DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF BROMHEXINE, GUAIFENESIN, AMBROXOL, SALBUTAMOL/TERBUTALINE, PSEUDOEPHEDRINE, TRIPROLIDINE AND CHLORPHENIRAMINE MALEATE IN COUGH-COLD SYRUPS

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

PAUL MAGUTU NJARIA
B. Pharm. (Nairobi)

U59/77619/2009

Department of Pharmaceutical Chemistry

School of Pharmacy

UNIVERSITY OF NAIROBI

2011

DECLARATION

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

_____   26th October 2011 .
PAUL MAGUTU NJARIA

This research thesis has been submitted with our approval as University supervisors.

_____   26th October 2011
DR. K.O. ABUGA, Ph.D.

Department of Pharmaceutical Chemistry,

University of Nairobi.

_____   27/10/2011
DR. F.N. KAMAU, Ph.D.

Department of Pharmaceutical Chemistry,

University of Nairobi.

_____   26th /Oct./2011.
DR. H.K. CHEPKWONY, Ph.D.

National Quality Control Laboratory and

Honorary lecturer,

Department of Pharmaceutical Chemistry,

University of Nairobi.

i

DEDICATION

This thesis is dedicated to my late parents, Mr. and Mrs. Njaria, for instilling the attitude of hard

work and pursuit of higher education and teaching me that even the largest task can be

accomplished if it is performed one step at a time.

To my family members who stood by me and for their moral support, motivation and

understanding.

To my fiancee, Jane Njere, for her unwavering encouragement, patience, understanding and

believing that I could do it.

To all those who believe in the richness of learning.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

	Title	Page

LIST OF TABLES

LIST OF ABBREVIATIONS

Å	Angstrom
Abs	Absorbance
ACE	Angiotensin converting enzyme
AMB	Ambroxol
API	Active Pharmaceutical Ingredient
A_s	Asymmetry factor
ATP	Adenosine triphosphate
BP	British Pharmacopoeia
BXN	Bromhexine
cAMP	Cyclic 3′,5′- adenosine monophosphate
CBM	Communication bus module
C_{18}	Octadecylsilane
°C	Degrees centigrade
CPM	Chlorpheniramine
FTIR	Fourier transform infra-red
GC	Gas chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GFN	Guaifenesin
g	Gram
HPLC	High Performance Liquid Chromatography
ICH	International conference on harmonization
ID	Internal diameter

k'	Capacity factor
Kg	Kilogram
LC	Liquid Chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
M	Molar
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
MPB	Methylparaben
MPB DEG	Methylparaben degradation product
m/z	Mass/charge ratio
μ	Micron
μL	Microlitre
μm	Micrometer
NF	National formulary
ng	Nanogram
nm	Nanometer
OTC	Over-the-counter
PED	Pseudoephedrine
pKa	Acid dissociation constant

ABSTRACT

'ough is one of the most common symptoms for which patients seek treatment from primary ealth care providers. Cough syrups are usually prescribed for relieve of coughs and most of 1em are formulated as combination of several ingredients. Currently there is no single high erformance liquid chromatography (HPLC) method for the analysis of the active ingredients imultaneously as the official methods available specify analysis of the active ingredients 1dividually. This makes the analysis of the components time consuming and expensive. In the resent study, a simple, reliable, accurate, precise, robust and isocratic HPLC method was eveloped for simultaneous determination of salbutamol (SBT)/terbutaline (TBT), seudoephedrine (PED), guaifenesin (GFN), ambroxol (AMB), chlorpheniramine (CPM), riprolidine (TPN) and bromhexine (BXN) in cough-cold mixtures in the presence of excipients.

he optimized chromatographic conditions were a mobile phase consisting of acetonitrile-0.25 \text{M} sodium hexanesulphonate-0.2 M ammonium acetate pH 3.0-water (35:4:10:51, % v/v/v/v) lelivered through the HPLC system at a flow rate of 1.0 mL/min. The stationary phase was a everesed phase octadecylsilane (C_{18}) measuring 250 mm in length, 4.6 mm internal diameter, 5 1m particle size and 110 Å pore size. The ultra-violet (UV) detection wavelength was set at 254 1m while the injection volume was 20 µL. The diluent consisted of acetonitrile-water (40:60, % /v). The retention time of salbutamol/terbutaline, pseudoephedrine, guaifenesin, ambroxol, :hlorpheniramine, triprolidine and bromhexine was 3.0, 3.5, 4.3, 5.9, 7.8, 9.4 and 18.3 minutes :espectively, giving a total run time of 21 minutes.

The developed method was validated for accuracy, precision, linearity, limit of detection, limit of quantitation and robustness. The validation was carried out according to the International Conference on Harmonization (ICH) guidelines. The accuracy of the method was demonstrated

by the recovery values obtained, being 99.9 % (SBT), 100.5 % (TBT), 99.2 % (PED), 99.8 % (GFN), 98.5 % (AMB), 99.7 % (CPM), 99.9 % (TPN) and 99.2 % (BXN). The precision of the method was shown through adequate repeatability or intra-day precision (RSD, 0.15-0.56 %) and intermediate precision (RSD, 0.45-1.53 %). The linearity range for the eight compounds was 25-200 % with coefficient of determination (R^2) being > 0.999 for all the compounds. The limit of detection values for salbutamol, pseudoephedrine, guaifenesin, ambroxol, chlorpheniramine, triprolidine, bromhexine and terbutaline were 1.28, 12.45, 7.68, 1.29, 3.27, 31.03, 1.67 and 10.08 ng, while the limit of quantitation values were 4.00, 18.68, 10.24, 3.22, 17.45, 51.72, 3.34 and 25.21 ng respectively. The robustness ranges for the three critical factors, mobile phase pH, column temperature and acetonitrile concentration, were 2.5-3.5 pH units, 35-45 ℃ and 33-37 % (v/v) respectively. The variations did not significantly affect separation of the components.

The method was applied in the analysis of nine commercial products obtained from pharmacy outlets within the city of Nairobi. Six products had three batches analyzed while two batches of the remaining three products were analyzed. All the samples complied with the general United States Pharmacopoeia (USP) specifications for assay (90.0-110.0 % label claim). The results obtained also demonstrated that there were minimal batch-to-batch variations. Extraction procedures were not applied during the assay of the samples thus significantly shortening the analysis time. It was concluded that the method is useful in quality control laboratories for the routine analysis of these compounds in cough-cold medications.

CHAPTER ONE

INTRODUCTION

1.1. Physiology of cough

Cough is a sudden and often repetitively occurring respiratory reflex mediated by sensory afferents of the vagus nerve [1]. It is a nonspecific symptom of respiratory disease with broad differential diagnosis and may cause serious complications and significantly affects a patient's lifestyle and sense of well-being. Cough is one of the most common symptoms for which patients seek medical attention from primary health care providers [2]. Cough helps to clear the airways from secretions, irritants, foreign particles and microbes, making it an important respiratory clearance mechanism that is stimulated by inflammatory and mechanical irritation of the airways and is especially important when normal mucociliary transport mechanisms are inadequate. Atelectasis, pneumonia, lung abscess, bronchiectasis and pulmonary fibrosis may occur when cough is ineffective in its protective role [3].

The cough reflex consists of three phases: an inhalation, a forced exhalation against a closed glottis and a violent release of air from the lungs following opening of the glottis, usually accompanied by a distinctive sound [1]. Coughing can happen voluntarily as well as involuntarily. The cough reflex is complex and it involves the central and peripheral nervous systems as well as the smooth muscle of the bronchial tree. It has been postulated that irritation of the bronchial mucosa causes bronchoconstriction, which, in turn, stimulates cough receptors located in tracheobronchial passages. Afferent conduction from these receptors occurs via fibers in the vagus nerve and central components of the reflex probably involve several mechanisms or centers that are distinct from the mechanisms involved in the regulation of respiration [4].

1.2. Classification of coughs

Cough is classified based on duration, quality and etiology. Cough can be acute, subacute or chronic based on duration; dry, productive, brassy or staccato based on quality and specific or nonspecific based on etiology [5, 6].

A cough is defined as productive when secretions expectorate during coughing. These secretions could be green, yellow, white and creamy, clear or blood-tinged [7]. A productive cough can be voluntary or involuntary and is an efficient way of clearing sputum, mucus, noxious substances and foreign particles from the airways. A non-productive cough, which is also classified as a dry cough, can be a prolonged, persistent and troublesome symptom [8].

Based on the duration, a cough that lasts less than 3 weeks is called an acute cough [9] while one lasting 3 to 8 weeks is called a sub-acute cough or postinfectious cough [10]. A cough persisting for more than 8 weeks is called a chronic cough [11].

1.3. Causes of cough

The cause of cough is dependent upon how long the symptom has been present. The most common causes of an acute cough include the common cold, acute sinusitis, pertussis (whooping cough), chronic obstructive pulmonary disease (COPD) exacerbations and allergic or non-allergic rhinitis [9].

Sub-acute cough generally arises after infection or due to acute sinusitis and asthma [10], while a chronic cough is mostly caused by post-nasal drip, asthma, gastroesophageal reflux disease, eosinophilic bronchitis, angiotensin converting enzyme inhibitor drugs or smoking [11].

1.4. Prevalence and incidence of cough

Cough is the most common condition for which patients seek health care in the United States of America [8,12]. The prevalence of chronic cough though difficult to determine, is estimated to occur in up to 40 % of the population worldwide [13]. Despite the incidence of chronic cough being uncertain, information has been gathered from large population surveys. In 2001, the European Community Respiratory Health survey on 18,277 subjects from 16 countries worldwide reported that the occurrence of nocturnal cough occurred in 30 % of the population while non-productive cough occurred in 10 % and productive cough in 10 % of the subjects [14]. These percentages were the median prevalence in the different centers. The incidence of chronic cough also appears to differ between sexes with a higher proportion being females [15]. Cough as a side effect of angiotensin converting enzyme (ACE) inhibitors is also more frequent in women than in men [16]. Furthermore, healthy females have a heightened sensitivity to inhalation of tussive agents and an augmented cough response compared to healthy males [17]. This gender difference in cough reflex sensitivity has also been described in patients with chronic cough [18].

1.5. Complications of coughing

The complications of coughing can be classified as either acute or chronic and usually arise from physiologic events. The magnitude of pressures, velocities and energy that are generated during vigorous coughing allow coughing to be an effective means of clearing the airways of excessive secretions and foreign material and providing cardiopulmonary resuscitation. However, they can also cause a variety of profound physical and psychosocial complications [19].

Acute complications include cough syncope characterized by fainting spells due to decreased blood flow to the brain when coughs are prolonged and forceful, insomnia, cough-induced

vomiting, rupture of blebs causing spontaneous pneumothorax, subconjuctival haemorrhage or "red eye", coughing defecation and in women with a prolapsed uterus, cough urination. Chronic complications are common and include abdominal or pelvic hernias, fractures of lower ribs and costocondritis [19].

1.6.   Treatment of dry cough

Suppression of cough should be avoided as it is a useful physiological mechanism that serves to clear respiratory passages of foreign material and excess secretions. However, there are many situations in which cough does not serve useful purpose but may instead only affect the patient negatively by disturbing normal rest and sleep. This is the case with dry coughs and the drugs used reduce the frequency and intensity of the coughing [4].

Antitussives are drugs that suppress a cough. Narcotics, primarily codeine are used as antitussives and work by depressing the cough center in the brain. Centrally acting cough suppressants are often associated with neurological side effects such as sedation, nausea, constipation and physical dependence, which can limit their use. Peripherally acting antitussives exert their effects by targeting the peripheral vagal sensory nerve afferents, which is a better approach that avoids unwanted side effects [8].

Dextromethorphan is the main ingredient in many Pharmacy-Only cough remedies that depresses the brain's cough center, but without the side effects associated with narcotics. Dextromethorphan is a congener of the narcotic analgesic levorphanol [20] and has no significant analgesic or sedative properties, does not depress respiration in usual doses and is non-addictive.

Codeine, which has antitussive, analgesic and slight sedative effects, is especially useful in relieving painful cough. It has useful antitussive action at doses lower than those required for analgesia. Thus, 15 mg is usually sufficient to relieve a cough [20, 21].

Demulcents are useful for coughs originating above the larynx whereby they form a protective coating over the irritated pharyngeal mucosa. Demulcents such as pectin, glycerine, honey are common ingredients in cough mixtures. In practice, they are used mainly for their placebo effect, although it has been suggested that the sweet taste of syrups might boost production of endogenous opioids in the brain [22]. They can be used to treat any type of cough but are particularly useful in treatment of dry coughs. Local anesthetics such as lidocaine, benzocaine, hexylcaine and tetracaine are also used to inhibit the cough reflex. Humidifying aerosols and steam inhalations exert an antitussive effect by acting as demulcents and by decreasing the viscosity of bronchial secretions.

1.7.   Treatment of productive cough

In a productive cough, mucus produced in the bronchial passages is moved upwards towards the pharynx by ciliary action and then expelled through coughing. Thus, treatment of productive cough involves treatment of the cause that entails a full assessment of the patient [23]. Simple interventions such as cessation of smoking, use of antibiotics in cases of upper respiratory tract infections or asthma treatment may be adequate. In most cases use of a productive cough mixture that aids in the healing process is preferred but such a mixture should not suppress the cough.

A productive, chesty cough, in which phlegm is coughed up, should be treated with an expectorant cough mixture to help loosen the phlegm and hence facilitate expectoration. Many

productive cough remedies are composed of a varied combination of anti-histamines, decongestants, mucolytics, expectorants and bronchodilators [24].

1.8.   Chemistry and pharmacology of the expectorants and mucolytics under study

Expectorants are drugs that increase the ciliary action in the bronchi and thin the mucus making it easy to cough up. Guaifenesin, terpin hydrate, ipecacuanha, ammonium chloride and squill are the major ingredients in most Pharmacy-Only expectorants [24]. These drugs are intended to help expel bronchial secretions from the respiratory tract by decreasing their viscosity, thus facilitating removal and by increasing the amount of respiratory tract fluid [25]. Examples of mucolytics include acetylcysteine, ambroxol, bromhexine, carbocysteine, sodium citrate and sobrerol [21]. Thiol containing compounds such as acetylcysteine interact with disulfide-containing mucins thereby breaking intra- and inter-molecular bonds [26]. Mucolytics may disrupt the gastric mucosal barrier and thus should be used with caution in patients with a history of gastric ulceration. The chemical structures of expectorants and mucolytics under study are shown in figure 1.1.
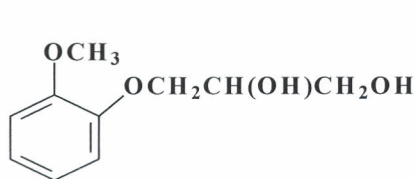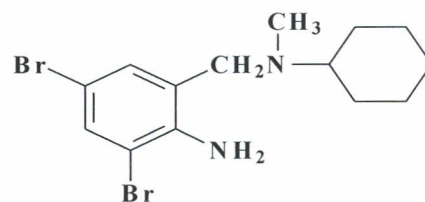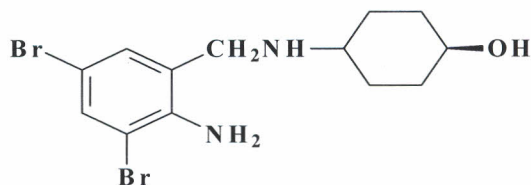
Figure 1.1: Chemical structures of expectorants and mucolytics under study

1.8.1. Guaifenesin

Guaifenesin is a natural substance that was isolated in the early 1500's for treatment of rheumatism. Though it has since been proven ineffective against rheumatism, it is widely used as a cough expectorant whereby it is taken orally [21]. It is readily absorbed after oral administration and metabolized by oxidation to β-(2-methoxyphenoxy)lactic acid which contributes about 40 % of metabolites excreted in the urine within 3 hours. It has a plasma half-life of about 1 hour and a volume of distribution of about 1 litre/kg [28].

Chemically, guaifenesin is 3-(2-methoxyphenoxy)-1,2-propanediol with the molecular formula $C_{10}H_{14}O_4$ and molecular weight 198.2 (figure 1.1). It is soluble in water, ethanol, chloroform, glycerol and practically insoluble in petroleum ether [27]. It is a white crystalline substance with a melting point of 78 - 82 °C [28].

1.8.2. Bromhexine

Bromhexine is an oral mucolytic agent with a low level of toxicity. It acts on the mucus at the formative stages in the glands, within the mucus-secreting cells and disrupts the structure of acid mucopolysaccharide fibres in mucoid sputum and produces less viscous mucus, which is easier to expectorate [21].

Bromhexine is well absorbed after oral administration but undergoes considerable first-pass metabolism including conjugation with glucuronic acid or sulphate. Ambroxol is an active metabolite of bromhexine. About 70 % of an oral dose is excreted in the urine in 24 hours as metabolites with less than 1 % as unchanged drug. It has a plasma half-life of about 6 hours [28].

Chemically, bromhexine is N-(2-amino-3,5-dibromobenzyl)-N-cyclohexylmethylamine with a molecular weight of 376.14 and molecular formula $C_{14}H_{20}Br_2N_2$ (figure 1.1). As a hydrochloride

7

salt, bromhexine is very slightly soluble in water and glacial acetic acid, slightly soluble in ethanol, chloroform and methanol but practically insoluble in acetone. It is a white crystalline powder with a melting point of 235 °C [27,28].

1.8.3. Ambroxol

Ambroxol is a metabolite of bromhexine with similar pharmacology. Chemically, ambroxol is *4-[[(2-amino-3,5-dibromophenyl)-methyl]amino]cyclohexanol* with molecular formula $C_{13}H_{18}Br_2N_2O$ and molecular weight of 378.11 (figure 1.1). Ambroxol hydrochloride has a melting point of 233 - 234.5 °C [27].

1.9. Chemistry and pharmacology of bronchodilators and decongestants under study

Bronchodilators are incorporated in cough syrups for management of cough associated with wheezing especially in cases of asthmatic cough. The β_2-adrenoceptor agonists, salbutamol and terbutaline are the two mainly used bronchodilators in cough syrups [24].

The pharmacologic effects of β-adrenergic agonists, are at least in part attributable to stimulation through β-adrenergic receptors of intracellular adenylate cyclase, the enzyme which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic 3',5'- adenosine monophosphate (cAMP). Increased cAMP levels are associated with relaxation of bronchial smooth muscle and inhibition of release of mediators of immediate hypersensitivity from cells, especially from mast cells. Response to a bronchodilator is characterised by an increase in forced expiratory flows and volumes and a decrease in airway resistance [29].

Majority of decongestants act via enhancing adrenergic activity by stimulating the α-adrenergic receptors. This induces vasoconstriction of the blood vessels in the nose, throat, and paranasal

sinuses, which results in reduced inflammation and mucus formation in these areas. Oral decongestants are effective in relieving symptoms of nasal congestion and a combination of a decongestant and an antihistamine has proven to be an optimal treatment regimen for allergic rhinitis. The most commonly used oral decongestants are ephedrine and pseudoephedrine [30]. The chemical structures of bronchodilators and decongestants under study are shown in figure 1.2.

HOCH$_2$
OH
CHCH$_2$NHC(CH$_3$)$_3$
HO
Salbutamol

HO
OH
CHCH$_2$NHC(CH$_3$)$_3$
HO
Terbutaline

OH
CH$_3$
NHCH$_3$
Pseudoephedrine
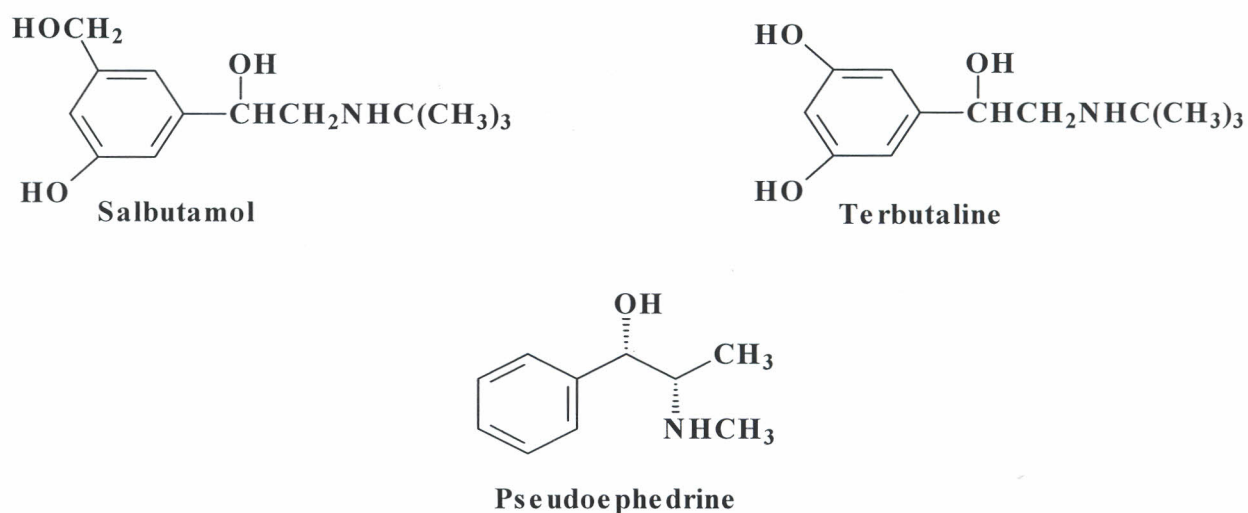
Figure 1.2: Chemical structures of bronchodilators and decongestant under study

1.9.1. Salbutamol

Salbutamol (albuterol) emerged in 1967 as the first agent selective for the β_2-adrenergic receptors of the lungs and bronchi, having negligible effect on the cardiac β_1-adrenergic receptors [31]. In 1969, salbutamol was launced by the Glaxo company as a longer lasting selective β_2-adrenoceptor agonist for the treatment of bronchial asthma [32]. Activation of the β_2-adrenoceptors results in bronchial smooth muscle relaxation which leads to dilatation of the airways [33]. It is rapidly absorbed after oral administration and after inhalation. About 60 to 90 % of a dose is excreted in the urine in 24 hours, of which approximately 50 % is unchanged

salbutamol and 50 % is the 4'-*O*-sulphate of salbutamol. Up to about 12 % is eliminated in the faeces. The plasma half-life is 2 to 7 hours [28].

Chemically, salbutamol is 4-hydroxyl-3-hydroxymethyl-α-[(*tert*–butylamino) methyl]-benzyl alcohol with a molecular weight of 239.3 and molecular formula $C_{13}H_{21}NO_3$ (figure 1.2). It is a white crystalline powder with a melting point of about 156 °C, sparingly soluble in water and ether and soluble in ethanol. Salbutamol sulfate ($C_{13}H_{21}NO_3.\frac{1}{2}H_2SO_4$) is used in cough syrups. It has a pKa of 9.3 and 10.3 and a melting point of 230 - 235 °C [28].

1.9.2. Terbutaline

Terbutaline is a direct-acting sympathomimetic agent with actions and uses similar to those of salbutamol. *In vitro* and *in vivo* pharmacologic studies have demonstrated that terbutaline exerts a preferential effect on β_2-adrenergic receptors that are the predominant receptors in bronchial smooth muscle. It is indicated for the prevention and reversal of bronchospasm in patients with asthma and reversible bronchospasm associated with bronchitis and emphysema [21].

Terbutaline is incompletely absorbed after oral administration and undergoes extensive first-pass metabolism in the liver and gut wall to produce the sulphate conjugate. Less than 15 % of an oral dose is present as free drug in the plasma. Up to about 50 % of a dose is excreted in the urine, predominantly as the sulphate conjugate, with up to 10 % as unchanged drug. It has a plasma half-life of about 3 to 4 hours and a volume of distribution of about 1 litre/kg [28].

Chemically, terbutaline is 2-*tert*-butylamino-1-(3,5-dihydroxyphenyl)ethanol with a molecular weight of 225.3, molecular formula $C_{12}H_{19}NO_3$ (figure 1.2) and a melting point of 119 - 122 °C. As a sulfate salt, terbutaline is a white powder with a melting point of 255 °C, molecular formula $C_{12}H_{19}NO_3.\frac{1}{2}H_2SO_4$ and a molecular weight of 274.3. Terbutaline sulfate is soluble in water,

slightly soluble in ethanol and practically insoluble in chloroform and ether. It has pKa values of 8.7, 10.0 and 11.0 at 20 °C [28].

1.9.3. Pseudoephedrine

Pseudoephedrine is used to relieve nasal congestion caused by colds, allergies, and hay fever. It is also used to temporarily relieve sinus congestion and pressure. It works by causing vasoconstriction in the nasal passages by acting directly on both α and β-adrenergic receptors with concomitant relaxation of the bronchioles and increased heart rate and contractility. It also enters the central nervous system readily [34]. It is an alkaloid obtained from *Ephedra spp* [28].

Pseudoephedrine is rapidly and completely absorbed after oral administration and up to 90 % of a dose is excreted unchanged in the urine in 24 hours with less than 1 % as norpseudoephedrine. It has a plasma half-life of about 5 to 8 hours and a volume of distribution of about 3 litres/kg [28].

Chemically pseudoephedrine is (1S,2S)-2-methylamino-1-phenylpropan-1-ol with a molecular weight of 165.2 and molecular formula $C_{10}H_{15}NO$ (figure 1.2). It has a melting point of 118 - 119 °C and is sparingly soluble in water but soluble in ethanol and ether. Pseudoephedrine hydrochloride is a white crystalline powder with a molecular weight of 201.7, molecular formula $C_{10}H_{15}NO.HCl$ and a melting point of 182 - 186 °C. It is soluble in water, ethanol and chloroform but slightly soluble in ether [28].

1.10. Chemistry and pharmacology of antihistamines under study

Antihistamines are competitive histamine antagonists at the H_1 receptors found in cells located throughout the body. The activity of these drugs depend on their chemical similarity to histamine [20]. Cough syrups commonly contain first generation antihistamines, including

brompheniramine, chlorpheniramine maleate, diphenhydramine, dexbrompheniramine and triprolidine [34]. The chemical structures of the antihistamines under study are shown in the figure 1.3.

Chlorpheniramine maleate   **Triprolidine**
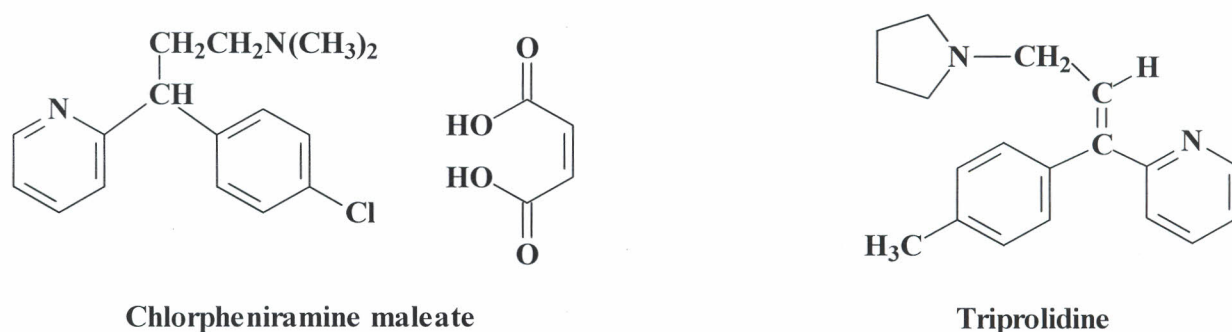
Figure 1.3: Chemical structures of antihistamines under study

1.10.1. Chlorpheniramine maleate

Chlorpheniramine is a competitive H_1 receptor antagonist indicated for relief of symptoms associated with perennial and seasonal allergic rhinitis, vasomotor rhinitis, allergic conjunctivitis, mild, uncomplicated urticaria and angioedema. It is a synthetic first generation alkylamine anti-histamine.

Chlorpheniramine is readily and almost completely absorbed after oral administration but extensively metabolized to polar and non-polar metabolites. The major metabolites are mono-desmethyl- and di-desmethylchlorpheniramine. After daily oral administration, about 20 % of a dose is excreted in the urine as unchanged drug, 20 % as monodesmethylchlorpheniramine, 5 % as didesmethylchlorpheniramine and less than 1 % is eliminated in faeces. It has a plasma half-life of about 18 to 40 hours and a volume of distribution of about 3 litres/kg [28].

Chemically, chlorpheniramine is 1-(p-chlorophenyl)-1-(2-pyridyl)-3-dimethylaminopropane ($C_{16}H_{19}ClN_2$) with a molecular weight of 274.80 and occurs as an oily liquid with a boiling point of 142 °C. Consequently, it is co-crystallized as the maleate salt ($C_{16}H_{19}ClN_2.C_4H_4O_4$) which is a white crystalline powder with a melting point of 130 - 135 °C and a molecular weight of 390.9. It is soluble in water, ethanol and chloroform but slightly soluble in ether [28].

1.10.2. Triprolidine

Triprolidine is a H_1 receptor antagonist used in allergic rhinitis, asthma and urticaria. It is a component of some cough and cold medicines. In most pharmaceutical formulations, it is used as triprolidine hydrochloride [24] which provides effective, temporary relief of sneezing, watery and itchy eyes and runny nose due to hay fever and other upper respiratory allergies. It is used in combination with pseudoephedrine hydrochloride for rhinitis and in other compound preparations for the symptomatic treatment of coughs and the common cold [21].

Chemically, triprolidine is (E)-2-[3-(pyrrolidin-1-yl)-1-p-tolylprop-1-enyl]pyridine with a molecular weight of 278.4 and molecular formula $C_{19}H_{22}N_2$ (figure 1.3). It is available as crystals with a melting point of 59 - 61 °C. In most pharmaceutical preparations, triprolidine hydrochloride monohydrate ($C_{19}H_{22}N_2.HCl.H_2O$, molecular weight 332.9) is used. It is a white crystalline powder with a melting point of 118 - 121 °C and soluble in water, ethanol, chloroform and slightly soluble in ether with a pKa value of 6.5 [28].

1.11. Literature review on the assay methods for the compounds under study

1.11.1. Guaifenesin

The British Pharmacopoeia (2007) specifies the titrimetric assay of guaifenesin raw material [35]. Although the method is cost effective, it suffers lack of selectivity.

The United States Pharmacopoeia (2009) has monographs for guaifenesin raw material, injection, capsules, oral solution, tablets and some compound finished products. The assay of guaifenesin raw material and capsules is by a HPLC method that utilizes an LC system equipped with a C_{18} reversed phase column (25 cm x 4.6 mm ID), mobile phase consisting of water-acetic acid (99:1, % v/v) and acetonitrile in a linear gradient elution and UV detection at 276 nm. Guaifenesin for injection is assayed by UV spectroscopy at 276 nm while the assay of guaifenesin oral solution, tablets, guaifenesin-pseudoephedrine hydrochloride capsules and guaifenesin-pseudoephedrine hydrochloride-dextromethorphan hydrobromide capsules involves a HPLC method that utilizes a C_{18} reversed phase column (25 cm x 4.6 mm ID), mobile phase consisting of methanol-glacial acetic acid-water (40:15:60, v/v/v) and UV detection at 276 nm. Guaifenesin-codeine phosphate oral solution is assayed using a GC method in a 1.2 m x 4 mm column containing 3 % liquid phase maintained at 170 °C and a flame ionization detector [36].

Several un-official methods for determination of guaifenesin in pharmaceutical preparations and bioanalysis in sera have been developed. The methods range from those of determining guaifenesin as a single entity to those of simultaneous analysis of guaifenesin in combination with other compounds.

Tapsoba *et al.* (2005) developed a voltammetric method for assay of guaifenesin in pharmaceutical formulations [37]. Determination of guaifenesin in human serum by capillary gas

chromatography and electron capture detection has also been reported by Sharaf and Stiff (2004) [38].

Chen *et al.* (2004) have reported a sensitive liquid chromatography-tandem mass spectrometry method for the simultaneous determination of paracetamol and guaifenesin in human plasma [39] while Eichhold *et al.* (2007) described simultaneous determination of dextromethorphan, dextrorphan and guaifenesin in human plasma using semi-automated liquid-liquid extraction and gradient liquid chromatography tandem mass spectrometry [40]. Another method for simultaneous determination of dextrorphan and guaifenesin in human plasma by liquid chromatography with fluorescence detection was also described by Stavchansky *et al.* (1995) [41].

Several other high performance liquid chromatographic methods for simultaneous determination of guaifenesin in combination with other compounds have been described. These include acetaminophen-guaifenesin-dextromethorphan combination in cough syrups [42], acetaminophen-guaifenesin-pseudoephedrine-pholcodine and paraben preservatives in cough mixtures [43], codeine phosphate-guaifenesin-chlorpheniramine maleate-phenylpropanolamine hydrochloride and pyrilamine maleate in expectorant preparations [44], guaifenesin-pseudoephedrine-dextromethorphan and guaifenesin-pseudoephedrine in commercial capsule dosage forms and guaifenesin-codeine in cough syrups [45].

1.11.2. Bromhexine hydrochloride

The BP (2007) bears a monograph for bromhexine hydrochloride raw material where a potentiometric titration assay method is specified [35]. This is the only official method for assay

of bromhexine hydrochloride. There are no official assay methods for finished products containing bromhexine.

Several un-official methods have been employed for the qualitative and quantitative determination of bromhexine in biological and pharmaceutical samples. Electron-capture gas-liquid chromatography (GLC) is described for determination of bromhexine in plasma and it involves derivatization with trifluoroacetic anhydride and quantified by GLC using a ^{63}Ni-electron capture detector [46]. Liquid chromatography-electrospray ionization mass spectrometry is described for the bioequivalence study of bromhexine after oral administration. The method involves use of simvastatin as the internal standard, a 150 mm x 4.6 mm ID, 5 μm C_{18} column, methanol-water (98:2, % v/v) as the mobile phase and detection performed in positive selected ion monitoring (SIM) mode at m/z 264.1 for bromhexine and m/z 441.7 for simvastatin [47].

Bromhexine in pharmaceutical preparations has been determined qualitatively and quantitatively using various techniques. Flow-injection extraction-spectrophotometry that involves formation of an ion-pair with orange IV, extraction into 1,2-dichloroethane and measurement of the absorbance at 412 nm of the organic phase has been reported [48]. Capillary isotachorphoresis with conductimetric detection has been described for determination of bromhexine or ambroxol in pharmaceuticals [49]. Potentiometric flow injection analysis of bromhexine hydrochloride and its pharmaceutical preparation has also been reported and utilizes bromhexine hydrochloride ion-selective electrodes based on bromhexinium tetraphenyl borate and bromhexinium-phosphotungstate [50]. Flow injection chemiluminescence method for determination of bromhexine hydrochloride has been established based on chemiluminescence reaction when cerium (IV) solution is injected into bromhexine hydrochloride-Morin solution [51]. Simultaneous determination of bromhexine in combination with other compounds has been

accomplished through several analytical techniques. Dextromethorphan hydrobromide–bromhexine hydrochloride tablets were analysed simultaneously using first derivative spectrophotometry, a technique that uses zero-crossing and peak to base measurement at 234 nm and 324 nm respectively [52].

Several HPLC methods for various combinations have been reported. These include bromhexine hydrochloride, methylparaben, propylparaben and dextromethorphan hydrobromide in cough-cold syrups that were determined simultaneously using a C_{18} reversed phase analytical column (150 mm x 3.9 mm ID), acetonitrile-15 mM triethylamine pH 3.9 (43:57, % v/v) mixture as the mobile phase and UV detection [53]. Clenbuterol, ambroxol and bromhexine in pharmaceutical formulations were also simultaneously determined by HPLC with potentiometric detection [54]. Determination of salbutamol sulphate and bromhexine hydrochloride combination in tablets by reversed phase liquid chromatography with a C_{18} column and UV detection at 224 nm has been reported [55] while reversed phase ion-pair HPLC was used for simultaneous determination of bromhexine hydrochloride and baicalin [56].

1.11.3. Ambroxol hydrochloride

The BP (2007) specifies potentiometric titration for the assay of ambroxol hydrochloride raw material. The method involves dissolving 0.3 g of ambroxol hydrochloride in 70 mL alcohol after which 5 mL of 0.01 M hydrochloric acid is added. The mixture is back titrated with 0.1 M sodium hydroxide to a potentiometric end point [35]. This is the only official method for assay of ambroxol hydrochloride. There are no pharmacopoeial specifications for finished products containing ambroxol.

Several un-official analytical methods have been employed for the individual or simultaneous quantitative determination of ambroxol in pharmaceutical formulations and biological samples. Ambroxol in body fluids is determined by capillary electrophoresis with fluorescent detection [57]. Non-destructive Raman spectroscopy has been reported for determination of ambroxol content in tablets [58]. Capillary isotachorphoresis is described for determination of ambroxol in pharmaceuticals and the technique utilizes conductimetric detection [49].

High performance liquid chromatography (HPLC) and UV spectroscopy have widely been developed for determination of ambroxol individually in pharmaceuticals [59-61]. Further, simultaneous determination of ambroxol and other compounds has been accomplished though utilization of various techniques, which include, simultaneous determination of clenbuterol, ambroxol and bromhexine in pharmaceutical formulations by HPLC with a cyano reversed phase silica column (250 mm x 4.6 mm ID), a mobile phase consisting of acetonitrile-ethanol-perchloric acid mixture and potentiometric detection [54]. High performance liquid chromatography for the determination of ambroxol in the presence of different preservatives in pharmaceutical formulations, utilizing C_{18} column (150 mm x 4.6 mm ID), mobile phase consisting of methanol-ammonium acetate buffer pH 3.45 (70:30, % v/v) and UV detection at 220 nm [62]. Simultaneous estimation of ambroxol hydrochloride and azithromycin in tablet dosage form by C_{18} reversed phase HPLC and a mobile phase consisting of acetonitrile-dipotassium phosphate (50:50, % v/v) mixture with UV detection at 215 nm has been achieved [63]. Simultaneous determination of roxithromycin and ambroxol hydrochloride in tablets by liquid chromatography utilizing a C_{18} column (200 mm x 4.6 mm ID, 5 μ), mobile phase consisting of acetonitrile-methanol-ammonium acetate (39:11:50, % v/v/v) mixture and UV detection at 220 nm [64] and quantitative determination of levofloxacin and ambroxol

hydrochloride by reversed phase high performance liquid chromatography have been reported [65]. Another combination is ambroxol hydrochloride and cetirizine hydrochloride where reversed phase high performance liquid chromatography and spectrophotometric estimation was used for analysis [66].

1.11.4. Pseudoephedrine hydrochloride

The BP (2007) contains monographs for pseudoephedrine hydrochloride raw material and pseudoephedrine hydrochloride tablets. The assay of the raw material involves dissolving 0.17 g of pseudoephedrine hydrochloride in 30 mL of alcohol and adding 5.0 mL of 0.01 M hydrochloric acid to this solution and carrying out potentiometric back titration using 0.1 M sodium hydroxide. Liquid chromatography is used for the assay of pseudoephedrine hydrochloride tablets and utilizes methanol as the diluent, C_{18} column (200 mm x 4.6 mm ID) as the stationary phase, 0.005 M dioctyl sodium sulphosuccinate in a mixture of methanol-water-glacial acetic acid (65:35:1, % v/v/v) as the mobile phase and UV detection at 258 nm [35].

The USP (2009) describes the assay of pseudoephedrine hydrochloride raw material, extended release capsules and tablets and oral solution. The assay of the raw material is by non-aqueous titration with 0.1 N perchloric acid to a crystal violet end point. Liquid chromatography is employed in the assay of pseudoephedrine extended release tablets and capsules and the oral solution. The chromatographic conditions include a normal phase silica column (4.6 mm x 150 mm ID), as mobile phase, alcohol-ammonium acetate solution (85:15, % v/v) and UV detection at 254 nm [36].

Several un-official methods for determination of pseudoephedrine in mixture have been described. Non-aqueous capillary electrophoresis is used for determination of pseudoephedrine,

dextromethorphan, diphenhydramine and chlorpheniramine in cold medicines [67] while reversed phase liquid chromatographic methods have been reported for simultaneous determination of pseudoephedrine in combination with other compounds. These include HPLC assay of loratadine and pseudoephedrine in pharmaceutical formulations with UV detection [68]. Ephedrine-pseudoephedrine-norephedrine-methylephedrine assay using HPLC with UV detection [69] and paracetamol-caffeine-pseudoephedrine-chlorpheniramine-cloperastine in human plasma with HPLC-tandem mass spectrometry [70]. The HPLC assay of pseudoephedrine-cetirizine in human plasma with ion trap spectrometry detection has also been reported [71]. Other methods for assay of pseudoephedrine hydrochloride-diphenhydramine hydrochloride-dextromethorphan hydrobromide in cough-cold formulations [72] and pseudoephedrine hydrochloride-brompheniramine maleate-dextromethorphan hydrobromide in cough-cold syrups [73] are found in the literature.

1.11.5. Salbutamol sulphate

The BP (2007) describes the assay methods for salbutamol sulphate raw material, injection, oral solution, pressurized inhalation, nebulizer solution and tablets. The assay of the raw material is by non-aqueous titration with 0.1 M perchloric acid to a potentiometric end point. Liquid chromatography under different conditions is employed for the assay of the other formulations except the nebulizer solution which is assayed by UV spectroscopy at 276 nm [35].

The USP (2009) has monographs for salbutamol sulphate raw material and tablets. It specifies non-aqueous titration for the assay of the raw material and HPLC for the assay of salbutamol sulphate tablets. The HPLC system utilizes a C_{18} column (150 mm x 4.6 mm ID) as the stationary phase, a mixture of 1.13 g of sodium 1-hexanesulphonate in 1200 mL water and 12

mL of methanol-glacial acetic acid (40:60, % v/v) as the mobile phase and UV detection at 276 nm [36].

Several non-pharmacopoeial techniques have been developed. Capillary electrophoresis with contactless conductivity detection is employed in determination of salbutamol in syrups [74]. Conductimetric method for the determination of reproterol hydrochloride, pipazethate hydrochloride and salbutamol sulphate in their pharmaceutical formulations is based on their ion associates with phosphotungstic and phosphomolybdic acids [75]. Ultra-violet (UV) spectroscopy is reported for determination of salbutamol sulphate using chlorinated quinines in the presence or absence of acetaldehyde [76]. Gas chromatography in determination of salbutamol in plasma after aerosol inhalation utilizes isotopically labeled albuterol as an internal standard and the samples are derivatized to trimethylsilyl ethers using N,O-bis (trimethylsilyl)trifluoro-acetamide with 1 % trimethylchlorosilane and then analyzed by GC-MS with selected-ion monitoring (SIM) for the ions m/z 369.15 and 370.15 [77].

Several high performance liquid chromatographic techniques have been developed for determination of salbutamol. Salbutamol in samples of urine collected post-inhalation has been determined by a HPLC technique that utilizes a C_{18} column (250 mm x 4.6 mm ID) as the stationary phase, a mixture of acetonitrile-tetrahydrofuran-methanol-buffer (10:8:14:62, v/v/v/v) as the mobile phase, fluorescent detection and terbutaline as the internal standard [78]. Salbutamol sulphate and its related substances in 0.5 % inhalation solution were determined by HPLC using a phenyl column (250 mm x 4.6 mm ID) and a mixture of 25 mM monobasic potassium phosphate pH 3.0-methanol (95:5, % v/v) as mobile phase with UV detection at 225 nm [79]. HPLC enantioselective analysis of bambuterol and albuterol using chiral HPLC columns has been reported [80] while quantitation of salbutamol in urine samples has been

achieved by an ultraperformance liquid chromatography tandem mass spectrometric technique [81] and liquid chromatography-electrospray ionization mass spectrometry [82]. Simultaneous determination of ipratropium bromide, fenoterol, salbutamol and terbutaline in nebulizer solution was accomplished by HPLC assay using a C_{18} (100 mm x 8 mm ID, 4 μm) column and UV detection at 225 nm [83].

1.11.6. Terbutaline sulphate

The BP (2007) describes the assay of terbutaline sulphate raw material and tablets. The assay of terbutaline sulphate raw material is by non-aqueous titration with 0.1 M perchloric acid to a potentiometric end point while the assay of tablets is carried out using HPLC on a C_{18} column (150 mm x 4.6 mm ID, 5 μm) and UV detection at 276 nm [35].

The USP (2009) bears monographs for terbutaline sulphate raw material, injection, tablets and inhalation aerosol whereby the assay for all the formulations is by HPLC. The assay of the raw material, injection, and tablets utilizes the same chromatographic system that is equipped with a C_{18} column (150 mm x 4.6 mm ID, 5 μm), ion pair-methanol (77:23, % v/v) mixture as the mobile phase and UV detection at 276 nm. The assay of terbutaline sulphate inhalation aerosol employs a HPLC equipped with a C_8 column (80 mm x 6.2 mm ID, 3 μm), a mixture of water-methanol-tetrahydrofuran-sodium 1-octane sulfonate as mobile phase with UV detection at 280 nm [36].

An un-official voltammetric method based on oxidation has been employed in the determination of terbutaline on an activated glassy carbon electrode [84]. Enzyme–linked immunosorbent assay and capillary electrophoresis are reported for use in terbutaline determination in urine after oral and inhaled administrations [85] and capillary electrophoresis with chemiluminescence detection

[86]. A novel chemiluminescence method for the determination of terbutaline based on the reaction of potassium ferricyanide with terbutaline sulphate in sodium hydroxide medium sensitized by the fluorescent dye rhodamine 6G has also been described [87].

Liquid chromatographic techniques have been developed for the determination of terbutaline either individually or in mixture with other compounds. Simultaneous analysis of bambuterol and its active metabolite terbutaline has been achieved by use of chiral liquid chromatography– tandem mass spectrometry [88]. Liquid chromatographic determination of terbutaline sulphate and its degradation products in pharmaceutical formulations has been reported. The method utilizes a C_{18} (150 mm x 4.6 mm ID, 5 μm) column, a mixture of 0.15 M ammonium acetate pH 4.0-glacial acetic acid (96:4, % v/v) as the mobile phase with UV detection at 270 nm [89]. Determination of terbutaline and salbutamol by HPLC on normal phase silica column with fluorescence detection at excitation wavelength of 200 nm has been described [90] while a stability indicating ion-pair HPLC specific for adrenaline, isoproterenol, dopamine, noradrenaline, methyldopa or terbutaline using a spectrofluorometric detector has also been reported [91].

1.11.7. Chlorpheniramine maleate

The BP (2007) bears monographs for chlorpheniramine maleate raw material, injection, oral solution and tablets. The assay of the raw material is by non-aqueous titration with 0.1 M perchloric acid to a potentiometric end point. The assay of the injection and tablets employs UV spectroscopy at 265 nm. The assay of chlorpheniramine maleate oral solution employs a GC equipped with a glass column (1.5 m x 4 mm) packed with acid-washed silanised diatomaceous support coated with 3 % w/w of dimethyl silicone fluid [35].

The USP (2009) bears monographs for chlorpheniramine maleate raw material, extended release capsules, injection, oral solution, tablets, chlorpheniramine maleate-pseudoephedrine hydrochloride capsules and oral solution. The assay of the raw material is by non-aqueous titration with 0.1 N perchloric acid to a crystal violet end point. An UV spectrophotometric method at 264 nm is employed in the assay of chlorpheniramine maleate injection, oral solution and tablets. The extended release capsules are assayed by HPLC equipped with a C_{18} column (150 mm x 3.9 mm ID), a mobile phase consisting of methanol-0.16 M sodium perchlorate-triethylamine-water (65:10:0.2:24.8, % v/v/v/v) and UV detection at 261 nm [36].

Non-aqueous capillary electrophoresis has been described in the non-pharmacopoeial assay of pseudoephedrine and determination of pseudoephedrine, dextromethorphan, diphenhydramine and chlorpheniramine in cold medicines [67].

Gas-Liquid Chromatography (GLC) has also been described for simultaneous quantitation of compound preparations such as chlorpheniramine, phenylpropanolamine, glyceryl guaiacolate and dextromethorphan [92, 93] while second derivative photodiode array spectroscopy is prescribed for the simultaneous determination of pseudoephedrine hydrochloride, chlorpheniramine maleate and dextromethorphan hydrobromide [94].

Several liquid chromatographic methods have been developed for simultaneous determination of chlorpheniramine and other compounds. Liquid chromatography–tandem mass spectrometry has been reported for the simultaneous quantitation of paracetamol, caffeine, pseudoephedrine, chlorpheniramine and cloperastine [70] and simultaneous analysis of codeine phosphate, ephedrine hydrochloride and chlorpheniramine maleate in cough–cold syrup formulation is described [95]. *Phenylpropanolamine hydrochloride, caffeine, paracetamol, glyceryl guaiacolate*

and chlorpheniramine maleate in tablets were assayed simultaneously using HPLC with diode array detection in the range of 210 to 400 nm [96].

A HPLC method utilizing tandem mass spectrometry detection, diphenhydramine as the internal standard, C_{18} column (250 mm x 4.6 mm ID) as the stationary phase and methanol-water-formic acid as the mobile phase, has been described for the simultaneous determination of chlorpheniramine and pseudoephedrine in human plasma [97]. Simultaneous determination of pseudoephedrine and chlorpheniramine in pharmaceutical dosage forms has been carried out using HPLC with UV detection at 254 nm [98] while simultaneous determination of paracetamol-pseudoephedrine-dextrophan-chlorpheniramine in human plasma has been accomplished by use of liquid chromatography-tandem mass spectrometry utilizing C_{18} column (100 mm x 2.0 mm ID, 3 μm) under gradient elution and quadrupole tandem mass spectrometry for detection [99].

1.11.8. Triprolidine hydrochloride

The assay of triprolidine hydrochloride raw material according to the BP (2007) is by non-aqueous titration with 0.1 M perchloric acid to a crystal violet end point while UV spectrophotometry at 290 nm is used in the assay of triprolidine hydrochloride tablets [35].

The USP (2009) has monographs for triprolidine hydrochloride raw material, oral solution, tablets and triprolidine-pseudoephedrine oral solution and tablets. The assay of the raw material is by non-aqueous titration using 0.1 N perchloric acid to a potentiometric end point. HPLC is employed in the assay of all the other triprolidine hydrochloride formulations using a normal phase silica column (250 mm x 4.6 mm), alcohol-0.4 % ammonium acetate solution (85:15, % v/v) as the mobile phase and UV detection at 254 nm [36].

Among the un-official methods, capillary electrophoresis is reported for determination of antihistamines, including triprolidine in serum and pharmaceutical products. This technique utilizes 24 cm capillary, 20 mM phosphate running buffer at pH 2.0, 2 psi.s^{-1} injection pressure and 5 kV applied voltage and UV detection at 214 nm [100]. A kinetic spectrophotometric method has been described for quantitation of triprolidine in bulk and drug formulations [101].

Liquid chromatographic techniques have been published for simultaneous determination of triprolidine and other compounds. Pseudoephedrine hydrochloride-codeine phosphate-triprolidine hydrochloride mixture in liquid dosage forms are assayed by HPLC using a C_{18} column (250 mm x 4.0 mm ID) and methanol-acetate buffer-acetonitrile (85:5:10, % v/v/v) as mobile phase with UV detection at 254 nm [102]. A liquid chromatography-ion trap mass spectrometry is described for the simultaneous determination of triprolidine and pseudoephedrine in human plasma [103] while triprolidine-pseudoephedrine-paracetamol-dextromethorphan mixtures have been assayed by HPLC using a C_{18} column, gradient elution and UV detection at 254 and 280 nm [104].

1.12. Study justification

Cough is one of the most common symptoms for which patients seek medical attention. It is a symptom that significantly and negatively affects a patient's lifestyle and sense of well being. Cough syrups are available for symptomatic relief of cough, although their use remains controversial. However, many pharmaceutical manufacturers continue to manufacture cough syrups for commercialization.

The quality of cough syrups is important because these products should have the correct quantities of the active pharmaceutical ingredients (API) and meet the other aspects of quality,

including packaging, for them to elicit the desired clinical outcomes. Cough syrups are generally classified as those used for treatment of productive coughs and those for dry coughs. The focus of the present study is to develop a liquid chromatographic method for simultaneous determination of content of the major APIs used in the treatment of productive coughs. These include guaifenesin, bromhexine, ambroxol, pseudoephedrine, salbutamol, terbutaline, chlorpheniramine maleate and triprolidine which are mostly formulated as combination products. Most productive cough syrups in the Kenyan market contain some of these compounds in combination, including salbutamol-bromhexine-guaifenesin, bromhexine-terbutaline-guaifenesin, ambroxol-terbutaline-guaifenesin, pseudoephedrine-triprolidine-guaifenesin, ambroxol-salbutamol-guaifenesin, pseudoephedrine-guaifenesin, pseudoephedrine-guaifenesin-chlorpheniramine maleate, pseudoephedrine-bromhexine, salbutamol-bromhexine and salbutamol-guaifenesin. Others also incorporate menthol, ammonium chloride and sodium citrate. There are also syrups containing either bromhexine or ambroxol as single entities used in the management of chronic obstructive pulmonary disease.

Currently in Kenya, cough syrups are not listed in the essential drug list. Consequently, most hospitals do not include them in their formularies but still prescribe cough syrups for patients who buy them from the community pharmacies. The official analytical methods available for assay of multi-component cough syrups are time consuming because the constituent compounds are assayed individually. There is no post-marketing surveillance study conducted to determine the quality of multi-component cough formulations sold in Kenya, yet they are among the most sold OTC medications. This could partly be due to lack of appropriate testing methods as literature review shows there are no official methods for assay of combined APIs in most cough syrups formulations. In addition, there are no national specifications for products unique to

Kenya. Thus, the drug regulatory authorities rely heavily on the analytical data and in-house methods of analysis provided by the manufacturers of these products as the basis for assessing their quality for market authorization purposes.

Several methods of analysis have been published for assay of a number of APIs in cough syrups. These include liquid chromatography, gas chromatography, UV-Visible spectroscopy, capillary electrophoresis and titrimetry. Of these, titrimetry and UV-Visible spectroscopy methods suffer the major drawback of lack of selectivity. Currently, the most commonly employed technique in routine assay is liquid chromatography that offers the advantages of high selectivity and sensitivity.

The development of a single isocratic liquid chromatographic method capable of determining the content of several compounds formulated as combinations in cough syrups would go a long way in enhancing the quality assurance of such pharmaceutical preparations. Such a method would also be advantageous to quality control laboratories, as it would simplify analysis of these preparations by permitting simultaneous analysis of components thus resulting in saving time and cost. The method may also be applied in post-marketing surveillance of these pharmaceuticals by the drug regulatory authorities. Pharmaceutical manufacturers can also adopt such a method for the quality assessment of the cough syrups during manufacture and release.

1.13. Study objectives

The general objective of the study was to develop and validate an isocratic reversed phase liquid chromatographic method for the simultaneous determination of guaifenesin, ambroxol hydrochloride, bromhexine hydrochloride, salbutamol sulphate/terbutaline sulphate, pseudoephedrine hydrochloride, triprolidine hydrochloride and chlorpheniramine maleate in cold-cough syrup formulations.

The specific objectives were:

a. To develop and optimize the LC conditions with UV detection for the simultaneous separation and quantification of guaifenesin, ambroxol hydrochloride, bromhexine hydrochloride, salbutamol sulphate/terbutaline sulphate, pseudoephedrine hydrochloride, triprolidine hydrochloride and chlorpheniramine maleate using HPLC.

b. To carry out validation of the LC method developed, in accordance to International Conference on Harmonization (ICH) guidelines.

c. To apply the developed method in the assay of commercial samples of cold-cough syrups obtained from the Kenyan market.

CHAPTER TWO

METHOD DEVELOPMENT

2.1. Introduction

High performance liquid chromatography (HPLC), particularly reversed phase HPLC, is the most popular analytical technique in the pharmaceutical industry. It is widely used for assay and impurity profiling during quality control tests. Pharmaceutical products formulated with more than one active pharmaceutical ingredient (API), typically referred to as combination products, are intended to meet patients' needs by combining the therapeutic effects of the drugs in one product. An analytical technique for simultaneous analysis of the APIs in a drug product is preferable to methods where these drugs are analysed individually. High performance liquid chromatography is the most practical technique used in the analysis of several drug compounds simultaneously which makes it cost effective. There are several purposes for which new HPLC methods are developed, namely; the drug or drug combination may not be official in any pharmacopoeia, analytical methods may not be available for the drug formulation due to the interference caused by excipients, analytical methods for a drug in combination with other drugs may not be available, the existing analytical procedures may require expensive reagents, solvents or may involve cumbersome extraction and separation procedures. The developed method should therefore overcome the shortcomings of existing techniques and further be accurate, reproducible, efficient, robust, cost-effective and reliable [105].

In the present study, a simple, reliable, accurate, precise, robust and isocratic HPLC method was developed for simultaneous determination of salbutamol/terbutaline, pseudoephedrine, guaifenesin, ambroxol, chlorpheniramine, triprolidine and bromhexine. With the exception of

guaifenesin, these are basic compounds commonly found in cough mixtures used for symptomatic relief of productive cough where they are formulated as combination products.

2.2. Reagents, chemicals and solvents

Analytical grade monobasic potassium phosphate, dibasic potassium phosphate, butanesulfonic acid sodium salt (Loba chemie PVT LTD, Mumbai, India), anhydrous sodium acetate, ammonium acetate, hexanesulfonic acid sodium salt, pentanesulfonic acid sodium salt, triethylamine (RFCL LTD, New Delhi, India), orthophosphoric acid (Merck Chemicals PTY Ltd., Gauteng, South Africa), potassium bromide (Merck, Darmstadt, Germany), heptanesulfonic acid sodium salt (Fischer Scientific, Leicestershire, UK) were used during method development.

The working standards used in the method development were ambroxol hydrochloride (AMB), bromhexine hydrochloride (BXN), chlorpheniramine maleate (CPM), guaifenesin (GFN), pseudoephedrine hydrochloride (PED), salbutamol sulphate (SBT), terbutaline sulphate (TBT) and triprolidine hydrochloride (TPN). These were kindly donated by the National Quality Control Laboratory (Nairobi, Kenya) from their stock of standards. The primary standards used were chlorpheniramine maleate, guaifenesin, pseudoephedrine hydrochloride, salbutamol sulfate, terbutaline sulfate and triprolidine hydrochloride, sourced from United States Pharmacopoeia (USP) reference standards.

Acetonitrile (Scharlab S.L. Sentmenat, Spain) used was HPLC grade while glacial acetic acid (Scharlau Chemie S.A, Spain) was analytical grade. Purified water was prepared in the laboratory using an Arium 61316 RO and Arium 611 VF water system (Sartorius AG, Göttingen, Germany) which utilizes reverse osmosis, ultra filtration through successive 0.45 μm and 0.2 μm membrane filters as well as UV irradiation.

31

2.3.   Instrumentation

2.3.1. Melting point apparatus

The melting points of the substances used as working standards were determined using a B-540 Buchi melting point apparatus (Buchi Labortechnik AG, Flawil, Switzerland). The apparatus was calibrated using certified primary chemical reference melting point standards, caffeine, vanillin and phenacetin obtained from the USP (Rockville, MD, USA).

2.3.2. Ultra-violet spectrophotometer

A double beam T90+ UV/VIS spectrophotometer controlled by the UVWIN software version 5.2.0 (PG Instruments, Leicestershire, United Kingdom) and quartz cuvettes of path length 10 mm, were used in obtaining UV spectra of the working standards over the range of 200 - 400 nm.

2.3.3. Infra-red spectrophotometer

A Shimadzu IRPrestige 21 Fourier Transform Infra-Red (FTIR) spectrophotometer (Shimadzu Corp., Kyoto, Japan) utilizing IRSolution Software Ver. 1.3 was used to record the infrared spectra for the compounds under study. Sample discs were prepared in potassium bromide using a manually operated hydraulic pellet press (Perkin Elmer GmbH, Uberlingen, Germany).

2.3.4. Liquid chromatographic system

A Shimadzu Prominence high performance liquid chromatographic (HPLC) system (Shimadzu Corp., Kyoto, Japan) was used for the study. The system was supported by a CBM-20A (S/N, L20234505135) Prominence communications bus module as the system controller and an LCSolutions Software Ver 1.22, SP1 and was equipped with an SPD-20A (S/N, L20134506373) Prominence UV/Visible detector which incorporated a deuterium lamp for ultraviolet and a tungsten lamp for visible region applications. A LC-20AT (S/N, L20114505973) Prominence

solvent delivery system with a dual-plunger tandem-flow solvent delivery module and a SIL-10AS (S/N, L20164503141) Prominence autosampler were part of the HPLC system assembly. The temperature was controlled using a CTO-10AS VP (S/N, C21044505693) column oven with a block heating thermostatic chamber and a preheater system. All mobile phase preparations were degassed using a Power Sonic 410 bench top ultrasonic bath (Daihan Labtech Ltd, Kyonggi-Do, Korea).

2.4. Characterization of the working standards

The working standards were characterized by means of melting point determination, potency determination by HPLC or titration, UV and IR spectroscopy.

2.4.1. Melting points of the working standards

The melting point ranges for all the eight working standards were determined as recorded in table 2.1. The values of melting points obtained fell within the ranges reported in the literature thus indicating the identity and purity of the substances.

Table 2.1: Melting points of the working standards

Working standard	Observed melting point (°C)	Literature melting point (°C) [28]
Ambroxol hydrochloride	233.8 - 234.1	233 - 234.5
Bromhexine hydrochloride	236.3 - 237.0	235 - 238
Chlorpheniramine maleate	133.7 - 134.3	130 - 135
Guaifenesin	79.9 - 80.4	78 - 82
Pseudoephedrine hydrochloride	184.0 - 184.6	182 - 186
Salbutamol sulphate	232.5 - 232.9	230 - 235
Terbutaline sulphate	245.7 - 246.2	246 - 248
Triprolidine hydrochloride	119.3 - 119.9	118 - 121

2.4.2. Infra-red absorption spectra

The Fourier Transform infra-red (FTIR) absorption spectra for the working standards and the primary chemical reference substances were recorded over a range of 4000 to 400 cm^{-1} using a 1 mm thick potassium bromide disc containing about 1 % w/w of each compound. The frequencies of the principal IR absorption bands were compared against those observed from primary chemical reference substances and those published in literature for all the substances to confirm their identities. All the spectra were concordant with the corresponding reference spectra (Appendices 1 to 8).

2.4.3. Ultra-violet absorption spectra

The ultra-violet (UV) absorption spectra were measured from 200 to 400 nm for all the working standards at concentrations of 0.025 mg/mL AMB, 0.025 mg/mL BXN, 0.02 mg/mL CPM, 0.048 mg/mL GFN, 0.5 mg/mL PED, 0.085 mg/mL SBT, 0.07 mg/mL TBT and 0.017 mg/mL TPN. These concentrations gave absorbances of about 0.5 at the λ_{max} and were arrived at using the A 1 %, 1 cm values reported in the literature [28]. The observed λ_{max} values were compared with the literature values as recorded in the table 2.2.

Table 2.2: **Absorption maxima for the working standards**

Working standard	Observed λ_{max} (nm)	Literature λ_{max} (nm) [28]
Ambroxol hydrochloride	244	244
Bromhexine hydrochloride	245	245
Chlorpheniramine maleate	265	265
Guaifenesin	273	273
Pseudoephedrine hydrochloride	257	257
Salbutamol sulphate	276	276
Terbutaline sulphate	276	276
Triprolidine hydrochloride	290	290

The solvent used was 0.1 N hydrochloric acid for better comparison with literature values where aqueous acid was used as the solvent. The observed absorption maxima were similar to those reported in the literature further confirming the identity of these compounds. An overlay of all the spectra is shown in figure 2.1 and individual spectra shown in appendices 9 to 16.
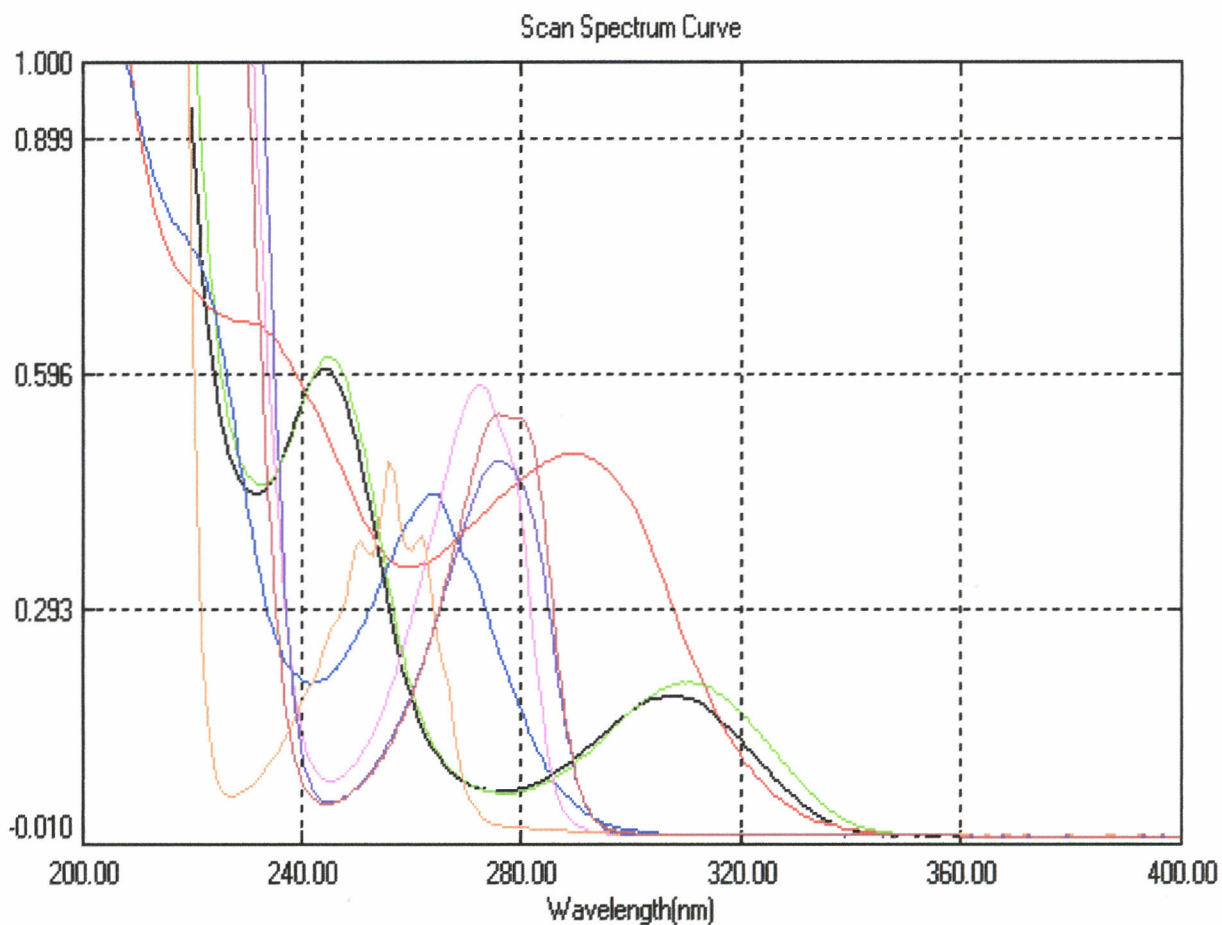
Figure 2.1: Combined UV spectra of the working standards

| Ambroxol | ———— | Bromhexine | ———— | Chlorpheniramine | ———— | Guaifenesin | ———— |
| Pseudoephedrine | ———— | Salbutamol | ———— | Terbutaline | ———— | Triprolidine | ———— |

35

2.4.4. Potency of the working standards

The potency of each of the eight working standards was determined using methods prescribed in the BP and USP [35, 36], either by HPLC or titration. The potencies of chlorpheniramine maleate, guaifenesin, pseudoephedrine hydrochloride, salbutamol sulfate, terbutaline sulfate and triprolidine hydrochloride were determined using HPLC since the primary standards were available while titration was used for ambroxol hydrochloride and bromhexine hydrochloride. The potencies obtained are recorded in table 2.3 on 'as is basis'.

Table 2.3: **Potencies of the working standards**

Working standard	Percentage potency (on 'as is' basis)
Ambroxol hydrochloride	99.4
Bromhexine hydrochloride	99.8
Chlorpheniramine maleate	99.9
Guaifenesin	99.6
Pseudoephedrine hydrochloride	100.8
Salbutamol sulphate	99.8
Terbutaline sulphate	99.7
Triprolidine hydrochloride	99.1

2.5. Method development

2.5.1. Introduction

Some chromatographic parameters were fixed at the onset of method development. These were the stationary phase, the detection wavelength, the flow rate and the injection volume. All other chromatographic conditions were varied during method development with emphasis on the

36

composition of the mobile phase. Chromatographic conditions that gave adequate separation of the compounds and minimum run time were considered optimum.

2.5.2. Fixed chromatographic parameters

2.5.2.1. Stationary phase

A reversed phase 25 cm x 4.6 mm ID Phenomenex Gemini® C_{18} column, S/N 491023-43, particle size 5 μ and pore size 110 Å (Phenomenex, Torrance, CA, USA) was selected as the stationary phase for the method development. Being a silica-based column, it offered the advantages of narrow pore size and diameter distribution, higher mechanical strength, higher column efficiency and superior peak parameters. The column brand used is claimed to be stable over an extended pH range of 1-12, hence giving room for mobile phase pH manipulation for improved separation of the components.

2.5.2.2. Mobile phase organic modifier

Acetonitrile was chosen as the organic modifier following preliminary work that favoured use of the solvent. The strong eluting power of acetonitrile supported the development of a single, isocratic LC method with relatively short analysis time. Additional advantages include low column back pressures, sharp peaks, low UV absorption wavelength cut off and miscibility with water at all proportions.

2.5.2.3. Detection wavelength

From the UV spectral scan of all the compounds, the wavelengths of maximum UV absorption ranged from 244-290 nm. All the compounds showed increased absorptivities at wavelengths below 240 nm (figure 2.1).

A detection wavelength of 254 nm was chosen because at this wavelength, most of the compounds under study showed marked UV absorption thus allowing larger responses of the compounds. However, guaifenesin absorption was at its minimum at 254 nm. Notably, in the commercial products, guaifenesin is available at concentrations about 50 times that of most compounds under study. A combination of these properties allowed UV detection of all the compounds. Additionally, the choice of 254 nm as the detection wavelength also offered the advantage of successful application of the developed method on HPLC instrumentation equipped with fixed wavelength UV detectors set at 254 nm, thus improving the versatility of the developed method.

2.5.2.4. Flow rate, injection volume and the reference working solution

The flow rate was fixed at 1.0 mL/min throughout the method development to ensure the column back pressures were maintained at below 150 bar while the injection volume was fixed at 20 μL as a compromise between peak responses and column loading.

The concentrations of the compounds in the reference working solution used during method development were 0.04 mg/mL (AMB), 0.08 mg/mL (BXN), 0.08 mg/mL (CPM), 0.2 mg/mL (GFN), 0.5 mg/mL (PED), 0.3 mg/mL (SBT), 0.3 mg/mL (TBT) and 0.04 mg/mL (TPN). These concentrations allowed comparable peak heights which enabled calculation of resolution and other chromatographic parameters during method development. The diluent used was acetonitrile-water (40:60 % v/v) since the compounds had varying solubilities in water with BXN being the least soluble.

2.5.3. Influence of chromatographic factors

2.5.3.1. Neutral conditions

A mobile phase consisting of acetonitrile-water (30:70, % v/v) was used as the starting mobile phase while the column temperature was maintained at 40 °C. Under these conditions, there was poor separation between most peaks making it impossible to calculate asymmetry factors for all the peaks except AMB (A_s = 0.901). Maleic acid (MAL) from chlorpheniramine maleate (CPM) co-eluted with SBT, TBT and the solvent peak with poor separation between this group of peaks and PED. The peaks of TPN and AMB were broad with poor symmetry whereas CPM peak split. Bromhexine (BXN) did not elute within 2 hours, probably due to its apolar character. Figure 2.2 shows a typical chromatogram obtained under these conditions.
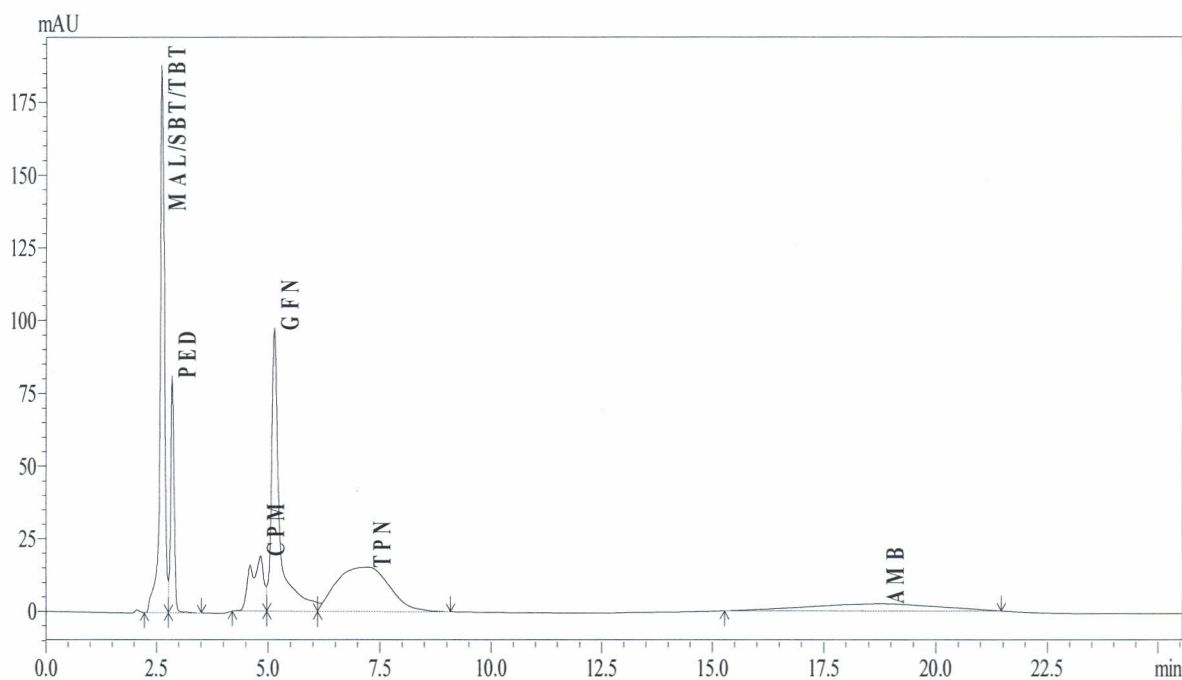
Figure 2.2: Typical chromatogram of the reference working solution obtained using unbuffered mobile phase. Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-water (30:70, % v/v). MAL-maleic acid, SBT-salbutamol, TBT-terbutaline, PED-pseudoephedrine, CPM-chlorpheniramine, TPN-triprolidine, GFN-guaifenesin, AMB-ambroxol

39

Effect of inorganic buffer on separation

The effect of monobasic potassium phosphate (KH_2PO_4) was investigated since it is the commonly used inorganic buffer in HPLC applications and hence readily available in the Kenyan market. The KH_2PO_4 was investigated at an effective concentration of 20 mM to avoid precipitation in the presence of acetonitrile. A stock solution of 0.2 M KH_2PO_4 was prepared and used in preparation of a mobile phase consisting of acetonitrile-0.2 M KH_2PO_4-water (30:10:60, % v/v/v). The pH of the buffer solution was not adjusted at this stage but was found to be 4.3. Under these conditions, all the peaks had improved shapes and BXN eluted at 85.54 minutes. The solvent front, SBT and TBT peaks co-eluted at 2.67 minutes while there was poor separation between SBT/TBT, MAL and PED peaks and GFN/AMB peaks. However, baseline separation was achieved for PED/GFN, AMB/CPM, CPM/TPN and TPN/BXN peak pairs. The asymmetry factors for the separated peaks ranged from 0.69 to 1.65 with BXN peak fronting (A_s = 0.69). Figure 2.3 is a typical chromatogram obtained under these conditions.
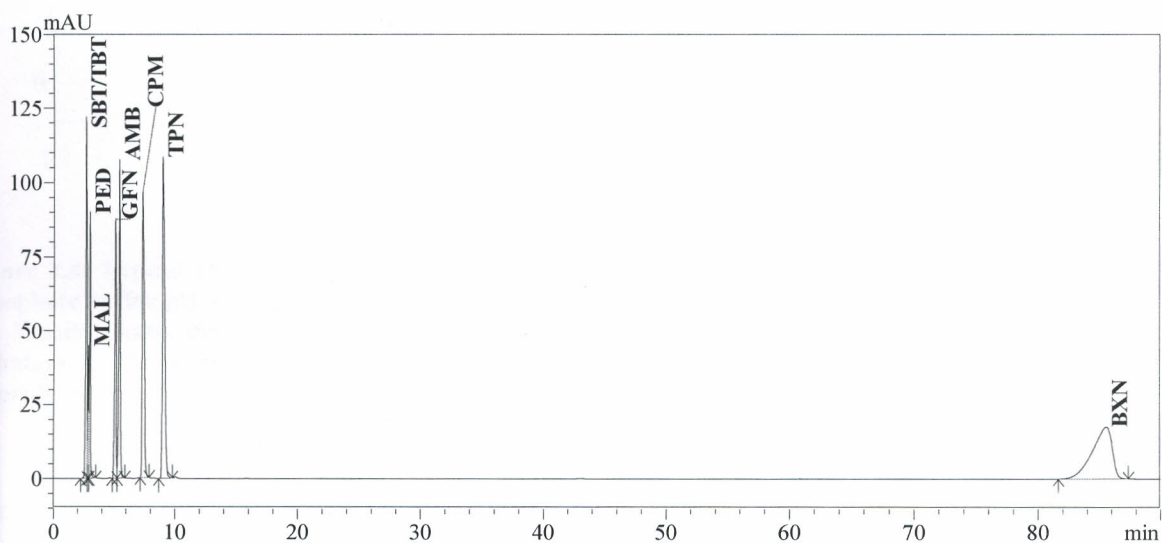
ure 2.3: **Typical chromatogram of the reference working solution obtained using monobasic potassium sphate buffer pH 4.3. Column: Phenomenex Gemini® 5 μ C_{18}, 250 × 4.6 mm ID. Column temperature: 40 Mobile phase: acetonitrile-0.2 M KH_2PO_4 pH 4.3-water (30:10:60, % v/v/v). TBT-terbutaline, SBT-utamol, MAL-maleic acid, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-rpheniramine, TPN-triprolidine, BXN-bromhexine.**

The effect of the phosphate buffer pH was investigated further to improve on the separation of the compounds and shorten the analysis time. Three mobile phase solutions containing acetonitrile-0.2 M KH_2PO_4-water (30:10:60, % v/v/v) were prepared at pH 3.0, 3.5 and 4.0 for the purpose of this investigation. The pH of each solution was adjusted accordingly using 0.2 M orthophosphoric acid. Buffer pH 3.0 yielded the best results (figure 2.4).

Figure 2.4: Typical chromatogram of the reference working solution obtained using monobasic potassium phosphate buffer pH 3.0. Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.2 M KH$_2$PO$_4$ pH 3.0-water (30:10:60, % v/v/v). TBT-terbutaline, SBT-salbutamol, MAL-maleic acid, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine.

The retention times and capacity factors of all the peaks increased with increase in pH from 3.0 to 4.0 with retention time of BXN exhibiting the greatest effect of pH variation (Table 2.4). The retention time of GFN was the least affected and this contributed to reversal of elution profile between AMB and GFN and reduced resolution of the peak pair GFN/AMB. The solvent peak,

SBT and TBT, MAL and PED co-eluted at all pH values investigated. The symmetry of the peaks remained unaffected except that of BXN which varied significantly over the pH range. The resolution in each case was calculated with reference to the peak eluting just before the component. Table 2.4 shows the effect of mobile phase pH on the retention time, asymmetry factor, resolution and capacity factor of the compounds under study.

Table 2.4: The effect of 0.2 M monobasic potassium phosphate buffer pH on the retention time, asymmetry factor, resolution and capacity factor of the compounds under study.

Mobile phase composition	Compound	Retention time (min)	Peak asymmetry factor	Resolution	k'
Acetonitrile-0.2 M KH$_2$PO$_4$ pH 3.0-H$_2$O (30:10:60, % v/v/v)	SBT/TBT	2.61	1.198	-	-
	PED	2.92	1.126	1.577	0.120
	AMB	4.68	1.239	8.322	0.794
	GFN	5.02	1.093	1.476	0.924
	CPM	6.29	1.588	5.214	1.411
	TPN	7.57	1.510	4.518	1.904
	BXN	16.19	1.811	19.718	5.212
Acetonitrile-0.2 M KH$_2$PO$_4$ pH 3.5-H$_2$O (30:10:60, % v/v/v)	SBT/TBT	2.60	1.195	-	-
	PED	2.92	1.073	1.560	0.121
	AMB	4.68	-	8.260	0.799
	GFN	4.99	-	1.362	0.919
	CPM	6.52	1.625	6.143	1.505
	TPN	7.89	1.516	4.606	2.030
	BXN	22.07	1.116	28.650	7.477
Acetonitrile-0.2 M KH$_2$PO$_4$ pH 4.0-H$_2$O (30:10:60, % v/v/v)	SBT/TBT	2.62	1.110	-	-
	PED	2.91	1.267	1.515	0.109
	AMB	4.84	-	6.617	0.846
	GFN	4.96	-	0.265	0.893
	CPM	6.67	1.672	4.283	1.545
	TPN	8.09	1.561	4.706	2.087
	BXN	42.83	0.663	35.929	15.346

Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.2 M KH$_2$PO$_4$-water (30:10:60, % v/v/v). TBT-terbutaline, SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, CPM-chlorpheniramine, AMB-ambroxol, TPN-triprolidine, BXN-bromhexine.

2.5.3.3. Effect of organic buffers on separation

The organic buffers investigated were sodium acetate (CH_3COONa) and ammonium acetate (CH_3COONH_4) at an effective concentration of 20 mM and pH 3.0.

2.5.3.3.1. Effect of sodium acetate

A mobile phase consisting of acetonitrile-0.2 M CH_3COONa pH 3.0-water (30:10:60) was prepared for purposes of this investigation. The pH of sodium acetate solution was adjusted with 0.2 M acetic acid.

With the exception of GFN, the retention times of all the peaks decreased significantly compared to those obtained with phosphate buffer with the BXN peak exhibiting the most profound effect in retention time which decreased from 16.19 to 6.86 minutes giving a total run time of about 12 minutes. There was change in elution order whereby CPM and TPN eluted before GFN and MAL eluted last at 9.56 minutes. The solvent peak, SBT and TBT co-eluted at 2.12 minutes while there was poor separation between SBT/PED, PED/AMB and CPM/TPN peak pairs. The separated peaks had adequate symmetry (A_s = 1.1 to 1.3) except BXN which exhibited fronting (A_s = 0.733).

2.5.3.3.2. Effect of ammonium acetate

A mobile phase consisting of acetonitrile-0.2 M CH_3COONH_4 pH 3.0-water (30:10:60) was investigated where the pH of ammonium acetate solution was adjusted with 0.2 M acetic acid.

The overall effect was similar to that of sodium acetate except reduction in the retention times of most components and the overall run time. Figure 2.5 is a typical chromatogram obtained with ammonium acetate mobile phase pH 3.0.

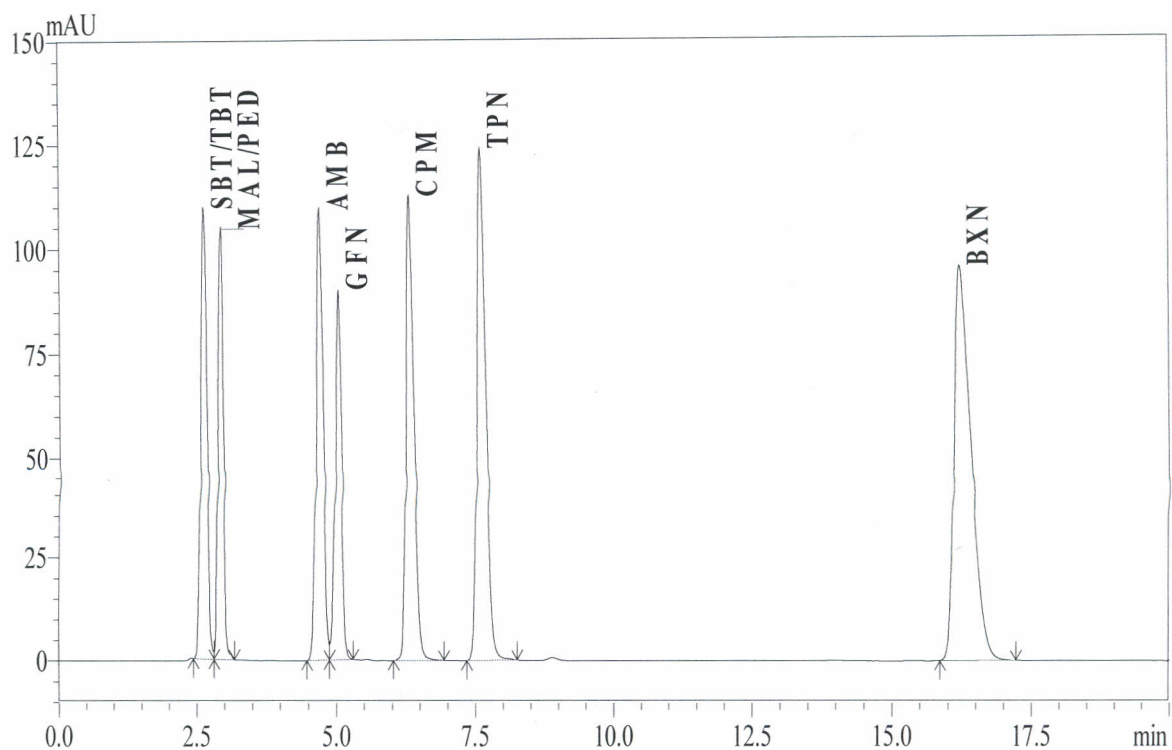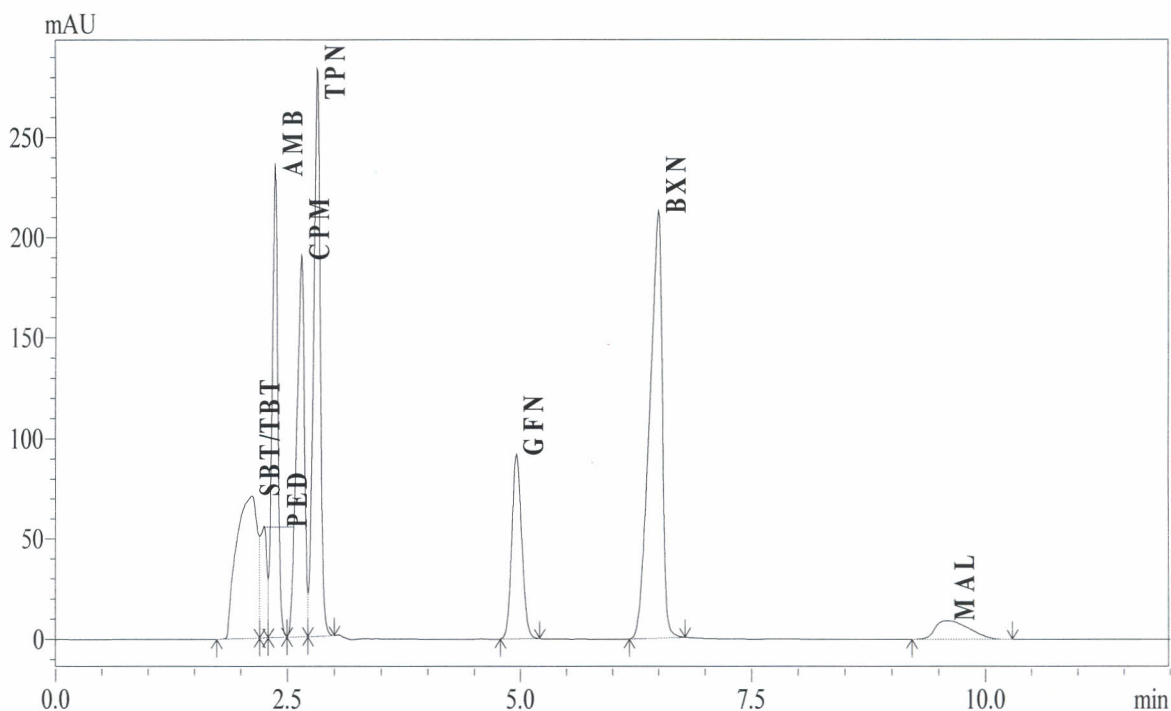**Figure 2.5: Typical chromatogram of the reference working solution obtained using ammonium acetate buffer pH 3.0. Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.2 M CH$_3$COONH$_4$ pH 3.0-water (30:10:60, % v/v/v). SBT-salbutamol, TBT-terbutaline, MAL-maleic acid, PED-pseudoephedrine, GFN-guaifenesin, CPM-chlorpheniramine, AMB-ambroxol, TPN-triprolidine, BXN-bromhexine.**

2.5.3.4.   Effect of ion-pairing agents

An ion-pairing agent is a large ionic molecule that carries a charge opposite to that of the analyte of interest. It usually has both a hydrophobic region to interact with the stationary phase and a charged region to interact with the analyte. The ion-pairing agent aligns itself on the surface of the stationary phase with the hydrophobic region interacting with the stationary phase while the charged region interacts with compounds that are oppositely charged. They are advantageous in separation of multi-component mixture in that they offer reduced separation times, sharper peak shapes, simultaneous separation of ionized and non-ionized analytes in one run and allow use of a wide range of additives to improve on separation.

44

With the exception of guaifenesin, all the compounds under study are basic and at pH 3.0, they were all protonated thus conferring a positive charge to them. To further improve the separation of the compounds, ion-pairing agents with negative charge were chosen as they would enhance the retention of these basic compounds. The ion-pairing agents chosen were the alkylsulphonic acid sodium salts that have the sulphonate portion as the negatively charged region and the alkyl portion as the hydrophobic region. In the present study, the effect of the ion-pairing agents was aimed at increasing the retention times of the compounds, hence improve separation. This supported the development of a single, isocratic LC method with relatively short analysis time. From the foregoing, none of the mobile phases produced complete separation of the compounds under study. The mobile phase consisting of acetonitrile-0.2 M CH_3COONH_4 pH 3.0-water (30:10:60, % v/v/v) was selected for incorporation of ion-pairing agents as it gave the shortest analysis time.

Hexanesulphonic acid sodium salt is the most commonly used anionic ion-pairing agent and hence readily available in the Kenyan market. A mobile phase consisting of acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH_3COONH_4 pH 3.0-water (30:4:10:56, % v/v/v/v) was prepared producing an effective concentration of 10 mM sodium hexanesulphonate.

In the chromatogram obtained (figure 2.6), all the peaks, except SBT/TBT peak pair, separated with resolution factors of > 4.0. The co-elution of the solvent peak and MAL was considered inconsequential because MAL was not under determination. Salbutamol (SBT) and terbutaline (TBT) are not formulated in the same drug product due to their similar pharmacological properties and thus it was not considered necessary to separate the peaks on the same chromatogram. In subsequent experiments, the two compounds were incorporated into the

reference working solution separately but for uniformity, only the SBT peak is shown in the figures presented.
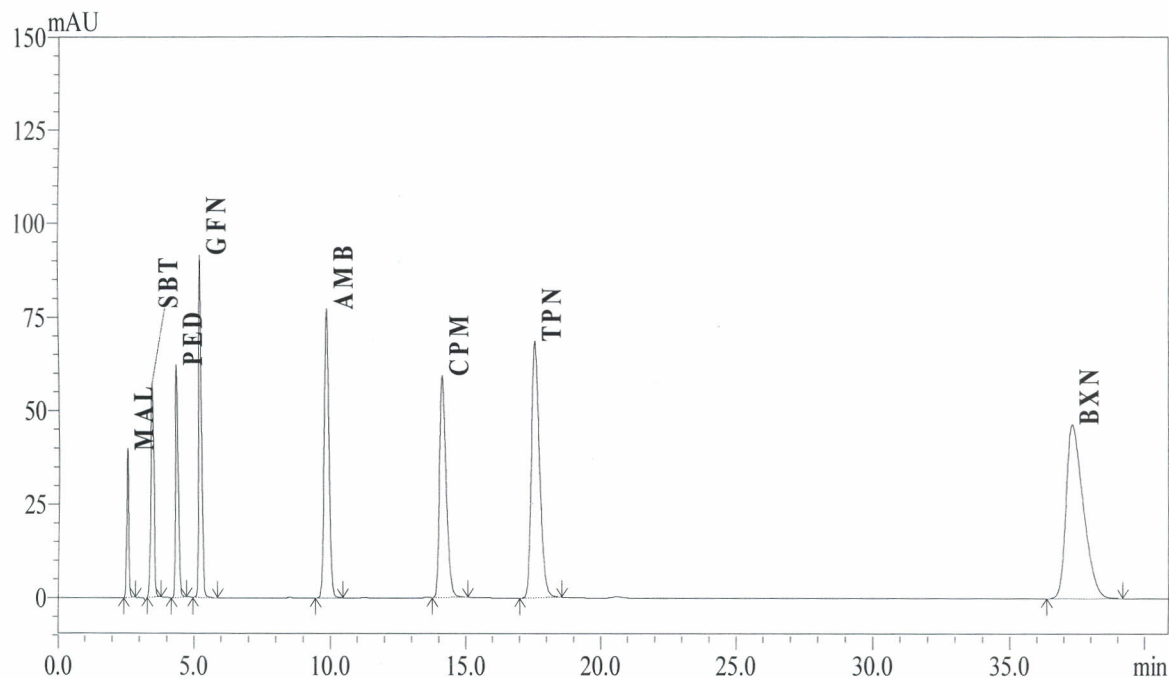
Figure 2.6: Typical chromatogram of the reference working solution obtained using 0.01 M sodium hexanesulphonate and acetonitrile (30 % v/v). Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (30:4:10:56, % v/v/v/v). MAL-maleic acid, SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine.

The retention times for all the peaks increased with BXN peak eluting last at 37.30 minutes. All the peaks exhibited improved shapes and symmetry with the highest asymmetry factor being 1.45 for BXN.

In order to develop a single isocratic LC method with a total run time of less than 30 minutes, the acetonitrile concentration was increased to 35 % v/v. All the peaks were adequately resolved with resolution factors for the peaks being greater than 2.5 (figure 2.7) with a concomitant reduction in the retention times of all the compounds. The BXN peak eluted at 18.28 minutes and hence was the most affected by the increase in acetonitrile concentration. The elution order

remained the same while the symmetry of the peaks was adequate with asymmetry factors ranging from 1.1 (SBT) to 1.66 (BXN).
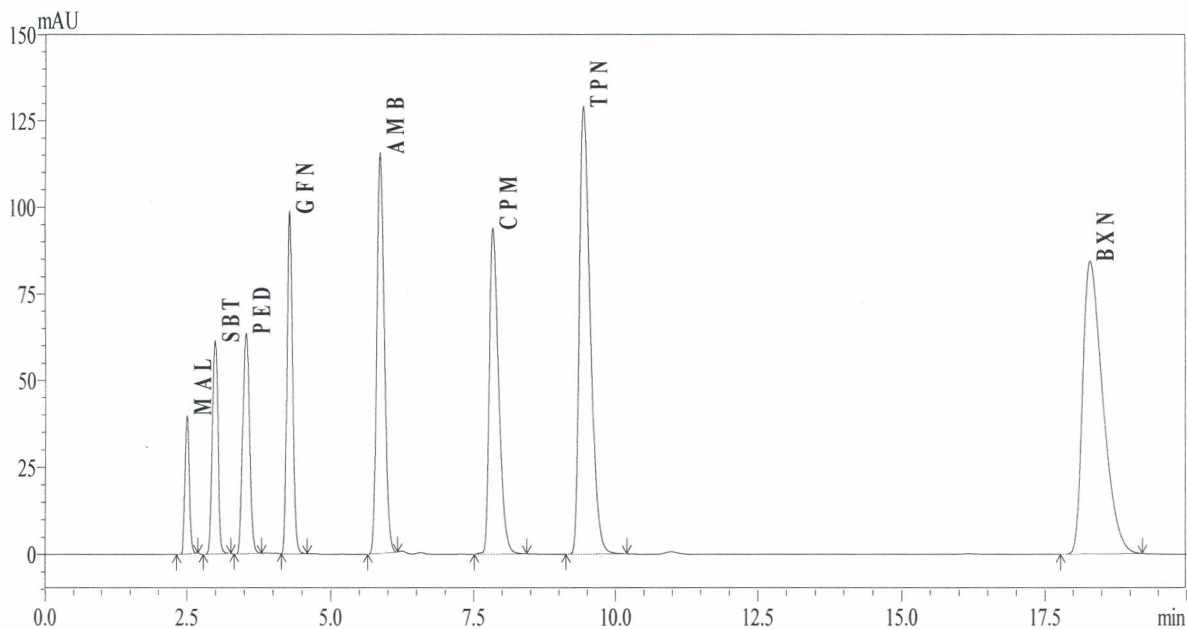
Figure 2.7: Chromatogram of the reference working solution obtained using 0.01 M sodium hexanesulphonate and acetonitrile (35 % v/v). Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:10:51, % v/v/v/v). MAL-maleic acid, SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine.

The influence of other alkylsulphonates was studied under the same conditions, each at an effective concentration of 10 mM. For this purpose, sodium butanesulphonate, sodium pentanesulphonate, sodium hexanesulphonate and sodium heptanesulphonate were used. Their effect on retention time, asymmetry factor, resolution and capacity factor of the compounds under study is shown on table 2.5 while figure 2.8 shows the effect of these alkylsulphonates on capacity factors of the compounds under study.

Table 2.5: The effect of ion pairing agents on the retention time, asymmetry factor, resolution and capacity factor of the compounds under study.

Ion pairing agent (0.01 M)	Compound	Retention time (min)	Peak asymmetry factor	Resolution	k'
Sodium butanesulphonate	SBT	2.682	0.956	-	-
	PED	2.970	1.687	1.647	0.108
	GFN	4.263	-	6.802	0.590
	AMB	4.457	-	0.908	0.662
	CPM	5.780	1.553	5.377	1.155
	TPN	6.820	1.524	3.747	1.543
	BXN	13.165	1.657	15.599	3.909
Sodium pentanesulphonate	SBT	2.814	1.309	1.200	0.066
	PED	3.239	1.079	1.933	0.226
	GFN	4.290	1.273	4.562	0.624
	AMB	5.129	1.300	3.820	0.942
	CPM	6.772	1.551	6.162	1.564
	TPN	8.081	1.524	4.222	2.060
	BXN	15.778	1.654	16.455	4.974
Sodium hexanesulphonate	SBT	2.980	1.134	2.813	0.196
	PED	3.520	1.203	2.597	0.413
	GFN	4.279	1.267	3.661	0.717
	AMB	5.857	1.274	7.194	1.351
	CPM	7.831	1.570	7.086	2.143
	TPN	9.428	1.582	4.798	2.784
	BXN	18.284	1.663	17.407	6.338
Sodium heptanesulphonate	SBT	3.406	0.771	3.935	0.438
	PED	4.253	0.984	2.647	0.796
	GFN	4.253	0.984	2.647	0.796
	AMB	7.922	1.272	12.923	2.344
	CPM	10.696	1.560	7.805	3.515
	TPN	13.046	1.503	5.412	4.508
	BXN	26.094	1.586	18.606	10.016

Column: Phenomenex Gemini® 5μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium alkylsulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:10:51, % v/v/v). SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine.
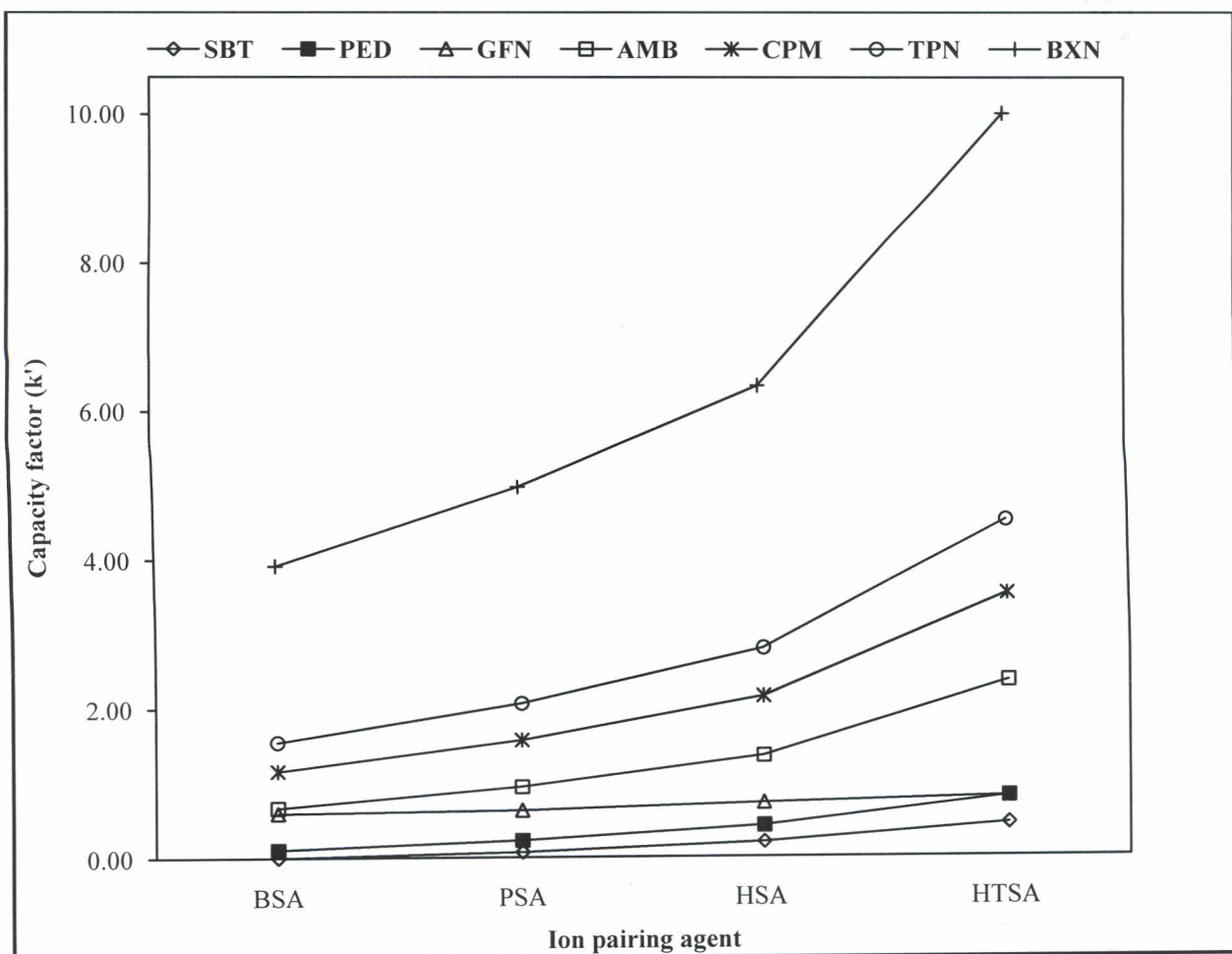
Figure 2.8: Effect of ion pairing agents on the capacity factors of the compounds under study. Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium alkylsulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:10:51, % v/v/v/v). SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine. BSA-sodium butanesulphonate, PSA-sodium pentanesulphonate, HSA-sodium hexanesulphonate, HTSA-sodium heptanesulphonate.

As the alkyl chain length of the ion pairing agent increased, the retention times and capacity factors of all the components except GFN increased and their asymmetry factors reduced. The chromatograms produced using the other sodium alkylsulphonates were compared to that produced using sodium hexanesulphonate. With sodium butanesulphonate, the retention times of all the components except GFN were reduced with BXN eluting last at 13.17 minutes. The peaks for SBT/TBT, MAL and the solvent peak co-eluted at 2.68 minutes and this group of peaks and

49

that of PED were not baseline separated. The resolution between GFN and AMB was poor with a resolution of 0.908 and it was thus not possible to calculate the asymmetry factor of each component. The asymmetry factors for BXN, TPN and CPM were not affected.

Sodium pentanesulphonate produced separation between SBT/TBT and MAL but this was not baseline ($R_s = 1.2$). Maleic acid (MAL) co-eluted with the solvent peak. The retention times for all the components except GFN were reduced with BXN eluting last at 15.78 minutes while peak symmetry was generally unchanged. The major drawback was the poor resolution between the fast eluting peaks of MAL, SBT/TBT and PED whose resolution factors were less than 2.0.

Sodium heptanesulphonate produced adequate separation with resolution > 2.5 for all the components except GFN and PED that co-eluted and produced a single peak at 4.25 minutes. Except for GFN, the retention times for all the compounds increased, with BXN peak eluting last at 26.09 minutes. The elution order remained the same while the symmetry of the peaks was generally improved except that of SBT that showed fronting ($A_s = 0.771$).

The effect of the four sodium alkylsulphonates studied as ion-pairing agents on the capacity factors of the compounds is shown in figure 2.8. The figure exhibits the degree of separation between the compounds under study when different ion-pairing agents are used and indicates GFN was generally unaffected by change in ion-pairing agent.

From the results obtained, sodium hexanesulphonate was selected as the ion-pairing agent of choice because it offered better resolution between the peaks. In all the experiments, terbutaline produced results similar to salbutamol with respect to retention time, capacity factor, symmetry and resolution.

2.5.4. Effect of sodium hexanesulphonate concentration

The effect of sodium hexanesulphonate concentration was investigated at concentrations of 0.001, 0.005 and 0.01 M which are commonly used in laboratory applications. A stock solution of 0.25 M was prepared and diluted in buffer solution and organic modifier to produce the desired concentration of sodium hexanesulphonate. Table 2.6 shows the effect of concentration of sodium hexanesulphonate on the retention time, asymmetry factor, resolution and capacity factor while figure 2.9 illustrates the effect on the capacity factors of the compounds under study.

Table 2.6: The effect of sodium hexanesulphonate concentration on the retention time, asymmetry factor, resolution and capacity factor of the compounds under study.

Ion pairing agent concentration (M)	Compound	Retention time (minutes)	Peak asymmetry factor	Resolution	k'
0.001	SBT	2.224	-	-	0.000
	PED	2.604	-	1.516	0.171
	GFN	4.305	-	1.140	0.935
	AMB	3.096	1.776	2.587	0.392
	CPM	3.909	0.728	3.150	0.758
	TPN	4.464	-	0.485	1.007
	BXN	9.111	1.352	13.289	3.096
0.005	SBT	2.687	1.289	-	0.000
	PED	3.225	0.829	1.993	0.200
	GFN	4.251	1.275	3.749	0.582
	AMB	5.019	1.308	3.448	0.868
	CPM	6.572	1.578	5.648	1.446
	TPN	7.809	1.572	3.885	1.906
	BXN	15.292	1.718	15.444	4.690
0.01	SBT	2.980	1.134	2.813	0.196
	PED	3.520	1.203	2.597	0.413
	GFN	4.279	1.267	3.661	0.717
	AMB	5.857	1.274	7.194	1.351
	CPM	7.831	1.570	7.086	2.143
	TPN	9.428	1.582	4.798	2.784

Ion pairing agent concentration (M)	Compound	Retention time (minutes)	Peak asymmetry factor	Resolution	k'
	BXN	18.284	1.663	17.407	6.338

Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:X:10:55-X, % v/v/v/v). SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine. X-% volume of ion pairing agent to make the desired concentration.

At a concentration of 0.001 M sodium hexanesulphonate, there was reduction in overall run time where the last eluting peak was BXN at 9.11 minutes. Except GFN, all the other compounds had their retention times reduced while the solvent peak co-eluted with SBT and MAL. There was a change in elution order whereby GFN eluted after AMB and CPM. The resolution between PED/SBT and GFN/TPN peak pairs was also unsatisfactory with R$_s$ of 1.516 and 0.485 respectively. Except BXN peak, all other peaks depicted poor shape and symmetry.

At a concentration of 0.005 M sodium hexanesulphonate, adequate resolution between GFN, AMB, CPM, TPN and BXN was achieved where resolution ranged from 3.749 to 15.444. The major drawbacks observed were the co-elution of SBT, MAL and the solvent peak and inadequate resolution between PED and SBT/MAL as the resolution was 1.993. Generally, there was reduction in retention times of all the compounds except GFN. Shorter overall run time was recorded with BXN peak eluting last at 15.25 minutes. Thus, the concentration of 0.01 M sodium hexanesulphonate was selected as the optimum ion pairing agent concentration as it offered adequate resolution and run time.
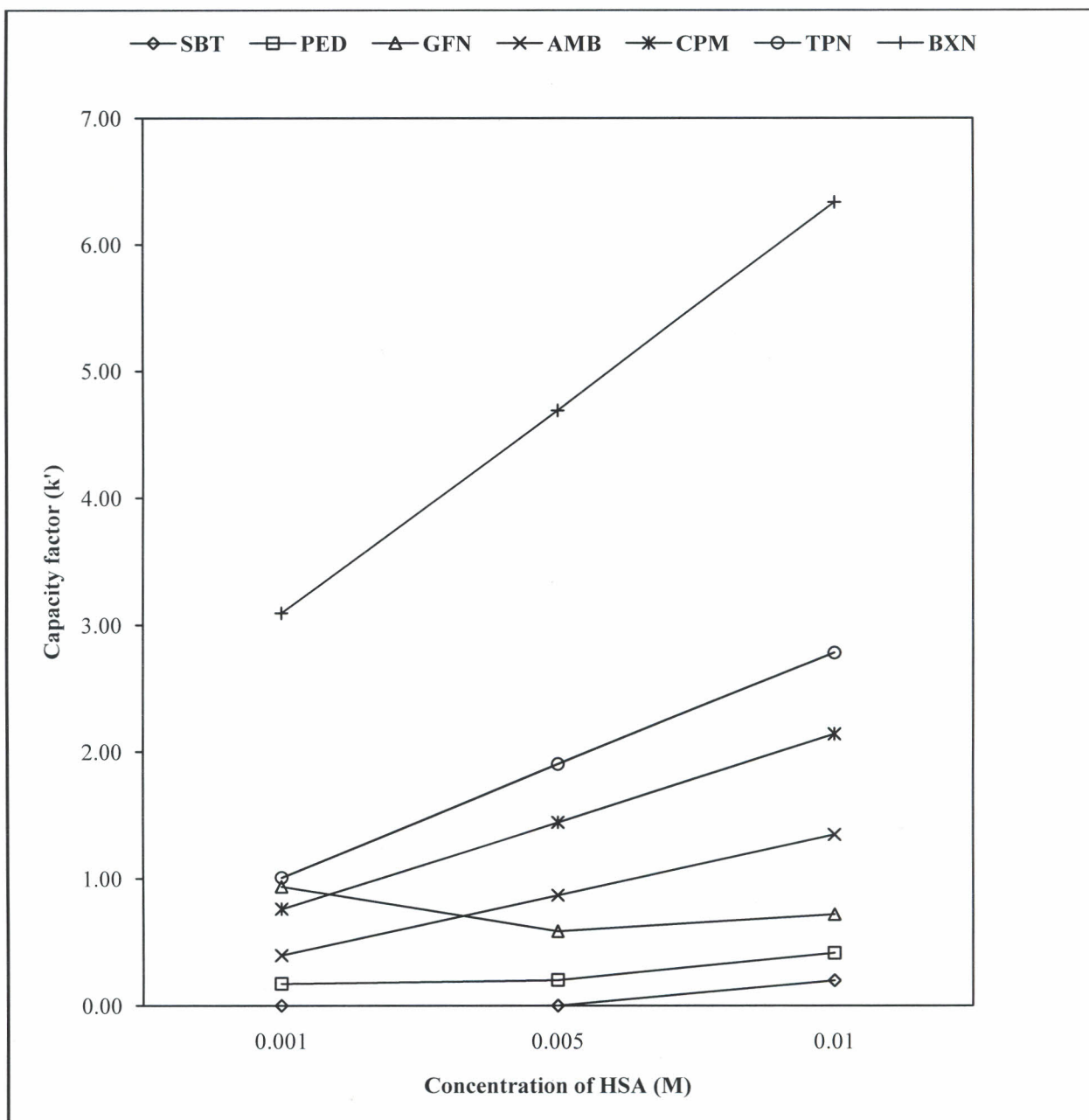
Figure 2.9: Effect of sodium hexanesulphonate concentration on the capacity factors of the compounds under study. Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:X:10:55-X, % v/v/v/v). SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine. HSA-sodium hexanesulphonate, X-% volume of ion pairing agent to make the desired concentration.

2.5.5. Effect of ammonium acetate concentration

This was investigated at 20 mM, 40 mM and 60 mM. A stock solution of 0.2 M ammonium acetate was prepared and diluted to the required concentration. Table 2.7 shows the effect of concentration of ammonium acetate on retention time, asymmetry factor, resolution and capacity factor of the compounds under study.

Table 2.7: The effect of ammonium acetate concentration on the retention time, asymmetry factor, resolution and capacity factor of the compounds under study.

Ammonium acetate concentration (M)	Compound	Retention time (minutes)	Peak asymmetry factor	Resolution	k'
	SBT	2.980	1.134	2.813	0.196
	PED	3.520	1.203	2.597	0.413
	GFN	4.279	1.267	3.661	0.717
0.02	AMB	5.857	1.274	7.194	1.351
	CPM	7.831	1.570	7.086	2.143
	TPN	9.428	1.582	4.798	2.784
	BXN	18.284	1.663	17.407	6.338
	SBT	3.035	1.225	4.125	0.233
	PED	3.642	1.191	3.201	0.479
	GFN	4.284	1.248	2.921	0.741
0.04	AMB	6.236	1.248	8.476	1.534
	CPM	8.348	1.552	7.162	2.392
	TPN	10.116	1.628	5.019	3.110
	BXN	19.512	1.690	17.463	6.927
	SBT	3.051	1.167	4.140	0.232
	PED	3.658	1.158	3.111	0.477
	GFN	4.339	1.245	2.976	0.752
0.06	AMB	6.387	1.240	8.704	1.579
	CPM	8.503	1.524	7.031	2.434
	TPN	10.286	1.600	4.992	3.154
	BXN	19.980	1.662	17.719	7.069

Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:X:61-X, % v/v/v/v). SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine. X-% volume of buffer to make the desired concentration.

Variation in ammonium acetate concentration did not significantly change retention times, resolution and asymmetry factors of the compounds under study. Considering the total analysis time and quality of separation, 20 mM was selected as the optimum ammonium acetate buffer concentration.

2.5.6. Effect of acetonitrile concentration

The effects of acetonitrile on the retention time, asymmetry factor, resolution and capacity factors of the compounds under study were investigated at concentrations of 30, 35 and 40 % v/v (Table 2.8). Figure 2.10 shows the effect of acetonitrile concentration on capacity factors of the compounds under study.

At 30 % v/v acetonitrile, the retention times and capacity factors of all the compounds increased significantly and the total run-time doubled to 40 minutes. The resolution among the peaks was greatly improved ($R_s > 4.2$) with baseline separation of all the peaks. The symmetry of all the peaks was adequate with asymmetry factors of 0.99-1.45. The major drawback with working at 30 % acetonitrile was the long analysis time.

At 40 % v/v acetonitrile, the retention times and capacity factors of all the compounds were reduced giving a total run-time of about 13 minutes with the last peak (BXN) eluting at 10.85 minutes. However, there was reduced resolution between SBT/TBT, MAL and PED whereby baseline separation was not achieved. There was also a reversal of elution order between MAL and SBT with SBT and the solvent peak co-eluting.

Consequently, 35 % v/v acetonitrile was taken as optimum in the mobile phase because it offered the shortest analysis time whereby all the compounds were baseline separated with $R_s > 2.5$.

Table 2.8: The effect acetonitrile concentration (% v/v) on the retention time, asymmetry factor, resolution and capacity factor of the compounds under study.

Acetonitrile concentration (% v/v)	Compound	Retention time (minutes)	Peak asymmetry factor	Resolution	k'
	SBT	3.463	0.994	5.014	0.365
	PED	4.360	1.312	4.297	0.718
	GFN	5.233	1.125	4.423	0.063
30	AMB	9.869	1.105	17.602	2.890
	CPM	14.129	1.443	10.871	4.569
	TPN	17.554	1.381	6.779	5.919
	BXN	37.323	1.450	22.761	13.712
	SBT	2.980	1.134	2.813	0.196
	PED	3.520	1.203	2.597	0.413
	GFN	4.279	1.267	3.661	0.717
35	AMB	5.857	1.274	7.194	1.351
	CPM	7.831	1.570	7.086	2.143
	TPN	9.428	1.582	4.798	2.784
	BXN	18.284	1.663	17.407	6.338
	SBT	2.627	-	0.787	0.044
	PED	3.118	1.024	1.337	0.239
	GFN	3.832	1.181	3.613	0.523
40	AMB	4.254	1.171	2.192	0.691
	CPM	5.285	1.300	4.766	1.100
	TPN	6.086	1.288	3.349	1.419
	BXN	10.848	1.271	14.973	3.312

Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 ºC. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (X:4:10:86-X, % v/v/v/v). SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine. X-% volume of acetonitrile to make the desired concentration.

Figure 2.10: Effect of acetonitrile concentration on the capacity factors of the compounds under study. Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (X:4:10:86-X, % v/v/v/v). SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine. X-% volume of acetonitrile to make the desired concentration.
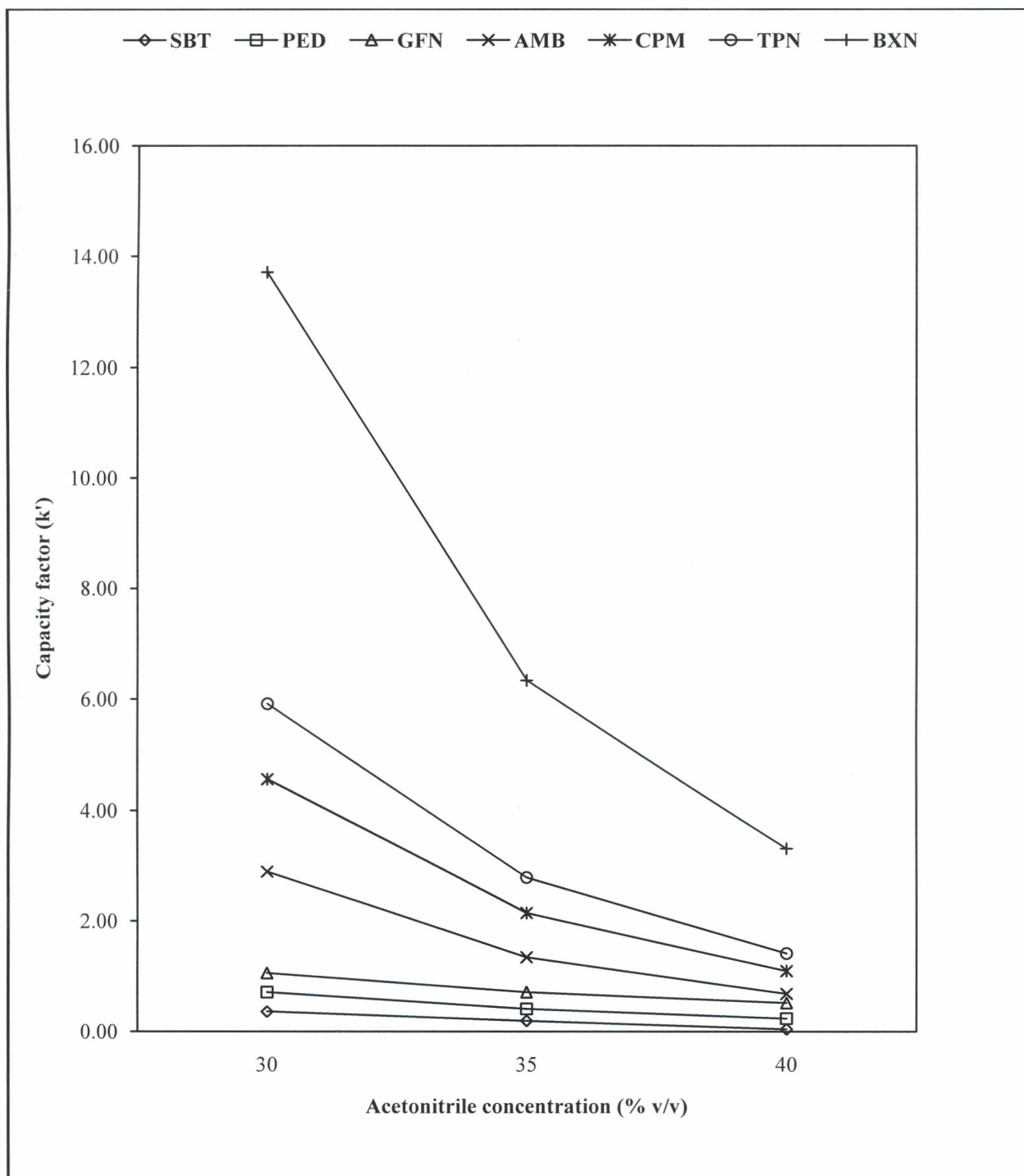
2.5.7. Effect of buffer pH

The effect of ammonium acetate buffer pH was investigated at pH 2.5, 3.0 and 3.5. Buffer of pH 2.5 was prepared using 0.2 M acetic acid without adding ammonium acetate. The effects on retention time, asymmetry factor, resolution and capacity factor at pH 2.5 and 3.5 were compared to those of pH 3.0. Generally there were slight changes in the chromatographic parameters of all the compounds except for BXN that exhibited profound effect of buffer pH variation, especially on its retention time and capacity factor. The retention time of BXN was 17.14, 18.28 and 21.85 minutes at pH 2.5, 3.0 and 3.5 respectively. However, at pH 2.5, the resolution between SBT and MAL was 2.3. Increased retention time of BXN at pH 3.5 was the major drawback because increased analysis time was undesirable. The effect of buffer pH on retention time, asymmetry factor, resolution and capacity factor of the compounds under study is shown in table 2.9 while figure 2.11 shows the effect of buffer pH on capacity factors of the compounds under study. Buffer pH 3.0 was selected as the optimum because it offered the best combination of total run time and separation of the compounds under study.

Table 2.9: The effect of ammonium acetate buffer pH on the retention time, asymmetry factor, resolution and capacity factor of the compounds under study.

Ammonium acetate buffer pH	Compound	Retention time (minutes)	Peak asymmetry factor	Resolution	k'
	SBT	2.959	1.097	2.331	0.178
	PED	3.492	1.160	2.423	0.389
	GFN	4.284	1.267	3.808	0.705
2.5	AMB	5.783	1.268	6.845	1.301
	CPM	7.471	1.519	6.223	1.973
	TPN	8.949	1.545	4.647	2.561
	BXN	17.140	1.696	16.878	5.821
3.0	SBT	2.980	1.134	2.813	0.196
	PED	3.520	1.203	2.597	0.413

58

Ammonium acetate buffer pH	Compound	Retention time (minutes)	Peak asymmetry factor	Resolution	k'
	GFN	4.279	1.267	3.661	0.717
	AMB	5.857	1.274	7.194	1.351
	CPM	7.831	1.570	7.086	2.143
	TPN	9.428	1.582	4.798	2.784
	BXN	18.284	1.663	17.407	6.338
	SBT	2.979	1.060	2.620	0.197
	PED	3.519	1.176	2.517	0.414
	GFN	4.281	1.281	3.723	0.720
3.5	AMB	5.876	1.282	7.279	1.361
	CPM	7.992	1.592	7.542	2.211
	TPN	9.631	1.604	4.822	2.870
	BXN	21.850	1.510	21.913	7.779

Column: Phenomenex Gemini® 5 μ C_{18}, 250 × 4.6 mm ID. Column temperature: 40 ºC. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH_3COONH_4-water (35:4:10:51, % v/v/v/v). SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine.

Figure 2.11: Effect of buffer pH on the capacity factors of the compounds under study. Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$-water (35:4:10:51, % v/v/v/v). SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine.
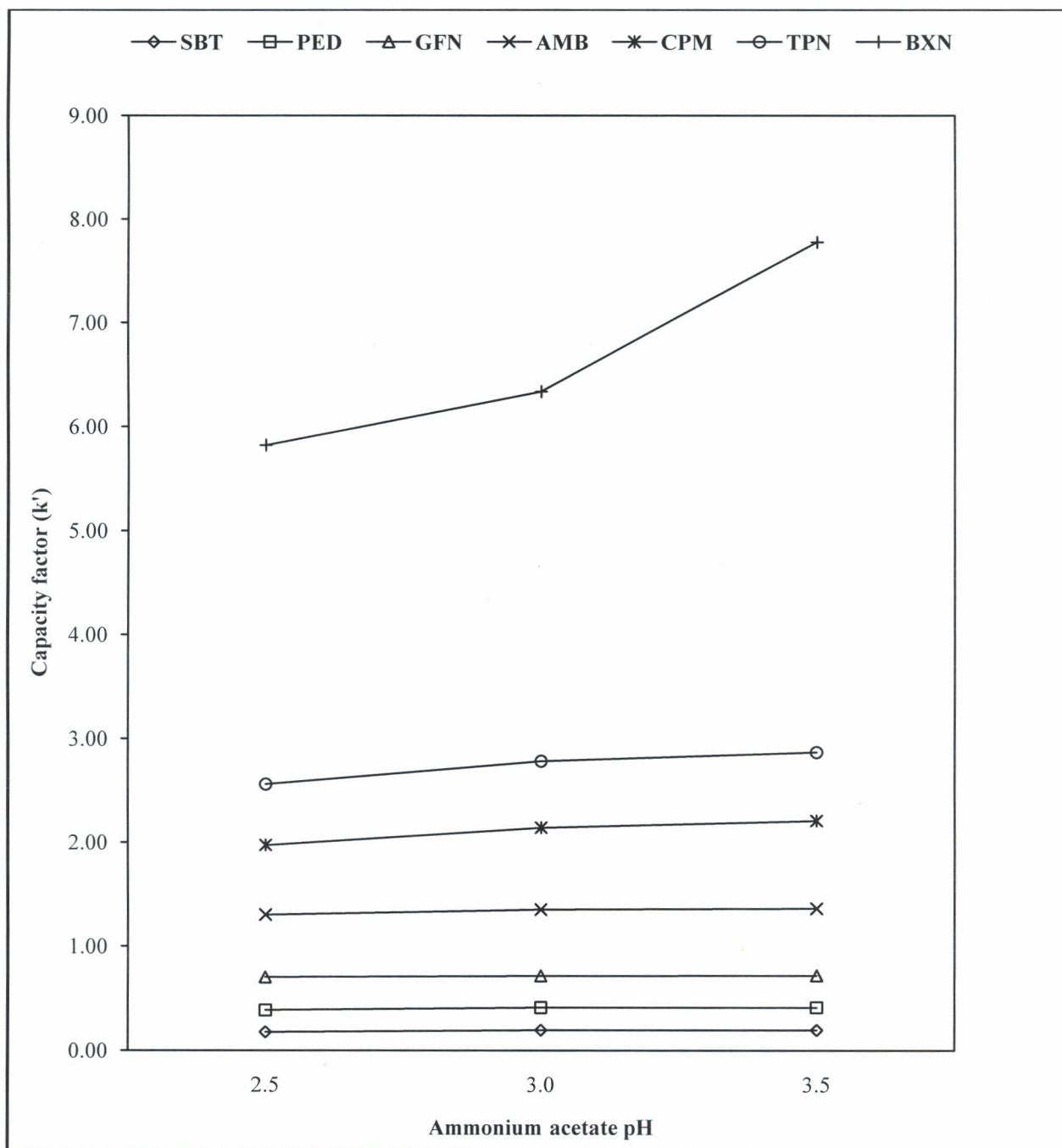
2.5.8. Effect of column temperature

Column temperature was varied from 30 to 50 °C at intervals of 5 °C and the effects on retention time, asymmetry, resolution and capacity factor recorded (table 2.10 and figure 2.12). Increase in column temperature from 30 to 50 °C reduced retention times and capacity factors of all the compounds. The symmetry and resolution were slightly affected while the column back pressures reduced with increase in temperature due to decrease in viscosity of the mobile phase. A temperature of 40 °C was selected as the optimum since it struck a balance between reasonable run time, resolution of the peaks and the stability of the column.

Table 2.10: The effect of column temperature on the retention time, asymmetry factor, resolution and capacity factor of the compounds under study.

Temperature (°C)	Drug	Retention time (minutes)	Peak asymmetry factor	Resolution	k'
	SBT	3.099	1.225	3.125	0.234
	PED	3.692	1.191	3.206	0.478
	GFN	4.584	1.248	2.970	0.742
30	AMB	6.244	1.249	8.654	1.384
	CPM	8.454	1.553	7.167	2.134
	TPN	10.116	1.626	5.029	2.707
	BXN	19.112	1.691	17.467	6.453
	SBT	3.093	1.156	2.399	0.165
	PED	3.656	0.981	2.376	0.337
	GFN	4.507	1.282	3.531	0.698
35	AMB	6.062	1.292	6.609	1.284
	CPM	7.966	1.554	6.486	2.001
	TPN	9.547	1.535	4.602	2.597
	BXN	18.837	1.662	17.640	6.097
	SBT	3.084	1.173	2.451	0.164
	PED	3.637	1.009	2.393	0.373
40	GFN	4.481	1.281	3.525	0.691
	AMB	5.960	1.282	6.333	1.250
	CPM	7.881	1.537	6.622	1.975

Temperature (°C)	Drug	Retention time (minutes)	Peak asymmetry factor	Resolution	k'
	TPN	9.421	1.516	4.554	2.556
	BXN	18.324	1.622	17.509	5.917
45	SBT	3.079	1.193	2.488	0.163
	PED	3.634	1.014	2.504	0.372
	GFN	4.474	1.291	3.590	0.689
	AMB	5.925	1.297	6.276	1.237
	CPM	7.880	1.559	6.793	1.975
	TPN	9.396	1.533	4.503	2.548
	BXN	18.133	1.617	17.400	5.847
50	SBT	3.001	1.199	2.356	0.164
	PED	3.599	1.065	2.499	0.350
	GFN	4.324	1.287	3.586	0.679
	AMB	4.876	1.254	6.234	1.211
	CPM	7.753	1.534	6.757	1.891
	TPN	9.298	1.521	4.459	2.469
	BXN	17.548	1.609	17.128	5.766

Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:10:51, % v/v/v/v). SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine.
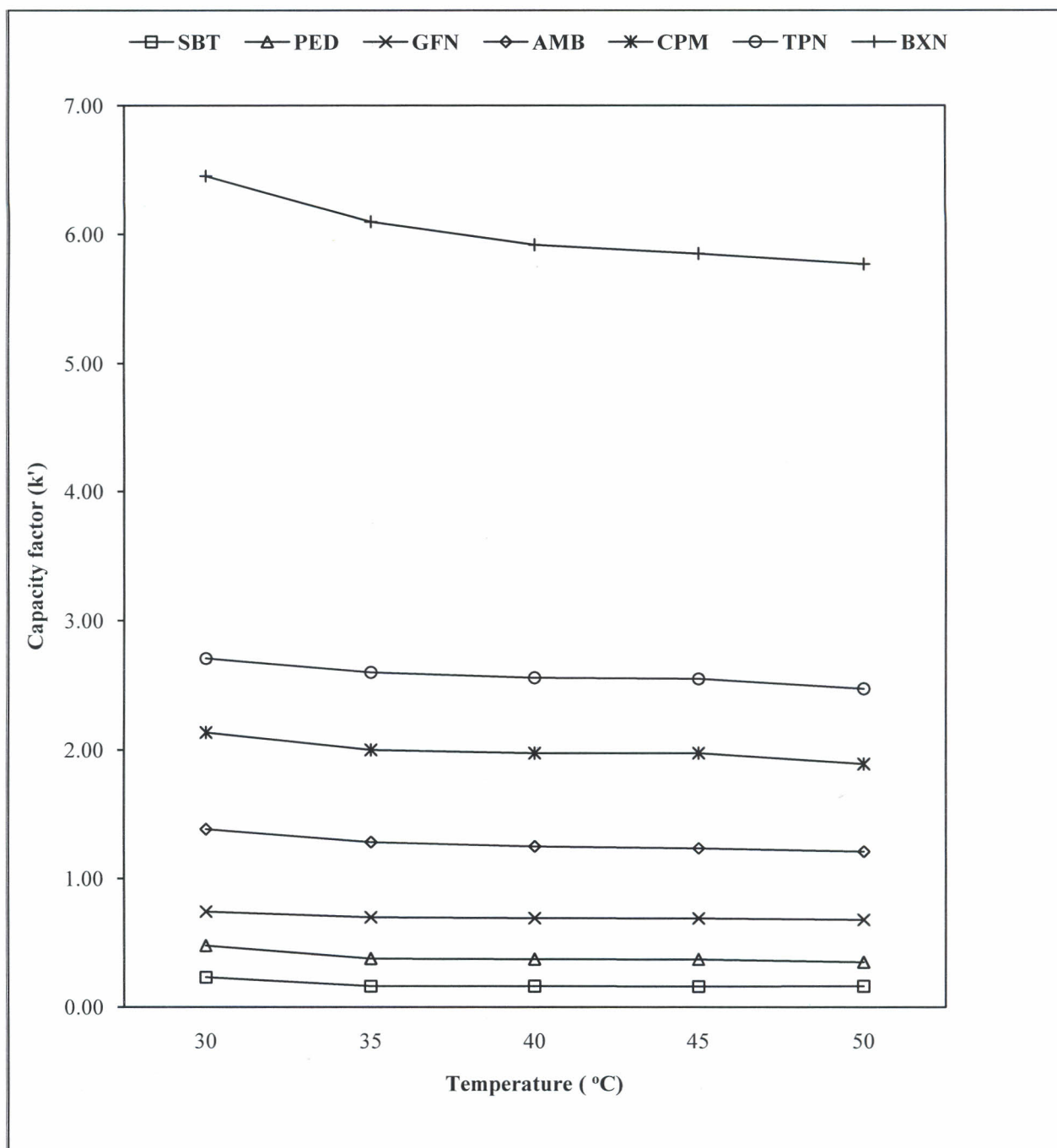
Figure 2.12: Effect of column temperature on the capacity factors of the compounds under study. Column: Phenomenex Gemini® 5 μ C$_{18,}$ 250 × 4.6 mm ID. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:10:51, % v/v/v/v). SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine.

2.5.9. Optimized chromatographic conditions

Following the investigation of the influence of the various factors on separation, the following were established as the optimized chromatographic conditions:

A mobile phase consisting of acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH_3COONH_4 pH 3.0-water (35:4:10:51, % v/v/v/v) delivered at a flow rate of 1.0 mL/minute; Octadecylsilane (C_{18}) with particle size 5 μm and pore size of 110 Å as the stationary phase packed in a column measuring 250 mm in length by 4.6 mm internal diameter (Phenomenex Gemini® C_{18}) and maintained at a temperature of 40 °C; The UV detector was set at a wavelength of 254 nm. Figure 2.13 is a typical chromatogram obtained under these conditions.
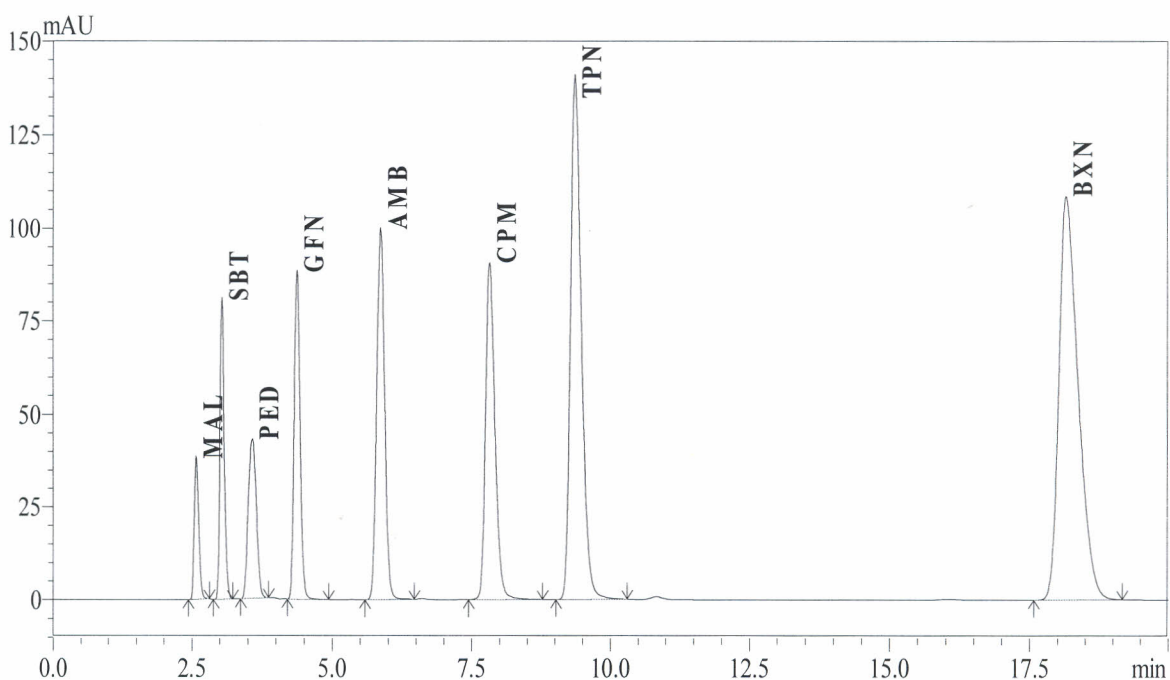

Figure 2.13: Typical chromatogram of the reference working solution obtained under the optimized chromatographic conditions. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH_3COONH_4 pH 3.0-water (35:4:10:51, % v/v/v/v). MAL-maleic acid, SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine.

2.5.10. Interference of preservatives

The developed LC method was meant to be used for analysis of the compounds under study in cough-cold syrups. Most manufacturers incorporate methylparaben or propylparaben as preservatives among other excipients and these may interfere with some of the compounds during analysis. Methylparaben and propylparaben were added to the reference working standard at concentration of 0.005 and 0.05 mg/mL respectively and run under the optimized chromatographic conditions to test the method's ability to separate the compounds under study in the presence of methyl- and propylparabens. All the ten compounds had baseline separation as shown in figure 2.14 and hence it was possible to analyse the compounds under study in the presence of methyl- and propylparabens.
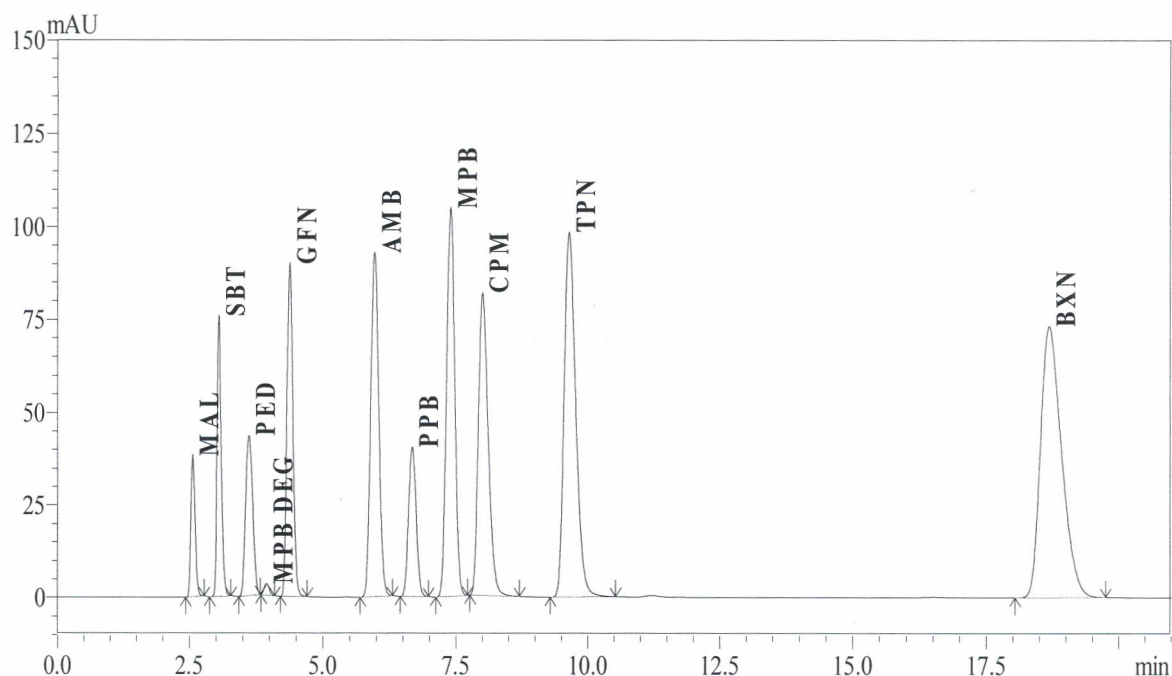

Figure 2.14: Typical chromatogram of the reference working solution spiked with methylparaben and propylparaben obtained under the optimized chromatographic conditions. Column: Phenomenex Gemini® 5μ C$_{18}$ 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:10:51, % v/v/v/v). MAL-maleic acid, SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine, PPB-propylparaben, MPB-methylparaben, MPB DEG-methylparaben degradation product.

CHAPTER THREE

METHOD VALIDATION

3.1   Introduction

Validation of an analytical method is the process by which it is established, by laboratory studies that the performance characteristics of the method meet the requirements for the intended analytical applications. According to International Conference on Harmonization (ICH) guidelines, the main objective of validation of an analytical method is to demonstrate that it is suitable for its intended purpose. The typical analytical characteristics used in method validation are accuracy, precision, limit of detection, limit of quantitation, linearity, range and robustness [36, 105].

In the present study, validation was carried out using a reference working solution with concentration of the compounds in the ratio at which they are formulated in the finished products and this was regarded as the 100 % sample. The concentrations used were 0.02 mg/mL (SBT), 0.025 mg/mL (TBT), 0.3 mg/mL (PED), 0.5 mg/mL (GFN), 0.3 mg/mL (AMB), 0.02 mg/mL (CPM), 0.0125 mg/mL (TPN) and 0.08 mg/mL (BXN).

3.2   Accuracy

The accuracy of an analytical method is the closeness of the test results obtained by that procedure to the true value. In the case of the assay of a drug substance, accuracy may be determined by application of the analytical procedure to an analyte of known purity or by comparison of the results of the procedure with those of a well characterized procedure, the accuracy of which has been stated or defined. In the case of the assay of a drug in a formulated product, accuracy may be determined by application of the analytical procedure to a placebo

mixture to which known quantities of the drug substances to be analysed have been added or adding known quantities of the analyte to the drug product or to compare the results obtained from a second, well characterized procedure, the accuracy of which is stated and/or defined. Accuracy is calculated as the percentage of recovery by the assay of the known added amount of the analyte in the sample. On the other hand, accuracy may also be inferred once precision, linearity and specificity have been established [36, 105].

The accuracy for the developed method was determined by spiking commercial products with working standards of the compounds under study. Several commercial samples were used since there was no single sample containing all the eight compounds under study. The standards were added to the samples at three different concentrations corresponding to 80, 100 and 120 % of the assay concentration and injected in triplicate. The standards were added at amounts that would increase the concentration of each compound in the sample by 20 %. The percentage recovery of each added working standard was regarded as the accuracy. Table 3.1 shows the percentage recoveries of the compounds under study.

Table 3.1: Percentage recoveries of the compounds under study.

Percentage recovery							
SBT	TBT	PED	GFN	AMB	CPM	TPN	BXN
99.9	100.5	99.2	99.8	98.5	99.7	99.9	99.2
(0.5)	(1.1)	(0.5)	(0.1)	(0.1)	(0.1)	(0.2)	(0.1)

SBT-salbutamol, TBT-terbutaline, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN- triprolidine, BXN-bromhexine. Values in parenthesis represent the RSD. n = 9.

The percentage recoveries of the compounds indicated adequate accuracy for the developed method.

3.3   Precision

The precision of an analytical method expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. Repeatability should be assessed using a minimum of 9 determinations covering the specified range for the procedure or a minimum of 6 determinations at 100% of the test concentration. Intermediate precision establishes the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts or equipment. Reproducibility is assessed by means of an inter-laboratory trial [36, 105].

In the present study, only repeatability and intermediate precision were assessed as reproducibilty was beyond the scope of the study. Repeatability or the intra-day precision was determined using the the percentage co-efficient of variation of six determinations at 100 % of the test concentration. The reference working solution was injected six times and the relative standard deviation (RSD) of the peak areas recorded as a measure of intra-day precision. The results were recorded in table 3.2. The RSD for the repeatability were < 2 % for all the eight compounds indicating the method had adequate repeatability.

Intermediate precision was assessed by use of inter-day variations on three consecutive days whereby fresh reference standard solutions and freshly prepared mobile phase were used. Six determinations were carried out on each day, the peak areas were normalised to the desired concentration for each compound and the RSD of the normalised peak areas taken as the measure of intermediate precision (Table 3.2).

Table 3.2: Repeatability and intermediate precision results for the compounds under study.

Compound	Repeatability peak areas RSD (n=6)	Intermediate precision peak areas RSD (n=18)
SBT	0.56	1.53
PED	0.15	1.09
GFN	0.25	0.45
AMB	0.16	0.59
CPM	0.23	0.82
TPN	0.28	0.63
BXN	0.28	0.72
TBT	0.41	0.75

SBT-salbutamol, TBT-terbutaline, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN- triprolidine, BXN-bromhexine.

The RSD for the intermediate precision were less than 2 % for all the eight compounds indicating the method had adequate precision.

3.4 Limit of detection

The limit of detection (LOD) of an individual analytical method is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. LOD is the concentration of an analyte that gives signal to noise ratio of 2 to 3 [105].

In the present study, the limit of detection was determined injecting standard solution of each compound under study in triplicate at different concentrations and calculating the signal to noise ratio. The concentration that gave signal to noise ratio of between 2 and 3 was considered the LOD and converted to ng. Table 3.3 shows the LOD for each compound under study.

Table 3.3: Limit of detection and quantitation for the compounds under study

Compound	Limit of detection (ng)	Limit of quantitation (ng)
SBT	1.28 (1.7)	4.00 (3.0)
PED	12.45 (1.4)	18.68 (3.2)
GFN	7.68 (3.9)	10.24 (1.5
AMB	1.29 (2.8)	3.22 (1.9)
CPM	3.27 (3.4)	17.45 (6.2)
TPN	31.03 (2.4)	51.72 (1.2)
BXN	1.67 (0.8)	3.34 (0.7)
TBT	10.08 (2.5)	25.21 (4.1)

SBT-salbutamol, TBT-terbutaline, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN- triprolidine, BXN-bromhexine. Values in parenthesis represent RSD.

3.5   Limit of quantitation

The limit of quantitation (LOQ) of an individual analytical method is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOQ is the amount of analyte that gives a signal to noise ratio of 10 [105].

In the present study, the same procedure as that of determination of LOD was applied in determination of LOQ and the concentration that gave a signal to noise ratio of 10 considered the LOQ and expressed as ng. The degree of precision considered adequate for LOQ determination from the peak areas of replicate injections (n=3) is RSD of less than 20 %. The results of LOQ and the degree of precision for each compound under study are shown in table 3.3.

Determination of LOQ is indicated for methods developed for determination of impurities and/or degradation products that are usually present in small quantities in the sample [105]. However, LOQ was determined for this method to give an indication of the amount of each compound which can be quantified with adequate precision and accuracy thereby facilitating the application

of the method in profiling of related substances and degradation products during stability studies. The LOQ values are low and thus the method can be applied in analysis of these compounds in biological samples.

3.6   Linearity and range

The linearity of an analytical method is its ability to obtain test results that are directly proportional to the concentration or amount of analyte in the sample within a given range. The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The ICH guidelines recommend the establishment of linearity at a minimum of five concentrations over the range of 80 to 120 % [105].

In the present study, a stock solution containing 0.04 mg/mL SBT, 0.05 mg/mL TBT, 0.6 mg/mL PED, 1.0 mg/mL GFN, 0.6 mg/mL AMB, 0.04 mg/mL CPM, 0.025 mg/mL TPN and 0.16 mg/mL BXN was prepared. This solution corresponded to 200 %. Terbutaline (TBT) was run separately due to co-elution with SBT. From the stock solution, serial dilutions were made to produce solutions with concentrations corresponding to 150, 120, 100, 80, 50 and 25 %. The solutions were analyzed at the optimum chromatographic conditions in triplicate and the corresponding peak areas recorded. Linearity plots of the peak areas against the concentration were plotted for each of the compounds and the data so obtained evaluated statistically using linear least squares regression analysis. Table 3.4 shows the regression analysis results for the compounds under study. The method was found to be linear over the range of 25 to 200 % for all the eight compounds.

71

Table 3.4: Linear regression analysis results for the compounds under study

Compound	Coefficient of determination (R^2)	y-intercept	Slope of regression line	Residual sum of squares
SBT	0.9995	+ 3492.67	1483668.52	2.6314×10^6
PED	0.9997	+ 5771.79	802964.85	1.0117×10^8
GFN	0.9992	+ 94295.70	2692215.66	8.9510×10^9
AMB	0.9991	+ 347681.01	21087964.04	2.4803×10^{11}
CPM	0.9999	+ 1285.93	12217846.93	3.2323×10^7
TPN	0.9999	+ 3450.17	32018374.27	1.1069×10^8
BXN	0.9998	- 17615.89	23761104.32	5.5821×10^9
TBT	0.9994	+ 3890.88	1423090.76	5.0542×10^6

SBT-salbutamol, TBT-terbutaline, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN- triprolidine, BXN-bromhexine.

3.7 Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [105]. In the case of liquid chromatography, typical variations include influence of variations of pH in a mobile phase, mobile phase composition, temperature and flow rate.

In the present study, influence of variation in mobile phase pH, acetonitrile concentration and temperature were investigated. These factors were varied over a narrow range at three levels to investigate the capacity of the method to remain unaffected by the variations. In each case as one factor was varied, the other two were held constant. The variations were mobile phase pH 3.0 ± 0.5 pH units, column temperature of 40 ± 5 °C and acetonitrile concentration of 35 ± 2 % v/v. Capacity factors were used to monitor the influence of these variations. Figures 3.1, 3.2 and 3.3 show the effect of varying the factors on capacity factors of the compounds under study.
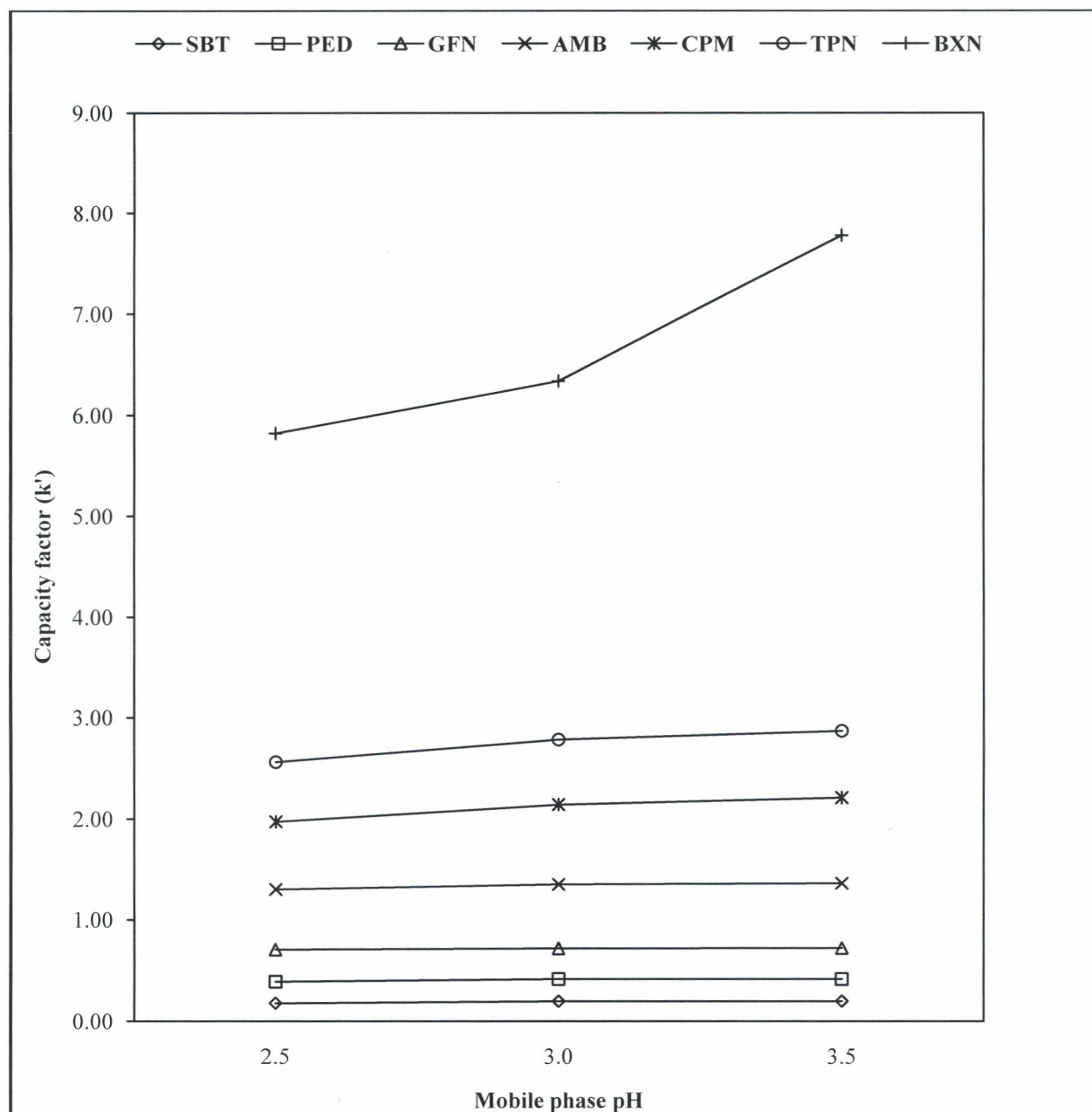
Figure 3.1: Effect of varying mobile phase pH on the capacity factors of the compounds under study. Column: Phenomenex Gemini 5 μ C_{18}, 250 × 4.6 mm ID. Column temperature: 40 ºC. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH_3COONH_4-water (35:4:10:51, % v/v/v/v). SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine.
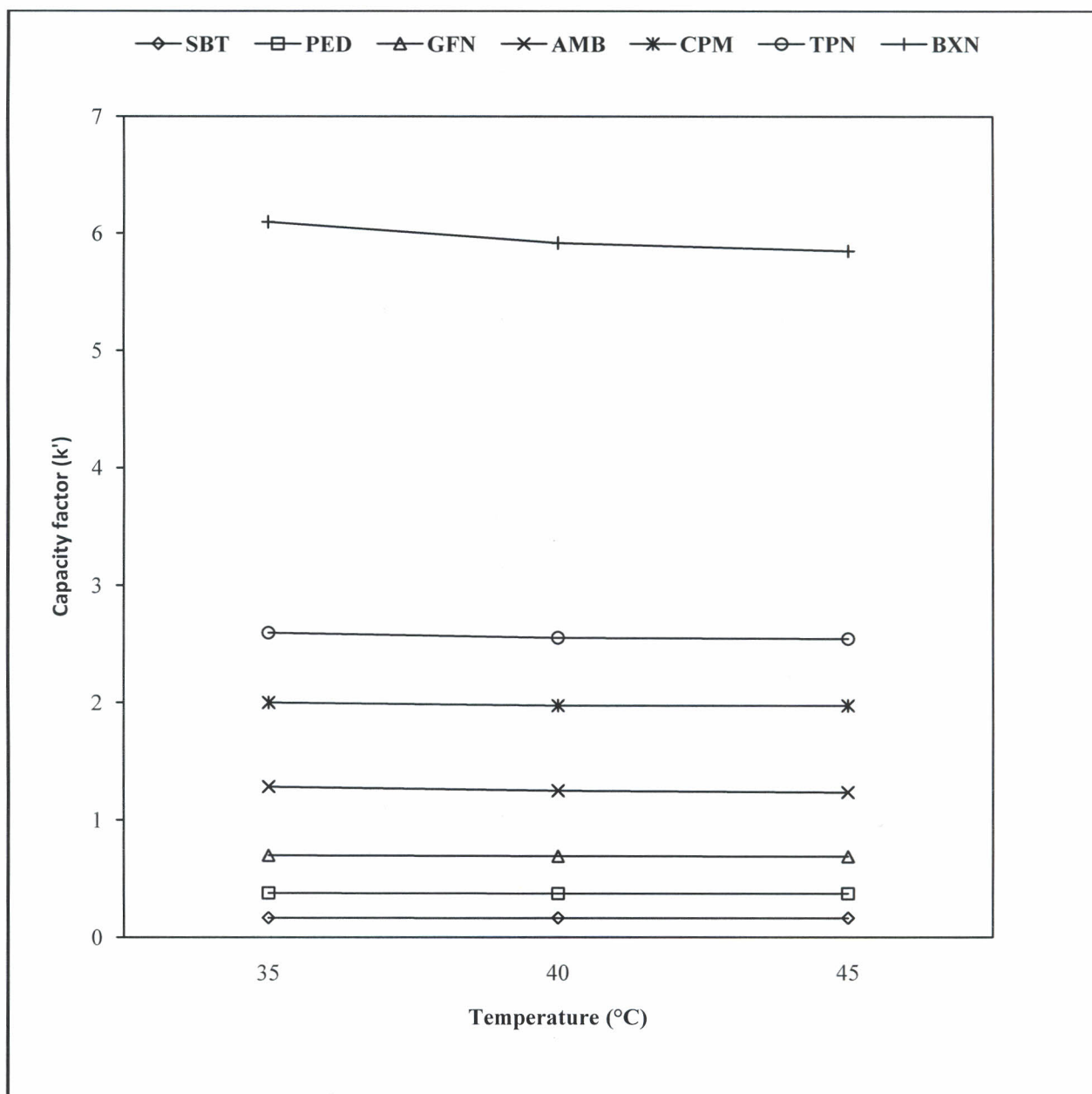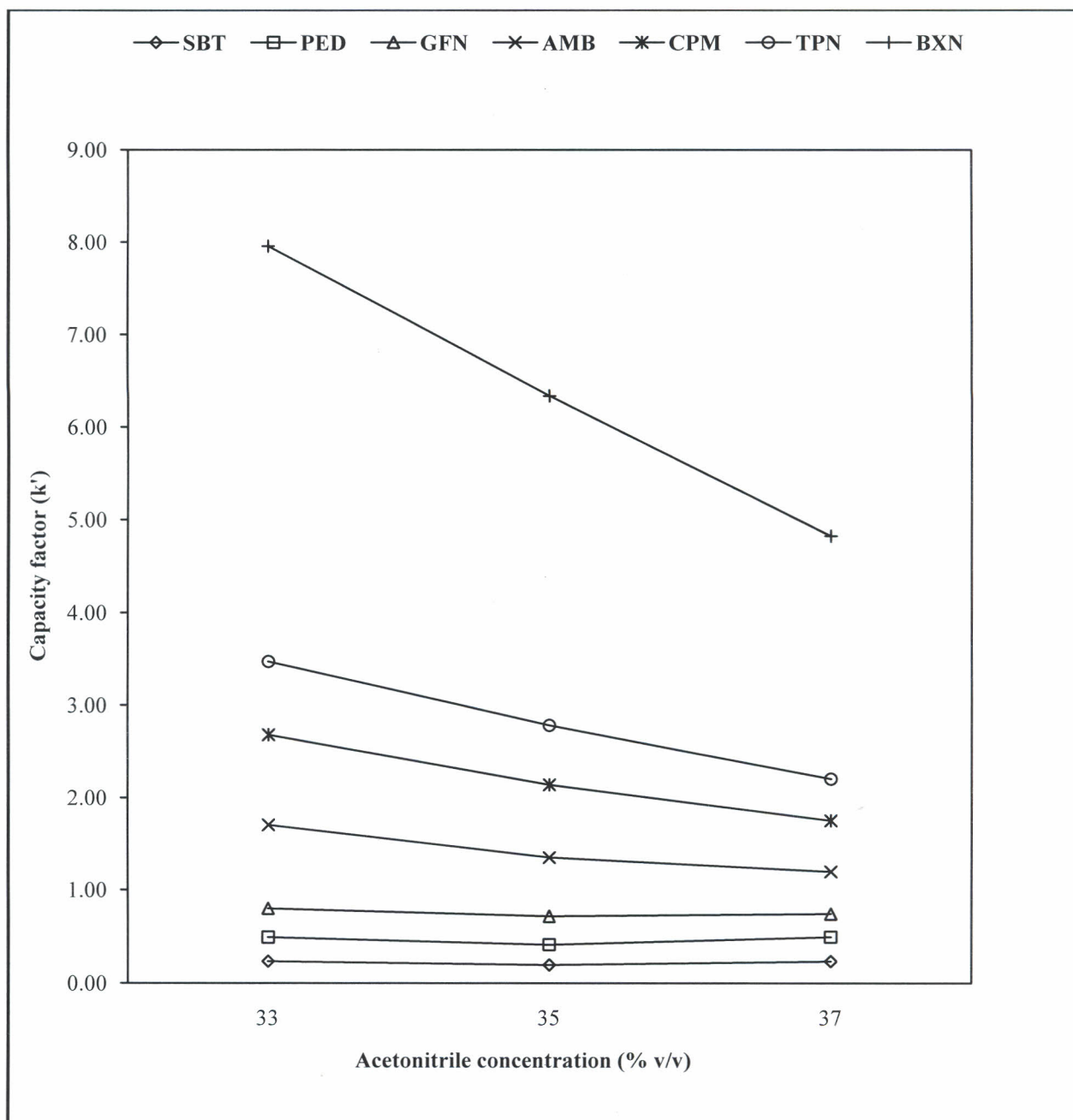
Figure 3.2: Effect of varying column temperature on the capacity factors of the compounds under study. Column: Phenomenex Gemini 5 μ C$_{18}$, 250 × 4.6 mm ID. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:10:51, % v/v/v/v). SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN- triprolidine, BXN-bromhexine.

Figure 3.3: Effect of varying the acetonitrile concentration on the capacity factors of the compounds under study. Column: Phenomenex Gemini 5 μ C_{18}, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH_3COONH_4 pH 3.0-water (X:4:10:86-X, % v/v/v/v). SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine. X-% volume of acetonitrile to make the desired concentration.

From figures 3.1, 3.2 and 3.3, the method remained largely unaffected by variation in temperature. However, the concentration of acetonitrile and the mobile phase pH variation had

the greatest impact on capacity factors especially on BXN and this calls for accurate measurement of mobile phase components and pH to minimize deviations from optimum during the application of the method. The retention time of BXN was most affected, being 14.51 minutes at acetonitrile concentration of 37 % v/v and 22.31 minutes at 33 % v/v, bearing significant impact on the total run time since BXN is the last eluting peak. The resolution of all the compounds remained largely unaffected by variation of these factors. It was concluded that the method was robust within the ranges of the critical factors investigated.

CHAPTER FOUR

ANALYSIS OF COMMERCIAL SAMPLES

4.1   Introduction

The developed method was applied in the analysis of finished products containing the compounds under study. Most of the cough syrups in the Kenyan market used for treatment of productive cough contain a combination of 2 - 4 of the compounds under study. In addition, some contain menthol whose determination is mainly by gas chromatography. There is no product in the market that contains all the compounds in combination and thus several products were analysed taking into consideration that all the compounds under study were tested and their content in the various products determined using the developed method.

4.2   Acquisition of commercial samples

The commercial finished products whose active ingredients contents were to be analyzed were sampled from community pharmacy outlets within the city of Nairobi. A total of nine commercial samples were obtained and coded A, B, C, D, E, F, G, H and I. Six products had three batches analyzed while two batches of the remaining three products were analyzed. For each sample, the batch number, manufacturing date, expiry date and the label claim were recorded as shown in table 4.1. Most of the samples had three of the compounds under study in combination and during sampling, it was necessary to ensure that all the compounds under study were analysed.

Table 4.1: Details of the samples analyzed

Product code	Batch number	Date of manufacture	Expiry date	Label claim (mg/5 mL)
A	102737A	July 2010	July 2013	Triprolidine HCl (1.25)
	111036A	March 2011	March 2014	Pseudoephedrine HCl (30)
	102738A	July 2010	July 2013	Guaifenesin (100)
B	01101238	May 2010	April 2012	Salbutamol (1) as sulphate
	01101987	August 2010	July 2012	Bromhexine HCl (2)
				Guaifenesin (50)
	01102448	April 2010	March 2012	Menthol (0.5)
C	M091028	October 2009	September 2011	Ambroxol HCl (30)
	M100686	August 2010	July 2012	Salbutamol (2) as sulphate
				Guaifenesin (50)
	M100154	February 2010	January 2012	Racementhol (1)
D	JK10580	June 2010	May 2013	Terbutaline sulphate (1.25)
	JK10976	September 2010	August 2013	Bromhexine HCl (4)
				Guaifenesin (50)
	JL10139	February 2011	January 2014	Menthol (2.5)
E				Ambroxol HCl (15)
	P90074	December 2009	May 2011	Salbutamol (1) as sulphate
	TP0002	October 2010	March 2012	Guaifenesin (50)
				Menthol (1)
F	101080	August 2010	July 2013	Chlorpheniramine maleate (2)
	090982	September 2009	August 2012	Pseudoephedrine HCl (30)
	101252	September 2010	August 2013	Guaifenesin (100)
G	HH0001R	January 2010	December 2012	Bromhexine HCl (4)
	HH0006R	September 2010	August 2013	Pseudoephedrine HCl (30)
	HH0007R	October 2010	September 2013	

Product code	Batch number	Date of manufacture	Expiry date	Label claim (mg/5 mL)
H	102879A	February 2011	February 2013	Pseudoephedrine HCl (30)
	090036A	January 2009	January 2012	Triprolidine HCl (1.25)
I	102089A	June 2010	December 2011	Guaifenesin (50)
	110081A	January 2011	July 2012	Salbutamol (1) as sulphate

4.3   Analysis of commercial samples

All the samples analysed were syrups that usually contain many excipients and preservatives, hence the possibility of peaks of the active ingredients being interfered with. The samples were screened during recovery determination to check for any interference of the peaks of interest by the excipients and whether there was need for extraction of the active ingredients from the sample matrix. It was found that the method could be used for the analysis of the ingredients in the presence of excipients as shown in the representative chromatograms of the samples in figures 4.1-4.8 where baseline separation was achieved and hence no need for extraction. The only peak that co-eluted with some excipients and solvent front was that of maleic acid and since it was not under determination, the co-elution was considered inconsequential.

The samples were prepared such that the solutions to be injected contained the active ingredients in concentrations of 0.02 mg/mL salbutamol, 0.025 mg/mL terbutaline sulphate, 0.3 mg/mL pseudoephedrine hydrochloride, 1 mg/mL guaifenesin, 0.3 mg/mL ambroxol hydrochloride, 0.02 mg/mL chlorpheniramine maleate, 0.0125 mg/mL triprolidine hydrochloride, 0.08 mg/mL bromhexine hydrochloride using acetonitrile-water (40:60 % v/v) as the diluent. A working standard solution with these concentrations excluding the terbutaline sulphate was prepared for use in the assay. Due to the fixed dose nature of the samples, sample C could only be prepared to

contain guaifenesin at a concentration of 0.5 mg/mL and samples B and G to contain bromhexine hydrochloride at a concentration of 0.04 mg/mL. A working standard solution composed of 0.5 mg/mL guaifenesin, 0.04 mg/mL bromhexine hydrochloride and 0.025 mg/mL terbutaline sulphate was prepared and used for analysis of guaifenesin in sample C, bromhexine hydrochloride in samples B and G and terbutaline sulphate in sample D.

During sample preparation, 10.0 mL or 5.0 mL of the sample, depending on the label claim was transferred into 100 mL volumetric flask and diluted to volume with acetonitrile-water (40:60 % v/v) mixture. The solution was filtered before injecting 20 µL in the LC system at the optimized chromatographic conditions. The samples were run in triplicate for each batch while the working standard solution was prepared in duplicate. A system suitability test was conducted before analyzing the products whereby the standard solution was injected six times and the RSD of the peak areas, retention times and the resolution (> 2.0) determined for all the components. The system was considered suitable only if the RSD was < 2.0. The peak areas of the standard solutions were normalized to the desired concentrations and to 100 % potency and the average peak areas compared to the sample peak areas in determination of the content of the active ingredients. The content was expressed as percentage label claim.

Specification limits of 90.0-110.0 % were adopted from the USP for all the compounds as those compounds with monographs for finished product products had these specifications. All the samples complied with the general United States Pharmacopoeia (USP) specifications for assay (90.0-110.0 % label claim) with the percentage content ranging from 92.8 to 107.0 %. The results obtained also demonstrated that there were minimal batch-to-batch variations. The results obtained are summarized in table 4.2.

Table 4.2: Assay report for the samples analyzed

Sample	Batch number	Percentage label claim							
		SBT	TBT	PED	GFN	AMB	CPM	TPN	BXN
A	102737A	-	-	99.3 (0.6)	98.7 (0.4)	-	-	99.4 (0.5)	-
	111036A	-	-	97.7 (0.3)	100.6 (0.2)	-	-	102.3 (0.2)	-
	102738A	-	-	98.2 (0.4)	99.3 (0.1)	-	-	101.7 (0.2)	-
B	01101238	97.4 (0.5)	-	-	97.8 (0.2)	-	-	-	96.3 (0.5)
	01101987	100.4 (0.9)	-	-	97.8 (0.6)	-	-	-	99.4 (1.1)
	01102448	98.2 (1.0)	-	-	97.2 (0.9)	-	-	-	98.9 (0.6)
C	M091028	98.5 (1.0)	-	-	97.7 (0.4)	92.8 (0.4)	-	-	-
	M100686	99.9 (1.1)	-	-	104.3 (1.1)	100.2 (1.3)	-	-	-
	M100154	99.3 (0.6)	-	-	99.5 (0.7)	99.8 (1.0)	-	-	-
D	JK10580	-	104.8 (0.9)	-	99.0 (0.5)	-	-	-	95.9 (0.3)
	JK10976	-	98.6 (1.5)	-	98.2 (0.8)	-	-	-	94.6 (0.6)
	JL10139	-	99.2 (1.1)	-	98.5 (0.7)	-	-	-	95.3 (0.2)
E	P90074	97.1 (0.7)	-	-	98.3 (0.5)	96.7 (0.4)	-	-	-
	TP0002	98.9 (1.4)	-	-	99.5 (0.8)	97.4 (0.7)	-	-	-
F	101080	-	-	98.5 (1.2)	98.0 (0.9)	-	97.3 (1.1)	-	-
	090982	-	-	97.0 (0.6)	100.1 (0.4)	-	96.6 (0.3)	-	-
	101252	-	-	97.4 (1.6)	99.7 (1.7)	-	95.6 (1.7)	-	-
G	HH0001R	-	-	107.0 (0.8)	-	-	-	-	101.2 (0.3)
	HH0006R	-	-	98.2 (0.5)	-	-	-	-	98.5 (0.6)
	HH0007R	-	-	101.6 (0.5)	-	-	-	-	102.1 (0.5)

Sample	Batch number	Percentage label claim							
		SBT	TBT	PED	GFN	AMB	CPM	TPN	BXN
H	102879A	-	-	95.7 (0.5)	-	-	-	99.7 (0.1)	-
	090036A	-	-	96.1 (1.0)	-	-	-	99.3 (0.9)	-
I	102089A	95.1 (1.1)	-	-	102.4 (0.7)	-	-	-	-
	110081A	96.2 (0.5)	-	-	101.7 (1.1)	-	-	-	-

Values in parenthesis represent the RSD

Figures 4.1-4.9 are typical chromatograms of the sample solutions while figures 4.10 and 4.11 are typical chromatograms of the working standard solution used during the assay.
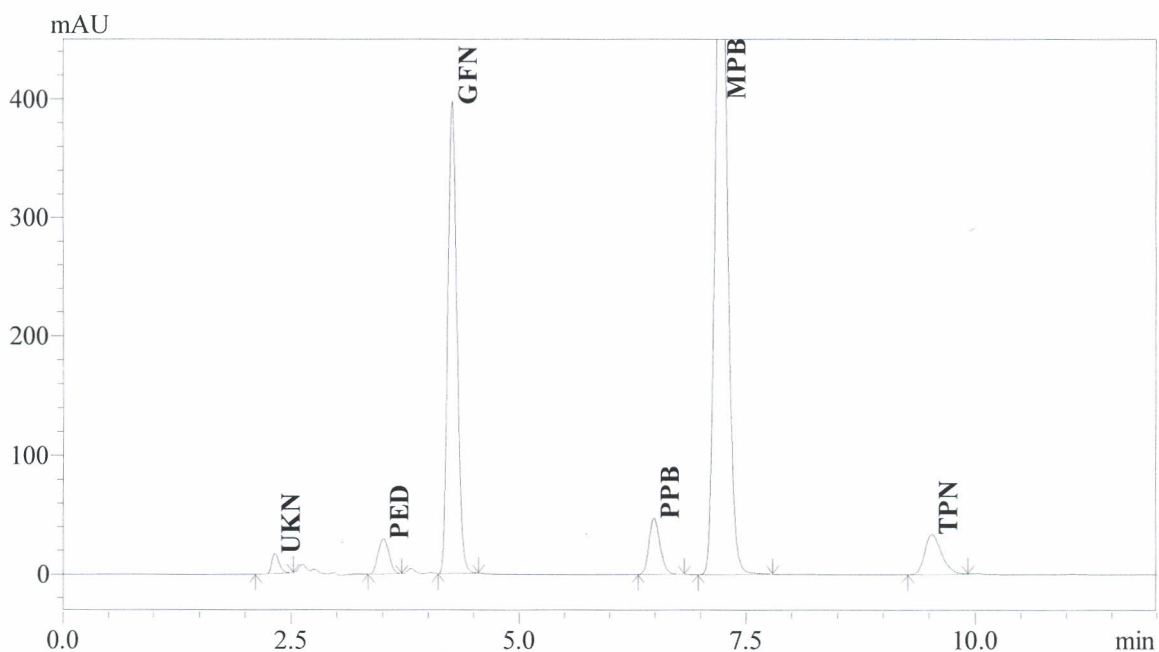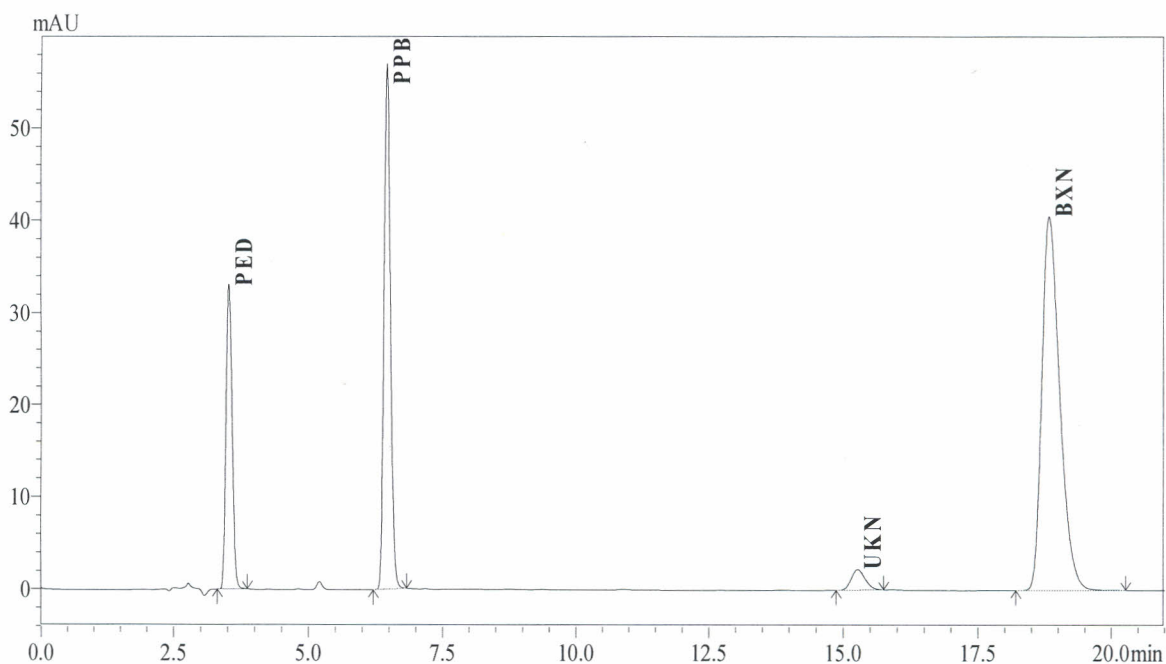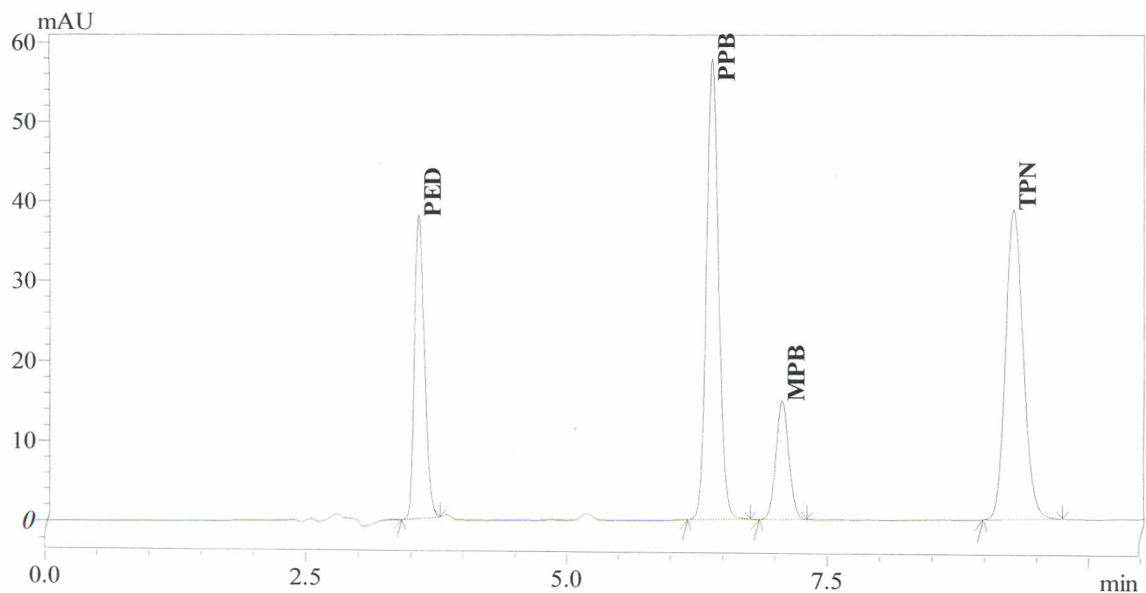

Figure 4.1: Typical chromatogram of sample A. Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:10:51, % v/v/v/v). UKN-unknown, PED-pseudoephedrine, GFN-guaifenesin, PPB-propylparaben, MPB-methylparaben, TPN-triprolidine.
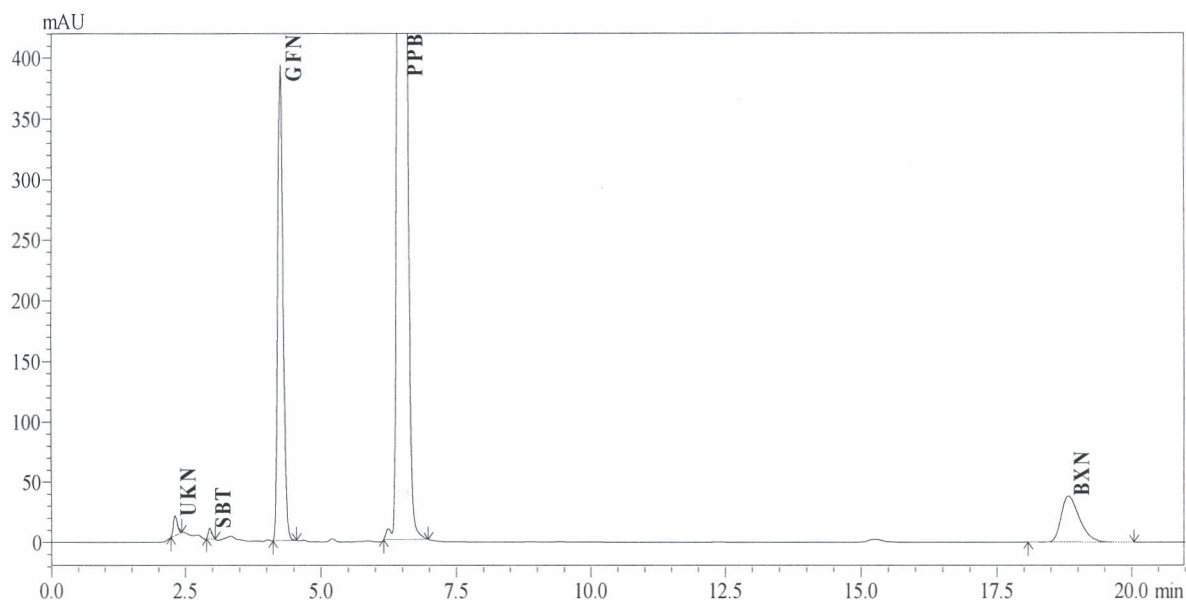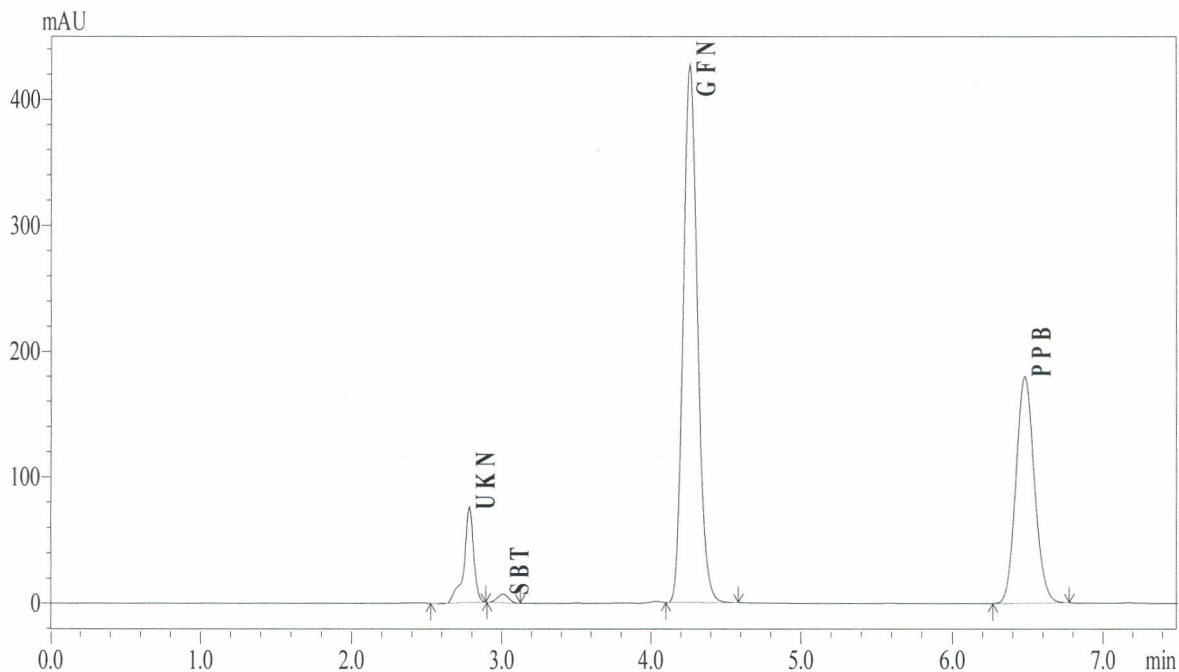
Figure 4.2: Typical chromatogram of sample B. Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:10:51, % v/v/v/v). UKN-unknown, SBT-salbutamol, GFN-guaifenesin, PPB-propylparaben, BXN-bromhexine.
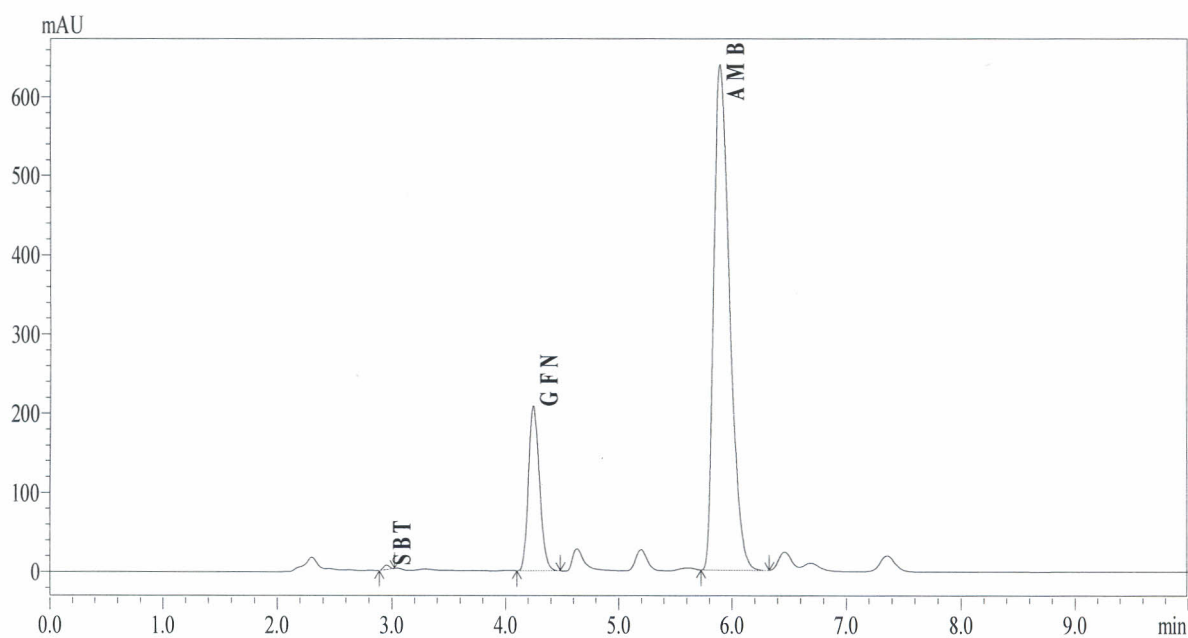
Figure 4.3: Typical chromatogram of sample C. Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:10:51, % v/v/v/v). SBT-salbutamol, GFN-guaifenesin, AMB-ambroxol.

83
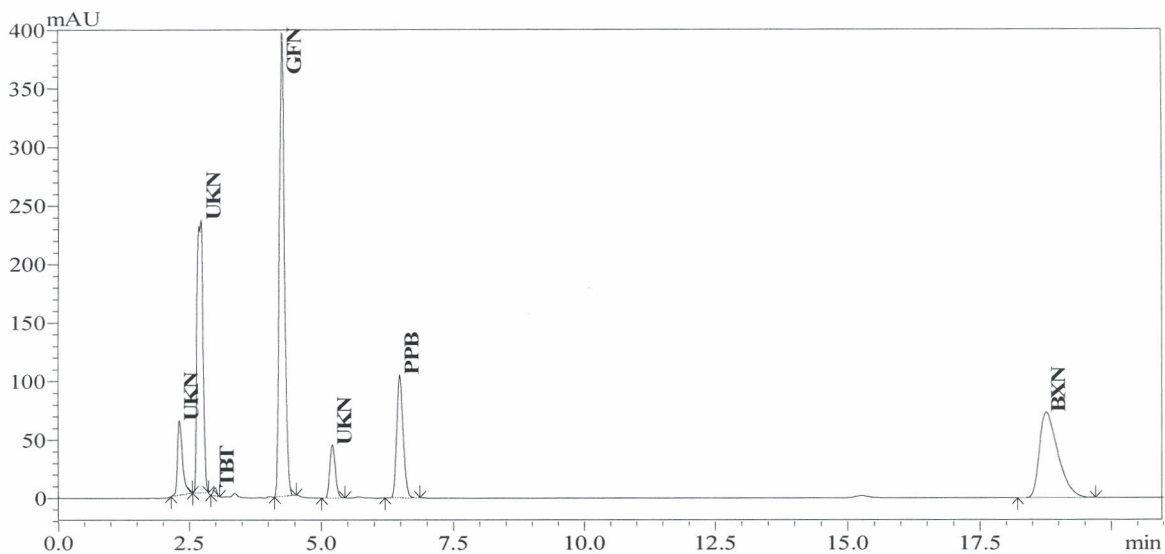
Figure 4.4: Typical chromatogram of sample D. Column: Phenomenex Gemini® 5 μ C₁₈, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH₃COONH₄ pH 3.0-water (35:4:10:51, % v/v/v/v). UKN-unknown, TBT-terbutaline, GFN-guaifenesin, PPB-propylparaben, BXN-bromhexine.
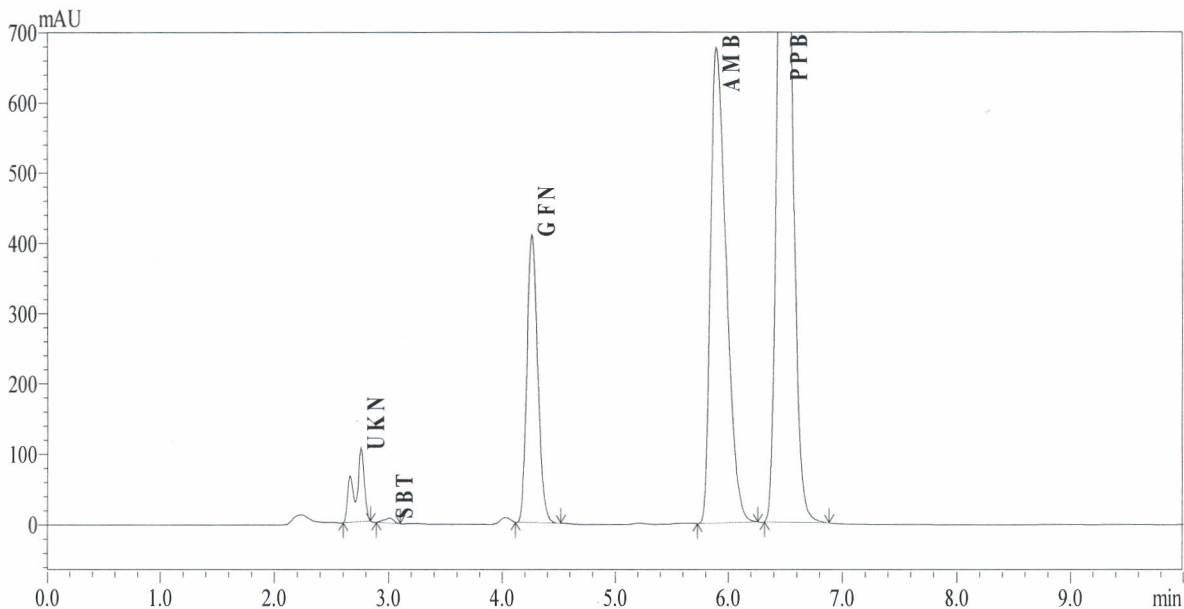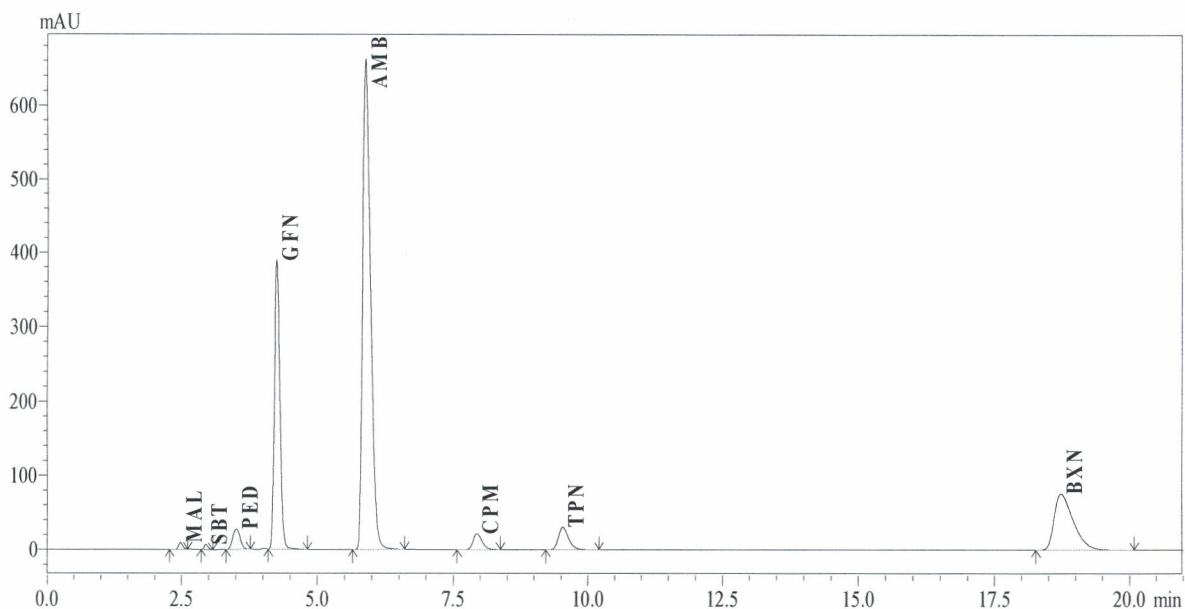

Figure 4.5: Typical chromatogram of sample E. Column: Phenomenex Gemini® 5 μ C₁₈, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH₃COONH₄ pH 3.0-water (35:4:10:51, % v/v/v/v). UKN-unknown, SBT-salbutamol, GFN-guaifenesin, AMB-ambroxol, PPB-propylparaben.
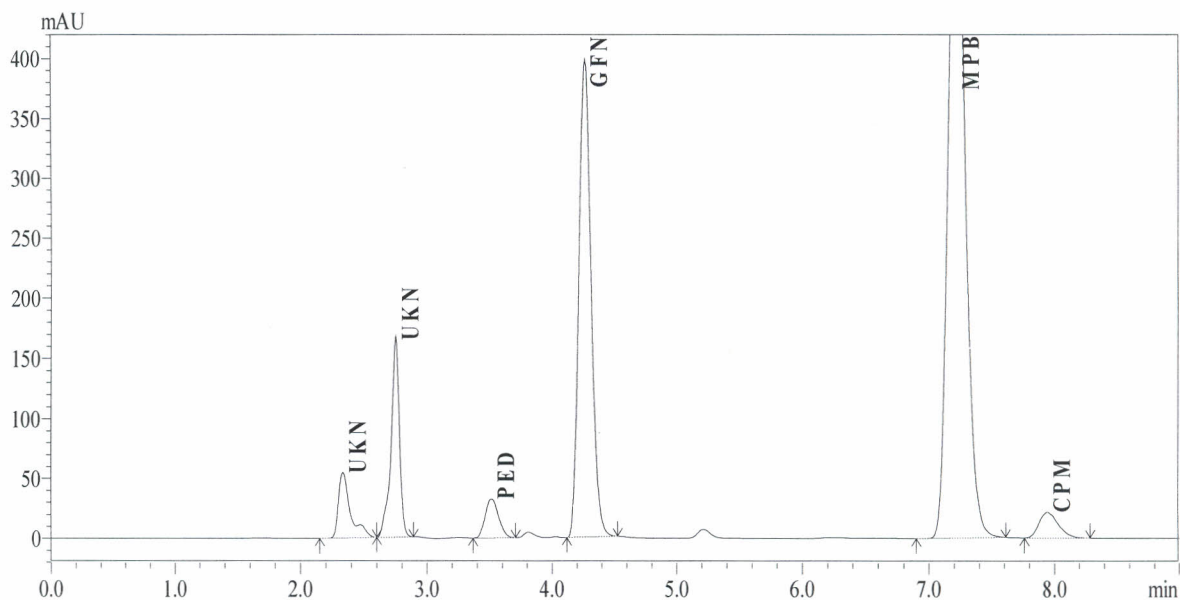
Figure 4.6: Typical chromatogram of sample F. Column: Phenomenex Gemini® 5 μ C₁₈, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH₃COONH₄ pH 3.0-water (35:4:10:51, % v/v/v/v). UKN-unknown, PED-pseudoephedrine, GFN-guaifenesin, MPB-methylparaben, CPM-chlorpheniramine.

Figure 4.7: Typical chromatogram of sample G. Column: Phenomenex Gemini® 5 μ C₁₈, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH₃COONH₄ pH 3.0-water (35:4:10:51, % v/v/v/v). PED-pseudoephedrine, PPB-propylparaben, UKN-unknown, BXN-bromhexine.

85

Figure 4.8: Typical chromatogram of sample H. Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:10:51, % v/v/v/v). PED-pseudoephedrine, PPB-propylparaben, MPB-methylparaben, TPN-triprolidine.

Figure 4.9: Typical chromatogram of sample I. Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 °C. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:10:51, % v/v/v/v). UKN-unknown, SBT-salbutamol, GFN-guaifenesin, PPB-propylparaben.

86

Figure 4.10: Typical chromatogram of the working standard solution containing SBT. Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 ºC. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:10:51, % v/v/v/v). MAL-maleic acid, SBT-salbutamol, PED-pseudoephedrine, GFN-guaifenesin, AMB-ambroxol, CPM-chlorpheniramine, TPN-triprolidine, BXN-bromhexine.
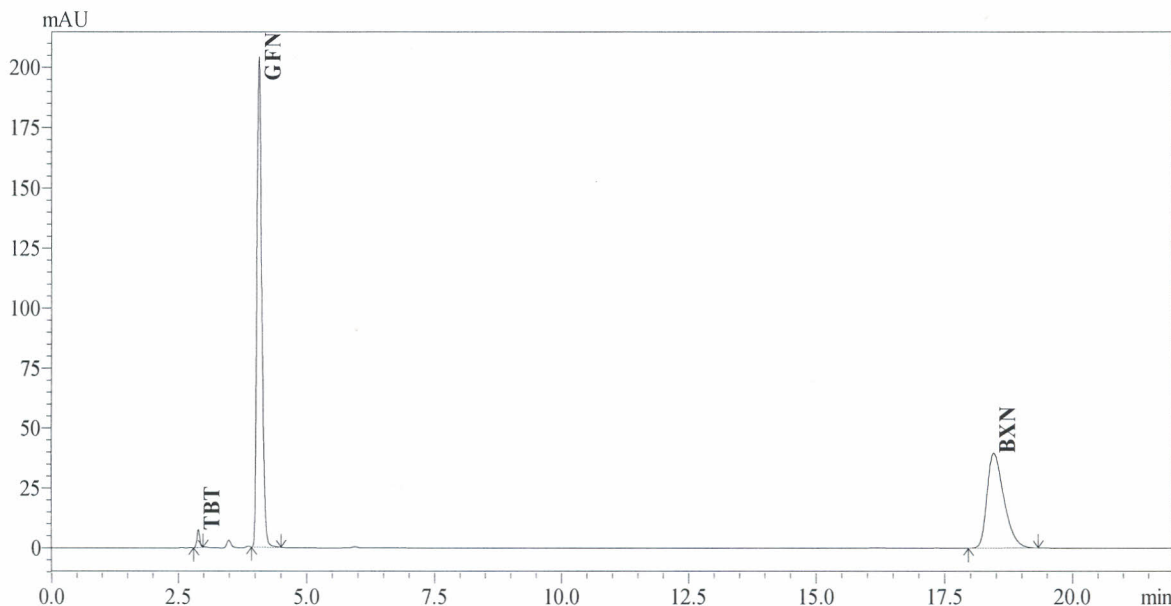
Figure 4.11: Typical chromatogram of the working standard solution containing TBT. Column: Phenomenex Gemini® 5 μ C$_{18}$, 250 × 4.6 mm ID. Column temperature: 40 ºC. Mobile phase: acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH$_3$COONH$_4$ pH 3.0-water (35:4:10:51, % v/v/v/v). TBT-Terbutaline, GFN-guaifenesin, BXN-bromhexine.

CHAPTER FIVE

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 General discussion

A HPLC method for the simultaneous determination of salbutamol/terbutaline, pseudoephedrine, guaifenesin, ambroxol, chlorpheniramine, triprolidine and bromhexine in cough-cold syrups was developed. This greatly simplifies the analysis of products containing these compounds whereby they can be determined simultaneously in the presence of excipients thus reducing the analysis time. Although there is no finished product containing all the compounds studied, combinations of these compounds can readily be analysed with the method.

The method utilizes readily available reagents making it easier to apply in most quality control laboratories. The use of reversed phase C_{18} column as the stationary phase makes the method readily applicable, since it is the most commonly used stationary phase in quality control laboratories. Additionally, the use of 254 nm as the detection wavelength offers the advantage of application of the method in HPLC systems with fixed wavelength UV detector and those with a variable wavelength UV detector.

During method validation, LOD and LOQ of the compounds were determined although not a requirement under the ICH guidelines. This was deemed important as it gave an indication of the smallest concentration of each analyte that could be quantified with adequate accuracy and precision thereby facilitating the application of the method in profiling of related substances and degradation products during stability studies. The method was found to be unaffected by small

88

variations in the mobile phase pH, column temperature and acetonitrile concentration and thus a robust method.

5.2   Conclusion

A simple, reliable, accurate, precise, robust and isocratic HPLC method was developed for simultaneous determination of salbutamol or terbutaline, pseudoephedrine, guaifenesin, ambroxol, chlorpheniramine, triprolidine and bromhexine in cough-cold mixtures in the presence of excipients. The optimized chromatographic conditions were mobile phase consisting of acetonitrile-0.25 M sodium hexanesulphonate-0.2 M CH_3COONH_4 pH 3.0-water (35:4:10:51, % v/v/v/v) delivered through the system at a flow rate of 1.0 mL/min. The stationary phase was reversed phase octadecylsilane measuring 250 mm in length, 4.6 mm internal diameter, 5 μm particle size and 110 Å pore size (Phenomenex Gemini[®]) maintained at a temperature of 40 ˚C. The ultra-violet detection wavelength was set at 254 nm while the injection volume was 20 μL. The diluent consisted of acetonitrile-water (40:60, % v/v).

Validation of the method demonstrated that the method was accurate with recoveries of 99.9 % (SBT), 100.5 % (TBT), 99.2 % (PED), 99.8 % (GFN), 98.5 % (AMB), 99.7 % (CPM), 99.9 % (TPN), 99.2 % (BXN), all of which were within the limits of 98.0-103.0 %. The precision of the method was shown through adequate repeatability or intra-day precision (RSD, 0.15-0.56 %) and intermediate precision (RSD, 0.45-1.53 %). The method also demonstrated adequate linearity over a range of 25-200 %. The linearity equations were $y = 1483668.52x + 3492.66$ ($R^2 = 0.9995$) for SBT, $y = 802964.85x + 5771.79$ ($R^2 = 0.9997$) for PED, $y = 2692215.66x + 94295.70$ ($R^2 = 0.9992$) for GFN, $y = 21087964.04x + 347681.01$ ($R^2 = 0.9991$) for AMB, $y = 12217846.93x + 1285.93$ ($R^2 = 0.9999$) for CPM, $y = 32018374.27x + 3450.17$ ($R^2 = 0.9999$) for TPN, $y = 23761104.32x - 17615.89$ ($R^2 = 0.9998$) for BXN and $y = 1423090.76x + 3890.88$ (R^2

= 0.9994) for TBT. The LOD values for salbutamol, pseudoephedrine, guaifenesin, ambroxol, chlorpheniramine, triprolidine, bromhexine and terbutaline were 1.28, 12.45, 7.68, 1.29, 3.27, 31.03, 1.67, 10.08 and the LOQ values were 4.00, 18.68, 10.24, 3.22, 17.45, 51.72, 3.34, 25.21 respectively. The low LOQ values indicate the method can be applied in analysis of these compounds in biological samples. The method was adequately robust with respect to small variations in mobile phase pH, column temperature and acetonitrile concentration.

The method was applied in analysis of commercial samples obtained from community pharmacy outlets within the city of Nairobi. All the samples complied with the general United States Pharmacopoeia (USP) specifications for assay (90.0-110.0 % label claim). The results obtained also demonstrated that there were minimal batch-to-batch variations. The method is useful in quality control laboratories for the routine analysis of these compounds in cough-cold medications.

5.3   Recommendations

The robustness of the method needs to be tested further using computer aided experimental design and data analysis using suitable software such as Statgraphics Plus and Modde 8.0. These software are able to plot surface response plots which are more accurate in assessing the robustness of the method.

Collaborative studies between laboratories need to be carried out to assess the method's ruggedness and reproducibility. This would improve the applicability of the method in other laboratories making it universal and more versatile.

REFERENCES

1. F.D. McCool. Global physiology and pathophysiology of cough: ACCP evidence-based clinical practice guidelines. *Chest*, 129 (1) (2006), 48S-53S.

2. J.M. Madison and R.S. Irwin. The Diagnosis and Treatment of Cough. *New Engl. J. Med.,* 343 (2000), 1715-1721.

3. J.M. Madison and R.S. Irwin. Cough: A Worldwide Problem. *Otolaryngol. Clin. North Am.,* 43 (1) (2010), 1-13.

4. J.G. Hardman and L.E. Limbirl d (Editors), Goodman and Gilman's The Pharmacological Basis of Therapeutics, 10th Edition, McGraw-Hill, New York, 2001. p 604.

5. A.B. Chang and W.B. Glomb. Guidelines for evaluating chronic cough in pediatrics: ACCP evidence-based clinical practice guidelines. *Chest,* 129(1) (2006), 260S-283S.

6. A.B. Chang, M.M. Eastburn and J. Gaffney. Cough quality in children: a comparison of subjective vs. bronchoscopic findings. *Respir. Res.,* 6 (2005), 3

7. M. Kraft. Approach to the patient with respiratory disease. *Cecil Medicine. 23rd ed. Philadelphia, Saunders Elsevier,* (2007), 83.

8. J. Nasra and G.M. Belvisi. Modulation of sensory nerve function and the cough reflex: Understanding disease pathogenesis. *Pharmacol. Ther.,* 124 (3) (2009), 354-375.

9. R.S. Irwin, L.P. Boulet, M.M. Cloutier, R. Fuller, P.M. Gold and V. Hoffstein. Managing cough as a defense mechanism and as a symptom. A consensus panel report of the American College of Chest Physicians. *Chest,* 114 (1998), 133S-181S.

APPENDICES
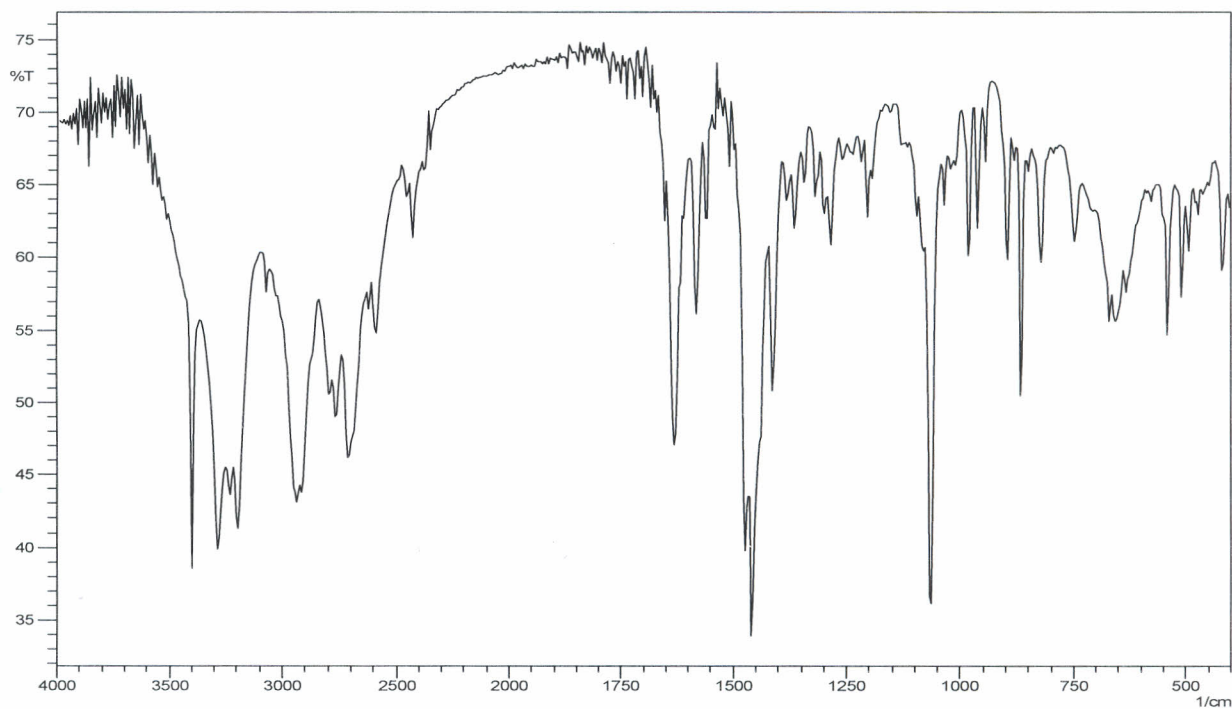
Appendix 1: FTIR spectrum of ambroxol hydrochloride (KBr disk)
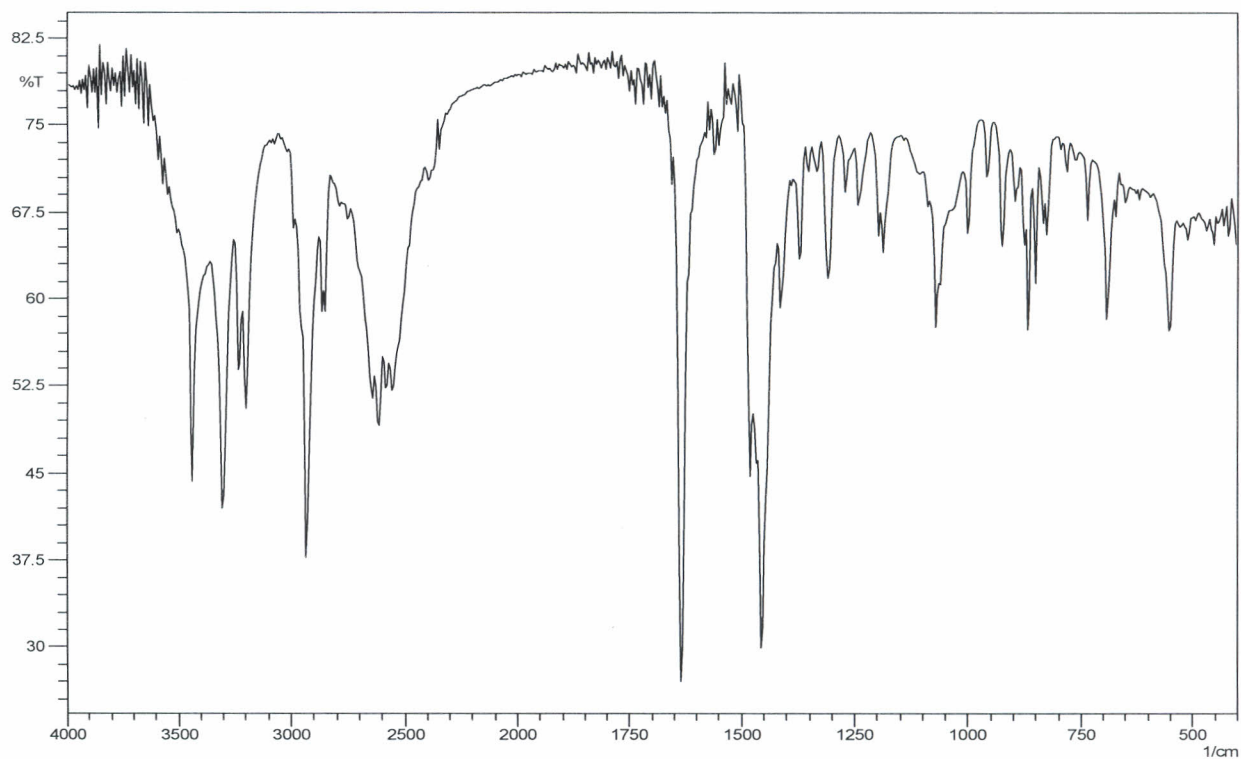
Appendix 2: FTIR spectrum of bromhexine hydrochloride (KBr disk)

106
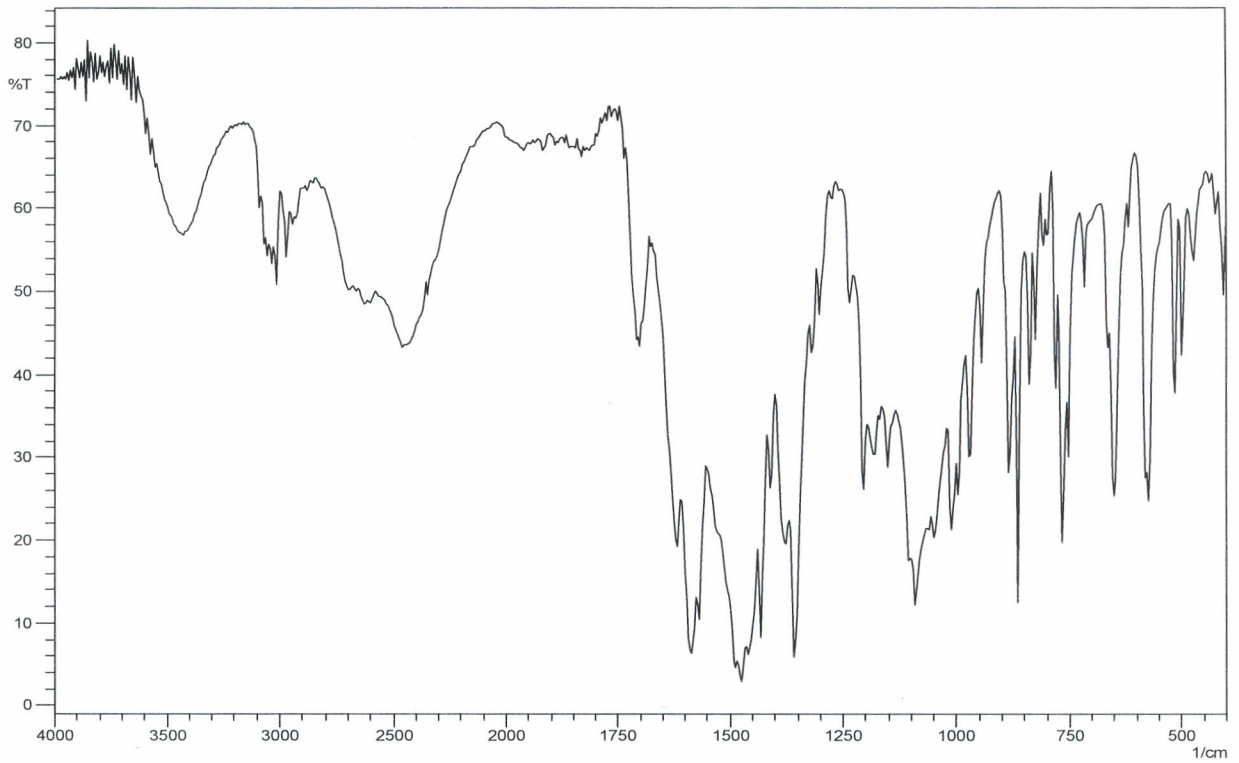
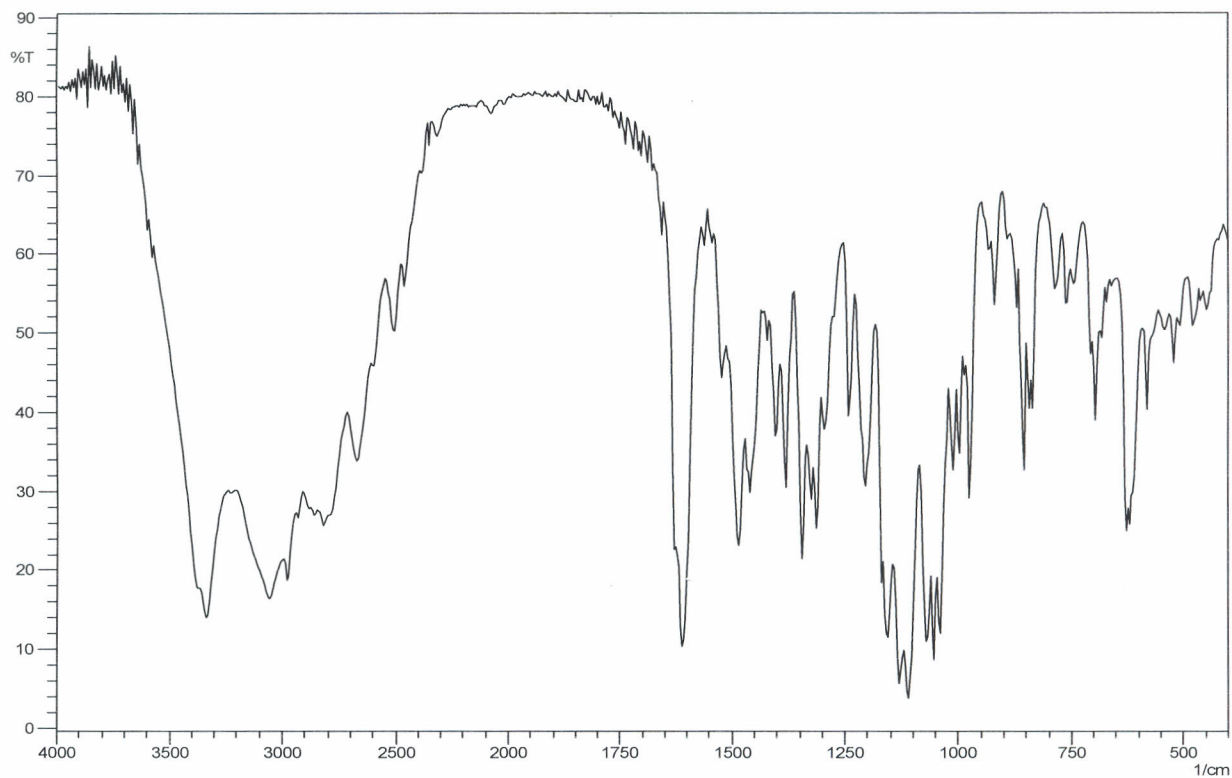**Appendix 3: FTIR spectrum of chlorpheniramine maleate (KBr disk)**
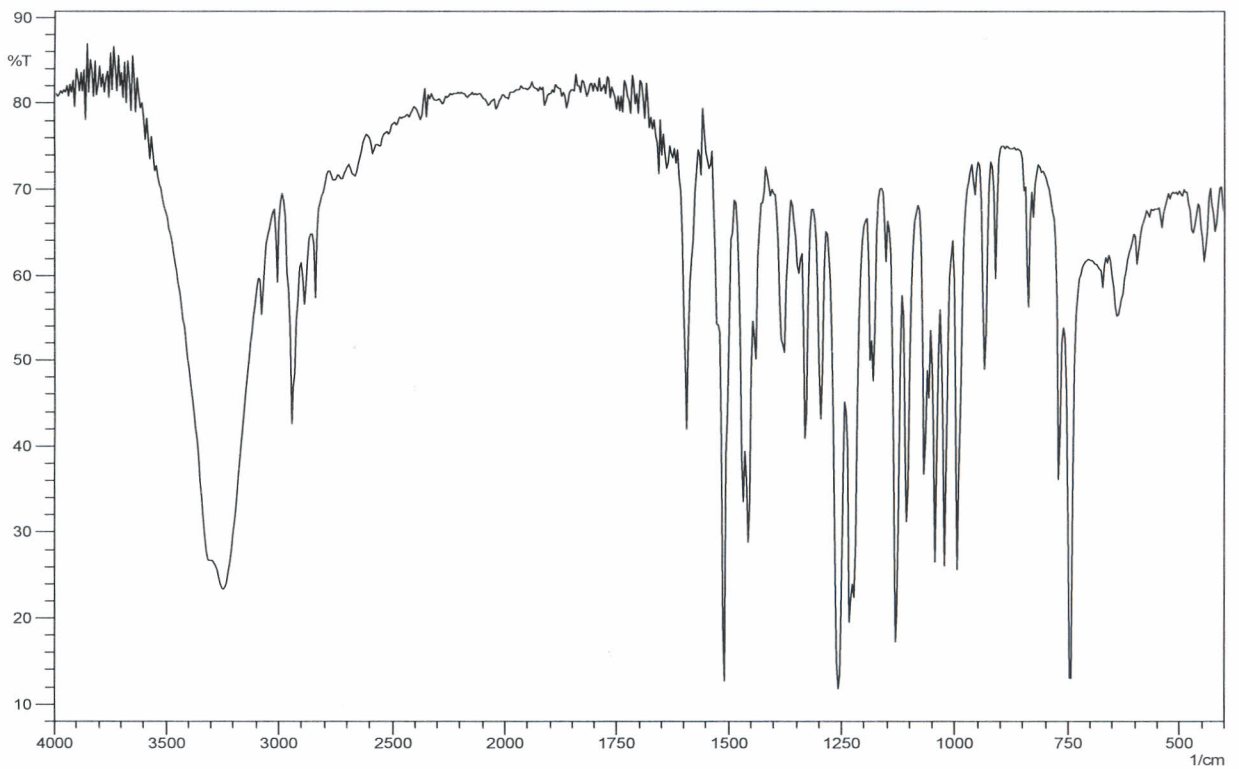
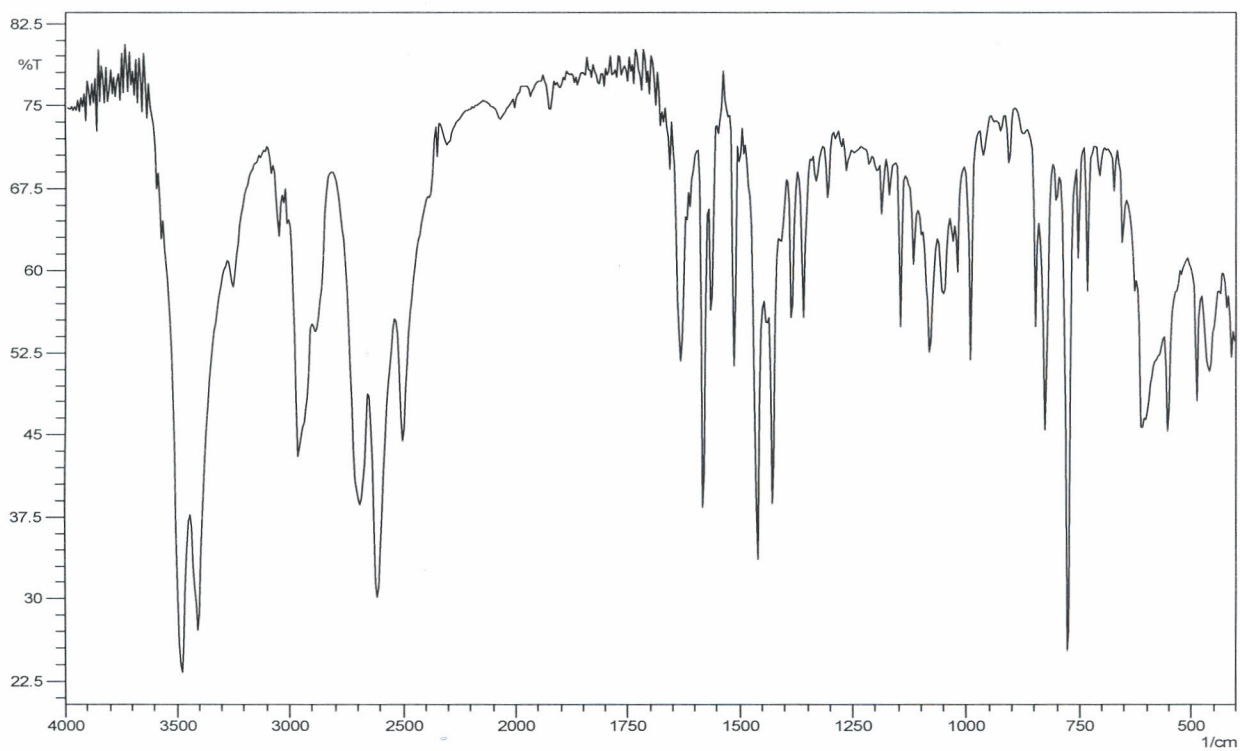**Appendix 4: FTIR spectrum of guaifenesin (KBr disk)**
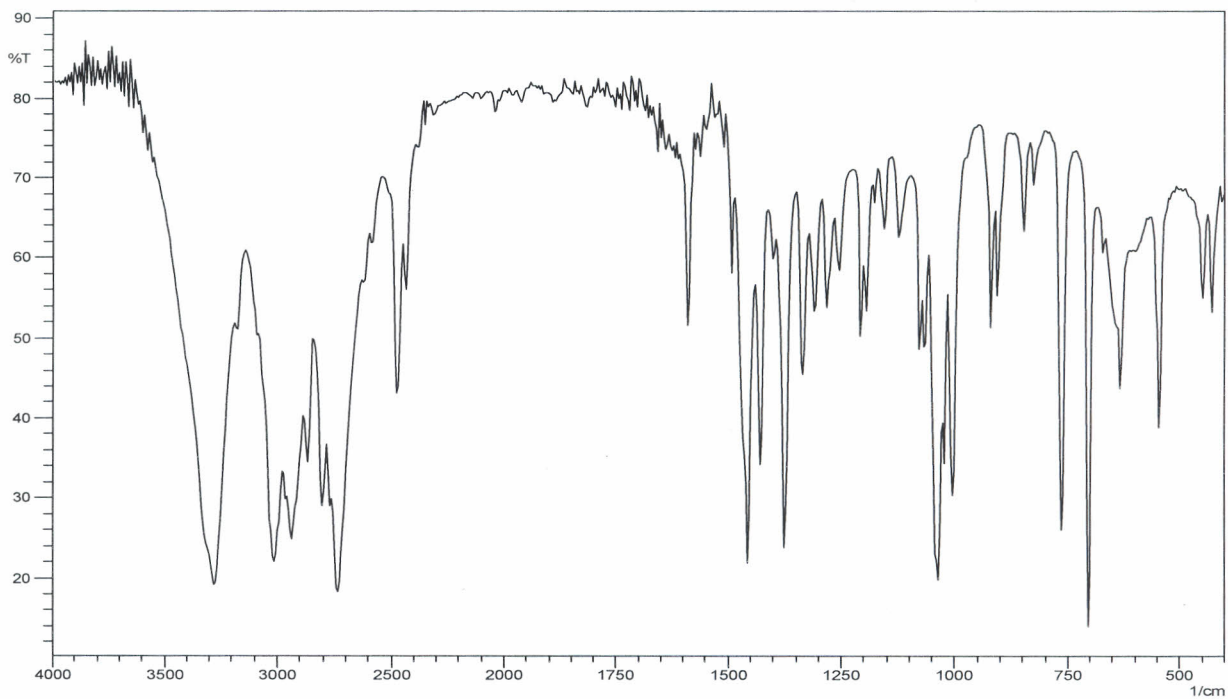
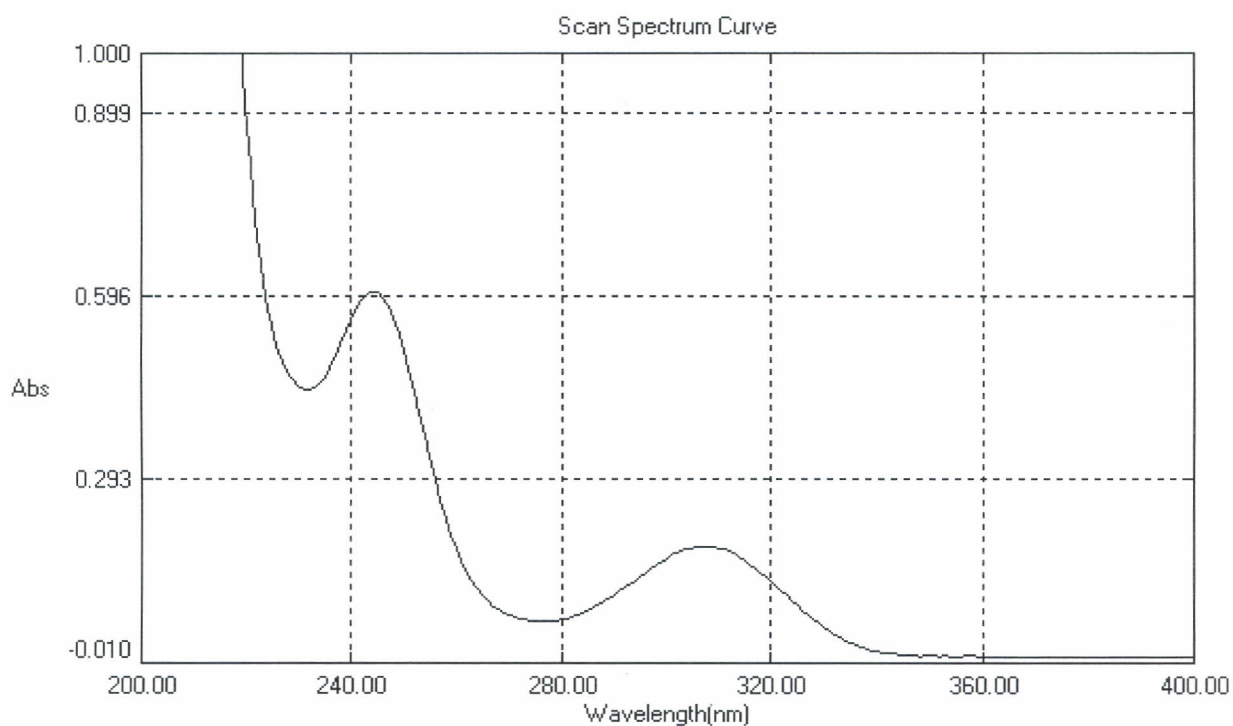**Appendix 5: FTIR spectrum of pseudoephedrine hydrochloride (KBr disk)**
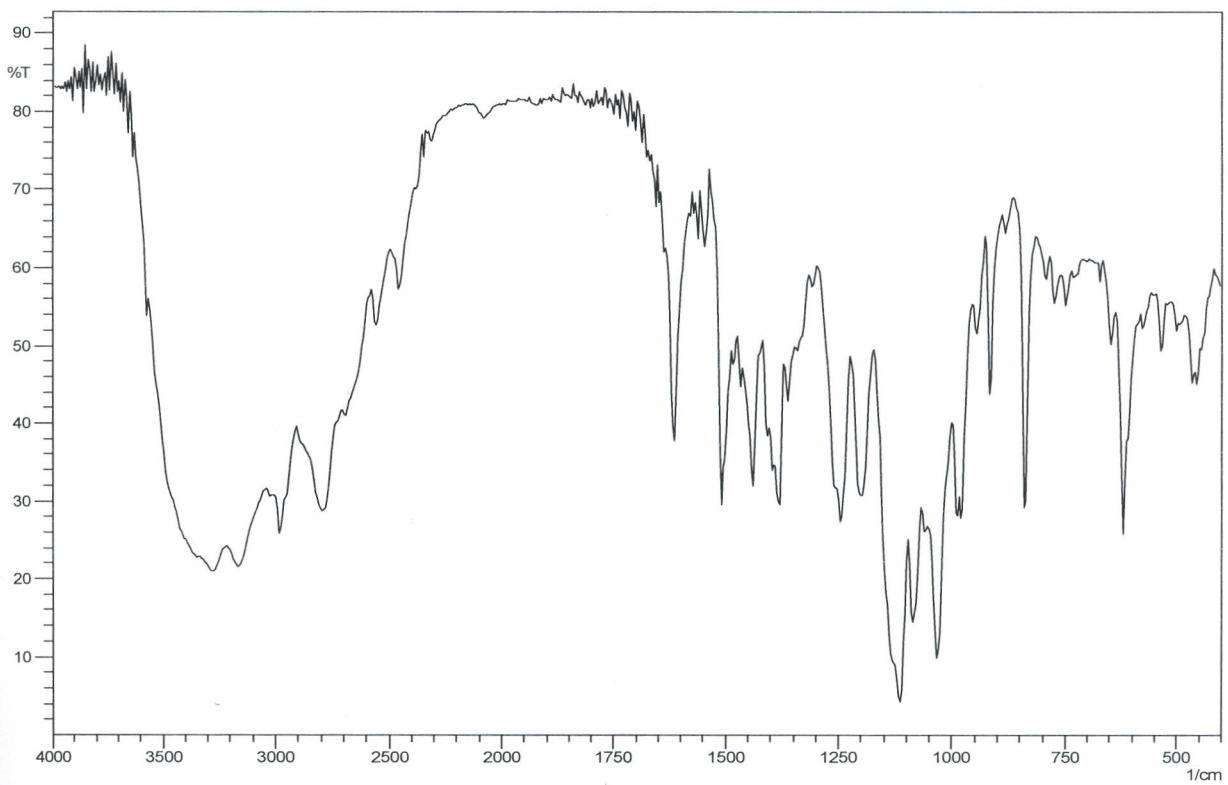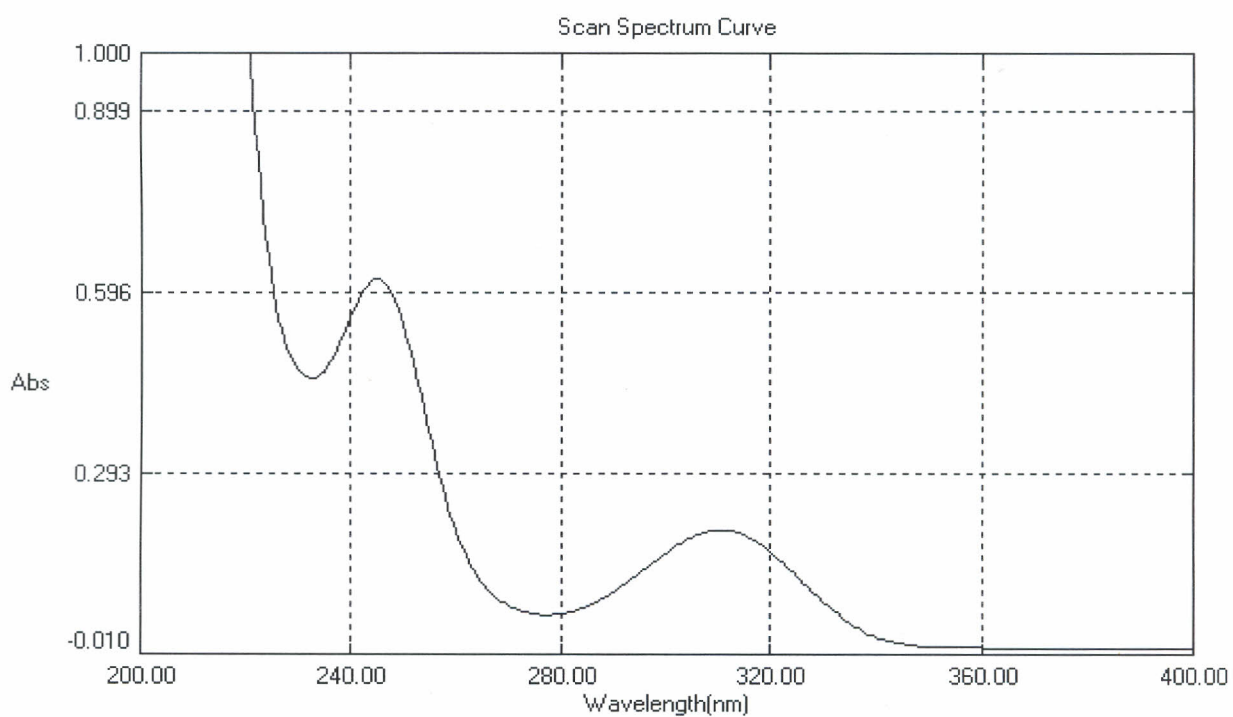

Appendix 6: FTIR spectrum of salbutamol sulphate (KBr disk)

Appendix 7: FTIR spectrum of terbutaline sulphate (KBr disk)

Appendix 8: FTIR spectrum of triprolidine hydrochloride (KBr disk)

109

Appendix 9: UV spectrum of ambroxol hydrochloride

Appendix 10: UV spectrum of bromhexine hydrochloride

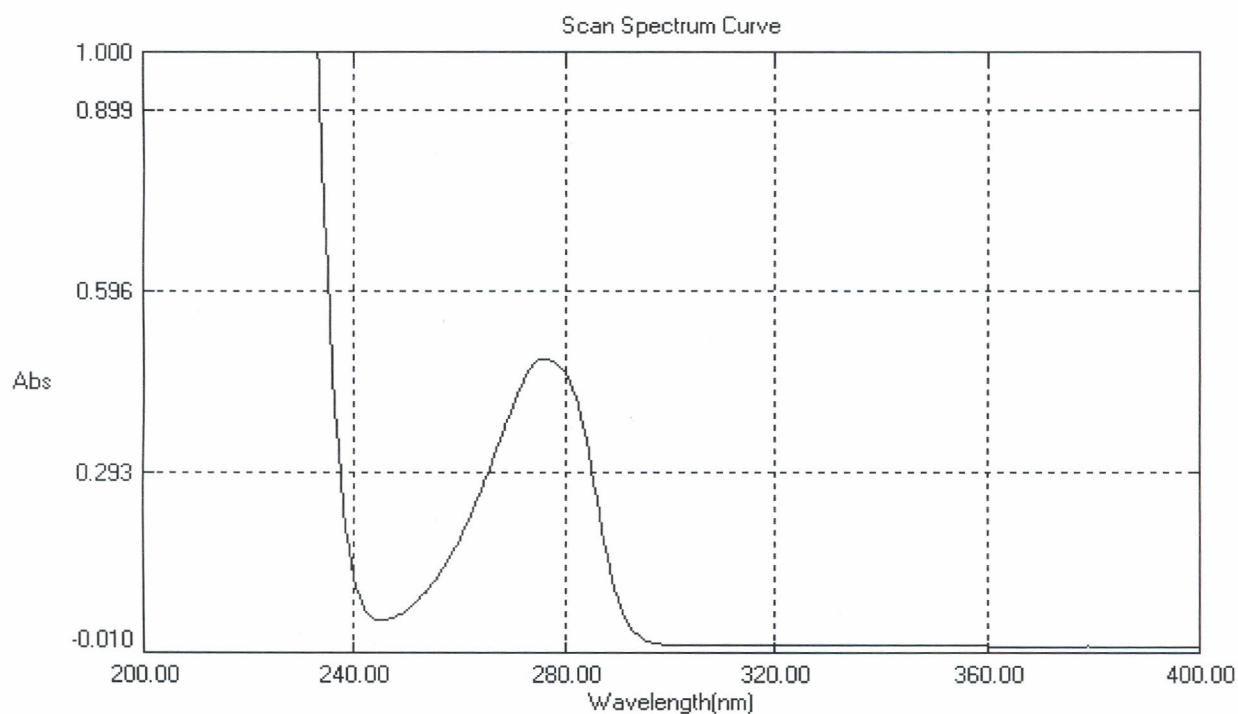110
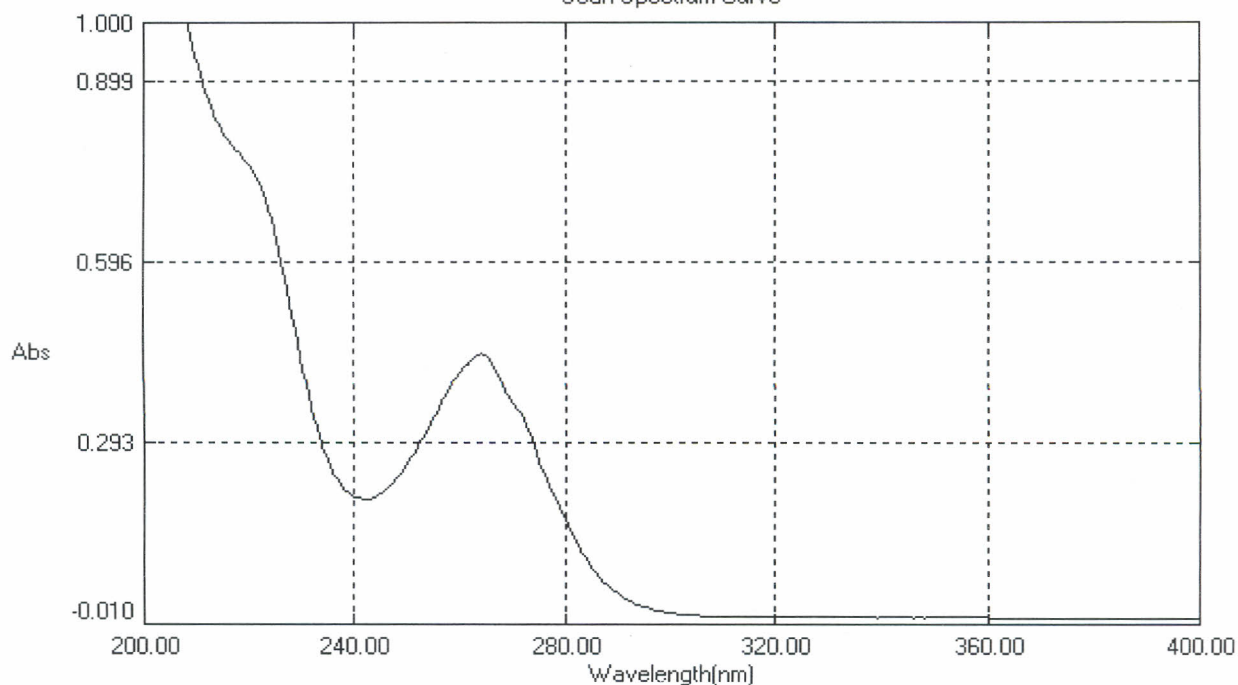
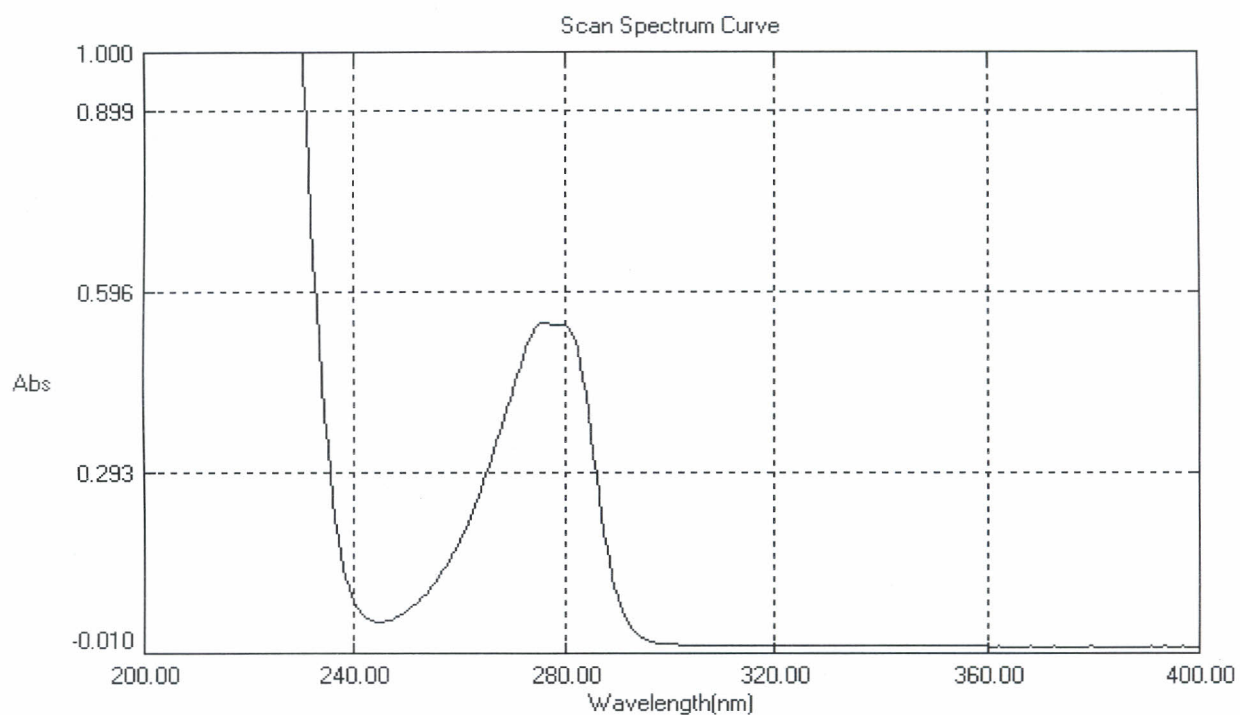**Appendix 11: UV spectrum of chlorpheniramine maleate**
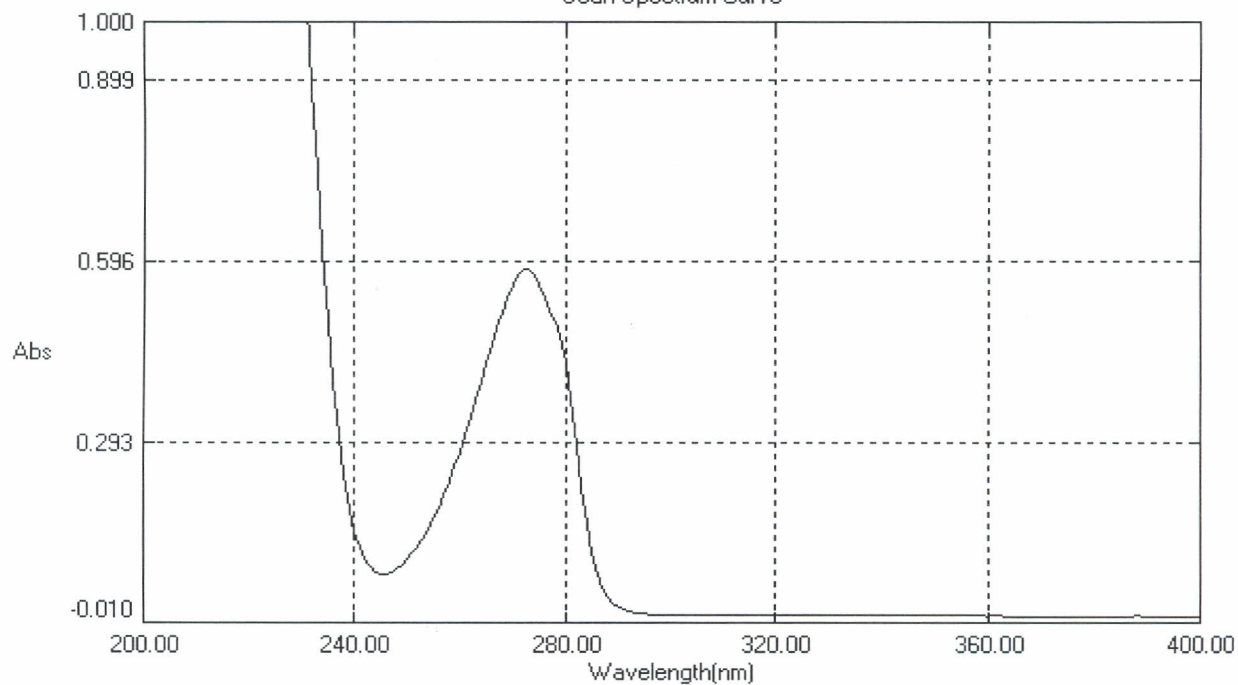
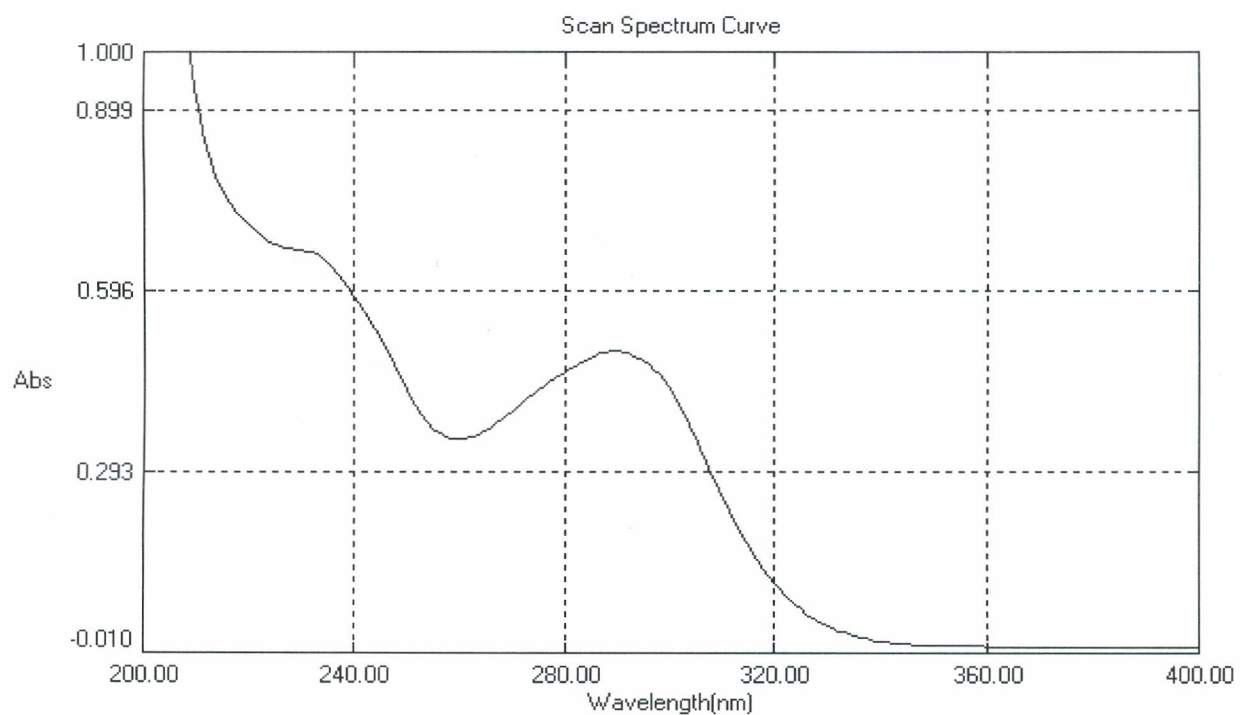**Appendix 12: UV spectrum of guaifenesin**

111
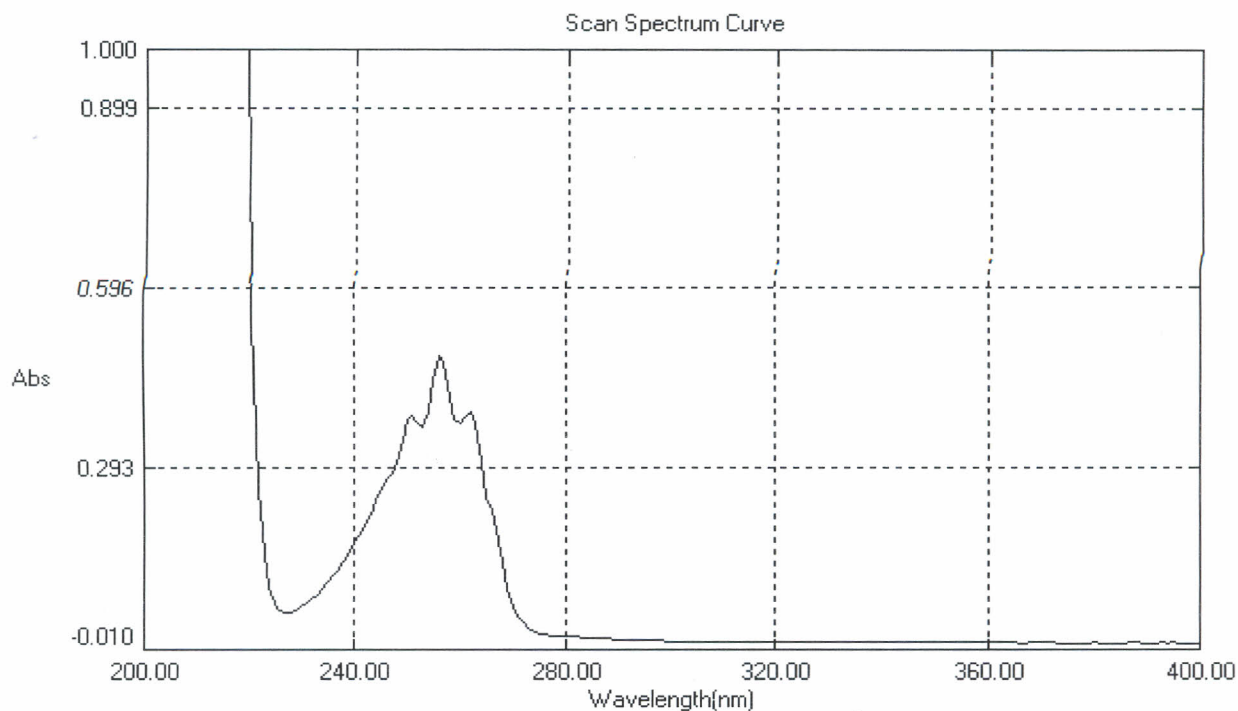
Appendix 13: UV spectrum of pseudoephedrine hydrochloride

Appendix 14: UV spectrum of salbutamol sulphate

112

Appendix 15: UV spectrum of terbutaline sulphate

Appendix 16: UV spectrum of triprolidine hydrochloride

113