REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

DEVELOPMENT AND VALIDATION OF AN ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHIC-TANDEM MASS SPECTROMETRY (UPLC-MS/MS) METHOD FOR DETERMINATION OF BETAMETHASONE OR DEXAMETHASONE IN PHARMACEUTICAL PREPARATIONS

(Mohammad Jamal) A. SHAMMOUT

Program of Analytical Chemistry
PhD THESIS

Ankara
2013

REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

DEVELOPMENT AND VALIDATION OF AN ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHIC-TANDEM MASS SPECTROMETRY (UPLC-MS/MS) METHOD FOR DETERMINATION OF BETAMETHASONE OR DEXAMETHASONE IN PHARMACEUTICAL PREPARATIONS

(Mohammad Jamal) A. SHAMMOUT

Program of Analytical Chemistry
PhD THESIS

THESIS SUPERVISOR
Prof.Dr.Nursabah E.BAŞÇI

ANKARA
2013

iii

Department: Analytical Chemistry

Program: Doctor of Philosophy

Title of thesis: Development and Validation of an Ultra-Performance Liquid Chromatographic-Tandem Mass Spectrometry (UPLC-MS/MS) Method for Determination of Betamethasone or Dexamethasone in Pharmaceutical Preparations.

Name and Surname: (Mohammad Jamal) A. SHAMMOUT

Date of Thesis Discussion: 25.01.2013

Our Jury committee approved this study (Dissertation) as thesis for Doctor of Philosophy in Analytical Chemistry.

Jury President:	Prof. Dr. Sibel A.Özkan Ankara University
Supervisor:	Prof. Dr. Nursabah E. Başcı Hacettepe University
Member:	Prof. Dr. Sacide Altınöz Hacettepe University
Member:	Prof. Dr. Sedef Kır Hacetepe University
Member:	Prof. Dr. Nuran Özaltın Hacettepe University

Approval:

This thesis is approved by the above mentioned jury members on the basis of Hacettepe University Graduate Education and Examination code`s articles and approved by institute of Board Members.

Prof. Dr. Ersin FADILLIOĞLU

Director of the Institute

DEDICATION

To Soul of My Father

To my loving Mother, my brothers, my sisters

And to my lovely wife, ASYA

ACKNOWLEDGEMENTS

I would like to thank my PhD supervisor Prof. Dr. Nursabah E. Başcı for her support, guidance, and unconditional assistance throughout the courses and Thesis supervision of my doctoral degree. Thank you to the individual members of my PhD committee for their ongoing academic assistance throughout the course of my PhD, to Prof. Dr. Sibel Özkan, Prof. Dr. Sacide Altınöz, Prof. Dr. Nuran Özaltın and Prof. Dr. Sedef Kır, thank you for supporting me not only throughout the final stages of my PhD but also during the initial coursework, cumulative exams, academic seminars, and oral proposals associated with the PhD program at department of Analytical Chemistry.

It would be impossible to appropriately thank the individual members of the Analytical Chemistry department in Faculty of Pharmacy at Hacettepe University, special thanks to Ayşegül Doğan, Ebru Üçaktürk, Emirhan Nemutlu and Mustafa Çelebier for all that they have done for me during PhD program for their assistance and expertise are greatly.

I would like to sincerely thank members of Turkish Doping Center specially, Sinan Onul,Betül Sağlam,Seda Olgaz, Şeyda Akpinar, Tuba Kaçar and Münevver Tandoğan for supporting during my doctoral research at Turkish Doping Center.

Great thankful to the Scientific and Technological Research Council of Turkey (TÜBİTAK) and Ministry of National Education for their scholarship that was supporting me during my study in Hacettepe University.

Finally, very special thanks for all my best Dear friends in Turkey specially:

Ahmed K.Alturk, Anis Sati, Ahmed M. Elturk, Adil Koray Yıldız, Mohammed Abu-Rumman and members Jordan Embassy in Turkey.

.

ÖZET

SHAMMOUT M.J., Betametazon veya Deksametazon'un Farmasötik Preparatlardan Analizi için bir Ultra-Performans Sıvı Kromatografisi-Tandem Kütle Spektrometrisi (UPLC-MS/MS) Yöntemi Geliştirilmesi ve Validasyonu, H.Ü.Sağlık Bil. Ens.,Analitik Kimya Anabilim Dalı Programı, Doktora Tezi, Ankara, 2013, Betametazon (BTM) ve Deksametazon (DXM) yüksek potentli florlanmış sentetik glukokortikosteroid ilaçlardır. Yaygın olarak anti-enflamatuvar, alerji ve adrenaralkorteks yetersizliği tedavisinde kullanılmaktadır.BTM veya DXM'un farmasötik preparatlarından (tablet, enjektabl ampul ve göz/kulak damlası) tayini için MRM modunda negatif Elektrosprey İyonizasyonv (-ESI) kullanarak Ultra Performanslı Sıvı Kromatografi - Tandem Kütle Spektrometresi (UPLC-MS/MS) bir kromatografi yöntemi geliştirilmiştir. Kromatografik analiz, UPLC Acquity BEH C_{18} (50 mm × 2.1mm, 1.7 µm) kolonunun asetonitril ve suda % 0.1 (h/h) asetik asit içeren hareketli faz ile basamaklı gradiyent sistemi kullanarak elue edilmesiyle gerçekleştirilmiştir. DXM ve BTM üçlü kuadrapol tandem MS'de negatif ESI kullanılarak MRM modunda m/z 451> 361 geçişi ile saptanmıştır. Mefrusid iç standart olarak kullanılmış ve MRM modunda m/z 381>189 geçişi izlenmiştir. Geliştirilen yöntemler ICH'nin analitik yöntem validasyonu rehberine göre valide edilmiştir.BTM ve DXM yöntemleri her madde için de 10 - 1500 ng mL^{-1} derişim aralığında doğrusaldır. Gözlenebilme sınırı (LOD) BTM veya DXM için 1 ng mL^{-1} iken, BTM ve DXM için alt tayın sınırı (LOQ) 5 ng mL^{-1} dir. Gün içi ve günler arası doğruluk bağıl hata (BH) olarak ifade edilmiş, BTM ve DXM için sırasıyla (- 1,66- 1.76) ile(- 0.93 - 1.98) dir. Diğer taraftan, gün içi ve günler arası kesinliği ifade edebilmek için kullanılan bağıl standart sapma (BSS) değerleri, BTM ve DXM için sırasıyla (0.28–1.80) ile 0.23- 1.02) dir. BTM ve DXM standart çözeltileri + 4 ºC'de 6 ay boyunca kararlıdır. Yöntemlerin aynı zamanda hızlı, özgün, tutarlı ve sağlam olduğu gösterilmiştir. Valide edilen yöntemler BTM (Celestone® Tablet ve Celestone® Enjektabl Ampul) ve DXM (Dekort® Tablet, Dekort® Enjektabl Ampul ve ONDARON® SIMPLE göz/kulak damlası) farmasötik preparatlarının analizine başarı ile uygulanmıştır. Bu nedenle, yöntemler BTM ve DXM'un farmasötik preparatlarından tayini için uygundur.

Anahtar kelimeler: Deksametazon, Betametazon, Mefrusid, UPLC/Tandem MS, tablet

ABSTRACT

SHAMMOUT M.J., Development and Validation of an Ultra-Performance Liquid Chromatographic-Tandem Mass Spectrometry (UPLC-MS/MS) Method for Determination of Betamethasone or Dexamethasone in Pharmaceutical Preparations, Hacettepe University, Institute of Health Sciences, Program of Analytical Chemistry, PhD Thesis, Anakara, 2013, Betamethasone (BTM) and Dexamethasone (DXM) are highly potent fluorinated synthetic glucocorticosteroids. They are widely used for the treatment of inflammation, allergies and adrenaral cortex insufficiency. Chromatographic methods for determination of BTM or DXM in pharmaceutical preparations (tablets, injectable ampoules and eye/ear drops) by Ultra-Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS) using negative Electrospray Ionization (- ESI) at MRM mode were developed. The chromatographic analysis were performed on UPLC Acquity BEH C_{18}(50 mm × 2.1mm, 1.7 µm) column was eluted using stepwise gradient with mobile phases of acetonitril and 0.1% (v/v) acetic acid in water. Detection of DXM and BTM was performed by triple quadrupole tandem MS using negative ESI at 451 > 361 m/z transition on MRM mode. Mefruside was used as an internal standard and traced at 381 > 189 m/z transition on MRM mode. Developed methods were validated according to analytical method validation guideline from ICH. The methods were linear over the concentration range of 10 to 1500 ng mL^{-1} for both BTM and DXM. Limits of detection (LOD) for BTM or DXM were1ng mL^{-1} while limit of quantitation (LOQ) was 5 ng mL^{-1} for BTM or DXM. The intra- and inter-accuracy values expressed as relative error (RE %) were –1.08 -1.76 and -0.93 – 1.98 for BTM and DXM, respectively. On the other hand, the intra- and inter-day precision expressed as relative standard deviation (RSD %) were 0.28-1.80 and 0.23-1.02 for BTM and DXM, respectively. The standard solutions of BTM and DXM were stable at + 4 ºC for 6 months. The method was also rapid, specific, rugged and robust. Validated methods were also successfully applied for the analysis of some commercially available pharmaceutical preparations of BTM (Celestone® Tablet and Celestone® Injectable Ampoule) and of DXM (Dekort® tablet, Dekort® injectable ampoule and Onadron ® Simple eye/ear drop). Therefore, this method is suitable for determination of BTM or DXM in their pharmaceutical preparations.

Key words: Dexamethasone, Betamethasone, Mefruside, UPLC/Tandem MS, tablet

TABLE OF CONTENTS

LIST OF ABBREVIATIONS, SYMBOLS/NOMENCLATURE

ACN	Acetonitril
APCI	Atmospheric Pressure Chemical Ion Ionisation
API	Active Pharmaceutical Ingredient
A_S	Peak Asymmetry Factor
BA	Betamethasone Acetate
BEH	Bridged Ethylsiloxane/Silica Hybrid
BP	Beatamethasone sodium Phosphate
BTM	Betamethasone
CAD	Collision Activated Dissociation
CI	Chemical Ionisation
CID	Collision Induced Dissociation
CSP	Chiral Stationary Phase
DAD	Diodo Array Detector
DXM	Dexamethasone
EI	Electron Impact
ESI	Elecrospray Ionisation
eV	Electron Volt
FAB	FAST Atom Bombardment
GC	Gas Chromatography
GR	Glucocorticoids7
HAc	Acetic Acid
HETP	Height Of Theoretical Plates
HPLC	High-Performance Liquid Chromatography
ICH	International Conference On Harmonization
ICP	Inductive Coupled Plasma
IEX	Ion Exchange
IPC	Ion Pairing Chromatography
IR	Infrared red
IS	Internal Standard

K	Equilibrium Constant
k`	Capacity Factor
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
LOD	Lowest Limit Of Detection
LOQ	Lowest Limit of quantification
MALDI	Matrix-Assisted Desorption Ionisation
Mef.	Mefruside
m/z	Mass To Charge Ratio
MRM	Multi Reaction Monitoring
MS	Mass Spectrometry
µg	Microgram
µg	Micrometer
N	Number of Theoretical Plates
n	Number of Measures
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NPLC	Norma Phase Liquid Chromatography
ODS	Octa Decyl Silane
PA ratio	Peak Asymmetry Ratio
r	Correlation Coefficient
R^2	Coefficient Of Determination
RE %	Relative Error
RP	Reversed Phase
Rs	Resolution
RSD	Relative Standard Deviation
SE	Standard Error
SEC	Size Exclusion Chromatography
SD	Standard Deviation
SPE	Solid Phase Extraction
t_0	Dead Time
TOF	Time of Fly

t_R	Retention Time
UPLC	Ultra-Performance Liquid Chromatography
UV	Ultra Violet
V_0	Dead Volume
V_R	Retention Volume
α	Selectivity Factor

INDEX OF FIGURES

INDEX OF TABLES

1. INTRODUCTION

Corticosteroids are a class of steroid hormones naturally synthesized in the adrenal cortex from cholesterol. According to their biological activity and pharmacological effects, corticosteroids can be divided into two groups: glucocorticosteroids (i.e. cortisol and cortisone), which regulate many aspects of metabolism and immune functions, and mineral corticosteroids, which regulate blood volume and electrolyte content (1).

Betamethasone (BTM) and Dexamethasone (DXM) are highly potent fluorinated synthetic glucocorticosteroids. They are widely used for the treatment of inflammation, allergies and adrenaral cortex insufficiency.BTM has been reported to have slightly stronger glucocorticod effect than DXM (2-4).

BTM and DXM are 20-25 times more potent than the naturally occurring glucocorticosteroid, Hydrocortisone. The potency of the anti-inflammatory property of BTM and DXM is contributed by the fluorination at the C-9 position. The difference in configuration of the methyl group at C-16 position is responsible for the diastereomeric isomerism associated with BTM and DXM epimers. The two epimers are very similar in chemical structure and properties (4, 5).

As drugs, synthetic corticosteroids are frequently used in human and veterinary medicine, often in combination with other compounds. Their use is related to their activity in replacement therapy for adrenal insufficiency, anti-inflammatory and immunosuppressant properties, which reduce the clinical manifestations of disease in a wide range of disorders. Treatment can be by oral administration, intramuscular, subcutaneous or intravenous injection, or as a topical application (6, 7).

Wide ranges of pharmaceutical preparations of different dosage forms that contain BTM or DXM or their ester derivatives were approved and commercially available, but none of these preparations has BTM and DXM in combination (3, 6-10).

Several analytical techniques have been published for the analysis of BTM and/or DXM in different matrices, e.g., high performance liquid chromatography (HPLC) (3, 11-14), High performance thin layer chromatography (HPTLC) (15), differential

pulse polarographic and voltammeteric (16, 17), Nuclear magnetic resonance (NMR) (18), UV/visible Spectrophotometrtic (19-22), Luminal chemiluminescences methods(23), micellar electrokinetic capillary chromatography (2,24), Gas chromatography with mass detection (GC–MS) (25, 26) or liquid chromatography with mass detection (LC–MS/MS) (7, 27-35).

Several HPLC methods for individual or simultaneous determination of BTM and/or DXM and related compounds in different types of samples were described in the literatures including official methods of the United State Pharmacopeia USP (4, 9).

The development of HPLC methods for assay/purity analysis of drugs and their related substances is a time-consuming process and is often a bottleneck in analytical labs. Separations scientists are thus continually driven to develop LC methods with ever-shorter analysis times. The benefits of faster analyses are clear: they allow for a greater number of analyses to be performed in a shorter amount of time, thereby increasing sample throughput and lab productivity. In addition, as test experiments are performed more quickly, the overall method development time is decreased (36).

UPLC is a new category of separation science that builds upon well-established principles of liquid chromatography, using sub-2μm porous particles. These particles operate at elevated mobile phase linear velocities to produce rapid separation with increased sensitivity and increased resolution. Several reports are available in the literature on the (HPLC-MS) and (UPLC-MS) for the different combination of drugs (37-39).

In the literature, a variety of different analysis methods for the determination of BTM and /or DXM in different matrixes and samples were reported. Nevertheless, some of them have some disadvantages. BTM and/or DXM were analyzed for clinical steroid analysis or sports doping laboratories for qualitative analysis where the specifity of the method is a primary importance. Therefore, most used analysis techniques are GC / MS and LC / MS, respectively. Analysis of glucocorticosteroids in biological samples using GC/MS is common (40). However, using this technique require a time and chemicals consuming derivatization process for the main compound to enhance the volatility of the analyte and require thermally stable molecule (23). Therefore, the ideal technique for quantitative determination of BTM

and /or DXM in variety of biological (urine, serum, plasma, milk, tissue, etc.) Pharmaceutical and ecological samples are LC-MS (See Table 2.3).

BTM or DXM and /or some of their esterficated derivatives(salts) such as phosphates, acetates, propionates and valerates are contained in several pharmaceutical preparations with different dosages form such as creams, ointment, inhalants, eye/ear drops, IV/IM injectable ampoules and tablets. Few studies about determination of amount BTM or DXM as an active ingredient in pharmaceutical preparationse were reported (3, 8, 12, 41-47). Thus, it's important to developing method for analysis of BTM or DXM in pharmaceutical preparations.

Liquid chromatography (LC) is currently considered as the gold standard in pharmaceutical analysis. Today, there is an increasing need for fast and ultra-fast methods with good efficiency and resolution for achieving separations in a few minutes or even seconds (38).

Ultra Performance Liquid Chromatography (UPLC) is a relatively new technique giving new possibilities in liquid chromatography, especially concerning decrease of time and solvent consumption. UPLC chromatographic system is designed in a special way to withstand high system back-pressures. Special analytical columns UPLC AcQuity UPLC BEH C18 packed with 1.7 μm particles are used in connection with this system. The UPLC system allows shortening analysis time up to nine times, comparing to the conventional system using 5 μm particle packed analytical columns used in LC (38, 39, 48, 49).

The extra resolution provided by the UPLC system reveals new information about the samples under investigation and reduces the risk of non-detection of potentially important co-eluting analytes. In order to address the very narrow peaks produced by UPLC, it is necessary to use a high data acquisition rate mass spectrometer such as a TOFMS or a triple quadrupole MS (Tandem MS) having a fast duty cycle (48).

Recently, few method of determination BTM and/or DXM or their related products in different biological or environmental samples using UPLC/MS were reported in the literature (22, 48-53)

There is no method to determination of BTM or DXM in pharmaceutical preparations using UPLC/Tandem MS was reported in the literatures.

The object of this thesis work is to develop, optimize and validate a new method to determine the amount of BTM or DXM in pharmaceutical preparation such as tablets, injectable ampoules and eye/ear drops by Ultra-Performance Liquid Chromatography combined with Triple Quadrupole Tandem Mass spectrometry (UPLC/MS/MS) using negative electrospray ionization (-ESI) in the Multi-Reaction-Monitoring mode(MRM).

2. GENERAL INFORMATIONS

2.1. Glucocorticosteroid Drugs (54-59)

The endocrine hormones can be divided into two chemical structural types: (1) the peptides and amino acid derivatives and (2) the cholesterol-based steroid compounds. By nature, steroidal hormones are produced by the male and female sex organs (testiculs, ovaries), the adrenal cortex and the placenta. As they are involved in the development of reproductive structures and secondary sexual characteristics, sex hormones are generally applied in veterinary medicine to regulate rut and improve fertility. Next to endogenous steroids, many semi-synthetic and synthetic analogues have been produced and administered to animals.

The steroid hormones can be grouped into six classes: glucocorticords, mineralocorticords, estrogen progestin, androgen, and vitamin D. The adrenal cortex synthesizes two classes of steroids: the corticosteroids, which have 21 carbon atoms, and the androgens, which have 19 carbon atoms. The actions of corticosteroids are classified as glucocorticoid (carbohydrate metabolism regulating) and mineral corticoid (electrolyte balance–regulating), reflecting their preferential activities.

BTM and DXM are epimeric synthetic glucocorticoids; their pharmacological and physiological properties are generally same for that to other drugs in glucocorticoids group.

2.1.1. Physical and Chemical Properties of Betamethasone (60)

Product name : Betmethasone (11β, 16β)-9-Fluoro-11, 17, 21-trihydroxy-16-methylpregna-1, 4-diene-3, 20-dione

IUPAC name : (8S, 9R, 10S, 11S, 13S, 14S, 16S, 17R)-9-fluoro-11,17-Dhydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-3-one.

Molecular Formula : $C_{22}H_{29}FO_5$

Elemental analysis : C 67.33%, H 7.45%, F 4.84%, O 20.38%

Molecular weight : 392.46 g/mol

Melting Point : 250-253 °C

Solubility : Betamethasone is practically insoluble in water. It is soluble in ethanol, methanol, acetone, dioxane and slightly soluble in chloroform

Refractive index : 76 ° (C=1, Dioxane)

Appearance : White or almost white crystalline powder

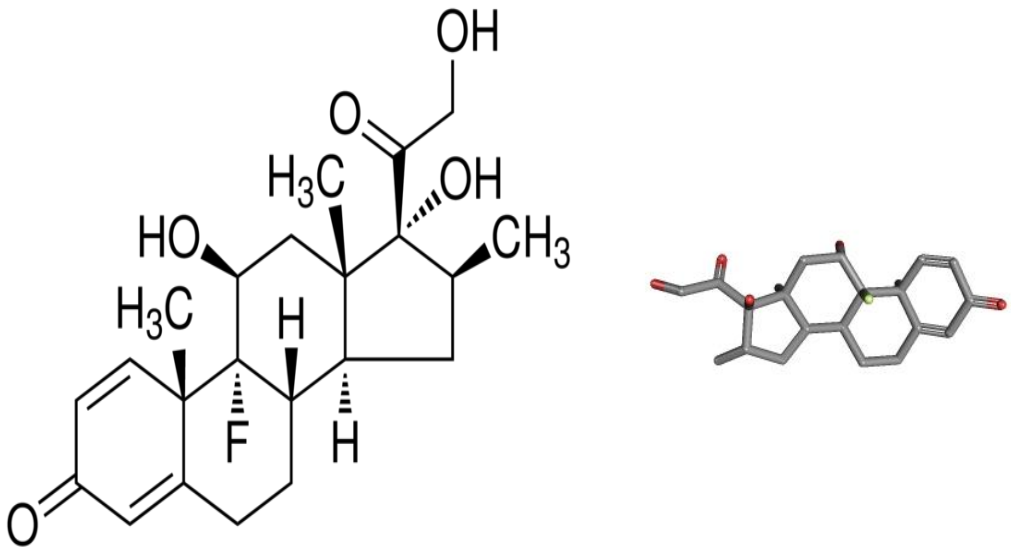


Figure 2.1. Chemical structure of betamethasone

2.1.2. Physical and Chemical Properities of Dexamethasone (60)

Product Name : Dexamethasone (11β, 16β)-9-Fluoro-11, 17, 21-trihydroxy-16-methylpregna-1, 4-diene-3, 20-dione

IUPAC : (8S,9R,10S,11S,13S,14S,16R,17R)-9-Fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta [a] phenanthren-3-one

Molecular Formula : $C_{22}H_{29}FO_5$

Elemental analysis : C 67.33%, H 7.45%, F 4.84%, O 20.38%

Molecular weight : 392.46 g/mol

Solubility : Practically insoluble in water; soluble 1 in 75 of ethanol, 1 in 15 of warm ethanol, and 1 in 1100 of chloroform; very slightly soluble in ether; sparingly soluble in acetone and methanol.

Melting Point : 235-237°C

Refractive index : 118 ° (C=1, Dioxane)

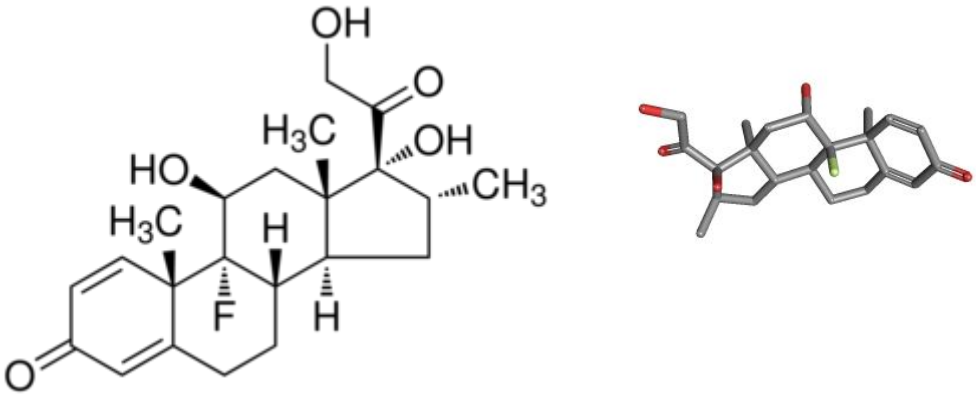


Figure 2.2. Chemical structure of dexamethasone

2.1.3. Mechanism of Action

As with other steroid hormones and drugs, glucocorticoids are thought to act primarily by turning on or off the expression of different genes. Glucocorticoids inter a target of cell and bind to specific receptor in the cell cytoplasm. The binding of the glucocorticoids activates the receptor, which allows the complex to enter the nucleus. In nucleus, the complex binds to selected DNA sites known as glucocorticoid response elements (GREs). This promotes or inhibits the transcription of specific mRNAs. In turn, the synthesis of the respective proteins is promoted or inhibited. A considerable delay may occur between uptake of the steroid into the cell and appearance of the effect in the target cell. More time may elapse before the effect ends and steroid is destroyed**.**

The dosage of steroid used clinically provides much more than the natural level of glucocorticoids activity. As a result, many of the effects produced are very different from the physiological effects of naturally occurring hormones. The pharmacologic(rather than physiologic) effects are achieved when glucocorticoids are required to treat disorders unrelated to adrenocortical functions, such as allergic reactions, autoimmune diseases, asthma and inflammation.

Cortisol and cortisone, the naturally occurring glucocorticoids, have relatively low affinity for the mineral corticoids receptors but still may produce some salt and water retention. Other glucocorticoids have relatively less mineral corticoid activity.

2.1.4. Physiological Functions and Pharmacological Effects

The effects of corticosteroids are numerous and widespread, and include alterations in carbohydrate, protein, and lipid metabolism; maintenance of fluid and electrolyte balance; and preservation of normal function of the cardiovascular system, the immune system, the kidney, skeletal muscle, the endocrine system and the nervous system. In addition, corticosteroids endow the organism with the capacity to resist such stressful circumstances as noxious stimuli and environmental changes.

The actions of corticosteroids are interrelated to those of other hormones. In the absence of glucocorticoids, epinephrine and nor epinephrine have only minor effects on lipolysis. Administration of a small dose of glucocorticoid, however, markedly potentiates their lipolytic action. Those effects of corticosteroids that involve concerted actions with other hormonal regulators are termed *permissive* and most likely reflect steroid-induced changes in protein synthesis that, in turn, modify tissue responsiveness to other hormones.

Pharmacological effects of glucocorticostertoids (GR) can be listed according to the site of action as the following:

Regulation of Gene Expression

The GR resides predominantly in the cytoplasm in an inactive form until it binds glucocorticoids. The inactive GR is complexed with other proteins, including heat-shock proteins and an immunophilin. After ligand binding, the GR dissociates from its associated proteins and translocates to the nucleus. There, it interacts with

specific DNA sequences called glucocorticoid responsive elements (GREs) within the regulatory regions of affected genes. These GREs thus provide specificity to the regulation of gene transcription by glucocorticoids.

Carbohydrate and protein metabolism

Corticosteroids profoundly affect carbohydrate and protein metabolism. These effects of glucocorticoids on intermediary metabolism can be viewed as protecting glucose-dependent tissues (*e.g.*, the brain and heart) from starvation. They stimulate the liver to form glucose from amino acids and glycerol and to store glucose as liver glycogen. In the periphery, glucocorticoids diminish glucose utilization, increase protein breakdown and the synthesis of glutamine, and activate lipolysis, thereby providing amino acids and glycerol for gluconeogenesis. The net result is to increase blood glucose levels. Because of their effects on glucose metabolism, glucocorticoids can worsen glycemic control in patients with overt diabetes and can precipitate the onset of diabetes in patients who are otherwise predisposed.

Lipid metabolism

Two effects of corticosteroids on lipid metabolism are firmly established. The first is the dramatic redistribution of body fat that occurs in settings of endogenous or pharmacologically induced hypercorticism, such as Cushing's syndrome. The other is the permissive facilitation of the lipolytic effect of other agents, such as growth hormone and adrenergic receptor agonists, resulting in an increase in free fatty acids after glucocorticoid administration. With respect to fat distribution, there is increased fat in the back of the neck (buffalo hump), face (moon faces), and supraclavicular area, coupled with a loss of fat in the extremities.

Electrolytes and water balance

Aldosterone is by far the most potent endogenous corticosteroid with respect to fluid and electrolyte balance. Thus, electrolyte balance is relatively normal in patients with adrenal insufficiency due to pituitary disease, despite the loss of glucocorticoid production by the inner cortical zones. Mineral corticoids act on the distal tubules and collecting ducts of the kidney to enhance reabsorption of Na^+ from the tubular fluid; they increase the urinary excretion of K^+ and H^+.

Glucocorticoids also exert effects on fluid and electrolyte balance, largely due to permissive effects on tubular function and actions that maintain glomerular filtration rate. In part, the inability of patients with glucocorticoid deficiency to excrete free water results from the increased secretion of vasopressin, which stimulates water reabsorption in the kidney.

In addition to their effects on monovalent cations and water, glucocorticoids also exert multiple effects on Ca^{2+} metabolism. Steroids interfere with Ca^{2+} uptake in the gut and increase Ca^{2+} excretion by the kidney. These effects collectively lead to decreased total body Ca^{2+} stores.

Cardiovascular system

The most striking cardiovascular effects of corticosteroids result from mineral corticoid-induced changes in renal Na^{+} excretion, as is evident in primary aldosteronism.The resultant hypertension can lead to a diverse group of adverse effects on the cardiovascular system. Consistent with the known actions of mineral corticoids in the kidney, restriction of dietary Na^{+} can lower the blood pressure considerably in mineral corticoid excess.

The second major action of corticosteroids on the cardiovascular system is to enhance vascular reactivity to other vasoactive substances. Hypoadrenalism is associated with reduced response to vasoconstrictors such as nor epinephrine and Ang II, perhaps due to decreased expression of adrenergic receptors in the vascular wall. Conversely, hypertension is seen in patients with excessive glucocorticoid secretion, occurring in most patients with Cushing's syndrome and in a subset of patients treated with synthetic glucocorticoids.

Skeletal muscles

Permissive concentrations of corticosteroids are required for the normal function of skeletal muscle, and diminished work capacity is a prominent sign of adrenocortical insufficiency. In patients with Addison's disease, weakness and fatigue are frequent symptoms. Excessive amounts of either glucocorticoids or mineralocorticoids also impair muscle function. In primary aldosteronism, muscle weakness results primarily from hypokalemia rather than from direct effects of mineralocorticoids on skeletal muscle. In contrast, glucocorticoid excess over

prolonged periods, either secondary to glucocorticoid therapy or endogenous hypercorticism, causes skeletal muscle wasting. This effect, termed *steroid myopathy*, accounts in part for weakness and fatigue in patients with glucocorticoid excess.

Central Nervous System (CNS)

Corticosteroids exert a number of indirect effects on the CNS, through maintenance of blood pressure, plasma glucose concentrations, and electrolyte concentrations. Increasingly, direct effects of corticosteroids on the CNS have been recognized, including effects on mood, behavior, and brain excitability and most patients receiving glucocorticoids respond with mood elevation, which may impart a sense of well-being despite the persistence of underlying disease. Some patients exhibit more pronounced behavioral changes, such as euphoria, insomnia, restlessness, and increased motor activity.

Circulation system

Corticosteroids also affect circulating white blood cells. Addison's disease is associated with an increased mass of lymphoid tissue and lymphocytosis. In contrast, Cushing's syndrome is characterised by lymphocytopenia and decreased mass of lymphoid tissue. The administration of glucocorticoids leads to a decreased number of circulating lymphocytes, eosinophils, monocytes, and basophils.

Anti-Inflammatory and Immune Suppressive Actions

Corticosteroids profoundly alter immune response. Glucocorticoids can prevent or suppress inflammation in response to multiple inciting events, including radiant, mechanical, chemical, infectious, and immunological stimuli. Although the use of glucocorticoids as anti-inflammatory agents does not address the underlying cause of the disease, the suppression of inflammation is of enormous clinical utility and has made these drugs among the most frequently prescribed agents. Similarly, glucocorticoids are of immense value in treating, diseases that result from undesirable immune reactions. The immunosuppressive and anti-inflammatory actions of glucocorticoids are inextricably linked, perhaps because they both involve inhibition of leukocyte functions.

2.1.5. Administration and Pharmacokinetics

Steroid formulations are available for oral, parenteral, and topical use. Many, including prednisone, prednisolone, methyl prednisolone and dexamethasone are well absorbed when administered orally and are particularly useful when anti-inflammatory treatment is required for a period of one to several weeks.

Other preparations are available for parenteral use. The sodium phosphate and succinate salts are highly water soluble, providing a rapid onset of action when given by intra venous and are often used in shock therapy. Other injectable formulations include esters such as methyl prednisolone acetate and triamcinolone acetonide, which have limited water solubility. The release of corticosteroids from these preparations is very slow and may result in anti-inflammatory effects and associated Hypothalamo-Pituitary-Adrenal Axis (HPAA) suppression for several weeks.

Corticosteroid preparations available for topical or intra-lesional administration can be effective in treating inflammation of the skin, eyes, or ears. Although controversial, intra-articular administration of glucocorticoids has been used in humans and animals, particularly horses, to manage inflammatory joint disease. Glucocorticoids are absorbed systemically from sites of local administration in amounts that may be sufficient to suppress the HPAA.

Hydrocortisone and numerous congeners, including the synthetic analogs, are orally effective. Certain water-soluble esters of hydrocortisone and its synthetic congeners are administered intravenously to rapidly achieve high concentrations of drug in body fluids. Effects that are more prolonged are obtained by intramuscular injection of suspensions of hydrocortisone, its esters, and congeners. Minor changes in chemical structure may markedly alter the rate of absorption, time of onset of effect, and duration of action.

Glucocorticoids also are absorbed systemically from sites of local administration, such as synovial spaces, the conjunctival sac, skin, and respiratory tract.

The corticosteroids also are well absorbed from the gastrointestinal tract following oral administration and quickly distributed to muscles, liver, skin, intestines and kidneys. In addition, oral corticosteroids cross the placenta and may be secreted in breast milk. Plasma protein binding of corticosteroids ranges from 61-95

%. Only unbounded drug is pharmacologically active. These drugs are metabolized in most tissues, mainly in the liver. Primarily the kidneys excrete the biologically inactive metabolites. Although the elimination half-lives of these drugs are relatively short (1-5 hours) the biological half lives are much longer, ranging from 8-60 hours.

Table 2.1. Pharmacokinetic parameters of some glucocorticoids.

	Anti-inflammatory potency	Sodium retaining potency	Plasma half-life (min)	Biological half-life (hrs)	Approximate equivalent dose (mg)
Short acting					
Cortisone	1.0	1.0	30	8-12	20
Hydrocortisone	0.8	0.8	80-118	8-12	25
Fludrocortisone**	10	125	short	--	--
Intermediate					
Prednisone	4.0	0.8	16	18-36	5
Prenisolone	4.0	0.8	115-212	18-36	5
Methylprednisolone	5.0	0.5	78-188	18-36	4
Triamcinolone	5.0	0	200+	18-36	4
Long acting					
Betamethasone	25.0	0	300+	36-54	0.75
Dexamethasone	25.0	0	110-210	36-54	0.75
Paramethasone	12.5	0	300+	36-54	2.0

'* Mineralocorticoid

2.1.6. Side Effects

Side effects caused mainly by high (pharmacological as compared to physiological) concentrations and for long times.

Most common side effects are;

- Development of Cushingoid habitus (trunkal obesity, moon faces, buffalo hump), salt retention, and hypertension (i.e. iatrogenic Cushing is syndrome)
- Suppression of the immune system (rendering the patient vulnerable to common and opportunistic infections)
- Osteoporosis (rendering the patient vulnerable to fractures)
- Peptic ulcers (resulting in gastric hemorrhages and /or intestinal perforation)

- Suppression of growth in children
- Behavioral problems
- Reproductive problems
- Prolonged suppression of the hypothalamic-pituitary-adrenal axis after drug discontinuation.

2.1.7. Therapeutic uses

Endocrine diseases

Pharmacological intervention in the treatment of endocrine malfunction or disease state generally takes one of three approaches:

(1) Replacement or supplementation of the natural hormone,

(2) Use of the hormone to obtain a specific response, or

(3) Use of drugs to modify the concentration or action of a specific hormone

Non-endocrine diseases

Outlined below are important uses of glucocorticoids in diseases that do not directly involve the Hypothalamo-Pituitary-Adrenal Axis. The disorders discussed are not inclusive; rather, they illustrate the principles governing glucocorticoid use in selected diseases for which these drugs are more frequently employed. The dosage of glucocorticoids varies considerably depending on the nature and severity of the underlying disorder. For convenience, approximate doses of a representative glucocorticoid (generally prednisone) are provided. This choice is not an endorsement of one particular glucocorticoid preparation over other congeners and is made for illustrative purposes only.

Rheumatic Disorders

Glucocorticoids are used widely in the treatment of a variety of rheumatic disorders and are a mainstay in the treatment of the more serious inflammatory rheumatic diseases, such as systemic lupus erythematosus, and a variety of vasculitic disorders, such as polyarteritis nodosa, Wegener's granulomatosis, Churg-Strauss syndrome, and giant cell arthritis.

Glucocorticoids are often used in conjunction with other immunosuppressive agents such as cyclophosphamide and methotrexate, which offer better long-term control than steroids alone. The exception is giant cell arthritis, for which glucocorticoids remain superior to other agents.

Renal Diseases

Patients with nephrotic syndrome secondary to minimal change disease generally respond to steroid therapy, and glucocorticoids clearly are the first-line treatment in both adults and children.

Allergic Disease

The onset of action of glucocorticoids in allergic diseases is delayed, and patients with severe allergic reactions such as anaphylaxis require immediate therapy with epinephrine.

Bronchial Asthma

Preterm Infants

Glucocorticoids such as betamethasone (12 mg intramuscularly every 24 hours for two doses) or dexamethasone (6 mg intramuscularly every 12 hours for four doses) are used frequently in the setting of premature labor to decrease the incidence of respiratory distress syndrome, intraventricular hemorrhage, and death in babies delivered prematurely.

Ocular Diseases

Gastrointestinal tract Diseases

Glucocorticoid therapy is indicated in selected patients with inflammatory bowel disease (chronic ulcerative colitis and Crohn’s diseas).

Malignancies

Glucocorticoids are used in the chemotherapy of acute lymphocytic leukemia and lymphomas because of their antilymphocytic effects.

Organ Transplantation

In organ transplantation, most patients are kept on a regimen that includes glucocorticoids in conjunction with other immunosuppressive agents.

Spinal Cord Injury

Diagnostic Applications of Adrenocortical Steroids

In addition to their therapeutic uses, glucocorticoids also are used for diagnostic purposes. The overnight dexamethasone suppression test is used to determine if patients with clinical manifestations suggestive of hypercortisolism have biochemical evidence of increased cortisol biosynthesis.

2.1.8. Betamethasone and Dexamethasone

Dexamethasone has a high potency and has minimal mineral corticoid activity. It is rapidly absorbed after oral administration with peak effects within 1-2 h. The duration of action is about 3 days after oral administration and up to weeks after injections of the sodium phosphate derivative. This long duration of action makes it unsuitable for alternate-day therapy. Parenteral administration is suitable for acute disorders, including anaphylaxis and cerebral edema.

Another indication is the prevention of respiratory distress syndrome (RDS) in situations where there is a special risk for the fetus. It is the given prior to delivery. The sodium phosphate of dexamethasone can be used for parenteral administrations and for intra-articular injections and injections in soft tissue lesions.

Betamethasone is hardly even used orally. It has a long duration and can therefore be used for alternate-day therapy. The parenteral formulation is also the sodium phosphate salt which when given by intravenous or intramuscular has a rapid onset of action. There are many similarities with dexamethasone such as their metabolic pathways and the indications for which both steroids are used like the prevention of Neonatal respiratory distress syndrome (RDS) and reduction of raised intracranial pressure.

Combinations of bethamethasone acetate and sodium phosphate have, when used for intra-articular and intra-lesional injections, the dual advantage of a rapid onset of action together with the long duration of action of a depot preparation.

2.2. Chromatographic Separations (61-70)

Chemical species are generally separated by converting them to different phases that can then be mechanically isolated. Chromatography is widely used for the separation, identification and determination of the chemical component in the complex mixtures. No other separation method is as powerful and as generally applicable as in chromatography that is find application to all branches of science.

2.2.1. History

Chromatography was invented and named by the Russian botanist Mikhail Tswett in 1906. He employed the technique to separate various plant pigments such as chlorophylls and xanthophylls by passing solutions of these compounds through a glass column pack with finely divided calcium carbonate. The separated species appeared as colored bands on the column, which accounts for the name he chose for the method, Greek *chroma* meaning ‘color`` and *graphine* meaning to ``write``.

2.2.2. Chromatographic Process Definition

Chromatographic separations are based on a forced transport of the liquid carrying the analyte mixture through the porous media and the differences in the interactions at analytes with the surface of this porous media resulting in different migration times for a mixture component.

In the above paragraph definition, the presence of two different phases is stated and consequently there is an interface between them. One of these phases provides the analyte transport and is usually referred to as the mobile phase, and the other phase is immobile and is typically referred to as the stationary phase.

A mixture of components, usually called analytes, are dispersed in the mobile phase at the molecular level allowing for their uniform transport and interactions with the mobile and stationary phases. High surface area of the interface between mobile and stationary phases is essential for space discrimination of different components in the mixture.

Analyte molecules undergo multiple phase transitions between mobile phase and stationary phase surface. Average residence time of the molecule on the stationary phase surface is dependent on the interaction energy. For different molecules with very small interaction energy difference the presence of significant

surface is critical since the higher the number of phase transitions that analyte molecules undergo while moving through the chromatographic column, the higher the difference in their retention. The nature of the stationary and the mobile phases, together with the mode of the transport through the column, is the basis for the classification of chromatographic methods.

2.2.3. Classification of Chromatographic Methods.

Chromatographic methods can be categorized in three ways (Figure 2.3):

A. The first classification is based upon the physical means by which the stationary and mobile phase s are brought into contact.

- Column chromatography: The stationary phase is held in a narrow tube through which the mobile phase is forced under pressure.
- Planary chromatography: The stationary phase is supported on a flat plate or in the interstices of a paper. The mobile phase moves under two forces: capillary action or under the influence of gravity.

B. Fundamental classification based upon the types of mobile and stationary phases:

- Liquid chromatography.
- Gas chromatography.
- Supercritical-fluid chromatography.

C. The kinds of the equilibria involved in the transfer of solutes between phases.

1. Adsorption chromatography
    (a) Gas–solid adsorption
    (b) Liquid–solid adsorption
2. Partition chromatography
    (a) Gas–liquid partition
    (b) Liquid–liquid partition
3. Size exclusion chromatography
    (a) Gas–solid exclusion
    (b) Liquid–solid exclusion
4. Ion-exchange chromatography
5. Ion-Pairing Chromatography

6. Size Exclusion Chromatography
7. Chiral Chromatography
8. Affinity Chromatography

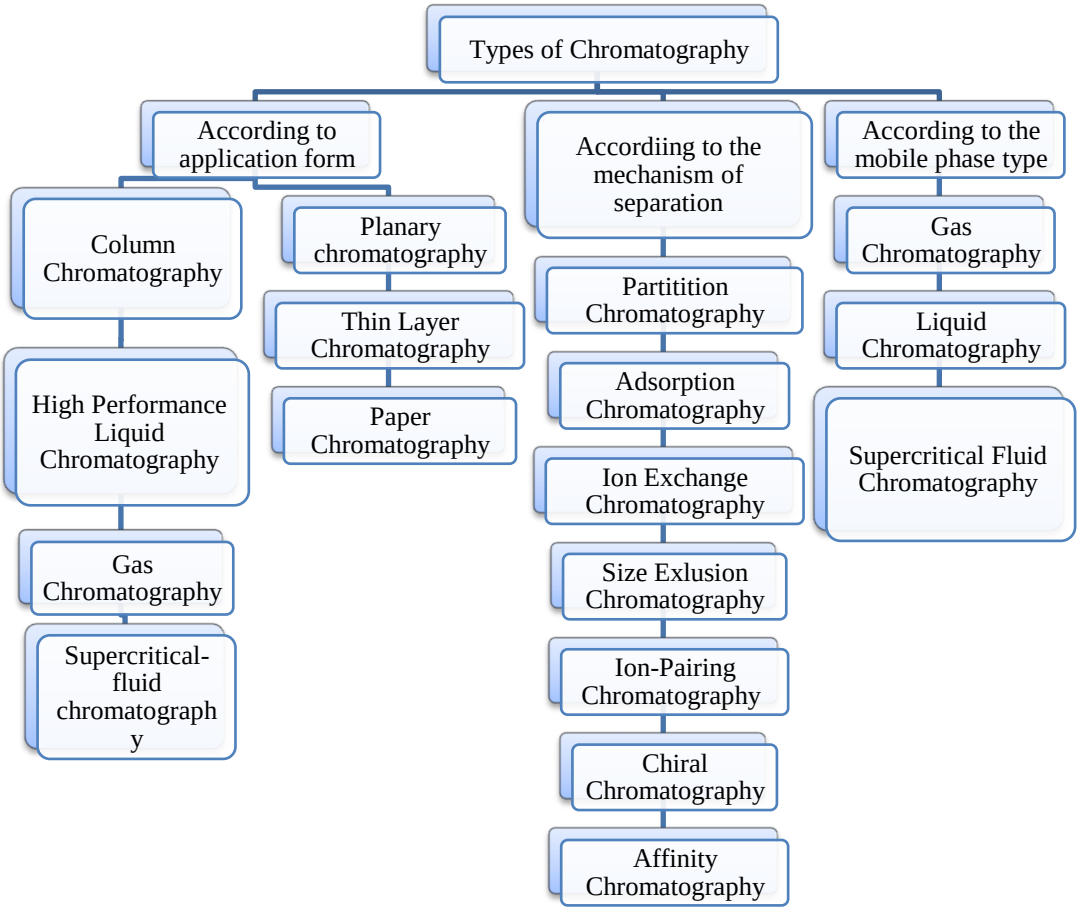


Figure 2.3. Chromatographic separation techniques

2.2.4. Elution Chromatography

Resolution of two components, A and B, on a column is demonstrated in Figure 2.4.

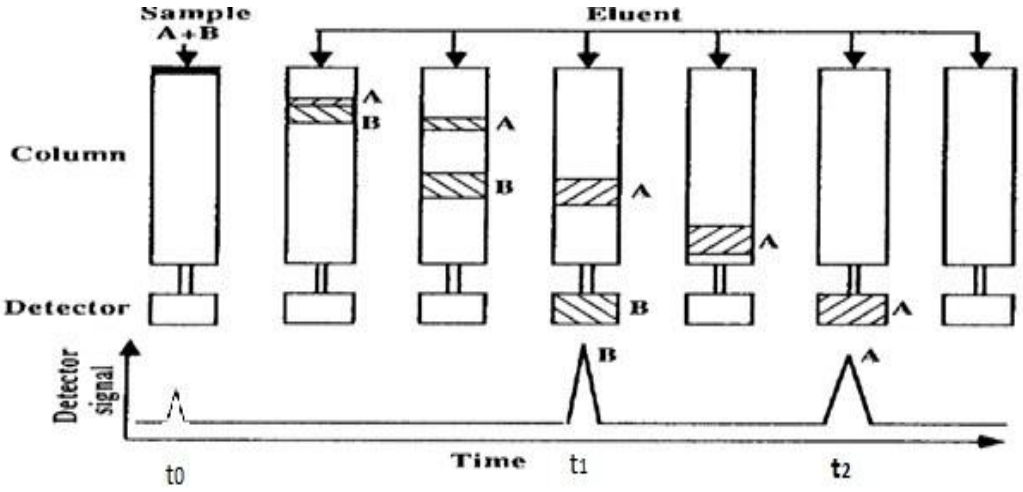


Figure 2.4. Elution chromatography scheme

Elution involves washing a solute through a column by addition of fresh solvent. A single portion of the sample dissolved in the mobile phase is introduced at the head of the column (at time t_0), whereupon components A and B distribute themselves between the two phases. Introduction of additional mobile phase (the eluent) forces the dissolved portion of the sample down the column, where further partition between the mobile phase and fresh portions of the stationary phase occurs (time t_1 for compound B in Figure2.4).

Further additions of solvent carry solute molecules down the column in a continuous series of transfers between the two phases. Because solute movement can occur only in the mobile phase, the average rate at which a solute migrates depends upon the fraction of time it spends in that phase. This fraction is small for solutes that are strongly retained by the stationary phase (component B in Figure 2.4, for example) and large where retention in the mobile phase is more likely (component A).Ideally, the resulting differences in rates cause the components in a mixture to separate into bands, or zones, along the length of the column.

Isolation of the separated species is then accomplished by passing a sufficient quantity of mobile phase through the column to cause the individual bands to pass out the end (to be eluted from the column), where they can be collected.

Isocratic and gradient elution in liquid chromatography:

Isocratic elution refers to the technique of using constant solvent composition throughout the run time in chromatographic analysis. An elution with a single solvent of constant composition is termed isocratic elution. In gradient elution, two (and sometimes more) solvent systems that differ significantly in polarity are employed. The ratio of the two solvent is varied in a programmed way, sometimes continuously and sometimes in a series of steps. Gradient elution frequently improves separation efficiency. Modern High-Performance Liquid Chromatography instruments are often equipped with proportioning valves that introduce liquids from two or more reservoirs at rates that vary continuously.

If a sample contains analytes that have widely divergent affinities for the column, a gradient elution is useful in shortening the analysis time and improving the shape of the peaks. In gradient elution, continuous change of mobile phase properties, e.g., composition, pH, flow rate, to increase eluent strength of the mobile phase is applied. As illustrated in figure 2.5, the first few peaks elute too close to the void volume of the column, suggesting that the mobile phase is too strong for these compounds. Also, the last peaks are short and broad with very long retention times, indicating that the mobile phase is too weak for these compounds. The solution to these problems is to begin with a weaker solvent and gradually increase the solvent strength throughout the course of the analysis.

The resolution of the early eluting peaks is improved, and the widths of the later peaks have been decreased while their heights have increased. The overall gradient separation yields more consistent peak widths, improved sensitivity, and shorter analysis times than would be possible for the corresponding isocratic separation.

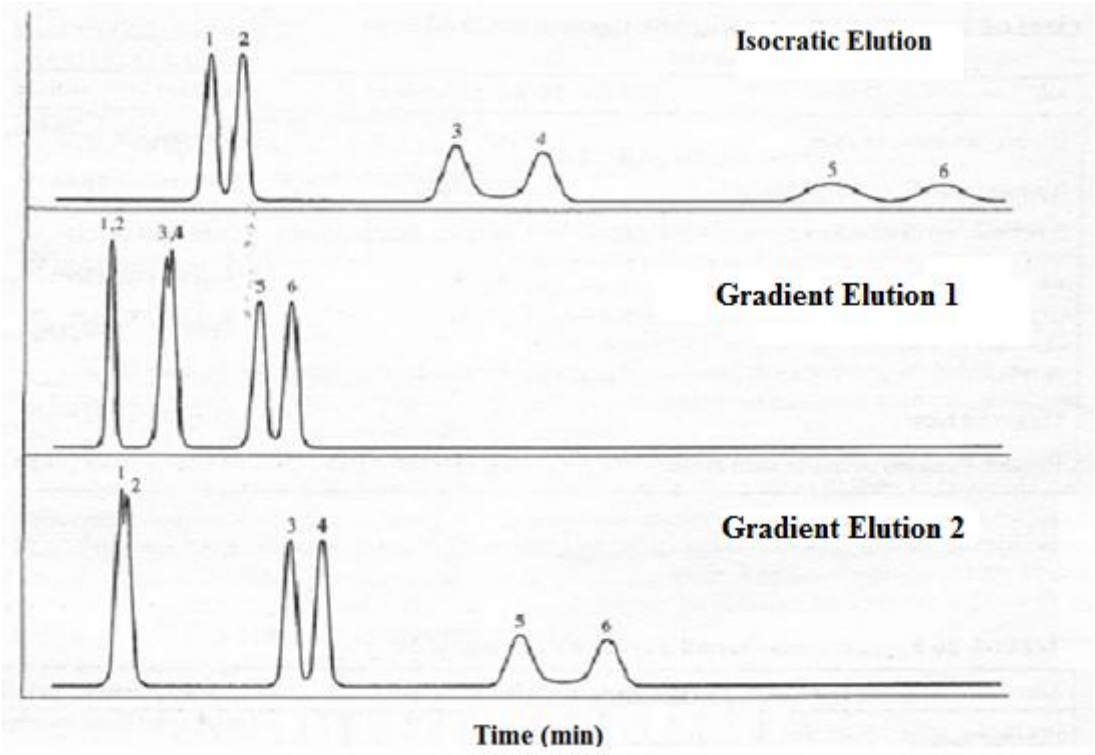


Figure 2.5. Isocratic and Gradient Elution Modes in Liquid Chromatography

The components of the gradient for the mobile phase can be binary, tertiary, or quaternary. The starting composition should be weak, which means that it should be very different in polarity from stationary phase. In reversed-phase HPLC, the solid phase is non-polar or hydrophobic, so the starting mobile phase should be very polar (usually water plus an organic modifier). During the gradient run, the mobile phase is made "stronger" by increasing the proportion of the less polar component usually acetonitrile, methanol, or tetrahydrofuran (THF). The opposite is required for a normal-phase gradient; namely, the proportion of the polar solvent is increased during the run. Polarity is probably not the best concept to use for chromatographic descriptions of the nature of phases.

Gradient elution gave a shorter overall analysis with similar resolution of the critical pair compared to isocratic elution without sacrificing repeatability in retention time, peak area and peak height or linearity of the calibration curve. In terms of separation speed, gradient elution is generally considered an inherently slower technique than isocratic elution since a widely accepted rule of thumb indicates that the column should be flushed

In gradient elution, a modulator is often used in the mobile phase to adjust eluent strength for better results in chromatographic separations. Compared with isocratic elution, the modulator concentration in the mobile phase in gradient elution is increased or decreased continuously with time. Therefore, gradient elution can be used to separate components, which have a wide range of retentively with no loss of resolution. Gradient elution is able to produce high peak heights in a shorter operation cycle compared with isocratic elution. For these reasons, gradient elution has been widely used in high performance liquid chromatography for analytical purposes.

An early goal of gradient elution was the reduction of peak tailing during isocratic separation. Because of the increase in mobile phase, strength during the time a peak is eluted in gradient elution, the tail of the peak moves faster than the peak front, with a resulting reduction in peak tailing and peak width.

2.3. Liquid Chromatography

Liquid chromatography (LC), which is one of the forms of chromatography, which is an analytical technique that is used to separate a mixture in solution into its individual components. As indicated by Tswett, the separation relies on the use of different phases or immiscible layers, one of which is held stationary, while the other moves over it. Liquid chromatography is the generic name used to describe any chromatographic procedure in which the *mobile phase is a liquid.* The separation occurs because, under an optimum set of conditions, each component in a mixture will interact with the two phases differently relative to the other components in the mixture.

High performance liquid chromatography (HPLC) is the term used to describe liquid chromatography in which the liquid mobile phase is mechanically pumped through a column that contains the stationary phase. An HPLC instrument, therefore, consists of an injector, a pump, a column, and a detector.

2.3.1. Modes of Liquid Chromatography

Adsorption Chromatography

The stationary phase is a solid on which the sample components are adsorbed. The mobile phase may be a liquid (liquid-solid chromatography) or a gas (gas solid chromatography); the components distribute between the two phases through a combination of sorption and desorption processes. Thin-layer chromatography (TLC) is a special example of sorption chromatography in which the stationary phase is a plan, in the form of a solid supported on an inert plate.

Partition Chromatography

The stationary phase of partition chromatography is a liquid supported on an inert solid. Again, the mobile phase may be a liquid (liquid-liquid partition chromatography) or a gas (gas-liquid chromatography, GLC).Paper chromatography is a type partition chromatography in which the stationary phases is a layer of water adsorbed on a sheet of paper.

In the normal mode of operation of liquid-liquid partition, a polar stationary phase (e.g, methanol on silica) is used with a non-polar mobile phase (e.g, hexane). This favors retention of polar compounds and elution of non-polar compounds and is called normal-phase chromatography. If a non-polar stationary phase is used, with a polar mobile phase, then non-polar solutes are retained more and polar solutes more readily eluted. This is called reversed-phased-phase chromatography.

a. ***Normal-Phase Liquid Chromatography (NP-LC)***

Normal-phase LC explores the differences in the strength of the polar interactions of the analytes in the mixture with the stationary phase. As with any liquid chromatography technique, NP-LC separation is a competitive process. Analyte molecules compete with the mobile-phase molecules for the adsorption sites on the surface of the stationary phase. The stronger the mobile-phase interactions with the stationary phase, the lower the difference between the stationary-phase interactions and the analyte interactions, and thus the lower the analyte retention.

Mobile phases in NP-LC are based on non-polar solvents (such as hexane, heptane, etc.) with the small addition of polar modifier (i.e., methanol, ethanol). Variation of the polar modifier concentration in the mobile phase allows for the

control of the analyte retention in the column. Typical polar additives are alcohols (methanol, ethanol, or isopropanol) added to the mobile phase in relatively small amounts. Since polar forces are the dominant type of interactions employed and these forces are relatively strong, even only 1 % (v/v) variation of the polar modifier in the mobile phase usually results in a significant shift in the analyte retention.

Packing materials traditionally used in NP-LC are usually porous oxides such as silica (SiO_2) or alumina (Al_2O_3). Surface of these stationary phases is covered with the dense population of OH groups, which makes these surfaces highly polar. Analyte retention on these surfaces is very sensitive to the variations of the mobile-phase composition. Chemically modified stationary phases can also be used in normal-phase LC. Silica modified with trimethoxy glycidoxypropyl silanes (common name: diol-phase) is typical packing material with decreased surface polarity. Surface density of OH groups on diol phase is on the level of 3–4 μmol $(m2)^{-1}$, while on bare silica silanols surface density is on the level of 8μmol/m^2. The use of diol-type stationary-phase and low-polarity eluent modifiers [esters (ethyl acetate) instead of alcohols] allow for increase in separation ruggedness and reproducibility, compared to bare silica.

Selection of using NP-LC as the chromatographic method of choice is usually related to the sample solubility in specific mobile phases. Since NP uses mainly non-polar solvents, it is the method of choice for highly hydrophobic compounds (which may show very stronger interaction in reversed phase LC), which are insoluble in polar or aqueous solvents.

b. Reversed-Phase Liquid Chromatography (RP-LC)

As opposed to normal-phase LC, reversed-phase chromatography employs mainly dispersive forces (hydrophobic or *van der Waals* interactions). The polarities of mobile and stationary phases are reversed, such that the surface of the stationary phase in RP-LC is hydrophobic and mobile phase is polar, where mainly water-based solutions are employed.

RP-LC is by far the most popular mode of chromatography. Almost 90% of all analyses of low-molecular-weight samples are carried out using RP-LC. One of the main drivers for its enormous popularity is the ability to discriminate very closely related compounds and the ease of variation of retention and selectivity. The origin

of these advantages could be explained from an energetic point of view: Dispersive forces employed in this separation mode are the weakest intermolecular forces, thereby making the overall background interaction energy in the chromatographic system very low compared to other separation techniques. This low background energy allows for distinguishing very small differences in molecular interactions of closely related analytes. In RP-LC, where its sensitivity to the minor energetic differences in analyte–surface interactions is very high attributed to the low background interaction energy.

Adsorbents employed in this mode of chromatography are porous rigid materials with hydrophobic surfaces. In all modes of LC with positive analyte, surface interactions (NP, RP and IEX) the higher the adsorbent surface area, the longer the analyte retention and in most cases the better separation.

The majority of packing materials used in RPLC are chemically modified porous silica. The properties of silica have been studied for many years, and the technology of manufacturing porous spherical particles of controlled size and porosity is well developed. Chemical modification of the silica surface was also intensively studied in the last 30 years, mainly as a direct result of growing popularity of RP-LC. Despite the intensive research and enormous growth of commercially available packing materials and columns, there is still no consensus on which properties the optimum RP stationary phase should have for the selective analysis of diverse sets of compounds such as pharmaceutical compounds that have a plethora of various ionizable functionalities, varying hydrophobicities, and different structural components (linear alkyl chains, aromatic rings, heterocyclic, etc.).

Ion-Exchange Chromatography (IEC)

Ion-exchange chromatography, as indicated by its name, is based on the different affinities of the analyte ions for the oppositely charged ionic centers in the resin or adsorbed counter ions in the hydrophobic stationary phase. Consider the exchange of two ions A^+ and B^+ between the solution and exchange resin E:

$$\boldsymbol{A{\cdot}E} + \mathbf{B}^+ \quad \rightarrow \quad \boldsymbol{B{\cdot}E} + \mathbf{A}^+ \tag{2.1}$$

The equilibrium constant for this process is shown in Equation 2.2:

$$K = \frac{[A^+]\,[BE]}{[AE]\,[B^+]} \qquad (2.2)$$

Equation 2.2 essentially determines the relative affinity of both cations to the exchange centers on the surface. If the constant is equal to 1, no discriminating ability is expected for this system. The higher the equilibrium constant (provided that it is greater than 1), the greater the ability of cation B^+ to substitute A on the resin surface.

Depending on the charge of the exchange centers on the surface, the resin could be either anion-exchanger (positive ionic centers on the surface) or cation-exchanger (negative centers on the surface).Cross-linked styrene-divinylbenzene is the typical base material for ion exchange resin. Exchange groups are attached to the phenyl rings in the structure and the degree of cross linkage is between 5% and 20%. The higher the cross linkage, the harder the material and the less susceptible it is to swelling, but the material usually shows lower ion-exchange capacity.

Four major types of ion-exchange centers are usually employed:

1. SO^-_3 strong cation-exchanger.
2. CO^-_2 weak cation-exchanger.
3. Quaternary amine—strong anion-exchanger.
4. Tertiary amine—weak anion-exchanger.

Analyte retention and selectivity in ion-exchange chromatography are strongly dependent on the pH and ionic strength of the mobile phase.

Size-Exclusion Chromatography (SEC)

SEC is the method for dynamic separation of molecules according to their size; as indicated by its name, the separation is based on the exclusion of the molecules from the porous space of packing material due to their steric hindrance. Hydrodynamic radius of the analyte molecule is the main factor determining its retention. In general, the higher the hydrodynamic radius, the shorter the retention. Historically, two different names are used for this method. In 1959, the molecular sieving principle was applied for the separation of biochemical polymers on dextran

gels, and it was called *gel-filtration chromatography* (GFC) which uses aqueous-based eluents with salts. In 1961, the same principle was applied for the molecular weight determination of synthetic polymers, and the name gel-permeation chromatography (GPC) which uses primarily organic solvents such as THF came into popular use among polymer chemists.

This is the only chromatographic separation method where any positive interaction of the analyte with the stationary phase should be avoided. In size-exclusion chromatography, the higher the molecular weight of the molecule, the greater its hydrodynamic radius, which results in faster elution. At the same time, if an analyte molecule interacts (undesired) with the stationary phase, thus increasing the retention of larger molecules, which may confound separation of molecules based solely on their hydrodynamic radius. Obviously, these two processes produce opposite effects, and analysis of the polymer molecular weight and molecular weight distribution would be impossible. This brings specific requirements to the selection of the column packing material and the mobile phase, where the mobile-phase molecules should interact with the surface of the stationary phase stronger than the polymer, thus preventing its interaction with the surface.

Polymer molecular weight determination is based on the relationship of the molecular hydrodynamic radius with the molecular weight. The radius is roughly proportional to the cubic root of the molecular weight, thus giving the impression that cubic root of the molecular weight should be proportional to the analyte retention volume. This is only observed in the regions of total exclusion and total permeation of the polymer molecules in the adsorbent porous space. A practically useful region for molecular weight determination is where partial permeation of the analyte molecules in the adsorbent porous space is observed. In this region, the adsorbent pore size distribution plays the dominant role in the adsorbent ability to discriminate molecules according to their molecular weight. It was found that the logarithm of analyte molecular weight has a linear relationship with the retention volume in this region.

Hydrodynamic radius of the polymer is also dependent on the analyte interaction with the solvent. Polymer conformation and degree of the salvation varies with the variation of the solvent properties.

Ion-Pairing Chromatography (IPC)

Although ionic compounds can be separated by ion-exchange chromatography, the ion-exchange mode may not give the desired selectivity. Instead, ion-pair chromatography can be used with, for example, a C_{18}-modified silica column. According to the simplest explanation, the analyte ion is paired with a counter-ion of opposite charge added to the eluent to give a neutral species (ion-pair) that can be separated by reversed-phase partition. Typical ion-pairing agents include alkane sulfonates and alkane quaternary ammonium compounds. Because the analytes and counter ions are ionic and thus hydrophilic, typical eluent solvents are methanol or acetonitrile–aqueous buffer mixtures.

Chiral Chromatography

Chiral chromatography is a branch of chromatography that is oriented towards the exclusive separation of chiral substances. Certain stereo isomers that differ only in the spatial arrangement of their atoms and in their capacity for rotating the plane of polarized light are termed optically active or chiral and the individual isomers are called enantiomers. Enantiomeric separations are achieved in chiral chromatography by the judicious use of chiral phases. The mobile phase can be a gas or liquid giving rise to chiral gas chromatography and chiral liquid chromatography. Chiral selectivity is usually achieved by employing chiral stationary phases (CSP) as shown in Figure 2.6, although, in chiral liquid chromatography, chiral mobile phases have been successfully employed. For any chiral separation, the stationary phase must be chosen so that the spatial arrangement of its composite atoms increases the probability or proximity of interaction differing significantly between the two enantiomers to be separated.

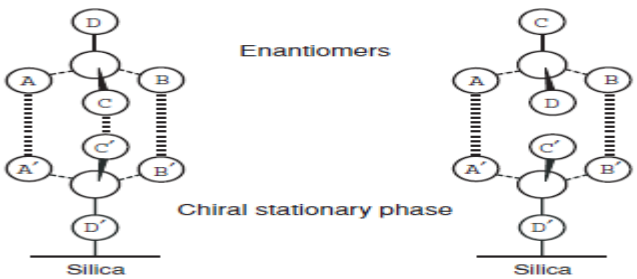


Figure 2.6. Enantiomers-chiral stationary phase reaction at chiral active site

Five general types of CSPs used in chromatography are;

- Polymer-based carbohydrates
- Pirkle or brush-type phases
- Cyclodextrins
- Chirobiotic phases
- Protein-based

Affinity Chromatography

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatographic matrix.

Affinity chromatography separates proteins by their binding specificities. As in Figure 2.7, the proteins retained on the column are those that bind specifically to a ligand cross-linked to the beads (in biochemistry, the term "ligand" is used to refer to a group or molecule that is bound). After nonspecific proteins are washed through the column, the protein of particular interest is eluted by a solution containing free ligand. The technique offers high selectivity, hence high resolution, and usually high capacity for the protein(s) of interest. Purification can be in the order of several thousand-fold and recoveries of active material are generally very high.

Affinity chromatography is unique in purification technology since it is the only technique that enables the purification of a biomolecule based on its biological function or individual chemical structure. Purification that would otherwise be time-consuming, difficult or even impossible using other techniques can often be easily achieved with affinity chromatography. The technique can be used to separate active biomolecules from denatured or functionally different forms, to isolate pure substances present at low concentration in large volumes of crude sample and to remove specific contaminants.

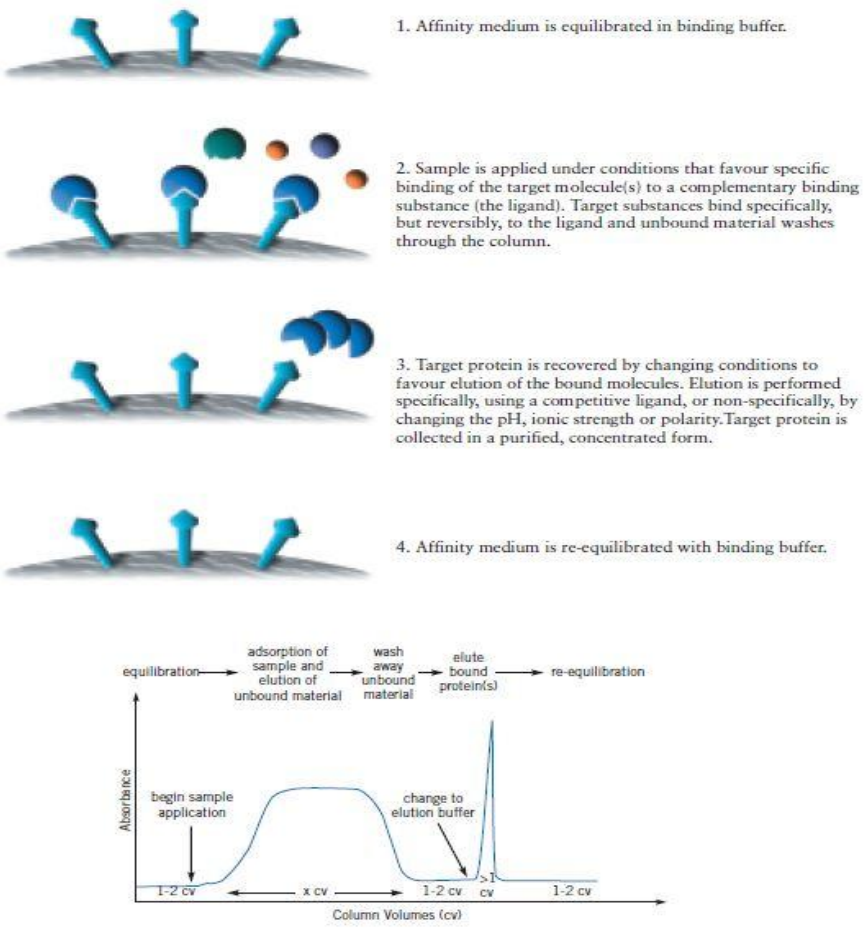


Figure 2.7. Mechanism of affinity chromatography

2.4. Principle Chromatographic Parameters

Chromatographic separations are based upon differences in the extent to which solutes are partitioned between the mobile (m) and stationary (s) phases. The equilibrium of solute A between the two phases is:

$$A_{mobile} \leftrightarrow A_{stationary} \qquad (2.3)$$

The equilibrium constant K called partition coefficient:

$$K = \frac{Cs}{Cm} \qquad (2.4)$$

C_s and C_m are molar concentrations of the solute in stationary and mobile phases, respectively

2.4.1. Retention Time (t_R)

The time it takes after sample injection for the analyte peak to reach the detector is called retention time t_R (Figure 2.8). The volume of mobile phase needed for the analyte to be eluted from stationary phase through the column until reaching detector is called retention volume (V_R) which can be calculated by:

$$V_R = t_R \times F \tag{2.5}$$

F is the volumetric flow rate of mobile phase.

2.4.2. Dead Volume Time (t_0)

The time taken by unretained species to pass through the column, also expressed by void volume (V_0).

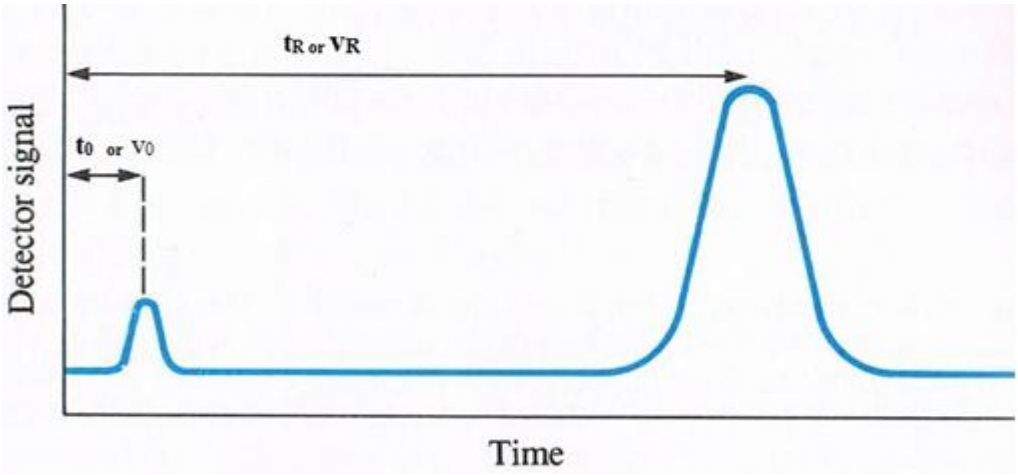


Figure 2.8.Demonstration of chromatographic parameters.

2.4.3. Capacity Factor (k`)

During the elution process, if the volumetric flow rate stays constant then the capacity factor can be written as following equation:

$$k` = \frac{(V_R - V_0)}{V_0} \tag{2.6}$$

$$k` = \frac{(t_R - t_0)}{t_0} \tag{2.7}$$

Capacity factor k` is an important parameter that describes the rate of solute migration on the column, value of k` is core unique for any substance and must be within the range 1 - 5 ideally.

2.4.4. Selectivity Factor (α)

Selectivity factor describe the differential migration rates between two eluents in the column.

$$\alpha = \frac{k`_2}{k`_1} = \frac{(t_{R2} - t_0)}{(t_{R1} - t_0)} \quad (2.8)$$

t_{R2} is belonging to the eluent that have the longer retention time must, α is expected to be greater than 1.

2.4.5. Column Efficiency (N)

Column efficiency depends on peak width eluted from the column and high efficiency the peak width will be narrow (sharp) than the separation value of the eluted materials will be more considerable and easier. Colum efficiency can be measured by the number of theoretical plate number (N).

$$N = 16 \times \left(\frac{t_R}{W}\right)^2 \quad (2.9)$$

W : Peak width calculated at base line

The number of theoretical plate (N) can also be calculated depending on the theoretical equivalent plate height (HETP) and the length of column packing material (L) as following:

$$HETP = \frac{L}{N} \quad (2.10)$$

Thus, column efficiency is directly proportional to greater column length and smaller plate height.

2.4.6. Resolution (R_s)

The resolution (R_s) of a column provides a quantitative measure of the column`s ability to separate two analytes eluted, consecutively.

$$R_s = 2 \times \frac{(t_{R2} - t_{R1})}{(W_1 + W_2)} \qquad (2.11)$$

For more comprehensive parameters that affect the resolution factor the following equation can be written as:

$$Rs = (\sqrt{N})/4\ ((\alpha-1)/\alpha)\ (k/\ (k+1)) \qquad (2.12)$$

As α increases resolution increase, vale of α must be larger than unity, but if α is ≤ 1 then peaks of combined analyte cannot be separable. At the same time, there is a limit for the effect of increasing α and k` on the resolution but column efficiency does (Figure 2.9 and 2.10).

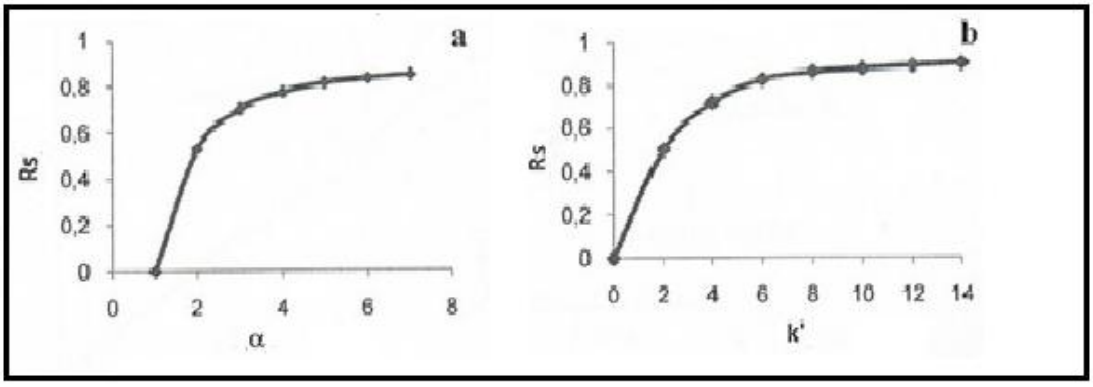


Figure2.9. The relationship between resolution and (a) selectivity and (b) capacity factor

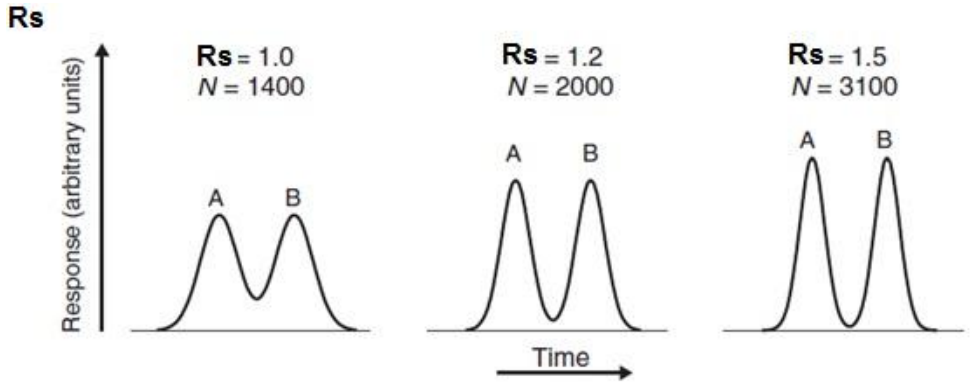


Figure 2.10. An illustration for the effect of column efficiency on resolution of analytes A and B

Efficiency factor is variable with flow rate, column packing material length, particle size and peak width, as resolution increases the peak width increase.

2.4.7. Peak Asymmetry Factor (A_s)

The chromatographic peak will be perfect in Gaussian shape and the elution isotherm is linear. Linearity of the isotherm means that the ratio of C_s/C_m is constant then all chromatographic bands at all concentration will be eluted at the same rate. In practice, ideal symmetrical peaks are not obtained, but their deviation from a true Gaussian peak that is called asymmetrical peak, asymmetry usually comes in two cases (Figure 2.11).

a- Fronting peak:

Often is resulting from overloading the column with analyte or eluent flow throw the channels within the column. In this case the capacity coefficient k` will increase and Langmuir isotherm will be obtained, the retention time of the eluted analyte will increase with higher concentration.

b- Peak tailing:

Usually, tailing in the peak is caused by adsorption of analyte to the reactive sites on the column walls and also by the increasing intermolecular forces. k` value will decrease leading to anti-Langmuir isotherm; the retention time in this case will decrease with higher concentrations.

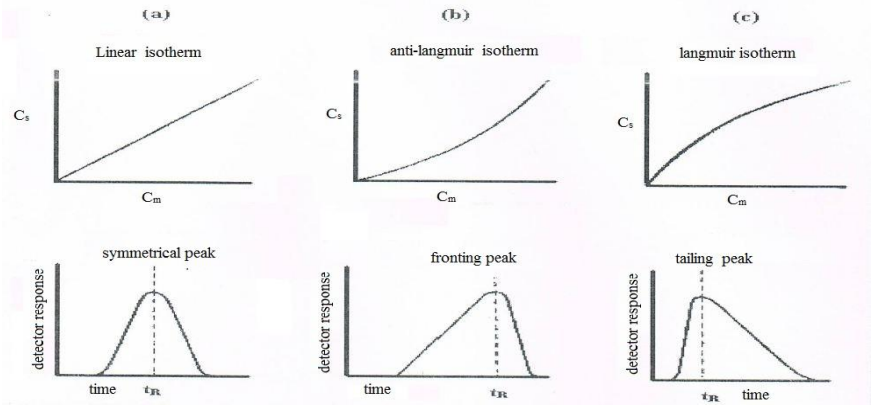


Figure 2.11. Elution isotherms and peak shapes; (a) ideal peak, (b) fronting peak and (c) tailing peak.

Peak asymmetry can be measured as shown in Figure 2.12, using equation 2.13.

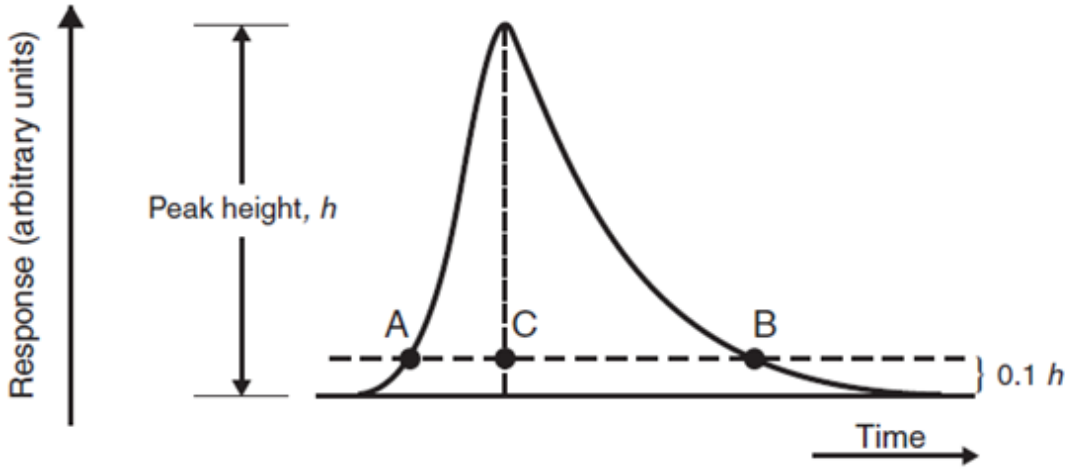


Figure 2.12. Calculation of peak asymmetry

AC and CB lengths indicated in Figure 2.12 are measured at 10% of the total peak height (*h*) above the baseline, and the asymmetry factor, A_s is calculated thus:

$$A_s = \frac{CB}{AC} \tag{2.13}$$

As = 1 Gaussian symmetrical peak, As < 1 Fronting peak, As > 1 Tailing peak**.**

2.5. Liquid Chromatography Instrumentations

High-performance liquid chromatography (HPLC) is well suited to the analysis of hydrophilic, thermally labile and/or high molecular weight compounds. In the analysis of drugs HPLC has additional practical advantages of flexibility, generally low running costs, a range of selective detectors, which can usually be linked in series, and ease of automation. These properties can often be exploited to facilitate the analysis of several compounds (e.g. drug and metabolites)

2.5.1. HPLC Instrumentation

Pumping pressures of several hundred atmospheres are required to achieve reasonable flow rates with packing in the 3 to10 µm size range, sizes common in modern liquid chromatography. As a consequence of these high pressures, the equipment for high-performance liquid chromatography tends to be considerably more elaborate and expensive than that encountered in other types of

chromatography. A typical diagram for a high-performance liquid chromatography is given in (Figure 2.13).

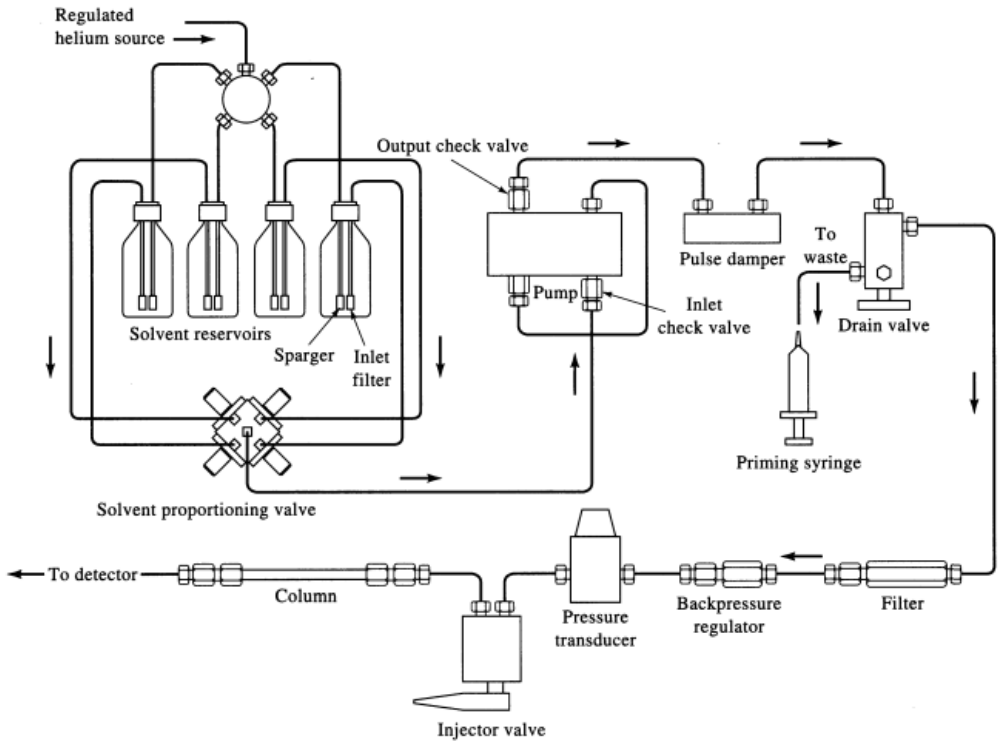


Figure 2.13. HPLC instrumental system

Mobile-Phase Reservoirs and Solvent Treatment Systems

A modern HPLC apparatus is equipped with one or more glass or stainless steel reservoirs, each of which contains 500 ml or more of a solvent. Provisions are often included to remove dissolved gases and dust from the liquids. The former produce bubbles in the column and thereby causes band spreading; in addition, both bubbles and dust interfere with detector performance. Degassers may consist of a vacuum pumping system, a distillation system, a device for heating and stirring, or a system for sparging, in which the dissolved gases are swept out of solution by fine bubbles of an inert gas that is not soluble in the mobile phase.

Pumping Systems

The requirement for the pumps used in liquid chromatography are severe and include (1) the generation of pressure up to 6000 psi, (2) pulse-free output,

(3) flow rates ranging from 0.1 to 10 ml min^{-1}, (4) flow reproducibility's of 0.5 % relative or better, and (5) resistance to corrosion by variety of solvent.

It should be noted that the high pressures generated by liquid-chromatography pumps do not constitute an explosion hazard because liquid are not very compressible. Thus, rupture of a component results only in solvent leakage. To be sure, such leakage may constitute a fire hazard.

Pumps are classified according to the mechanism of eluent displacement by which the liquid is forced through the chromatograph as illustrated in Figure 2.14. Although a wide variety of pump designs have been developed over the years, nearly all LC pumps since the 1980s are based on some variation of the reciprocating piston pump.

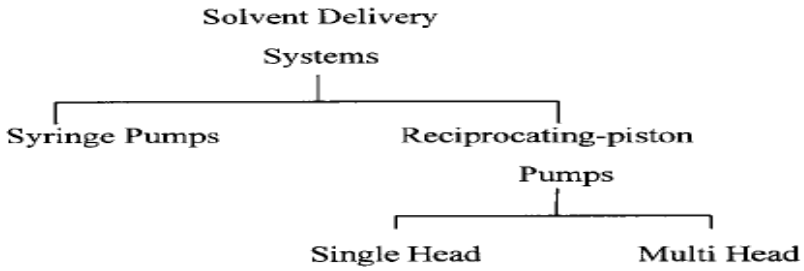


Figure 2.14. Classification of pumps according to the mechanism of eluent displacement

Sample-Injection Systems

Although syringe injection through an elastomeric septum is often used in liquid chromatography, this procedure is not very reproducible and is limited to pressures less than about 1500 psi. In stop-flow injection, the solvent flow is stopped momentarily, a fitting at the column head is removed, and the sample is injected directly onto the head of the packing by means of a syringe.

Although syringe injection finds considerable use owing to its simplicity, the most widely used method of sample introduction in liquid chromatography is based upon sampling loops (valve) like the one shown in (Figure 2.15).

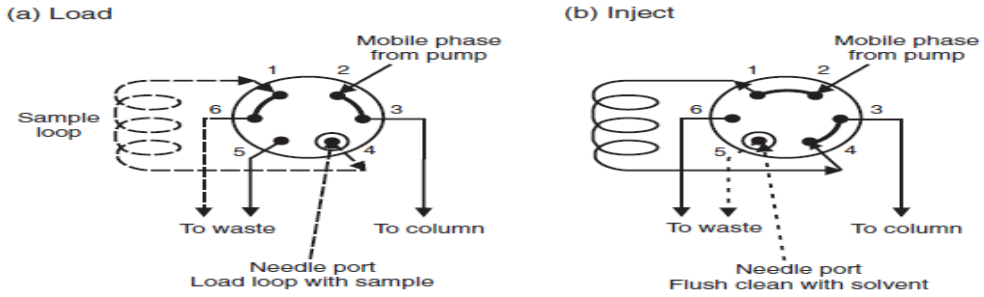


Figure 2.15. HPLC injection valve showing (a) load and (b) inject positions.

These devices are often an integral part of modern liquid–chromatography equipment and have interchangeable loops that provide a choice of sample sizes ranging from 5 to 500 ml. The reproducibility of injections with a typical sampling loop is a few tenths of a percent relative.

Columns for High –Performance Liquid Chromatography

Liquid–chromatography columns are usually constructed from stainless steel tubing. Although heavy-walled glass tubing is sometimes employed for lower pressure applications (< 600 psi), most columns are made from stainless steel range in length from 10-30 cm and have inside diameters of 4 to 10 mm with column packing typically in particle sizes of 5 or 10 µm. Columns of this type often provide 40000 – 60000 plates/m. Recently, high-performance micro columns with inside diameter of 1 to 4.6 mm and length of 3 to 7.5 cm have been available. These columns, which are packed with 3 or 5 µm particles, contain as many as 10 000 plates/m and have the advantage of speed and minimal solvent consumption.

The most common packing for liquid chromatography is prepared from silica particle, which are synthesized by agglomerating sub-micrometer silica particles under conditions that lead to larger particles with highly uniform diameters. The resulting particles are often coated with thin organic film, which is chemically or physically bonded to the surface (Table 2.2 and Figure 2.16). Other packing materials include alumina particles, porous polymer particles, and ion-exchange resins. Octadecyl silane (ODS)-coated silica gel, which is a reverse-phase packing, where the mechanism of retardation is due to partitioning of the lipophilic portion of molecule into the stationary phase.

Table 2.2. Common types of HPLC stationary phases and particle sizes

Mode	Material	Particle size (μM)	Treatment
Adsorption	Silica, irregular	2–20	Unreacted
	Silica, spherical	5–10	Unreacted
	Alumina, irregular	3–12	Unreacted
	Alumina	5–20	Unreacted
Reversed-phase	Silica with long C chain	3–15	C-18
	Silica with intermediate C chain	5–10	C-8
	Silica with short C chain	5–10	C-1, C-3
Normal-phase	Silica (weak)	5–15	Ester, ether, diester
	Silica (medium)	5–15	NO_2, CN
	Silica (high)	5–15	Alkylamino, amino
Ion-exchange	Silica (anion)	5–15	NMe^{3+}, NR^{3+}, NH_2
	Resin (anion)	7–20	NMe^{3+}, $-NH^{3+}$
	Silica (cation)	5–10	$-SO^{3-}(H^+)$, $-SO^{3-}$, $(NH_4)^+$
	Resin	5–20	$-SO^{3-}$

Silica gel and ODS coated silica gel are two of the most commonly used packings for normal- and reverse-phase chromatography application, respectively. But there is a variety of normal- and reverse-phase packings available, most of which are based on chemical modification of the silica gel surface, although in recent years stationary phase which are based on organic polymers have become available. The extent to which a compound is retained will depend primarily upon its polarity, in the case of silica gel, and primarily upon its lipophilicity in the case of a reverse-phase, packing such as ODS silica gel. Most drug molecules have both lipophilic and polar groups.

The other factor to consider with regard to the degree of retention of a particular compound, apart from the stationary phase, is the nature of the mobile phase. The more polar a mobile phase, the more quickly it will elute a compound from a silica gel column, and the more lipophilic a mobile phase, the more quickly it will elute a compound from a reverse-phase column.

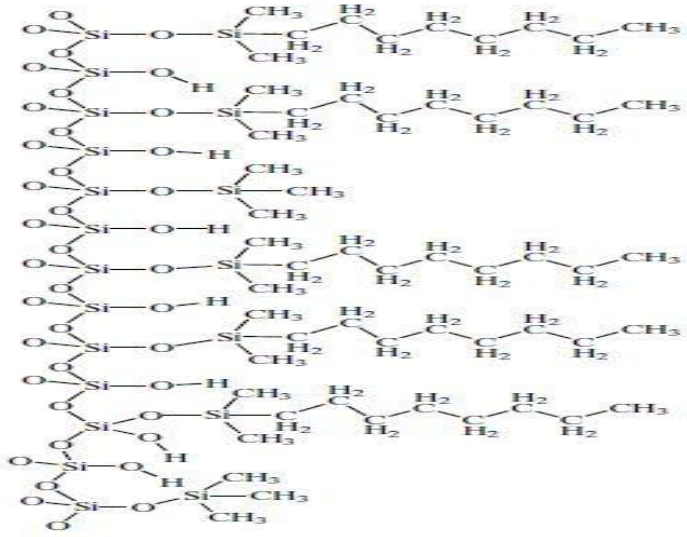


Figure 2.16. Schematic representation of the surface of a typical reversed-phase packing, with C_8 bonded phase

HPLC Detectors (70)

The ideal HPLC detector would have: (i) high sensitivity, (ii) universal or specific response as required, (iii) wide linear dynamic range, (iv) minimal band broadening, (v) a stable response with temperature or flow-rate change and non-destructive. Currently, no detector fulfils all of these requirements of the sample.

HPLC Detectors Classified according to analyte chemical and physical properties as;

A) Bulk or Solute Property Detectors:

*Bulk property detectors***:** Respond to a mobile phase bulk property such as: Refractive index, dielectric constant or density

*Solute property detectors***:** Respond to some property of solute such as: UV absorbance, fluorescence or diffusion current

b) Mass Sensitive and Concentration Sensitive Detectors:

The mass sensitive detector responds to the mass of solute passing through it per unit time and is thus independent of the volume flow of mobile phase such as: transport detector

Concentration sensitive detectors: provide an output that is directly related to the concentration of solute in the mobile phase passing through it, e.g., UV absorbance detectors.

c) Specific and Non–Specific Detectors:

Specific detectors respond to a particular type of compound or a particular chemical group; fluorescence detector would be a typical specific detector that responds only to those substances that gives fluoresce

Non-specific detector responds to all solutes present in the mobile phase and its catholic performance makes it a very useful and popular type of detector such asrefractive index detectors.

Common detectors used inliquid chromatographic separations are;

1. UV/Visible and Diode array detectors
2. Florescence detectors
3. Infrared spectrometer detectors (IR)
4. Electrochemical detectors
5. Radioactive detectors
6. Conductive detectors
7. Dielectric constant detectors
8. Refractive index detectors
9. Density detectors
10. Transport detector
11. Nuclear magnetic resonance (NMR) detectors
12. Diffusion current detectors
13. Mass spectrometer detector
14. Evaporative light scattering detector
15. Circular dichroism (Chiral Detection) detectors
16. Immunoassay detectors

2.6. Ultra Performance Liquid Chromatography –UPLC (36, 39, 70, 71)

High performance liquid chromatography (HPLC) has proven to be the predominant technology used in laboratories worldwide during the past 30-plus years. One of the primary drivers for the growth of this technique has been the evolution of packing materials used to effect separations.

The underlying principles of this evolution are governed by the van Deemter equation, with which any student of chromatography is intimately familiar. The van Deemter equation is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency). And, since particle size is one of the variables, *Van Deemter* curve can be used to investigate chromatographic performance

Ultra performance liquid chromatography (UPLC) is an emerging analytical technique, which draws upon the principles of chromatography to run separations at higher flow rates for increased speed, while simultaneously achieving superior resolution and sensitivity. UPLC was developed through the recognition that a reduction in the stationary phase particle size will have the greatest benefit to any chromatographic process.

While the increased efficiency of small particle chromatography has long been recognized, development of techniques utilizing this science is complicated by the large increases in system backpressures encountered when pumping mobile phase through sub 2μm particles. Traditional HPLC systems are unable to operate at backpressures typically afforded by small particle chromatography. While there is no single separation parameter that distinguishes between ‘high performance’ and ‘ultra performance’ liquid chromatography, UPLC refers to chromatographic separations employing sub 2μm stationary phase particles of high mechanical strength.

2.6.1. Principles of UPLC

According to the *Van Demeeter* equation, as the particle size decreases to less than 2μm, not only is there a significant gain in efficiency, but the efficiency does not diminish at increased flow rates or linear velocities.

$$H = A + \frac{B}{\mu} + C\mu \qquad (2.14)$$

The *van Deemter* equation is an empirical formula that describes the relationship between mobile linear velocity (μ) and column efficiency (HETP or H). The van Deemter plot illustrates the principles of the van Deemter equation and is used to predict and determine the mobile phase flow rate where column efficiencies will be maximized. How particle diameter can significantly reduce the HETP resulting in higher separation efficiencies is illustrated in Figure 2.17.

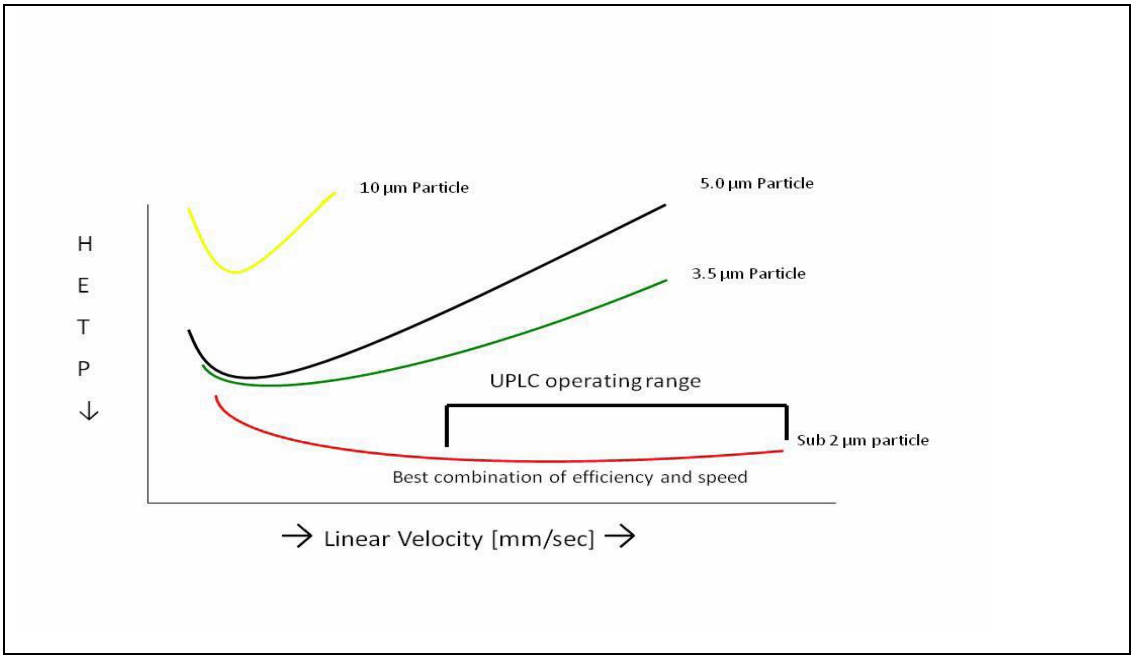


Figure 2.17. A diagram for van Deemter equation plots

The extended minimum of the sub 2 µm indicates that increases in mobile phase flow rates do not have the same negative influences on separation efficiency as seen with the larger particles. This means that increased efficiencies are available over a much wider range of flow rates and the speed of analysis can be increased without sacrificing efficiency or resolution.

Efficiency is the primary separation parameter behind UPLC since it relies on the same selectivity and retentively as HPLC. Review of the fundamental resolution (R_s) equation reveals that chromatographic resolution is directly proportional to the square root of column efficiency (Equation 2.12). Resolution is proportional to the square root of theoretical plate number (N), but since N is inversely proportional to particle size (d_p):

$$N \alpha \quad \frac{1}{dp} \tag{2.15}$$

As the particle size is lowered by a factor of three, from, for example, 5 µm (HPLC scale) to 1.7 µm (UPLC-scale), N is increased by three and resolution by the square root of three or 1.7 factors. N is also inversely proportional to the square of

$$N \alpha \ \frac{1}{w^2} \tag{2.16}$$

The narrower the peaks are, the easier they are to separate from each other. Also, peak height is inversely proportional to the peak width:

$$H \alpha \ \frac{1}{w} \tag{2.17}$$

So as the particle size decreases to increase N and subsequently R_s, an increase in sensitivity is obtained, since narrower peaks are taller peaks. Narrower peaks also mean more peak capacity per unit time in gradient separations. Moreover, van Deemter theory states that optimum flow rate (F_{opt}), corresponding to maximum separation efficiencies, and increases as particle size decreases:

$$F_{opt} \alpha \ \frac{1}{dp} \tag{2.18}$$

As can be seen from the van Deemeter plot, as particle size decreases, the corresponding HETP also decreases, resulting in higher efficiencies. It is also evident from the van Deemter plot that the highest efficiencies are available over a much wider range of flow rates with smaller particles than with larger particles.

The separation efficiency of HPLC increased as the particle size of column packing decreased from 10 µm in the 1970s down to 3.5 µm in the 1990s. One consistent observation with all of these particlesizes, as well as the 2.5 µm particles used in the early 2000s was that HETP decreased to a minimum value and then increased with increasing flow rate. When employing 1.7 µm stationary phase particles such as those used in UPLC, the resulting van Deemter plot not only

exhibits a decreased HETP relative to the larger particles but also offers an extended minimum over a wider range of linear velocities.

As a result, flow rate or speed of analysis can be optimized without sacrificing resolution. This means that when transitioning from 5μm to 1.7μm particles, not only can column length be reduced by a factor of 3, but the separation can be run at three times the flow rate. This translates to a nine-fold increase in throughput with no loss in efficiency or resolution.

Since backpressure is proportional to flow rate, achieving small particle, high peak capacity separations requires fully redesigned HPLC systems capable of operating at backpressures beyond the capabilities of today's system designs. To take full advantage of the increased speed, superior resolution and sensitivity afforded by smaller particles, instrument technology had to be fully redesigned.

To fully realize the potential speed, sensitivity and resolution of UPLC separations, new pressure – tolerant reversed phase particle had to be developed. Production of extremely small, efficient particles with high mechanical strength would allow the analyst to surpass the performance standards of current HPLC column technology.

In more recent years, first, Waters Corporation has utilized a bridged ethylsiloxane/silica hybrid (BEH) structure with a narrow particle size distribution, produced by the condensation of 1,2-bis (triethoxysilyl) ethane and tetra-ethoxysilane. This new hybrid material was developed in a 1.7 μm particle to improve efficiency, ruggedness, pH range, peak shape and loading capacity, as well as the ability to run at elevated backpressures and temperatures. The interconnection of silica atoms with ethyl groups whilst maintaining a silica backbone has proven to be a key success factor for Waters UPLC columns because it means maintaining the strength of silica, while achieving reduced silanol activity and improved resistance to alkaline conditions. Peak shape is further optimized using trifunctional C_{18} bonding chemistry and a proprietary end-capping procedure.

High efficiency separations employing 1.7 μm solid phase particles routinely produce peaks with a half-height width of less than 1 second, which poses significant challenges for the detection system. One major concern when operating with very narrow peaks is the ability of the MS to obtain a sufficient number of data points

across a peak to perform peak integration and data-dependant MS/MS analysis. To accurately and reproducibly quantify analyte peaks with half-height widths of less than one second, detection systems capable of rapid data acquisition are required to ensure that sampling rates are high enough to capture sufficient data points across such narrow peaks.

2.6.2. UPLC instrumentation

Due to several but limited type or manufactures for UPLC systems, the instrumentation terms have still not been classical as in HPLC. Thus, the system utilized for the experiments in the thesis presented here is referred for UPLC instrumentation part. AQUITY UPLC consists of a binary solvent manager, sample manager (including the column heater), detector, and optional sample organizer. The binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient mixed under high pressure. There is a built-in solvent degassing as well as solvent select valves to choose from up to four solvents. There is a 15000 pressure limit (about 1000 bar) to take full advantage of the sub 2 μm particles. The sample manager also incorporates several technology advancements.

Low dispersion is maintained through the injection process using pressure assist sample introduction, and a series of pressure transducers facilitate self-monitoring and diagnostics. It uses needle-in-needle sampling for improved ruggedness and needle calibration sensor increases accuracy.

Injection cycle time is 25 s without a wash and 60 s with a dual wash used to further decrease carry over. A variety of micro titer plate formats (deep well, mid-height, or vials) can also be accommodated in a thermostatically controlled environment. Using the optional sample organizer, the sample manager can inject from samples from up to 22 micro titer plates. The sample manager also controls the column heater. Column temperatures up to 65 °C can be attained. A “pivot out” design provides versatility to allow the column outlet to be placed in closer proximity to the source inlet of an MS detector to minimize excess tubing and sample dispersion.

2.6.3. UPLC Columns

In 2000, Waters introduced XTerra®, a first generation hybrid chemistry that took advantage of the best of both the silica and polymeric column worlds. XTerra columns are mechanically strong, with high efficiency and operate over an extended pH range. They are produced using a classical sol-gel synthesis that incorporates carbon in the form of methyl groups. But in order to provide the necessary mechanical stability for UPLC, a second generation bridged ethyl hybrid (BEH) technology was developed. These 1.7 µm particles derive their enhanced mechanical stability by bridging the methyl groups in the silica matrix. Some of Aquity UPLC columns as example to UPLC column stationary phases are given in Figure 2.18.

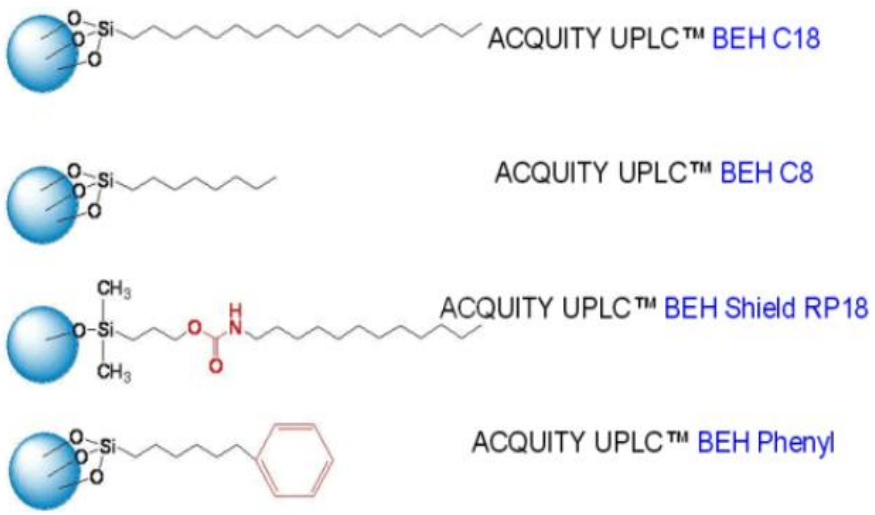


Figure 2.18 . The ACQUITY UPLC columns

2.6.4. UPLC Detectors

Although the detectors utilized in HPLC may also be used in UPLC, the most detector types used with UPLC systems are Mass Spectrometer (MS) and diode-array spectrophotometer detectors. MS detection is significantly enhanced by UPLC.

By the mid-1990s, HPLC directly coupled to MS was in routine use in drug metabolism laboratories for these types of studies. Enhanced selectivity and sensitivity, and rapid, generic gradients made LC–MS the predominate technology for both quantitative and qualitative analyses. However, with the ever-increasing numbers and diversity of compounds entering development, and the complex nature

of the biological matrices being analyzed, new analytical procedures and technology was required to keep peace with the testing demands. Unexpected, reactive or toxic metabolites must be identified as early as possible to reduce the very costly attrition rate. This quest for more accurate data meant improving the chromatographic resolution to obtain higher peak capacity, reducing the co-elution of metabolites, while enhancing the sensitivity and decreasing ion suppression in the MS.

2.7. Mass Spectrometry (61, 66-68, 72-73)

Mass spectrometry is concerned with the electron ionization and subsequent fragmentation of molecules and with the determination of the mass to charge ratio (m/z) and the relative abundances of the ions were produced. Functional groups in the molecule direct the fragmentation in such a way that knowing the structure of the molecule it is possible to predict the fragmentation pattern.

A mass spectrometer works by generating charged molecular fragments either in a high vacuum or in immediately prior to the sample entering the high-vacuum region. The ionized molecules have to be generated in the gas phase. In classical mass spectrometry, there was only method of producing the charged molecules but now there are quite a number of alternatives.

Once the molecules are charged and in the gas phase, they can be manipulated by the application of either electric or magnetic fields to enable the determination of their molecular weight and the molecular weight of any fragments which are produced by the molecule breaking up.

2.7.1. MS Instrumentation

In a MS, operat ions under high vacuum, analytes are ionized, sometimes fragmented, and then directed to a mass analyzer where they are separated according to their mass-to-charge ratios, m/z. The ion current generated is plotted versus m/z ratios to produce a mass spectrum, which is characteristic of the original analyte and can be used for both qualitative and quantitative analysis. Usually the z value of the ions is 1, so the m/z ratio actually is the same as the mass for that ion. The parts of a mass spectrometer are shown diagrammatically in Figure 2.19.

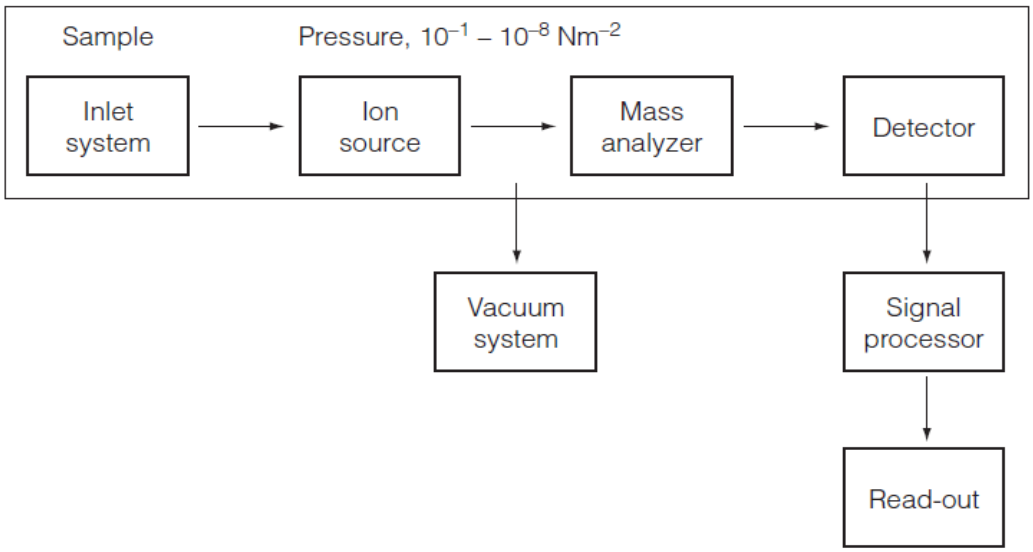


Figure 2.19. The major parts of a mass spectrometer

General MS main components are:

- **Sample inlet,** which facilitates the controlled introduction of gaseous or vaporized liquid samples via a molecular leak (pinhole aperture) and solids via a heated probe inserted through a vacuum lock.
- **Ion source** to generate ions from the sample vapor;
- **Mass analyzer** which separates ions in space or time according to their mass-to-charge ratio. Ions generated in the source are accelerated into the analyzer chamber by applying increasingly negative potentials to a series of metal slits through which they pass.
- **Detector System**for detecting the ions and recording the relative abundance of each of the resolved ionic species.

2.7.2. Ionization Techniques in MS

Electron impact ionization (**EI**) employs a high-energy electron beam (~70 eV). Collisions between electrons and vaporized analyte molecules initially result in the formation of molecular ions, which are radical cations:

$$M + e^- \rightarrow M^{+\bullet} + 2e^-$$

These then decompose into smaller fragments.

Chemical ionization (**CI**) is a softer technique than EI, ions being produced by collisions between sample molecules and ions generated by a reagent gas such as methane or ammonia. Three stages are involved. For methane, for example:

(i) reagent gas ionized by EI:

$$CH_4 + e^- \rightarrow CH_4^{+\bullet} + 2e^-$$

(ii) secondary ion formation:

$$CH_4^{+\bullet} + CH_4 \rightarrow CH_5^+ + CH_3^\bullet$$

(iii) formation of molecular species:

$$CH_5^+ + M \rightarrow MH^+ + CH_4$$

(pseudomolecular ion)

Desorption techniques are used mainly for solid samples that can be deposited on the tip of a heatable probe that is then inserted into the sample inlet through vacuum locks. Molecules are ionized by the application of a high potential gradient (**field desorption, FD**) or by focusing a pulsed laser beam onto the surface of the sample.

Matrix-assisted laser desorption (**MALDI)**, the sample is mixed with a compound capable of absorbing energy from the laser and which results in desorption of protonated sample molecules. These techniques are very soft, give little fragmentation and are especially useful for compounds with a high RMM.

Fast atom bombardment and plasma (californium-252) desorption techniques deal rather effectively with polar substances (usually of higher molecular weight) and salts. Samples may be bulk solids, liquid solutions, thin films, or monolayers.

Field ionization and field desorption: are techniques used for studying surface phenomena, such as adsorbed species and trapped samples, and the results of chemical reactions on surfaces; they are also suitable for handling large lipophilic polar molecules.

Atmospheric pressure chemical ionization (APCI) interfaces, nitrogen is introduced to nebulize the mobile phase producing an aerosol of nitrogen and solvent

droplets, which are passed into a heated region. Desolvation occursand ionization is achieved by gas phase ion-molecule reactions at atmospheric pressure, electrons and the primary ions being produced by a corona discharge.

Inductively coupled plasma (ICP) mass spectrometry*:* In this technique, the necessary ionization of the analyte is achieved within the plasma itself. In a typical configuration, the plasma is directed axially on to the apex of a cone with a small orifice at its Peak. The cooler outside sheath of the plasma is diverted away radially by the cone, and the core plasma can then pass into the **MS** analyser

2.7.3. Interfacingin Mass Spectrometry

With other analytical techniques necessitates the use of specially designed interfaces and ionizing sources.These include thermospray, electrospray and ionspray for liquid chromatography-mass spectrometry (LC-MS), and an inductively coupled plasma (ICP) for ICP-MS.

Thermo spray Method

The HPLC effluent is fed into a microfurnace maintained at up to 400ºC that protrudes into a region of reduced pressure (approximately 1 torr). The heat creates a supersonic, expanding aerosol jet that contains a mist of fine droplets of solvent vapor and sample molecules. The droplets vaporize downstream and the excess vapor is pumped away. Ions of The sample molecules are formed in the spray either by direct desorption or by chemical ionization when used with polar mobile phases that contain buffers such as ammonium acetate. A conventional electron beam is used to provide gas-phase reagent ions for the chemical ionization of solute molecules

Electrospray ionization (ESI)

Also operating at atmospheric pressure, the liquid mobile phase is ejected from a metal capillary tube into an electric field obtained by applying a potential difference of 3−6 kV between the tube and a counter electrode. The drops accumulate charge on their surface, and as they shrink by evaporation they break into ever smaller charged droplets. The uncharged solvent molecules are pumped away and the charged ions pass into the mass spectrometer (Figure 2.20).

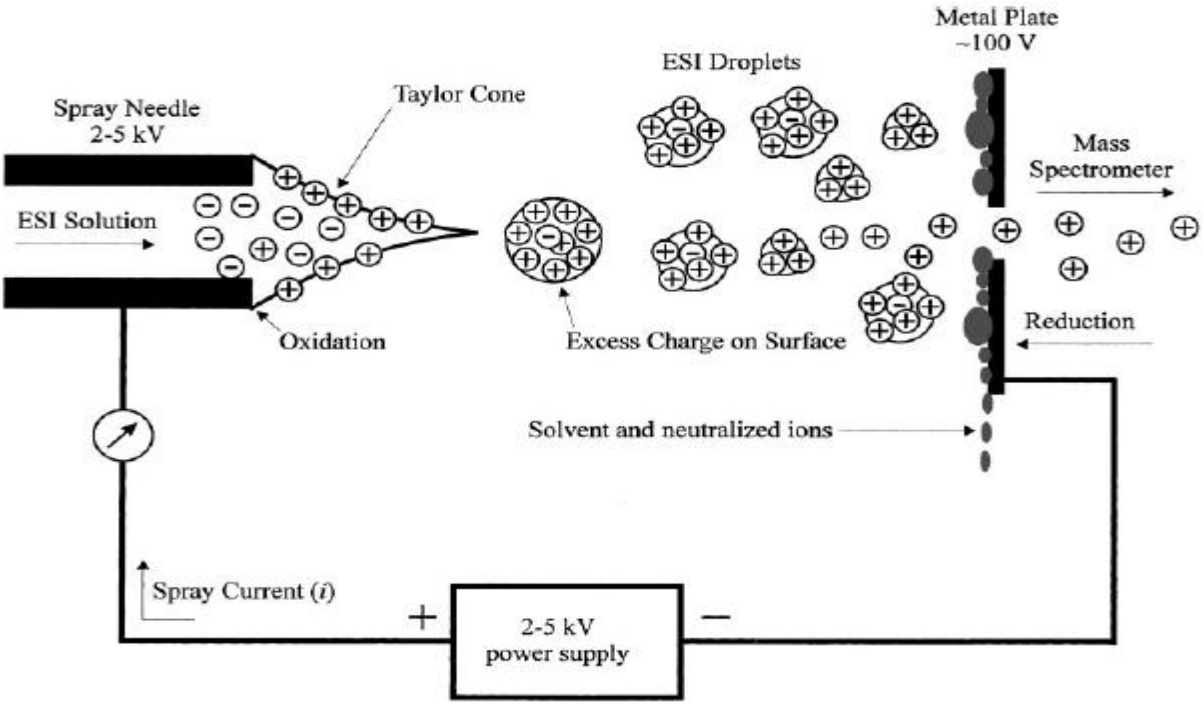


Figure 2.20.ESI LC/MS interface diagram

2.7.4. Mass Analyzers

The function of the mass analyzer is to separate the ions produced in the ion source according to their different mass–charge ratios. The analyzer section is continuously pumped to a very low vacuum so that ions may be passed through it without colliding with the gas molecules. The energies and velocities v of the ions moving into the mass analyzer are determined by the accelerating voltage V from the ion source slits and the charge *z* on the ions of mass *m*

The interfacing of a liquid chromatography to mass spectrometer proved much more than interfacing a gas chromatograph since each mole of solvent introduced into the instrument produces 22.4 liter of solvent vapor, even at atmospheric pressure. The technique has made huge advances in last 10 years and there are many types of interface available, the most successful of which are the electro spray and atmospheric pressure ionisation sources.

Magnetic sector instruments

In a magnetic sector instrument, the ions generated are pushed out of the source by a repeller potential of same charge as the ion itself. They are then accelerated in an electric field of *ca* 3-8 kV and travel through an electrostatic field region so that they are forced to fall in to a narrow range of kinetic energies prior to entering the field of a circular magnet. They then adopt a flight path through the magnetic field depending on their charge to mass (m/z) ratio; the large ions are deflected less by a magnetic field:

$$m/z = H^2 r^2/2V \qquad (2.19)$$

Where H is magnetic field strength, r is the radius of circular path in which the ion travels, and V is the acceleration voltage.

As particular values of H and V , only ions of particular mass adopt a flight path that enable them to pass through the collector slit and be detected. If the magnetic field strength is varied, ions across a wide mass range can be detected by the analyzer; a typical sweep time for the magnetic field across a mass range of 1000 is 5-10 s but faster speed are required if high-resolution chromatography is being used in conjunction with mass spectrometry. The accelerated voltage can also be varied while the magnetic field is Field constant, in order to produce separation of ions on the basis of their kinetic energy

(1) The sample is introduced into the instrument source by heating it on the end of a probe until it evaporates, assisted by the high vacuum within the instrument.

(2) Once in the vapor phase, the analyte is bombarded with the electrons produced by a rhenium or tungsten filament, which are accelerated towards positive target energy of 70 eV. The analyte is introduced between the filament and the target, and the electrons cause ionisation as follows:

$$M + e \rightarrow M^+ \qquad (2.20)$$

(3) Since the electrons used are of mush higher energy than the strength of the bonds within the analyte (4-7eV), extensive fragmentation of the analyte usually occurs.

(4) Two types of system are commonly used to separate ions on the basis of their charge to mass ratio.

Double-focusing Mass Spectrometer

Double-focusing mass spectrometers use a magnetic field to select ions based on their *m*/*z* values and an electric field to select ions based on their energy. These instruments became the workhorse of MS from the 1930s through the end of the 1970s. These instruments are capable of separating ions with very small differences in *m*/*z* values allowing for the determination of the elemental composition of the ion based on these millimass measurements (2.21).

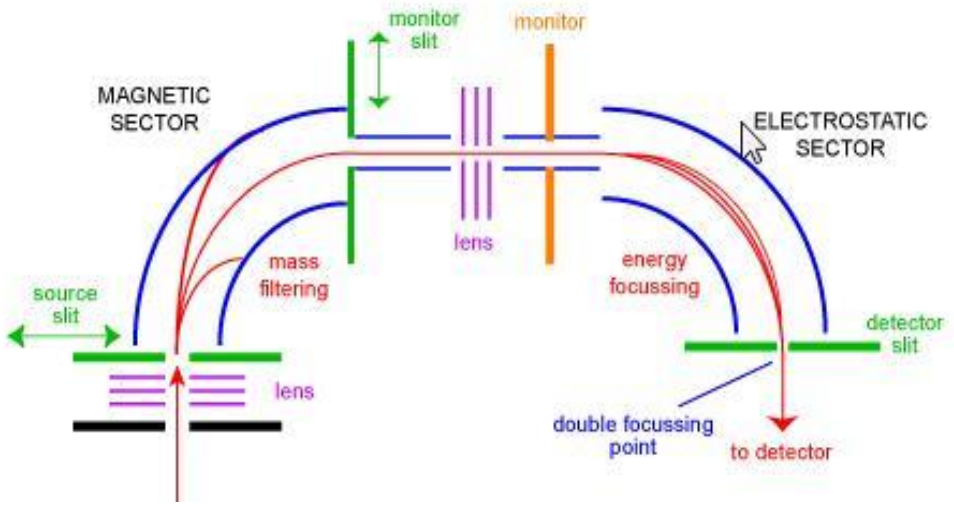


Figure 2.21. Scheme of a double focusing magnetic spectrometer

Quadrupole instruments

A cheaper and more sensitive mass spectrometer than magnetic sector instrument is based on the quadruple analyzer (Figure 2.22) which used two electric field applied at right angles to each other, rather than a magnetic field, to separate ions according to their m/z ratios. One of the fields used is DC and the other oscillates as radiofrequency (Figure 2.22).

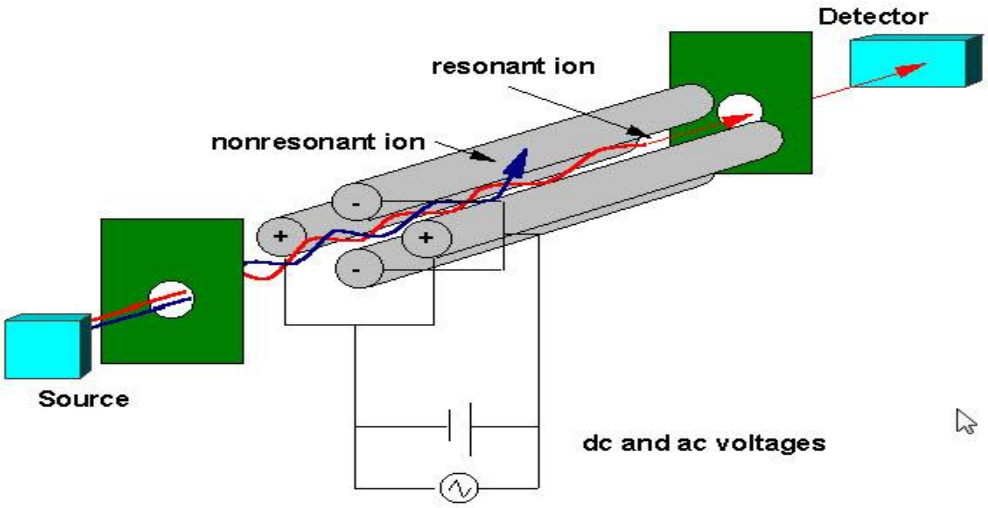


Figure 2.22 . Scheme of a quadrupole analyzer

The effect of applying the two electrostatic fields at right angles to each other, one of which is oscillating (resonating), is to create a resonance frequency for each *m/z* value, ions which resonate at the frequency of the quadrupole are to pass through it and be detected. Thus ions across the mass range of the mass spectrum are selected as the resonance frequency of the quadrupole is varied. A quadrupole instrument is more sensitive than a magnetic sector instrument since it is able to collect ions with a wider range of kinetic energies.

The disadvantage of a simple quadrupole mass spectrometer is that it cannot resolve ions to an extent > 0.1 amu whereas a magnetic sector instrument can resolve ions to a level of 0.0001 amu or more. This enables the latter to be used to determine accurate masses for unknown compounds and thus assign their elemental compositions.

Matrix-assisted laser desorption with time of flight (MALDI-TOF)

MALDI-TOFcan be used for very large proteins >200000 amu. The sample is dissolved in a light-absorbing matrix; soft ionisation is promoted by a pulsed laser; and ions are ejected from the matrix and accelerated using an electrostatic field into field-free region. The lighter ions travel fastest. In order to improve resolution, a device called `reflactron` is used to focus the kinetic energies of a population of a particular ion prior to its field-free region. The length of time taken for ions to reach

the detector gives their molecular weight (MW). The pulsed nature of the ionisation ensures there is no overlap between spectra. Ideal technique for characterization of the MW of large proteins (Figure2.23).

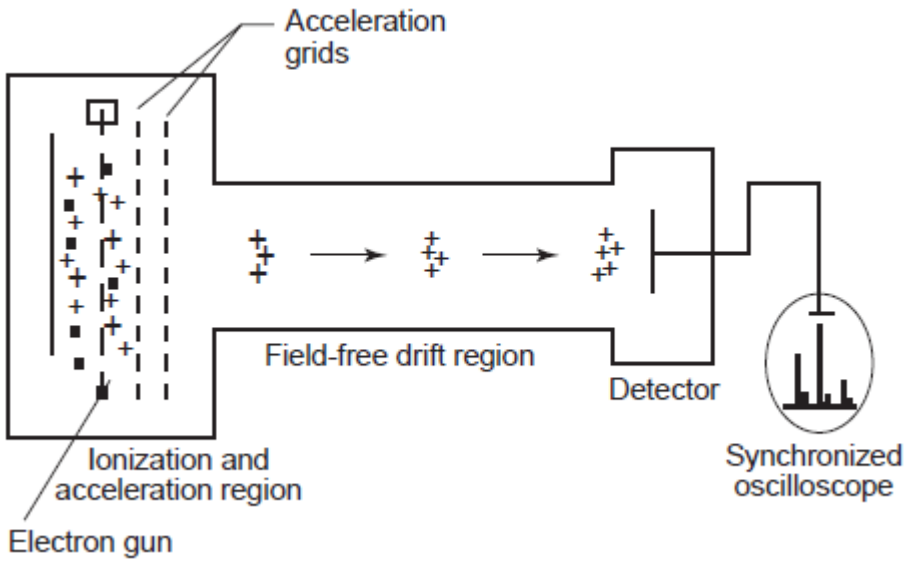


Figure 2.23. Diagram of TOF MS Analyzer

Ion-Trap Mass Spectrometer

A quadrupole ion trap consists of three electrodes; two end-cap electrodes normally are held at ground potential and between them a ring electrode to which an rf potential, often in the megahertz range, is applied to generate a quadrupole electric field. These components can be held in the palm of the hand. Ionization in ion traps is commonly achieved by electron ionization, which occurs within the trap.

Chemical ionization uses the variable time scale of the ion trap first to generate reagent ions *via* electron impact and then allows these reagent ions to react with the vaporized analyte molecules. Both ionization methods are limited to gaseous samples.Desorption ionization methods enable mass spectrometry application to fragile nonvolatile compounds,which can be implemented by forming ions in an external source by fast ion bombardment or secondary ion mass spectrometry, and then injecting them into the trap. Although trapped ions can be mass-analyzed by several methods, a mass-selective instability scan is used most commonly. In this procedure, a change in operating voltages is used to cause trapped ions of a particular

m/*z* ratio to adopt unstable trajectories. By scanning the amplitude of the rf voltage applied to the ring electrode, ions of successively increasing *m*/*z* are made to adopt unstable trajectories and to exit the ion trap, where they can be detected by using an externally mounted electron multiplier.

Tandem Mass Spectrometry (MS/MS)

Since soft ionisation technique such as ESI produces very little diagnostic fragmentation, it is often used in conjunction with tandem mass spectrometry. The types of mass spectra obtained by using collision-induced dissociation (CID) in a tandem mass spectrometer are similar to those, which are obtained under EI conditions.

Typically,the molecular ion of the molecule is selected (the precursor ion) by the first quadrupole(Figure 2.24). The selected ion is then fragmented using a second quadrupole, into which argon gas is introduced, which acts as collision cell. The fragment produced (product ions) are separated using third quadrupole. The technique can sometimes be used without chromatographic separation, making it very rapid technique in areas such as clinical screening for diagnostic marker compounds.

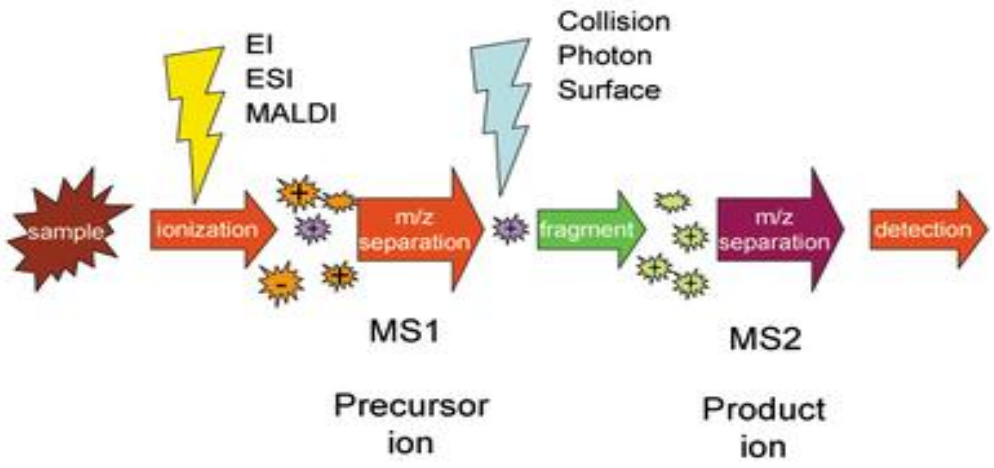


Figure 2.24. Tandem Mass spectrometry

2.7.5. Mass Spectrometer Detectors

Electron Multiplier

In the electron multiplier, the ion beam strikes a conversion dynode, which converts the ion beam to an electron beam. A discrete dynode multiplier has 15 to 18 individual dynodes arranged in a venetian blind configuration and coated with a material that has high secondary-electron-emission properties. A magnetic field forces the secondary electrons to follow circular paths, causing them to strike successive dynodes.

Faraday Cup Collector

The Faraday cup collector consists of a cup with suitable suppressor electrodes, to suppress secondary-ion emission, and guard electrodes. It is placed in the focal plane of the mass spectrometer.

2.8. Methods for analysis of BTM and DXM

The methods reported for the analysis BTM and/ or DXM in a different matrices using Liquid Chromatography (LC) or Ulta-Perfomnce Liquid Chromatography (UPLC) techniques were briefly given in Table 2.3.

Table2.3 . Liquid chromatographic methods for analysis BTM and/or DXM in different matrices.

Drug	Matrix/ purification	Column	Mobile phase	Detector	Findings	Ref.
BTM + DXM	Tablet Derivatization with N-Carbobenzoxy-l-phenylalanin.	Novapack silica (75mm× 3.9mm), 4 µm Particle Size.	n-hexane:dichloromethane: isopropanol (100:100:3) /1mL/min	-UV/VIS 240 nm -MS-FAB -NMR C^{13}	**Retention time (min)**: 2 peaks within 4-6 min **- Selectivity** α: 1.5. **- Resolution Rs**: 1.9 **- Percentage of Manufacture`s claim:** BTM: 93.5 ,SD= 1.58 DXM: 99.0 ,SD= 2.14 **LOD (pmol in 20µL):** BTM 4.2 , DXM 2.1 **Linearity (r):** 0.999	3
BTM + DXM	Bovine Liver	Xterra RP C18 (150mm×2.1mm) , 3.5 µm	0.1% HAc :ACN Gredient(75:25) within 27 min at 0.2 ml/min	Tandem MS/ - ESI	**Retention time (min)**: At 451/360 *m/z* BTM 10.9, DXM 11.2	6

					ecovery: BTM 73-77% DXM 75-76% **LOD (µg/kg)** BTM 1.46,DXM 1.20 **LOQ(µg/kg)** BTM 1.76, DXM 1.33	
BTM and its phospha te and acetate esters	Human Plasma LLE + SPE	- Xterra MS C8 (100×2.1mm), 5 µm - Xterra C18 (150×3.9), µm	1-Ammonuim formate buffer: MeOH (35:65) at 0.3 ml/min 2.Ammonium formate:MeOH:ACN (60:20:20) at 1 ml/min	Tandem MS /+ESI	**Retention time(min)** BTM 1.75 Acetate 3.6 Phosphate 2.1 **LOD:** BTM 0.50 ng/ml BTM phosphate 1.00 ng/ml **Accuracy at LOD** BTM 92.89% Acetate 107.15% Phosphate 96.11 % **Linearity R^2>0.999**	7

BTM Diprop-ionate	Cream and ointment	Waters SymmetryShield RP18 column (150×4.6 mm), 3.5 µm	A:Water B:Acetonitrile Gradient, 1.5ml/min	DAD 240 nm	**Retention time (min):** BTM dipropionate 27.1 **LOD** : 0.02 µg mL^{-1} **LOQ:** 0.5 µg mL-1 **Accuracy** (0.165 mg mL-1): 99.5% - 102.6% **Precision**: RSD < 0.3%	8
BTM **DXM**	Bovine Urine Enzymatic Hydrolysis	Chromsphere C_{18} (200mm×3mm), 5 µm Particle size.	H_2O:MeOH 50:50 →35:65 in 12 min	-UV/VIS 264nm -GC/MS-FAB-+ EI(detection)	**-Retention time (min):** BTM 15.08,DXM 15.18 - **LOD at 590 *m/z*** BTM 0.2 ng/ml DXM 0.1ng/ml	11
BTM **DXM**	Pharmaceutical Active ingredients powder	ACE C8 10 cm	a) ACN:20mM Cyclodextrine 15:85 b)ACN:H_2O , ACN% 19-25 c) THF:H_2O , THF% 16-21	DAD at 240nm	**Retention time (min):** BTM 12-12.5,DXM 14.5 **Resolution Rs** 3.3 Recovery: DXM 98.4%. R^2= 0.9999 **LOD**: 0.01% of 1mg/mL (API)	12

BTM acetate + DXM	Bovine liver	Kromsil RP C18 (150mm×4mm), 5 µm	MeOH: Water (80:20 v/v)	DAD 240 nm	**Retention time (min):** BTM acetate 2.78, DXM 2.37 **LOD**(µg mL-1): BTM acetate 0.034 DXM 0.021 **LOQ(**µg mL-1): BTM acetate 0.0410 DXM 0.027	13
BTM + DXM	Equine urine Enzymatic hydrolysis	Supelco RP DB-8 (75mm×4.6mm i.d), 3 µm Particle size.	1% HAc : MeOH	MS/MS with APCI ionization.	**-Retention time (min):** BTM 24.02,DXM 24.43 **Recovery of 10 ng/ml at 393*m/z*** BTM = 86% DXM= 87%	27
BTM DXM	Bovine Urine LLE + C_{18} SPE	Hpercarb (100mm×4.6mm) ,7 µm	ACN : H2O +0.3% HCH 90:10 , 1.0ml/min,15 min	Tandem MS /API ion source	**Retention time (min):** BTM 8.52 DXM 6.6 **Accuracy:**	28

					BTM 98.6 -100 % DXM 97.5-98.9% **LOD/LOQ (µg kg^{-1}):** BTM 0.2/0.3 DXM 0.2/0.4 **Recovery:** BTM 62.5-68.7 % DXM 56.2- 62 %	
BTM **DXM**	Bovine urine - Enzymatic hydrolysis/ C18 SPE	Symetry C18 (150mm×4.6mm) , 5 µm Particle size.	H_2O:ACN 80:20 Gradient 80:20→ 50:50 in 15 min, 50:50 for 5 min.	MS/Douple quadrupole-APCI, 2^{nd} quadrupole 41 *m/z* loss mode	**-Retention time (min):** BTM 11.86 DXM 12.06 -LOD : 1µg kg^{-1} **- Recovery:** BTM 113.0 % DXM 106.4 %	29
BTM **DXM**	Bovine liver Enzymatic	Hpercarb (100mm×2mm), 5 µm	ACN:H_2O (90:10) + 0.3% (v/v) Formic acid	MS/MS Triple quadrupole	**Retention time (min):** BTM 5.53 DXM 4.37	30

	hydrolysis+ C18 SPE			with ± ESI	**LOD/ LOQ:** 0.1-0.4 µg kg^{-1}	
BTM + DXM	Hair, acidic digestion	Uptisphere ODSB C18 (150mm×2.0mm) , 5 µm	2 mM ammonium formate pH 3:ACN (10:90) Gradient: ACN% 10→100 in 9 min,for 6 min Flow rate 0.32ml/min→0.42ml/ min in 9 min,for 6 min.	Tandem MS/Triple quadrupole with +ESI	**Retention time (min):** BTM/DXM : 8.9 **LOD:** BTM = 18.2 ng ml^{-1} DXM not detected	31
DXM	Milk, Eggs Enzymatic hydrolysis+ C18 SPE	UPLC /ACQUITY BEH C18 (100mm*2.1mm), 1.7 µm	MeOH:H_2O 0.1% v/v formic acid (35:65) Gradient : MeOH 35%→40% in 6 min→80% in 6min→95% in	Tandem MS/-ESI	**-Recovery:** 99.3%- 112.3% At 0.4 and 2 µg kg^{-1} spiked milk. - **LOD** = 0.01µg kg^{-1} - **LOQ** = 0.04 µg kg^{-1}	32

			3min→ 35% in 0.1min.			
BTM **DXM**	Sewage/river water SPE	Zorbax XDB C18 (50mm×4.6mm), 1.8 µm	A) Water:ACN (78:22)+0.1%HCH B)MeOH:ACN(78:22 +0.1%HCH Multi step Gredient B% 0.8→99.9 within 13min,1 ml/mi	Tandem MS/ ESI	**Retention time (min)** BTM 10.4,DXM 11.1 **LOD** ng mL^{-1}:BTM/DXM River :0.5/0.5 Efluent sewage 0.3/0.3 Influent sewage7.5/7.5 **Recovery%** BTM,DX River :94,92 Efluent sewage 92,90 Influent sewage 95/91	33
BTM **DXM**	Edible tissues Prussurized liquid extraction	Hypersil Gold C18 (150×2.1mm), 5µm	A: Water/ B:ACN/ C:0.2% HCH Gradient: t_0(65:30:5), t_{17}(65:30:5) t_{20}(45:50:5), t_{24}(45:50:5)	MS-Triple quadrapole/E SI	**Retention time (min)** BTM 16 DXM 17.5 **Recovery %** BTM 71.4-79.3 DXM 71.3-75.7 **LOQ**: 0.5–2µgkg^{-1} in muscle	34

			$t_{24.}$(65:30:5), F.R: 0.2ml/min			
BTM **DXM**	Cosmetic products	Zorbax Eclipse XDBC18 (50*4.6mm),1.8µ	0.1% formic acid in water (A) ACN (B) Gradient at 0.25 ml/min, B % = 20 initialy	Tandem MS	**Retention time(min)** BTM 11.69, DXM 11.76 **LOD/LOQ ng ml^{-1} :** BTM 1.55/5.17 DXM 2.33/7.78 **Precision RSD%** BTM 1.7-4.8, DXM 2.4-5.7	35
BTM-dipropionate **DXM**	Cream, ointment, suspension. LLE	Symmetry C18 (75×4.6mm), 3.5µm	Water:ACN (50:50) F.R = 1.5 ml/min	UV/DAD 240 nm	**Retention time (min):** BTM dipropionate 6.34 DXM 0.92 **Accuracy:** BTM 97.0% DXM 98.7%	42
BTM **DXM**	Related substance and impurities	ACE 3 C18 (150×4.6mm),3µm	A: H2O/Methan sulfuric acid 0.1% B: Tert.butanol:1,4-dioxane 7:93 %	UV/DAD 254 nm	**Retention time (min):** BTM 21.83, DXM 24.84 **Accuracy** (4mg mL^{-1})	43

		Develosil ODS UG3 (150×4.6mm), 3µm	Gradient:(80:20)»(75: 25)»(48:52)»(80:20)		BTM 98.1-99.9% DXM 103.6-116.3% **LOD**: 0.02% API conc. **LOQ:** 0.05% API conc. level respectively relative to the API analytical concentration (mg mL^{-1}**)**	
BTM **BTM 17-mono-propion-ate (B17P)**	Human Plasma After i.m injection	Hanbon Lichrospher C18 (150×4.6mm), 5 µm	A) Ether: cyclohexane (4:1) B) MeOH :Water (85:15), 0.7ml/min Gradient:	MS Quantum/ ESI	**Retention time (min)** BTM 2.72, B17P 3.30 **Recovery:** BTM 82.7-85.9% B17P 83.685.7 % **LOQ ng mL^{-1}** BTM 0.1 ,B17P 0.05 **Linearty range** BTM 0.1-50 ng mL^{-1} B17P 0.05-5 ng ml^{-1}	44
BTM	**Injectable ampoule:**	ACE 3 C18 (150mm×4.6mm)	a)1,4-dioxane:THF:Buffer	UV/DAD 254 nm	**-Retention time (min):** BSP 17.52, BA 42.56	45

DXM	Betamethasone acetete (BA), Betamethasone soduim Phosphate (BSP)	, 3 µm	pH 3.5 (4:4:92) b) 1,4-dioxane:THF:Buffer pH 3.5 (14:28:58) Gredient B% 0→100 in 60 min F.R 1ml/min		**-Recovery %:** BSP 100 +/- 0.3% BA 99 +/- 0.2% **LOD** BSP 0.1 µg mL^{-1} BA 0.03 µg mL^{-1} **LOQ** BSP 0.04 µg mL^{-1} BA 0.075 µg mL^{-1} **Linearity R^2**: 1.000	
DXM **+** **Related substa-nces**	Dexamethasone coated drug	Zorbax eclips XDP,C8 (250mm×4.6mm) ,5 µm	A) 20mM Ammonium formate pH 3.8:ACN(73:27 v/v), B)ACN Gradient A % 100 for 12 min 100→55 in 28 min, 55→100 in 10 min F.R: 1.5 ml/min	DAD At 239 nm	**Retention time (min):** DXM 12.92 **Recovery:** 89.6-105.8 % **R^2** = 0.999 **LOD:** 0.008 µg ml^{-1} **LOQ:** 0.025 µg ml^{-1}	46

BTM **DXM**	Equine urine Enzymatic Hydrolysis +SPE	UPLC BEH C18 (100×2.1mm), 1.7 µm	0.1% HAc in H_2O:MeOH Gradient: 70:30 for 0.5 min,70:30→20:80 in 8min,→0:100 in 2.4,0.2 to 0.3 ml/min 0:100→70:30 in 0.1 min,eq. for 2.4min	Tandem MS API-ESI	**Retention time (min):** BTM 7.49 DXM 7.50 **LOD(ng mL^{-1}):** 0.3 **Recovery:** BTM 73.2% ,DXM 82%	49
BTM **DXM**	Doping urine - LLE/SPE	Intersil ODS-3 (150mm×3mm), 3 µm Particle size.	1 mM Ammonium Acetate pH 6.8 :ACN , F.R= 0.4 ml/min Gradient 60:40 →0:100 in 2 min for 5 min	MS/single quadrapole with turbo ESI	**-Retention time (min):** BTM 6.4 DXM 6.6 **-LOD** : 1 ng ml^{-1} - **LOQ**: 5 ng ml^{-1}	74

BTM	Urine - Enzymatic hydrolysis	1st: chrompack C18(50mm×4.6mm), 3 µm 2nd : Zorbax TMS (250mm*4.6mm), 5 µm	1st :(50:50) 0.1 M ammonium acetate:MeOH 50:50 2nd:(37:63) ACN: 0.1 M ammonium acetate	Tandem MS With thermal spray ionization(TSP)	**Retention time (min):** At m/z 393 , BTM 6.5 **LOD:** 0.2 ng mL^{-1} **LOQ**: 1 ng mL^{-1}	75
BTM **DXM**	Bovine and Brocaine Urine Enzymatic hydrolysis+ SPE	Zorbax Eclipxs XDB (100mm×2.1mm), 1.8 µm	ACN:0.1% FA Isocritic 15 min,0.22 ml/min	Tandem MS/triple quadrapole MS with -ESI	**Retention time (min):** One peak 7.7-8 min **Recovey** at 2,3 ,4 ng mL^{-1} BTM 82-109 % DXM 76-115 %	76
BTM **DXM**	Human Urine Hydrolyisi +LLE	-Zorbax Eclipse C18(100×2.1mm), 1.8 µm	5mM ammonium formate 0.01% formic acid : ACN (90:10), 0.3ml/min	MS/TOF-ESI	At m/z393 S/N ratio: BTM: 164, 0.2 µg mL^{-1} DXM: 192, 2.6 µg mL^{-1}	77

BTM **DXM**	Urine Enzymatic hydrolysis+ LLE	normal LC: Discovery C18 (50×2.1),5 µm Fast LC: Zorbax (50×2.1), 1.8 µm	H_2O+0.1% HAc:ACN +0.1%HAc Gradient: ACN% 15→60 in 5 min 60→100 in 7 min→100 for 1 min 100→15 in 2 min	Tandem MS/Triple quadrapole MS -ESI	**Retention time (min):** Normal LC: BTM/DXM 9.0-10.4 Fast LC: BTM/DXM 5.2-6.1 **LOD/LOQ** 1ng mL^{-1} **Recovery**: 88%	78
BTM **DXM**	BTM/DXM Active gradient mixture	Symmetry C18 (150×2.1), 5 µm	MeOH:H_2O+0.5% HAc (60:40) isocritic, 0.3 ml/min	Ms/Ion Trap with ±ESI	Ratio of product ions of DXM is 15% higher than that for BTM $R \geq 0.86$	79
BTM **DXM**	Active gradient powder	Symmetry C18 (150×4.6mm),5µ J.shper C18 (150*4.6mm),4µ	ACN:H_2O (35:65) ACN: H_2O(35:65) + Cyclodextrine	UV/DAD	**Retention time (min):** 5.5-6.5 **Rs Resolution < 2**	80

BTM **DXM**	Doping Urine LLE	Neclosil C18 (100×3mm),5µm	1% HAc in H_2O: ACN Gradient: %ACN 25 for 22 min,25→65 in 0.5 65 for 7.5 min,65→25 in 10 min Flow rate 0.3 ml/min	MS/ion trap + ESI	**Relative Retention time.** BTM 0.771,DXM 0.734 **LOD (ng ml^{-1})** BTM 2 DXM 1 **Ion product ratio:** m/z BTM DXM 307 100 66.4 325 33.8 23.4 345 86 100	81
BTM **DXM**	Milk Protien precipitation-LLE	UPLC BEH C18 (100mm×2.1mm, 1.7 µm	(A) 0.1% of formic acid in water (B).0.1% of formic acid in MeCN Gradient (95:5), 0.4 mL 9 min,40 C	MS/TOF-Double ESI	**Retentiontime (min)** BTM 3.55,DXM 3.58 **LOD µg mL^{-1}:** BTM 0.5, DXM 1 **Accuracy % (30 ng mL^{-1})** BTM 64 , DXM 54	82
BTM **DXM**	Liver Enzymatic hydrolysis+	UPLC BEH C18 (100mm×2.1 mm),1.7 µm	A)Water + 0.5%HAc B)ACN+ 0.5% HAc Gradient: 75:25 for 4min,100:0	Tandem MS Tripe quadrapole -ESI	**Retention time(min)** BTM 3.32,DXM 3.50 **LOD** (µg kg^{-1}) BTM 2.35 ,DXM 2.31	83

	C18 SPE		in 0.3min,100:0 for 0.7 min,75:25 in 0.1 min,75:25 for 1.9 min F.R 0.6 ml/min		**LOQ**(µg kg^{-1}**)** BTM 2.63, DXM 2.57	
BTM **DXM**	Bovine Milk Deprotienization and LLE	Symmerty C8 (150*3.9mm),5µm	A: 1.5mM NH_4COONa pH 4.75 B: ACN Gradient, t_0(72:28), 0.6 ml/min	Tandem MS/-ESI	**Retention time(min)** BTM 14.7,DXM 15.8 **LOD/LOQ** (µg kg^{-1}**)** BTM 0.33 ,0.36 DXM 0.34,0.37 **Accuracy(MRL level)** 96.6% ,96.8%	84
BTM **DXM**	Hair -Acid digestion - SPE	Novapack C18 (150*2mm),4 µm	A: ACN B: 2mM NH4COOH,pH=3 Gradient ACN% (30→70) F.R = 0.2 ml/min	MS/ion spray	**Retention time (min):** BTM 4.8 DXM 5.3 **Recovery: 85.7%** **LOD** : 0.05 µg mg^{-1}	85

BTM + DXM	Milk LLE+ gel permeation chromatography	ZORBAX SB-C18 (150 ×2.1 mm),3.5 µm	acetonitrile (A) and 0.1% formic acid (B) gradient at 0.3 ml/min	Tandem MS	**Retention time(min)** BTM 10.55 DXM 10.89 **LOD/LOQ µg/kg:** BTM 0.1/0.4 DXM 0.05/0.2 **Linarity R^2** BTM 0.9995 DXM 0.999	86
BTM DXM	Bovine Milk SPE	Hypercarb (30×2.1 mm),5 µm	ACN:Water: Formic acid (95:5:0.5) at 0.25 ml/min	MS/MS +APCI	**Retention time(min)** BTM 2.03,DXM 2.49 **Recovery** % BTM 89.6-93.6 DXM 96.8-103.4 **Assay CV%** BTM 4.43 DXM 3.21	87
BTM DXM	Bovine tissue Hydrolysis,SL	Kinetex pheny-hexyl (100×4.6	A: 5mM ammonium acetate with 0.1%	Tandem MS	**Linearity R^2** BTM 0.9982 -,0.9995 **Recovery:**	88

	E and SPE	mm),2.6µm	HAc Ph= 5.4 B: MeOH Gradient,0.8ml/min, 24 min run time		BTM 102-119**%** DXM 93-96**%** **Reproducibility(RSD):** BTM 2.5-16.7 % DXM 6.7-12.5 %	
BTM **DXM**	Human Urine LLE	Restek Ultra C18 (100×2.mm),5 µm	0.05% formic acid+ 20mM ammonium acetate pH= 3 (A) ACN (B)	Ion trap + ESI	**Retention time(min)** BTM 14.7 ,DXM 15.0 **LOD/LOQ ng/mL :** BTM/DXM 3/5 **Linear range ng/mL** 5 – 80	89
DXM	Postmortem tissues LLE+SPE	UPLC BEH C_{18} (50×2.1mm), 1.7 µm	**A:**MeOH:Water: Formic acid (10: 89.9: 0.1) **B**:MeOH:FA (99.9: 0.1) Gradient (50:50) at 0.5ml/min Run time : 8 min	Tandem MS	Mass spectrum library identification	90

BTM **DXM**	Tablet,plasma, serum and urine	SYNERGI MAX-RP, 4 µm (50*4.6 mm)	A: ACN B: 0.1 Mm ammonium acetate Gradient 0.75 mL/min, 14 min	Tandem MS +ESI	**Retention time(min)** BTM 5.56,DXM 5.92 **LOD (S/N= 4-4.3**): 0.3-0.7 µg mL^{-1} (in serum and urine) **Recovery :** 75% of the synethetic steroids in suspected drugs.	91

3. MATERIALS AND METHODS

3.1. Chemicals and Reagents

• Glacial acetic acid	Merck
• Methanol (HPLC grade)	Sigma-Aldrich
• Acetonitirile (HPLC grade)	Merck
• Betamethasone	Sigma-Aldrich
• Dexamethasone	Sigma-Aldrich
• Mefruside	Donated by German Doping Control Centre

3.2. Instruments and Apparatus

• UPLC/Tandem MS	
▪ Ultra Pressure Pump	Waters® ACQUITY
▪ Auto-Sampler	Waters® ACQUITY
▪ Column Oven	Waters® ACQUITY
▪ Triple Quadruple / ESI ion source Mass Spectrometer Detector	Waters® ACQUITY
• Automatic micropipette	Ependrof (10 – 100 µl and 100 -1000 µl)
• Injector	Hamilton (1 – 10 µl)
• pH meter	Mettler Toledo
• Balance	Mettler Toledo
• Ultrasonic bath	Bandelin, Sonorex ,RK 154 BH
• Centrifuge	Hettich EBA 20
• Vortex-mixer	IDL RS2, Heidolph
• Milli-Q water system	Branstead NanoPure Diamond
• Refrigerator	Bosch
• Deep-freezer	Bosch

3.3. Glassware and Materials

- Volumetric Flask — 5-500 mL, Pyrex®
- Beakers — 50-100 mL, Pyrex®
- Mobile Phase Bottle — 2 L, Borosilicate glass
- Tubes — 10 ml, screw-capped borosilicate
- Vials — 2 mL, Glass

3.4. UPLC Columns

ACQUITY BEH C18 (50 mm × 2.1 mm i.d), 1.7µm Particle size, WATERS®

ACQUITY BEH C18 (150mm ×2.1 mm i.d), 1.7µm Particle size, WATERS®

3.5. Pharmaceutical Preparations

Table 3.1. List of pharmaceutical preparations that contain BTM or DXM and/or their ester derivatives.

Drug name	Active ingredients	Supplier	Market
Celestone® tablet	BTM 0.5 mg / Tablet	Schering-Plough	Jordan
Celestone®, Chronodose® 1 mL injectable ampoule (BTM sodium phosphate and BTM acetate containing suspension in water.)	3.947 mg BTM disodium phosphate equivalent to 3 mg BTM and 3 mg BTM acetate / 1 mL	Schering-Plough	Turkey
DEKORT® tablet	0.5 mg DXM/tablet	Deva Holding A.Ş.	Turkey
DEKORT® 2 mL injectable ampoule	DXM 21-phosphate equivalent to 8 mg DXM 21- phosphate disodium / ampoule	Deva Holding A.Ş.	Turkey
ONADRON® SIMPLE 5 mL eye/ear drop	DXM 21-phosphate equivalent to 5 mg DXM 21-phosphate disodium / drop	I.E. Ulagay A.Ş.	Turkey

3.6. Stock and Working Solutions

3.6.1. Mobile Phase Contents

Mobile phase was containing acetonitrile (ACN) and 0.1 % (v/v) acetic acid (HAc) in water. For the preparation of 0.1 %(v/v) HAc, 100 µL of glacial HAc is added in 100 mL volumetric flask and diluted to the volume with HPLC grade water.

3.6.2. Preparation of Stock and Working Standard Solutions

- *BTM stock solution (1000 µg mL^{-1})*

A 10 mg of BTM standard powder was transferred to 10 mL volumetric flask, methanol about 5 mL added and flask well shaked until all of powder was dissolved then the volume was completed to 10 mL with methanol.

- *BTM working standard solution (1000 ng mL^{-1})*

A 100 µL of stock solution was put into a 100 mL volumetric flask and filled up to the volume with methanol.

- *DXM stock solution (1000 µg mL^{-1})*

A 10 mg of DXM standard powder was transferred to 10 ml volumetric flask, methanol about 5mL added and flask well shaked until all of powder is dissolved then the volume was completed to 10 mL with methanol.

- *DXM working solution (1000 ng mL^{-1})*

100 µL of stock solution was put into a 100 mL volumetric flask and filled up to the volume with methanol.

- *Mefruside (Internal Standard, 20* µg mL^{-1}*)*

An amount of10 mg of Mefruside standard powder was transferred to 100 mL volumetric flask, methanol about 50 mL added and flask well shaked until all of powder is dissolved then the volume was completed 100 mL with methanol (100µg mL^{-1}).A 20 mL from solution of 100µg mL^{-1}Mefruside was transferred to 100 mL volumetric flask and completed to the volume with methanol. The final concentration will be 20µg mL^{-1} Mefruside (IS) standard solution.

3.6.3. Synthetic Preparation and Placebo Solutions

*BTM Synthetic Preparation*s: 0.5 mg of BTM standard, 20 mg starch, 150 mg lactose, 9 mg magnesium citrate and 10 mg of gelatin, which were equivalent to the amounts in one commercial tablet, were weighted then put into a 100 mL volumetric flask. Then 50mL volume of methanol was added and the flask was stated in an ultrasonic bath for maximum solubility (30-60 min). The flask was left until to be cooled and then volume was completed to 100 mL with methanol.

BTM Tablet Placebo Solution: The same procedure in synthetic preparation without adding 0.5 mg of BTM standard was carried out.

*DXM Synthetic Preparation***:** 0.5 mg of DXM standard, 20 mg starch, 150 mg lactose, 20 mg magnesium citrate and traces (5mg) of yellow dye that were equivalent to the amounts in one commercial tablet were weighted and put into a100 mL volumetric flask. Then some volume of methanol was added to and the flask was left in an ultrasonic bath for maximum dissolution (30-60 min). The flask was waited until to be cooled and then the volume was completed to 100 mL with methanol.

DXM Tablet Placebo Solution: The same procedure for synthetic preparation was performed without adding 0.5 mg of DXM standard powder.

3.6.4. Solutions of Commercial Pharmaceutical Preparation

Celestone Tablet: 10 tablets were weighted, grounded by a mortar until to be homogeneous soft powder. The amount equal to one tablet (199.2 mg) was weighted and transferred to a 100 mL volumetric flask, filled partially with methanol and well mixed on a vortex-mixer. Then the flask was left in the ultrasonic bath until maximum dissolution (30 - 60 min) and waited for cooling then completed to the flask volume with methanol. A 100 µL of prepared solution was transferd to 1 mL vial complete volume with methanol then spiked with 25 µL of µg IS (500 ng mL^{-1} BTM).

*Dekort Tablet***:** 10 tablets were weighted, grounded by a mortar until to be homogeneous soft powder. The amount equal to one tablet (198.8 mg) was weighted and transferred to a 100 mL volumetric flask, filled partially with methanol and well

mixed on a vortex-mixer. Then the flask was left in the ultrasonic bath until maximum salvation (30 - 60 min) and waited for cooling then completed to the flask volume with methanol. A 100 µL of prepared solution was transferred to 1 mL vial complete volume with methanol then 25 µL of µg IS was added (500 ng mL^{-1} DXM).

Celestone Chronodose Ampoule: Celestone ampoule contains 3.947 mg Betamethasone sodium phosophate equivalent to 3 mg Betamethasone and 3 mg Betamethasone acetate in suspension. The content of an ampoule (1 mL) was transferred into a tube and centrifuged at 3500 rpm. The aqueous layer that containing Betamethasone sodium phosophate was taken into a 100mL flask and the volume is completed to 100 mL with methanol, then well mixed on a vortex-mixer.A 25 µL of this solution was transferred to a 1 mL vial and the was completed to 1 mL with methanol then spiked with 25 µL of µg IS (986.75ng mL^{-1}BTM sodium phosphate equivalent to 750 ng mL^{-1} BTM)

Dekort I.V/I.M Injectable Ampoule: The content of an ampoule (2 mL) was transferred into 100 mL flask volume was completed to 100 mL by gradually adding methanol under gentle shaking. A 10 µL of this solution was transferred to 1 mL vial, the volume was completed to 100 mL with methanol then 25 µL of µg IS was added (800 ng mL^{-1}DXM sodium phosphate equivalent to 608 ng mL^{-1}DXM).

Onadron eye/ear drop: The content of one drop (5 mL) was transferred into a 100 mL volumetric flask and 50 mL methanol was added then was well mixed. The volume was completed to 100 mL with methanol.A 20 µL of this solution was transferred to a 1 mL vial then the volume was complete to 1 mL with methanol then 25 µL of µg IS was added (1000 ng mL^{-1} DXM sodium phosphate equivalent to 760 ng mL^{-1} DXM).

3.7. Method Development and Optimization

3.7.1. Method Optimization:

Tested parameters in the optimization study were given below:

- Different mobile phases organic modifier types and ratios (15, 40, 50 and 80%) in the mobile phase,
- Different mobile phase flow rates (0.1, 0.2, 0.3 and 0.4 $mLmin^{-1}$)
- Different analytical column lengths (50 and 150 mm)
- Different gradient profiles (A, B, C, D and E).

3.7.2. UPLC /Tandem MS parameters

UPLC/Tandem MS parameters decided after method optimization were;

- **Column technology** : UPLC AcQueity BEH C_{18} , 1.7µm particle size
- **Column dimensions** : 50 mm x 2.1 mm internal diameter (i.d.)
- **Injector volume** : 10 µL
- **Column oven temperature** : 40 ^{o}C
- **Mobile phase components** : A: 0.1% (v/v) HAc in H_2O, B: ACN
- **Flow rate** : 0.3 mL min^{-1}
- **Gradient elution program** :

Time (Min)	A%	B%
0.0	85	15
0.4	85	15
8.0	15	85
8.5	15	85
9.2	85	15
10	85	15

- **Run or analysis time** : 10 min
- **Carrier gas** : Argon
- **Ion selection mode** : Multi Reaction Monitoring (MRM)
- **Ionization mode** : Electrospray ionization source (ESI) in negative mode.

- **Collusion energy:**

BTM or DXM	m/z 451>451	8.0 eV
BTM or DXM	m/z 451>391	8.0 eV
BTM or DXM	m/z 451>361	8.0 eV
Mefruside(IS)	m/z 381>381	25 eV
Mefruside(IS)	m/z 381>345	25 eV
Mefruside(IS)	m/z 381>189	25 eV

- **Selected ions m/z transitions** : For BTM or DXM (451> 361 m/z),
  : For Mefruside (381>189 m/z)

The m/z transitions were selected by increasing collusion energy in the 2^{nd} MS then select the ions of highest signals.

3.8. Method Validation Procedures

The analytical methods that were developed for analysis of BTM and DXM in pharmaceutical preparations were validated according to ICH Analytical method validation guideline (92). The applicability of the methods presented in this study for the analysis pharmaceutical preparations of DXM or BTM were also demonstrated. The MRM ion pairs of 451>361 for BTM or DXM and 381>189 for Mefruside (IS) which were having highest signal were monitored in validation studies.

3.8.1. Specificity

Specificity of the method is the ability to measure accurately the concentration of an analyte in the presence of all other sample. In other words, specific method refers to a method that produces a response for a single analyte only. Testing specificities of the methods were tested by comparison of the peaks of placebo, synthetic preparations, BTM standard, DXM standard, Mefruside (IS) and pharmaceutical preparations.

3.8.2. Range and Linearity

The ranges for developed methods were determined as the interval between the upper and lower concentrations of BTM or DXM. Although a minimum of 5 concentrations is recommended for the establishment of linearity in ICH guideline, seven different concentrations of working standards (10, 50, 100, 250, 500, 1000 and

1500 ng mL^{-1}) of BTM or DXM were prepared from the stock solutions, and 1 mL was transferred into the vial and then spiked with 25 µL of 20 µg mL^{-1} Mefruside (IS). The peak area ratio of BTM or DXM to the peak area of IS was plotted against to the concentrations of BTM or DXM to construct the calibration curves. Linearity of the method with analyte concentration evaluated statistically by using one-way ANOVA analysis of variations in Microsoft Excel 2007 (v.12.0.4518.1014).

3.8.3. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value that is accepted either as a conventional true value or as an accepted reference value and the found value. In this thesis, accuracy was represented as intra-day accuracy, inter-day accuracy and recovery for DXM and BTM.

Working standards at 3 concentration levels for DXM or BTM within linearity range (50, 500 and 1000 ng mL^{-1}, n = 6 for each concentration level) were prepared from stock solutions and 1 mL of the standards was transferred into a vial and then spiked with 25 µL of 20 µg mL^{-1} IS. Then the standards were analyzed in the same day to determine intra-day accuracy and in 5 days for inter-day accuracy. The indicator for accuracy was percentage of the relative error of BTM or DXM (Appendix 1).

The absolute recovery of an analyte is comparison for the detector response obtained from an amount of the analyte added to and extracted from the matrix. BTM or DXM standard solutions were added to the tablet placebo solution at a final concentration of 1000 ngmL^{-1} in 100 mL volumetric flask. Then 1 mL of this solution was transferred into a vial, spiked with 25 µL of 20 µg mL^{-1} IS and analyzed. The absolute recovery was calculated as given in Appendix 1.

3.8.4. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision of an analytical procedure is usually expressed as the variance, standard

deviation or coefficient of variation of a series of measurements. In this thesis, the precision was presented as intra-day precision, inter-day precision and repeatability.

For DXM or BTM, working standards at 3 concentration levels within linearity range (50, 500 and 1000 ng mL^{-1}, n=6 for each concentration level) were prepared from stock standard solutions, 1 mL of standard was transferred into a vial then spiked with 25 µL of 20µg mL^{-1} IS. Then the standards were analyzed in the same day to determine intra-day precision and in 5 days for inter-day precision. Precision was given as relative standard deviation values at each concentration levels for DXM or BTM (Appendix 1).

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Instrument or injection repeatability testing were worked out by preparing 10 working standard solutions at 150 ng mL^{-1} of each BTM and DXM containing I.S then injected at short level of time (e.g. same batch).

3.8.5. Limit of Detection (LOD)

The smallest amount of an analyte that can be detected by a particular method is named as limit of detection. According to ICH analytic method validation guideline, LOD had been found depending on the signal-to-noise ratio determination. The signal-to-noise ratio values for DXM or BTM were performed by comparing measured signals obtained from their standards with the baseline signal level. A signal-to-noise ratio at about 3 was considered for estimating detection limits for DXM and BTM.

3.8.6. Limit of Quantification (LOQ)

LOQ describes the smallest amount of analyte that can be quantified reliably. Signal-to-noise ratio at 10:1 was considered to determine the LOQ levels of DXM or BTM (92).

3.8.7. Ruggedness

Ruggedness is the degree of reproducibility of the results obtained under a variety of conditions, which include different laboratories, analysts, instruments, reagent or days. To determine the ruggedness for DXM or BTM two sets of working standards at 100 ngmL^{-1} (n=6) were prepared and of 1 mL solution was transferred

into a vial then 25 µL of 20 µg mL^{-1} IS was added. Each set of DXM or BTM standards were analyzed by two different analysts, and differentiation in the results were statistically evaluated using F-test for two sample variation in Microsoft excel 2007 (v. 12.0.4518.1014).

3.8.8. Robustness

Robustness is evaluated in order to show the reliability of an analysis with respect to deliberate variations in method parameters. Changes in the values of ACN percentage by ±1 ACN (14 and 16 ACN %) and in flow rate by ± 10% (0.027 and 0.033 mL min^{-1} were tested. Statistical evaluation of differences was performed by t-test (Appendix 2).

3.8.9. Stability

Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term and short-term. The stability was tested using BTM or DXM standards at a concentration of 100 ng mL^{-1} (n=6) under the following conditions:

1. Short-term stability: 24 hours waiting in sunlight, darkness at room temperature and in refrigerator at + 4°C.
2. Long-term stability: 6 months waiting in refrigerator at + 4°C.

3.8.10. System Suitability

System suitability tests are most often applied to analytical instrumentation. They are designed to evaluate the components of the analytical system in order to show that the performance of the system meets the standards required by the method (9).

For this purpose, working solutions of BTM or DXM at 150 ng mL^{-1} was used. The parameters and acceptance criteria stated in European and US Pharmacopeia and ICH guidelines were applied (9, 10, and 92).

4. FINDINGS

4.1. Optimization of Chromatographic Conditions

The effects of different chromatographic parameters in UPLC leading to the best chromatographic assays of DXM or BTM in their pharmaceutical preparations were investigated in this study. Thus, content and combination of mobile phase, column length, flow rate of mobile phase and gradient profile properties in UPLC system were considered in optimization study.

4.1.1. Effect of Mobile Phase Organic Modifier

In initial ACN effect studies, percentage of ACN in mobile phase in the range from 0 to 0.4 minutes was adjusted to 15, 40, 50 and 80 % (v/v), respectively. In each trial, ACN % was 85at 8.2 min and consequent steps were not changed which were given in Section 3.7.2. Then chromatographic parameters of BTM or DXM peaks were calculated at all tested %ACN (Table 4.1).

Table 4.1. Effect of mobile phase initial ACN content on the chromatographic parameters

	BTM			**DXM**		
ACN (%)	**t_R (min)**	**PA ratio**	**N**	**t_R (min)**	**PA ratio**	**N**
15	3.34	1.00	27889	3.37	1.00	28392
40	0.93	1.25	819	0.95	1.25	854
50	0.66	1.50	860	0.66	1.50	860
80	No peak					

t_R: Retention time, PA: Peak asymmetry: Number of theoretical plates.

Using gradient system, the capacity factor values for BTM or DXM peaks will decrease or increase according to the change percentage of ACN during gradient profile. In addition, the t_0 value is not fix in the gradient system. Thus, capacity factor was not calculated as chromatographic parameters while using the gradient elution system. In other hand, numbers of theoretical plates N for DXM or BTM were decreased by increasing % ACN (Figure 4.1).

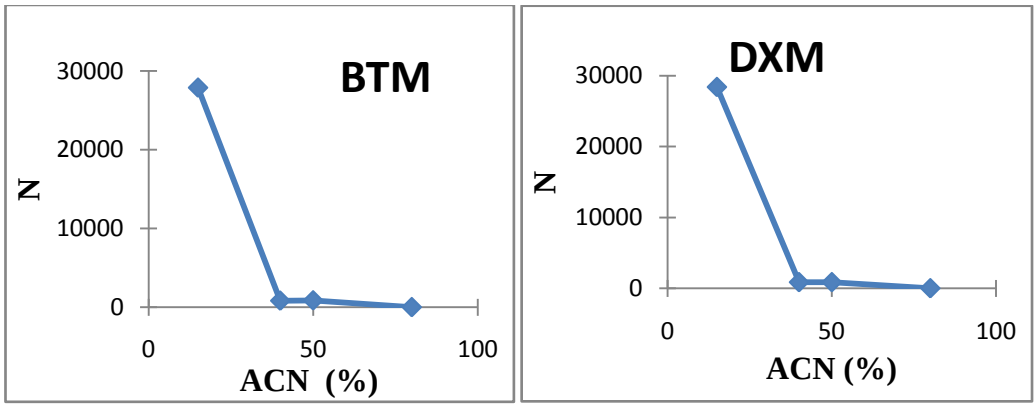


Figure 4.1. Effect of initial ACN percentage of mobile phase on N values for BTM or DXM

Methanol was also tested as organic modifier under the same chromatographic conditions with ACN in UPLC, but no considerable peaks for DXM or BTM was detected.

4.1.2. Effect of Gradient Profile

Gradient profile slope at the first ramp was changed as 30.43, 46.67, 26.92, 19.44 and 9.21 % ACN min^{-1} in profiles A, B, C, D and E (Figure 4.2, Table 4.2), other parameters were adjusted as described in Section 3.7.2. Correspondingly, total analysis time was altered as 3.5, 4.5, 6, 8 and 10 min respectively (Figure 4.2). Then chromatographic parameters of BTM or DXM peaks were calculated for all gradient profiles tested (Table 4.3).

Table 4.2. Properties of Applied Gradient Profiles

Gradient Profile Code	**Profile properties**						
A	**Time(min)**	0	0.4	2.5	2.7	3	3.5
	ACN%	15	15	85	85	15	15
B	**Time(min)**	0	0.4	2	3.1	4	4.5
	ACN%	15	15	85	85	15	15
C	**Time(min)**	0	0.4	3	4.2	5.5	6
	ACN%	15	15	85	85	15	15
D	**Time(min)**	0	0.4	4	5.2	6.5	8
	ACN%	15	15	85	85	15	15
E	**Time(min)**	0	0.4	8	8.5	9.2	10
	ACN%	15	15	85	85	15	15

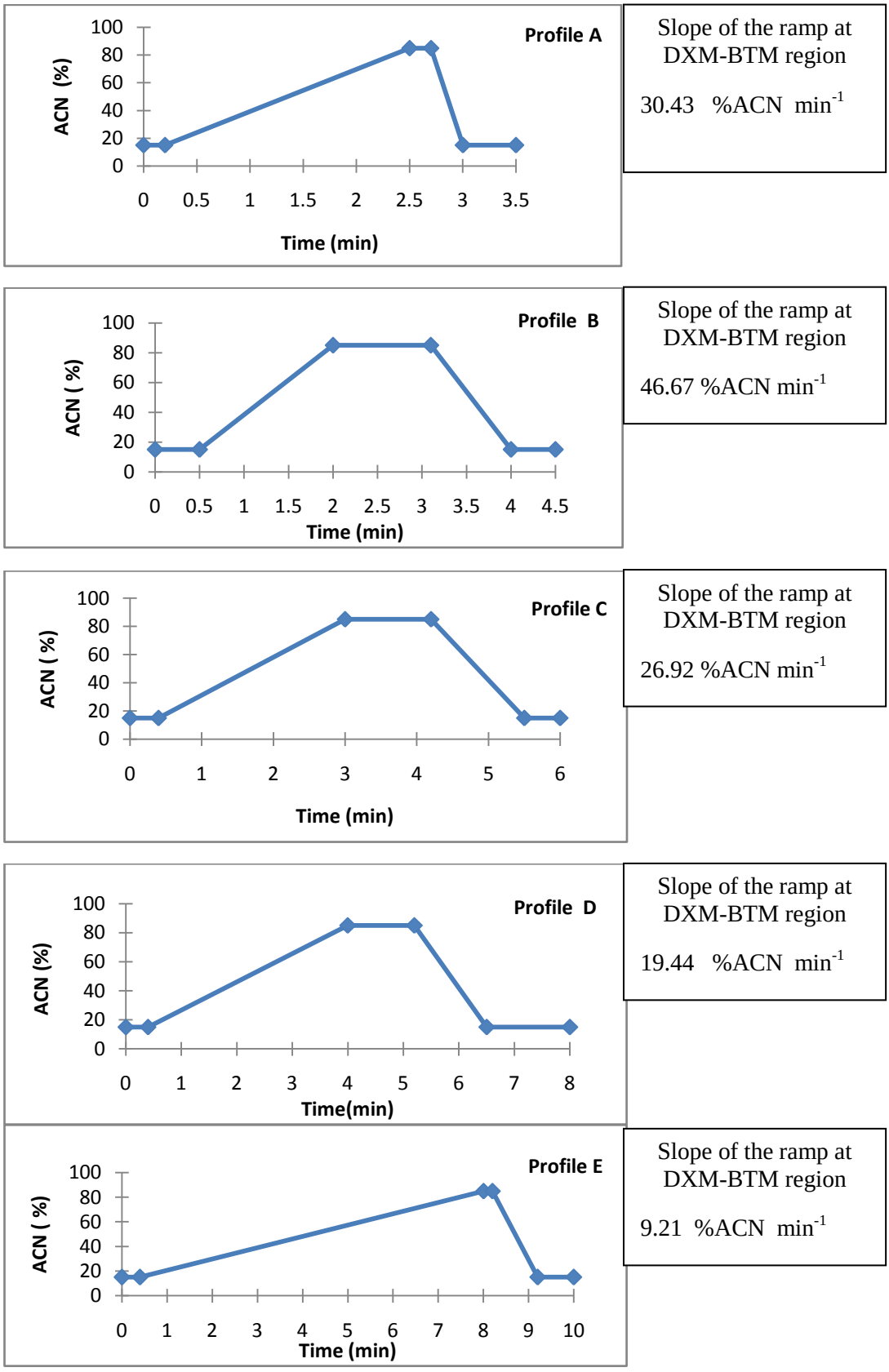


Figure 4.2. Gradient profile types applied for DXM or BTM elution

Table 4.3. Effect of gradient profile on chromatographic parameters for DXM and BTM

	BTM			**DXM**		
Gradient Profile	**t_R (min)**	**PA ratio**	**N**	**t_R (min)**	**PA ratio**	**N**
A	1.87	1.00	6981	1.88	1.00	7056
B	2.04	1.00	8220	2.04	1.00	8220
C	2.19	0.88	11990	2.19	0.88	11990
D	2.99	1.00	9933	3.01	1.00	10138
E	3.37	1.00	10752	3.40	1.00	10944

t_R: Retention time, PA: Peak asymmetry: Number of theoretical plates.

4.1.3. Effect of Mobile Phase Flow Rate

The effect of mobile phase flow rate on the analysis performances for DXM or BTM in UPLC were tested at 0.1, 0.2, 0.3 and 0.4 mL min^{-1}, respectively. Other parameters were adjusted as described in Section 3.7.2. Then chromatographic parameters of BTM or DXM peaks were calculated for all flow rates tested (Table 4.4**).**

Table 4.4. Effect of flow rate on chromatographic parameters for DXM and BTM

	BTM			DXM		
Flow Rate (mL min^{-1})	**t_R (min)**	**PA ratio**	**N**	**t_R (min)**	**PA ratio**	**N**
0.1	8.44	1.00	28493	8.46	1.00	28628
0.2	4.05	0.80	11664	4.07	0.80	11779
0.3	3.37	1.00	10752	3.40	1.00	10944
0.4	2.98	1.00	9867	3.02	1.00	10133

t_R: Retention time, PA: Peak asymmetry, N: Number of theoretical plates.

Number of theoretical plates (N) for BTM and DXM peaks was decreased by increasing of mobile phase flow rate (Figure 4.3).

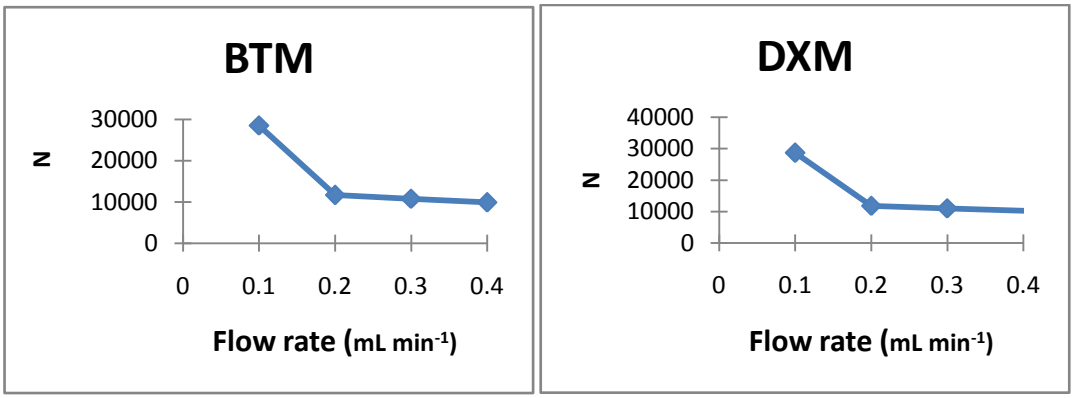


Figure 4.3. Effect of flow rate on N values of BTM or DXM

4.1.4. Effect of Column Length

Two UPLC columns containing C_{18} functional group were tested; both were in the same internal diameter (2.1 mm i.d) and filled with packing material in same particle size (1.7µm) but in different length as 50 mm and 150 mm. Other parameters were adjusted as described in Section 3.7.2 and then chromatographic parameters of BTM or DXM peaks using both columns were calculated (Table 4.5).

Table 4.5. Effect of column length

	BTM			**DXM**		
Column Length(mm)	**t_R (min)**	**PA ratio**	**N**	**t_R (min)**	**PA ratio**	**N**
50	3.34	1.00	27889	3.37	1.00	28392
150	5.05	0.80	40804	5.09	0.70	41452

After optimization of chromatographic conditions, UPLC Acquity BEH C_{18} , 1.7µm particle size (50 mm x 2.1 mm i.d.) was eluted by a mobile phase containing 0.1% (v/v) HAc in H_2O (A) and ACN (B) under gradient profile E (Table 4.2) at a flow rate of 0.3 mL min^{-1}.for analysis of BTM or DXM.

4.2. Method Validation

4.2.1. Specify

In order to qualitative and quantitative determination of BTM or DXM, selection of specific ions or fragments resulted by Tandem MS using negative ESI was performed. In negative ion mode the acetate adduct ([M- H + CH_3CO_2H]-, m/z 451) is fragmented, resulting in only two product ions; [M-H] and [M-H-CH_2O]$^-$ (391, 361) for DXM and BTM, respectively (93, 94).Mass spectrum for DXM and BTM by Tandem MS using negative ESI in scan mode were given in Figure 4.4 and 4.5.

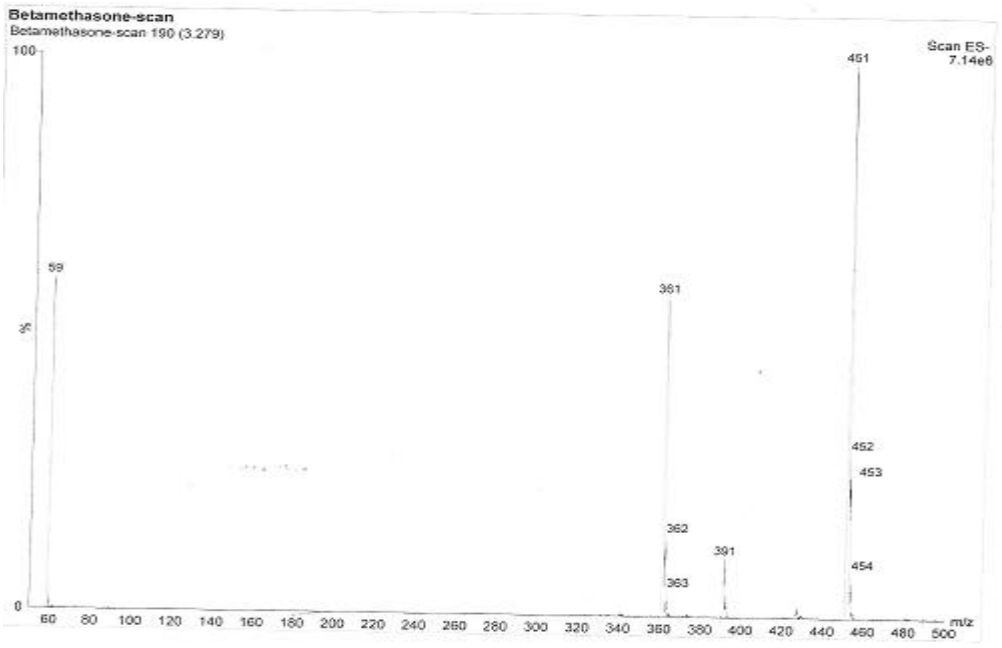


Figure 4.4. Mass spectrum of BTM

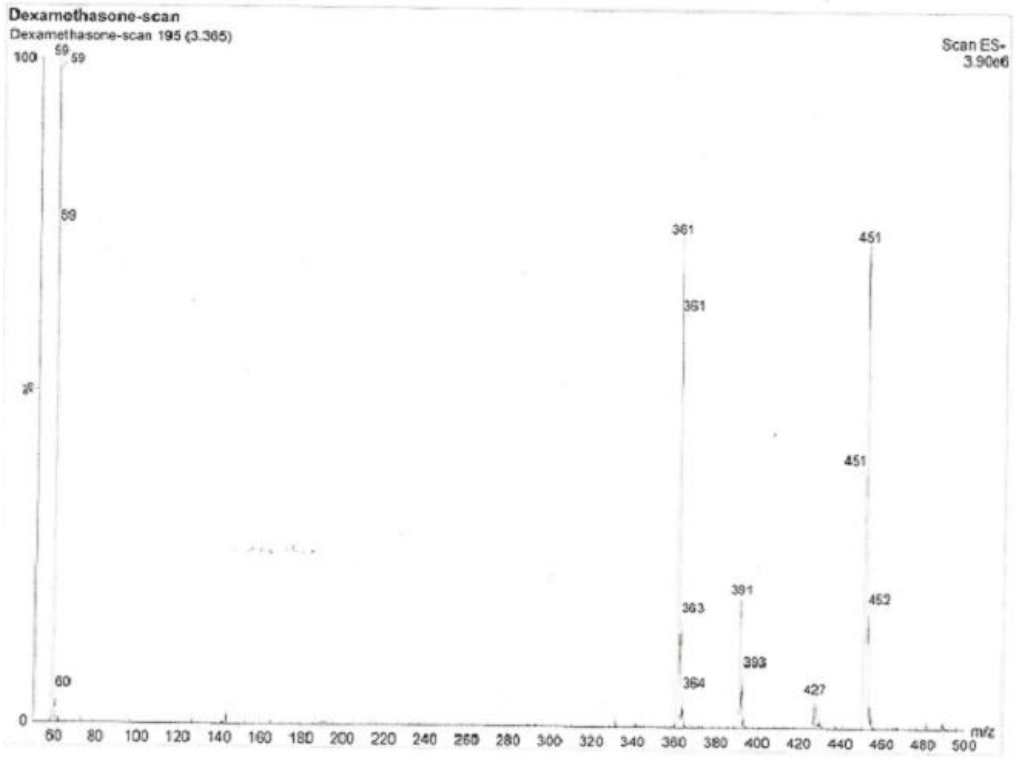


Figure 4.5. Mass spectrum of DXM

Major ions for DXM and BTM were 451, 391 and 361, in which the m/z 451 was pseudo fragment ion (Table 4.6), consequently using Multi Reaction Monitoring (MRM) mode, major diagnostic transitions were 451>361 and 451>391 for BTM and DXM. In this work, is the higher one, then 361 and 391 respectively, thus transition was used in quantitation for DXM and BTM.

Table 4.6. Fragment ions of BTM and DXM.

Type of ion	**Fragment ion**		***m/z*** **of Ion**
Acetate adduct	$[M- H + CH_3CO_2H]^-$	$[M - H + 60]^-$	451
Product ion	$[M-H]^-$	$[M-1]^-$	391
Product ion	$[M –H-CH2O]^-$	$[M -1-30]^-$	361

To demonstrate specify of developed methods for analysis of DXM or BTM, the chromatograms of standard solution of BTM or DXM monitored under optimized conditions, and were compared with the chromatograms obtained from placebo and synthetic preparations (Figure 4.4 and 4.5). BTM or DXM peaks were found to be well resolved from the baseline and the peaks of BTM or DXM were free of any interference from matrix or synthetic sample or pharmaceutical components (Figure 4.6 and Figure 4.7), thus developed methods were specific.

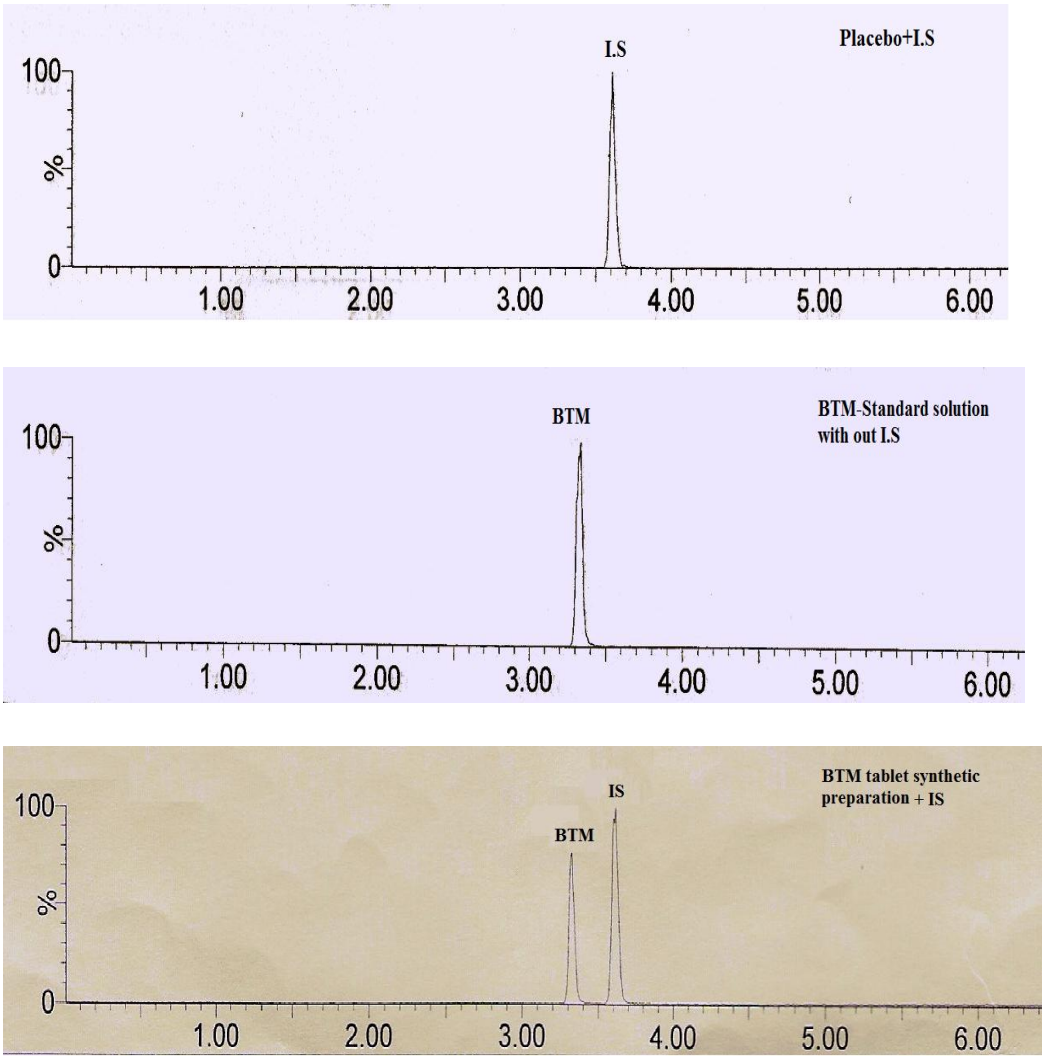


Figure 4.6. Representative chromatograms for BTM (100 ng mL^{-1}), [IS (25µL 20µg mL^{-1}].

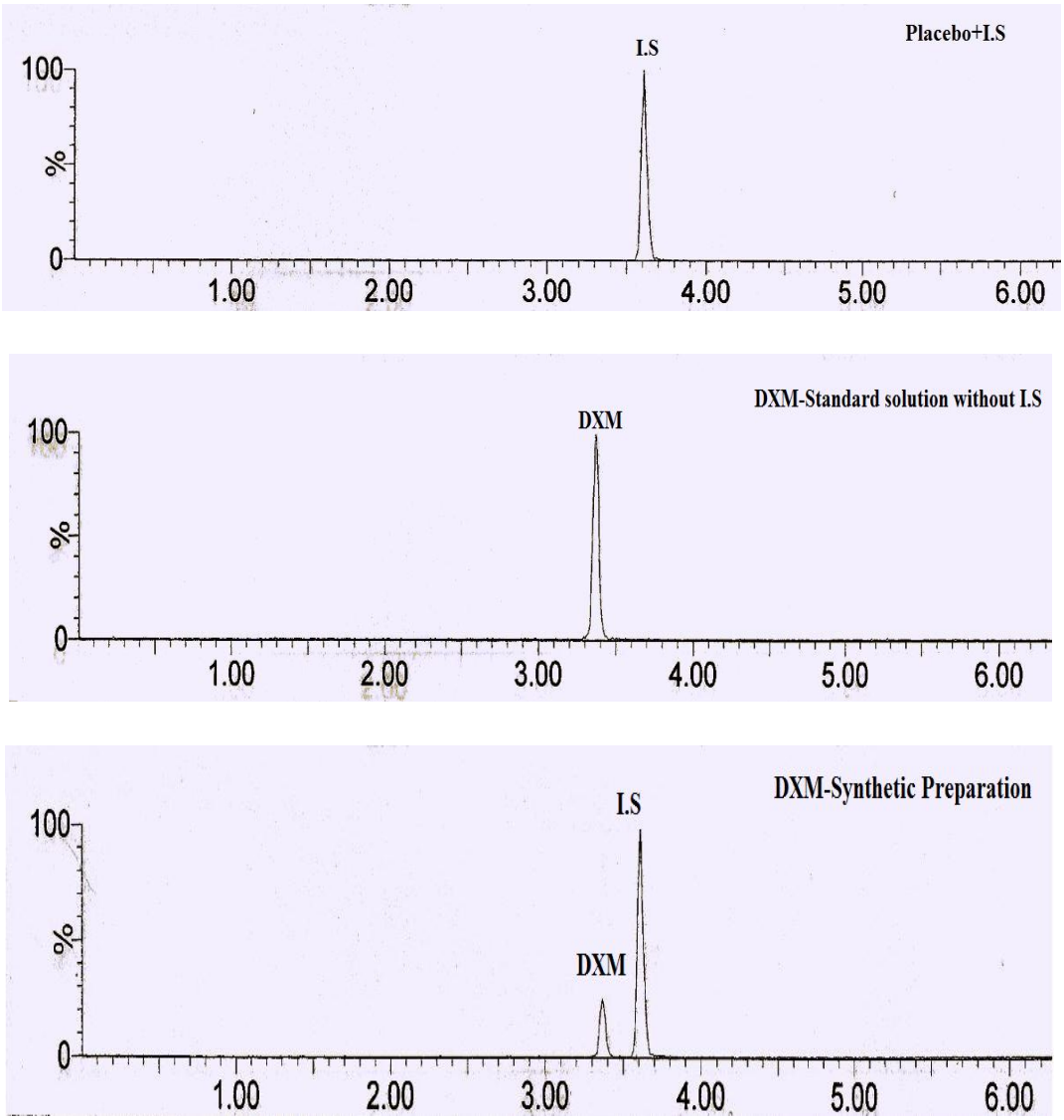


Figure 4.7. Representative chromatograms for DXM (100 ng mL^{-1}), [IS (25µL 20µg mL^{-1}].

4.2.2. Sensitivity

Sensitivity of the method was evaluated by determination of LOD and LOQ of BTM and DXM using signal-to-noise (S/N) ratio method. LOD was the concentration of analyte at which its peak gives S/N ≥ 3 while LOQ was the concentration of the analyte, which its peak gives S/N ≥ 10 (Table 4.7).

Table 4.7. LOD and LOQ values for BTM and DXM**.**

Sensitivity	BTM	DXM
LOD	1 ng mL^{-1}	1 ng mL^{-1}
LOQ	5 ng mL^{-1}	5 ng mL^{-1}

4.2.3. Range and linearity

Calibration curves for calculating the amount of BTM and DXM in pharmaceutical preparation were constructed separately at optimized linear dynamic concentration range. Seven-point calibration curves were obtained in concentration range from 10 to 1500 ng mL^{-1} (10, 50, 100, 250, 500, 1000, and 1500 ng mL^{-1}) for BTM or DXM standards in methanol spiked with I.S. Six independent determinations were performed at each concentration.

The ratio of peak area (BTM or DXM peaks area / I.S. peak area) as response were plotted versus the concentrations (ng mL^{-1}) for each analyte (Figures 4.8 and 4.9) to construct calibration curve. DXM and BTM calibration curves were linear over the concentration range from 10 to 1500 ng mL^{-1} (Table 4.8).

In order to control the linearity of the calibration curve, the expected values of BTM or DXM concentration were plotted against the values that calculated from their calibration curves (Figures 4.10 and 4.11).

Statistical analysis parameters were calculated using One-Way ANOVA analysis of variance to evaluate the linearity of the method in tested concentration range (Table 4.9).

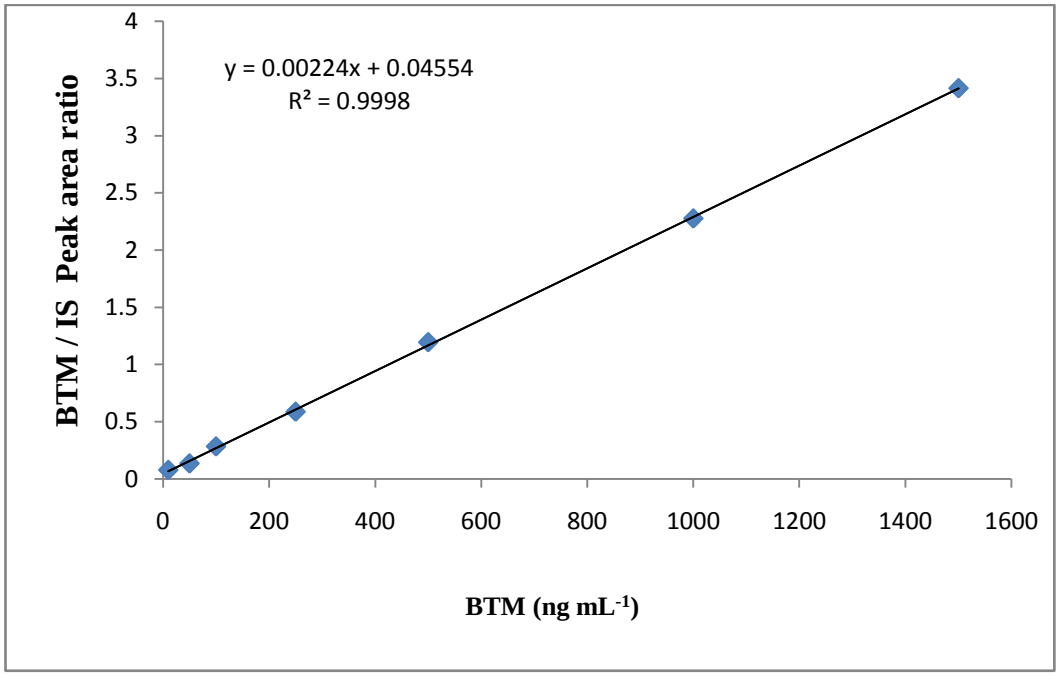


Figure 4.8. Calibration curve of BTM

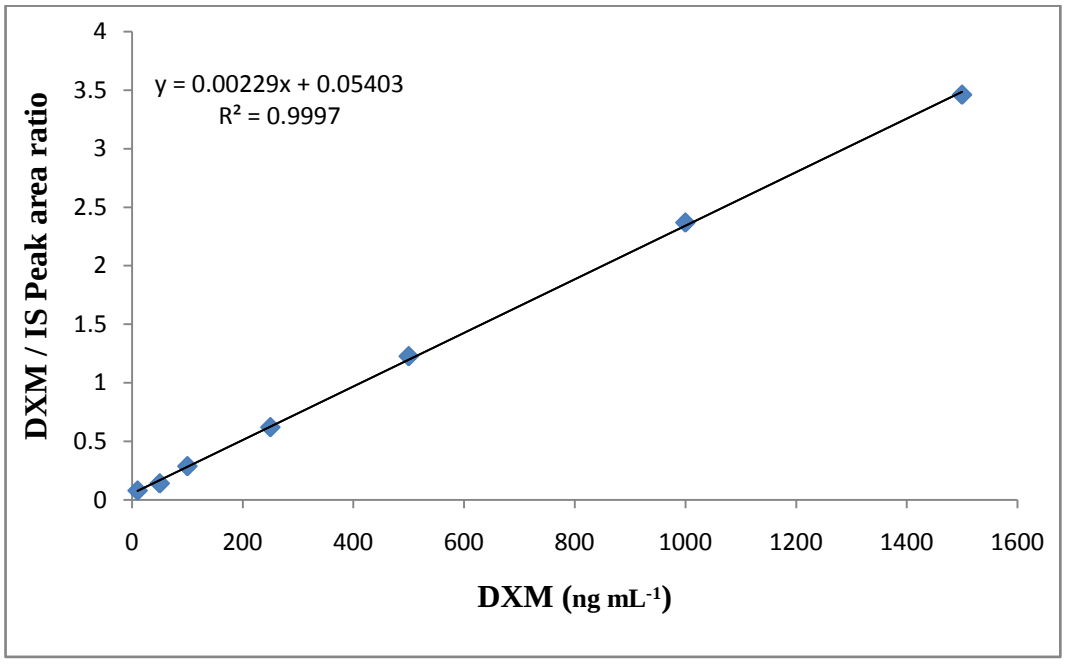


Figure 4.9. Calibration curve of DXM

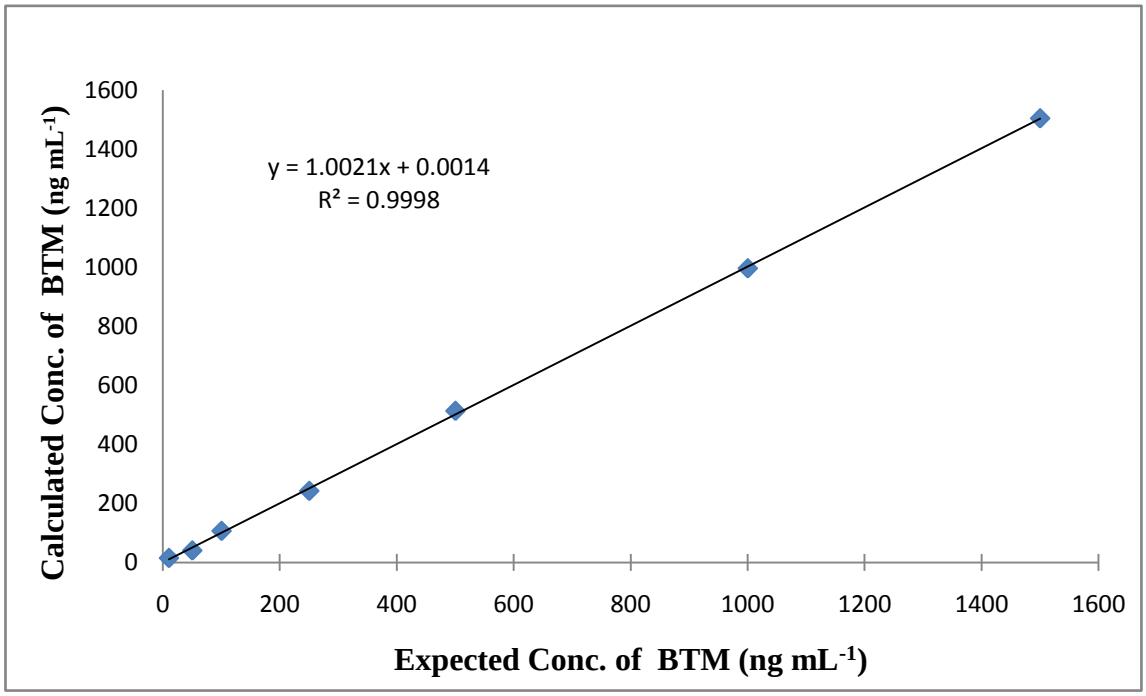


Figure 4.10. Method Linearity Control for BTM

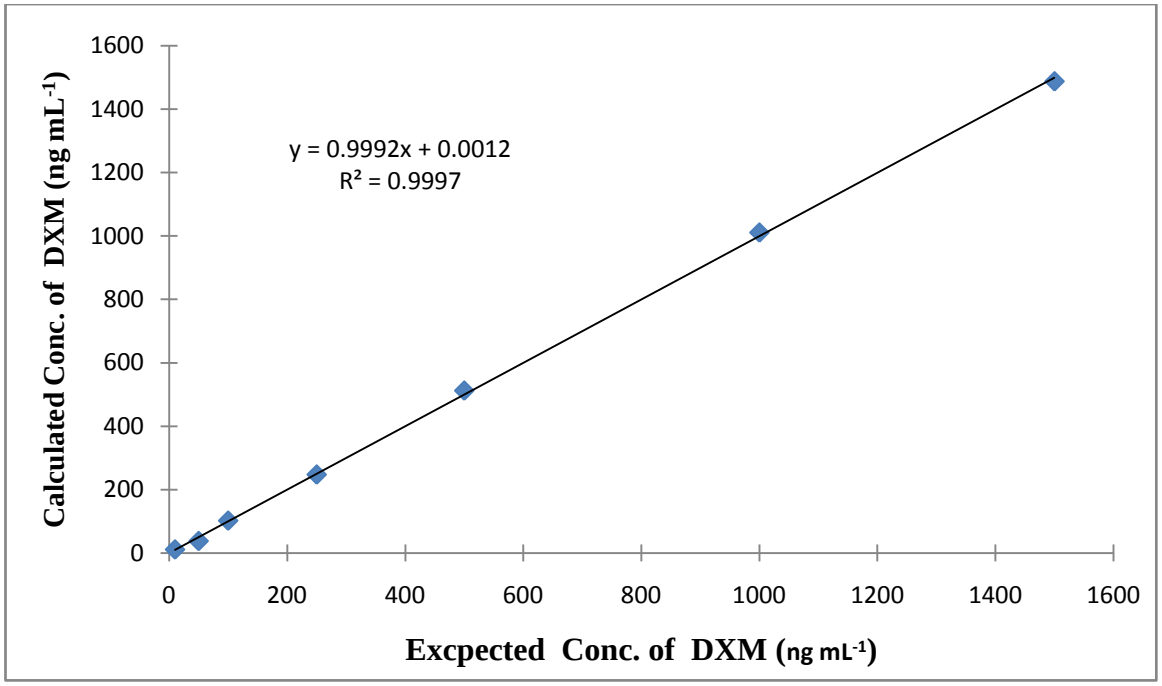


Figure 4.11. Method linearity control for DXM

Table 4.8. Regression line parameters for calibration curves of BTM and DXM

Regression line Parameters	**BTM**	**DXM**
Regression Equation	y =0.00224x + 0.0455	y =0.00229x + 0.0540
Standard Error of Slope	1.4781 X 10^{-5}	1.773 x 10^{-5}
Standard Error of Intercept	0.0106	0.0126
Correlation Coefficient (r)	0.9999	0.9998
Coefficient of Determination (R^2)	0.9998	0.9997
Linearity Range	10.0-1500 ng mL^{-1}	10.0-1500 ng mL^{-1}

Table 4.9. One-Way ANOVA analysis of variance results for BTM and DXM.

BTM	**$F_{calculated}$**	**p value**	**Decision**
	23063.67	2.35 X10^{-10}	p < 0.05, the relationship between *x* and *y* is linear
	$t_{calculated}$	**p value**	**Decision**
	151.87	2.35 X10^{-10}	p <0.05, The value of Correlation coefficient (r) is significant
DXM	**$F_{calculated}$**	**p value**	**Decision**
	16653.51	5.30 X10^{-10}	p < 0.05, the relationship between *x* and *y* is linear
	$t_{calculated}$	**p value**	**Decision**
	129.05	5.30 X10^{-10}	p <0.05, The value of Correlation coefficient R is significant

4.2.4. Accuracy

The intra-day and inter-day accuracy data that obtained by developed UPLC/MS method were determined by calculating relative error (RE) values for 3 level of concentrations for BTM or DXM as 50,500 and 1000 ngmL^{-1} (Table 4.10 and Table 4.11).

4.2.5. Precision

The inter-day and intra-day precision data that obtained using developed UPLC/MS method were determined by calculating values of standard error (SE)of the mean and relative standard deviation (RSD%). Precision values at 3 level of concentration (50,500 and 1000 ng mL^{-1}) were given in Tables 4.12 and 4.13.

Table 4.10. Intra-day accuracy studies for BTM and DXM (n=6).

Concentration (ng mL^{-1})	**BTM**		**DXM**	
	Measured (ng mL^{-1})	**RE (%)**	**Measured (ng mL^{-1})**	**RE (%)**
50	49.80	-0.39	50.14	0.28
	50.33	0.66	49.53	-0.93
	50.80	1.59	50.50	1.00
	50.79	1.59	50.36	0.72
	49.32	-1.36	50.00	0.02
	49.35	-1.30	51.07	2.13
500	499.27	-0.15	500.36	0.073
	498.40	-0.32	505.70	1.14
	501.51	0.30	504.62	0.92
	501.50	0.30	509.90	1.98
	501.50	0.30	503.22	0.64
	499.27	-0.15	496.56	-0.69
1000	1009.93	0.99	993.12	-0.69
	983.44	-1.66	1003.14	0.31
	1002.03	0.20	1001.69	0.17
	989.18	-1.08	994.71	-0.53
	1015.29	1.52	1004.43	0.44
	1005.17	0.52	1008.66	0.87

RE (%): Relative error

Table 4.11. Inter-day accuracy studies for BTM and DXM (n=5)

Concentration (ng mL^{-1})	**BTM**		**DXM**	
	Measured (ng mL^{-1})	**RE (%)**	**Measured (ng mL^{-1})**	**RE (%)**
50	49.60	-0.80	49.59	-0.82
	50.88	1.76	50.29	0.58
	50.50	0.99	50.20	0.41
	50.20	0.41	50.27	0.54
	50.24	0.48	49.77	-0.46
500	501.26	0.25	498.35	-0.33
	499.92	-0.01	501.55	0.31
	500.27	0.05	502.38	0.48
	504.33	0.86	503.40	0.68
	500.24	0.048	501.12	0.22
1000	1003.77	0.38	1005.04	0.50
	1008.63	0.86	1003.90	0.39
	1005.45	0.55	1002.15	0.21
	1003.95	0.40	1000.96	0.09
	999.66	-0.03	999.25	-0.07

RE (%): Relative error

Table 4.12. Intra-day precision studies for BTM and DXM (n=6).

Concentration (ng mL^{-1})	**BTM**		**DXM**	
	$\bar{X}$ ± SE	**RSD(%)**	**$\bar{X}$ ± SE**	**RSD (%)**
50	50.24 ± 0.37	1.80	50.27 ± 0.21	1.02
500	500.24 ± 0.58	0.28	503.39± 1.87	0.91
1000	1000.84 ± 5.00	1.22	1000.96 ± 2.42	0.59

$\bar{X}$ ± SE: Mean ± Standard Error, RSD%: Relative standard deviation

Table 4.13. Inter-day precision studies for BTM and DXM (n=5).

Concentration (ngmL^{-1})	**BTM**		**DXM**	
	$\bar{X}$ ± SE	**RSD (%)**	**$\bar{X}$± SE**	**RSD (%)**
50	50.28 ± 0.066	0.93	50.02 ± 1.44	0.64
500	501.21 ± 0.81	0.36	501.35± 0.85	0.38
1000	1004.5± 1.27	0.28	1002.26 ± 1.03	0.23

$\bar{X}$ ± SE: Mean ± Standard Error, RSD%: Relative standard deviation

4.2.6. Repeatability

Ten standard solution of each at concentration of 150 ng mL^{-1} of BTM or DXM were prepared and injected in the same batch. Repeatability was expressed by determination of relative standard deviation RSD % of the results (Table 4.14).

Table 4.14. Repeatability results of BTM or DXM at 150 ng mL^{-1} (n=10)

Concentration (150 ng mL^{-1})	**BTM**	**DXM**
	Measured (ng mL^{-1})	**Measured (ng mL^{-1})**
1	152.0092	146.2192
2	149.2772	149.6639
3	150.9671	152.3315
4	150.852	152.3364
5	149.4083	148.8481
6	148.0347	148.0351
7	155.8438	151.6277
8	151.9423	152.2704
9	153.4347	147.2887
10	151.4531	148.3607
$\bar{x}$ ± SE	**151.32 ± 0.71**	**149.70 ± 0.73**
SD	**2.23**	**2.29**
RSD (%)	**1.47**	**1.53**
95% CI	**149.73 - 152.92**	**148.06 - 151.34**

$\bar{X}$ ± SE: Mean ± Standard Error, SD: Standard deviation, RSD (%): Relative standard deviation, CI: Confidence interval.

4.2.7. Recovery

Recovery is percentage for the amount of BTM or DXM that was recovered from spiked solution of synthetic tablet`s preparation that contains 1000 ng $mL^{-1}$of BTM or DXM. The results were shown in Table 4.15 and RE (%) of the mean and RSD (%) values were calculated.

Table 4.15. Recovery results of BTM and DXM spiked to the tablet`s synthetic solutions (n=6).

Number of replicators (n)	Amount of BTM spiked (1000 ng mL^{-1})		Amount of DXM spiked (1000 ng mL^{-1})	
	Measured (ng/mL)	Recovery (%)	Measured (ng/mL)	Recovery (%)
1	966.57	96.66	970.05	97.00
2	995.32	99.53	984.83	98.48
3	978.44	97.84	991.68	99.17
4	993.41	99.34	956.88	95.69
5	1008.29	100.83	1005.12	100.51
6	1017.53	101.75	1000.27	100.03
$\bar{X}$ ± SE	**993.26 ± 7.64**	**99.33 ±0.76**	**984.81 ± 7.52**	**98.48**
SD	**18.72**	**1.87**	**18.42**	**1.84**
RSD (%)	**1.88**	**1.88**	**1.87**	**1.87**
CI	**973.62 - 1012.99**		**965.47 - 1004.14**	

$\bar{X}$ ± SE: Mean ± Standard Error, SD: Standard deviation, RSD%: Relative standard deviation, CI: Confidence interval.

4.2.8. Ruggedness

Standard solutions of 100 ng mL^{-1} for BTM or DXM were prepared and analyzed by two analysts at the same optimized chromatographic and instrumental conditions (Table 4.16 and 4.17)**.**

Table 4.16. Results for the analysis of BTM by two analysts (n = 5)

	1st Analyst		**2nd Analyst**	
Added Amount	**Measured (ngmL^{-1})**	**Recovery (%)**	**Measured (ngmL^{-1})**	**Recovery (%)**
BTM (100 ng mL^{-1})	98.56	98.56	102.35	102.35
	100.88	100.88	98.30	98.30
	100.54	100.54	101.49	101.49
	97.40	97.40	99.48	99.48
	101.14	101.4	102.84	102.84
$\bar{x}$ ±SE	**99.71 ± 0.733**		**100.89 ± 0.866**	
SD	**1.64**		**1.94**	
RSD (%)	**1.64**		**1.92**	
95 % CI	**101.14 - 98.27**		**99.20 – 102.59**	
F-Test	**F calculated = 1.397 < F tabled = 6.388 , p = 0.376 > α = 0.05 No significant difference between results of two analysts**			

$\bar{X}$ ± SE: Mean ± Standard Error, SD: Standard deviation, RSD %: Relative standard deviation, CI: Confidence interval.

Table 4.17. Results for the analysis of DXM by two analysts (n = 5)

	1st Analyst		**2nd Analyst**	
Added Amount	**Measured (ng mL^{-1})**	**Recovery (%)**	**Measured (ng mL^{-1})**	**Recovery (%)**
DXM (100 ng mL^{-1})	101.67	101.67	99.26	99.26
	98.73	98.73	100.68	100.68
	101.66	101.66	102.25	102.25
	99.71	99.71	101.77	101.77
	101.34	101.34	98.80	98.80
$\bar{x} \pm$ SE%	**100.62 ± 0.59**		**100.55 ± 0.67**	
SD	**1.34**		**1.51**	
RSD (%)	**1.33**		**1.50**	
95% CI	**99.45 –101.79**		**99.22 – 102.06**	
F-Test	**F calculated= 0.925 < F tabled =6.388 , p = 0.471 > α = 0.05** **No Significant difference between results of two analysts**			

$\bar{X}$ ± SE: Mean ± Standard Error, SD: Standard deviation, RSD%: Relative standard deviation, I: Confidence interval.

4.2.9. Robustness

Evaluation of the method robustness is performed by measuring its capacity to remain unaffected after small but deliberate variations in method parameters. Analysis of BTM or DXM with small changes (± 1.0) in the values of amount of organic modifier (% ACN) and (± 10 %) in flow rate of the mobile phase were carried out, and then the results statistically compared with that of normal method conditions (Table 4.18 and 4.19).

Table 4.18. Robustness studies of BTM (n = 6)

BTM	**Optimum Conditions**		**Changes in ACN%**		**Changes in Flow Rate**	
(150ng mL^{-1})	**15 % ACN**	**0.3 mL min^{-1}**	**14% ACN**	**16% ACN**	**0.27 mL min^{-1}**	**0.33 mL min^{-1}**
Measured ($ngmL^{-1}$) ± SE	151.66 ± 1.34		152.08 ± 1.82	149.24 ± 0.81	152.19 ± 1.77	151.06 ± 0.92
RSD (%)	2.15		2.93	1.30	2.85	1.48
t-Test: Two-Sample Assuming Unequal Variances			p = 0.90 > 0.05	p = 0.36 > 0.05	p = 0.87 > 0.05	p = 0.81 > 0.05

RSD%: Relative standard deviation, p = p value of t test, $\bar{X}$ ± SE: Mean ± Standard Error

Table 4.19. Robustness studies of DXM (n = 6)

DXM	**Optimum Conditions**		**Changes in %ACN**		**Changes in Flow Rate**	
(150 ng mL^{-1})	**15 % ACN**	**0.3 mL min^{-1}**	**14% ACN**	**16% ACN**	**0.27 mL min^{-1}**	**0.33 mL min^{-1}**
Measured ($ngmL^{-1}$) ± SE	151.21 ± 0.80		153.52 ± 0.63	150.19 ± 1.96	154.49 ± 1.64	152.41 ± 1.24
RSD (%)	1.29		1.21	3.18	2.58	1.99
t-Test: Two-Sample Assuming Unequal Variances $T_{Calculated} < T_{tabled}$			p = 0.18 > 0.05	p = 0.75 > 0.05	p = 0.29 > 0.05	p = 0.61 > 0.05

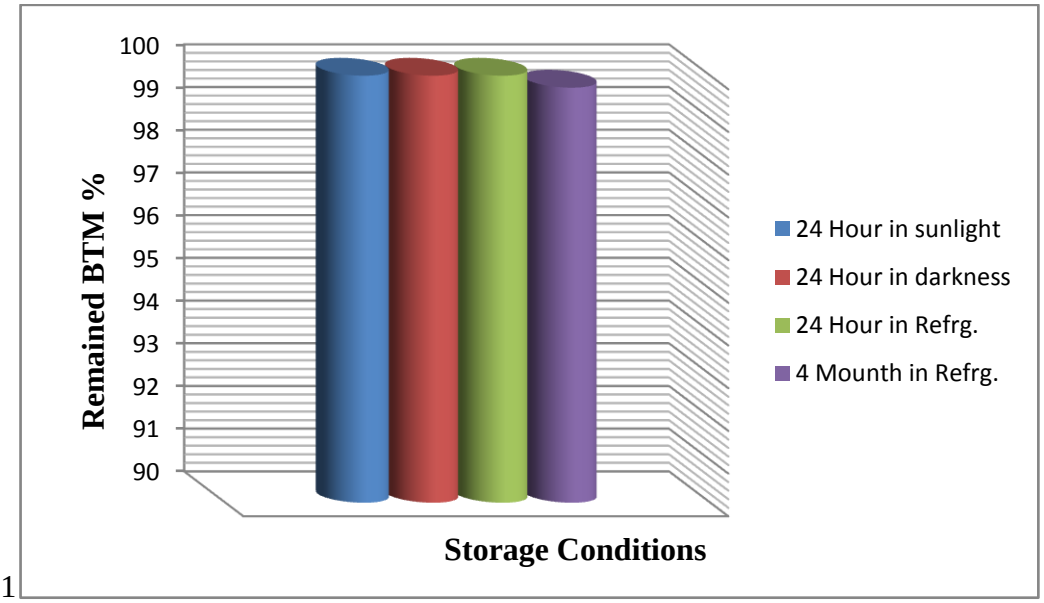
RSD%: Relative standard deviation, p = p value of t test, $\bar{X}$ ± SE: Mean ± Standard Error

4.2.10. Stability

Investigations on BTM and DXM standard solutions stability were performed under several storage conditions as mentioned in section 3.8.10 were calculated as percentage of remained analyte amount after certain time of storage (Table 4.20, Figure 4.12 and 4.13).

Table 4.20. Stability of BTM and DXM solutions (n =6)

Storage condition		**Remained Amount %**	
		BTM	**DXM**
Short Term	**After 24 Hour at + 25 ^{0}C in Sunlight**	100.29	100.89
	After 24 Hour at + 25 ^{0}C in Darkness	99.93	100.52
	After 24 Hour at +4 ^{0}C in Refrigerator	100.79	100.08
Long Term	**After 6 Months at +4 ^{0}C in Refrigerator**	99.87	99.72



1

Figure 4.12. BTM stability Studies

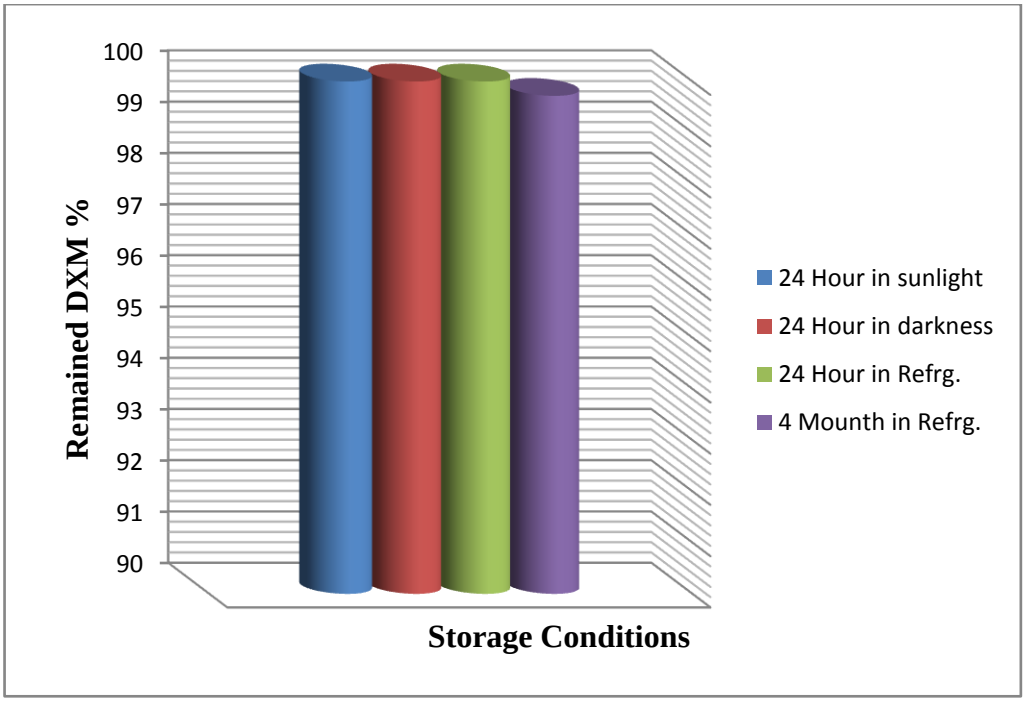


Figure 4.13. DXM stability Studies

4.2.11. System Suitability

System suitability parameters for the developed method were determined from the peaks of BTM vs. I.S and DXM vs. I.S; criteria of acceptable values were done according USP pharmacopeia guidelines (Table 4.21).

Table 4.21. System suitability tests for BTM and DXM

System suitability parameters	BTM	DXM
Precision of Injections (RSD %) for n=10	0.99	0.67
Peak asymmetry ratio(PA)	1.00	1.00
Number of theoretical plats (N)	26406	27225
Resolution (Rs) (between each analyte and IS)	BTM vs. I.S 1.69	DXM vs. I.S 1.58

4.3. Pharmaceutical Preparation Analysis

In this study, method of analysis BTM and DXM using UPLC/Tandem MS instrumentation was developed and validated. Commercial Pharmaceutical preparations containing BTM (Celestone 0.5 mg tablets and Celestone chronodose Injectable ampoule) and DXM (Dekort 0.5 mg tablet, Dekort injectable ampoule and Onadron eye/ear drop) were analyzed by validated UPLC/Tandem MS method for analysis of BTM and DXM (Figure 4.14 Figure 4.15, Tables 4.22 and 4.23).

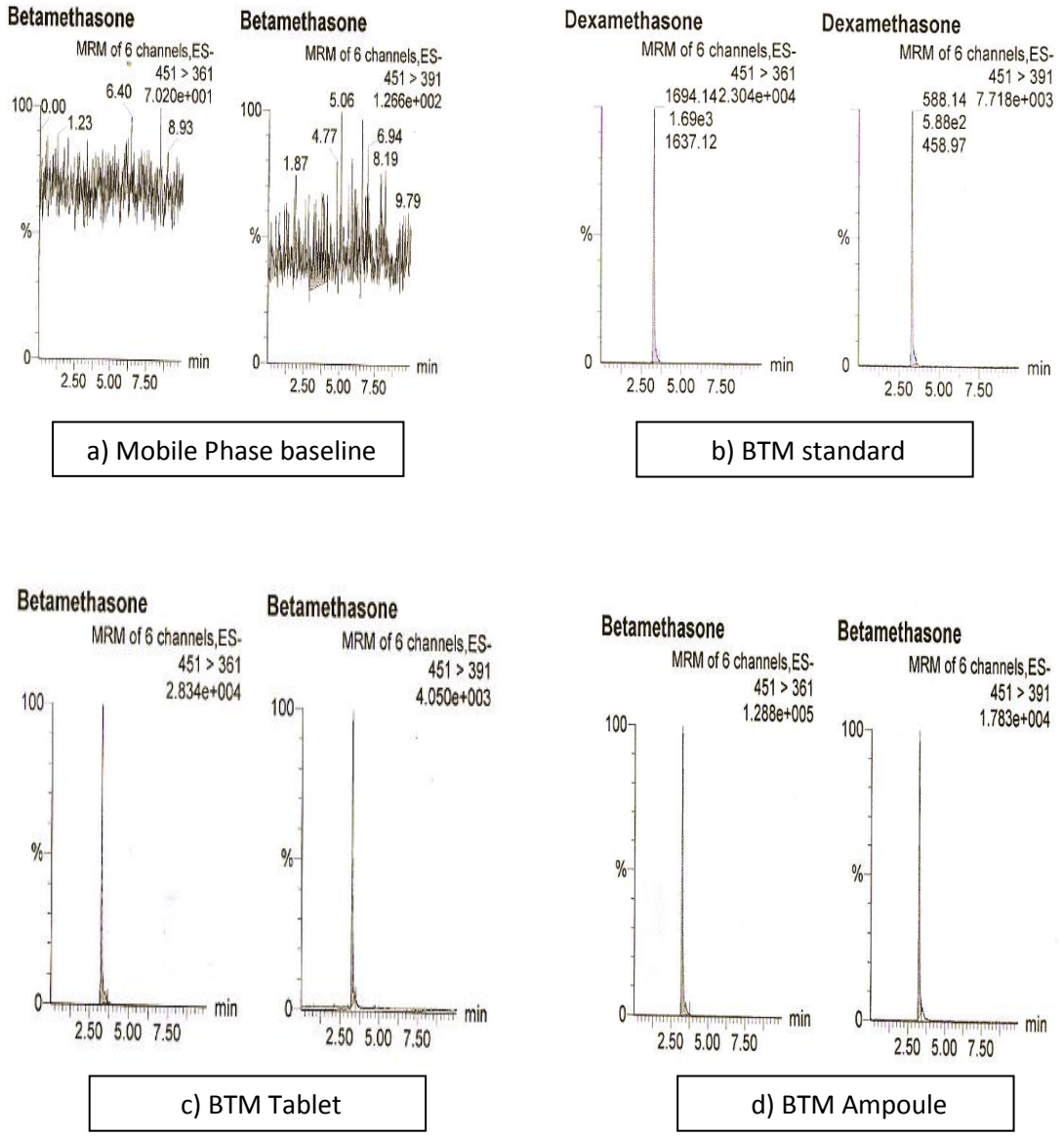


Figure 4.14. Representative macro outputs obtained from (a) mobile phase, (b) BTM standard (100 ng mL^{-1}), (c) BTM tablet and (d) BTM ampoule preparations.

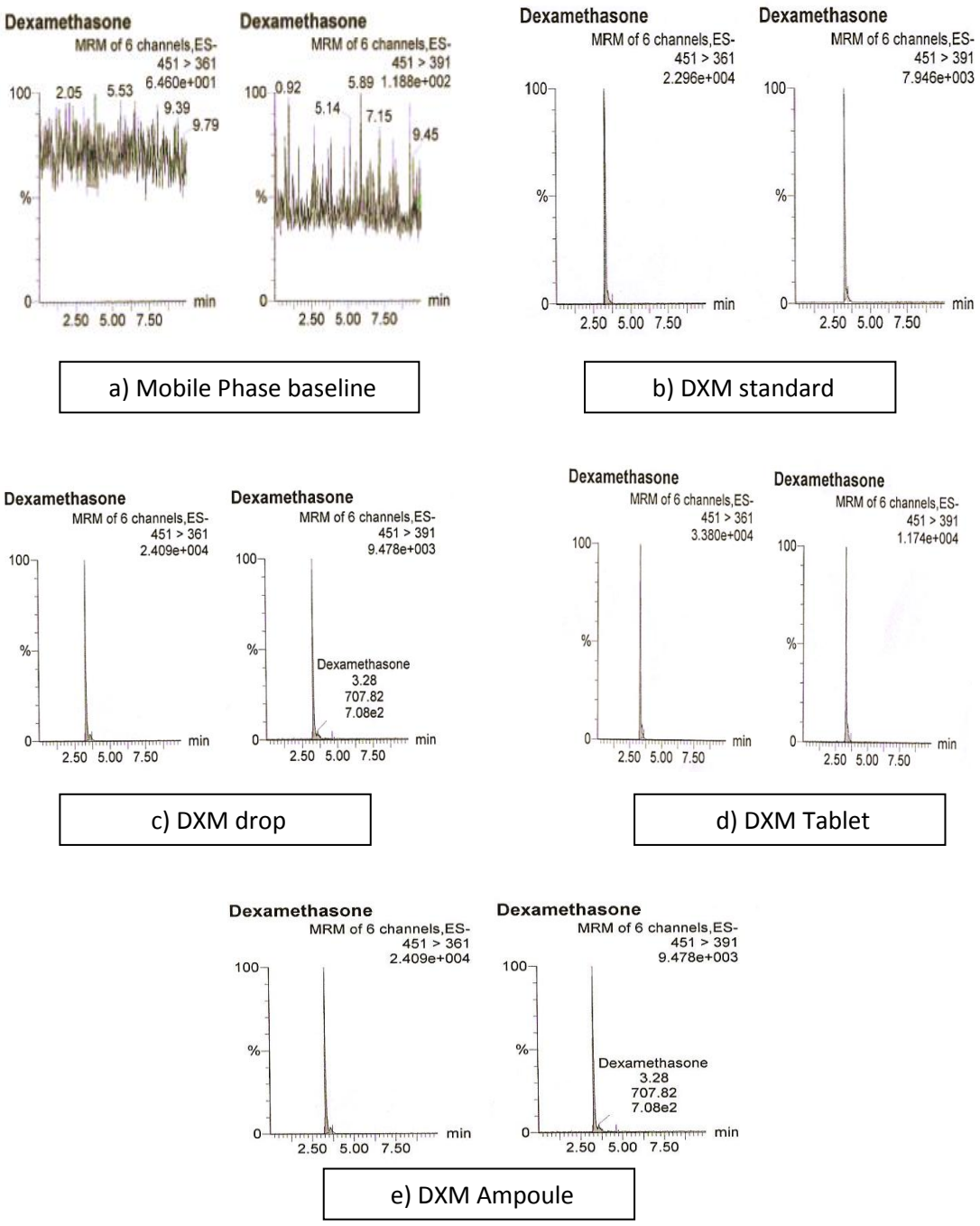


Figure 4.15. Representative macro outputs obtained from (a) mobile phase, (b) DXM standard (100 ng mL^{-1}), (c) DXM tablet, (d) DXM ampoule and (e) DXM drop preparations.

Table 4.22. Analysis of BTM containing pharmaceutical preparations.

Pharmaceutical Form	**Celestone ® Tablet**	**Celestone Chronodose® inectable ampoule**
Amount of active ingredient	**0.5 mg BTM/tablet**	**3.947 mg BTM disodium phosphate**
Found (mg)	0.500	4.020
	0.508	3.972
	0.489	4.078
	0.480	3.989
	0.498	3.921
	0.493	4.020
$\bar{X}$	**0.495 ± 0.004**	**3.997 ± 0.022**
SD	**0.010**	**0.059**
RSD (%)	**1.989**	**1.46**
CI 95%	**0.486 ± 0.502**	**3.957 - 4.042**
Bias	**0.014**	**0.010**

$\bar{X}$ ± SE: Mean ± Standard Error, SD: Standard deviation, RSD%: Relative standard deviation, CI: Confidence interval.

Table 4.23. Analysis of DXM containing pharmaceutical preparations.

Pharmaceutical Form	**Dekort ® tablet**	**Dekort ® inectable 2 mL ampule**	**Onadron® 0.1% 5 mL eye/ear drop**
Amount of active ingredient	**0.5 mg DXM /tablet**	**8 mg DXM 21-phosphate**	**5 mg DXM 21-phosphate**
Found (mg)	0.503	7.962	5.123
	0.497	8.036	5.064
	0.487	7.715	4.945
	0.489	8.146	5.246
	0.508	8.076	5.168
	0.488	8.069	5.088
$\bar{X}$ ± SE	**0.495±0.004**	**8.001± 0.062**	**5.105± 0.042**
SD	**0.009**	**0.152**	**0.102**
RSD (%)	**1.81**	**1.90**	**1.99**
CI	**0.488 - 0.502**	**7.879 -8.122**	**5.024 -5.187**
Bias	**0.005**	**0.001**	**0.105**

$\bar{X}$ ± SE: Mean ± Standard Error, SD: Standard deviation, RSD%: Relative standard deviation,CI: Confidence interval.

5. DISCUSSION

Corticoids belong to a group of hormones produced by the suprarenal cortex, although this term is also used to describe all their metabolites that are removed by urine. Depending on its biological action, corticoids have been classified in glucocorticoids and mineral corticoids. Both, DXM and BTM are included in the group of the glucocorticoids. Their activity is in replacement therapy for adrenal insufficiency, and as an anti-inflammatory and immunosuppressant (13).

BTM is used to treat many conditions including dermatitis, arthritis, inflammatory bowel disease, reactive airways disease, and respiratory distress syndrome in preterm infants and pruritus in corticosteroid-responsive dermatomes. BTM is formed by hydrolysis of the phosphate or acetate esters after intravenous or intramuscular administration to human (7). Other esters and salts of BTM are available for other routes of administration or applications, e.g., valerate, butyrate, propionate benzoate salts. There are several approved products formulated based on a fast releasing BTTM phosphate ester or as a dual acting suspension formulation containing BTM phosphate and BTM acetate, both esters are expected (7, 8).

BTM and DXM are epimers with identical chemical structures except the orientation of the methyl group at C-16 position is in the opposite direction from the plane. Thus, they have similar physiological and pharmacological effects with different activities (12).

Many pharmaceutical preparations containing BTM or DXM and/or their ester derivatives are commercially available. None of these pharmaceutical preparations contains BTM and DXM in combination (6-8, 43).

In recent years, use of UPLC-MS technique increased due to its ability to reduce analysis time with higher efficiencies even at very low concentration to the level of few ng mL^{-1} (39, 48).

Several LC methods have been reported for determination of BTM and DXM in different types of samples. None of these methods was based on the assay of BTM or DXM in pharmaceutical preparations using Ultra Performance Liquid Chromatography combined with Tandem Mass Spectrometer (UPLC/Tandem MS). Thus, the aim of the presented thesis was to optimize, develop and validate a method

for BTM and DXM determination in their pharmaceutical preparations as tablets, injectable ampoules and eye/ear drops by UPLC/Tandem MS.

5.1. Method optimization

In order to optimize any analytical method, different experimental conditions affecting on its analysis performance should be tested to achieve optimum separation and detection conditions within a reasonable run time. In this thesis, mobile phase type and its combination, mobile phase flow rate, gradient profile, analytical column type and internal standard to be used were tested as experimental parameters under the conditions given in Section 3.7.2.

5.1.1. Selection of Mobile Phase Content

Methanol and ACN were widely used as organic modifier in mobile phase, particularly for reversed phase liquid chromatography, thus both solvents were tested for the elution of BTM and DXM at the same conditions. Because there was no resolution from the baseline for both BTM and DXM, methanol was not selected as an organic modifier for the mobile phase used in UPLC/Tandem MS. Therefore, further steps for chromatographic optimization for BTM and DXM were carried out using ACN as organic modifier.

5.1.2. Effect of Mobile Phase Organic Modifier Percentage

Optimization of initial mobile phase combination was performed by increasing the ratio of organic modifier (% ACN) in the mobile phase in the range from 15 to 80 %. The results clearly demonstrated that increasing of % ACN decreases the polarity of the mobile phase in other words increasing its hydrophobicity.

Analyte partitions take place between the two phases depending upon its chemistry (hydrophobicity). BTM and DXM have hydrophobic behavior (50), so with increasing the mobile phase hydrophobicity they will retained in mobile phase more than in stationary phase and will eluted earlier because of low interaction with the stationary phase leading in decreasing in the retention time and number of theoretical plates (Figure 4.1 and Table 4.1).

According to the obtained data from table 4.1, gradient mobile phase system with initial mobile phase combination that have 15 % ACN: 85 % HAc 0.1% (v/v)

was selected because highest number of theoretical plates (N= 27889 and 28392 for BTM and DXM respectively) and peak symmetry ratio was 1.00 for both BTM and DXM. At 40 and 50 % ACN, N values were less than 2000, which is not suitable for system suitability test.

5.1.3 Selection of Gradient Profile

Five gradient profiles were designed in order to have different slopes in BTM or DXM elution region, which were resulted in different run times while the initial ACN ratio was fixed at 15% (Figure 4.2 and Table 4..2). While the run time of gradient profile increased the efficiency of chromatographic method increased, as retention time and number of theoretical plate increased (Table 4.3). Because of increasing the time between the initial and final ratio will decrease the steepness of the gradient resulting in more interaction between analyte molecule and column stationary phase rather than with mobile phase, more retention of analyte in solid phase will increasing the effectiveness of column. Moreover, the symmetry of peaks were not affected significantly and were about 1.00.

Gradient profile E as t_0: 15% ACN, $t_{0.4}$:15% ACN, $t_{8.0}$: 85% ACN, $t_{8.5}$: 85% ACN $t_{9.2}$: 15% ACN, t_{10}:15% ACN (Table 4.2 and Table 4.3) with total analysis time of 10 minutes provided retention times of 3.37 and 3.40 min for BTM and DXM, respectively, highest number of theoretical plates with symmetrical peak shapes were selected as gradient profile for validation step.

5.1.4. Selection of Flow Rate

Increasing the flow rate of gradient system will decreased the time of interaction between the analyte and solid phase of the column. As a result, decreasing in retention time and number of theoretical plate of BTM and DXM was obtained by increasing flow rate (See Figure 4.3 and Table 4.4).

Conversely, increasing of the flow rate will increase the column back pressure which allow the analyte molecule occupating more pours space between packing material particles which mean more surface area of interaction but the effect linear velocity mobile phase which decrease the time of interaction is the predominant.

The mobile phase flow rate of 0.1 mL min^{-1}, although the number of theoretical plate value was the highest (N= 28493 and 28628 for BTM and DXM respectively) but the retention time obtained is longest (t_R= 8.44 and 8.46 for BTM and DXM respectively) which is not agree with aim of this study to develop fast method for analysis of BTM and DXM. Flow rate of 0.3 mL min^{-1} which provide high N values with acceptable retention times and symmetrical peaks (t_R= 3.37 and 3.40 for BTM and DXM respectively were selected as the flow rate of the method for further method development steps.

5.1.5 Analytical Column Selection

The stability and reproducibility of the columns during method development, a C_8 or C_{18} column made from specially purified, less acidic silica (minimal metal contamination), designed specifically for separation of basic compounds is generally suitable for all samples, and strongly recommended.

The column`s length, internal diameter and packing material particle size are other factors affecting the efficiency of the method to separate sample analytes. According to the van Deemeter equation, the smaller column packing material particle size can significantly reduce the HETP resulting in higher separation efficiencies at lower flow rates of mobile phase.

The initial column used with UPLC instrument was AcQuity BEH C_{18} which is length is 50 mm and internal diameter of 2.1 mm with particle size of 1.7µm. Columns of BEH (bridged ethyl hybrid) technology with 1.7µm particles derive their enhanced mechanical stability by bridging the methyl groups in the silica matrix. These columns also provide high efficiency (narrower and higher peaks with adequate resolution at shorter time) and operate over an extended pH range.

During method optimization, another available column was tested. This column was the same as initially used but only in different length which was 150 mm. Although the longer column produce higher number of theoretical plates (N), the shorter column produce fine symmetrical peaks in shorter retention times of the analytes (Table 4.5). So the column of 50 mm length was used in the next steps of method development, validation and sample analysis.

5.1.6. Internal Standard

The internal standard is a different compound from the analyte but should be well resolved in the separation (Rs must be > 1.5), and the internal standard can compensate for changes in sample size or concentration due to instrumental variations. The ratio of analyte to internal standard peak areas serves as the analytical parameter.

During thesis experiments, Mefruside was used as the internal standard with BTM and DXM because it found routinely used in analysis of glucocorticosteroids drugs (BTM and DXM included) in doping human urine samples as internal standard due it's have no interference with glucocorticosteroid drugs. In system suitability test (Table 4.21), Resolution (Rs) values between BTM or DXM and Mefruside (IS) were 1.69 and 1.58, respectively

5.2. Discussion of the Method Validation Results

Specifity

In this study, a validated method for assay of BTM and DXM in pharmaceutical preparations using Ultra-Performance Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry (UPLC-ESI-MS/MS) was developed. Although that molecular weight of BTM or DXM is 392 g/mol, the expected molecular ion of 392 m/z could not be seen in scan mode of MS. Instead, multiple reaction monitoring (MRM) measurements using ESI-MS/MS with a negative ion mode, acetylated molecular ion(or adduction ion) with 451 m/z derived from adduction reaction with acetate group from the acetic acid in the mobile phase was used as a precursor ion [M-H- $CH_3COOH]^-$ because its most abundant ion peak in the mass spectra. In 2^{nd} MS, the precursor ion was undergoing further fragmentation process by collisionaly activated dissociation (CAD) producing smaller fragment as product ions (Figure 5.1). In order to select diagnostic product ions [Daughter ions] characteristic for each analyte for the quantitative analysis the collision energy were optimized to obtain maximal intensities for all diagnostic product ions were d the collision energy was optimized for each ion which called; The product ions at 391 and 361 m/z have the highest signal intensities (Table 4.6, Figure 4.4 and 4.5). Although that the precursor ion have the highest energy,

selection one of the products ions as diagnostic ion provide higher specifity and selectivity of the method toward the analytes. So, the product ion of highest intensity at 361 m/z was selected to be an diagnostic ion 451>361 m/z transition (Table 4.6).

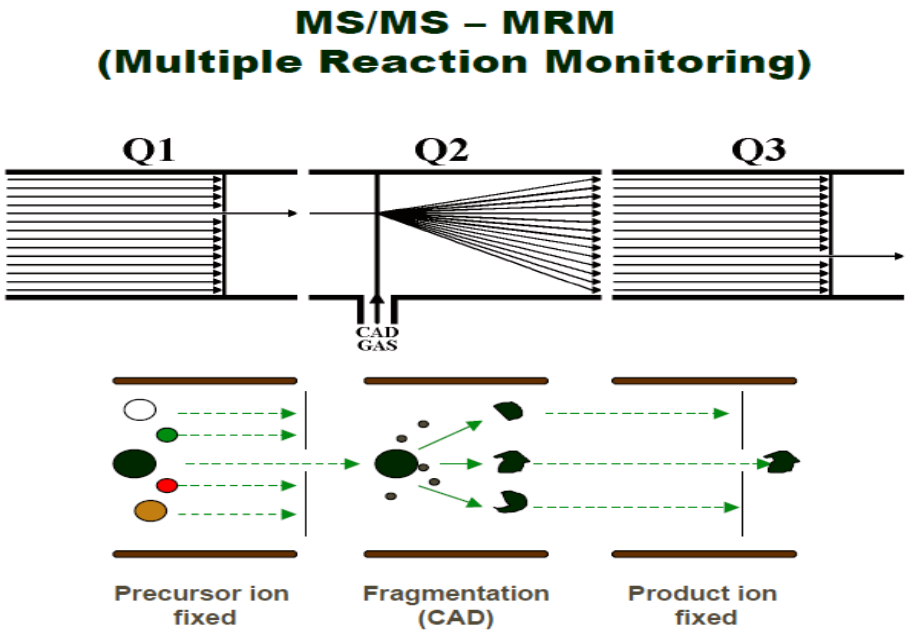


Figure 5.1.MS/MS Multiple Reaction Monitoring (MRM) scheme

The comparison between the chromatograms of standard solutions of BTM or DXM with that of placebo, synthetic preparation and pharmaceutical samples demonstrated that the peaks for the analytes of interest were well resolved from the baseline and not affected or interfered by any other peak of any matrix or sample components (Figures 4.6 and 4.7). Therefore, the methods were specific to assay of BTM and DXM in pharmaceutical preparations under the optimized conditions.

Sensitivity

Using the method of Signal-to-Noise ratio to determine the LOD and LOQ values for BTM or DXM, the method was found sensitive to minimum detectable concentration (LOD) of 1 ng mL^{-1} for either BTM or DXM with a signal to noise ratio of 3 the minimum concentration that can be quantitatively sensed by the developed method (LOQ) was 5 ng/mL for either BTM or DXM at a signal to noise ratio of 10 (Table 4.7). According to the ICH method validation guideline (92), the

LOD and LOQ values are quite enough in purpose of pharmaceutical preparation analysis for BTM or DXM.

Range and Linearity

The relationship between the concentration of standard solutions of BTM or DXM and the response of the detector (as the BTM or DXM peak area to peak area of I.S ratio) were expressed by plotting a calibration curve using seven points of concentrations ranged from 10 to 1500 ng mL^{-1} for BTM or DXM (Figures 4.8 and 4.9). The parameters of the regression line of the calibration curve constructed for BTM and DXM were shown in Table 4.8. The value of coefficient of determination R^2 for BTM and DXM are 0.9998 and 0.9997, respectively. The values describe how high the linear regression line is fits a set of data (R^2 range 0-1) for BTM or DXM.

In order to prove the linearity of the calibration curve and that the correlation coefficient value is significant value and different from zero, Statistical analysis using One-Way ANOVA analysis of Variance was done (Table 4.9)

At confidence level of 95% ($\alpha = 0.05$), the value of correlation coefficient (r) for BTM or DXM is significant value and not equal to zero ($t_{calculated} > t_{table}$, $p < 0.05$, then reject H_0: value of r is not equal to zero).In other hand, the relationship between concentration and detector response for BTM or DXM is linear (F calculated > F table, $p < 0.05$, then reject H_0: No linear relationship between x and y values).

In order to control the linearity of the calibration curve constructed, a graphic curve illustrate the relationship between the added concentrations of BTM or DXM and that calculated from the regression equation of the calibration curve as illustrated in Figure 4.10 and 4.11. The slope of resulted curve is near unity (0.999 for DXM and 1.000 for BTM) and the correlation coefficient is > 0.999 for both BTM and DXM, So, the method is linear at test range of concentrations.

Accuracy

Six injections, of three different concentrations (50,500 and 1000 ng mL^{-1}) for BTM or DXM were given on the same day, the values of relative error (RE) for every injection were calculated (Table 4.10). RE values for all injections at all concentration levels for either BTM or DXM did not exceed the value of ± 2.00 %.

These studies were also repeated in five different days to determine inter-day accuracy. The values of RE were calculated to the mean of found concentration for every day. Again, the RE values for inter-day accuracy studies did not exceed ± 2.00 % (Table 4.11).

Accuracy can also be reported as percent recovery by the assay of known added amount of analyte in the sample. Studying the ability of the developed method to recover the spiked amount of BTM or DXM (1000 ng mL^{-1}) from a synthetic pharmaceutical preparations (Table 4.15) show that the recovered amount of BTM ranged from 96.66 to 101.75 % within the interval of 973.62 - 1012.99 $ngmL^{-1}$at 95 % confidence level for BTM and 95.96 – 100.51 % within the interval of 965.47 - 1004.14 ng mL^{-1}at 95 % confidence level for DXM.

Precision

Both intra-day and inter-day precision studies indicated a high degree of precision over the three concentrations investigated in the accuracy studies. The values of relative standard deviation did not exceed 2.00 % for either BTM or DXM (Tables 4.12 and 4.13).

Repeatability expresses the precision under the same operating conditions over a short interval of time. Ten injections of 150 $ngmL^{-1}$ concentration for BTM or DXM were given on the same day (Table 4.14).RSD values were 1.47 % for BTM within intervals of 149.73 - 152.92 $ngmL^{-1}$for BTM and 148.06 - 151.34 $ngmL^{-1}$for DXM.

Ruggedness

Standard solutions of 100 ng mL^{-1} concentration for BTM and DXM were prepared and injected by two different analysts under the same conditions using different wet lap and the same instrument (Table 4.16 and 4.17). The results were statistically analyzed showing that there is no significant difference between results of the two analyst (F calculated < F table and $p > 0.05$). Therefore, the method was not affected and still accurate and precise even with different analysts.

Robustness

According to the statistical analysis using (t test) developed method was not affected by small but deliberate changes in the amount of organic modifier (14 and

16% ACN) and flow rate of the mobile phase 0.27 and 0.33 mL min^{-1}), showing that all p values of t test for tested samples were larger than α ($p > 0.05$, Table 4.18 and 4.19).So, there was no difference between the means of the sample.

Stability

Fresh standard solutions of BTM or DXM at concentration of 100 ng mL^{-1} were hours and 6 months under different storage conditions as shown in Table 4.20 different times under different storage conditions. The mean of the results (remained amount of the analyte) at each storage condition were compared to that of freshly prepared solutions that assumed to be 100%. The lowest and highest means of remained amount of the analyte are (99.87-100.79 %) for BTM and (99.72-100.89 %) for DXM. These results are within the acceptance range of 98-102 % which considered within it the method is been considered stable.

System Suitability

According to the acceptance criteria of USP, the system is suitable for the developed method. All calculated chromatographic parameters were within the acceptance limits of system suitability test for developed method: RSD of Injections < 1%, Number of theoretical plates N > 2000, peak asymmetry ratio PA ≤ 1.5 and resolution Rs > 1.5 (Table 4.21).

5.3. Pharmaceutical Preparation Analysis

After the developed method has been validated, this method was applied to analysis of some pharmaceutical preparations that contains BTM, DXM, or their salts to assay the amount of the analyte of interest in that pharmaceutical product.

Celestone ® Tablet containing 0.5 mg BTM was found to have 0.480-0.508 mg in six samples. But analyzing of 1mL ampoule of Celestone Chronodose® injection shown that was containing 3.972 – 4.078 mg of BTM as BTM disodium phosphate while in leaflet is 3.947 mg (Table 4.22).

Dekort ® tablet found it was containing 0.488 – 0.508 mg DXM (Leaflet: 0.5 mg). Dekort® 2mL injectable ampoule and Onadron® 0.1% 5 mL drop contain 7.715-8.146 mg (leaflet: 8.00 mg) and 4.945 -5.246 mg (leaflet 5.00 mg) DXM as DXM 21-phosphate respectively (Table 4.23).

A number of methods have been reported for analysis of pharmaceutical preparations that contain BTM or DXM and/or their ester derivatives singularly and/or in combination with other active ingredients.The most popular of these methods, have been liquid chromatography, especially in RP separation. Many LC methods with spectrophotometric detection were reported for analyses of tablet containing BTM or DXM. Generally, UV/Vis or DAD spectrophotometers as a detector at wavelength 240-254 nm and different column type like silica or RP-C_{18} were used. Methods include derivatization process using silica column for analysis of BTM and DXM in tablets (3), using cyclodextrine as mobile phase additive to analysis from BTM and DXM pharmaceutical active ingredients (API) (12, 80). LC-UV/Vis or DAD methods for analysis of BTM/DXM and related products in API (43,46),DXM in ointment(42), BTM actetae and BTM di-phosphate esters in API and ampoule (45),DXM diphosphate in eye drop(95), BTM and DXM in cosmetics (35) were reported.

LC-spectrophotomeric methods are generally less sensitive, less specific and longer run time than LC-Mass spectrometric methods. In the other hand, LC-UV/Vis, DAD, chemilumisance methods sometimes require time consumable sample preparation and derivatization process.

Few LC-MS or LC-MS/MS for analysis of BTM or DXM and/or their ester derivatives singularly and/or in combination with other active ingredients were also reported. Analysis of BTM and DXM in tablet using LC-MS-FAB after dervatization process resulted in separation of BTM and DXM (R_s = 1.9) with decreasing in run time (3), separation of BTM and DXM in API mixture by LC-MS-Ion Trap-ESI were determined by the differences in the ratio of m/z 391 product ion (79). Determination of BTM and DXM traces in cosmetic products using RP-C18 column with 1.8 µm particle size, by LC-Tandem MS, resulted in retention times of BTM and DXM was 11.69 and 11.76 respectively. LOD was 1.5-2.33 ng mL^{-1} and LOQ was 5.17-7.78 ng mL^{-1} (35). Another article show that analysis of BTM and DXM in tablets by LC/Tandem MS using gradient system at 0.75 mL min^{-1} flow rate, the retention times was 5.56 and 5.92 min for BTM and DXM respectively(91). Therefore, the LOD and LOQ values obtained by this method were the lowest and provide higher

sensitivity according to that obtained by previously reported methods for analysis of BTM and DXM in pharmaceutical preparations.

UPLC/Tandem-MS has not been used for the analysis of BTM or DXM and/or acetate or sodium phosphate ester derivatives in tablets, ampoule and drops, then in this study; analysis of pharmaceutical preparations containing BTM or DXM and/or acetate or sodium phosphate ester derivatives by UPLC/Tandem-MS was performed. The advantages of UPLC-MS technique over HPLC-MS are that UPLC provide faster retention time of the analyte using slower flow rate. As example, in this study the retention time of BTM or DXM was < 3.5 min at 0.3 mL min^{-1}, while in other LC-MS methods for analysis of BTM or DXM in tablets the retention time is > 4.0 min at flow rates > 0.75 mL min^{-1} (45, 91). This advantage means reduction in solvent consumption, which is very important for routine analysis. In addition, presented method provides compatible sensitivity obtained by LC/Tandem MS methods. In this study, simple sample preparation led to high accuracy, precision, and recovery values (98-102%) and sharp peaks with peak width reach to 0.06 min.

Ultra Performance Liquid Chromatography (UPLC) could be considered a new direction of liquid chromatography. The new trend in the pharmaceutical analysis is to methods transfer from HPLC to UPLC. As efficiency and speed of analysis has become of a great importance in many application of liquid chromatography, especially on a field of pharmaceutical, toxicological and clinical analysis, where there it is important to increase throughput and reduce analysis costs, UPLC could play a significant role in the future of liquid chromatography (96,97).

At a time when many scientists have, reached separation barriers pushing the limits of conventional HPLC, UPLC present the possibility to extend and expand the utility of this widely used separation science. UPLC begins to fulfill the promise of increased speed, resolution and sensitivity predicted for liquid chromatography. This achievement enables method development to be more efficient, allowing products to be brought to market faster. Hence, use of such UPLC systems will become the option of choice for the development of fast LC methods in pharmaceutical development in the near future (97).

6. RESULTS AND SUGGESTIONS

In this thesis works, a chromatographic method for determination two drugs from the group of Glucorticosteroids (BTM and DXM) in pharmaceutical preparations (Tablets, Injectable ampoules and eye/ear drops) by Ultra-Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS) using negative Electrospray ionization (- ESI) at MRM mode were developed.

During method development, the chromatographic and spectrometric conditions were optimized in order to obtain shorter retention times of the analytes with best analysis efficiency and detector response. The optimal chromatographic conditions obtained were: 15% ACN: 0.1% HAc in water (v/v) as mobile phase initial combination solution for the gradient system (t_0: 15% ACN, $t_{0.4}$:15% ACN, $t_{8.2}$: 85% ACN, $t_{9.5}$: 15% CAN and t_{10}:15% ACN) within 10 minutes total analysis time, 0.3 mL min^{-1} as flow rate of the mobile phase and UPLC AcQuity BEH RP C18 column with 50 mm length,2.1 mm internal diameter and 1.7 µm particle size as an analytical column. While the best mass spectrometric conditions obtained by selecting the product ion at 361 m/z (451 m/z > 361 m/z mass transition) at MRM mode using –ESI.

The developed method was validated according to ICH guideline for specifity, stability, range and linearity, sensitivity, accuracy, precision, ruggedness and robustness. The results were evaluated that validation findings meet the acceptance criteria considering the methods were be validated.

Developed and validated methods were successfully applied for analysis of commercially available pharmaceutical preparations for BTM (Celestone® Tablet and Celestone®injectable Ampoule) and for DXM (Dekort® tablet, Dekort® injectable Ampoule and Ondaron® Simple eye/ear drop). The obtained results (amount of BTM or DXM found in pharmaceuticals) are very similar to that mentioned in the leaflets. Therefore, this method is suitable for determination of BTM or DXM in pharmaceutical preparations.

In the literatures, none of the previously published studies on the analysis of BTM or DXM using UPLC/MS/MS involved determination of BTM or DXM in pharmaceutical preparations.

Suggestions

Developing this method to be capable for determination BTM or DXM in other pharmaceutical preparations forms such as topical creams and ointments or oral suspension syrups or inhalation dosage forms is recommended. These pharmaceutical forms need to special attention during sample preparations and it is important to have high recovery percentage.

REFERENCES

1. Gentili A. (2007). LC-MS methods for analyzing anti-inflammatory drugs in animal-food products. *Trends Anal. Chem*., 26, 595–608.
2. Song L., Bai J., Zhou W. (2008)Determination of Betamethasone and Dexamethasone in Human Urine and Serum by MEKC After an Experimental Design. *Chromatographia*, 68, 287-293.
3. Chen SH., Wu SM., Wu HL. (1992) Stereochemical analysis of betamethasone and dexamethasone by derivatization and high-performance liquid chromatography. *Journal of Chromatography*, 595(1-2), 203-208.
4. Santos-Montes A., Gasco-López A. I., Izquierdo-Hornillos R. (1994) Simultaneous Determination of Dexamethasone and Betamethasone in Pharmaceuticals by Reversed-Phase HPLC. *Chromatographia*, 39(9/10), 539-542.
5. Luo Y, Uboh CE, Soma LR, Guan F, Rudy JA, Tsang DS. (2005) Resolution, Quantification and Confirmation of Betamethasone and Dexamethasone in Equine Plasma by Liquid Chromatography/Tandem Mass Spectrometry. *Rapid Communcation in Mass Spectrometry*, 19, 825-832.
6. Dusi G, Gasparini M, Curatolo M, Assini W, Bozzoni E, Tognoli N, Ferretti E. (2011) Development and validation of a liquid chromatography-tandem mass spectrometry method for the simultaneous determination of nine corticosteroid residues in bovine liver samples. *Analytica Chimica Acta*, 700, 49-57.
7. Salem II, Alkhatib M, Najib N. (2011) LC-MS/MS determination of betamethasone and its phosphate and acetate esters in human plasma after sample stabilization. *Journal of Pharmaceutical and Biomedical Analysis*, 56,983-991.
8. Johnston SE, Gill NL, Wei YC, Markovich R, Rustum AM.(2010) Development and Validation of a Stability-Indicating RP-HPLC Method for Simultaneous Assay of Betamethasone Dipropionate, Chlorocresol,

and for the Estimation of Betamethasone Dipropionate Related Compounds in a Pharmaceutical Cream and Ointment. *Journal of Chromatographic Science*, 48, 733-741.

9. The United States Pharmacopeia. (2005) Rockville: The National Formulary, United States Pharmacopial Convention.
10. European Pharmacopeia (3rd Ed.). (2001).Strasbourg: Council of Europe.
11. Bagnati R, Ramazza V, Zucchi M, Simonella A, Leone F, Bellini A, Fanelli R. (1996) Analysis of Dexamethasone and Betamethasone in Bovine Urine by purification With an "On-Line" Immunoaffinity Chromatography-High- performance Liquid Chromatography System and determination by Gas Chromatography -Mass Spectrometry. *Analytical Biochemistry*, 235(2), 119-126.
12. Xiao KP, Xiong Y, Rustum AM. (2008) Quantitation of Trace Betamethasone or Dexamethasone in Dexamethasone or Betamethasone Active Pharmaceutical Ingredients by reversed-Phase High-Performance Liquid Chromatography. *Journal Chromatographic Science*, 46, 15-22.
13. Quintana M. C., Blancoa M. H., Lacala J., Hernándeza L. (2001) Analysis of Pharmaceutical Residues In Bovine Liver By HPLC. *J. Liq. Chrom. & Rel. Technol,* 24(5), 735-745.
14. Samtani M.N., Schwab M., Nathanielsz P.W., Jusko W.J. (2004) Stabilization and HPLC Analysis of betamethasone sodium phosphate in plasma. *J. Pharm. Sci*, 93, 726–732.
15. Wulandari L, Indrayanto G. (2003) HPTLC Determination Betamethasone in Tablets and its Validation. *Journal of Liquid Chromatography and Related Technologies*, 26(16), 2709-2717.
16. Jeyaseelan, C., Joshi A. (2002) Trace determination of dexamethasone sodium phosphate in pharmaceutical formulations by differential pulse polarography. *Analytical and Bioanalytical Chemistry*, 373(8), 772-776.
17. Balaji K., Raghunadha Reddy G. V., Madhusudana Reddy T., Jayarama Reddy S. (2008) Determination of prednisolone, dexamethasone and

hydrocortisone in pharmaceutical formulations and biological fluid samples by voltammetric techniques using β-cyclodextrin modified carbon paste electrode. *African Journal of Pharmacy and Pharmacology,* 2(8), 157-166,

18. McEwen I, Elmsjö A, Lehnström A, Hakkarainen B, Johansson M. (2012) Screening of counterfeit corticosteroid in creams and ointments by NMR spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis*, 70, 245–250.
19. Kalpana J., Shivakumar T. Anbarasu C. (2011) Development & Validation of an *in Vitro* Dissolution Method With HPLC Analysis for Betamethasone Acetate and Betamethasone Sodium Phosphate in Formulated Dosage Form. *IJPSR*, 2(7), 1869-1874.
20. Awen B., Hwisa N., Chandu B., Nama S., Katakam P.(2010) Development and validation of a new spectrophotometric method for the determination of betamethasone in bulk and pharmaceutical dosage forms. *Int J Pharm Biomed Res* 2010, *1*(3), 87-89.
21. Miolo G, Gallocchio F, Levorato L, Dalzoppo D, Beyersbergen van Henegouwen G.M., Caffieri S. (2009) UVB photolysis of betamethasone and its esters: characterization of photoproducts in solution in pig skin and in drug formulations. *J Photochem Photobiol B.*, 96(1), 75-81.
22. Amin A. S., Issa Y. M. (1997) Extraction-Spectrophotometric Method for the Determination of Betamethasone in Pure Form and in Pharmaceutical Formulations. *Analytical Letters*, 30(1), 69-78.
23. Iglesias Y, Fente C, Vazquez B.I, Franco C, Cepeda A, and Mayo S (2002). Application of the luminal chemiluminesence reaction for the determination of nine corticosteroids, *Anal.Chem.Acta.,* 468(1), 43-52.
24. Noe S, Bholer J, Keller E, Frahme A.W (1998) Evaluation and optimisation of separation buffers for the determination of corticosteroids with micellar electrokinetic capillary chromatography (MECC). *J.Pharm.Biomed.Ana.,* 18 (4 - 5), 911-918.

25. Rodchenkov G.M., Uralets V.P., Semenov V.A., Gurevich V.A. (1988) Gas Chromatographic and mass spectral study of betamethasone synthetic corticosteroid metabolism, *J. Chromatogr*, 432, 283–289.
26. Watson DG, McGhee CN, Midgley JM, Dutton GN, Noble MJ. (1990) Penetration of topically applied betamethasone sodium phosphate into human aqueous humour, *Eye (Lond.)*, 4, 603–606.
27. Tang PW, Law WC, Wan TS. (2001) Analysis of corticosteroids in equine urine by liquid chromatography - mass spectrometry. *Journal Chromatography B*, 754,229-244.
28. Van Den Hauwe O, Perez JC, Claereboudt J, Van Peteghem C. (2001) Simultaneous Determination of Betamethasone and Dexamethasone Residious in Bovine Liver by Liquid Chromatography Tandem Mass Spectrometry. *Rapid Communcation in Mass Spectrometry*, 15, 857-861.
29. Sangiorgi E.,Curatolo M., Assini W., Bozzoni E. (2003) Application of neutral loss in liquid chromatography-mass spectrometry for the determination of corticosteroids in bovine urine. *Analytica Chimica Acta*, 483, 259-267.
30. Van den hauwe, O., Dumoulin, F., Antignac, J.P., Bouche, M.P., Elliott, C., Van Peteghem, C. (2002) Liquid chromatographic-mass spectrometric analysis of 11 glucocorticoid residues and an optimization of enzymatic hydrolysis conditions in bovine liver. *Analytica Chimica acta*, 473, 127-134.
31. Gaillard Y, Vayssette F, Pépin G. (2000) Compared interest between hair analysis and urinalysis in doping controls Results for amphetamines, corticosteroids and anabolic steroids in racing cyclists. *Forensic Science Internationa*l, 107, 361-379.
32. Cui X, Shao B, Zhao R, Yang Y, Hu J, Tu X. (2006) Simultaneous determination of seventeen glucocorticoids residues in milk and eggs by ultra-performance liquid chromatography Electrodpray Tandem Mass Spectrometry. *Rapid Communication in Mass Spectrometry*, 20, 2355-2364.

33. Herrero P, Borrull F, Pocurull E, Marcé RM. (2012) Determination of glucocorticoids in sewage and river waters by ultra-high performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, 1224, 19-26.
34. Chen D, Tao Y, Liu Z, Zhang H, Liu Z, Wang Y, Huang L, Pan Y, Peng D, Dai M, Wang X, Yuan Z. (2011) Development of a liquid chromatography-tandem mass spectrometry with pressurized liquid extraction for determination of glucocorticoid residues in edible tissues. *Journal of Chromatography B*, 879,174-180.
35. Nam YS, Kwon IK, Lee Y, Lee KB.(2012) Quantitative monitoring of corticosteroids in cosmetic products manufactured in Korea using LC-MS/MS. *Forensic Science International*, 220, 23-28.
36. Separation Science Redefined (2005)The Use Of Acquity UPLC In Pharmaceutical Development, Web Link: ***www.chromatographyonline.com***
37. Dongre VG, Karmuse PP, Rao PP, Kumar A. (2008). Development and validation of UPLC method for determination of primaquine phosphate and its impurities. *J. Pharm.Biomed. Anal*, 46, 236-242.
38. Lakshmi Narasimham Y.S., Barhate V.D. (2010) Development and validation of stability indicating UPLC method for the simultaneous determination of beta-blockers and diuretic drugs in pharmaceutical dosage forms. *J. Chem. Metrol*, 4(1), 1-20.
39. Swartz, M. E. (2005). UPLC: An Introduction and Review. *J. Liq. Chrom. & Rel. Technol*, 28, 1253-1263.
40. Arthur KE, Wolff JC, Carrier DJ. (2004). Analysis of betamethasone, dexamethasone and related compounds by liquid chromatography electrospray mass spectrometry. *Rapid Commun Mass Spectrom*, 18, 678–68.
41. Nam YS, Kwon IK, Lee Y, Lee KB.(2012) Quantitative monitoring of corticosteroids in cosmetic products manufactured in Korea using LC-MS/MS. *Forensic Science International*, 220, 23-28.

42. Gaudiano MC, Lucente D, Antoniella E, Bertocchi P, Muleri N, Manna L, Bartolomei M, Alimonti S, Valvo L, Rodomonte AL. (2010) For export only medicines come back to Europe: A RP-LC method for the screening of six glucocorticoids in illegal and counterfeit anti-inflammatory and lightening creams. *Journal of Pharmaceutical and Biomedical Analysis*, 53, 158-164.
43. Fu Q, Shou M, Chien D, Markovich R, Rustum AM. (2010) Development and validation of a stability-indicating RP-HPLC method for assay of betamethasone and estimation of its related compounds. *Journal of Pharmaceutical and Biomedical Analysis*, 51, 617-625.
44. Zou JJ, Dai L, Ding L, Xiao DW, Bin ZY, Fan HW, Liu L, Wang GJ. (2008) Determination of betamethasone and betamethasone 17-monopropionate in human plasma by liquid chromatography-positive negative electrospray ionization tandem mass spectrometry. *Journal of Chromatography B*, 873, 159-164.
45. Lu J, Wei Y, Rustum AM. (2010). A stability-indicating reversed-phase high performance liquid chromatography method for simultaneous assay of two corticosteroids and estimation of their related compounds in a pharmaceutical injectable formulation. *Journal of Chromatography A*, 1217, 6932-6941.
46. Chen Q, Zielinski D, Chen J, Koski A, Werst D, Nowak S.(2008) A validated, stability-indicating HPLC method for the determination of dexamethasone related substances on dexamethasone-coated drug-eluting stents. *Journal of Pharmaceutical and Biomedical Analysis*, 48, 732-738.
47. Peña-García-Brioles D, Gonzalo-Lumbreras R, Izquierdo-Hornillos R, Santos-Montes A. (2004) Method Development for betamethasone and dexamethasone by micellar liquid chromatograpy using acetyl trimethyl ammonium bromide and validation in tablets Application cocktails. *Journal of Pharmaceutical and biomedical Analysis*, 36, 65-71.
48. Touber ME, van Engelen MC, Georgakopoulus C, van Rhijn JA, Nielen MW. (2007) Multi -detection of corticosteroids in sports doping and

veterinary control using high-resolution liquid chromatography /time-of-flight mass spectrometry. *Analytica Chimica Acta*, 586, 137-146

49. Wong CH, Tang FP, Wan TS. (2011) A broad-spectrum equine urine screening method for free and enzyme-hydrolysed conjugated drugs with ultra performance liquid chromatography/tandem mass spectrometry. *Analytica Chimica Acta*, 697, 48-60.
50. Ortelli D, Cognard E, Jan P, Edder P. (2009) Comprehensive fast multiresidue screening of 150 veterinary drugs in milk by ultra-performance liquid chromatography coupled to time of flight mass spectrometry. *Journal of Chromatography B*, 877, 2363-2374.
51. Deceuninck Y, Bichon E, Monteau F, Antignac JP, Le Bizec B. (2011) Determination of MRL regulated corticosteroids in liver from various species using ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC). *Analytica Chimica Acta,* 700**,** 137-143.
52. Becue I, Van Poucke C, Van Peteghem C. (2011) The use of library identification and common fragments for the identification of corticosteroids in forensic samples" *Anal Bioanal Chem*, 400, 2837–2846.
53. Batt AL, Kostich MS, Lazorchak JM. (2008) Analysis of Ecologically Relevant Pharmaceuticals in Waster Water and Surface Water Using Selective Solid-Phase Extraction and UPLC-MS/MS. *Anal. Chem,* 80, 5021-5030.
54. Theodore M. Brody, Joseph Larner and Kenneth P. Minneman. (1991) Human Pharmacology: molecular to Clinical (International student Ed.). New York. Mosby by Wolf Publishing limited.
55. Cedric M. Smith, Alan M. Reynard. (1992) Text Book of Pharmacology (1^{st}Ed.). United Kingdom: W.B.Saunders.
56. Hardman JG, Limbird LE, Gilman AG. (2001).GOODMAN & GILMAN`s Pharmacological Basis of Therapeutics (10^{th} ed.). United State of America: McGraw-Hill.
57. C. J. van Boxtel, B. Santoso & I. R. Edwards (2001).Drug benefits and risks: International textbook of clinical pharmacology .UK: John Wiley & sons.

58. Sir Colin Dollery. (1991). Therapeutic Drugs (Vol.1).UK: Churchill Livingstone.
59. Minneman Kenneth P., Wecker L. (2005). Brody`s Human Pharmacology: Molecular to Clinical (4th Ed.) Usa: Elsevir Mobsy.
60. The Merk Index. (1996).New Jeresy: Merk and Co. Inc.
61. Brady C. (1994). Analytical Chemistry (5th edition). UK: John Wiley & sons.
62. Skoog D.,West. D., Holler,F. J. (1998).Principles of Instrumental Analysis(5th Ed.) Brooks Cole.
63. Issaq Haleem J. (2002). A Century of Separation science (E-Book) New York: Marcel Dekk
64. Yuri Kazakrvich and Rosario Lobrutto (2007).HPLC for Pharma ceutical Scientists. New Jersey: John Wiley & sons.
65. Robert J Flanagan, Andrew Taylor, Ian D Watson, Robin Whelpton (2007). Fundamentals of Analytical Toxicology. England: John Wiley and sons Inc.
66. Satinder Ahuja. (2003). Chromatography and Separation Scince (V.4),USA: Elsevir Science
67. James M. Miller. (2005).Chromatography: Concepts and Contrasts.(2nd Ed)John Wiley and sons inc. Canada
68. Andera Weston and Phyllis R.Brown. (1997). HPLC and CE Principls And Practice.W.B.Saundars company,California.
69. David G. Watson. (2005). Pharmaceutical Analysis (2nd Ed) Elsevier Health Sciences,Canada
70. Raymond P. W. Scot. (1996). Chromatographic Detectors Design, Function, and Operation). Chromatografic scince seris v.73, Marcel Dekker, Inc, New York
71. Jerkovich Anton D., LoBrutto R., Vivilecchia R. (2005) The use of ACQUITY UPLC in Pharmaceutical Development. SeparationScienceRedefined, 215-217. **ww.chromatographyonline.com**

72. Moffat A.C. (1986) Clarke's Isolation and Identification of Drugs: In Pharmaceuticals, Body Fluids and Post Mortem Material. (2nd Ed.) London : The Pharmaceutical Press
73. Wilfried M.A. Niessen. (2006). Liquid Chromatography–Mass Spectrometr(3rd edtion). London: Taylor and Francis,
74. Fluri K, Rivier L, Dienes-Nagy A, You C. , Maître A. , Schweizer C. Saugy M., Mangin P. (2001) Method for confirmation of synthetic corticosteroids in doping urine samples by liquid chromatography-electrospray ionization mass spectrometry. *Journal of Chromatography A*, 926.
75. Polettini A, Marrubini Bouland G, Montagna M. (1998) Development of coupled-column liquid chromatography-tandem mass spectrometric method for the direct determination of betamethasone in urine. *Journal of Chromatography B*, 713, 339-352.
76. Andersen JH, Hansen LG, Pedersen M. (2008) Optimization of solid phase extraction clean up and validation of quantitative determination of corticosteroids in urine by liquid chromatography-tandem mass spectrometry. *Analytic Chimica Acta*, 617, 216-224.
77. Georgakopoulos CG, Vonaparti A, Stamou M, Kiousi P, Lyris E, Angelis YS, Tsoupras G, Wuest B, Nielen MW, Panderi I, Koupparis M.(2007) Preventive doping control analysis: liquid and gas chromatography time-of-flight mass spectrometry for detection of designer steroids. *Rapid Communcations in Mass Spectrometry*, 21, 2439-2446.
78. Mazzarino M, Botrè F (2006) A Fast liquid chromatographic mass spectrometric screening method for the simultaneous detection of synthetic glucocorticoids, some stimulants,anti-oestrogen drugs and synthetic anabolic steroids. *Rapid Communcation in Mass Spectrometry*, 20, 3465-3476.
79. De Wasch K, De Brabander HF, Van de Wiele M, Vercammen J, Courtheyn D,Impens S. (2001) Differentiation between dexamethasone andbetamethasone in a mixture using multiple mass spectrometery. *Journal of Chromatography A*, 926, 79-86.

80. Flood KG, Reynolds ER, Snow NH. (2000) Characterization of inclusion complexes of betamethasone-related steroids with cyclodextrines using high-performance liquid chromatography. *Journal of Chomatography A*, 903, 49-65.
81. Deventer K, Delbeke FT. (2003) Validation of screening method for corticosteroids in doping analysis by liquid chromatography/tandem mass spectrometry. *Rapid Communication in Mass Spectrometry*, 17, 2107-2114.
82. Ortelli D, Cognard E, Jan P, Edder P. (2009) Comprehensive fast multiresidue screening of 150 veterinary drugs in milk by ultra-performance liquid chromatography coupled to time of flight mass spectrometry. *Journal of Chromatography B*, 877, 2363-2374.
83. Deceuninck Y, Bichon E, Monteau F, Antignac JP, Le Bizec B. (2011) Determination of MRL regulated corticosteroids in liver from various species using ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC). *Analytica Chimica Acta*, 700, 137-143.
84. MaloneE.M.,Dowling G., Elliott C.T., Kennedy D.G., Regana L. (2009) Development of a rapid, multi-class method for the confirmatory analysis of Anti-inflammatory drugs in bovine milk using liquid chromatography tandem mass spectrometry. *Journal of Chromatography A*, 1216, 8132-8140.
85. Cirimele V, Kintz P, Dumestre V, Goullé JP, Ludes B. (2000) Identification of ten corticosteroids in human hair by liquid chromatography-ionspray mass spectrometry. *Forensic Science International*, 107, 381-388.
86. Sun, H. Kang, Z. Li, H. Zhang, J. Lv, Y. (2012) Quantitative Determination and Confirmation of Five Synthetic Glucocorticoid Residues in Milk Powder by Gel Permeation Chromatography-Liquid Chromatography-Tandem Mass Spectrometry. *Food Anal. Methods*, 5, 643-650.
87. Li C, Wu Y, Yang T, Zhang Y. (2010) Rapid simultaneous determination of dexamethasone and betamethasone in milk by liquid chromatography

tandem mass spectrometry with isotope dilution. *Journal of Chromatography A*, 1217, 411-414.

88. Tölgyesi A, Sharma VK, Fekete S, Lukonics D, Fekete J. (2012) Simultaneous determination of eight corticosteroids in bovine tissues using liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B*, 906, 75– 84.
89. Dikunets, M., Appolonova, S. Rodchenkov, G. (2010) Structure of chemical compounds, methods of analysis and process control identification of endogenous and exogenous glucocorticoids by HPLC-MS in human urine. *Pharmaceutical Chemistry Journal*, 44(6), 43-49
90. Becue I, Van Poucke C, Van Peteghem C. (2011) The use of library identification and common fragments for the identification of corticosteroids in forensic samples. *Anal Bioanal Chem*, 400, 2837–2846.
91. Taylor Robert L., Grebe Stefan K., Singha Ravinder J.(2004)Quantitative, Highly Sensitive Liquid Chromatography–Tandem Mass Spectrometry Method for Detection of Synthetic Corticosteroids. *Clinical Chemistry*, 50(12), 2345-2352.
92. ICH Committee S. (2005). Validation Of Analytical Procedure: Text And Methodology Q2 (R1) Harmonized Tripartite Guidelines.
93. Croes K., Goeyens L., Baeyens W., Van Loco J., Impen S.(2009) Optimization and validation of a liquid chromatography tandem mass spectrometry (LC/MS^n) method for analysis of corticosteroids in bovine liver: Evaluation of Keyhole Limpet ß-glucuronidase/sulfatase enzyme extract. *Journal of Chromatography B*, 877, 635–644.
94. Schanzer W., Geyer H., Gotzmmann A., Mareck U. (2003) Recent advances in doping analysis (11). Sport and Buch StauB, Kölen, 23-31.
95. M. Pendela, E. Adams, J. Hoogmartens. (2004). Development of a liquid chromatographic method for ear drops containing neomycin sulphate, polymyxin B sulphate and dexamethasone sodium phosphate. *Journal of Pharmaceutical and Biomedical Analysis*, 36 , 751–757

96. Novakova L., Matysova L, Solich P. (2006) Advantages of application of UPLC in pharmaceutical analysis. *Talanta*, 68, 908–918.
97. Roge A B., Firke S N., Dhane R M., Gunjkar V J., Vadvalkar S M.(2011)Novel Achievement of HPLC: UPLC. *International Journal of Pharm Tech Research*, 3(3), 1423-1429.

Appendix 1. Statistical coefficients calculations

- Relative Standard Deviation (RSD %) = $\frac{Standard\ \ deviation\ \ (S.D)}{Mean\ \ (\overline{x})}$ X 100

- Relative Error (RE %)

$$= \frac{true\ \ value\ \ of\ \ a\ quantity\ -observed\ \ value\ \ of\ \ the\ quantity}{true\ \ value\ \ of\ \ a\ quantity} \times 100$$

- Standard Error (SE) = $\frac{Standard\ \ Deviation\ (SD)}{\sqrt{Mesures\ \ Number\ (n)}}$

- Recovery % = $\frac{observed\ \ value\ \ of\ \ the\ quantity}{true\ \ value\ \ of\ \ a\ quantity} \times 100$

- Remained Amount % = $\frac{Concentation\ \ \ of\ \ the\ analyte\ \ solution\ \ \ after\ \ storage\ \ Time}{Concent\ ration\ \ of\ \ Fres\,hly\ \ prepared\ \ \ analye\ \ \ solution\ \ x\ 100}$ x 100

- Bias = True value - Measured mean value

Appendix 2.T-Test: Two-Sample Assuming Unequal Variances

T test testing if there significant difference between two means

- Standard error. Compute the standard error (SE) of the sampling distribution.

  $SE = sqrt[(s_1^2/n_1) + (s_2^2/n_2)]$

  where s_1 is the standard deviation of sample 1, s_2 is the standard deviation of sample 2, is the size of sample 1, and is the size of sample 2.

- Degrees of freedom. The degrees of freedom (DF) is: $n_1 + n_2 - 2$
- Test statistic. The test statistic is a t-score (t) defined by the following equation.

  $t = [(x_1 - x_2) - d] / SE$

  where x_1 is the mean of sample 1, x_2 is the mean of sample 2, d is the hypothesized difference between population means, and SE is the standard error.

P-value. The P-value is the probability of observing a sample statistic as extreme as the test statistic. Since the test statistic is a t-score, use the t Distribution Calculator to assess the probability associated with the t-score, having the degrees of freedom computed above.

State the hypotheses. The first step is to state the null hypothesis and an alternative hypothesis.

Null hypothesis: $\mu_1 - \mu_2 = 0$
Alternative hypothesis: $\mu_1 - \mu_2 \neq 0$

Note that these hypotheses constitute a two-tailed test. The null hypothesis will be rejected if the difference between sample means is too big or if it is too small.

IF $t_{calculated} > t_{table}$, $p < \alpha = 0.05$ then the null hypothesis will be rejected

IF $t_{calculated} < t_{table}$, $p > \alpha = 0.05$ then the null hypothesis will be Accepted

C.V

I was born in Al-Salt-Jordan in 1977; I had my B.Sc. degree in Chemistry from Al-Yarmouk University in 2000. After 3 years I was graduated from University of Jordan with a master degree inAnalytical Toxicology. I had jobs in area of analytical chemistrymainly inArab Pharmaceuticals ManufacturingCompany (2003-2004) and Jordanian ministry of Health (2005-2006). In 2004, I came to Ankara (Turkey) to learnTurkish language for 2 semesters. Then, in 2006 i was a Ph.D. student in analytical chemistry at Faculty of Pharmacy. In this year 2013 I will present my Ph.D. thesisto have the Ph.D degree.