DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF CAFFEINE, CHLORZOXAZONE, CODEINE, DICLOFENAC, DOXYLAMINE, IBUPROFEN AND PARACETAMOL IN ANALGESIC PREPARATIONS

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

BY

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DECLARATION

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

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This work is dedicated to my daughter Kendi for bringing a smile to my face after the hardest of sessions in the Laboratory and to my wife Elizabeth, my rock and support when things got tough.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

µ	Micron
µL	Microliter
λ_{max}	Wavelength of maximum absorbance
A_s	Asymmetry factor
BP	British Pharmacopoeia
°C	Degrees centigrade
C_8	Octylsilane stationary phase for chromatography
C_{18}	Octyldecylsilane stationary phase for chromatography
CAF	Caffeine
cAMP	Cyclic adenosine monophosphate
CHR	Chlorzoxazone
CNS	Central nervous system
COD	Codeine
DIC	Diclofenac
DOX	Doxylamine
FTIR	Fourier transform infrared
GC	Gas chromatography
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immune Deficiency syndrome
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
IBP	Ibuprofen
ICH	International conference on harmonization
k'	Capacity factor
LC	Liquid chromatography
LC-MS	Liquid chromatography coupled to mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
m	Meter

M	Molar
mg	milligram
Min	Minute
mL	Milliliter
MS	Mass spectrometry
NF	National formulary
ng	Nanogram
NIR	Near Infrared
nm	Nanometer
NSAIA	Non-Steroidal anti-inflammatory agent
pK_a	Acid dissociation constant
PAR	Paracetamol
Ph. Int.	International Pharmacopoeia
R^2	Coefficient of determination
R_s	Resolution
s	Second
TLC	Thin layer chromatography
t_R	Retention Time
µm	micrometer
USP	United States Pharmacopoeia
UV	Ultraviolet
WHO	World Health Organization

ABSTRACT

Pain is a common menace afflicting many people worldwide. There are a myriad of medications that are available commercially to combat all forms of pain. The quality of medicines is an important aspect in healthcare provision. However, more focus is put on the quality of drugs for the major diseases such as tuberculosis, HIV/AIDS and malaria than for analgesics. There is little quality surveillance of the pain medications that are available commercially in Kenya. There are no official methods for the simultaneous analysis of most of the drug compounds present in the commonly available pain medications. Only the methods for the analysis of paracetamol and caffeine preparations and paracetamol and codeine phosphate preparations are available. This makes the analysis of samples expensive and time consuming as each component would be analyzed individually.

In the present study, a simple, rapid, precise, sensitive and robust isocratic elution reversed-phase liquid chromatographic method was developed for the simultaneous determination of caffeine, chlorzoxazone, codeine, diclofenac, doxylamine, ibuprofen and paracetamol. These seven compounds are present in some commonly available formulations used for pain management in Kenya.

A mixture of these seven compounds was separated using a liquid chromatographic system with a mobile phase consisting of methanol-0.25 M sodium octanesulfonate-0.2 M ammonium acetate pH 6.5-water (50:2:10:38, % v/v/v/v). This was delivered at a flow rate of 1 mL/min through a column with the dimensions 250 mm in length and 4.6 mm internal diameter packed with an octyldecylsilane stationary reverse phase (Phenomenex Gemini® 5μ, C_{18}) maintained at a temperature of 40 °C using a column oven and a UV detection wavelength of 220 nm.

Validation of the method demonstrated that the limit of detection for caffeine, chlorzoxazone, codeine, diclofenac, doxylamine, ibuprofen and paracetamol were 11.99 ng, 20.72 ng, 25.08 ng, 12.50 ng, 32.93 ng, 12.02 ng, 22.36 ng, and the limit of quantitation were 79.92 ng, 103.60 ng, 104.50 ng, 62.49 ng, 164.64 ng, 48.08 ng, 111.80

ng respectively. The method was accurate with recovery rates of 100.3% (caffeine), 101.6% (chlorzoxazone), 98.3% (codeine), 98.1% (diclofenac), 102.7% (doxylamine), 99.2% (ibuprofen) and 98.1% (paracetamol). The method was linear over a concentration range of 75% to 125% for all seven compounds with the respective coefficient of determination (R^2) values being 0.9995 (caffeine), 0.9986 (chlorzoxazone), 0.9992 (codeine), 0.9993 (diclofenac), 0.9975 (doxylamine), 0.9998 (ibuprofen) and 0.9960 (paracetamol). The method also demonstrated adequate intra-day and intermediate precision with intra-day precision coefficients of variation ranging from 0.15-0.37% and intermediate precision coefficients of variation ranging from 0.91-1.96% for the seven compounds.

The method developed was used for the analysis of four randomly selected commercially available pain medications containing varying combinations of the seven compounds. Three batches of each of the drug samples were analyzed and the results obtained demonstrated that there was minimal batch variation. The assay values for caffeine, chlorzoxazone, diclofenac and ibuprofen ranged from 95.3-102%, 92.0-96.6%, 95.5-98.6% and 96.7-99.3% respectively. The assay values of paracetamol ranged from 99.6-117%. One product was found to have values of paracetamol consistently higher than the adopted limits with assay values ranging from 115-117%. The levels of codeine and doxylamine were found to be consistently below the adopted specifications with assay values ranging from 51.3-53.2% and 65.2- 67.2% respectively.

The method can be used in the analysis of pain medications containing any combination of the seven compounds and it can therefore be adopted by quality control laboratories for routine analysis and in the post market surveillance of pain medications.

CHAPTER ONE

INTRODUCTION

1.1. Pain

Pain is an unpleasant and unique physical and psychological experience ranging from mild discomfort to agonized distress, associated with real or potential tissue damage. Pain is a protective mechanism and occurs whenever body tissues are damaged and results in an individual's attempt to remove the pain stimulus. Pain is usually in response to impulses from the peripheral nerves in damaged tissues. Attention is a crucial component of pain and thus distraction can act as a basis for pain therapy and on the other hand, anxiety and depression can focus attention and exaggerate the pain. If the nerve pathways are damaged the brain can increase the amplification in the pathway, maintaining the pain sensation as a protective mechanism [1-3].

Many ailments of the human body cause pain. The ability to diagnose different diseases depends largely on the physician's knowledge of the different qualities of pain. Pain is classified into fast pain and slow pain. Fast pain is also referred to as sharp pain, pricking pain, acute pain and electric pain. It is felt about 0.1 seconds after application of a pain stimulus and is not felt in deeper tissues of the body. Slow pain is also referred to as slow burning pain, aching pain, throbbing pain, nauseous pain and chronic pain. It is felt one second or more after application of pain stimulus and then increases slowly over time. It usually results in tissue damage on the skin and other deep organs in the body and can lead to prolonged unbearable suffering [4].

1.2. Physiology of pain

The sense organs for pain are naked nerve endings. They are widespread in the superficial layers of the skin and in certain internal tissues, such as the arterial walls, the joint surfaces and the falx and tentorium in the cranial vault. Most other deep tissues are sparsely supplied with pain endings. Nevertheless, any widespread tissue damage can summate to cause the slow-chronic-aching type of pain in most of these areas [3, 4].

Mechanical, thermal and chemical pain stimuli excite the pain receptors. Some of the chemicals that can excite the pain receptors are bradykinin, serotonin, histamine, cytokines, potassium ions, acids, acetylcholine and proteolytic enzymes. Prostaglandins and substance P enhance the sensitivity of the naked nerve endings but do not directly excite them [4, 5].

Pain receptors use two separate pathways for transmitting pain signals into the CNS. These two pathways partially correspond to the two types of pain, fast-sharp pain pathway and slow-chronic pathway. The fast-sharp pain signals are elicited by either mechanical or acute thermal pain stimuli. They are transmitted in the peripheral nerves to the spinal cord by small myelinated type Aδ fibers 2-5 μm in diameter, at velocities of between 6 and 30 m/s. In the spinal cord the type Aδ fibers excite neurons of the neospinothalamic tract. A few fibers of the neospinothalamic tract terminate in the reticular areas of the brain stem, but most pass all the way to the thalamus [3, 4].

The slow-chronic pain is specifically elicited by chemical type of stimuli but at times by persisting mechanical or thermal stimuli. It is transmitted in the peripheral nerves to the spinal cord by unmyelinated type C fibers 0.4-1.2 μm in diameter, at velocities of about 0.5 and 2 m/s. Slow-chronic pain is transmitted to the brain by the paleospinothalamic pathway. It is a phylogenetically much older system and transmits pain mainly carried in the peripheral type C fibers, although it also transmits some signals from type Aδ fibers as well. The paleospinothalamic pathway terminates widely in the brain stem. Only about one-tenth to one-fourth of the fibers pass to the thalamus [3, 4].

A sudden onset of painful stimulus often gives a double pain sensation due to this double system of pain innervations; a fast-sharp pain followed by a slow pain. The sharp pain alerts an individual of a damaging influence and therefore plays an important role in making the individual react immediately to remove oneself from the stimulus. The slow pain tends to become more and more painful over a period of time. This sensation eventually gives one the intolerable suffering of long continued pain [4].

1.3. The natural pain suppression system

The degree to which different individuals react to pain varies. This results partly from the capacity of the brain to suppress the input of pain signals to the nervous system by activating a pain control system. This system transmits signals down the spinal cord to a pain inhibitory complex in the spinal cord. At this point, the analgesia signals can block pain before it is relayed to the brain [4].

Several transmitter substances are involved in the analgesia system including enkephalins and serotonin. Serotonin causes local cord neurons to secrete enkephalin which in turn elicits both presynaptic and postsynaptic inhibition of incoming type C and type Aδ pain fibers. Thus, the analgesia system can block pain signals at the initial entry point to the spinal cord [4].

1.3.1. The opiate system in the brain

Morphine-like agents act at many points in the analgesia system causing pain relief. These opiate-like substances include β-endorphin, met-enkephalin, leu-enkephalin and dynorphin. The two enkephalins are found in the brain stem and spinal cord, in the analgesia system and β-endorphin is present in both the hypothalamus and the pituitary gland. Dynorphin is found mainly in the same areas as the enkephalins but in much lower quantities. Although the fine details of the brain's opiate system are not completely understood, activation of the analgesia system by nervous signals or inactivation of pain pathways by morphine-like drugs can suppress many pain signals [4].

1.3.2. The tactile sensory signals

Stimulation of large type Aβ sensory fibers from the peripheral tactile receptors can depress the transmission of pain signals. Thus, touching or shaking an injured area decreases the pain of the injury. The relief is primarily due to the inhibition of pain pathways in the dorsal horn gate by stimulation of large-diameter touch-pressure afferents. This mechanism and simultaneous psychogenic excitation of the central analgesia system probably form the basis of pain relief by acupuncture [3, 4].

1.4. Artificial pain suppression

This is achieved in several ways, the most common being pharmacological interference using drugs that have intrinsic analgesic activity. These drugs can be divided into three main classes as described in the WHO three-step pain ladder for the management of cancer pain. Pain therapy should start with non-opioid drugs like paracetamol and the non-steroidal anti-inflammatory agents followed by weak opioids like codeine, dextropropoxyphene and combinations of codeine with paracetamol. Only when there is still is no pain relief should strong opioid drugs such as morphine and diamorphine be used [6].

Drugs that have no intrinsic analgesic activity can also be used. These drugs are referred to as co-analgesics. These drugs have a primary use in other conditions but are also effective, either alone or in combination with conventional analgesics. Tricyclic antidepressants and anticonvulsants are used in deafferentation pain while calcium channel blockers are used in sympathetically mediated pain. Muscle relaxants, antibiotics and steroids each also relieve pain when used in appropriate situations [2].

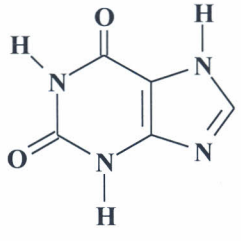
Acupuncture, ice, heat, ultrasound, massage and spinal cord stimulation can all achieve analgesia [2]. Several clinical procedures have been developed for suppressing pain by electrical stimulation of large sensory nerve fibers. The stimulating electrodes are placed on selected areas of the skin or the brain. The patient personally controls the degree of stimulation. Dramatic relief has been reported in some instances. The pain relief often lasts as long as 24 hours after only a few minutes of stimulation [4]. Pain pathways can also be blocked either temporarily or permanently by local anesthetics [2].

Four of the drugs in the current study namely codeine, diclofenac, ibuprofen and paracetamol have intrinsic analgesic activity while three of the drugs are co-analgesics namely caffeine, chlorzoxazone and doxylamine.

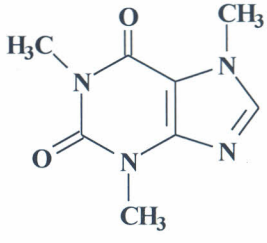
1.5. Chemistry and pharmacology of the co-analgesic compounds under study

1.5.1. Caffeine

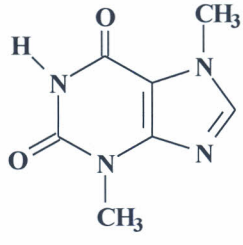
Caffeine is probably the most widely used social drug worldwide. It is obtained from coffee or from the dried leaves of *Camellia sinensis*, or prepared synthetically. It is also present in *guarana, maté, and kola* [7, 8]. Caffeine together with theophylline and theobromine are three closely related methylxanthine alkaloids that occur in plants widely distributed geographically. Caffeine is ingested through soft drinks, coffee, tea, cocoa, chocolate and prescription or over the counter drugs. The basis for the popularity of caffeine containing beverages is the ancient belief that they have stimulant and antisoporific actions that elevate mood, decrease fatigue and increase capacity for work [9].



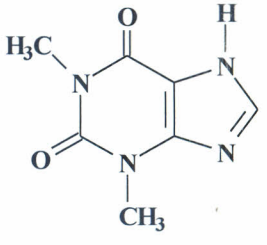
Xanthine



Caffeine



Theobromine



Theophylline

Figure 1.1: Chemical structures of caffeine and some related xanthines

Caffeine is a dioxypurine methylated at positions 1, 3 and 7. Its chemical name is 3,7-Dihydro-1,3,7-trimethyl-1H-purine-2,6-dione, molecular formula $C_8H_{10}N_4O_2$ and a molecular weight 194.19 (Figure 1.1). It occurs as silky white crystals, usually matted together or a white crystalline powder. It is soluble in water, alcohol, acetone, pyrrole,

tetrahydofuran and ethyl acetate and slightly soluble in ether and benzene. Caffeine has a melting point of 238 °C and is basic with a pK_a of 14.0 at 25 °C [8, 10, 11].

Caffeine exerts its central actions by blocking adenosine receptors. Adenosine modulates adenylyl cyclase activity thus causing contraction of isolated airway smooth muscle as one of its peripheral actions. At high concentrations, the methylxanthines inhibit phosphodiesterase, thereby inhibiting the breakdown of cAMP and increasing its concentration inside cells. The increased intracellular cAMP levels mediate most of caffeine's pharmacological actions [7, 10].

Caffeine is absorbed readily after oral administration while absorption following rectal administration by suppository may be slow and erratic. It is also absorbed through the skin. Absorption through intramuscular route is slower than oral formulations [12]. In the absence of food, solutions or uncoated tablets of caffeine produce maximal concentrations in plasma within 1 hour. Caffeine is distributed into all body compartments including crossing the placenta and in breast milk. The apparent volume of distribution is 0.4-0.6 L/kg body weight. Caffeine is eliminated primarily by metabolism in the liver with less than 5% of administered caffeine being recovered in urine unchanged. Metabolism takes place via oxidation, demethylation and acetylation to 1-methyluric acid, 1-methylxanthine, 7-methylxanthine, 1,7-dimethylxanthine, 5-acetylamino-6-formylamino-3-methyluracil and other metabolites that are excreted in urine. Neonates lack the capacity to metabolize it. Caffeine has a plasma half-life of 3-7 hours which increases by about twofold in women during the later stages of pregnancy or with long-term use of oral contraceptive steroids and 100 hours in neonates [9, 12].

Clinically caffeine is used orally alone and in combination with analgesics for the treatment of headache. Caffeine exerts no intrinsic analgesic activity. Analgesic-caffeine combinations have however been reported to produce a synergistic effect and a beneficial effect on mood. These results however, have not always been reproducible in well-controlled studies [10]. Caffeine has also been used for the management of apnea of preterm infants [9]. Caffeine is normally administered by mouth in doses of 50 to 250

mg. Therefore most of the pharmaceutical formulations available in the market contain between 50-100 mg caffeine in combination with other active pharmaceutical ingredients, mainly analgesics.

1.5.2. Chlorzoxazone

Chlorzoxazone was first manufactured by McNeil Pharmaceutical Company USA in 1958 and was marketed as Paraflex®. Chlorzoxazone is a metabolite of zoxazolamine whose hepatotoxicity excludes it from commercial application.

Chlorzoxazone **Zoxazolamine**

Figure 1.2: Chemical structures of chlorzoxazone and zoxazolamine

Chlorzoxazone is 5-chloro–2(3*H*)-benzoxazolone, a benzoxazole derivative of molecular formula $C_7H_4ClNO_2$ and molecular weight 169.58 (Figure 1.2). It occurs as colourless crystals or white crystalline powder with a bitter taste and a melting point of 190-194 °C. The compound is slightly soluble in water, soluble in methanol, ethanol and isopropanol. It is freely soluble in aqueous solutions of alkali hydroxides and ammonia. Chlorzoxazone is basic with a pK_a of is 8.0 at 20 °C [8, 10, 11].

Chlorzoxazone is a centrally acting skeletal muscle relaxant with sedative effects. Its actual mode of action has not been identified but it is thought to inhibit muscle spasms by acting at the level of the spinal cord and subcortical areas of the brain.

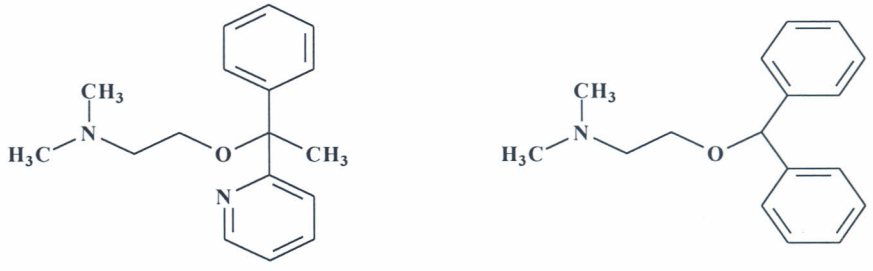
After oral administration, chlorzoxazone is rapidly and completely absorbed. Peak plasma concentrations are attained within 1-4 hours. The onset of action is usually within 1 hour while the duration of action is 3-4 hours. It is metabolized in the liver to 6-

hydroxychlorzoxazone and excreted in urine as the glucuronide. Less than 1% is excreted as unchanged drug in urine. It has a plasma half-life of about 1 hour [10, 12].

Clinically, chlorzoxazone is used as an adjunct in the symptomatic treatment of musculoskeletal conditions associated with painful muscle spasms [12]. It is usually marketed as oral tablets containing 250 and 500 mg of chlorzoxazone [7] and in compound analgesic preparations of 250 mg.

1.5.3. Doxylamine

Doxylamine is an ethanolamine-derivative H_1 histamine receptor antagonist [7].



Doxylamine Diphenhydramine

Figure 1.3: Chemical structures of doxylamine and diphenhydramine

The chemical name of the compound is *N*, *N*-Dimethyl–2-[1–phenyl–1-(2–pyridinyl)] ethoxy ethanamine (Figure 1.3) with molecular formula $C_{17}H_{22}N_2O$ and molecular weight 270.4. Doxylamine is commercially available as the succinate salt with the molecular formula $C_{17}H_{22}N_2O.C_4H_6O_4$ (molecular weight 388.5). Doxylamine succinate is a white or creamy white powder with a characteristic odor and a melting point of 100-104 °C. It is soluble in water, ethanol and chloroform and slightly soluble in benzene. Doxylamine is basic with pK_a values of 4.4 and 9.2 [8, 10].

Following oral administration, doxylamine is rapidly absorbed and peak plasma concentrations are achieved within 2-3 hours. It readily crosses the blood brain barrier [7, 10] and it has a half-life of about 10 hours in healthy adults [10].

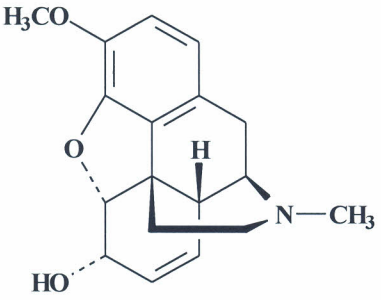
Doxylamine is a first-generation H_1 receptor antagonist which exerts its effects in the body by competitive, reversible blockade of H_1 histamine receptors. It also has antimuscarinic and sedative effects, due to its similarity to the general structure of drugs that have muscarinic cholinoceptor, α-adrenoceptor, serotonin and local anesthetic receptor binding [7, 12]. It is used for the symptomatic relief of hypersensitivity reactions and pruritic skin disorders, as a hypnotic in the short term treatment of insomnia and as an ingredient of compound preparations for symptomatic treatment of coughs, common cold and in pain preparations [12]. Compound analgesic preparations usually contain doxylamine succinate 5 mg.

1.6. Chemistry and pharmacology of the analgesics compounds under study

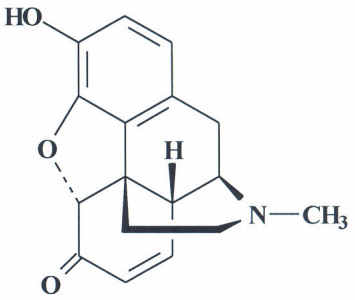
1.6.1. Codeine

Codeine also known as methylmorphine is an alkaloid present in the poppy plant, *Papaver somniferum.* The milky juice of the plant contains several of alkaloids including morphine, codeine and papaverine. Codeine was first isolated in 1832 in France by Pierre Robiquet, a French chemist and pharmacist. It is currently the most therapeutically used opiate in the world [9].

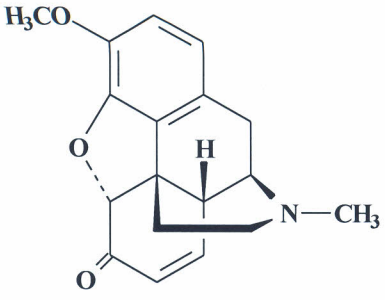
The chemical nomenclature of codeine is (5α,6α)-7,8-Didehydro–4,5–epoxy–3–methoxy–17–methylmorphinan–6–ol monohydrate, a phenanthrene derivative opiate agonist (Figure 1.4). It has the molecular formula $C_{18}H_{21}NO_3$ (molecular weight 299.36). It occurs as colourless or white crystals with a melting point of 154-156 °C. It is basic with a pK_a of 8.2 at 20 °C [8, 10, 11].



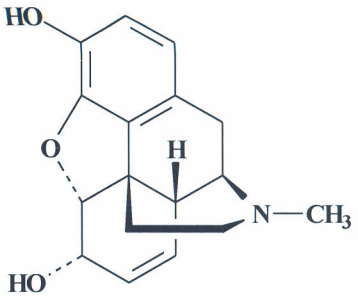
Codeine



Hydromorphone



Oxycodone



Morphine

Figure 1.4: Chemical structures of codeine and some related opioids

Codeine produces analgesia through interaction with opioid receptors for which it has very low affinity. The analgesic effect of codeine is due to its conversion to morphine. About 10% of administered codeine is O-demethylated to form morphine. This conversion is effected by the cytochrome P450 isoenzyme CYP2D6. This enzyme undergoes genetic polymorphism that leads to the inability to convert codeine to morphine by 10% of the caucasian population thus renders codeine ineffective as an analgesic. Other polymorphisms can lead to enhanced metabolism and thus increasing the sensitivity to codeine's effects. Codeine also has antitussive effects [9].

Codeine is approximately 60% as effective orally as parenterally. Administration of codeine phosphate orally produces peak plasma concentrations in about 1 hour. Codeine is metabolized by O- and N-demethylation in the liver to morphine, norcodeine, normorphine and hydrocodone. Codeine and it metabolites are excreted almost entirely in urine, mainly as glucuronide conjugates. The plasma half-life is 3-4 hours after oral or intramuscular administration [9, 12].

Clinically codeine is used in the relief of mild to moderate pain that cannot be relieved by non-opiate analgesics. It is used in combination with other analgesics to produce an additive analgesic effect. The analgesics commonly used in combination include aspirin and paracetamol [10].

Codeine is usually marketed as tablets containing codeine phosphate 15 mg, 30 mg and 60 mg. Codeine is also an ingredient in some compound analgesic preparations. The content of codeine in these preparations varies from manufacturer to manufacturer but rarely exceeds 50 mg. Codeine is also present in antitussive preparations; usually in the concentrations of 3 mg/ 5 mL and 15 mg/ 5 mL.

1.6.2. Diclofenac

Diclofenac was first marketed by the Ciba-Geigy company in 1973 under the brand name Voltaren®.

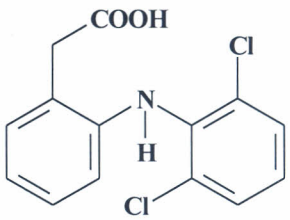


Figure 1.5: Chemical structure of diclofenac

The chemical name is 2-[(2,6-Dichlorophenyl) amino] benzeneacetic acid (Figure 1.5) is a phenylacetic acid derivative non-steroidal anti-inflammatory agent (NSAIA) with molecular formula $C_{14}H_{11}Cl_2NO_2$ and molecular weight 292.2. Diclofenac occurs as the sodium or potassium salt and is faintly yellowish white, practically odorless, slightly hygroscopic crystalline powder. It is acidic with a pK_a of 4.2 at 25 °C and a melting point of 156-158 °C. It is soluble in water, methanol and acetone and slightly soluble in acetonitrile and cyclohexane [8, 10, 11].

Diclofenac exhibits anti-inflammatory, analgesic and antipyretic activity. It is a potent, relatively non-selective cyclooxygenase inhibitor. It also decreases arachidonic acid levels probably by altering release or uptake of fatty acids [7, 9, 10].

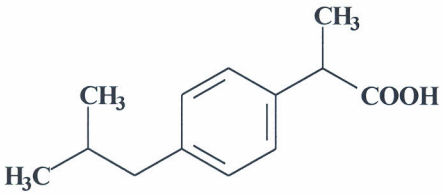
Diclofenac is rapidly absorbed when administered orally, rectally or intramuscularly. Administration with food slows down the absorption but does not alter the extent of absorption. Orally administered diclofenac is subjected to substantial first pass effect with 50-60% of the drug reaching systemic circulation unchanged. At therapeutic doses it is more than 99% plasma protein bound with a plasma half-life of 1-2 hours. Diclofenac penetrates synovial fluid and has been detected in breast milk. It is metabolized in the liver by a cytochrome P450 CYP2C subfamily to 4-hydroxydiclofenac (the major metabolite), 5-hydroxydiclofenac, 3-hydroxydiclofenac and 4, 5-dihydroxydiclofenac. It is excreted as the glucuronide and sulphate conjugates in urine (65%) and bile (35%) [9, 12].

Diclofenac is used for the relief of pain and inflammation in conditions such as rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, renal colic, acute gout and following some surgical procedures [10]. It may also be used in the management of primary dysmenorrhea where it relieves pain and reduces the frequency and severity of uterine contractions [10].

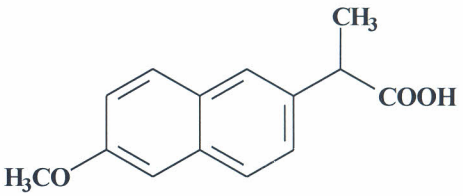
Diclofenac is marketed as tablets containing 25 mg, 50 mg, 75 mg and 100 mg of the active ingredient. Suppositories containing 100 mg and intramuscular injection with 25 mg/mL of the drug are also available. Diclofenac is usually present in the strength of 50 mg in compound analgesic preparations.

1.6.3. Ibuprofen

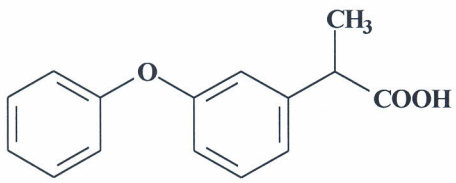
Ibuprofen belongs to the class of arylpropionic acid NSAIA. It was developed by the research arm of Boots Group UK during the 1960s and marketed as Brufen® in the United Kingdom.



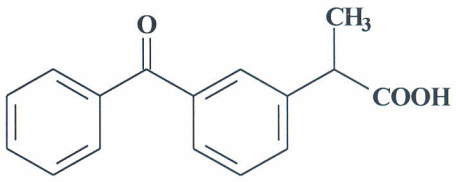
Ibuprofen



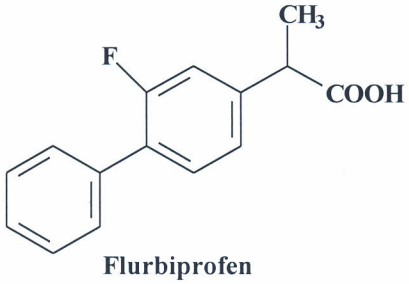
Naproxen



Fenoprofen



Ketoprofen



Flurbiprofen

Figure 1.6: Chemical structures of ibuprofen and related arylpropionic acid derivatives

Ibuprofen is α-methyl–4-(2–methylpropyl) benzeneacetic acid and is commercially available as a racemic mixture of two optical isomers whereby only the *l* isomer has clinical activity. In adults, 60% of the *d* isomer is converted slowly to the *l* isomer. The molecular formula of the compound is $C_{13}H_{18}O_2$ (Figure 1.6) while the molecular weight is 206.27. The drug occurs as a white to off white crystalline powder with a melting point of 75-77 °C. It has a slight, characteristic odor and is practically insoluble in water, soluble in ethanol, chloroform and ether. It is acidic with pKa values of 4.4 and 5.2 [8, 10, 11].

Ibuprofen is rapidly absorbed after oral administration and achieves peak plasma levels within 15-30 min with a plasma half-life of 2 hours. Ibuprofen is extensively bound to

plasma proteins (about 99%). It is rapidly excreted in urine mainly as the hydroxylated and carboxylated metabolites and their conjugates [9, 12].

It is an effective cyclooxygenase inhibitor. It also alters platelet function and prolongs bleeding time. It has useful anti-inflammatory, analgesic and antipyretic activities [9]. It has been postulated that it produces antipyresis by acting on the hypothalamus with heat dissipation being increased because of vasodilation and increased peripheral blood flow [10].

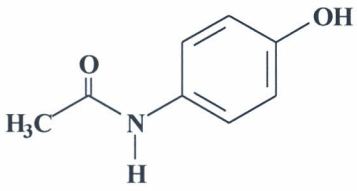
Ibuprofen is used for the relief of mild to moderate pain in conditions such as primary dysmenorrhea, acute and chronic rheumatoid arthritis and osteoarthritis, ankylosing spondylitis and other musculoskeletal and joint disorders such as sprains and strains. The compound also possesses antipyretic properties [10, 12].

Ibuprofen is usually marketed as tablets containing 200 mg, 400 mg and 600 mg of the drug. Pediatric oral solutions are also available containing 100 mg/5mL ibuprofen. Compound analgesic preparations usually contain ibuprofen 200 mg in tablets or capsules.

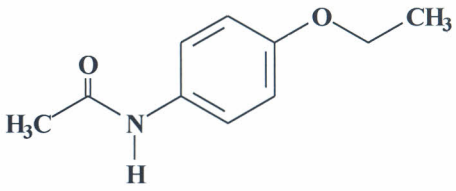
1.6.4. Paracetamol

Paracetamol is a synthetic derivative of p-aminophenol. The parent member of this group of drugs is acetanilide which was first introduced in 1886 by A. Cahn and P. Hepp. Paracetamol was first introduced in medicine by Joseph Von Mering in 1893 but only gained popularity after 1949 when it was recognized as a major active metabolite of both acetanilide and phenacetin [9].

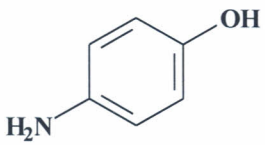
Paracetamol is *N*-(4-hydroxyphenyl) acetamide with molecular formula $C_8H_9NO_2$ and molecular weight 151.16 (Figure 1.7). It occurs as a white crystalline powder with a melting point of 169-170.5 °C and a slight bitter taste. Paracetamol is soluble in water and alcohol. Paracetamol is basic with a pK_a of is 9.5 at 25 °C [8, 10, 11].



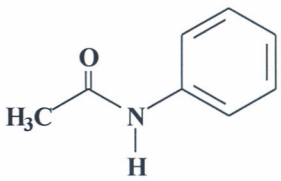
Paracetamol



Phenacetin



4-Aminophenol



Acetanilide

Figure 1.7: Chemical structures of paracetamol and some structurally related compounds

Paracetamol is used for the treatment of mild to moderate pain when an anti-inflammatory effect is not necessary. It is a weak prostaglandin inhibitor in peripheral tissues and possesses no significant anti-inflammatory effects. It produces analgesia and antipyresis by inhibition of prostaglandin synthesis. The compound does not affect uric acid levels and lacks platelet-inhibiting properties [7, 9, 10].

Paracetamol is readily absorbed from the gastro-intestinal tract with peak plasma concentrations occurring about 10-60 min after oral administration and a plasma half-life of about 2 hours. It is distributed into most body tissues, crosses the placenta and is present in breast milk. Plasma-protein binding is negligible at normal therapeutic doses but increases with increasing concentrations with 20-50% bound in acute intoxication concentrations. Paracetamol is metabolized mainly in the liver and excreted in urine mainly as the glucuronide (60%) and sulphate (35%) conjugates. Less than 5% is excreted unchanged. A small proportion undergoes cytochrome P450-mediated N-hydroxylation to form N-acetyl-benzoquinoneimine which during an over dosage may accumulate and cause tissue damage [9, 12].

Paracetamol on its own is ineffective in the management of inflammatory conditions such as rheumatoid arthritis, but it can be used as an adjunct to anti-inflammatory therapy. It is

the drug of choice for mild analgesia in patients who are allergic or intolerant to aspirin and other non-steroidal anti-inflammatory agents (NSAIAs). Paracetamol does not antagonize the effects of uricosuric agents and thus can be concomitantly used with probenecid in the treatment of gout [7, 10].

Paracetamol is available commercially in various dosage forms. It is usually present as 500 mg, dispersible or chewable tablets, flavored syrups for pediatrics containing paracetamol 120 mg and 250 mg/5 mL and 60 mg suppositories [7]. In compound analgesic preparations it is usually present in concentrations ranging from 200 mg-500 mg.

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

Several methods for the analysis of caffeine, chlorzoxazone, codeine phosphate, diclofenac sodium, doxylamine succinate, ibuprofen and paracetamol have been described in official pharmacopoeias and in published scientific papers.

1.7.1. Caffeine

The USP (2009) specifies a liquid chromatographic method for the analysis of caffeine raw material. No official method for any caffeine finished product is prescribed [13]. The BP (2007) specifies a non-aqueous titration method using 0.1 M acetous perchloric acid for the analysis of caffeine raw material with no official method prescribed for any caffeine finished product [14]. The Ph. Int. (2006) also specifies a non-aqueous titration method for the analysis of anhydrous caffeine raw material however, no method for caffeine finished products are prescribed [15].

Several methods for the analysis of caffeine have been reported in literature. Abourashed and Mossa (2004) described a HPTLC–UV densitometric method of analysis [16] while Alkaysi *et al.* (2008) used a rapid HPLC method for the determination of caffeine in plasma and saliva [17]. Senetskaya and Sell (1974) described a spectrophotometric titration method without the use of an indicator for the determination of amidopyrine, caffeine, phenacetin and their mixtures using 0.1 N perchloric acid as a titrant. The

method utilized the difference in absorption spectra of the compounds in their protonated and nonprotonated forms [18]. Song and Ashley (1998) also described a method for the determination of caffeine using gas chromatography coupled to mass spectrometry [19]. Ford *et al.* (2005) also reported a method for quantitative determination of caffeine on reversed-phase C_8 TLC using a surface sampling electrospray ionization system with tandem mass spectrometry detection [20]. Another HPTLC method for screening and quantitative estimation of caffeine in different extracts of tea samples was described by Misra *et al.* (2009). Separation was performed on silica gel 60 F_{254} HPTLC plates with ethyl acetate-methanol (90:10 % v/v) as a mobile phase. The determination was carried out using UV 274 nm [21]. A micellar electrokinetic chromatographic method was described by Toshiro *et al.* (1998) for the quantitative determination of individual catechins, caffeine and ascorbic acid from commercially canned green and black teas [22].

1.7.2. Chlorzoxazone

The USP (2009) specifies a UV spectrophotometric method using a wavelength of detection of 282 nm for the assay of chlorzoxazone raw material. A LC method using a C_{18} reverse phase column and water-acetonitrile-glacial acetic acid (70:30:1 % v/v/v) as the mobile phase is used for the analysis of the chlorzoxazone tablets [13].

There are several non-pharmacopoeial methods for the analysis of chlorzoxazone. Haque and Stewart (1998) used a semi permeable surface HPLC column to develop a direct injection method for the simultaneous determination of chlorzoxazone and its 6-hydroxy metabolite in serum [23]. Bari *et al.* (1997) developed a method for the analysis of ibuprofen, chlorzoxazone and acetaminophen using packed column supercritical fluid chromatography [24] while Shaikh and Devkhile. (2008) developed a reversed-phase LC method for the simultaneous determination of paracetamol, aceclofenac and chlorzoxazone in commercial pharmaceutical preparations. The method employs a 250 x 4.6 mm C_{18}, 5μ analytical column and a mobile phase consisting of acetonitrile-50 mM orthophosporic acid at a pH of 6 (40:60 %v/v) with a flow rate of 1 mL/min and the detector wavelength set at 270 nm [25]. Mu-Chang *et al.* (1997) used a two-dimensional

TLC for qualitative analysis of caffeine, indomethacin, ethoxybenzamide, chlorzoxazone and diazepam. For quantitative analysis, a reversed-phase HPLC method was established for simultaneous determination of the five drugs [26]. Pawar *et al.* (2009) developed a method for the simultaneous determination of aceclofenac, paracetamol and chlorzoxazone. Chromatographic separation of the three drugs was performed on a C_{18} column (250 mm x 4.6 mm, 5μ) with a mobile phase comprising of 10 mM potassium dihydrogen phosphate at pH 5.55-acetonitrile (60:40 % v/v) at a flow rate of 1.0 mL/min and UV detection at 205 nm [27] while Anju and Sandeep (2007) developed a HPLC method for determination of paracetamol, chlorzoxazone and diclofenac using a reversed phase C_8 column with UV detection at 280 nm. The mobile phase consisted of acetonitrile-0.05 M ammonium dihydrogen phosphate at pH 4.06 (60:40 % v/v) and a flow rate of 1.5 ml/min [28]. Stewart and Chan (2006) developed a flourimetric method for the determination of chlorzoxazone. The method is based on the intrinsic drug fluorescence in chloroform using excitation and emission wavelengths of 286 and 310 nm respectively [29].

1.7.3. Codeine

The USP (2009) prescribes a non-aqueous titration using 0.1 N perchloric acid and a potentiometric end point for the analysis of the raw material. For codeine phosphate tablets, the USP prescribes a titration using 0.02 N sulphuric acid and methyl red with an initial extraction step for the alkaloid using 6 N ammonium hydroxide and chloroform [13]. The BP (2007) also specifies a non-aqueous titration method using 0.1 M Perchloric acid and crystal violet for the analysis of the raw material. For codeine phosphate tablets, the BP specifies a titration using 0.1 M sodium hydroxide and methyl red with an initial extraction step of the alkaloid using 5 M ammonia and chloroform [14]. The Ph. Int. (2006) specifies a non-aqueous titration using 0.1 M perchloric acid for the assay of the raw material. For codeine phosphate tablets a non-aqueous titration using 0.05 M perchloric acid with a prior extraction step using 2 M sodium hydroxide and chloroform is prescribed [15].

Various non-official methods have been developed for the determination of codeine phosphate. Değim *et al.* (2001) developed a HPLC method for the simultaneous determination of codeine and dionin in antitussive analgesic tablet formulations using a C_{18} column and a mobile phase consisting of methanol-water (33:67 % v/v) with a detection wavelength of 210 nm [30]. Sisco *et al.* (1985) also developed a HPLC method for the evaluation of codeine phosphate drug substance derived from poppy straw or opium concentrate [31]. Another HPLC method was described by Murat (2001) for the simultaneous determination of paracetamol, caffeine and codeine phosphate. It used a C_8 column and as mobile phase 0.01 M potassium dihydrogen phosphate-methanol-acetonitrile-isopropyl alcohol (84:4:6:6 % v/v/v/v) and spectrophotometric detection at a wavelength of 215 nm [32].

1.7.4. Diclofenac

The USP (2009) specifies a non-aqueous titration method using 0.1N perchloric acid and a potentiometric end point for the assay of diclofenac sodium raw material. Further the USP specifies a liquid chromatographic method for the assay of diclofenac sodium delayed release tablets with the mobile phase consisting of phosphate buffer (pH 2.5)-methanol (30:70 % v/v) using a C_8 reverse phase column [13]. The BP (2007) specifies a non-aqueous titration method using 0.1 M perchloric acid and a potentiometric end point for the assay of the raw material. It also specifies a LC method using a C_8 reverse phase silica based column, as mobile phase with a flow rate of 1 mL/minute, 0.1 % w/v phosphoric acid-0.16 % w/v sodium dihydrogen phosphate pH 2.5-methanol and (17:17:66) and a detection wavelength of 254 nm for the analysis of gastro-resistant diclofenac tablets and prolonged-release diclofenac tablets [14].

Various researchers have described other methods for the analysis of diclofenac sodium. Donato *et al.* (1994) described two methods for the quantification of ibuprofen, indomethacin, ketoprofen, piroxicam and diclofenac in suspension, suppositories, capsules, injection solutions and tablets by capillary zone electrophoresis and micellar electrokinetic capillary chromatography [33]. de Micalizzi *et al.* (1998) used a spectrophotometric method to determine diclofenac and benzyl alcohol in injectable

formulations by applying the first-derivative method of crossing zero for diclofenac sodium and second derivative for benzyl alcohol [34]. Mohammed *et al.* (2001) developed a LC-MS method for the determination of diclofenac sodium, flufenamic acid, indomethacin and ketoprofen [35]. Pimenta *et al.* (2002) implemented two independent methods for the simultaneously determination of diclofenac in an automated analytical system. Potentiometric detection was carried out with an ion-selective electrode based on a cyclodextrin and a fluorimetric determination with the sample previously subjected to in-line irradiation with UV light [36]. Matthieu and de Souza (2006) presented a quantitative analytical method for the determination of diclofenac in pharmaceutical preparations by diffuse reflectance in the visible region of the spectrum [37]. Pérez-Ruiz *et al.* (1997) described spectrophotometric determination of diclofenac by liquid–liquid extraction using acridine yellow with a flow system [38].

1.7.5. Doxylamine

The USP (2009) specifies a non aqueous titration to a crystal violet end point for the raw material, a UV spectrophotometric method with a detection wavelength of 262 nm for doxylamine oral solution and a LC method with a mobile phase composed of a mixture of monobasic potassium phosphate, triethylamine, sodium lauryl sulphate and acetonitrile for the tablets [13]. The BP (2007) specifies a non-aqueous titration with a potentiometric end point for the pharmaceutical substance [14].

Other methods for analysis of doxylamine include that reported by Pathak and Rajput (2008) for a simultaneous derivative spectrophotometric analysis of doxylamine succinate, pyridoxine hydrochloride and folic Acid in combined tablet formulations [39]. Argekar and Sawant (1999) developed a simple, precise and rapid ion pair reversed-phase high-performance liquid chromatography method for the simultaneous determination of pyridoxine hydrochloride and doxylamine succinate in tablets [40]. They also described another simple, precise, accurate, rapid and stability indicating high performance thin layer chromatography method for the simultaneous determination of pyridoxine hydrochloride and doxylamine succinate in tablets [41].

1.7.6. Ibuprofen

The USP (2009) prescribes a LC method with a mobile phase consisting of 4 mg/mL chloroacetic pH 3-acetonitrile (40:60 % v/v), a C_{18} reverse phase column and a detection wavelength of 254 nm for the assay of ibuprofen raw material and ibuprofen tablets [13]. The BP (2007) specifies a titration method using 0.1 M sodium hydroxide and phenolphthalein solution for the analysis of the raw material. The BP specifies a LC method with phosphoric acid-water-methanol (0.3:24.7:75 % v/v/v) as the mobile phase with a flow rate of 1.5 mL/min, a 25 cm × 4.6 mm, C_{18} reverse phase column and a detection wavelength of 264 nm for the assay of ibuprofen tablets, cream and gel. For the oral suspension it specifies a LC method with acetonitrile-0.01 M phosphoric acid (40:60 % v/v) as the mobile phase with a flow rate of 2 mL/min, a C_{18} reverse phase column (30 cm × 3.9 mm) and a detection wavelength of 220 nm [14]. The Ph. Int. (2006) specifies a direct titration method using 0.1 M sodium hydroxide to a phenolphthalein end point for the assay of the raw material and to a potentiometric end point for the assay of ibuprofen tablets [15].

Other researchers have developed methods for the analysis of ibuprofen. Sádecká *et al.* (2001) described a capillary isotachophoresis method for the quantification of ibuprofen and naproxen [42] while Persson-Stubberud and Åström (1998) developed a micellar electrokinetic capillary chromatographic method for the separation of ibuprofen, codeine phosphate and their main degradation products and impurities [43]. Hergert and Escandar (2003) developed a spectrofluorimetric method for the determination of ibuprofen in the presence of β-cyclodextrin [44]. Ravisankar *et al.* (1998) described a reversed-phase HPLC method for the simultaneous estimation of paracetamol, ibuprofen and chlorzoxazone in formulations. The method was carried out on a C_8 column using 0.2% triethylamine-acetonitrile (pH 3.2) as the mobile phase and detection was carried out at a wavelength of 215 nm [45].

1.7.7. Paracetamol

The USP (2009) specifies a UV spectrophotometric method at a wavelength of 244 nm using 1-cm cells for the assay of the raw material. It specifies a LC method with water-

methanol (75:25 % v/v) as the mobile phase with a flow rate of 1.5 mL/min, 30 cm x 3.9 mm C_{18} reverse phase column and a detection wavelength of 243 nm for the assay of paracetamol tablets, oral solution, effervescent oral solution, suppositories, oral suspension and capsules. For paracetamol extended release tablets a LC method with mobile phase as methanol-water (70:30 % v/v) spiked with 1 mL solution of a water-phosphoric acid using a 15 cm x 3.9 mm C_{18} reverse phase column and a detection wavelength of 295 nm is prescribed. The USP specifies a LC method with a mixture of water-methanol-glacial acetic (69:28:3 % v/v/v) as mobile phase with a flow rate of 2 mL/min using a 10 cm x 4.6 mm C_{18} reverse phase column and a detection wavelength of 275 nm for the assay of paracetamol and caffeine tablets while for paracetamol and codeine phosphate tablets and capsules a LC method with monobasic potassium phosphate buffer pH 2.35-methanol (98:2 % v/v) as mobile phase with a flow rate of 1.5 mL/min using a 25 cm x 4.6 mm C_{18} reverse phase column and a detection wavelength of 214 nm is prescribed. For paracetamol and codeine phosphate oral solution a separate LC method for each of the components is prescribed. It prescribes a mixture water-methanol (70:30 % v/v) as mobile phase with a flow rate of 2 mL/min using a 30 cm x 3.9 mm C_{18} reverse phase column and a detection wavelength of 280 nm is for paracetamol. For codeine phosphate 4.44 mg/mL of docusate sodium dissolved in methanol-water-tetrahydrofuran-phosphoric acid (60:36:4:0.1 % v/v/v/v) as mobile phase with a flow rate of 1.5 mL/min using a 30 cm x 3.9 mm C_{18} reverse phase column and a detection wavelength of 280 nm is prescribed. It prescribes a LC method with 0.216 mg/mL of sodium-1-octanesulfonate in 4.9 mg/mL monobasic potassium phosphate pH 3.9-acetonitrile (90:10 % v/v) as mobile phase with a flow rate of 2 mL/min using 15 cm x 4.6 mm phenylsilyl reverse phase column and a detection wavelength of 220 nm for the assay of paracetamol and codeine phosphate oral suspension [13].

The BP (2007) prescribes a titration method with 0.1 M cerium sulphate to a ferroin indicator end point for the assay of the raw material. It describes a UV spectrophotometric method with the absorbance taken at a maximum of 257 nm using 1 cm cells for the assay of paracetamol tablets, dispersible tablets and soluble tablets. For paracetamol oral solution and oral suspension a LC method with 0.01 M sodium

butanesulphonate in formic acid-methanol-water (0.4:15:85 % v/v/v) as mobile phase at a flow rate of 2 mL/min using a 20 cm × 4.6 mm, C_{18} reverse phase column and a detection wavelength of 243 nm is prescribed. It prescribes two titration methods for the assay of paracetamol suppositories depending on the strength of paracetamol in the product. For suppositories containing more than 150 mg of paracetamol, 0.2 M ammonium cerium (IV) sulphate is used as the titrant and 0.1 M ammonium cerium (IV) sulphate is used for suppositories containing 150 mg or less of paracetamol. The BP also prescribes a LC method with 0.01M sodium pentanesulphonate in water pH 2.8-methanol (78:22, % v/v) as mobile phase at a flow rate of 1.5 mL/min using a 10 cm × 4.6 mm, C_{18} reverse phase column and a detection wavelength of 243 nm for paracetamol and 220 nm for codeine phosphate for paracetamol and codeine phosphate tablets and effervescent tablets [14]. The Ph. Int. (2006) specifies a nitrite titration method using 0.1M sodium nitrite for the assay of the raw material. It specifies a UV spectrophotometric method with at a wavelength maximum of 257 nm for the assay of paracetamol tablets [15].

There are other methods that have been developed for the analysis of paracetamol. Chunli and Baoxin (2004) proposed a spectrophotometric method for the determination of paracetamol. The method is based on the microwave assisted alkaline hydrolysis of paracetamol to *p*-aminophenol that reacts with S^{2-} in the presence of Fe^{3+} as oxidant to produce a methylene blue-like dye having an absorptivity maximum at 540 nm [46]. Another method was developed by Moreira *et al.* (2005) for the direct analysis of paracetamol pharmaceutical formulations based on the native fluorescence of paracetamol [47]. A chemiluminometric method was illustrated by Ruengsitagoon *et al.* (2006) using flow injection for the determination of paracetamol, based on the chemiluminescence produced by the reduction of tris(2,2′-bipyridyl)ruthenium(III) which is obtained by oxidation of tris(2,2′-bipyridyl)ruthenium(II) by potassium permanganate in dilute sulphuric acid in the presence of paracetamol [48]. McEvoy *et al.* (2006) developed an oil-in-water microemulsion LC method for the analysis of paracetamol in a *suppository formulation [49]. A near-infrared reflectance spectroscopic method was* reported by Trafford *et al.* (1999) for the non-destructive determination of the content of paracetamol in bulk batches of intact tablets by collecting NIR spectra in the range 1100–

2500 nm and using a multiple linear regression calibration method [50]. Bouhsain *et al.* (1996) developed an analytical procedure for the simultaneous determination of paracetamol, acetylsalicylic acid and caffeine in pharmaceuticals by partial least-squares treatment of Fourier transform infrared spectrometric data obtained in the wavenumber range 823 and 1775 cm^{-1}, using the 14 characteristic bands included in this interval for the three compounds [51]. A spectrophotometric method was also proposed by Bouhsain *et al.* (1996) for the determination of paracetamol in pharmaceuticals. The method is based on the on-line alkaline hydrolysis of paracetamol to *p*-aminophenol and the reaction with 8-hydroxyquinoline (8-quinolinol) in the presence of potassium periodate as oxidant to form a blue indophenol dye which absorbs at 608 nm [52]. Franeta *et al.* (2002) reported a LC method for simultaneous determination of acetylsalicylic acid, paracetamol, caffeine and phenobarbital in tablets. Separation was achieved using a C_{18}, 5 μm 250 × 4.6 mm column, acetonitrile-water (25:75 % v/v) adjusted to pH 2.5 with phosphoric acid as the mobile phase at a flow rate of 2.0 mL/min and UV detection at 207 nm [53].

1.8. Study justification

There are many pharmaceutical preparations that are used in the management of pain. Many of these pain medications are available to the general public as over the counter medication. Several of the formulations available commercially are a combination of two or more drug compounds; which can all be analgesics or a mixture of analgesics and co-analgesics. The use of two or more analgesics in the same formulation is controversial as there is no conclusive data on their improved effectiveness over individual drug therapy. On the other hand they do offer a means of controlling pain from separate mechanisms of action that can be additive or synergistic. This means that the drugs can be administered in lower doses thereby reducing the chances of adverse effects.

Currently, there are very few compendial methods that are available for the analysis of combination products. Most of the methods available specify the analysis of individual components in a combination using different methods of analysis. Only combinations

containing paracetamol and caffeine and paracetamol and codeine phosphate have compendial methods.

The aim of the present study was to develop a single method for the simultaneous determination of caffeine, chlorzoxazone, codeine phosphate, diclofenac sodium, doxylamine succinate, ibuprofen and paracetamol. These compounds are present in some of the most commonly used pain medications in Kenya as combinations that incorporate paracetamol and caffeine, paracetamol and codeine phosphate, paracetamol, caffeine and codeine phosphate, paracetamol, caffeine, doxylamine succinate and codeine phosphate, diclofenac sodium and paracetamol, diclofenac sodium, paracetamol and chlorzoxazone, ibuprofen and paracetamol, ibuprofen, paracetamol and codeine phosphate and ibuprofen, paracetamol and chlorzoxazone.

Some of these combination formulations do not have methods of analysis in any of the officially recognized pharmacopoeias and the regulatory authority is forced to rely on the methods provided by the manufacturers, details of which often refer to respective monographs for individual compounds. In addition, the country lacks an effective mechanism to monitor and curb the influx of counterfeit and substandard pharmaceutical products. Pain medications are among some of the most widely used pharmaceuticals and therefore are more likely to attract counterfeit and substandard products. A single method for the analysis of any variety of combination of these compounds would go a long way in ensuring their quality. Quality control laboratories can use it to rapidly gauge the quality of such medications before market authorization in conjunction with the drug regulatory authority, during post market surveillance exercises and in batch release testing.

The method can also be adopted for the routine analysis of any product that may contain any combination of the seven compounds under study. This can be done easily without the need to change the LC system from product to product or the use of a new method for each product.

1.9. Study objectives

The general objective of this study was the development and validation of a single method for the simultaneously determination of caffeine, chlorzoxazone, codeine phosphate, diclofenac sodium, doxylamine succinate, ibuprofen and paracetamol in analgesic preparations.

The specific objectives of the study were:

1. To develop and optimize an isocratic LC method with UV detection for the simultaneous separation and quantification of caffeine, chlorzoxazone, codeine, diclofenac, doxylamine, ibuprofen and paracetamol.
2. To carry out validation of the LC method developed.
3. To apply the developed method in the analysis of commercially available drug samples.

CHAPTER TWO

METHOD DEVELOPMENT AND OPTIMIZATION

2.1 Reagents and solvents

Analytical grade monobasic potassium phosphate (Loba Chemie PVT Ltd., Mumbai, India), dibasic potassium phosphate , anhydrous sodium acetate (RFCL Ltd, New Delhi, India), ammonium acetate (Loba Chemie PVT Ltd., Mumbai, India), glacial acetic acid, hexanesulfonic acid sodium salt, butanesulfonic acid sodium salt and octanesulfonic acid sodium salt (Fischer Scientific UK Ltd., Leicestershire, UK) were used during method development.

Methanol HPLC grade (RFCL Ltd, New Delhi, India) was used for all analytical work. All aqueous solutions were prepared using purified water prepared from an Arium water system which consisted of a reverse osmosis module and an ultrafiltation module with a UV irradiation component (Sartorius AG, Göttingen, Germany).

2.2 Instrumentation

2.2.1 Melting point apparatus

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

2.2.2 Ultra-violet spectrophotometer

Ultra-violet absorption spectra over the range 200-400 nm were measured for each of the working standards using a calibrated double beam T90+ UV/VIS Spectrometer controlled via a computer using UVWIN spectrophotometer software version 5.2.0 (PG Instruments, Leicestershire, United Kingdom) using quartz cuvettes with a path length of 10 mm.

2.2.3 Infra-red spectrophotometer

The InfraRed spectra for each of the working standards was measured using a Shimadzu IRPrestige 21 Fourier Transform Infra-Red (FTIR) spectrophotometer (Shimadzu Corp., Kyoto, Japan) running on IRSolution Software Ver. 1.3.

2.2.4 Liquid chromatographic system

Shimadzu Prominence high performance liquid chromatographic system (Shimadzu Corp., Kyoto, Japan) was used for the study. It consisted of a CBM-20A Prominence communications bus module, a SPD-20A Prominence UV/Visible detector which incorporated a deuterium lamp for ultraviolet applications and a tungsten lamp for applications in the visible region, a LC-20AT Prominence liquid chromatography solvent delivery system which was a dual-plunger tandem-flow solvent delivery module and a SIL-10AS Prominence autosampler. The temperature was controlled using a CTO-10AS VP column oven which was a block heating type thermostatic chamber equipped with a preheater system. The LC system was controlled via a computer using LCSolutions Software Ver 1.22, SP1. All mobile phase preparations were degassed using a Power Sonic 410 bench top ultrasonic bath (Daihan Labtech Ltd, Kyonggi-Do, Korea).

2.3 Working standards

Anhydrous caffeine (CAF), ibuprofen (IBP) and paracetamol (PAR) raw materials were kind donations from Dawa Limited (Baba Dogo Road, Nairobi, Kenya) while diclofenac sodium (DIC) and codeine phosphate (COD) raw materials were donations from Cosmos Limited (Lunga Lunga Road, Nairobi, Kenya). Chlorzoxazone (CHR) working standard was donated by the National Quality Control Laboratory (Hospital Road, Nairobi, Kenya). Doxylamine succinate (DOX) working standard was purchased from Sigma-Aldrich (St Louis, MO, USA). The raw materials were characterized and used as working standards for the study. The tests involved in the characterization were determination of their melting points, measurements of UV and IR absorption spectra and determination of potency.

2.3.1 Determination of melting point

The melting points of the seven working standards were determined and compared with values published in literature. The results are recorded on Table 2.1. All the values obtained were comparable to the literature values indicating that the compounds identity was as stated.

Table 2.1: Working standards melting points

Working standard	Observed melting point (°C)	Literature melting point (°C) [8]
Caffeine	237.4 - 237.6	234 - 239
Chlorzoxazone	190.7 - 191.3	190 - 194
Codeine phosphate	238.2 - 238.4	238 - 240
Diclofenac sodium	283.3 - 283.6	283 - 285
Doxylamine succinate	104.3 - 105.0	103 - 108
Ibuprofen	75.4 - 76.6	75 - 77
Paracetamol	170.9 - 171.0	168 - 172

2.3.2 Determination of ultra-violet absorption spectra

The ultraviolet absorption spectra for the seven working standards were measured over a range of 200-400 nm. For this purpose, solutions of 0.01 mg/mL CAF, 0.016 mg/mL CHR, 0.09 mg/mL COD, 0.016 mg/mL DIC, 0.02 mg/mL DOX, 0.27 mg/mL IBP and 0.0075 mg/mL PAR were prepared in water-methanol (50:50 % v/v).

Table 2.2: Absorption maxima for the working standards

Working standard	Observed λ_{max} (nm)*	Literature λ_{max} (nm) [8]
Caffeine	272.0	273.0
Chlorzoxazone	280.0	280.0
Codeine phosphate	284.0	285.0
Diclofenac sodium	280.0	275.0
Doxylamine succinate	260.0	260.0
Ibuprofen	264.0	265.0
Paracetamol	246.0	245.0

*Diluent: Methanol-Water (50:50 % v/v)

The wavelengths of maximum absorbance (λ_{max}) observed were compared with those reported in literature. The λ_{max} and λ_{min} were used in establishing the detection wavelength used in the method development. The spectra obtained are shown in Appendices 8-14 while the λ_{max} values observed are shown in Table 2.2 and an overlay of the spectra of the seven compounds is shown in Figure 2.1. The differences between the values observed and those in literature are most likely due to the differences in the diluents used. Aqueous acid was used as the diluent for paracetamol, doxylamine succinate, chlorzoxazone, codeine phosphate and caffeine and aqueous alkali for diclofenac sodium and ibuprofen in obtaining the literature values.

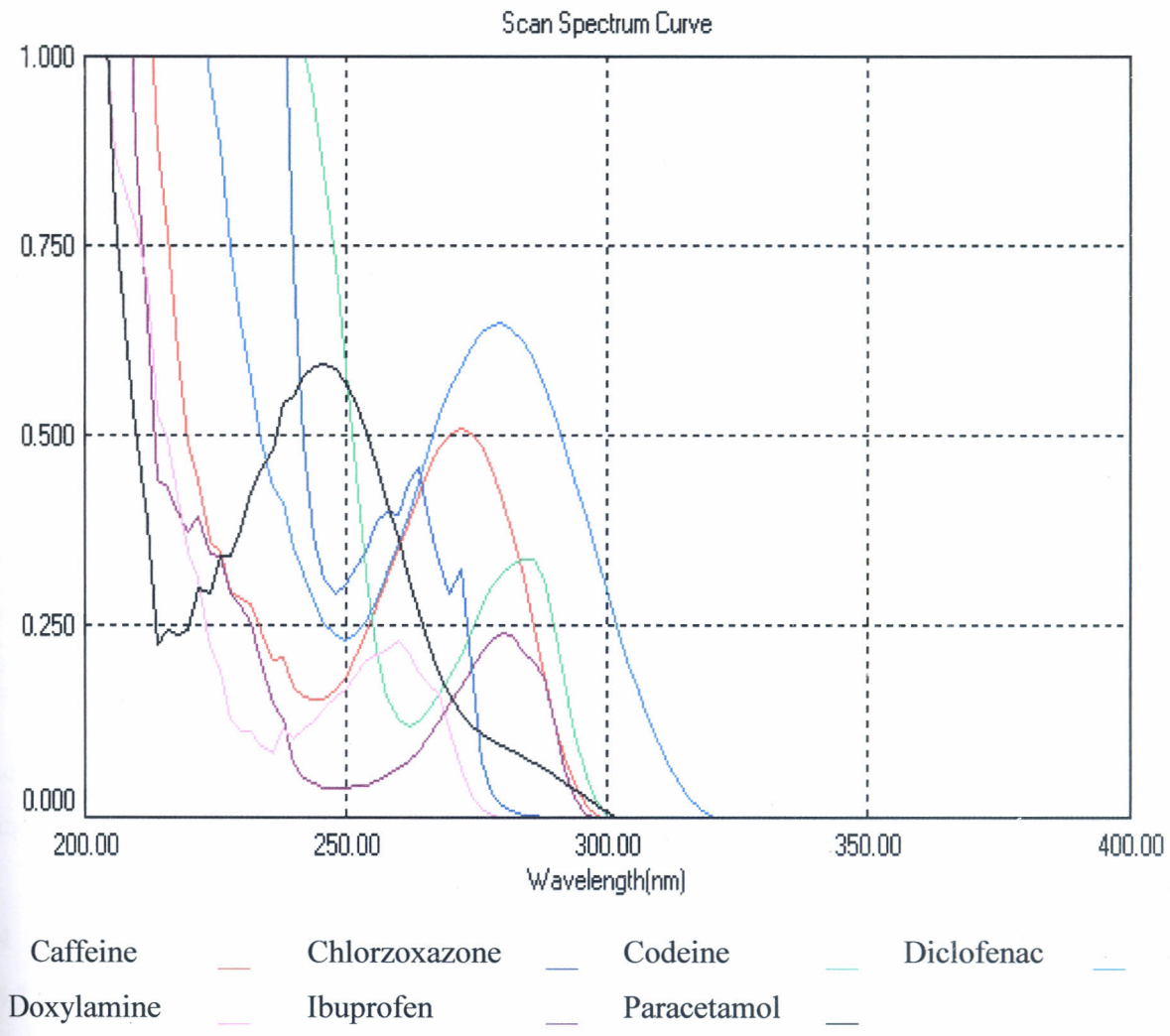


Figure 2.1: Superimposed UV spectra of the compounds under study

2.3.3 Determination of infra-red absorption spectra

The infrared absorption spectra were measured over a range of 2000 to 400 cm^{-1}. A 1 mm thick potassium bromide disc containing about 1% w/w of each of the compounds was used. The spectra so obtained (Appendices 1–7) were compared with those of primary standards similarly prepared and concurrently measured and with published values [7, 14]. The principle absorption bands were in concordance with literature values and were comparable with those of the primary standards.

2.3.4 Determination of potency

The potency of the seven working standards was determined using the methods prescribed in the USP or BP [13, 14]. The potency of caffeine, ibuprofen and paracetamol was determined using HPLC analysis which also served as an additional step in the identification of the compounds. The determined percentage potencies of the working standards on 'as is' basis are shown in Table 2.3.

Table 2.3: Percentage Potencies of the working standards

Working standard	Percentage potency (on 'as is' basis)
Caffeine	99.8
Chlorzoxazone	99.5
Codeine phosphate	99.4
Diclofenac sodium	99.8
Doxylamine succinate	100.0
Ibuprofen	99.2
Paracetamol	101.3

The above four tests carried out all served to confirm the identity of the working standards as the seven compounds to be studied.

2.4 Method development

2.4.1 Introduction

The detector wavelength, stationary phase, organic modifier, flow rate and injection volume were fixed from the onset and experiments were carried out systematically to

deduce the optimum chromatographic conditions. An isocratic system was preferred over a gradient system so as to eliminate the inherent complications that come with it. The chromatographic conditions that gave the best balance between separation of the seven compounds and the overall retention time were taken as the optimum.

2.4.2 Fixed chromatographic conditions

2.4.2.1 Choice of detector wavelength

The observed UV spectra indicated that all the compounds had a λ_{max} between 245 and 285 nm (Figure 2.1). The intensity of absorption for CAF, CHR, COD, DIC, DOX and IBP was found to increase markedly below 240 nm except PAR which showed a minimum 216 nm before markedly increasing.

When used in combination with any other of the compounds being studied DOX and COD are always present in the least quantities, being about 5-10 times lower than the other components. Paracetamol, when used in combination with any of the compounds under study is usually present in the highest concentrations. These two factors influenced the choice of the detection wavelength to ensure that the absorption intensities of the seven compounds were not so different as to render simultaneous UV detection impractical.

From Figure 2.1, a wavelength of 220 nm shows that the absorption intensities are increased for COD, CAF, CHR, DIC, DOX and IBP and decreased for PAR. This wavelength offered the best compromise between the absorption intensities of the compounds under study and the working reference solution concentrations used in the method development. One disadvantage of working at such a low wavelength is the possibility of the excipients in the drugs being analyzed exhibiting some absorbance.

2.4.2.2 Choice of stationary phase

A 25 cm × 4.6 mm ID Phenomenex Gemini® C_{18} column S/N 505049-2, particle size of 5µ and a pore size of 110Å was selected as the stationary phase for the method development (Phenomenex, Torrance, CA, USA.). This brand of column was selected

because it is claimed to be stable over an extended pH range of 1-12. A unique silica-organic layer grafting introduced as the final stage in the silica manufacture achieves this wide pH stability. This silica-organic shell protects the particle from chemical attack. Another added advantage is that this column offers increased loading and retention of basic compounds. This was especially important since five of the compounds under study were bases and thus offered a wide room to maneuver during method development.

2.4.2.3 Choice of organic modifier, flow rate and injection volume

The organic modifier was fixed as methanol. This was due to its greater availability, lower cost and lower elution power when compared to acetonitrile. A weaker eluting solvent is desirable because of the number of compounds under study. Furthermore, the most commonly used buffer salts are more soluble in water-methanol mixtures than they are in water-acetonitrile mixtures hence there is less risk of buffer salt precipitation.

The flow rate was fixed at 1 mL/min. This was chosen because it offered the best mix in terms of column back pressures and retention times. The preferred backpressure limit was 150 bar. Methanol-aqueous mixtures are normally viscous and therefore result in high column backpressures. A high flow rate reduces the retention time of the components hence is undesirable for the separation of multi-component preparations.

The injection volume was fixed at 10 μL. This was selected to avoid overloading the column especially for PAR because a relatively high concentration was to be used.

2.4.3 Reference working solution

A reference working solution containing 0.2 mg/mL of CAF, 1 mg/mL of CHR, 0.04 mg/mL of COD, 0.2 mg/mL of DIC, 0.02 mg/mL of DOX, 0.8 mg/mL of IBP and 2 mg/mL PAR working standards was prepared in water-methanol (50:50 % v/v). The ratio of the concentrations represented the relative concentrations of the compounds in commercial drug samples.

2.4.4 Influence of chromatographic factors

2.4.4.1 Effect of inorganic buffer on separation

A mobile phase containing water-methanol (50:50, % v/v) was used as the starting point. Under these conditions COD, DOX and PAR co-eluted whereas DIC and IBP did not eluted out even after 60 min, probably because they are not ionized. The chlorzoxazone peak was also broad. Figure 2.2 is a typical chromatogram obtained.

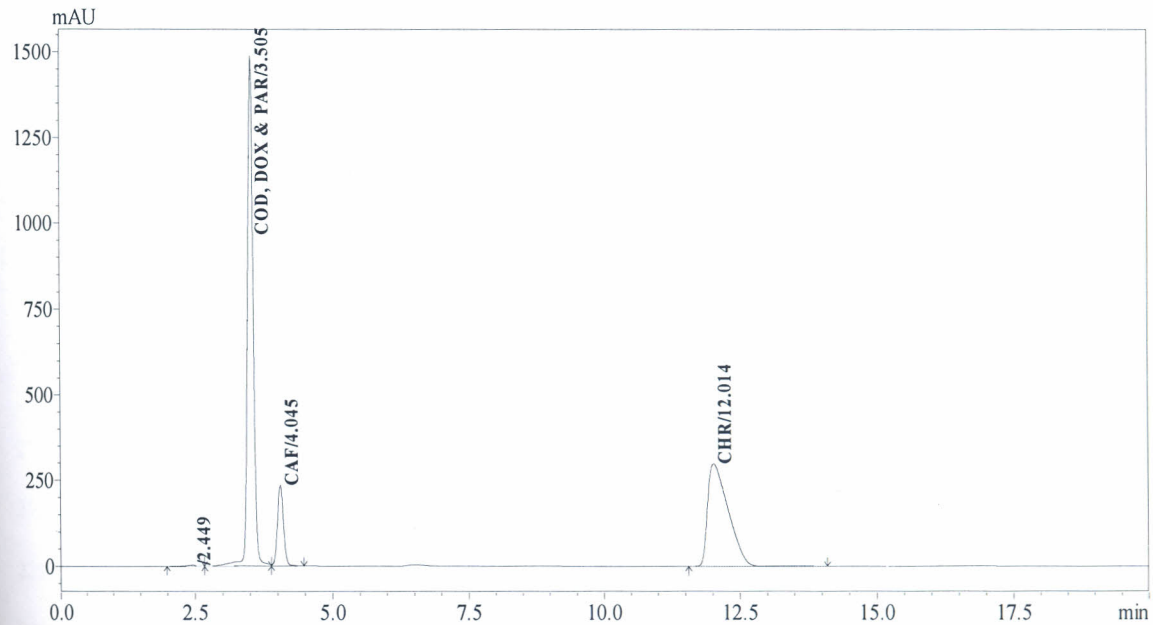


Figure 2.2: Chromatogram of the reference working solution obtained using unbuffered mobile phase. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-water (50:50 % v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine.

Consequently, the effect of buffered mobile phase was investigated. For this purpose monobasic potassium phosphate (KH_2PO_4) was selected because it is commonly used and thus is readily available and is known to yield good separation. Furthermore, phosphate buffers have negligible UV absorbance at the selected wavelength of detection (220 nm). Considering the methanol concentration in the mobile phase (50 %) a concentration of 20 mM of the buffer was selected to preclude precipitation of the buffer salts in the mobile phase. This is in line with the recommended buffer concentration of 10-50 mM for routine analysis to prevent any incompatibilities with the organic modifier. From the pK_a values (Table 2.4) of DIC and IBP, a starting pH of 5.0 was selected. The mobile phase

contained methanol-0.2 M KH_2PO_4 pH 5.0-water (50:10:40 % v/v/v). Figure 2.3 shows that there was improved separation of the seven compounds.

Table 2.4: The pK_a values of the working standards

Working standard	pK_a value [8]
Caffeine	14.0
Chlorzoxazone	8.0
Codeine phosphate	8.2
Diclofenac sodium	4.2
Doxylamine succinate	4.4 and 9.2
Ibuprofen	4.4 and 5.2
Paracetamol	9.5

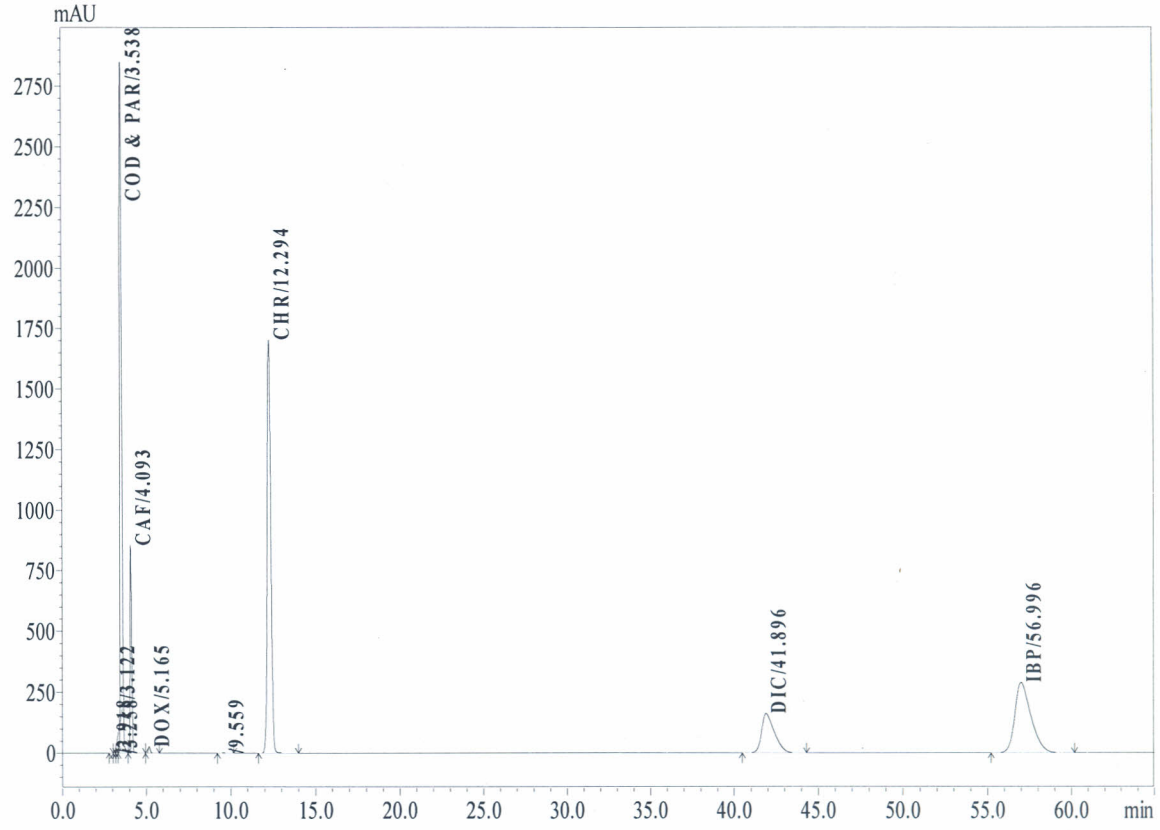


Figure 2.3: Chromatogram of the reference working solution obtained using inorganic phosphate buffer mobile phase at pH 5.0. Column: Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.2 M KH_2PO_4 pH 5.0-water (50:10:40, % v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

At pH 5.0 all the seven compounds were eluted out with an overall run time of 65 min. Two of the compounds, PAR and COD co-eluted with a retention time of 3.52 min. The elution order of the other four compounds was DOX, CHR, DIC and IBP respectively (Figure 2.3). There was adequate separation between PAR and CAF with a resolution of 2.77. The asymmetry factors of all the separated compounds were between 0.8 and 2.

2.4.4.1.1 Effect of buffer pH

In an effort to improve the separation of the co-eluted compounds and reduce the overall run time, the pH of the mobile phase was varied over the range 5.5-6.5 and the effects on the separation recorded. For this purpose, three mobile phase solutions containing methanol-0.2 M KH_2PO_4-water (50:10:40 % v/v/v) were prepared at pH 5.5, 6.0 and 6.5. Figure 2.4 depicts a typical chromatogram obtained using the mobile phase of pH 6.5.

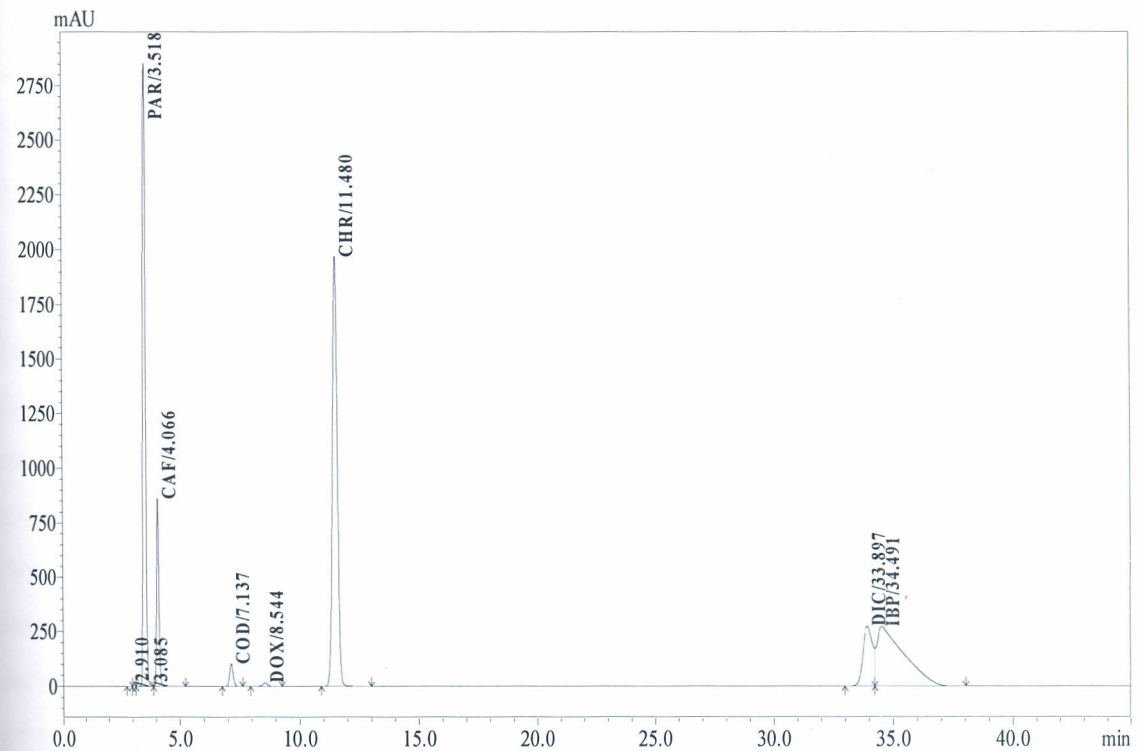


Figure 2.4: Chromatogram of the reference working solution obtained using inorganic phosphate buffer mobile phase at pH 6.5. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.2 M KH_2PO_4 pH 6.5-water (50:10:40, % v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

The effect of buffer pH on the retention time, asymmetry factor, resolution and capacity factors of the compounds under study are summarized in Table 2.5. The resolution in each case calculated with reference to the peak eluting immediately before the component. The data demonstrated that raising the pH of the mobile phase gradually increased retention times of COD and DOX though the increase was more marked in COD where the retention time increased from 3.5 min at pH 5.0 to 7.2 min at pH 6.5. The capacity factors of PAR, CAF and CHR were not significantly affected as demonstrated by Figure 2.5. However, there was a marked decrease in the retention times of DIC and IBP. The resolution between these two peaks decreased as the pH increased until they merged at pH 6.5. The asymmetry factor of IBP increased at high pH values. The resolution between PAR and CAF did not change significantly as the pH was increased. The overall run time decreased progressively as the pH increased from 5.0 to 6.5. Codeine co-eluted with PAR at pH 5.0 and with CAF at pH 5.5. At pH 6.0, all compounds were completely separated though the resolution between DIC and IBP was poor being 0.85. At pH 6.5, DIC and IBP were not baseline separated (resolution 0.35) while the rest of the compounds were completely separated.

As the pH increases the retention time for all acidic compounds decreases. This is because the compounds are progressively getting ionized. Generally the retention time of the ionized form of a drug is shorter than its non-ionized form. At pH 5.0 the apparent mobile phase pH is very close to the pK_a values of both DIC and IBP, the two compounds are only partially ionized hence the longer retention times. As the pH increases, the degree of ionization increases until at pH 6.5 DIC is completely ionized since the pH is more than 2 units from its pK_a. The increase in ionization as the pH increased could account for the increase in peak symmetry of IBP. The ionized form of IBP exchange or compete with hydrogen ions on the silica surface of the column increasing the asymmetry of the peak. As the pH increased, there was more ionized form of the drug present hence the observed increase in the asymmetry factor. The basic compounds, PAR, CAF, CHR, COD and DOX have dissociation constants ranging from 8.0-14.0 thus are completely ionized at pH 5.0. As the pH was increased to 6.5, the degree of ionization is not affected significantly.

Table 2.5: 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	Drug	Retention time (min)	Peak Asymmetry factor	Resolution	k'
MeOH-0.2 M KH_2PO_4 pH 5.0-H_2O (50:10:40, % v/v/v)	PAR	3.52	1.09	1.18	0.21
	CAF	4.07	1.19	2.77	0.40
	COD	3.52	1.09	1.18	0.21
	DOX	5.12	1.28	5.09	0.76
	CHR	12.24	1.02	7.49	3.21
	DIC	41.93	1.49	32.82	13.42
	IBP	56.90	1.40	9.44	18.57
MeOH-0.2 M KH_2PO_4 pH 5.5-H_2O (50:10:40, % v/v/v)	PAR	3.52	1.11	1.74	0.24
	CAF	4.07	1.34	2.66	0.44
	COD	4.07	1.34	2.66	0.44
	DOX	5.40	1.27	5.96	0.91
	CHR	12.04	1.02	9.72	3.25
	DIC	36.77	1.69	29.11	11.99
	IBP	41.92	3.22	2.81	13.81
MeOH-0.2 M KH_2PO_4 pH 6.0-H_2O (50:10:40, % v/v/v)	PAR	3.56	1.11	2.56	0.21
	CAF	4.11	1.18	2.81	0.40
	COD	5.63	1.10	7.09	0.92
	DOX	6.53	1.34	3.48	1.22
	CHR	12.04	1.02	12.52	3.09
	DIC	35.10	1.55	30.64	10.93
	IBP	36.60	3.59	0.85	11.45
MeOH-0.2 M KH_2PO_4 pH 6.5-H_2O (50:10:40, % v/v/v)	PAR	3.54	1.10	0.96	0.21
	CAF	4.09	1.18	2.77	0.40
	COD	*7.17*	*1.05*	*13.20*	*1.45*
	DOX	8.58	1.00	4.50	1.93
	CHR	11.57	1.03	8.24	2.95
	DIC	34.19	-	33.75	10.68
	IBP	34.77	-	0.35	10.88

Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.2 M KH_2PO_4-water (50:10:40 % v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

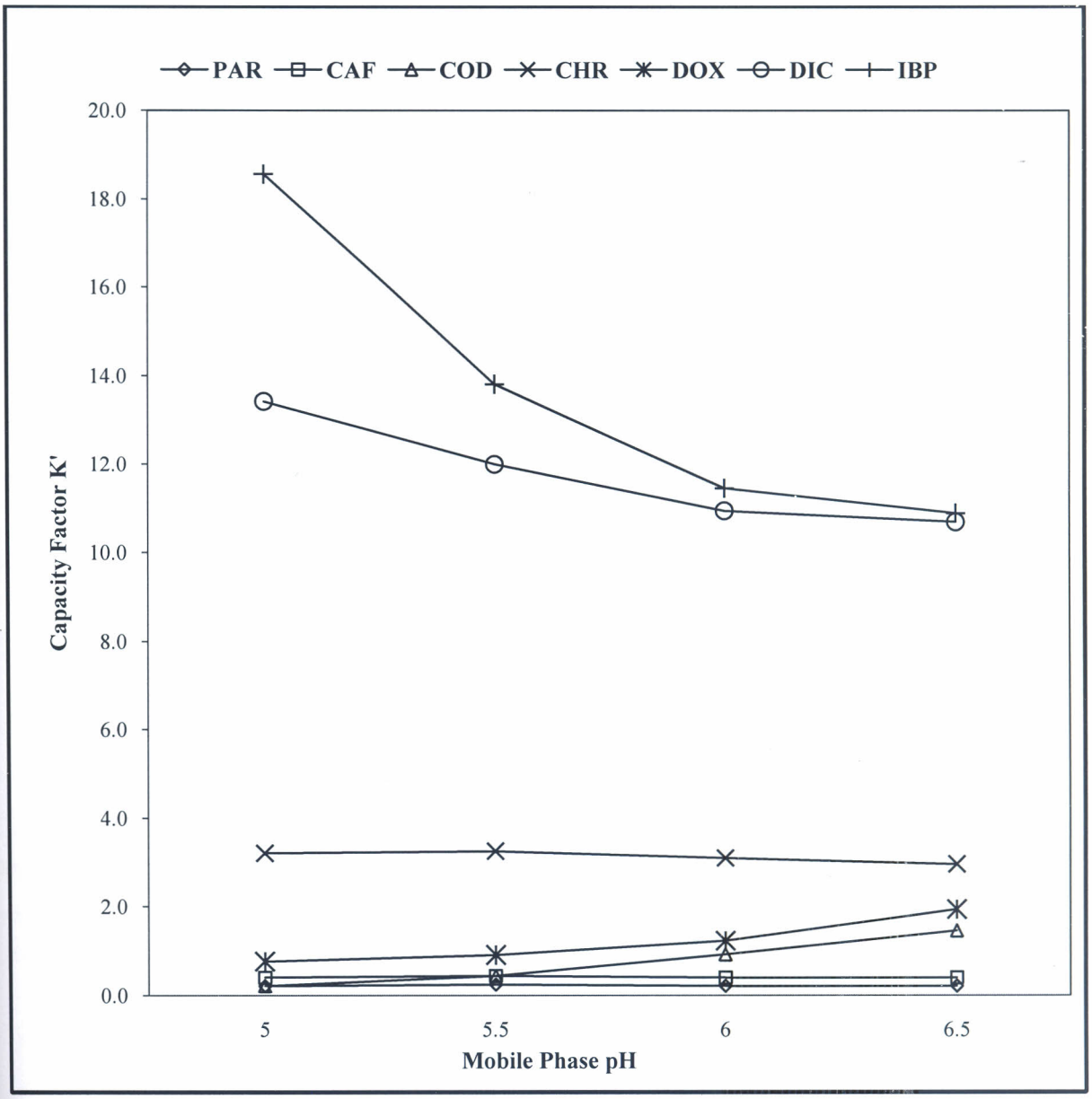


Figure 2.5: Effect of 0.2 M monobasic potassium phosphate buffer pH on the capacity factors of the compounds under study. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.2 M KH_2PO_4-water (50:10:40 % v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

2.4.4.2 Effect of organic buffers on separation

2.4.4.2.1 Effect of sodium acetate buffer

Further changes were made to reduce the overall run time and improve the peak shapes using an organic buffer. Four mobile phase solutions were prepared containing methanol-

0.2 M sodium acetate-water (50:10:40 % v/v/v) at pH 5.0, 5.5, 6.0 and 6.5. The pH was adjusted to the required value using 0.8 % v/v acetic acid. Figure 2.6 is a typical chromatogram obtained using mobile phase of pH 6.5.

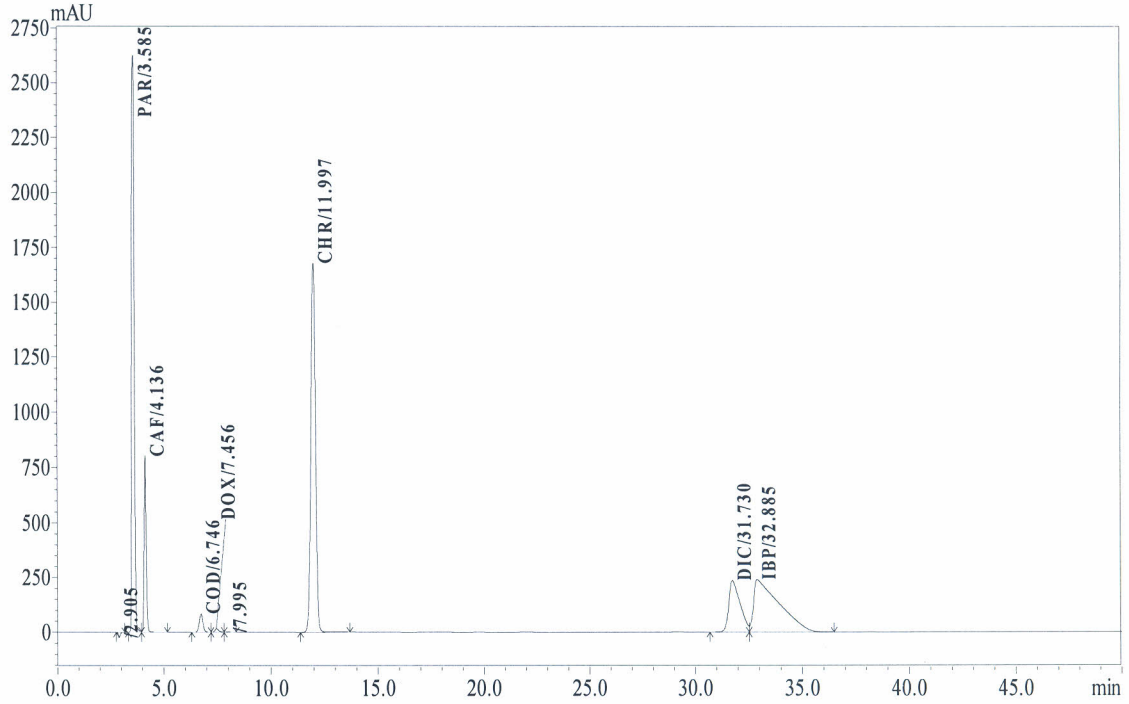


Figure 2.6: Chromatogram of the reference working solution obtained using sodium acetate buffer mobile phase at pH 6.5. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.2 M CH_3COONa-water pH 6.5 (50:10:40, % v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

The effect of the buffer pH on the retention time, asymmetry factor, resolution and capacity factors of the compounds under study are summarized in Table 2.6. Using mobile phase with a buffer at pH 5.0, the retentions times of PAR, CAF, COD, DOX and CHR did not change significantly compared to those obtained using the phosphate buffer. There was however, an increase in the capacity factors of DIC and IBP from 13.4 and 18.6 to 15.1 and 23.4 respectively (Table 2.6). Increased buffer pH caused a gradual decrease in the retention time and capacity factors of DIC and IBP with a concomitant decrease in the resolution of these two peaks reaching 0.66 at pH 6.5. The asymmetry factors were found to increase with pH with the value for IBP increasing above 2. An increase in buffer pH had no effect on PAR, CAF and CHR peaks while COD and DOX exhibited increased retention times.

Table 2.6: **The effect of 0.2 M sodium acetate buffer pH on the retention time, asymmetry factor, resolution and capacity factor of the compounds under study.**

Mobile phase composition	Drug	Retention time (min)	Peak Asymmetry factor	Resolution	k'
MeOH-0.2 M CH_3COONa pH 5.0-H_2O (50:10:40, % v/v/v)	PAR	3.51	0.95	3.62	0.24
	CAF	4.05	1.19	2.69	0.42
	COD	3.51	0.95	3.62	0.24
	DOX	5.19	1.46	5.52	0.83
	CHR	12.07	1.03	4.94	3.25
	DIC	45.85	1.36	36.17	15.14
	IBP	69.20	1.58	12.37	23.36
MeOH-0.2 M CH_3COONa pH 5.5-H_2O (50:10:40, % v/v/v)	PAR	3.51	1.09	3.59	0.23
	CAF	4.04	1.16	1.36	0.42
	COD	3.77	0.00	1.26	0.32
	DOX	5.37	1.40	6.15	0.89
	CHR	11.94	1.00	9.26	3.19
	DIC	36.28	1.58	28.42	11.74
	IBP	46.32	2.53	5.73	15.26
MeOH-0.2 M CH_3COONa pH 6.0-H_2O (50:10:40, % v/v/v)	PAR	3.56	1.09	3.65	0.24
	CAF	4.12	1.17	2.75	0.43
	COD	4.89	1.11	3.55	0.70
	DOX	6.10	1.44	4.62	1.12
	CHR	12.30	0.99	12.21	3.27
	DIC	34.34	1.72	26.15	10.93
	IBP	37.87	3.48	1.91	12.15
MeOH-0.2 M CH_3COONa pH 6.5-H_2O (50:10:40, % v/v/v)	PAR	3.55	1.10	3.60	0.23
	CAF	4.09	1.17	2.74	0.42
	COD	6.67	1.04	10.34	1.32
	DOX	7.38	1.49	2.21	1.57
	CHR	11.88	0.96	11.50	3.13
	DIC	31.41	-	27.31	9.93
	IBP	32.56	-	0.66	10.33

Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.2 M CH_3COONa-water (50:10:40 % v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

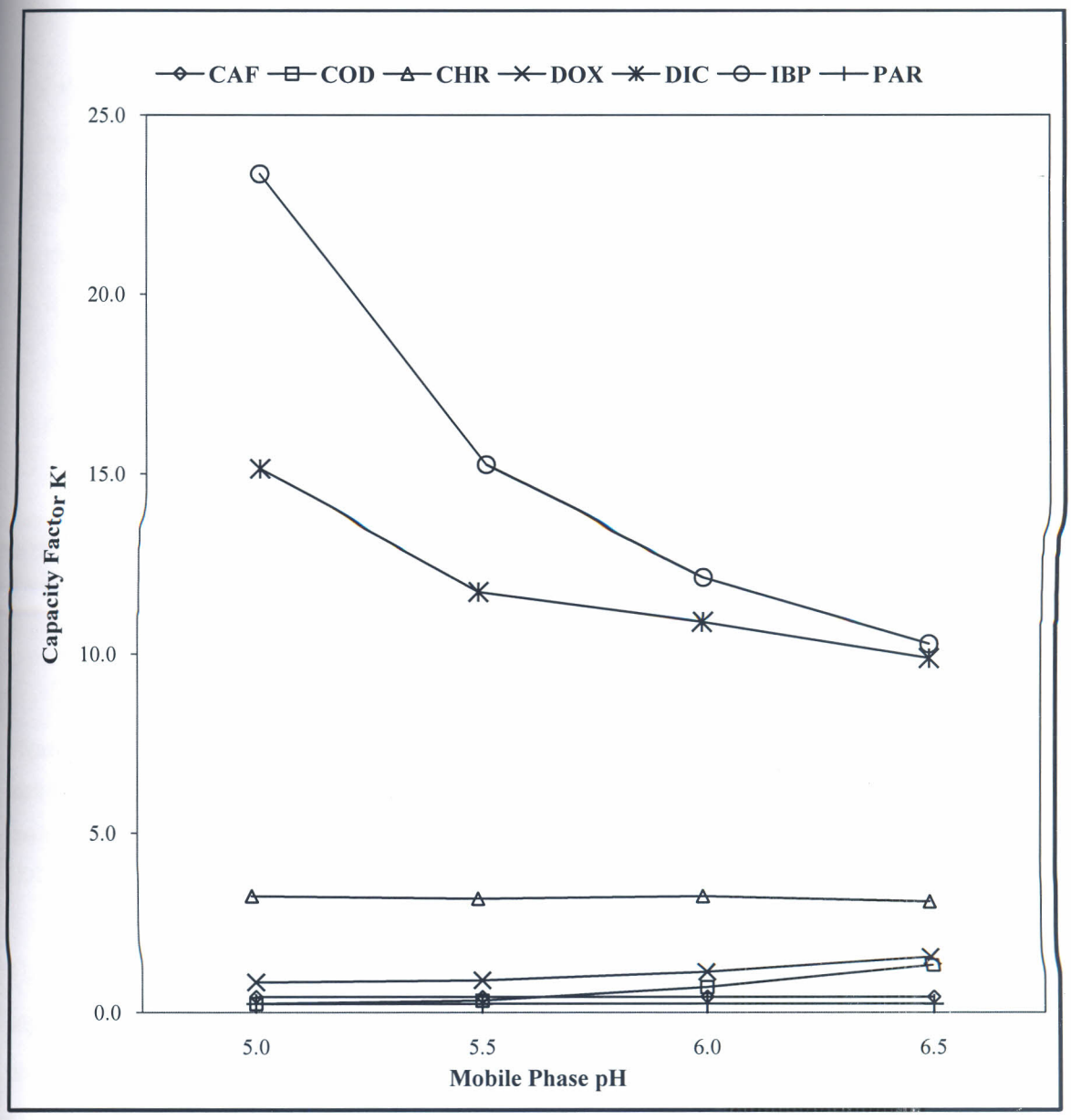


Figure 2.7: Effect of 0.2 M sodium acetate buffer pH on the capacity factors of the compounds under study. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.2 M CH_3COONa-water (50:10:40, % v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

2.4.4.2.2 Effect of ammonium acetate buffer

The effects of the volatile organic buffer ammonium acetate were investigated using four mobile phase solutions containing methanol-0.2 M ammonium acetate-water (50:10:40 % v/v/v) at pH 5.0, 5.5, 6.0 and 6.5. Figure 2.8 is a typical chromatogram obtained at pH 6.5.

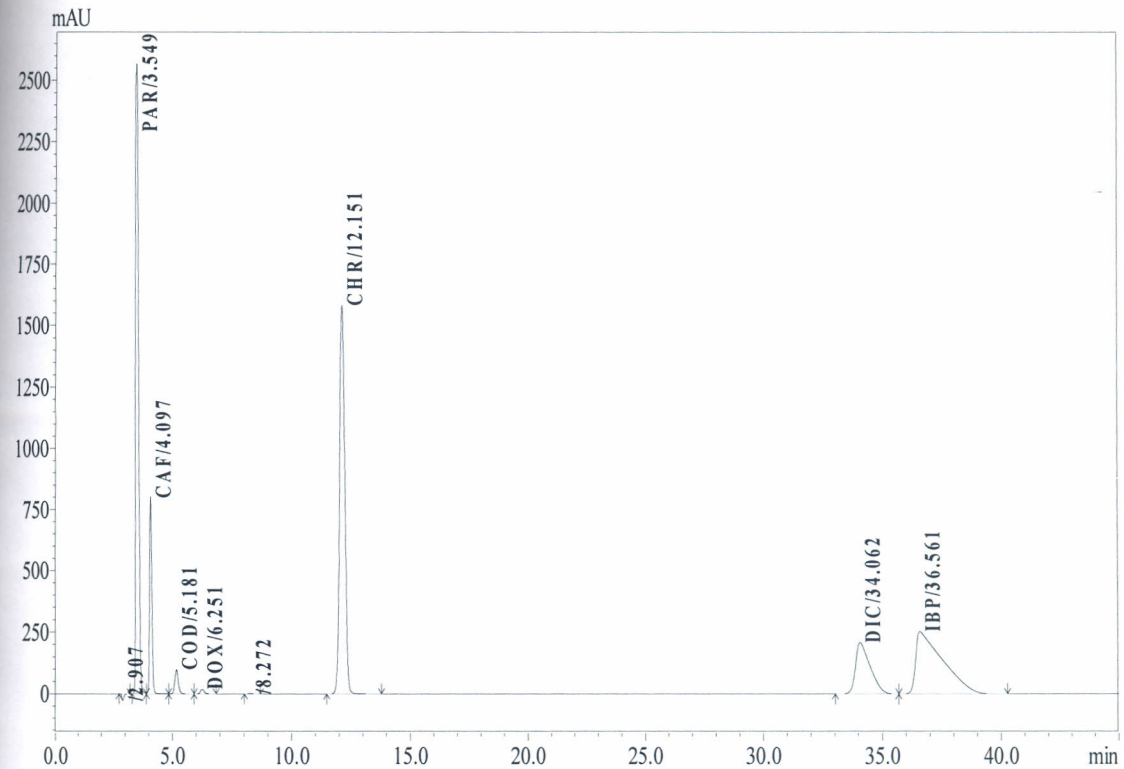


Figure 2.8: Chromatogram of reference working solution obtained using ammonium acetate buffer mobile phase at pH 6.5. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.2 M CH_3COONH_4 pH 6.5-water (50:10:40, % v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

The effect of the buffer pH on the retention time, asymmetry factor, resolution and capacity factors of the compounds under study are summarized in Table 2.7. The elution profile as shown in Table 2.7 and Figure 2.9 was very similar to that observed with the phosphate buffer, the major difference being the increased retention times of DIC and IBP. At pH 6.5 all the peaks are completely resolved with the resolution between DIC and IBP being 1.45. The asymmetry factors were found to increase with pH with the value for IBP increasing above 2.

Table 2.7: **The effect of 0.2 M ammonium acetate buffer pH on the retention time, asymmetry factor, resolution and capacity factor of the compounds under study**

Mobile phase composition	Drug	Retention time (min)	Peak Asymmetry factor	Resolution	k'
MeOH-0.2 M CH_3COONH_4 pH 5.0-H_2O (50:10:40, % v/v/v)	PAR	3.49	1.09	0.94	0.22
	CAF	4.02	1.19	2.69	0.41
	COD	3.29	0.00	2.17	0.15
	DOX	5.09	1.30	5.22	0.78
	CHR	11.99	1.02	10.01	3.20
	DIC	46.57	1.30	36.75	15.29
	IBP	69.40	1.53	12.16	23.33
MeOH-0.2M CH_3COONH_4 pH 5.5-H_2O (50:10:40, % v/v/v)	PAR	3.51	1.12	3.19	0.22
	CAF	4.06	1.18	1.53	0.41
	COD	3.72	0.00	0.90	0.29
	DOX	5.40	1.26	6.37	0.87
	CHR	12.21	1.01	8.86	3.23
	DIC	39.04	1.55	30.44	12.52
	IBP	49.55	2.50	5.85	16.17
MeOH-0.2M CH_3COONH_4 pH 6.0-H_2O (50:10:40, % v/v/v)	PAR	3.53	1.10	3.34	0.22
	CAF	4.08	1.18	2.78	0.41
	COD	4.53	1.15	2.05	0.56
	DOX	5.91	1.30	5.47	1.04
	CHR	12.23	1.01	11.14	3.22
	DIC	35.46	1.65	28.09	11.23
	IBP	39.84	3.30	2.48	12.74
MeOH-0.2M CH_3COONH_4 pH 6.5-H_2O (50:10:40, % v/v/v)	PAR	3.52	1.10	3.38	0.22
	CAF	4.07	1.18	2.73	0.41
	COD	5.16	1.14	4.81	0.79
	DOX	6.23	1.25	3.91	1.16
	CHR	12.05	1.01	11.44	3.17
	DIC	33.66	1.66	27.86	10.65
	IBP	36.17	3.64	1.45	11.52

Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.2 M CH_3COONH_4-water (50:10:40 % v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

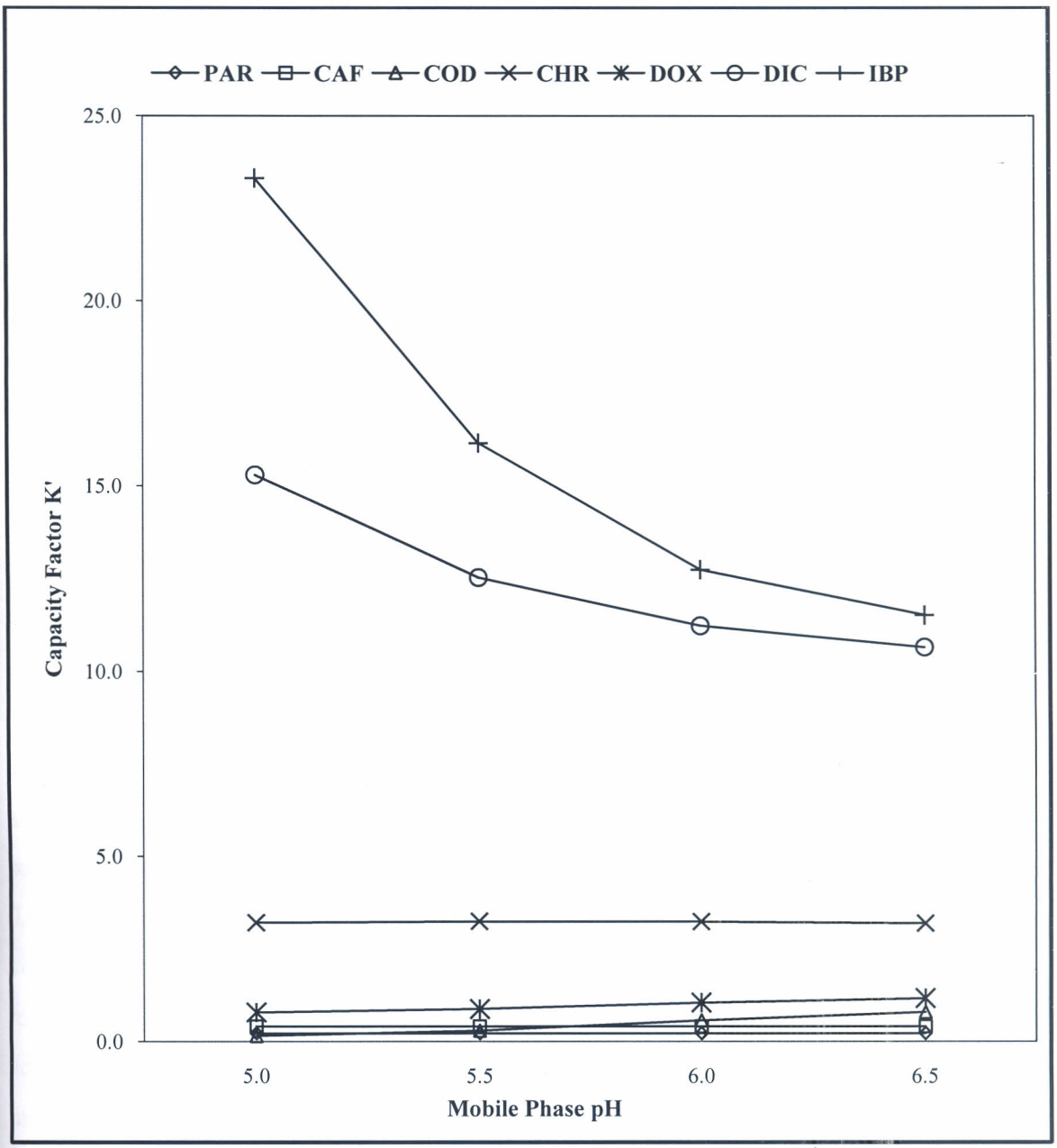


Figure 2.9: Effect of 0.2 M ammonium acetate buffer pH on the capacity factors of the compounds under study. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.2 M CH_3COONH_4-water (50:10:40, % v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

From the study of the three buffer types at the four pH values, it was observed that the overall run time decreased as the pH was increased from 5.0 to 6.5. Using a buffer pH above 6.5 would yield better separation but silica based columns are unstable at pH values greater than 7. Therefore to ensure that the method is as widely applicable as

possible, a buffer pH of 6.5 was selected as optimum. Further, the ammonium acetate buffer offered the best separation at pH 6.5. This is useful as it allows the coupling of the LC method to MS. Since it is more miscible with high concentrations of organic modifiers, it allows experimental versatility compared to inorganic phosphate buffers.

2.4.4.3 Effect of ion pairing agents.

Ion pairing agents influence retention times and peak symmetries through ionic interaction with ionic analyte compounds. Ion pairing agents are commonly present as cationic or anionic reagents. Cationic ion pairing agents interact with anionic samples to increase their retention times and with cationic samples to decrease their retention times while anionic ion pairing agents have the opposite effect. From Figure 2.8, it can be inferred that the ideal effect of the ion pairing agent would be to increase the retention times of CAF and PAR and reduce the retention times of DIC and IBP. The compounds PAR, CAF, COD, DOX and CHR are cations at pH 6.5 and DIC and IBP are anions thus the best ion pairing agent to theoretically improve the overall retention times would be an anionic ion pairing agent. The most commonly used anionic ion pairing agents are alkylsulfonic acid salts. Three alkylsulfonic acid salts namely sodium butanesulfonate, sodium hexanesulfonate and sodium octanesulfonate were used at a concentration of 0.005 M. Stock solutions of 0.25 M of each of the ion pairing agent were prepared and a volume to make up the required concentration added to a volume of 0.2 M CH_3COONH_4 required to give the final buffer concentration, this was then diluted with water and adjusted to pH 6.5. This was mixed with an equal volume of methanol to make the mobile phase. Table 2.8 depicts the effect of these ion pairing agents on the retention times, capacity factors and asymmetry factors of the seven compounds and Figure 2.10 shows the effects on the capacity factors.

The introduction of the three ion pairing agents had minimal effect on the retention time of PAR, CAF and CHR. The retention time of COD increased slightly after introduction of the ion pairing agent and as the alkyl side chain of the reagents increased. The retention time of DOX increased with the introduction of the ion pairing agent and as the alkyl side chain of the reagents increased. The retention times of the DIC and IBP

decreased with the introduction of the ion pairing agent and as the alkyl side chain increased. Alkylsulfonic acid salts consist of a polar negatively charged head and a non-polar alkyl side chain. On introduction onto the stationary phase they align themselves such that the alkyl side chain interacts with the non polar stationary phase and the polar head is left at the surface of the stationary phase to interact with the mobile phase components. The negatively charged head attracts positively charged molecules through ionic interactions thus increasing their transit time through the column. On the other hand, the negative charge repels any negatively charged mobile phase constituents reducing their transit time and hence their retention times above. This explains the observations above. The interaction of the ion pairing agent with the stationary phase increases as the carbon chain length of the alkyl side chain increases. This explains why the effects of the ion pair on the drug compounds increased as the alkyl side chain increased.

The asymmetry factors of PAR, CAF, COD, CHR and DOX were slightly reduced by the introduction of the ion pairing agents. The increase in the alkyl side chains did not affect the symmetry of PAR, CAF and CHR with a slight decrease in the asymmetry factor of COD. The asymmetry factor of IBP was the most affected as it decreased from 2.68 with sodium butanesulfonate to 0.99 with sodium octanesulfonate.

There was a significant decrease in the overall run time with the introduction of the ion pairing agent and as the alkyl side chain increased. Additionally, the asymmetry factors of all the compounds were between 0.8 and 2.0. Sodium octanesulfonate was chosen as the ion pairing agent to be utilized in the method.

Table 2.8: The effect of ion pairing agents on the retention time, asymmetry and capacity factors of the compounds under study

Ion pairing agent (0.005M)	Drug	Retention Time (min)	Peak Asymmetry factor	k'
No Ion pairing agent	PAR	3.52	1.10	0.22
	CAF	4.07	1.18	0.41
	COD	5.16	1.14	0.79
	DOX	6.23	1.25	1.16
	CHR	12.05	1.01	3.17
	DIC	33.66	1.66	10.65
	IBP	36.17	3.64	11.52
Sodium Butanesulfonate	PAR	3.52	1.08	0.22
	CAF	4.06	1.15	0.40
	COD	5.22	1.10	0.81
	DOX	6.76	1.20	1.34
	CHR	11.87	0.98	3.11
	DIC	29.95	1.32	9.36
	IBP	32.81	2.68	10.35
Sodium Hexanesulfonate	PAR	3.51	1.07	0.23
	CAF	4.03	1.15	0.41
	COD	5.48	1.09	0.23
	DOX	8.07	1.23	1.82
	CHR	11.66	0.99	3.07
	DIC	24.41	1.19	7.53
	IBP	27.40	2.05	8.57
Sodium Octanesulfonate	PAR	3.54	1.07	0.28
	CAF	4.03	1.14	0.45
	COD	6.46	1.05	1.33
	DOX	13.26	1.07	3.78
	CHR	11.62	0.98	3.19
	DIC	16.95	1.01	5.11
	IBP	20.59	0.99	6.42

Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.2 M CH_3COONH_4 pH 6.5-water *(50:10:40 % v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-*doxylamine, DIC-diclofenac, IBP-ibuprofen.

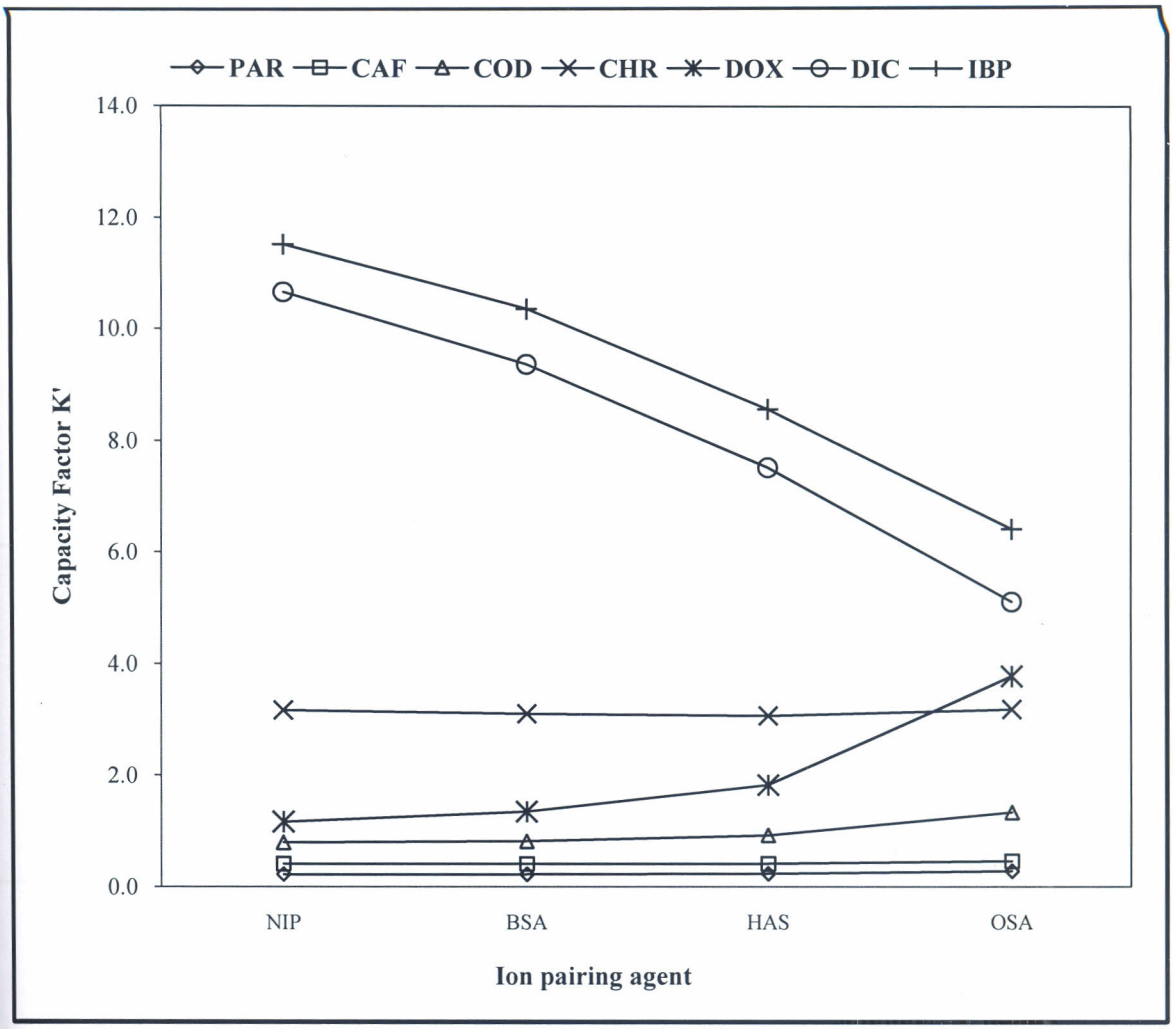


Figure 2.10: Effect of ion pairing agents on the capacity factors of the compounds under study. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol--0.2 M CH_3COONH_4 pH 6.5-water (50:10:40 % v/v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen, NIP-no ion pairing agent, BSA-sodium butanesulfonate, HAS-sodium hexanesulfonate, OSA-sodium octanesulfonate.

2.4.5 Method optimization

2.4.5.1 Effect of buffer and ion pairing agent concentrations

Three different concentrations of sodium octanesulfonate were investigated, 0.001 M, 0.005 M and 0.01 M. For this purpose, a stock solution of 0.25M was prepared and appropriately diluted in the buffer solution and the organic modifier. Table 2.9 shows the effect of the ion pair concentration on the retention times, peak symmetry and capacity factor.

Table 2.9: The effect of sodium octanesulfonate concentration on the retention time, asymmetry and capacity factors of the compounds under study

Ion pairing agent Concentration (mM)	Drug	Retention Time (min)	Peak Asymmetry factor	k'
0.001	PAR	3.51	1.09	0.22
	CAF	4.03	1.18	0.39
	COD	5.51	1.10	0.90
	DOX	8.01	1.29	1.77
	CHR	11.51	0.99	2.98
	DIC	23.63	1.21	7.17
	IBP	26.59	2.21	8.20
0.005	PAR	3.51	1.09	0.22
	CAF	4.03	1.18	0.39
	COD	6.46	1.05	0.28
	DOX	13.26	1.07	3.78
	CHR	11.62	0.98	3.19
	DIC	16.95	1.01	5.11
	IBP	20.59	0.99	6.42
0.01	PAR	3.51	1.08	0.30
	CAF	3.96	1.15	0.47
	COD	6.94	1.02	1.58
	DOX	17.81	0.81	5.61
	CHR	11.33	0.99	3.21
	DIC	14.20	0.98	4.27
	IBP	17.81	0.81	5.61

Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.25 M Sodium Octanesulfonate-0.2 M CH_3COONH_4 pH 6.5-water (50:x:10:40 % v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

The increase in concentration of the ion pairing agent had no significant effect on PAR, CAF and CHR. The retention of COD increased with increasing ion pair concentration and the asymmetry factor slightly reduced. The retention time of DOX increased from 8.0 to 18.7 min while its peak symmetry reduced from 1.3 to 0.8. The retention times of DIC and IBP each decreased by about 9 min and the symmetry factors also decreased. The

overall retention time reduced as the ion pair concentration increased. At a concentration of 0.01 M of the ion pairing agent, DOX and IBP peaks co-eluted.

In view of the foregoing, the optimal ion pairing agent concentration was taken as 0.005 M. At this concentration, all the seven compounds were adequately separated and all the asymmetry factors were between 0.8 and 2.

The effect of the acetate buffer concentration were also investigated using four concentrations; 0.02 M, 0.04 M, 0.06 M and 0.08 M. Table 2.10 shows the effects of the buffer concentration on the retention times, asymmetry factors and capacity factors of the seven compounds.

The most remarkable effect observed was the increase in the retention times of DIC and IBP and the decrease in the retention times of DOX as the buffer concentration increased. The asymmetry factor of IBP increased with an increase in buffer concentration while those of the other compounds were not significantly affected.

From table 2.10 it can be observed that increasing the buffer concentration did not improve the separation significantly. Therefore a buffer concentration of 0.02 M was taken as the optimal concentration.

Table 2.10: The effect of ammonium acetate buffer concentration on the retention time, asymmetry and capacity factors of the compounds under study

Buffer Concentration (M)	Drug	Retention Time (min)	Peak Asymmetry factor	k'
0.02	PAR	3.53	1.07	0.28
	CAF	4.01	1.17	0.45
	COD	6.55	1.05	1.37
	DOX	13.49	1.04	3.89
	CHR	11.46	0.98	3.15
	DIC	16.26	1.02	4.89
	IBP	19.66	0.97	6.13
0.04	PAR	3.52	1.07	0.23
	CAF	4.00	1.17	0.40
	COD	6.08	1.06	0.23
	DOX	11.36	0.99	2.98
	CHR	11.36	0.99	2.98
	DIC	18.71	1.00	5.56
	IBP	21.70	1.20	6.60
0.06	PAR	3.51	1.08	0.21
	CAF	3.99	1.17	0.38
	COD	5.75	1.08	0.98
	DOX	10.55	1.05	2.64
	CHR	11.33	0.98	2.91
	DIC	20.69	0.99	6.13
	IBP	23.52	1.21	7.11
0.08	PAR	3.52	1.11	0.21
	CAF	4.00	1.18	0.37
	COD	5.66	1.10	0.94
	DOX	10.34	0.94	2.55
	CHR	11.38	1.01	2.91
	DIC	21.49	1.01	6.38
	IBP	24.28	1.25	7.34

Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.25 M Sodium Octanesulfonate-0.2 M CH_3COONH_4 pH 6.5-water (50:x:10:40 % v/v/v. PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

2.4.5.2 Effect of methanol concentration

Five mobile phase solutions were prepared containing 40, 45, 50, 55 and 60 % v/v of methanol. The buffer and ion pairing agent concentrations were fixed at 0.02 M ammonium acetate pH 6.5 and 0.005 M sodium octanesulfonate. Table 2.11 and Figure 2.11 show the effects of increasing organic modifier concentration on the chromatographic parameters.

Increasing the methanol concentration had the effect of reducing the retention time of all the compounds. This is because as the methanol concentration is increased, the elution power of the mobile phase increased and hence the compounds eluted faster. The least affected were PAR and CAF while DOX was the most affected whereby its elution order was changed as the methanol concentration increased. There was an increase in asymmetry factors as the methanol concentration was increased.

Although the increasing methanol concentration had the advantageous effect of reducing the overall run time it reduced the resolution between PAR and CAF and between DOX and IBP and CHR. Doxylamine merged with IBP at 40 % v/v methanol and with CHR at 55 % v/v methanol. A methanol concentration of 50% v/v gave the best balance of overall run time, retention times and resolution.

Table 2.11: The effect of methanol concentration on the retention time, asymmetry factor, resolution and capacity factor of the compounds under study

Methanol Concentration (% v/v)	Drug	Retention Time	Peak Asymmetry factor	Resolution	k'
40	PAR	3.91	1.06	6.37	0.50
	CAF	4.78	1.12	3.97	0.83
	COD	11.57	0.98	14.10	3.43
	DOX	38.49	0.85	5.02	13.73
	CHR	21.50	0.96	17.59	7.22
	DIC	32.75	0.99	13.30	11.53
	IBP	38.49	0.85	5.02	13.73

Methanol Concentration (% v/v)	Drug	Retention Time	Peak Asymmetry factor	Resolution	k'
45	PAR	3.70	1.07	5.09	0.38
	CAF	4.34	1.16	3.04	0.61
	COD	8.45	1.04	11.23	2.14
	DOX	24.94	1.08	2.23	8.27
	CHR	15.63	0.97	18.91	4.81
	DIC	23.11	1.01	12.12	7.59
	IBP	27.69	0.90	3.07	9.29
50	PAR	3.53	1.07	4.02	0.28
	CAF	4.01	1.17	2.37	0.45
	COD	6.55	1.05	6.46	1.37
	DOX	13.49	1.04	4.78	3.89
	CHR	11.46	0.98	15.23	3.15
	DIC	16.26	1.02	5.50	4.89
	IBP	19.66	0.97	5.74	6.13
55	PAR	3.41	1.08	3.26	0.21
	CAF	3.79	1.18	1.92	0.34
	COD	5.48	1.11	4.53	0.21
	DOX	8.75	0.98	11.67	2.10
	CHR	8.75	0.98	11.67	2.10
	DIC	11.76	1.05	8.30	3.16
	IBP	14.11	1.09	5.20	3.99
60	PAR	3.31	1.08	2.81	0.16
	CAF	3.62	1.19	1.66	0.28
	COD	4.83	1.18	3.42	0.70
	DOX	6.45	1.14	7.06	1.27
	CHR	7.20	1.03	2.89	1.54
	DIC	9.19	1.07	6.61	2.24
	IBP	10.96	1.14	4.84	2.86

Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.25 M Sodium Octanesulfonate-0.2 M CH_3COONH_4 pH 6.5-water (x:2:10:88-x, % v/v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

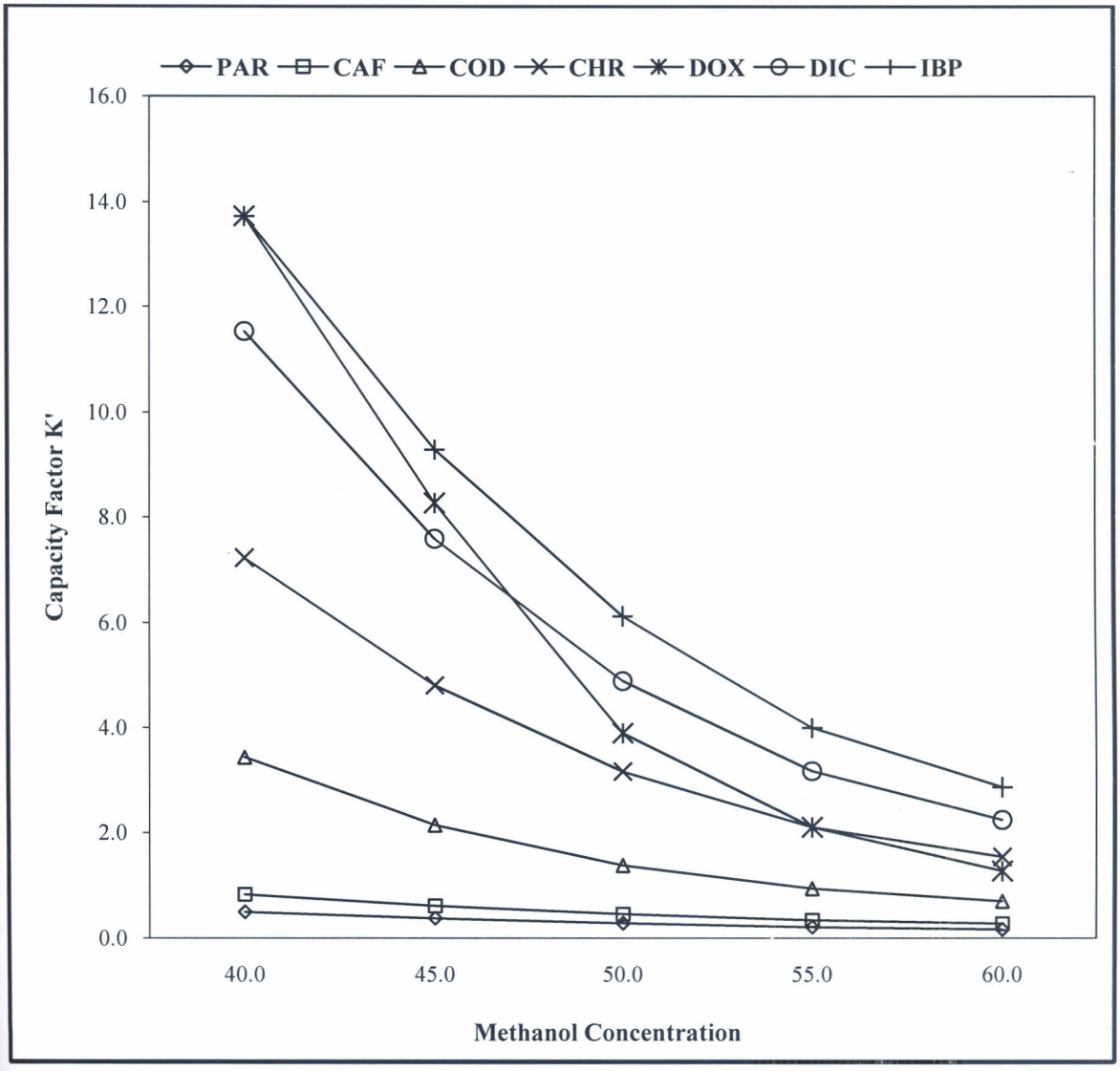


Figure 2.11: Effect of methanol concentration on the capacity factors of the compounds under study. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.25 M Sodium Octanesulfonate-0.2 M CH_3COONH_4 pH 6.5-water (x:2:10:88-x, % v/v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

2.4.5.3 Effect of column temperature

The optimal column temperature was determined using a mobile phase consisting of methanol-0.25 M Sodium Octanesulfonate-0.2 M CH_3COONH_4 pH 6.5-water (50:2:10:38 % v/v/v). The oven was set at four different temperatures, 21 °C, 30 °C, 40 °C and 50 °C. The effect on the monitored chromatographic parameters and the column backpressure are recorded in Table 2.12.

Table 2.12: The effect of temperature on the column back pressure, retention time, asymmetry factor, resolution and capacity factor of the compounds under study

Temperature (°C)	Column Backpressure (Bar)	Drug	Retention time (min)	Peak Asymmetry factor	Resolution	k'
Room Temperature	170 - 173	PAR	3.66	1.02	4.63	0.35
		CAF	4.18	1.09	2.40	0.54
		COD	7.22	1.00	7.10	1.66
		DOX	18.00	1.07	5.80	5.63
		CHR	14.59	0.92	17.89	4.37
		DIC	21.25	0.96	4.52	6.82
		IBP	23.57	0.93	2.84	7.68
30	150 - 153	PAR	3.55	1.03	4.33	0.32
		CAF	4.05	1.11	2.37	0.50
		COD	6.87	1.01	6.93	1.55
		DOX	15.78	1.07	5.53	4.85
		CHR	12.96	0.93	16.28	3.80
		DIC	18.70	0.97	4.71	5.93
		IBP	21.51	0.95	3.93	6.97
40	127 - 130	PAR	3.46	1.04	3.99	0.28
		CAF	3.94	1.12	2.33	0.46
		COD	6.55	1.01	6.49	1.43
		DOX	13.71	1.06	5.13	4.08
		CHR	11.41	0.94	14.31	3.23
		DIC	16.26	0.97	4.81	5.03
		IBP	19.47	0.96	5.08	6.22
50	113 - 114	PAR	3.37	1.05	3.67	0.26
		CAF	3.82	1.13	2.28	0.43
		COD	6.24	1.01	6.68	1.33
		DOX	11.99	1.05	4.75	3.48
		CHR	10.10	0.95	12.38	2.77
		DIC	14.22	0.97	4.81	4.31
		IBP	17.62	0.96	6.07	5.58

Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.25 M Sodium Octanesulfonate-0.2 M CH_3COONH_4 pH 6.5-water (50:2:10:38, % v/v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

An increase in temperature reduced the overall run time and the retention times of all the seven compounds to varying degrees with minimal effect on the asymmetry factors of the peaks. In order to strike a balance between the overall run time and column stability the operating temperature was chosen as 40 °C.

2.4.5.4 Optimized chromatographic conditions

From the experiments carried, the optimum chromatographic conditions were deduced to be a mobile phase containing methanol, 0.25M sodium octanesulfonate, 0.2M ammonium acetate at a pH of 6.5 and water (50:2:10:38 % v/v/v/v). This was delivered at a flow rate of 1 mL/min through an octyldecylsilane (C_{18}) stationary reverse phase with the dimensions 250 mm in length and 4.6 mm internal diameter (Phenomenex Gemini® 5μ C_{18}) maintained at a temperature of 40 °C. A volume of 10 μL of the reference working solution was injected into the LC system and the UV detector was set at 220 nm. Figure 2.12 shows a typical chromatogram obtained at these optimum chromatographic conditions.

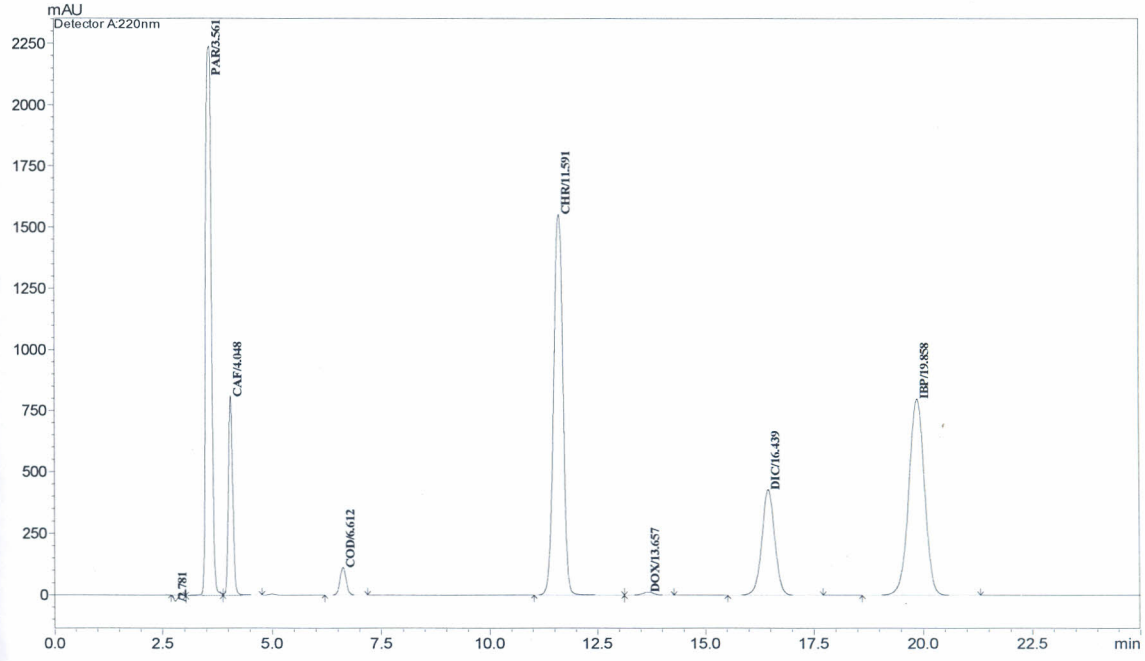


Figure 2.12: Chromatogram of the reference working solution at the optimized chromatographic conditions. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.25 M Sodium Octanesulfonate-0.2 M CH_3COONH_4 pH 6.5-water (50:2:10:38 % v/v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

CHAPTER THREE

METHOD VALIDATION

3.1 Accuracy

The accuracy of an analytical method can be defined as the agreement between the result obtained with method being validated and an accepted reference value. The accuracy can be inferred from precision, linearity and specificity. It can also be defined as closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found [54].

In the present study, the accuracy was determined by spiking commercial drug samples with known amounts of the seven compounds under study and calculating the percentage recovery. Since there was no available placebo mixture and no single commercially available drug product contains all of the seven compounds, several drug samples were used. Each drug sample was spiked with the working standard equivalent to 20% of the labeled amount of the component whose recovery was being determined at the optimum chromatographic conditions. The calculated recoveries are depicted in table 3.1. The recoveries were within the specified limits of 98-103 % [54].

Table 3.1: Percentage recoveries of the compounds under study.

Percentage Recovery						
PAR	**CAF**	**COD**	**DOX**	**CHR**	**DIC**	**IBP**
98.1 (0.3)	100.3 (0.1)	98.3 (0.5)	102.7 (0.7)	101.6 (1.2)	98.1 (0.8)	99.2 (0.5)

PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen. Values in parenthesis represent the RSD, n=4.

3.2 Linearity

The linearity of an analytical procedure is its ability to obtain a response that is directly proportional to the concentration of an analyte in the sample within a specified range. The range is the interval between the upper and lower concentration of an analyte in the

sample for which it has been demonstrated that the analytical procedure has an acceptable level of precision, accuracy and linearity. The ICH guidelines recommend that a minimum of five concentrations be utilized in the determination of linearity over the range of 80-120% [54].

A stock solution containing 5 mg/mL PAR, 0.5 mg/mL CAF, 0.1 mg/mL COD, 2.5 mg/mL CHR, 0.05 mg/mL DOX, 0.5 mg/mL DIC and 2 mg/mL IBP was prepared. From this stock solution, six different concentration levels were prepared with reference to the working standard concentrations of the seven compounds used in method development. The system was run at the optimum chromatographic conditions. The peak areas obtained were plotted against the concentration in mg/mL.

The data so obtained were evaluated statistically using a linear least squares regression analysis. The results of the analysis are depicted in table 3.2.

Table 3.2: Linear regression analysis results for the compounds under study.

Drug	Coefficient of determination	y-intercept	Slope of regression line	Residual sum of squares
PAR	0.9960	+13006317.90	3919343.68	1.5772×10^{11}
CAF	0.9995	+274065.03	25200967.68	2.4360×10^{10}
COD	0.9992	+10690.97	23197718.41	1.3342×10^{9}
DOX	0.9975	-667.67	9101469.99	1.4676×10^{8}
CHR	0.9986	+4681821.32	17497181.80	2.6677×10^{11}
DIC	0.9993	+76240.33	39115105.06	7.2740×10^{10}
IBP	0.9998	+621592.37	22942174.64	1.5889×10^{11}

PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

The method was found to be linear over a range of 75-125% for all the seven compounds.

3.3 Precision

The precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. It is considered at three levels namely repeatability, intermediate precision and reproducibility [54].

Repeatability expresses the precision under the same operating conditions over a short interval of time [54]. This can also be termed as intra-day or intra-assay precision. This was determined by injecting six replicate injections of the working standard mixture into the LC system at the optimum chromatographic conditions and determining the relative standard deviation for each of the seven compounds. This is depicted in table 3.3.

Intermediate precision expresses within-laboratory variations [54]. This can be through use of different analysts, different days or different analysts. For the purposes of this study, intermediate precision was performed on three different days. Fresh mobile phase and working standard mixture was prepared each day and a minimum of six replicate injections determined. The peak areas for each of the seven compounds were normalized for each day and subjected to statistical analysis. The relative standard deviation for the normalized peak areas for the three days was then calculated (Table 3.3).

Table 3.3 Repeatability and intermediate precision results for the compounds under study.

Drug	Repeatability Peak areas RSD (n=6)	Intermediate Precision Peak areas RSD (n=26)
PAR	0.32	1.95
CAF	0.16	0.91
COD	0.14	1.04
DOX	0.23	1.89
CHR	0.17	1.17
DIC	0.14	1.96
IBP	0.14	1.29

PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

Reproducibility expresses the precision between laboratories and is normally applicable in collaborative studies and in standardization of methodology [54]. This was not performed since it is beyond the scope of the study. The RSD for the repeatability and intermediate precision tests were less than 2 % for all the seven compounds indicating the method had adequate precision.

3.4 Limit of detection

The detection limit of an analytical method is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value [54]. Statistically, the limit of detection (LOD) in HPLC is defined as the peak that gives a signal to noise ratio of 3:1 [55]. The LOD was determined by making progressive dilutions of each of the working standards solutions. Each dilution was injected in quadruplet and the signal to noise ratio determined. The dilution that gave a signal to noise ratio of 2:1 to 3:1 was taken as the detection limit for the method developed. The LOD values so determined are shown in table 3.4.

Table 3.4 The limits of detection and quantitation for the compounds under study.

Drug	Limit of Detection (ng)	Limit of Quantitation (ng)*
PAR	22.36 (1.7)	111.8 (6.2)
CAF	11.99 (1.1)	79.92 (1.9)
COD	25.08 (1.5)	104.5 (0.3)
DOX	32.93 (6.1)	164.64 (1.4)
CHR	20.72 (4.9)	103.6 (0.6)
DIC	12.50 (4.3)	62.49 (0.2)
IBP	12.02 (6.1)	48.08 (4.4)

PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen. Values in parenthesis represent the RSD, n=3.

3.5 Limit of quantitation

The limit of quantitation (LOQ) of an analytical method is the lowest amount of the analyte in a sample which can be quantitatively determined with suitable precision and

accuracy. It is particularly necessary for methods developed to be used in the determination of impurities and degradation products which are usually present in very small amounts in the sample [54]. The LOQ is statistically defined as a peak giving a signal to noise ratio of about 10:1 [55]. Progressive dilutions of the working standards were made and injected in quadruplet and the signal to noise ratio determined. The dilution that gave a signal to noise ratio of around 10:1 was taken as the LOQ. Table 3.4 shows the LOQ values so determined.

3.6 Robustness

Robustness of an analytical method is a measure of its capacity to remain unaffected by small, but deliberate variations in method functions and provides an indication of its reliability during normal usage [54]. HPLC method robustness testing involves varying various chromatographic factors within a realistic range and assessing the influence. The factors normally varied include organic modifier concentration, mobile phase pH, column temperature, column manufacturer and the mobile phase flow rate. For the purposes of the study, the methanol concentration, the mobile phase pH and the column temperature were varied.

For each of the factors, the effects were studied at two levels. The factors were varied over a narrow range then over a wider range. In each case as one factor was varied the other two were held constant. Methanol concentration was studied at ± 1 % and ± 5 % of the optimum methanol concentration. The pH was varied from ± 0.1 pH units and ± 0.5 pH units of the optimum pH value. The temperature was varied from ± 1 ^{o}C and ± 5^{o}C of the optimum temperature. The working standard concentrations were applied as determined during the method development. The runs were made in quadruplet and the influence of the factors on the separation observed and recorded. From observations made during method development, several system suitability parameters were set and used to evaluate the robustness data obtained. The critical peak pairs were identified as CAF and PAR, DOX and CHR and DOX and DIC. The resolution between CAF and PAR was set at greater than 2, the retention time of DOX was set to be between that of CHR and DIC,

the tailing factor of IBP to be less than 1.5 and the retention time of the last eluting peak to be less than 25 min.

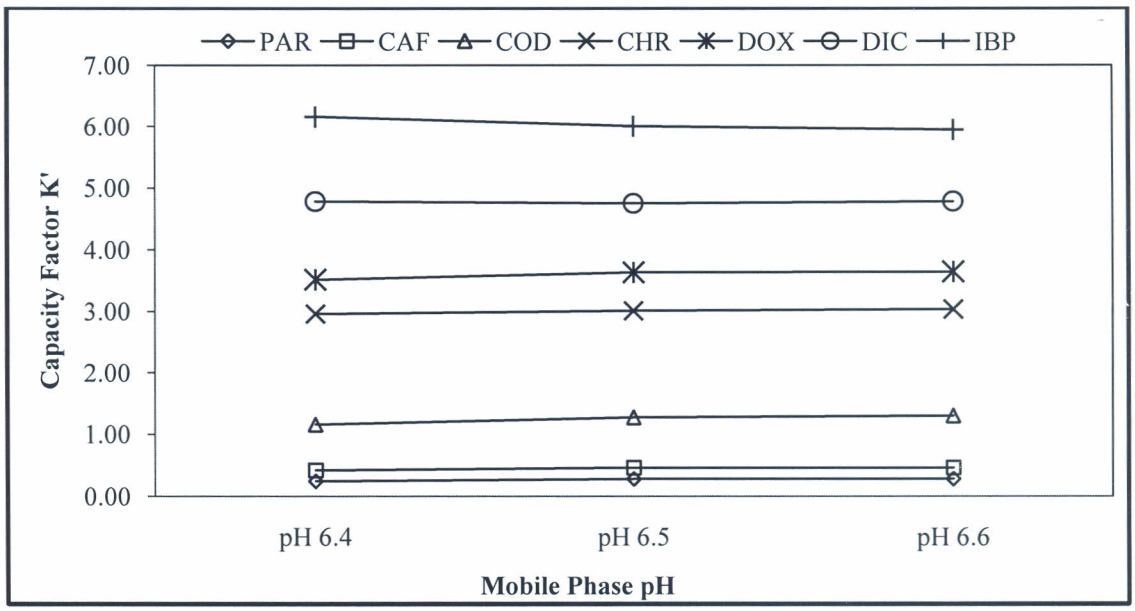


Figure 3.1: Effect of varying mobile phase pH by ± 0.1 pH units on the capacity factors of the compounds under study. Column: Phenomenex Gemini 5μ C_{18} 250 × 4.6 mm. PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

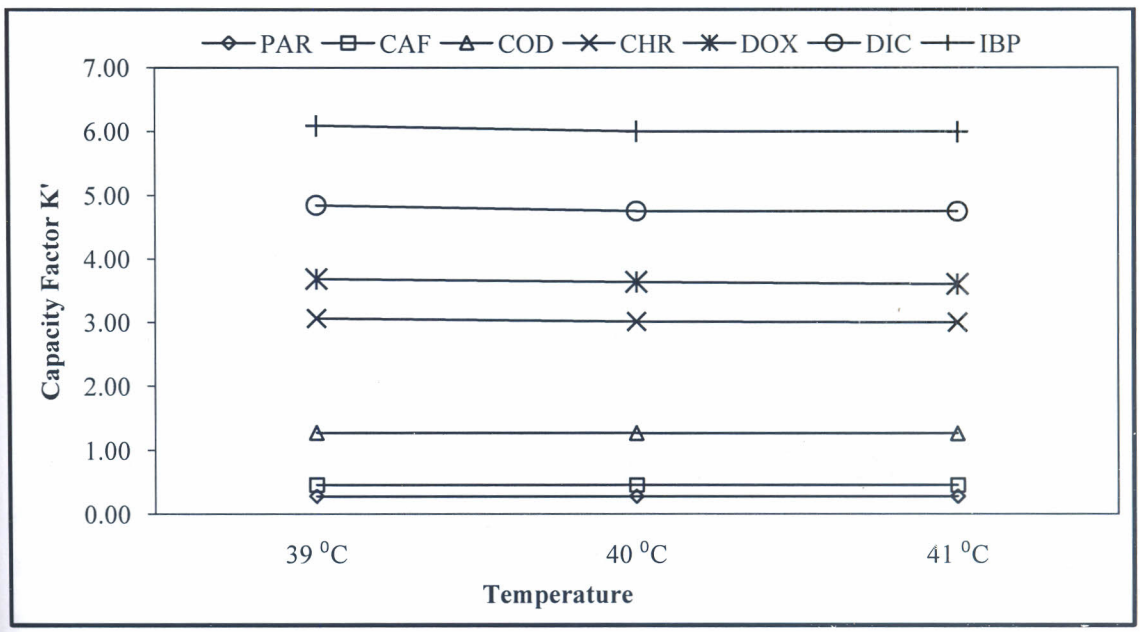


Figure 3.2: Effect of varying temperature by ± 1 °C on the capacity factors of the compounds under study. Column: Phenomenex Gemini 5μ C_{18} 250 × 4.6 mm. PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

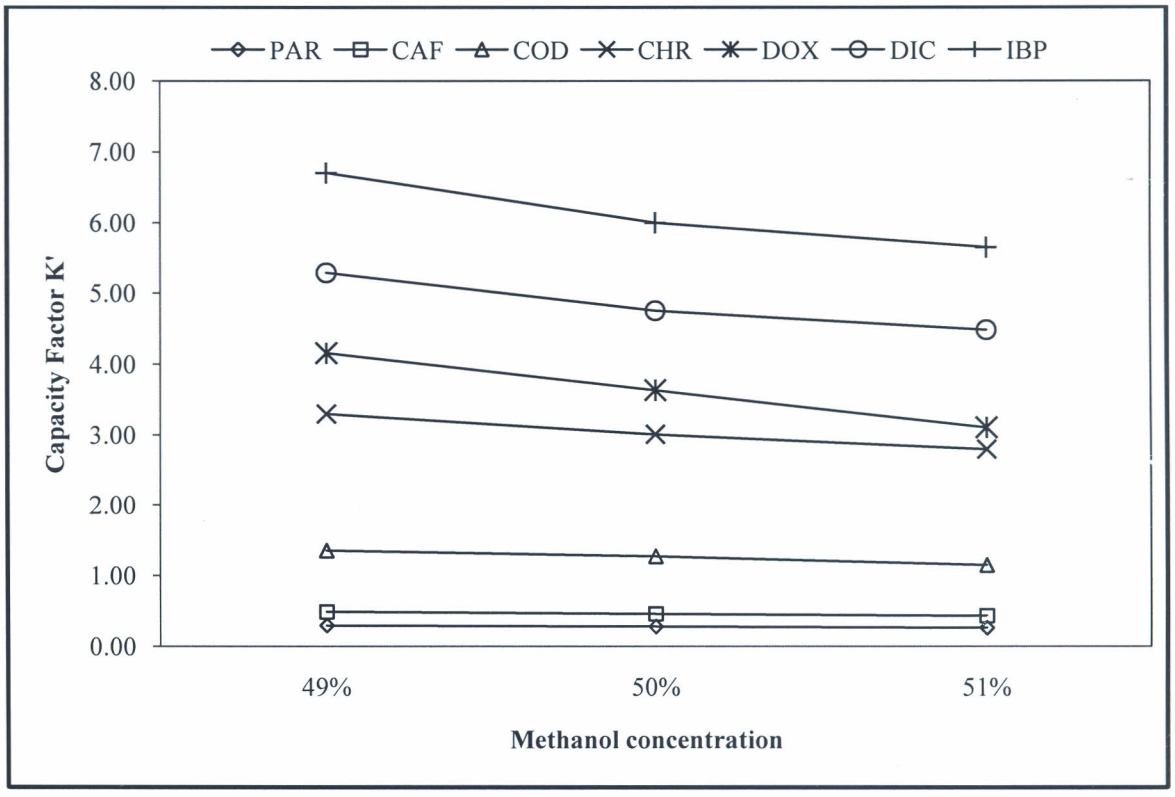


Figure 3.3: Effect of varying methanol concentration by ± 1% on the capacity factors of the compounds under study. Column: Phenomenex Gemini 5μ C_{18} 250 × 4.6 mm. PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

From Figures 3.1-3.3 it was discernible that the method was robust with regard to small variations in the three factors. Nonetheless, the method demonstrated sensitivity to small changes in the methanol concentration. This became more evident when the variation ranges were widened as shown in Table 3.5 and Figure 3.6.

Figure 3.1-3.6 demonstrate that as each of these factors is changed, the capacity factors change. There is a need take measures during method application to minimize their effects. This can be done by accurate measurement of all the mobile phase components, using a calibrated pH meter to read and adjust the pH of the mobile phase and by using a system that has a column oven that is programmable and has been validated. All these measures are aimed at reducing the inherent variations that can arise from these procedures.

Table 3.5: Effect of varying methanol concentration, mobile phase pH and temperature on the selected system suitability parameters of caffeine, doxylamine, chlorzoxazone, diclofenac and ibuprofen.

Varied Chromatographic Conditions		R_s CAF	t_R CHR	t_R DOX	t_R DIC	t_R IBP	A_s IBP
Methanol Concentration (% v/v)	45	3.04	15.63	24.94	23.11	27.69	0.90
	50	2.37	11.46	13.49	16.26	19.66	0.97
	55	1.92	8.75	8.75	11.76	14.11	1.09
Mobile Phase pH	6.0	2.35	12.57	14.90	19.35	25.26	1.03
	6.5	2.18	11.14	13.49	15.55	18.74	1.06
	7.0	2.12	11.07	13.17	15.41	17.92	1.16
Column Temperature	35°C	2.20	12.00	14.70	16.80	19.90	1.05
	40°C	2.18	11.14	13.49	15.55	18.74	1.06
	45°C	2.16	10.47	12.54	14.53	17.80	1.06

Column: Phenomenex Gemini 5μ C_{18} 250 × 4.6 mm 110 Å. Column temperature 40 °C. Flow rate: 1 mL/min. Detection: 220 nm. Injection volume 10 μL. PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

From the results, it was discernible that methanol concentration had the highest effect on the method. At a methanol concentration of 55 % v/v, the resolution between CAF and PAR was below the set limit of 2 and at a concentration of 45 % v/v, the retention time of the last eluting component was greater than 25 min. Of the seven compounds under study, the capacity factor of DOX was the most affected by the variation in the methanol concentration. At a methanol concentration of 45 % v/v its elution order was changed and at 55 % v/v, it co-eluted with CHR (Figure 3.3).

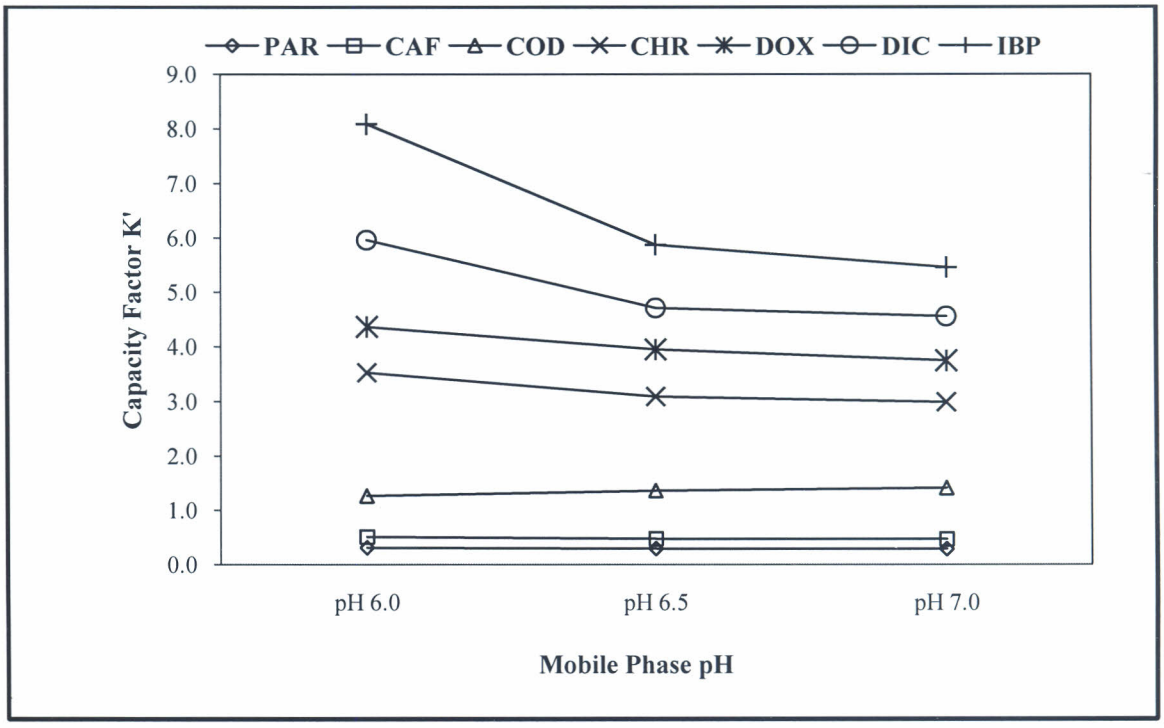


Figure 3.4: Effect of varying mobile phase pH by ± 0.5 pH units on the capacity factors of the compounds under study. Column: Phenomenex Gemini 5μ C_{18} 250 × 4.6 mm. PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

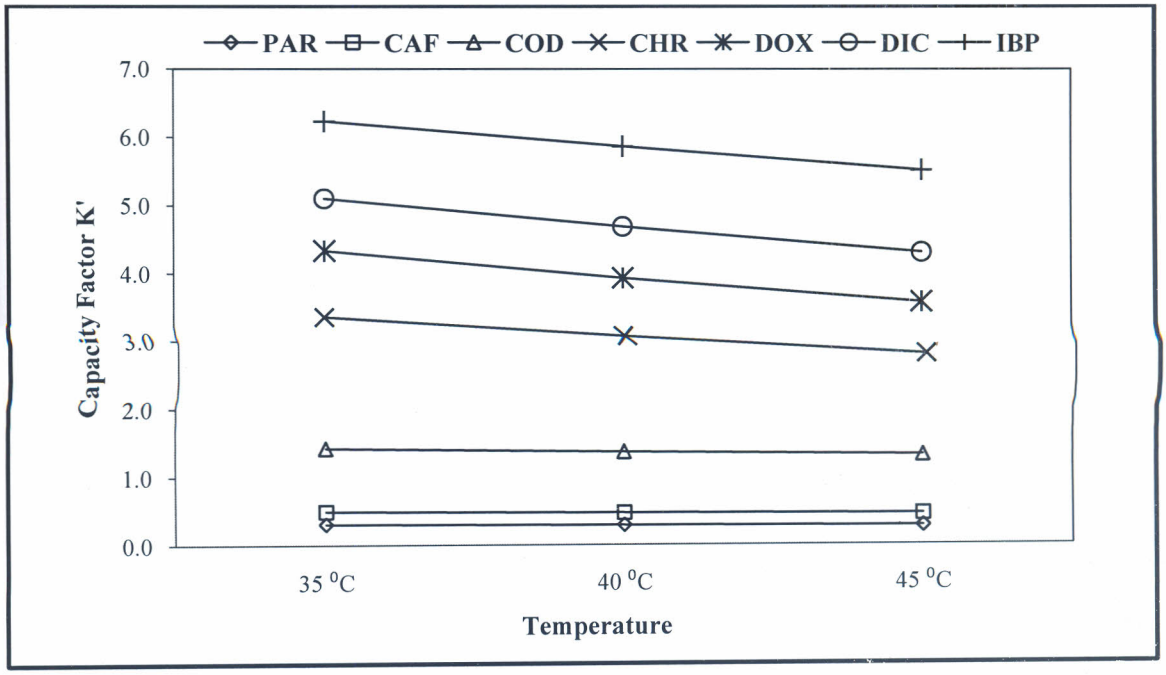


Figure 3.5: Effect of varying temperature by ± 5 °C on the capacity factors of the compounds under study. Column: Phenomenex Gemini 5μ C_{18} 250 × 4.6 mm. PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

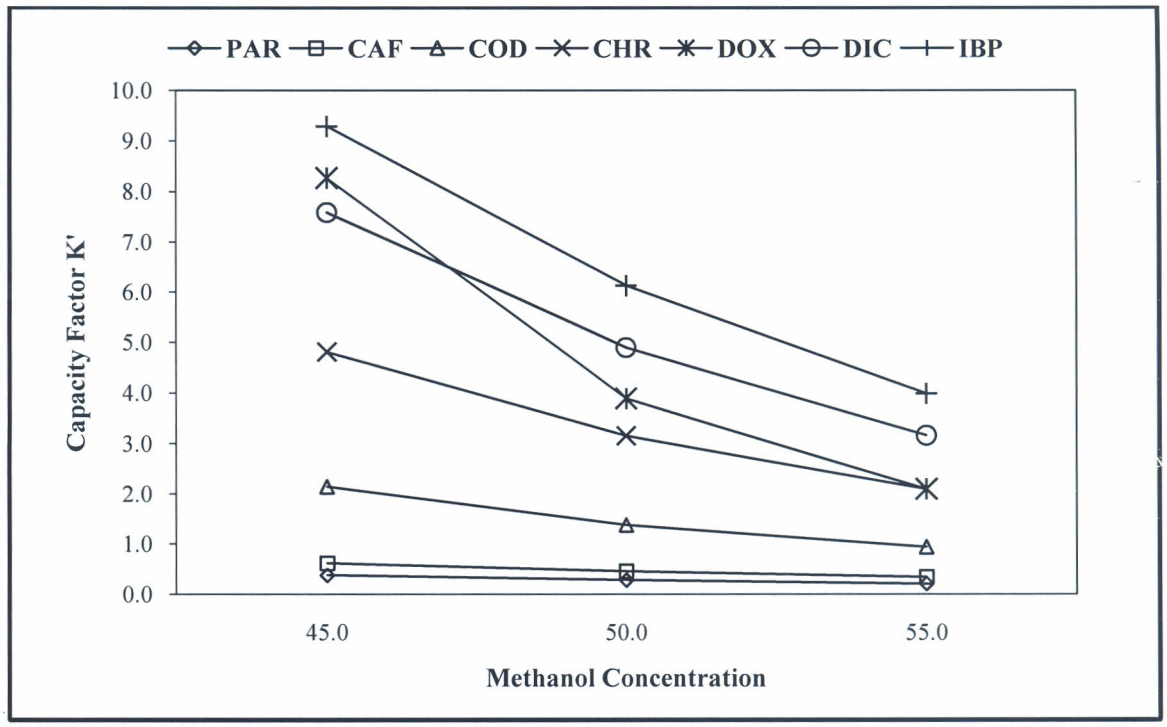


Figure 3.6: Effect of varying methanol concentration by ± 5 % on the capacity factors of the compounds under study. Column: Phenomenex Gemini 5μ C_{18} 250 × 4.6 mm. PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

CHAPTER FOUR

ANALYSIS OF COMMERCIAL SAMPLES

4.1 Introduction

The method developed was intended for the analysis of various analgesic preparations that are available in the market. It is applicable to as many combinations of the seven study components as may be available in the market. Before its recommendation for general use it has to be verified that the method can actually analyze these components. This chapter is therefore aimed at verifying that the method can actually perform the task it was developed for.

4.2 Acquisition of commercial samples

The samples to be tested were obtained from pharmacy outlets within the city of Nairobi. Since no single drug product contains all seven compounds, careful choice was made to ensure that all the compounds were represented in the samples taken. To this end four different samples were obtained; coded A, B, C and D. The composition of the four brands is shown in Table 4.1.

Table 4.1: Details of the samples analyzed

Sample	Batch No:	Label Claim
A	A9697 A9698 A91276	Each tablet contains: Paracetamol 450 mg; Codeine Phosphate 10 mg; Doxylamine Succinate 5 mg; Caffeine 50 mg.
B	X103EE X104EE X107EE	Each caplet contains Paracetamol 500 mg; Caffeine 65 mg.
C	C00110AP L0039AP L0049AP	Each uncoated tablet contains Chlorzoxazone 250 mg; Diclofenac potassium 50 mg; Paracetamol 500 mg.
D	81003 91002 101001	Each tablet contains Ibuprofen 400 mg; Paracetamol 325 mg; Chlorzoxazone 250 mg.

4.3 Analysis of the samples

Three batches of each of the samples were analyzed using the developed method. Twenty tablets of each batch were weighed and pulverized. A quantity of powder was weighed such that the drug concentrations fell within the determined range after dilution (75% - 125%) of the determined reference working solution concentrations. This was then filtered and injected into the LC system. Water-methanol (50:50 v/v) was used as the diluent for all samples. In each case standard preparations containing the drug components to be analyzed were prepared concurrently and at similar concentrations as the drug samples. The prepared samples and standards were run at least three times and the peak areas recorded were used to calculate the percentage content of the drug components in each of the drug samples. The results are shown in table 4.2. Figures 4.1-4.4 are typical chromatograms of the samples analyzed.

The specifications were set at between 90 – 110% of the labeled claim. This was an adoption of the USP specifications for paracetamol and caffeine tablets and paracetamol and codeine phosphate tablets and capsules. The method was used successfully in the analysis of the four samples products. Sample D had paracetamol values above the limits ranging from 115-117%. The other three samples had assay values of paracetamol ranging from 99.6-102%. Sample A and B contained caffeine with assay values ranging from 95.3-102%. Sample C and D contained chlorzoxazone with assay values ranging from 92.0-96.6%. Sample C contained diclofenac with assay values ranging from 95.5-98.6% and sample D contained ibuprofen with assay values ranging from 96.7-99.3%. Sample A contained codeine and doxylamine and the content was less than the adopted specifications ranging from 51.3-53.2% and 65.2-67.2% respectively. The RSD for these two compounds were also greater than 2%, this could be due to the fact that these two compounds are present in very small amounts in the drug product and are therefore not uniformly distributed in the crushed powder.

Generally the batch to batch variation was minimal in the samples analyzed. The greatest variation was between 95.3-102% for caffeine content of sample B.

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Generally the batch to batch variation was minimal in the samples analyzed. The greatest variation was between 95.3-102% for caffeine content of sample B.

Table 4.2: Assay results for the samples analyzed

Sample	Batch No:	Percentage content						
		PAR	CAF	COD	DOX	CHR	DIC	IBP
A	A9697	101.8	100.5	51.3	67.0	-	-	-
		(1.7)	(0.7)	(0.7)	(0.8)			
	A9698	100.7	98.1	53.2	67.2	-	-	-
		(1.8)	(0.9)	(3.4)	(1.8)			
	A91276	100.1	99.4	51.6	65.2	-	-	-
		(1.7)	(1.0)	(5.6)	(5.5)			
B	X103EE	101.1	97.1	-	-	-	-	-
		(1.5)	(1.9)					
	X104EE	99.6	102.4	-	-	-	-	-
		(1.6)	(0.4)					
	X107EE	101.1	95.3	-	-	-	-	-
		(1.8)	(1.0)					
C	C00110AP	101.0	-	-	-	95.9	95.5	-
		(1.1)				(0.8)	(2.0)	
	L0039AP	101.3	-	-	-	96.6	98.6	-
		(1.4)				(1.8)	(1.4)	
	L0049AP	100.0	-	-	-	95.2	97.0	-
		(2.0)				(1.0)	(0.2)	
D	81003	115.2	-	-	-	92.6	-	99.3
		(1.8)				(1.7)		(1.8)
	91002	117.1	-	-	-	92.0	-	96.7
		(1.3)				(0.6)		(0.2)
	101001	117.1	-	-	-	93.8	-	97.6
		(1.3)				(0.7)		(1.6)

The values in the parenthesis are the %RSD.

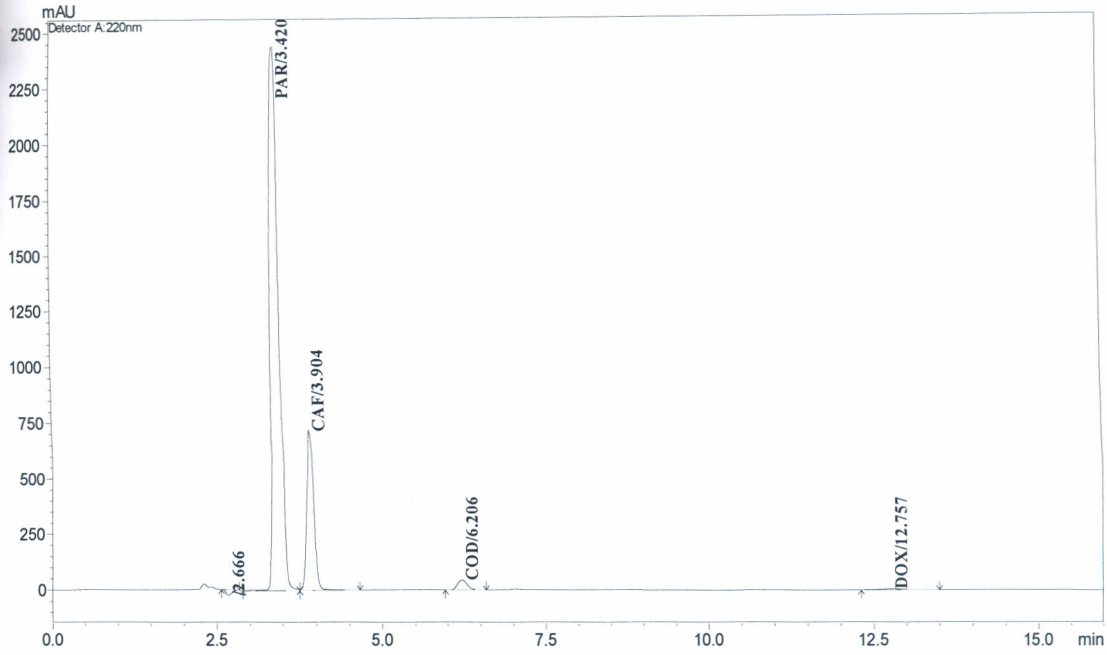

Figure 4.1: Typical chromatogram of sample A. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.25 M Sodium Octanesulfonate-0.2 M CH_3COONH_4 pH 6.5-water (50:2:10:38 % v/v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

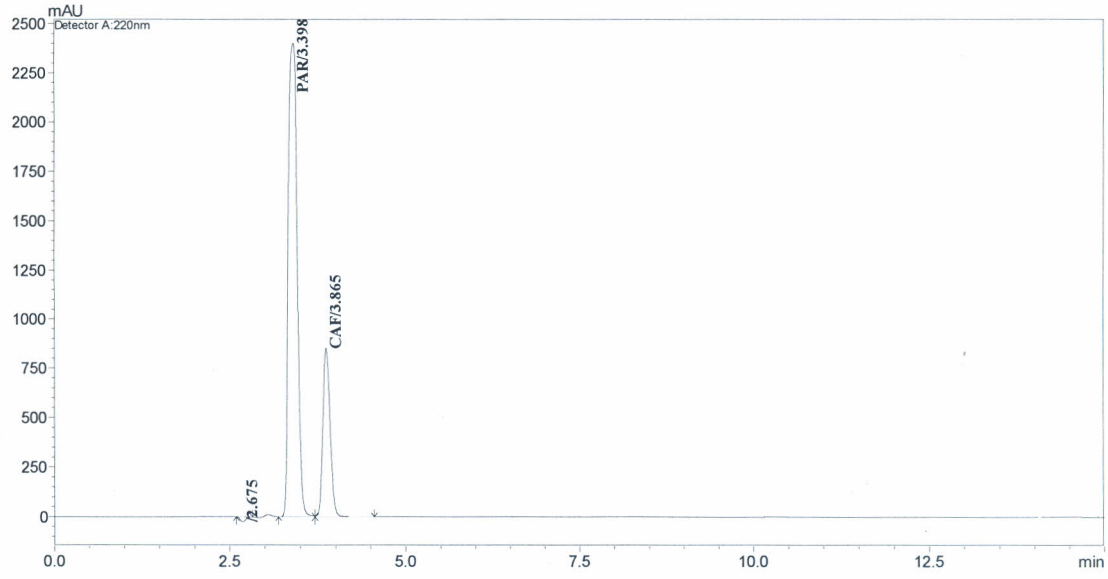

Figure 4.2: Typical chromatogram of sample B. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.25 M Sodium Octanesulfonate-0.2 M CH_3COONH_4 pH 6.5-water (50:2:10:38 % v/v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

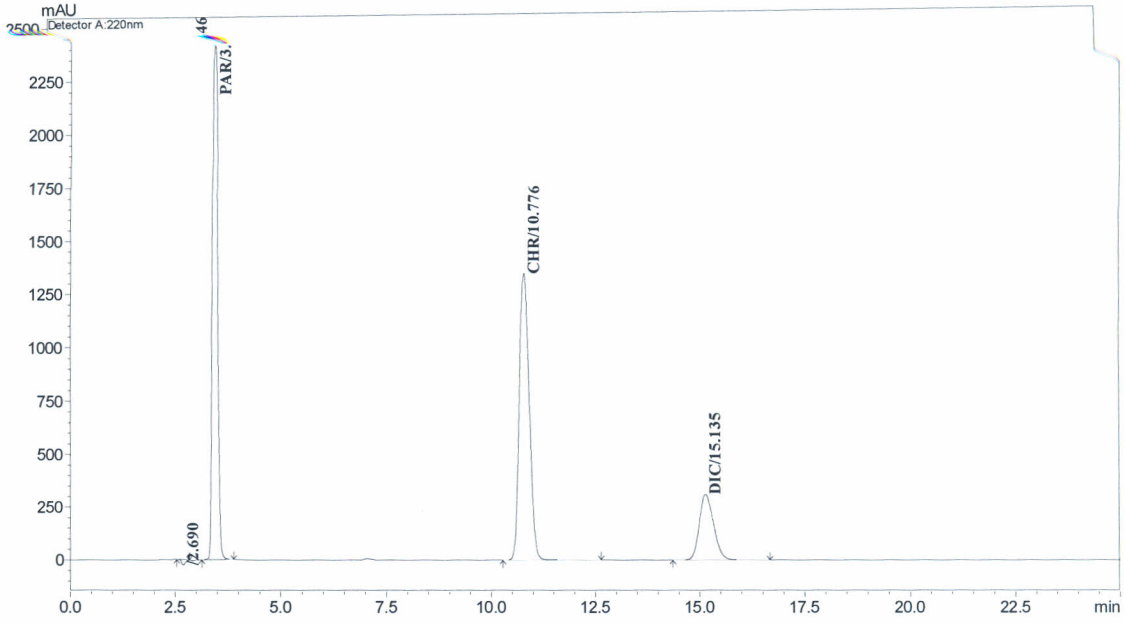

Figure 4.3: Typical chromatogram of sample C. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.25 M Sodium Octanesulfonate-0.2 M CH_3COONH_4 pH 6.5-water (50:2:10:38 % v/v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

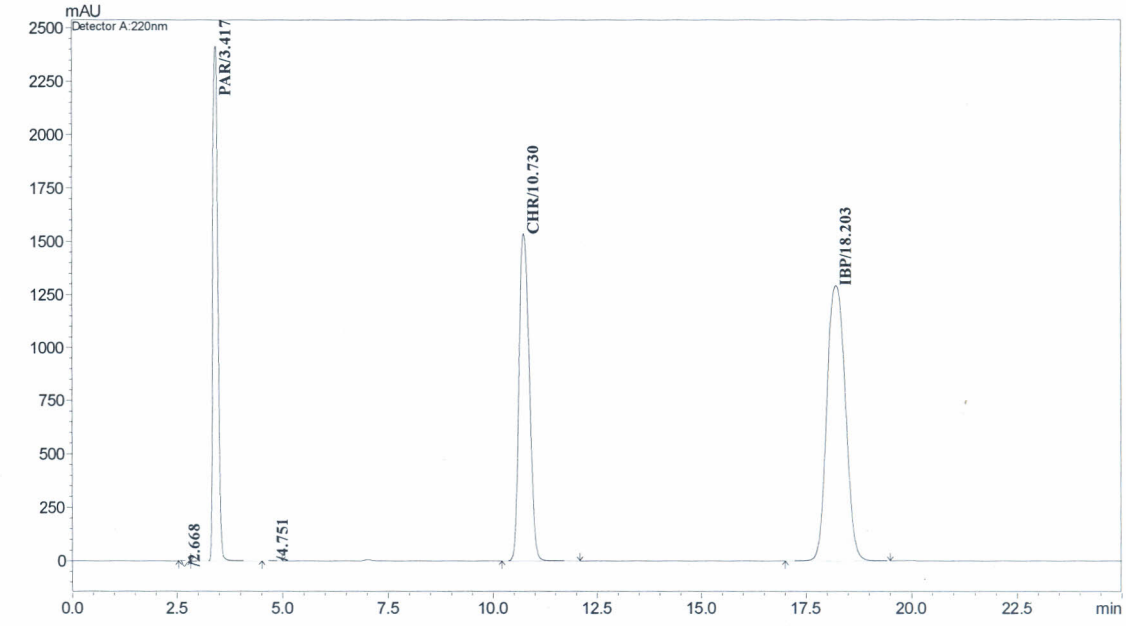

Figure 4.4: Typical chromatogram of sample D. Column: Phenomenex Gemini® 5μ C_{18} 250 × 4.6 mm. Mobile phase: methanol-0.25 M Sodium Octanesulfonate-0.2 M CH_3COONH_4 pH 6.5-water (50:2:10:38 % v/v/v/v). PAR-paracetamol, CAF-caffeine, COD-codeine, CHR-chlorzoxazone, DOX-doxylamine, DIC-diclofenac, IBP-ibuprofen.

CHAPTER FIVE

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 General Discussion

A method for the analysis of several drug components in analgesic preparations was developed. It was found to have an adequate degree of accuracy and linearity to be used in the quantitative determination of PAR, CAF, COD, DOX, CHR, DIC and IBP in the various pharmaceutical preparations sampled. The method utilizes commonly available reagents. It can therefore be utilized in most quality control laboratories.

The method also employed a silica based C_{18} stationary phase that is widely used in analysis of pharmaceutical products and is thus readily available in QC laboratories. The detection wavelength was 220 nm which necessitated the use of a variable wavelength UV detector. This is not a major setback as most modern HPLC machines have variable wavelength UV detectors.

Currently, there is no single method available to analyze these seven drug components simultaneously. This developed method affords a great advantage both in times of analysis and cost because several drug products containing any of the seven compounds in different combinations can be analyzed together. This is particularly useful in post market surveillance activities of analgesic preparations as many samples can be tested simultaneously thus reducing both time of analysis which is always vital in post market surveillance exercises and also reduces the cost of analysis. The ideal situation for a pharmaceutical analyst is one method that analyzes all pharmaceutical products. The method developed is a step towards development of such a super method.

5.2 Conclusion

A high performance liquid chromatographic method was developed to be used in the analysis of caffeine, chlorzoxazone, codeine, Diclofenac, doxylamine, ibuprofen and

paracetamol present in analgesic preparations available for sale in Kenya. The optimum chromatographic conditions were a mobile phase containing methanol, 0.25M Sodium Octanesulfonate, 0.2M CH_3COONH_4 at a pH of 6.5 and water in the ratios (50:2:10:38, % v/v/v/v). This was delivered at a flow rate of 1 mL/min through an octadecylsilane stationary reverse phase with the dimensions 250 mm in length and 4.6 mm internal diameter (Phenomenex Gemini 5μ C_{18}) maintained at a temperature of 40 °C. A volume of 10 μL of the working standard mixture was injected into the LC system and UV detector was set at 220 nm.

Validation of the method demonstrated that the limit of detection for CAF, CHR, COD, DIC, DOX, IBP and PAR were 11.99 ng, 20.72 ng, 25.08 ng, 12.50 ng, 32.93 ng, 12.02 ng, 22.36 ng, and the limit of quantitation were 79.92 ng, 103.60 ng, 104.50 ng, 62.49 ng, 164.64 ng, 48.08 ng, 111.80 ng respectively. The method was accurate with recoveries of 100.3% (CAF), 101.6% (CHR), 98.3% (COD), 98.1% (DIC), 102.7% (DOX), 99.2% (IBP) and 98.1% (PAR). It also demonstrated adequate linearity over a range of 75% to 125% and accuracy. The linearity equations for the components were: PAR $y = 3919343.68x + 13006317.90$ ($R^2 = 0.9960$), CAF $y = 25200967.68x + 274065.03$ ($R^2 = 0.9995$), COD $y = 23197718.41x + 10690.97$ ($R^2 = 0.9992$), DOX $y = 9101469.99x - 667.67$ ($R^2 = 0.9975$), CHR $y = 117497181.80x + 4681821.32$ ($R^2 = 0.9986$), DIC $y = 39115105.06x + 76240.33$ ($R^2 = 0.9993$) and IBP $y = 22942174.64x + 621592.37$ ($R^2 = 0.9998$). The method also demonstrated adequate intra-day and intermediate precision with intra-day precision coefficients of variation ranging from 0.15-0.37% and intermediate precision coefficients of variation ranging from 0.91-1.96% for the seven compounds. Robustness studies demonstrated that the chromatographic functions were largely unaffected by small changes in pH and temperature. Changes in the methanol concentration affected DOX significantly but the other components were largely unaffected.

The developed method was used to successfully analyze 12 batches of samples collected from pharmacy outlets within Nairobi city. The product containing COD and DOX gave assay values below 90% for both components. This was seen in all the three batches

analyzed. One of the samples also had its values of PAR greater than 110% (115-117%). with minimal variation in the three batches analyzed. This indicates there is a need to further study the content of PAR, COD and DOX in these two pharmaceutical products through a post market surveillance exercise encompassing many more samples and locales. The other components were within the stated limits of 90-110% with minimal inter batch variation. The method developed was found to be simple, precise and fast enough to offer a practical means for routinely analyzing analgesic preparations containing any of the seven components in a QC laboratory.

5.3 Recommendations

This method was developed to be used mainly for post market surveillance exercises. To make it more applicable, more work needs to be done to increase the number of drugs that can be analyzed with greater focus on the more readily available over the counter analgesics such as aspirin. This would allow a single method to analyze as many components simultaneously as may be possible.

The resolution between the CAF and PAR is greater than 2 but work needs to be done to try and improve this further. This is because the column used was new and used only for this method development. As a column ages the chromatographic parameters worsen; improving the resolution increases how long a particular column can be used with this particular method. Collaborative studies need to be carried out to determine the reproducibility of the method. This would improve the applicability of the method as it could be used in other laboratories.

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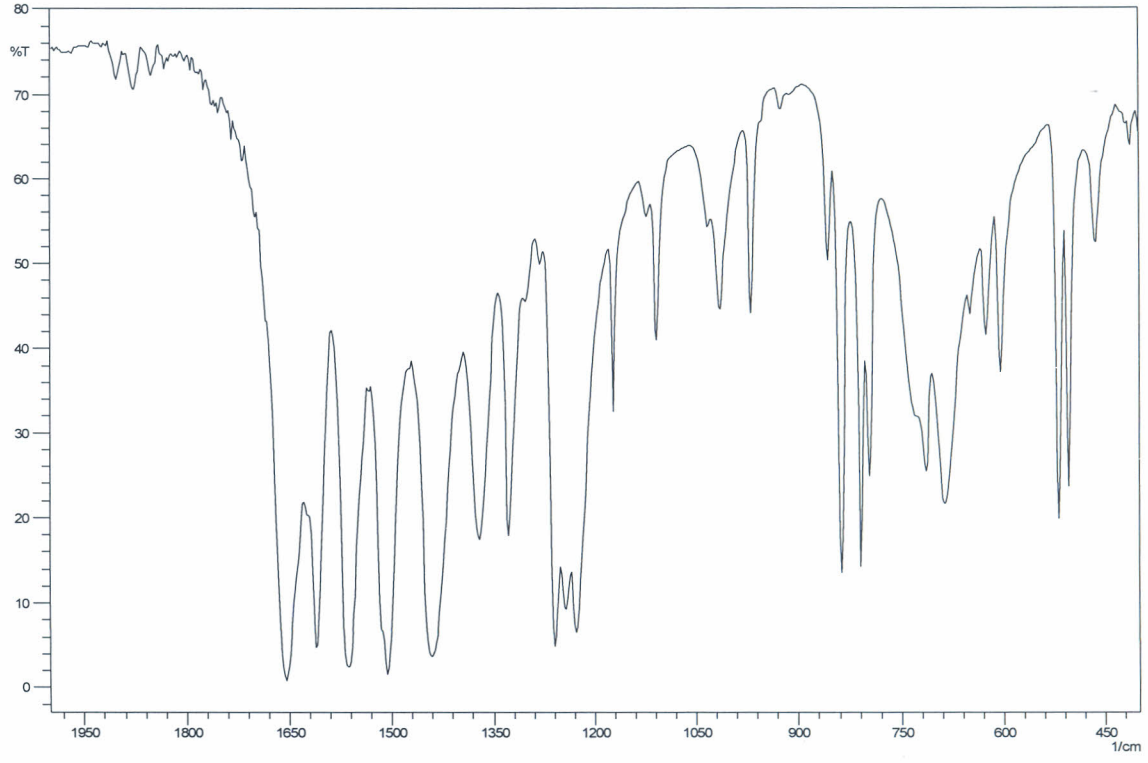
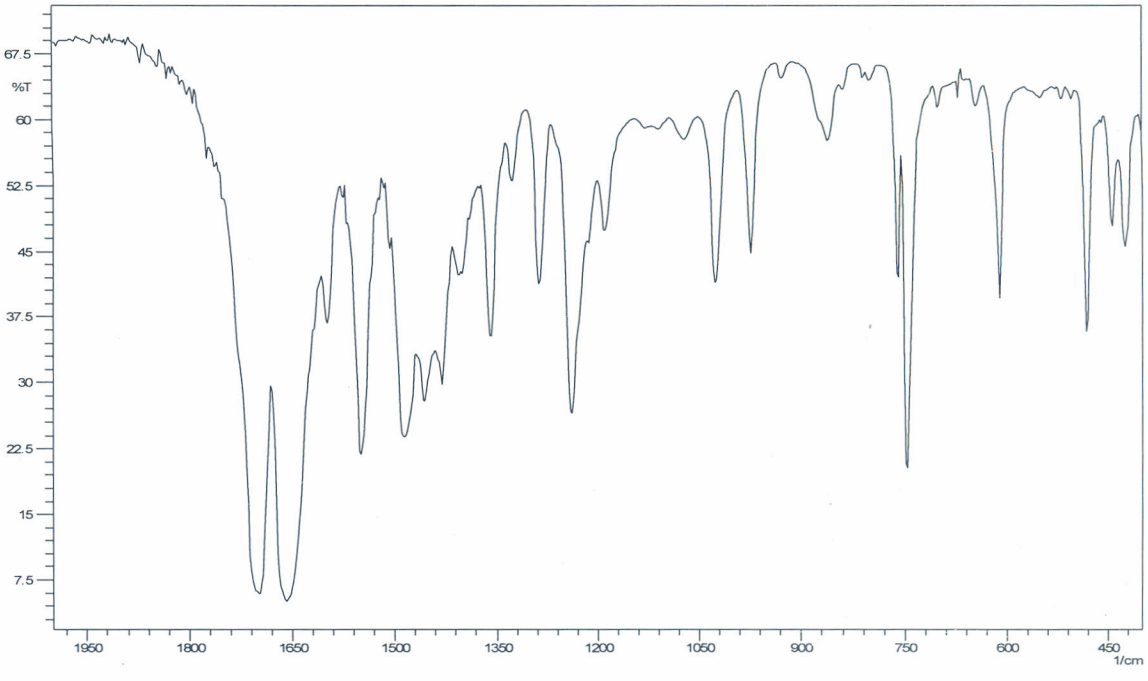
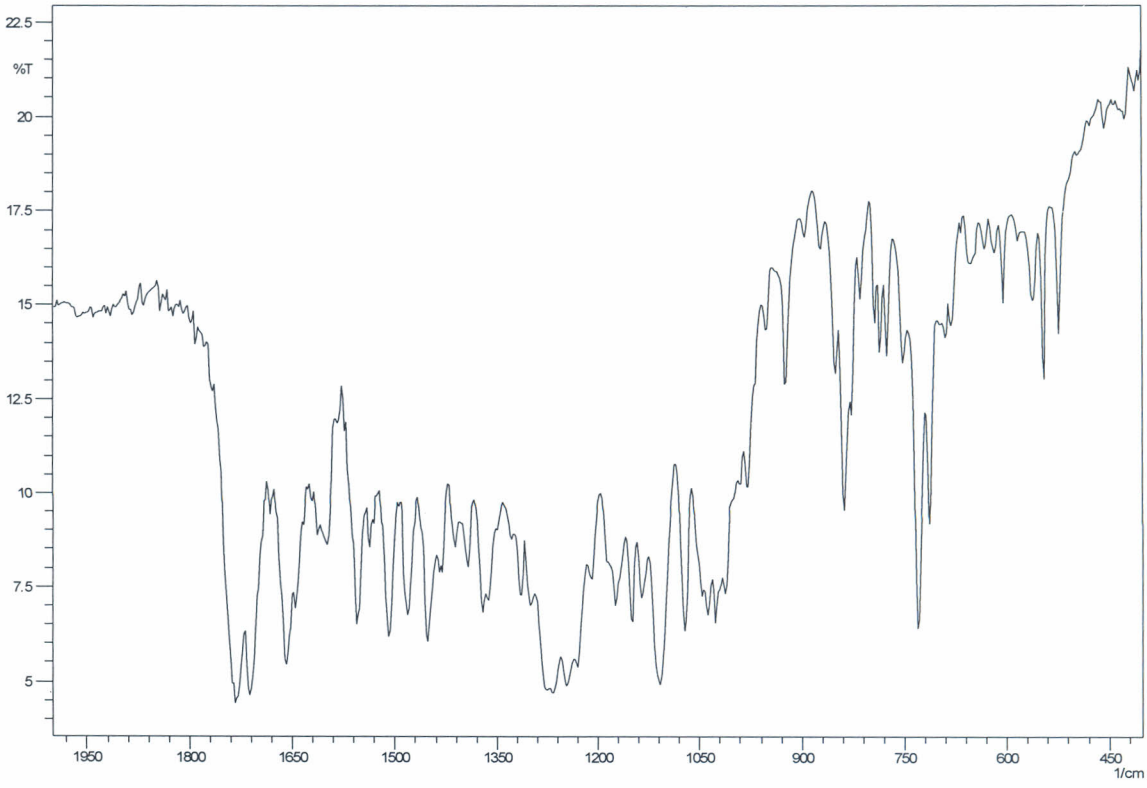
APPENDICES



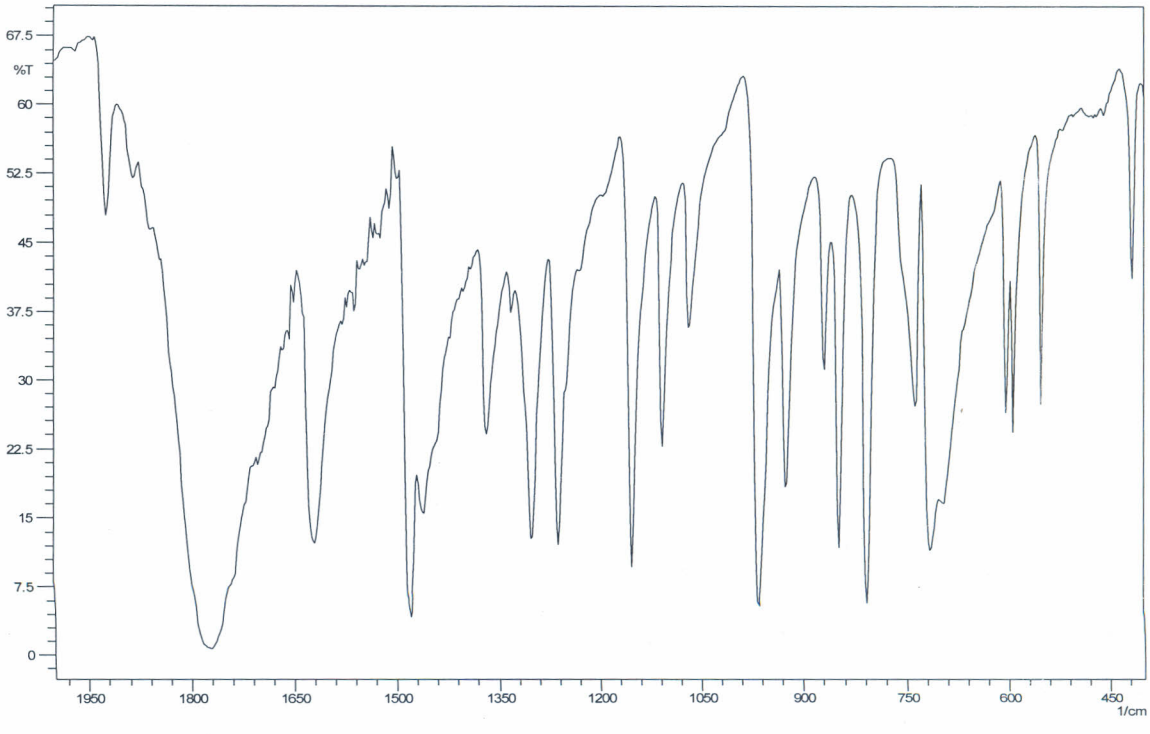
Appendix 1: FTIR spectrum of paracetamol (KBr Disk)



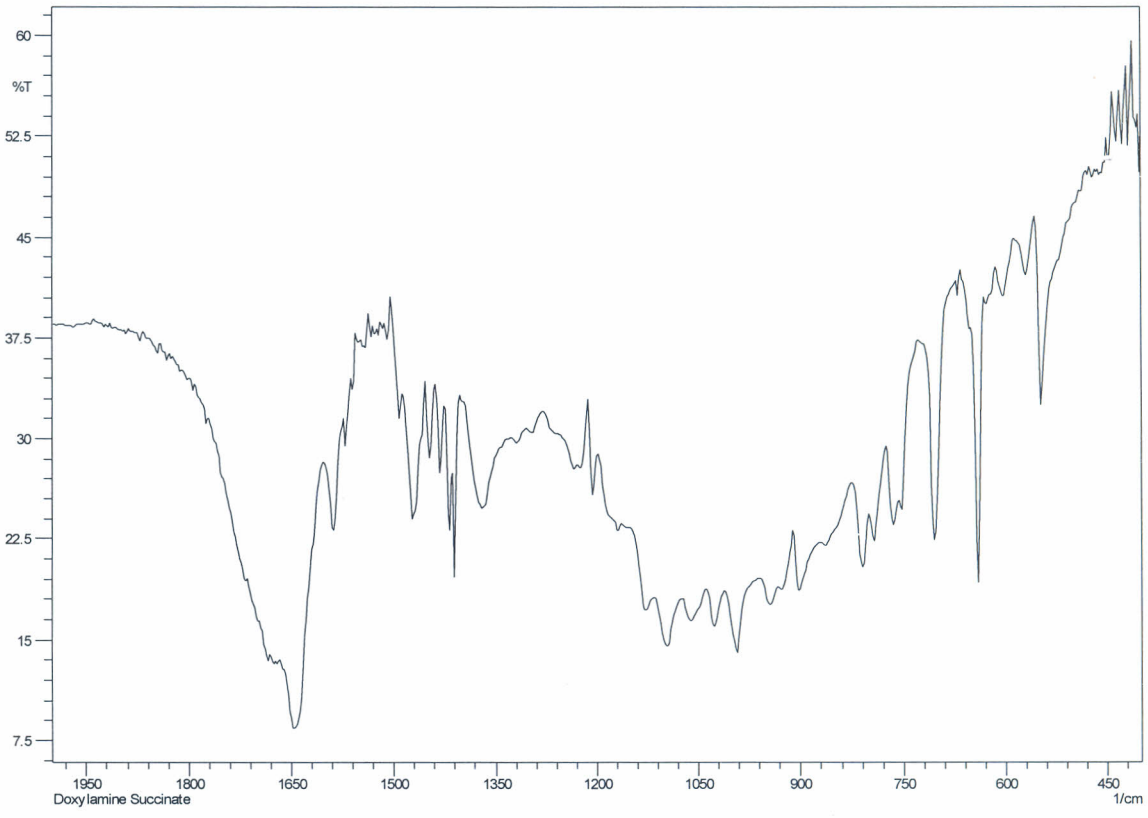
Appendix 2: FTIR Spectrum of caffeine (KBr Disk)



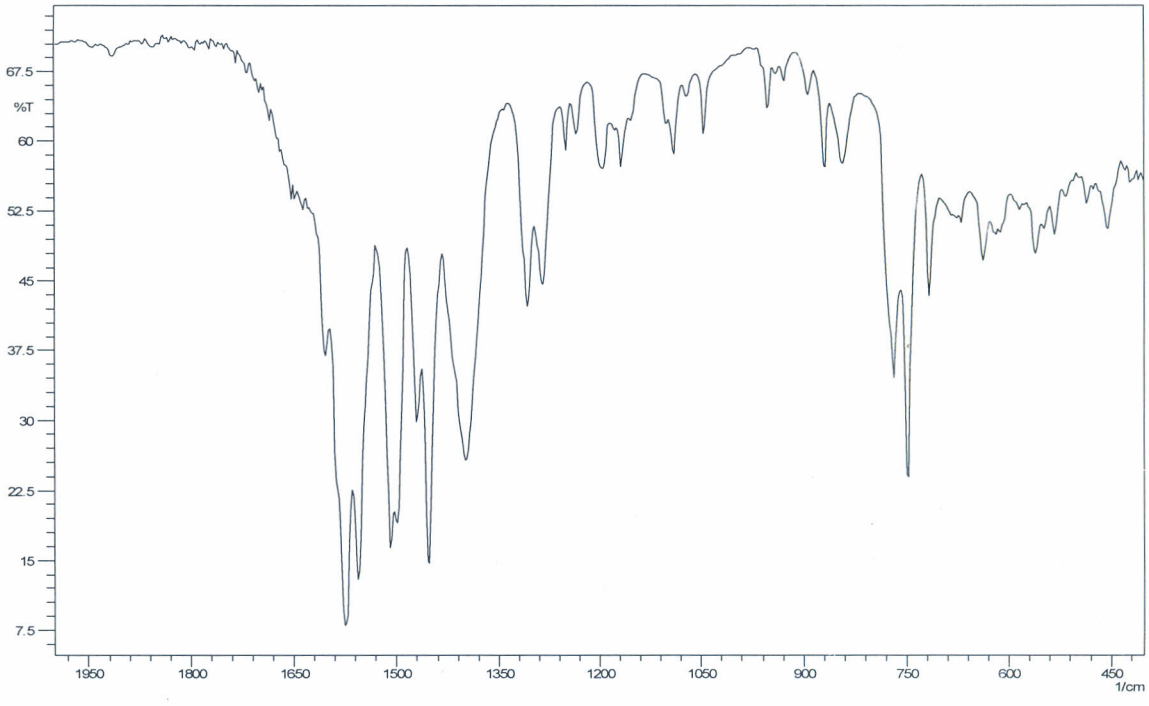
Appendix 3: FTIR Spectrum of codeine phosphate (KBr Disk)



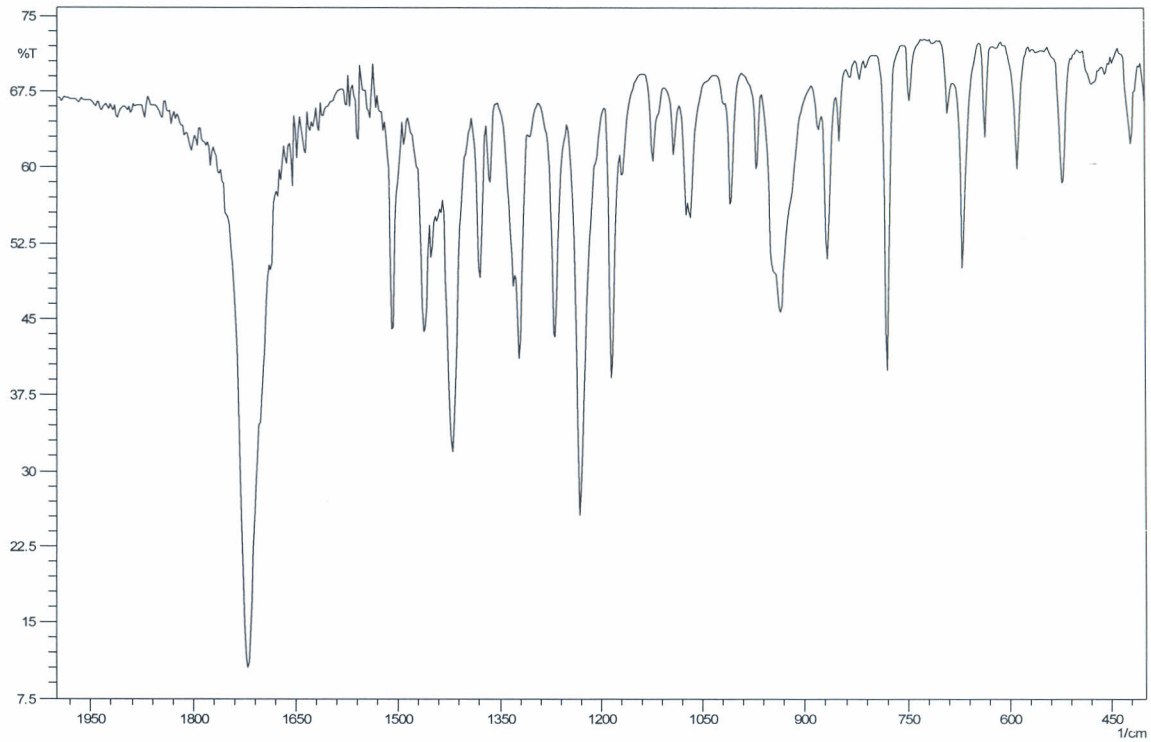
Appendix 4: FTIR Spectrum of chlorzoxazone (KBr Disk)



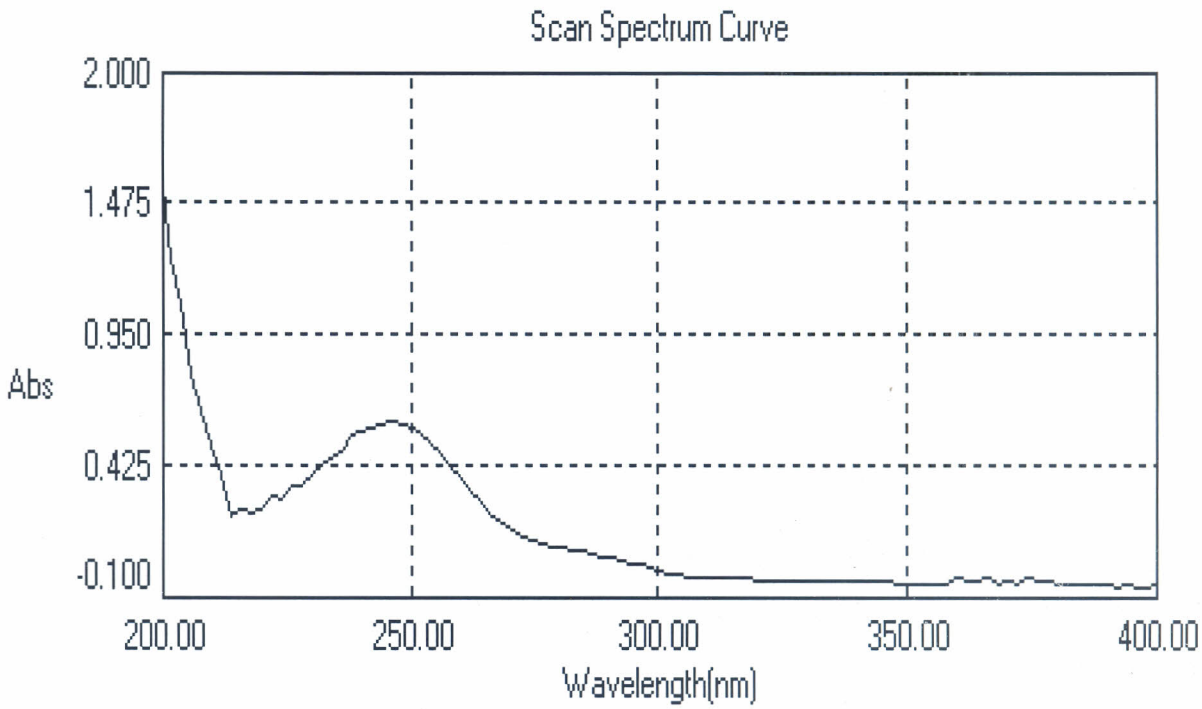
Appendix 5: FTIR Spectrum of doxylamine succinate (KBr Disk)



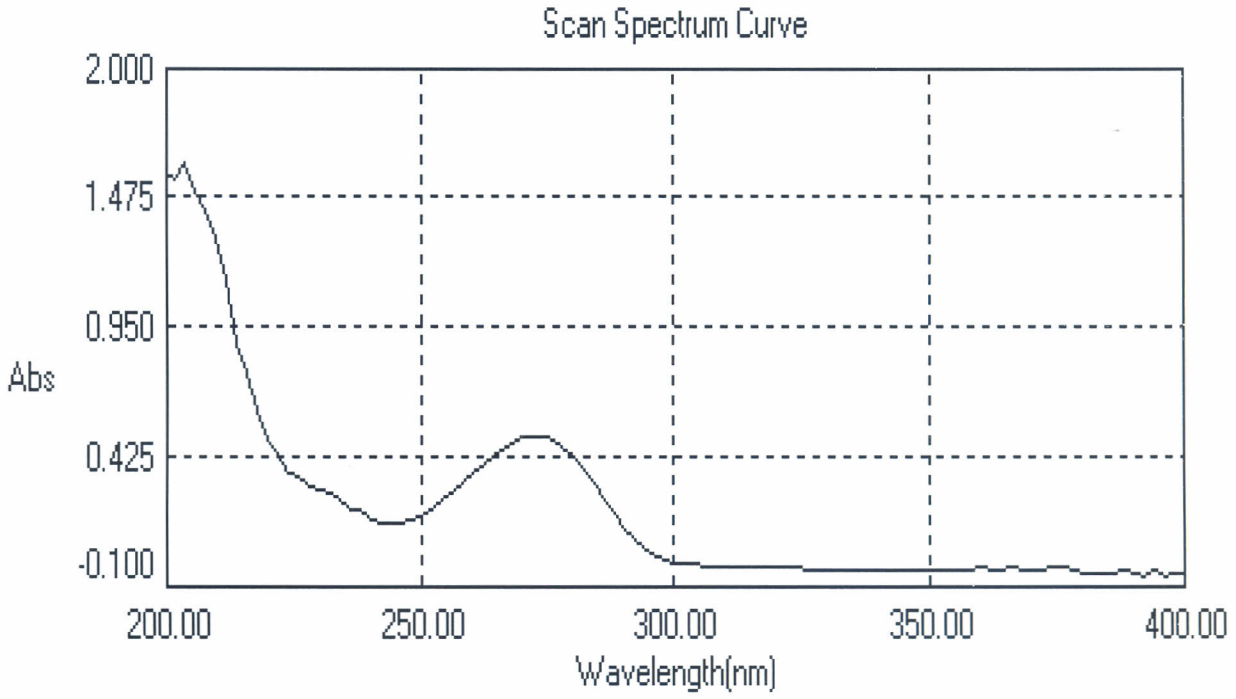
Appendix 6: FTIR Spectrum of diclofenac sodium (KBr Disk)



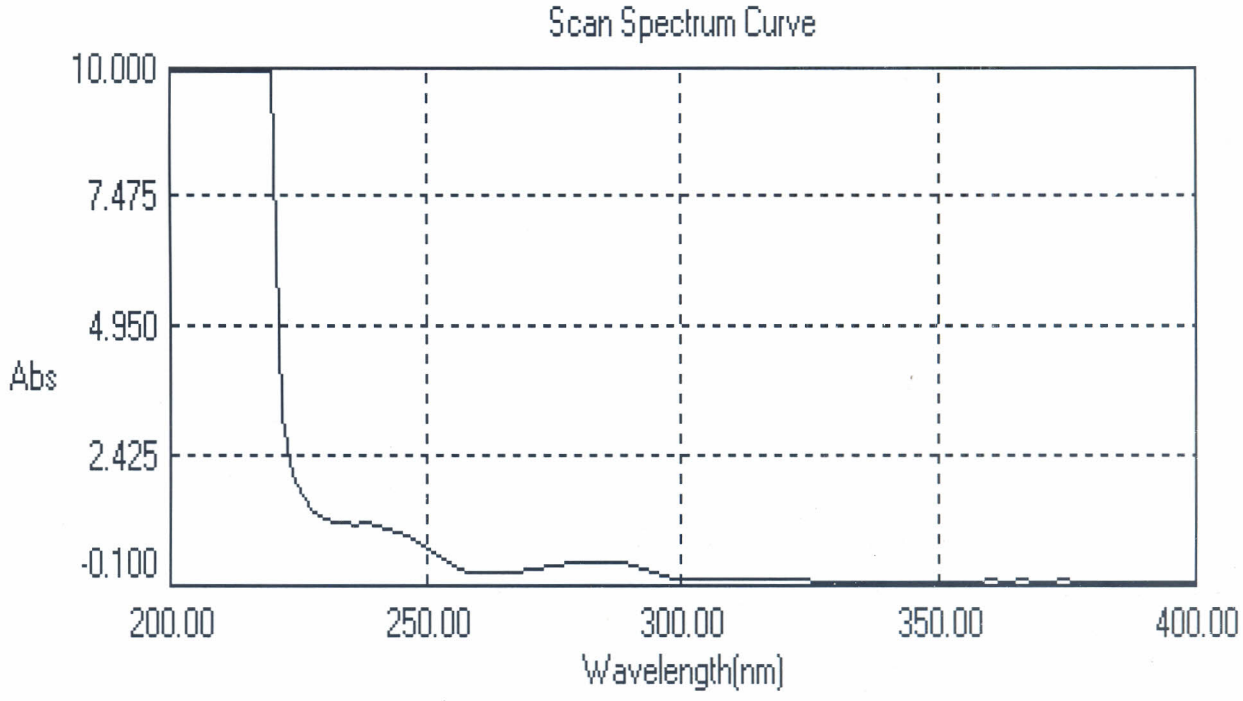
Appendix 7: FTIR Spectrum of ibuprofen (KBr Disk)



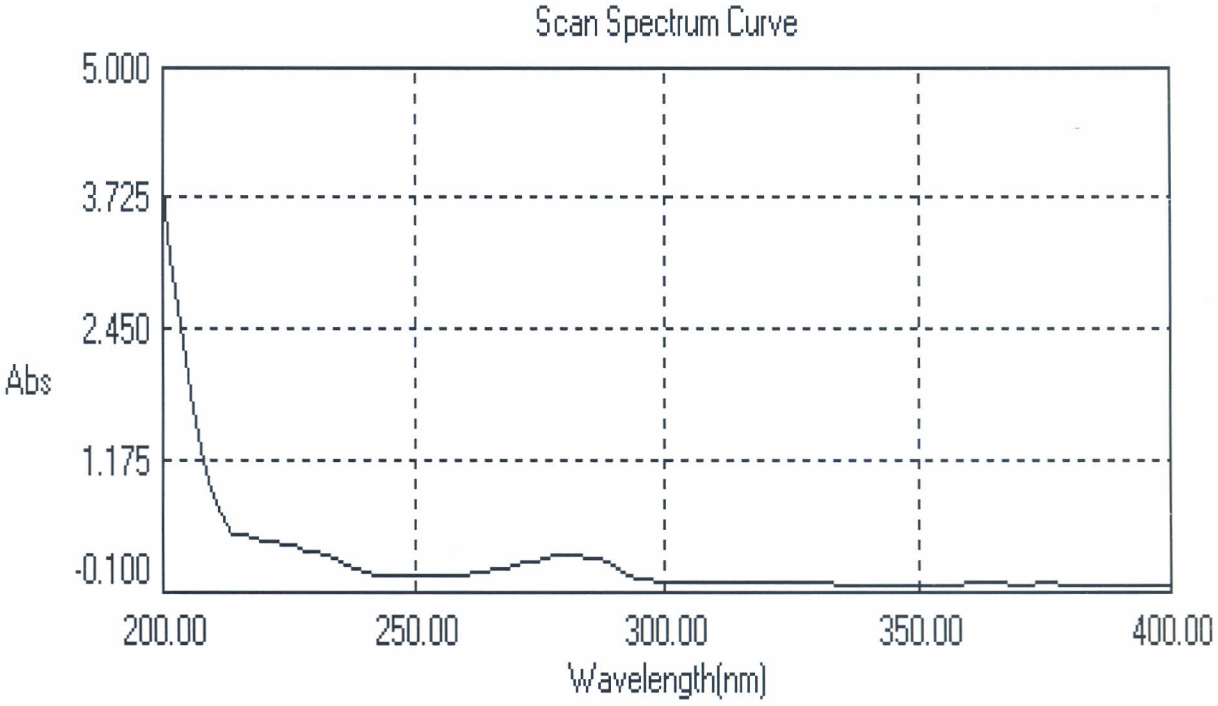
Appendix 8: UV Spectrum of paracetamol



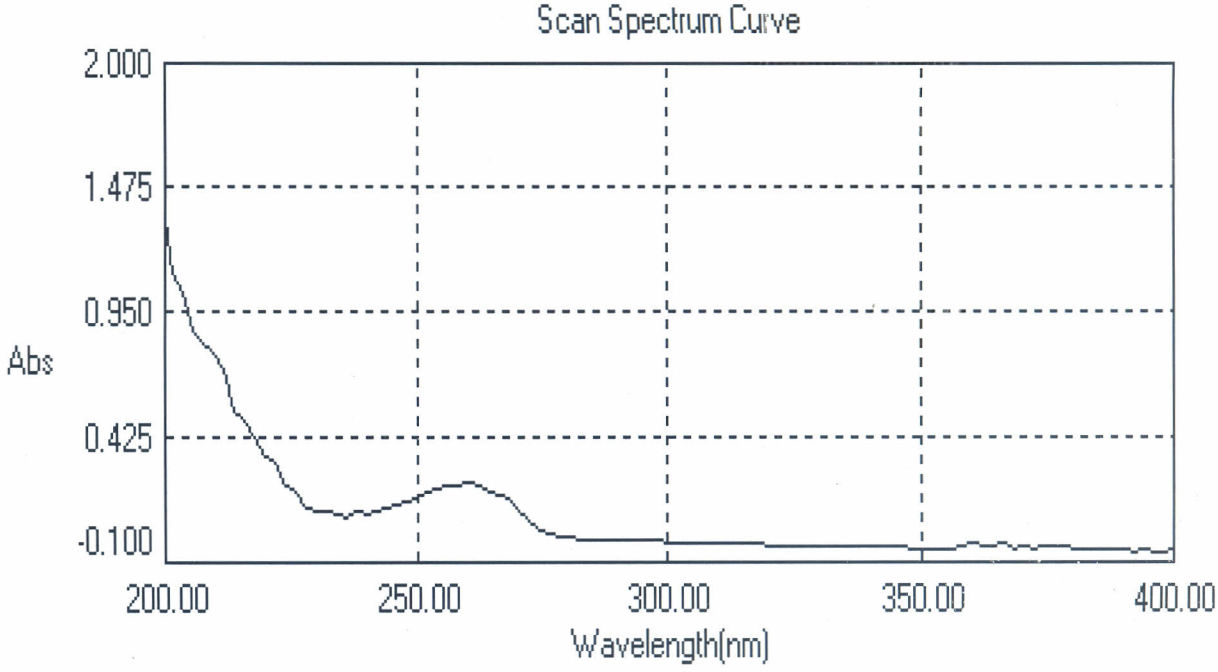
Appendix 9: UV Spectrum of caffeine



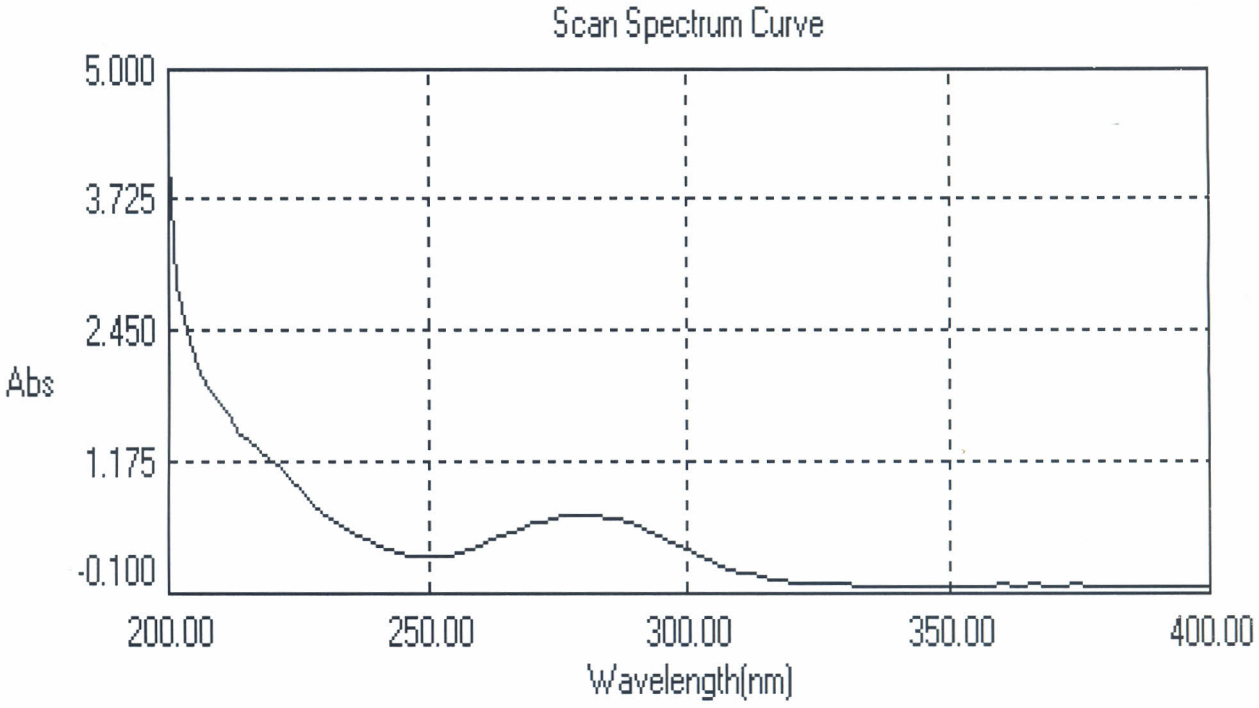
Appendix 10: UV Spectrum of codeine phosphate



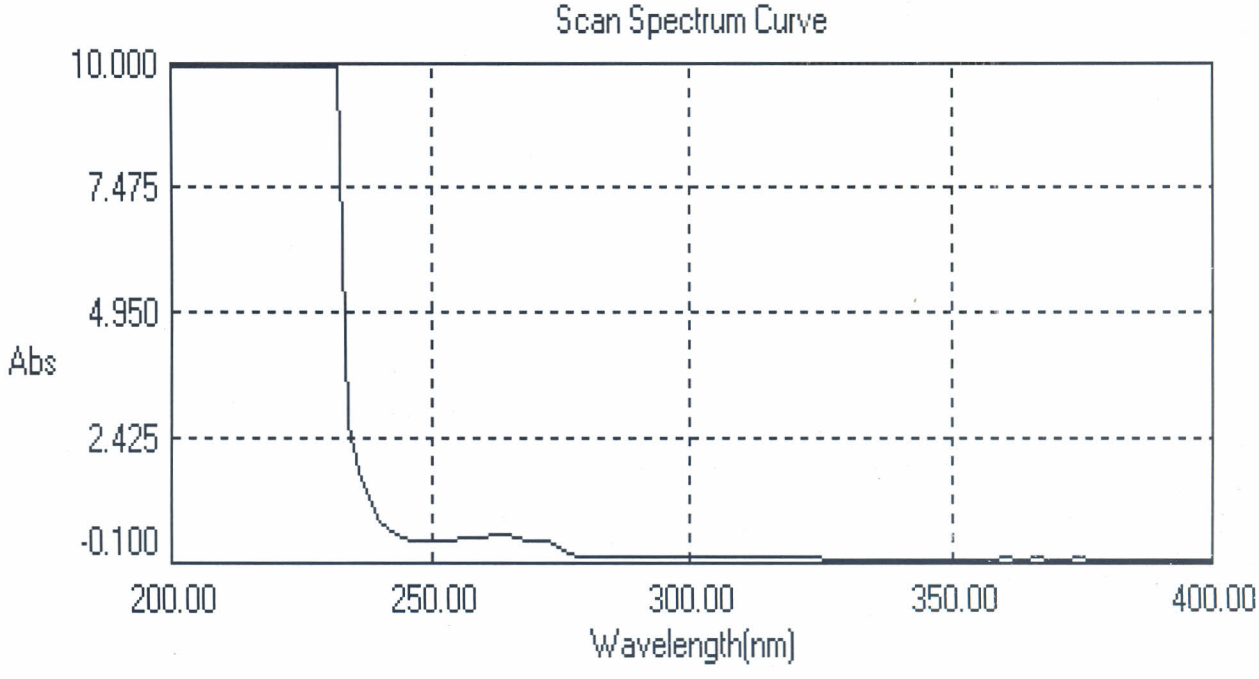
Appendix 11: UV Spectrum of chlorzoxazone



Appendix 12: UV Spectrum of Doxylamine succinate



Appendix 13: UV Spectrum of diclofenac sodium



Appendix 14: UV Spectrum of ibuprofen