination from peak areas of replicate injections (n=3) is RSD less than 10-20% and at a signal to noise ratio of 10 [28,61].

LOQ of the developed method was determined using the same procedure for LOD with serial dilutions of the stock solutions containing EPD 0.6 mg/mL, CPM 0.2 mg/mL, DPH 0.4 mg/mL and PRM 0.05 mg/mL being injected into the chromatograph. Results obtained are summarized in Table 3.1.

Table 3.1. Limit of Detection and Limit of Quantitation for ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM)

Drug	Limit of Detection (ng)	Limit of Quantitation (ng)	Peak Areas RSD (%) at LOQ
EPD	234.4	1125.4	1.3
CPM	0.2	1.1	4.2
DPH	13.0	32.6	4.4
PRM	0.04	0.7	6.0

Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:40:9.85, % v/v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 50 µL.

From the results obtained, the method was found to exhibit satisfactory precision at the limit of quantification for all four analyte compounds, with each peak area yielding RSD values less than 20%. In the case of EPD, precision was highest with RSD value 1.3%.

3.3 Linearity and range

The linearity of an analytical method is defined as its ability to elicit detector responses that are directly proportional to the concentration of analyte in samples within a given range. In the case of LC methods, the component peak areas should be directly proportional to analyte concentrations over the method's specified linear range. The ICH guidelines on method validation recommend that linearity be demonstrated at a minimum of five concentration levels with particular emphasis on the range from 80% to 120% of the analytical working concentration [28,61].

Linearity of the developed method was determined by preparing a standard stock solution from which working solutions were prepared by diluting appropriately to yield solutions containing 25%, 50%, 75%, 100%, 125% and 150% of the working standard solution concentration (EPD 0.6 mg/ml, CPM 0.08 mg/ml, DPH 0.4 mg/ml and PRM 0.2 mg/ml).

Each of these solutions was then analyzed in triplicate and the peak areas obtained from each analyte compound plotted against concentration.

The data obtained from the linearity determination experiments was subjected to linear regression analysis with the concentration of drug compound (in mg/mL) injected being plotted against the peak areas obtained on replicate injections. A summary of the linearity analysis results obtained is shown in Table 3.2:

Table 3.2. Parameters for linearity of detector response for ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM)

Drug	Slope	y - intercept	R^2 Value	Standard error of estimate $S_{y,x}$
EPD	1,358,372	- 1,487.15	0.9999	4,381
CPM	20,875,948	- 24,156.85	0.9997	12,410
DPH	2,552,972	- 13,199.40	0.9997	8,360
PRM	109,676,910	+ 1,536,244.95	0.9979	459,671

Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:40:9.85, % v/v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 50 µL.

3.4 Precision

The precision of an analytical method refers to the degree to which individual test results from repeated analysis of the same sample are similar to one another. Precision is expressed as the relative standard deviation (coefficient of variation) of the results obtained from analysis of a series of replicate determinations of a homogenous sample using the method being evaluated. Precision is measured using three parameters: Repeatability, intermediate precision and reproducibility [61]. The coefficient of variation of six replicate injections run on the same day is used as a measure of repeatability. This parameter is also called intra-day precision. Intermediate precision is determined using the coefficient of variation of various assays done on different days,

using different equipment or by different analysts. This study only focused on inter-day variability as a measure of intermediate precision. Reproducibility is assessed by means of inter-laboratory trials but this was not done during the course of this study because it was not one of the objectives.

The intra-day variation was determined by preparing 3 different standard solutions containing 0.6 mg/mL EPD, 0.08 mg/mL CPM, 0.4 mg/mL DPH and 0.2 mg/mL PRM on the same day and subsequently injecting each solution into the chromatography system six times. The peak areas of all four components from the injections run were normalized to the desired component concentration and the relative standard deviation of the corrected areas calculated as summarized in Table 3.3.

The inter-day precision of the method was determined by running 6 replicate injections of a freshly prepared standard solution on different days for a 3 day interval. Fresh mobile phase was prepared for each day of analysis. The component peak areas obtained were all normalized to the desired concentration and the relative standard deviation of normalized areas calculated to determine the inter-day precision of the method. The results obtained are summarized in Table 3.3.

Table 3.3: Intra-day and inter-day method precision for ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM)

Drug	Within-day peak areas % coefficient of variation (n=18)	Between-day peak areas % coefficient of variation (n=36)
EPD	1.26	1.76
CPM	1.48	2.13
DPH	0.91	1.70
PRM	1.71	1.96

Column: Phenomenex Gemini-NX· 5 µm. Column temperature: 40 °C. Mobile phase: methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:40:9.85, % v/v/v/v). Flow rate: 1.00 mL/ min. Detection: 254 nm. Injection volume: 50 µL.

3.5 Robustness

The capacity of an analytical method to remain unaffected by small changes in parameters is defined as its robustness [28,61]. To determine the robustness of the developed method, the effect of making small but deliberate adjustments in the optimized chromatographic factors was investigated. The factors adjusted were pH of the buffer, column temperature and methanol concentration. These were selected based on observations made during method development that showed all three to have a significant influence the separation of the analytes and especially on the selectivity between critical peak pairs MAL/EPD and CPM/DPH.

Ideally, experimental designs are employed in testing method robustness, involving the use of special software that determines the number of experiments and factors to be adjusted as well as evaluating the data obtained. In the absence of such a tool for comprehensive robustness experimental design, simple robustness of the method was determined from the degree of variation observed in peak areas and retention times from the same working standard solution analyzed while adjusting each of the LC factors indicated. Six replicate injections of the same working standard solution were run after having adjusted a single chromatographic parameter and the relative standard deviations of both peak areas and retention times of component peaks calculated. The degree of variation observed was then used to infer the method's robustness. The influence of changing each of the three chromatographic factors was tested at 3 levels. The buffer pH was studied at 4.5, 5.0 and 5.5, column temperature at 35 °C, 40 °C and 45 °C while influence of methanol concentration was tested at 45%, 50% and 55% v/v. The findings are summarized in Table 3.4. The working standard solution used in the robustness study

contained EPD 0.6 mg/mL, CPM 0.08 mg/mL, DPH 0.4 mg/mL and PRM 0.2 mg/mL in a mixture of methanol-water (50:50, % v/v) as solvent.

From the results obtained, the method appeared to be largely unaffected by changes in all three chromatographic parameters on the quantification of component peak areas. Of the four test compounds, only PRM areas seemed to be significantly affected by changes methanol concentration and to a lesser degree, by buffer pH. This change was probably due to increased retention times resulting in peak broadening and thus adversely affecting peak integration. Ephedrine, chlorpheniramine and diphenhydramine exhibited little change in peak areas with variation of the three LC factors.

Table 3.4. Effect of column temperature, mobile phase buffer pH and mobile phase methanol concentration on peak areas and retention times of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM)

Chromatographic Parameter Altered	Drug	Peak Area RSD (%)	Retention Time RSD (%)
Column Temperature (35, 40, 45 °C)	EPD	0.33	1.94
	CPM	0.43	5.61
	DPH	0.74	6.40
	PRM	2.75	7.98
Mobile Phase Buffer pH (4.5, 5.0, 5.5)	EPD	0.55	0.39
	CPM	1.66	3.09
	DPH	0.60	4.52
	PRM	3.17	11.27
Mobile Phase Methanol Concentration (45%, 50%, 55% v/v)	EPD	1.96	6.09
	CPM	0.67	27.38
	DPH	0.77	30.26
	PRM	4.75	35.76

Column: Phenomenex Gemini-NX 5 µm. Flow rate: 1.00 mL/ min. Detection: 254 nm. Injection volume: 50 µL.

Figure 3.1 illustrates the effects of changing methanol concentration, buffer pH and column temperature on peak capacity and resolution factors. Resolution in all cases was

determined with reference to immediately preceding peak. For ephedrine, resolution was calculated relative to maleic acid peak.

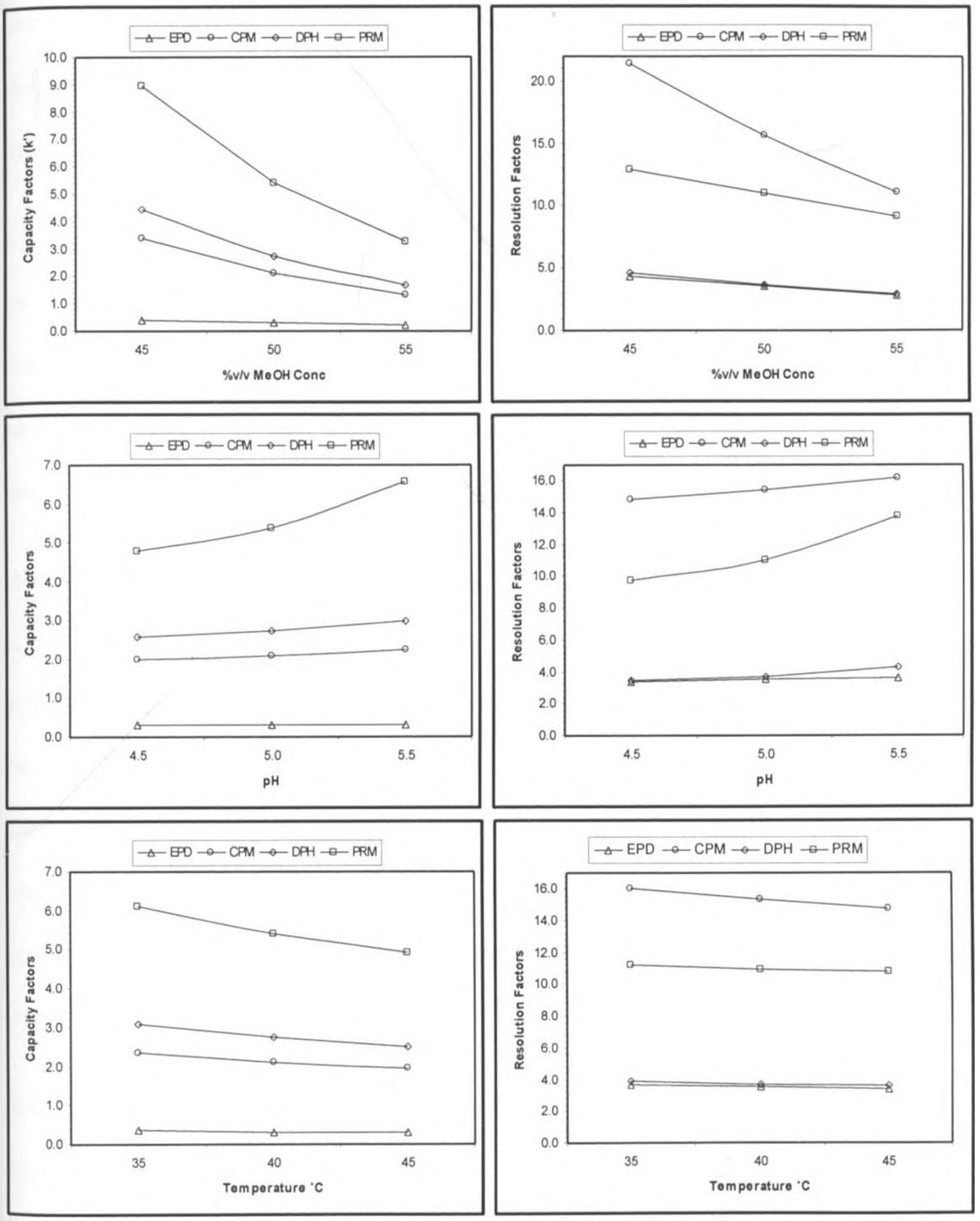


Figure 3.1. Effect of methanol concentration, mobile phase buffer pH and column temperature on capacity and resolution factors

Column: Phenomenex Gemini-NX 5 µm. Flow rate: 1.00 mL/ min. Detection: 254 nm. Injection volume: 50 µL.

Methanol concentration had the greatest impact on both capacity factors and peak resolution, thereby underscoring its effect on analysis run time and separation between peaks. Nevertheless, even over the entire robustness range investigated for this factor, resolution between critical peak pairs MAL/EPD and CPM/DPH was greater than 2.5 indicating the method's robustness.

Mobile phase pH and column temperature were both noted to exert appreciable influence on resolution and capacity factors but to a much less extent compared to methanol concentration. Like in the case with methanol concentration, selectivity of the method remained within acceptable limits (resolution > 2.5) for all component peaks over the entire pH and temperature robustness testing range.

Robustness data revealed that although the accuracy of quantifying peak areas was not significantly affected by changing key LC factors within relatively wide ranges, it was still critical to take precautions during mobile phase preparation to avoid fluctuations in peak retention times. Such precautions include accurate adjustment of buffer pH using a calibrated pH meter and accurate measurement of methanol volume. Temperature had the least pronounced impact on both peak area and retention time variation. This impact could be reduced by ensuring that column temperature is maintained using a thermostatic oven, heating block or water bath.

3.6 Stability of working standard solution

Stability of a working standard solution containing approximately CPM 0.08 mg/mL, DPH 0.4 mg/mL, EPD 0.6 mg/mL and PRM 0.2 mg/mL dissolved in a mixture of methanol-water (50:50, % v/v) was monitored daily over a 72 h period under the following storage conditions:

- Solution A: Stored at ambient room temperature (17 °C to 21 °C) in a clear glass container unprotected from light;
- Solution B: Stored at ambient room temperature in an amber coloured glass container protected from light;
- Solution C: stored in a refrigerator (2 °C to 8 °C) in a clear glass container.

The stability of these solutions was determined by running triplicate injections of each at 24 h intervals and computing the mean areas of each component peak relative to the freshly prepared solution from Day 1. To avoid possible peak area fluctuations resulting from slight changes in mobile phase composition, the same mobile phase was used to run all the solutions throughout the entire duration of the test. Table 3.5 is a summary of the results obtained.

Table 3.5. Stability of working standard solutions stored under different conditions for 72 h

Working Standard Solution	Drug	Percentage Peak Area of Original Solution		
		After 24 h	After 48 h	After 72 h
A	EPD	100.8	101.6	101.1
	CPM	105.5	111.3	117.3
	DPH	100.1	99.7	99.0
	PRM	97.1	96.3	92.6
B	EPD	101.0	101.5	99.6
	CPM	101.2	102.9	103.5
	DPH	100.8	102.1	99.8
	PRM	100.0	99.0	96.3
C	EPD	101.8	101.1	100.0
	CPM	99.9	99.8	99.8
	DPH	100.5	99.9	99.1
	PRM	102.0	101.8	100.6

Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:40:9.85, % v/v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 50 µL.

Percentage peak areas obtained from the working standard stored under different conditions revealed that PRM was most susceptible to light. Degradation products from PRM eluted close to the EPD peak and possibly also co-eluted CPM peak, resulting in greater peak areas for this component than had been determined from the freshly prepared solution (Figure 3.2). Degradation of PRM in the working solution was also evidenced by a colour change in the solution when left to stand for several hours at ambient temperature exposed to light as the solution gradually turned violet-pink. The content of PRM was found to decrease by almost 8% when stored under these conditions over 72 h whereas EPD and DPH were unaffected.

Working standard solution stored at ambient temperature but protected from light showed much greater stability with all components showing little change in peak areas over the initial 48 h after preparation. However, after 72 h, the peak area of PRM reduced by 4% indicating that this compound was also sensitive to temperature, and to a lesser degree, to light as well.

The working standard solution stored in the refrigerator exhibited virtually no change in all component peak areas over the entire 72 h test period. This indicated that the most ideal practical precaution to be taken when analyzing samples using this method would be to ensure that all test solutions are freshly prepared and protected from light possibly through the use of amber coloured low actinic glassware.

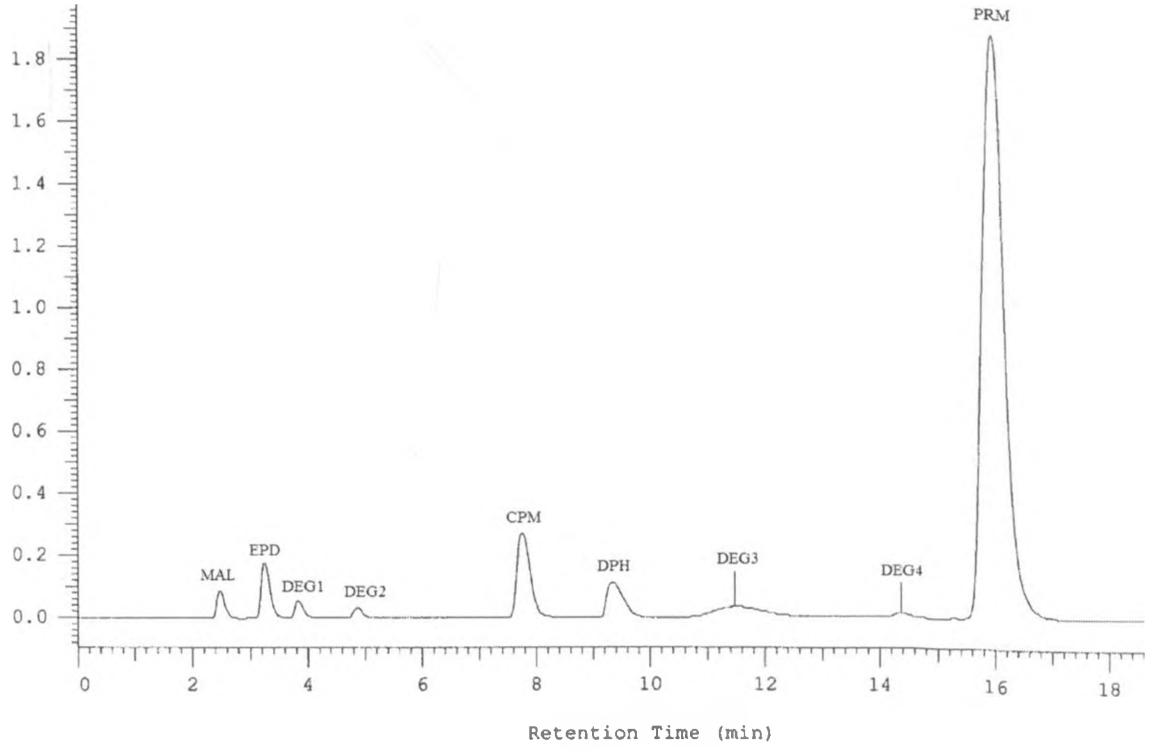


Figure 3.2. Chromatogram of a mixture of ephedrine (EPD), chlorpheniramine (CPM), diphenhydramine (DPH) and promethazine (PRM) 72 h after preparation
DEG1, DEG2, DEG3 and DEG4 represent peaks arising from unknown degradation compounds.
Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:40:9.85, % v/v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 50 µL.

Findings from these experiments implied that there was a critical need in formulating syrups containing PRM to take precautions to protect the products from exposure to light throughout their shelf life. This can be achieved by packaging the syrups in amber coloured bottles. Additionally, since degradation of PRM appeared to result from oxidation in the presence of light, the incorporation of anti-oxidants such as ascorbic acid could be a vital step in the formulation of these syrups.

CHAPTER FOUR

ANALYSIS OF COMMERCIAL SAMPLES

4.1 Introduction

The aim of any analytical method development and validation is to create a reliable technique that may be employed in the analysis of commercial samples. The method developed in this study was intended for use in evaluating the quality of cold-cough syrups marketed in Kenya containing the four compounds of interest as active ingredients. The reliability of the method for its intended use was tested by using it to evaluate the quality of commercially marketed cold-cough syrups available from pharmacies in Nairobi city, Kenya.

4.2 Acquisition of samples

Test samples were purchased from randomly selected retail pharmacies located within the central business district and suburbs of the city of Nairobi. From the Drug Register maintained by the Pharmacy and Poisons Board, four different registered products were identified that contained at least three of the four analyte compounds studied. The four product samples were coded A, B, C and D (Table 4.1).

Table 4.1. Product samples collected for analysis

Product Code	Batch Number	Date of Manufacture	Expiry Date	Label Claim (mg / 5 mL)
A	09-04007	April 2009	March 2012	Ephedrine HCl (7.5) Diphenhydramine HCl (5.0) Promethazine HCl (2.5)
	09-05065	May 2009	April 2012	
	08-11014	November 2008	October 2011	
B	0609057	June 2009	May 2012	Ephedrine HCl (7.5) Diphenhydramine HCl (5.0) Promethazine HCl (2.5)
	0509009	May 2009	April 2012	
	0209049	February 2009	January 2012	
C	00672T	July 2008	June 2011	Ephedrine HCl (7.5) Diphenhydramine HCl (5.0) Chlorpheniramine Mal. (1.0) Promethazine HCl (2.5)
	00766T	May 2009	April 2012	
	00745T	March 2009	February 2012	
D	48976	June 2007	June 2010	Ephedrine HCl (5.0) Diphenhydramine HCl (5.0) Chlorpheniramine Mal. (2.5) Promethazine HCl (2.5)
	50830	January 2009	January 2012	
	50412	September 2008	September 2011	

4.3 Sample preparation

4.3.1 Analysis of unextracted samples

Test sample solutions were prepared as follows: 10.0 mL of the product was transferred into a 25 mL volumetric flask and diluted to volume using methanol-water (50:50, % v/v). The resultant solution was filtered and injected (50 µL) into the developed HPLC system. Two representative chromatograms of the products analyzed are shown (Figures 4.1 and 4.2).

Chromatograms obtained showed notable interference in the elution of component peaks by the presence of unidentified excipients present in the samples. Most affected by the interference was the EPD peak, owing to its short retention time and small peak area. These interferents may be strongly UV-absorbing excipients. The presence of these additional peaks in the test sample chromatograms greatly hindered the accurate

determination of active ingredient peak areas and undermined quantification of sample components.

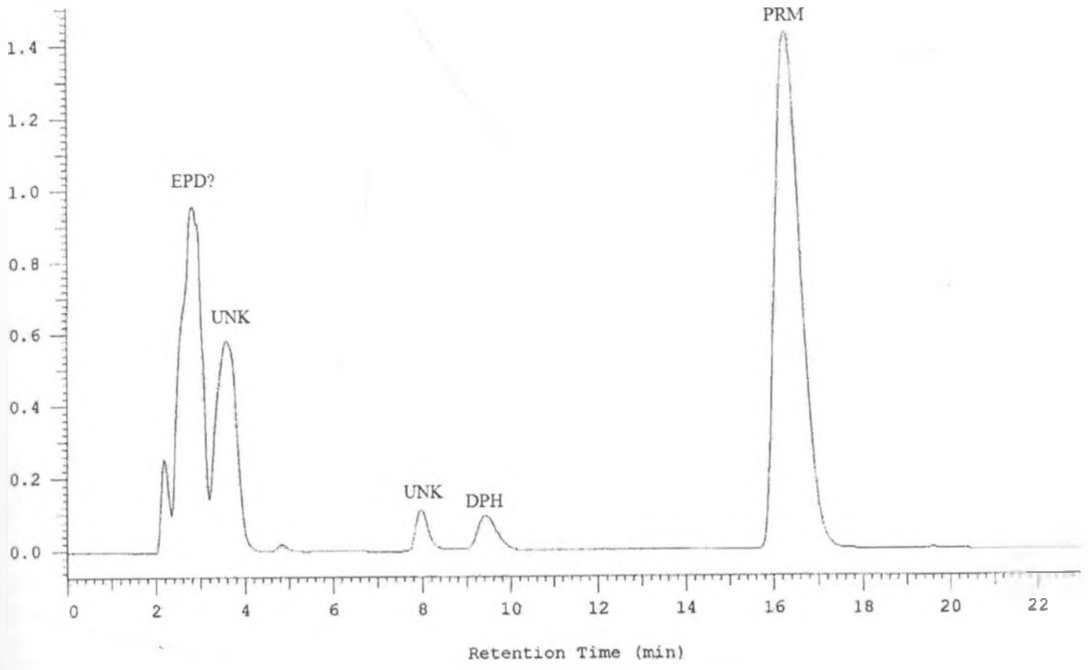


Figure 4.1. Chromatogram of unextracted Product B analysis sample
UNK: unidentified compounds, EPD: Ephedrine, DPH: Diphenhydramine, PRM: Promethazine
Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:40:9.85, % v/v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 50 µL. Nominal concentrations: EPD 0.6 mg/mL, DPH 0.4 mg/mL and PRM 0.2 mg/mL.

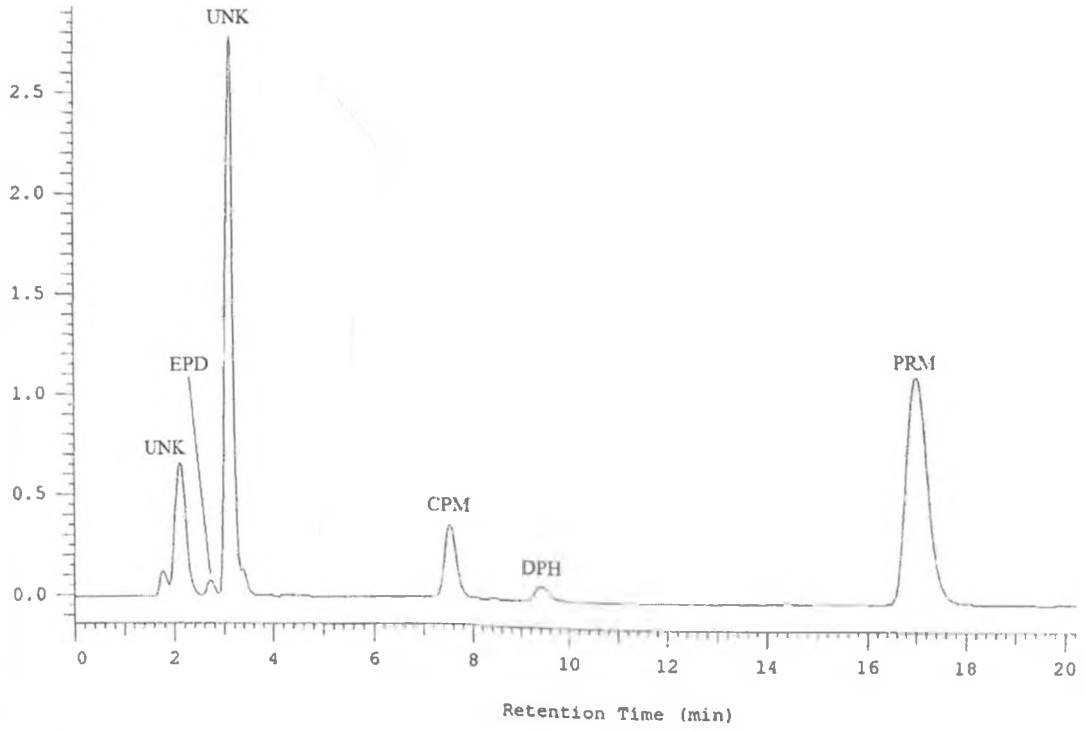


Figure 4.2. Chromatogram of unextracted Product D analysis sample
UNK: unidentified compounds, EPD: Ephedrine, DPH: Diphenhydramine, CPM: Chlorpheniramine, PRM: Promethazine
Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:40:9.85, % v/v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 50 µL. Nominal concentrations: EPD 0.4 mg/mL, CPM 0.2 mg/mL, DPH 0.4 mg/mL and PRM 0.2 mg/mL.

4.3.2 Sample extraction procedure

To circumvent the problem of interferences, a sample preparation procedure was designed that was aimed at eliminating the interfering excipient components. Literature review had revealed that similar extraction procedures had been employed in the analysis of similar active pharmaceutical ingredients in biological samples [62]. Sample preparation was carried out as follows: 10.0 mL of syrup was pipetted into a 25 mL volumetric flask and made to volume using a solution of 0.2 M NH_4OH resulting in the precipitation of the analyte free bases. This preparation was then transferred into a 250 mL separating funnel with the volumetric flask being carefully rinsed out using the extraction solvent to ensure none of the analytes remained in the flask. The transferred

preparation was then extracted with three 75 mL portions of extraction solvent, comprising a mixture of hexane-dichloromethane-isopropyl alcohol (20:10:1 v/v). The combined organic extracts were then reduced to dryness using a rotary evaporator at 40 °C. The residue obtained was dissolved in methanol-water (50:50, % v/v) and made to volume in a 25 mL volumetric flask. This solution was then injected into the HPLC system and run against a standard solution containing known concentrations of the analytes. Figures 4.3 and 4.4 are representative chromatograms of the product analysis samples after extraction showing the elimination of most of the interferents.

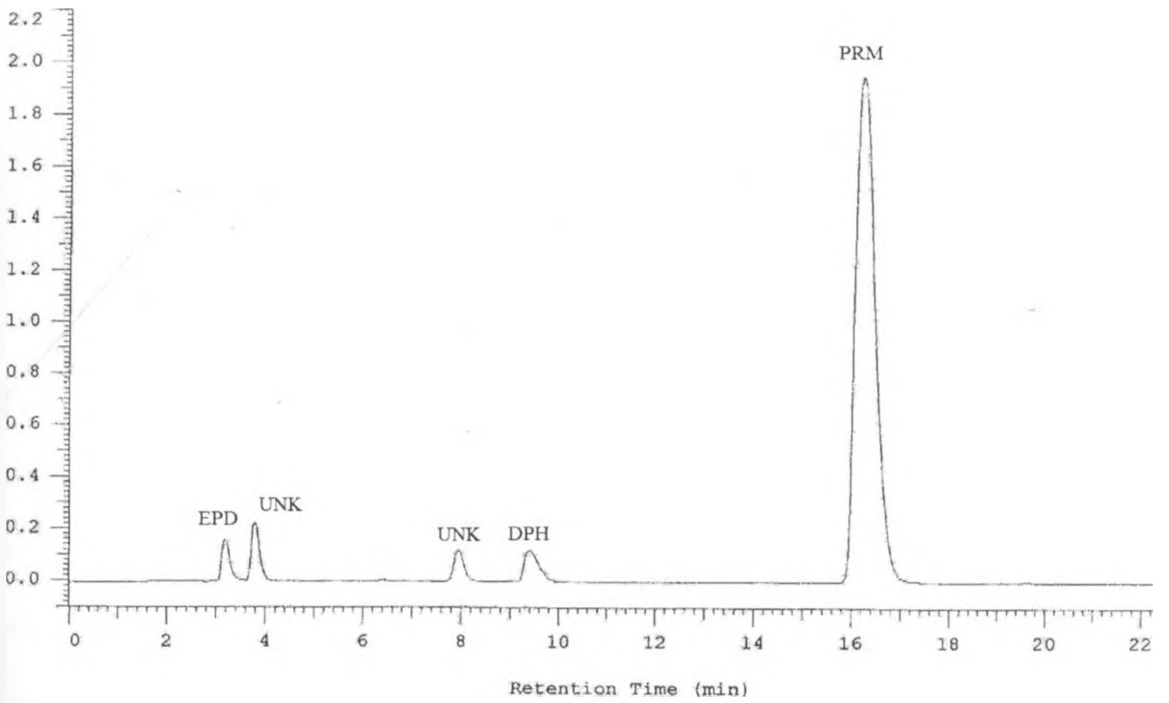


Figure 4.3. Chromatogram of Product B analysis sample after extraction
UNK: Unidentified compounds, EPD: Ephedrine, DPH: Diphenhydramine, PRM: Promethazine
Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:40:9.85, % v/v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 50 µL. Nominal concentrations: EPD 0.6 mg/mL, DPH 0.4 mg/mL and PRM 0.2 mg/mL.

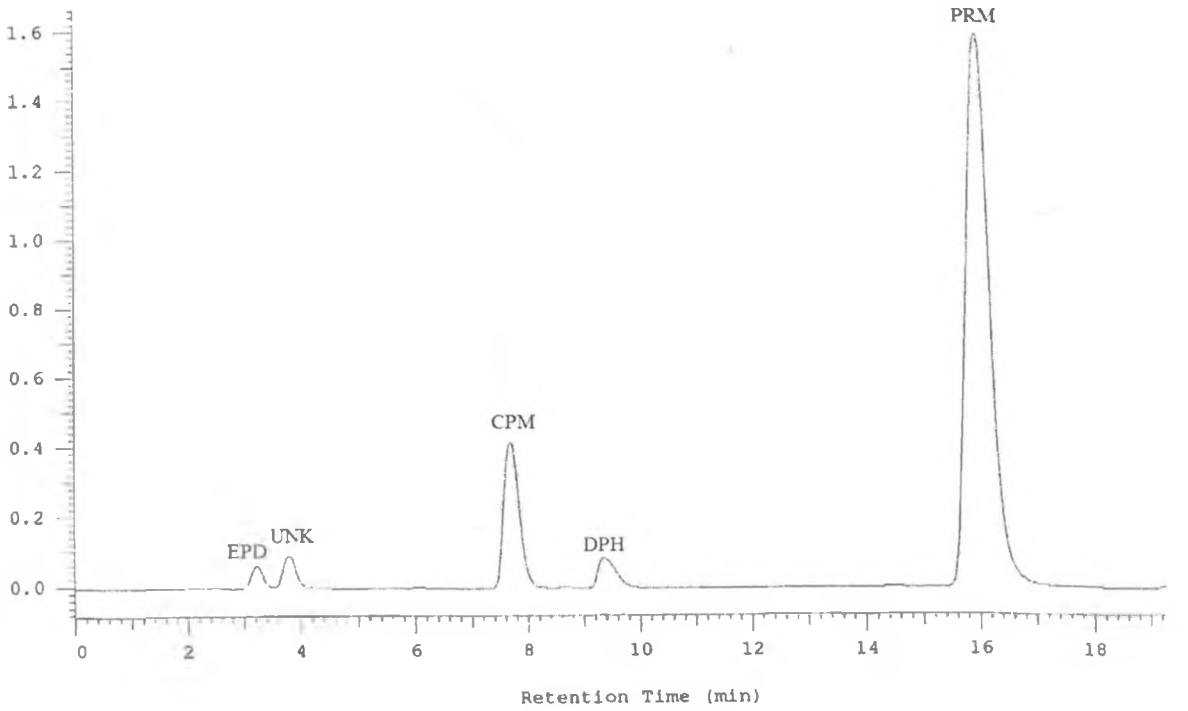


Figure 4.4. Chromatogram of Product D analysis sample after extraction
UNK: Unidentified compounds, EPD: Ephedrine, DPH: Diphenhydramine, CPM: Chlorpheniramine, PRM: Promethazine
Column: Phenomenex Gemini-NX 5 µm. Column temperature: 40 °C. Mobile phase: methanol-triethylamine-0.2 M ammonium acetate pH 5.0-water (50:0.15:40:9.85, % v/v/v/v). Flow rate: 1.00 mL/min. Detection: 254 nm. Injection volume: 50 µL. Nominal concentrations: EPD 0.4 mg/mL, CPM 0.2 mg/mL, DPH 0.4 mg/mL and PRM 0.2 mg/mL.

The effectiveness of the sample preparation procedure was tested on an aqueous solution of the working standards prepared to contain the same labeled concentration of ingredients as the undiluted products. Replicate 10.0 mL aliquots of this solution were taken through the sample preparation procedure described above and analyzed against 10.0 mL aliquots of the same solution directly diluted to 25 mL using MeOH-Water (50:50, % v/v) serving as reference solutions. The extraction recovery (Table 4.2) from the synthetic preparation was calculated from the percentage ratio of the component peak areas in the extracted solutions chromatograms to peak areas in the directly diluted solution thus:

$$\% \text{ Recovery} = \frac{\text{Component Peak Area in Extracted Solution}}{\text{Component Peak Area in Directly Diluted Solution}} \times 100$$

Table 4.2 summarizes the percentage recoveries of the four active ingredients from the aqueous working standard solution.

Table 4.2. Percentage recovery of active ingredients from aqueous working standard solution

Active Ingredient	EPD	CPM	DPH	PRM
% Recovery	99.4 (0.8)*	98.9 (0.7)	98.5 (0.6)	101.8 (0.6)

*Figures in parenthesis represent the percentage relative deviation of the mean percentage recovery

Recovery of the active ingredients from the working standard solution ranged from a high of 101.8% in the case of PRM to a low of 98.5% for DPH indicating that the sample preparation method appeared to be a reliable means of separating the ingredients from possible interfering components present in the commercial products.

4.4 Analysis of samples

Three batches of each product were sampled for testing and analyzed using the developed HPLC method. The samples were all subjected to the developed clean-up procedure described previously. Replicate injections of both test and standard solutions were run with at least three injections for each solution. The HPLC analysis injections were made in a sequence that bracketed the sample preparations between the standards. Each HPLC injection run was recorded for a minimum of 20 min, to allow sufficient elution time.

4.5 Results

Table 4.3 is a summary of the assay results obtained from analysis carried out on the 12 batches.

Table 4.3. Assay of active ingredients in analyzed product samples expressed as percentages of stated labeled amounts

Product Code	Batch Number	Percentage assay values*			
		EPD	CPM	DPH	PRM
A	09-04007	80.8 (0.3)	-	93.9 (0.5)	98.2 (0.9)
	09-05065	87.8 (1.9)	-	91.6 (0.5)	95.2 (0.6)
	08-11014	83.5 (1.0)	-	96.0 (1.9)	87.3 (1.0)
B	0609057	84.9 (0.9)	-	93.8 (1.5)	101 (0.9)
	0509009	79.6 (1.3)	-	92.5 (0.9)	96.2 (2.0)
	0209049	85.0 (1.9)	-	95.6 (1.9)	82.3 (0.9)
C	00672T	77.3 (1.6)	93.4 (1.2)	91.0 (1.4)	81.3 (1.4)
	00766T	89.7 (1.6)	96.9 (1.3)	95.3 (1.2)	103 (1.1)
	00745T	87.5 (1.7)	97.2 (1.4)	95.3 (1.7)	98.3 (1.7)
D	48976	71.8 (0.5)	100 (0.5)	86.1 (1.1)	69.1 (0.7)
	50830	80.9 (0.6)	97.4 (1.0)	92.2 (1.8)	94.7 (1.9)
	50412	76.6 (0.7)	99.8 (0.9)	93.7 (0.9)	91.6 (0.9)

*Assay results are expressed as percentages of stated labeled amounts, figures in parentheses represent the percentage relative standard deviation, n=3.

From the precision obtained (RSD < 2.0%), it was observed that the assay technique was reliable. Although no monograph for any of the sample combinations are present in official pharmacopoeia, the assay limits specified in the British and United States Pharmacopoeia for single component oral syrups containing any of the four ingredients were used as a basis for determining whether the products met quality specifications. In both cases, the pharmacopoeia specified assay limits of 90%-110% for each drug component [26,28]. The most noticeable feature was the low content in all twelve samples of the ephedrine component whose assay value was found to range from 71.8% to 89.7% thus failing to comply with the assay limits defined in the B.P. and U.S.P.

Chlorpheniramine in all 6 cases where it had been incorporated as an active ingredient was found to be present at levels within the 90%-110% assay limits with the content ranging from 93.4% to 100% of the label claim. Diphenhydramine was present in all 12 product batch samples and was found to exhibit assay values greater than 90% of the labeled amount in all except one batch (86.1%). Promethazine too was present in all product samples and was noted to exhibit the greatest degree of variation of the active ingredient components with content ranging from a low of 69.1% to a high of 103%. A total of four of the batches tested were found to contain less than the lower 90% limit for content of this ingredient with one batch from each of the four different products tested exhibiting this anomaly.

Assay results indicated that there was significant inter-batch variation in the content of active ingredients in the products tested. Ephedrine appeared to be the component most affected by this variation with promethazine also exhibiting similar disparity, albeit to a lesser extent. Diphenhydramine and chlorpheniramine showed the least degree of variation and only one product batch out of the 12 tested was found to contain less than 90% of these two ingredients.

Possible reasons for inter-batch variability may be due to poor Good Manufacturing Practices or instability of the active ingredients. Methodological inconsistency of recovery was ruled out as a possible cause of inter-batch and inter-product variability because validation procedures showed there was a low coefficient of variation in recovery (less than 2%).

4.6 Determination of the accuracy of assay results

To confirm the efficiency and accuracy of the sample preparation extraction procedure and its possible influence on the assay values obtained, a series of experiments were run. One batch of each product was spiked with a known amount of the active ingredients and the recovery determined. The standard solution used to spike the samples was prepared by dissolving in water amounts of each of the 4 active ingredient components that would yield a solution containing approximately the same concentration as the undiluted syrups (EPD 1.5 mg/mL, CPM 0.2 and 0.5 mg/mL, DPH 1.0 mg/mL and PRM 0.5 mg/mL). The product batch samples were spiked with 2 mL of this aqueous standard solution corresponding to 20% of the labeled amounts of active ingredients being added to 10 mL aliquots of the product samples. The sample preparation process was then performed on the spiked aliquots as described previously. The concentration level chosen to spike the samples were selected on the basis that it lay within the linearity range of the method. Table 4.4 illustrates the percentage recovery from the product samples spiked with 20% of the active ingredients.

Table 4.4: Percentage recovery of active ingredient components from samples spiked with 20% of stated labeled amounts

Product Code	Batch Number	Percentage component recoveries (% C.V); n=3			
		EPD	CPM	DPH	PRM
A	09-05065	96.0 (0.8)	-	100.2 (1.0)	98.6 (0.9)
B	0609057	98.7 (0.9)	-	100.9 (0.3)	99.0 (0.2)
C	00766T	99.3 (1.7)	103.2 (1.2)	102 (0.9)	100 (0.9)
D	50830	101.6 (1.2)	101.9 (1.0)	103.5 (1.1)	98.0 (1.0)

The results obtained indicated that when samples were spiked with approximately 20% of the active ingredients, the recovery rate for CPM, DPH and PRM was acceptable, ranging from 98.0% to 103.5%. Ephedrine exhibited a greater degree of variance in recovery

percentages that ranged from a low of 96.0% in the case of Product A to a high of 101.6% in the case of Product D. The variation in EPD recovery suggested that perhaps extraction of this ingredient during the sample preparation procedure was more affected by excipients incorporated into the syrup formulations than the other active ingredients. However, the results indicated also that the extraction method was able to definitely quantify to a good extent increased amounts of EPD intentionally added to the samples.

CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSION

5.1 General discussion

The method developed for the analysis of multiple anti-histamine compounds in cold-cough preparations exhibited good sensitivity, precision and linearity to allow for its use in the accurate determination of even low levels of these ingredients in commercial samples. By allowing the four compounds of interest to be analyzed in a single HPLC run, the method greatly reduces overall analysis time.

The development of a HPLC method that uses readily available and cheap reagents was designed to make the procedure more universally applicable and cost effective. The choice of methanol as the organic modifier in the mobile phase provided a relatively cheap and less toxic method for routine analysis of commercial cold-cough syrups in both manufacturing industries and regulatory authority laboratories.

The selection of detection wavelength at 254 nm offered the possibility of employing the method to test samples on HPLC systems equipped with fixed wavelength UV detectors. Another factor that was aimed at making the method more readily applicable in a wide range of settings is its use of silica based reversed-phase C18 column that is most commonly employed in the analysis of majority of drug compounds using LC techniques.

In carrying out the validation process for the developed method, it was not possible to comprehensively determine robustness owing to lack of experimental design method validation software capable of analyzing and interpreting robustness data. Nevertheless, indicative procedures on the method's robustness were tested by investigating the

quantitative impact of varying key LC factors including pH, organic modifier concentration and column temperature on the variation of component peak areas and retention times. More comprehensive studies aimed at quantifying the impact of simultaneous variation of these as well as other LC factors in the form of surface response plots would be a more accurate way of assessing the method's robustness.

5.2 Recommendations and further work

Coupling the LC method to mass spectroscopy was one of the factors considered in selecting mobile phase buffers. Volatile ammonium acetate buffer was used to allow for easier coupling of the LC method with MS detection. The high sensitivity of LC-MS would help in monitoring the stability of the active ingredients and indicating the presence of degradation products.

Development of a simpler, less time consuming sample preparation procedure should be considered. Assessment of the method's accuracy could best be conducted by preparing placebo products in the laboratory containing all the excipients incorporated in commercial samples and spiking these with known amounts of the active ingredients prior to analysis. From the recovery determined using this procedure, the method's accuracy in assaying commercial samples could be more reliably established.

Methanol was used as the organic modifier in developing the analytical method because of its relatively low cost and toxicity, however, the main drawback to its use is the relatively low eluting power compared to acetonitrile. The use of acetonitrile as an alternative to methanol could be investigated to determine if it could be used to reduce the total analytical run time while maintaining good selectivity.

Collaborative studies between different laboratories could establish the method's ruggedness across varying environments. Ruggedness is a pre-requisite for the successful transfer of any analytical technique since it allows for widespread application of the method with a predictable degree of reliability and accuracy.

Identification and characterization of the degradation products observed when testing the stability of working standard solutions could be investigated to determine their origin and ways of minimizing their impact on the method's accuracy.

5.3 Conclusion

A high performance liquid chromatography method was developed that, for the first time, could be used to quantify a mixture of ephedrine, chlorpheniramine, diphenhydramine and promethazine commonly incorporated in cold-cough syrups marketed in Kenya.

The optimum chromatographic conditions established for the developed method were: Analytical column: Phenomenex Gemini-NX C18, 250 mm length and 4.6 mm internal diameter, 5 µm particle size maintained at a temperature 40 °C. Mobile phase composition: methanol-triethylamine-0.2 M ammonium acetate buffer pH 5.0-water (50:0.15:40:9.85, % v/v/v/v) delivered at a flow rate of 1.00 mL/min and ultraviolet detection at 254 nm. Validation of the analytical method indicated that it exhibited good linearity over a wide range of concentrations as well as presenting good sensitivity and selectivity. The linearity equations for each of the analyte drugs were as follows: EPD y=1358372x-1487, regression coefficient (R^2=0.9999); CPM y=20875948x-24156 (R^2=0.9997); DPH y=2552972x-13199 (R^2=0.9997) and PRM y=109676910x+1536245 (R^2=0.9979). Simple robustness studies indicated that the quantitative accuracy of the

method was largely unaffected by small changes in the key LC factors of mobile phase pH, methanol concentration and column temperature.

The developed method was effectively applied in the analysis of commercial samples. Analysis results of 12 batches of commercially available cold-cough syrup samples using the developed method indicated that all twelve batches had content of EPD less than 90% of what was stated on the label. The other components in the samples tested were found to be present at different amounts ranging from 93.4% to 100% for CPM, 86.1% to 96.0% for DPH and 69.1% to 103% in the case of PRM. This variance in assay values from the products tested indicated that the samples analyzed exhibited significant batch-to-batch variation. This might be worth investigating further by carrying out analysis on a larger sample size that would be more indicative of the overall quality of these products circulating in the market. These findings underscore the need for a regular and sustained market surveillance program to ensure that consistently good quality products are available in circulation and also a good and reliable method for use in industry QC laboratories.

The method developed was found to be simple, precise and fast enough to offer a practical means for carrying out routine quality control analysis on cold-cough syrups in both pharmaceutical industry quality control and regulatory laboratories.

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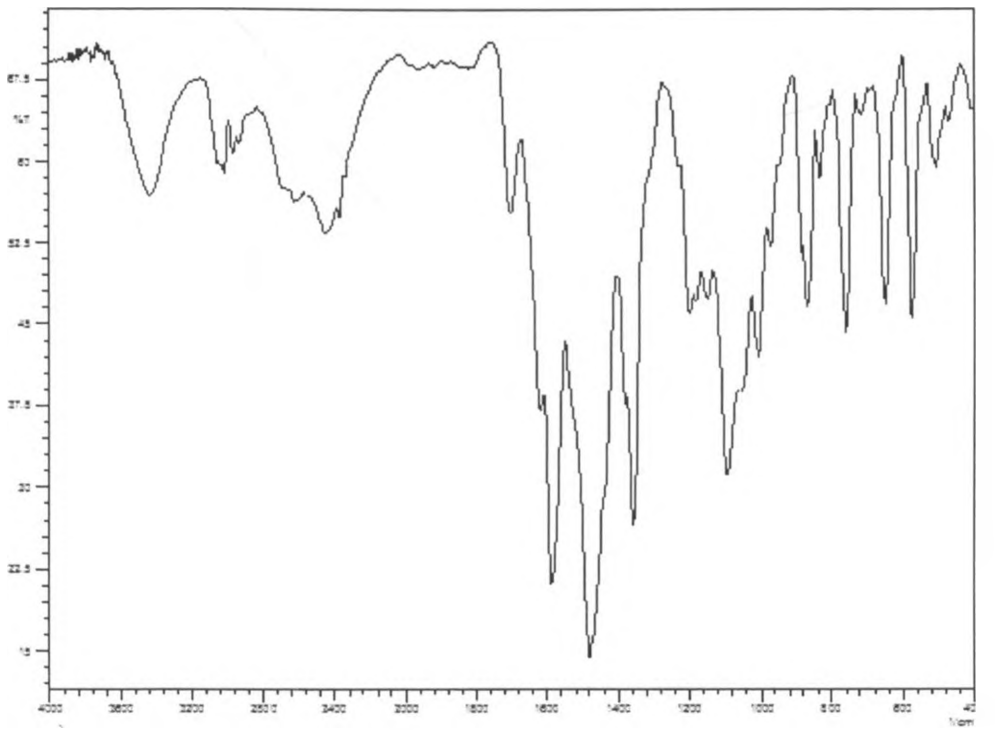
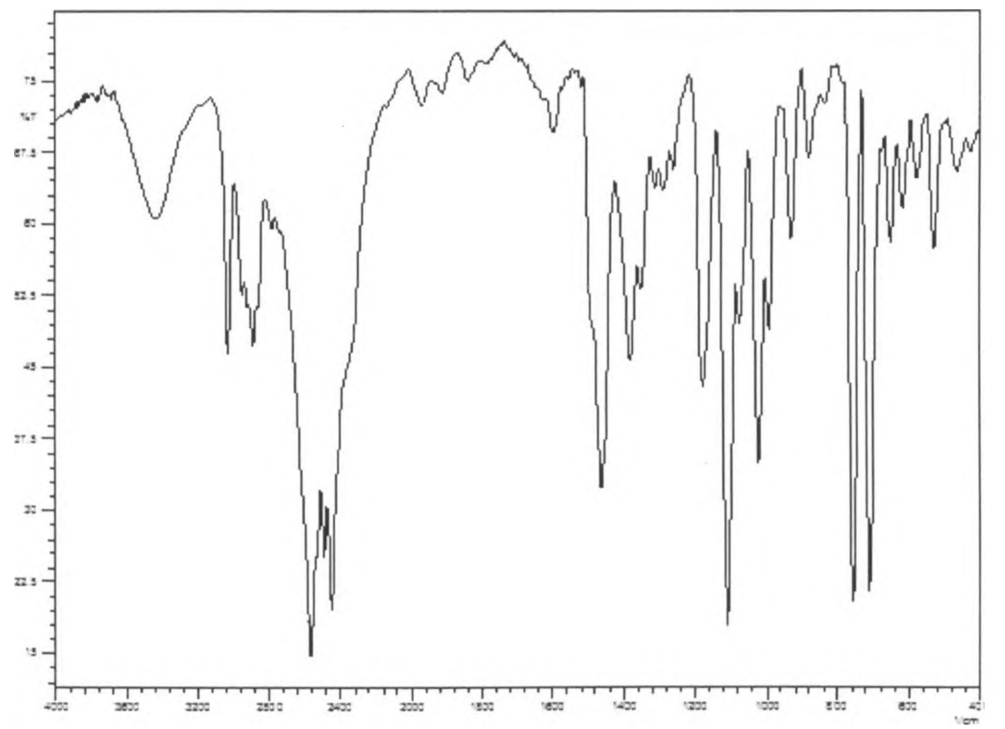
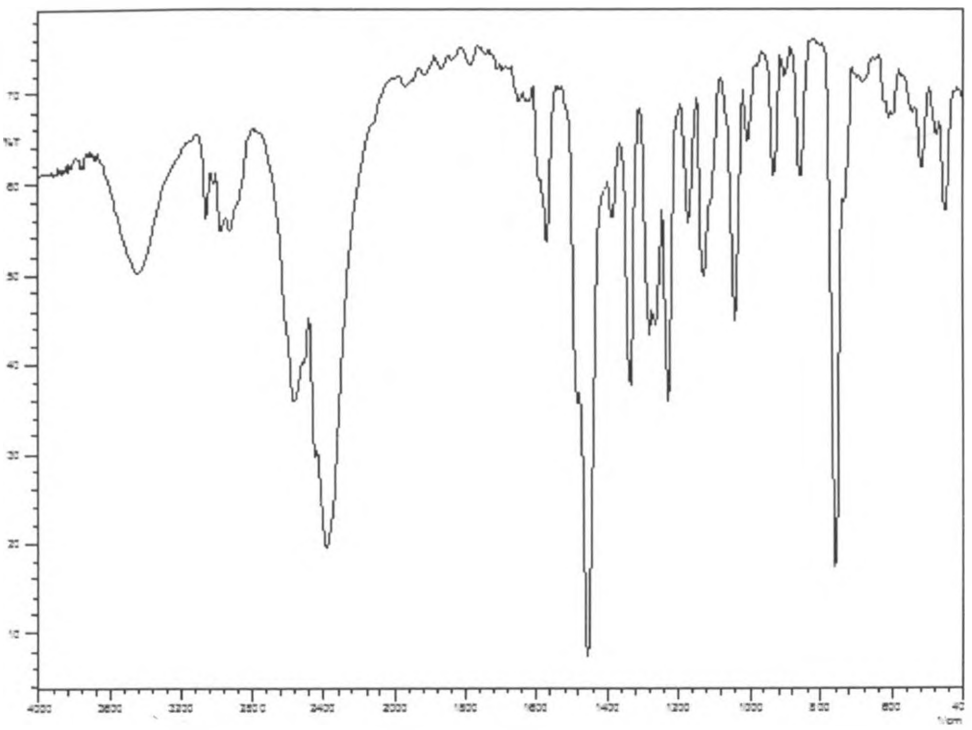
APPENDICES



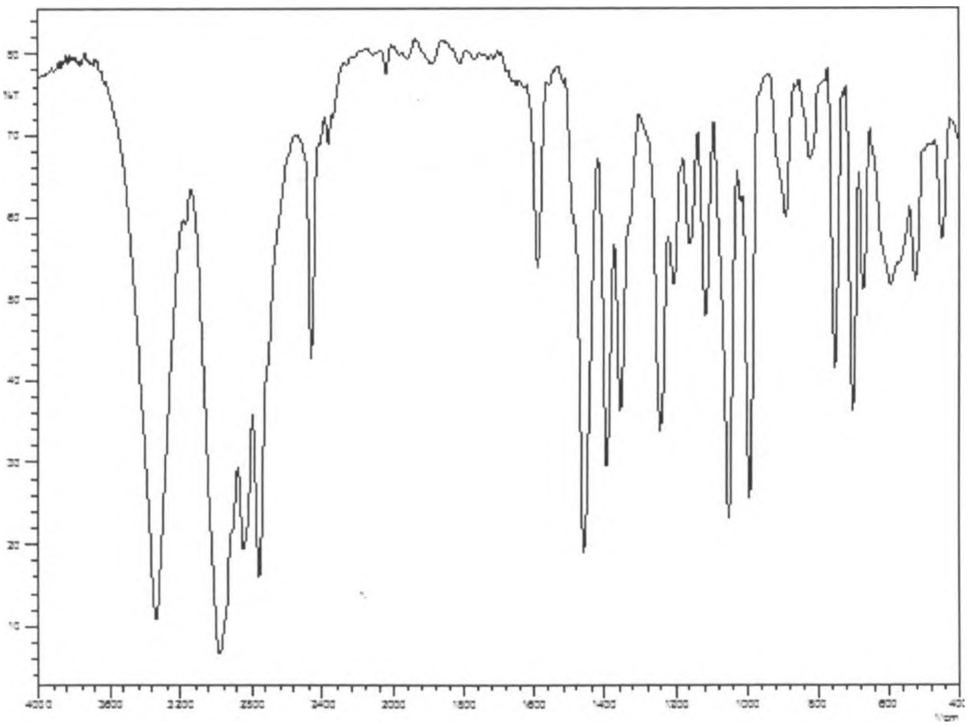
Appendix 1: FTIR spectrum of Chlorpheniramine maleate (KBr disk)



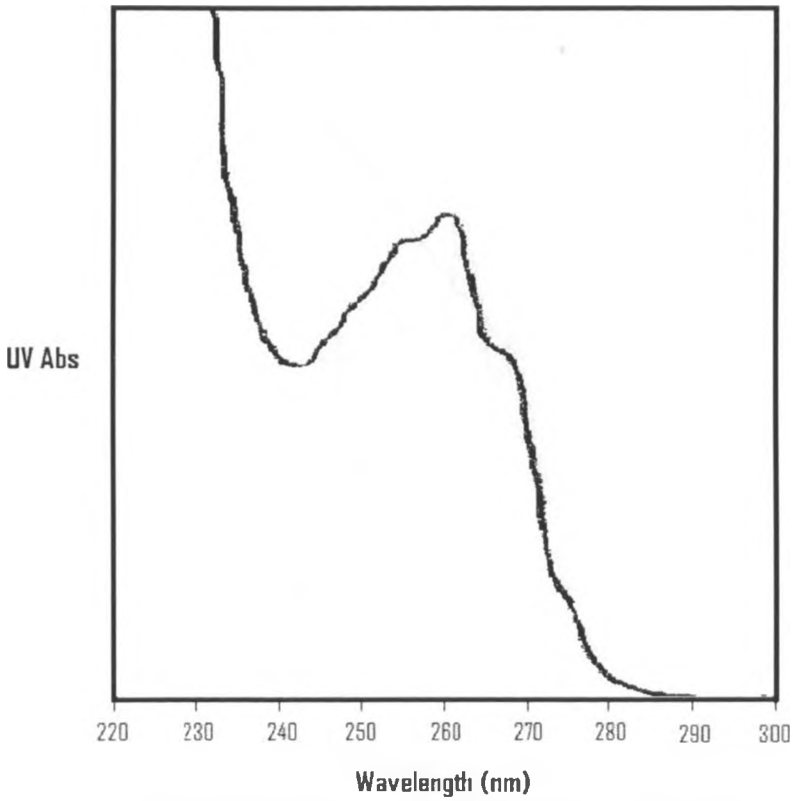
Appendix 2: FTIR spectrum of Diphenhydramine hydrochloride (KBr disk)



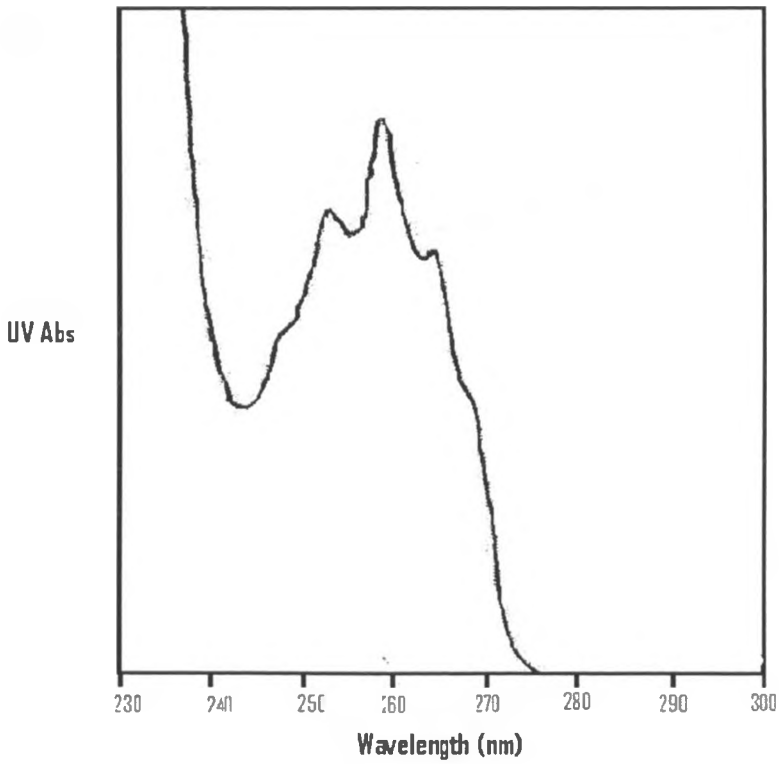
Appendix 3: FTIR spectrum of Promethazine hydrochloride (KBr disk)



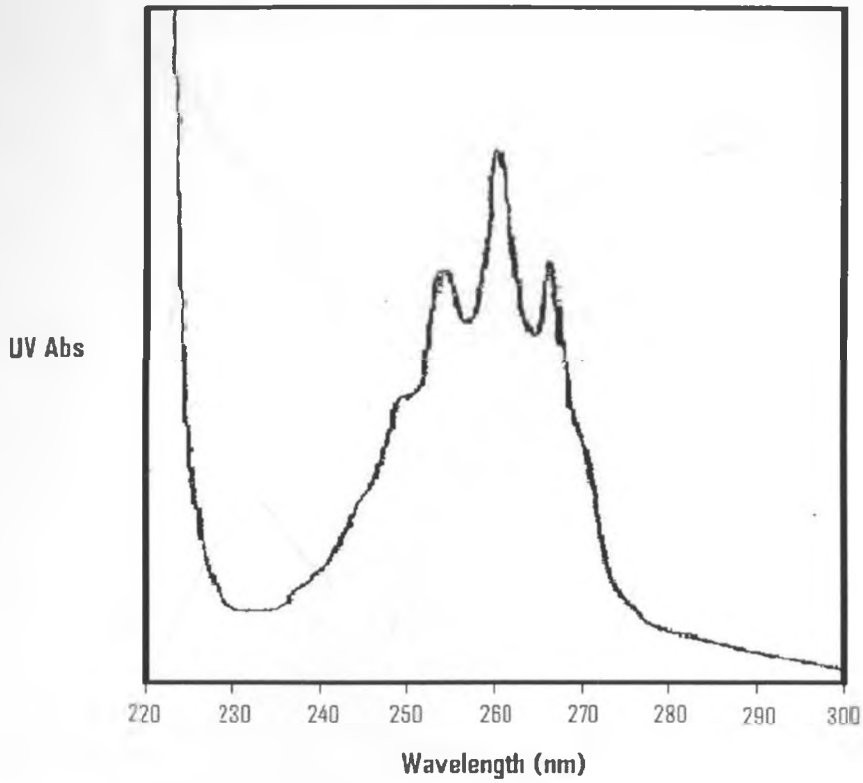
Appendix 4: FTIR spectrum of Ephedrine hydrochloride (KBr disk)



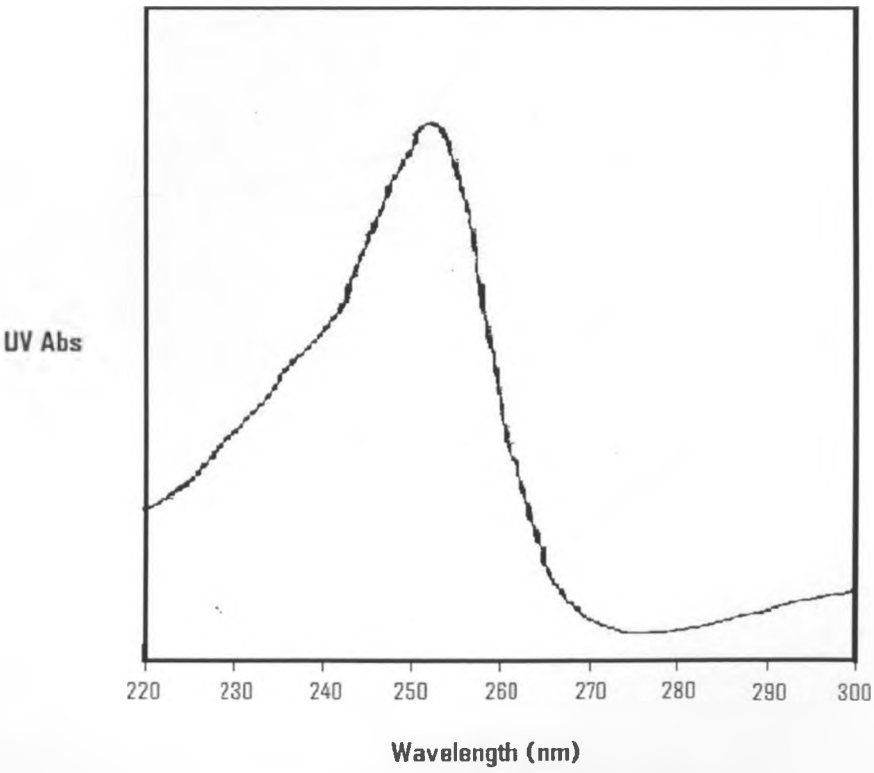
Appendix 5: Chlorpheniramine maleate UV spectrum



Appendix 6: Diphenhydramine HCl UV spectrum



Appendix 7: Ephedrine HCl UV spectrum



Appendix 8: Promethazine HCl UV spectrum