ENANTIOSPECIFICITY OF POLYCLONAL ANTIBODIES RAISED AGAINST RACEMIC AND PURE ENANTIOMERS OF SALBUTAMOL AND THE IMPLICATIONS OF THEIR USE IN ENZYME IMMUNOASSAY

By

CHAN SUE HAY

Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy

February 2015

ACKNOWLEDGEMENT

I wish to convey my deepest gratitude to my supervisors Dr. Tan Soo Choon and Prof. Mohd. Zaini Asmawi for their constant advice, guidance, support, patience, and friendship throughout the course of this project. They have made the experience fulfilling and worth the while.

I would like to acknowledge the support of School of Pharmaceutical Sciences and the financial support provided by Universiti Sains Malaysia. I also wish to thank Veterinary Forensic Laboratory, Usains Biomics Sdn. Bhd., and Institute for Molecular Medicine (INFORMM) and staff for the use of their facilities and equipment during the tenure of my work. Also my deepest gratitude to Dr. Ramlan Mohamed and his staff from Veterinary Research Institute (VRI), Ipoh for enabling us to carry out the animal work at the VRI.

This project is completed with the unbelievable friendship, support and assistance of people who have made a difference in it. I would like to take this chance to thank Mr. Ng Chek Wan, Mr. Foo Thin Choon, Ang Chee Wei, Yeong Keng Yoon, Ms. Victoria Koay, Yeoh Seng Hoe, Fauziah Rastam, Jason Tan Yu Liang, Tan Yu Qian, Warren Lee, Mdm. Yeoh Nona, Mr. Goon, Dr. Mervyn Liew, Dr. Asaraf Ali, Dr. Soh Peng Kai, Lee Kee Keat, Chia Su Chien, and Tan Hsien Hui for their contributions. This work cannot be carried out without the friendship and support from colleagues and friends: Mr. Goh Jip Yin, Mr. Sivanandan Pillai, Khoo Hooi Tee, Emy Khoo, Wong Boon Keat, June Sim Mei Lin, Lye Jin Sean, Kuan Ya Shen, Sim Hann Liang, Ng Shy Yee, Karen Ong Bee Lian, Chua Hui Peen, Norasidah Rashit, Mohd. Abd. Muin, Pn. Azimah, Dr. Gurjeet Kaur, Siti Jannah Iman, and many others that have not been mentioned here.

Finally, I dedicate my work and achievement to my ever-supporting family and loved ones for their understanding and patience throughout the course of these studies. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS& SYMBOLS

Å	Angstrom
ADME	Absorption, distribution, metabolism, and excretion
AGP	α_1-Glycoprotein
AP	Alkaline phosphatase
ATP	Adenosine-5'-triphosphate
AUC	Area under the curve
BDDE	Butane-1,4-diol diglycidyl ether
BSA	Bovine serum albumin
cAMP	Cyclic adenosine-3',5'-monophosphate
COPD	Chronic obstructive pulmonary disorder
CSP	Chiral stationary phase
CV	Coefficient of variance
DMF	Dimethylformamide
DPI	Dry powder inhaler
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
fmoles	Femto moles
g	Gram
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometer
G_s	Guanine nucleotide regulatory protein
HAS	Human serum albumin
HCG	Human chorionic gonadotrophin
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HPTF	High performance tangential flow filtration
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IS	Internal standard

KCl	Potassium chloride
kDa	Kilo Dalton
KH_2PO_4	Potassium di-hydrogen phosphate
KLH	Keyhole limpet haemocyanin
KOH	Potassium hydroxide
L	Litre
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometer
LOD	Limit of detection
LOQ	Limit of quantification
MA	Mixed anhydride
MALDI	Matrix assisted laser desorption ionization
MDI	Metered dose inhaler
mg	Milligram
mL	Millilitre
mmole	Millimoles
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Di-sodium hydrogen phosphate
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate / sodium bicarbonate
NaOH	Sodium hydroxide
ng	Nanogram
$(NH_4)_2SO_4$	Ammonium sulphate
OD	Optical density
OVA	Ovalbumin
PAPS	3’-phosphoadenosine-5’-phosphosulphate
PBA	Phenylboronic acid
PEG	Polyethylene glycol
PST	Phenolsulphotransferase
RIA	Radioimmunoassay

RT	Room temperature
SD	Standard deviation
SPE	Solid-phase extraction
SULT	Sulphotransferase
TEA	Triethylamine
TG	Thyroglobulin
TMB	3,3',5,5'-tetramethylbenzidine
TMCS	Trimethylchlorosilane
TOF	Time of flight
UDP	Uridine-5'-diphospho-
UHQ	Ultrapure water
WADA	World Anti-Doping Agency
β	Beta
µg	Microgram
µL	Microlitre

GLOSSARY OF TERMINOLOGIES FOR CHIRALITY

Achiral	A molecule that is superimposable on its mirror image and has at least one plane of symmetry.
Antipode	Synonym of enantiomer.
Asymmetric carbon atom	A carbon atom that has four different atoms/groups/ligands attached.
Chiral	Having the property of chirality. A chiral molecule is a molecule that is not superimposable on its mirror image. It has no plane of symmetry.
Chiral centre	A tetrahedral atom in a molecule bearing four different ligands. Lone pair of electrons is treated as ligands. If a chiral centre is a carbon atom, it can also be called an asymmetric carbon. Synonym: chiral atom, chirality centre, and centre of chirality.
Chirality	A fundamental property of three-dimensional objects.
Constitutional isomers	Compounds with the same molecular formula but different structural formulas. Synonym: Structural isomers
Diastereomers	Stereoisomers that are not mirror images of each other. Cis- and trans- isomers are a subset of diastereomers. All cis- and trans-isomers are diastereomers but not all diastereomers are of the cis- and trans- orientation.
Enantiomer	One of a pair of molecule entities that are related to each other by a reflection. They are mirror images of each other and non-superimposable.
Enantiomeric ratio	The ratio of the percentage of one enantiomer in a mixture to that of the other, e.g. 70(+):30(-).
Eudismic ratio	The difference in pharmacologic activity between two enantiomers of a drug.
Optical activity	A sample of material able to rotate the plane of polarisation of a beam of transmitted plane-polarised light is said to possess optical activity (or to be optically active). This optical rotation is the classical distinguishing characteristic (sufficient but not necessary) of systems containing unequal amounts of corresponding enantiomers. An enantiomer causing rotation in a clockwise direction (when viewed in the direction facing the oncoming light beam) under specified conditions is called dextrorotatory and its chemical name or formula is designated by the prefix (+)-; one causing rotation in the opposite sense is laevorotatory and designated by the prefix (-)-. Materials with optical activity also exhibit other chiroptic phenomena.
Racemic/racemate	An equimolar mixture of a pair of enantiomers. It does not exhibit optical activity. The chemical name or formula of a racemate is distinguished from those of the enantiomers by the prefix (-)- or (+)- or *rac*- (*racem*-) or by the symbols *RS* and *SR*.

Stereochemistry	A subdiscipline of chemistry involving the study of the relative spatial arrangement of atoms that form the structure of molecules and their manipulation. An important branch of stereochemistry is the study of chiral molecules. Stereochemistry is also known as 3D chemistry because the prefix 'stereo' means 'three-dimensionality'.
Stereoisomer	Compounds with the same molecular formula and the same structural formula but different from each other in their three-dimensional configuration of their atoms in space.
Stereoselectivity	The preferential formation in a chemical reaction of one stereoisomer over another. When stereoisomers are enantiomers, the phenomenon is called enantioselectivity and is quantitatively expressed by the enantiomer excess. When they are diastereomers, it is called diastereoselectivity and is quantitatively expressed by the diastereomer excess.
Stereospecificity	A reaction is termed stereospecific if starting materials differing only in their configuration are converted into stereoisomeric products. According to this definition, a stereospecific process is necessarily stereoselective but not all stereoselective processes are stereospecific.

KESPESIFIKAN ENANTIOMER ANTIBODI POLIKLONAL YANG DIBANGUNKAN TERHADAP ENANTIOMER RASEMIK DAN ENANTIOMER TULEN SALBUTAMOL SERTA IMPLIKASI PENGGUNAANNYA DALAM IMUNOASAI ENZIM

ABSTRAK

Salbutamol (albuterol) adalah agonis β_2-adrenergik yang popular digunakan dalam rawatan asma dan gangguan obstruktif pulmonari kronik (COPD). Salbutamol biasanya disalahgunakan sebagai peningkat prestasi dalam sukan serta penggalak tumbesar yang berkesan pada ternakan. Pengawasan terhadap penyalahgunaan salbutamol bergantung kepada kejayaan pelaksanaan kaedah penapisujian air kencing untuk mengesan bahan tersebut di mana immunoasai enzim memainkan peranan penting. Oleh kerana kecenderungan untuk menukar kepada enantiomer tunggal sebagai ajen terapeutik yang lebih selamat dan berkesan, (*R*)-salbutamol telah diperkenalkan untuk kegunaan terapeutik pada manusia serta sebagai penggalak tumbesar dalam perubatan veterinar. Pertukaran ini menimbulkan tanda tanya tentang penggunaan imunoasai tradisional yang menggunakan antibodi rasemik (*RS*)-salbutamol. Kereaktifan-silang dan keterpilihan-enantio antibodi menggunakan immunogen yang disintesiskan melalui kaedah campuran anhidrid (MA) dan pengaktifan epoksi (BDDE) pada arnab, telah dikaji. Antibodi-antibodi menunjukan keterpilihan-enantio samaada terhadap (*R*)-salbutamol atau (*S*)-salbutamol bergantung kepada individu haiwan, jadi antibodi-antibodi ini tidak sesuai digunakan dalam pemantauan aras (*R*)-salbutamol. Sehubungan itu, tiga jenis antibodi enantio-khusus menggunakan (*R*)- dan (*S*)-salbutamol sebagai hapten juga telah dihasilkan. Antibodi (*R*)-salbutamol menunjukkan kereaktifan-silang sebanyak 3.94-7.13% terhadap antipodnya. Manakala antibodi (*S*)-salbutamol menunjukkan kereaktifan silang sebanyak 3.28-5.25% terhadap enatiomer-(*R*). Antibodi-antibodi ini berpotensi digunakan untuk pemantauan aras setiap individu enantiomer dalam air kencing. Sampel air kencing khinzir yang dianalisis menggunakan kaedah ELISA menunjukkan 17.71% sampel positif palsu dan 0% sampel

negatif palsu, apabila dibandingkan dengan kaedah kiral LC-MS/MS yang disahkan. Sampel positif air kencing khinzir didapati kandungan utmanya adalah (*S*)-salbutamol, sekaligus mencadangkan bahawa penyingkiran salbutamol adalah secara stereo-terpilih kepada (*S*)-salbutamol. Empat sampel air kencing dari ekuin (kuda) dan dua sampel dari manusia yang dikenalpasti positif mengandungi salbutamol juga dianalisa. Keputusan menunjukkan proses metabolisme utama salbutamol pada ekuin adalah melalui proses glukuronidasi, manakala pada manusia melalui proses sulfasi. Metabolit glukuronida dan sulfat diasingkan daripada air kencing ekuin dan manusia, masing-masing. Metabolit sulfat sangat rintang terhadap hidrolisis enzim β-glukuronidase/arilsulfatase dan keadaan piawai hidrolisis asid. Sejumlah metabolit sulfat diasingkan dari sel HepG2 yang dieramkan dengan salbutamol. Metabolit glukuronida dan sulfat yang diasingkan kemudiannya digunakan untuk mencirikan kereaktifan-silang antibodi rasemik salbutamol. Kereaktifan-silang antibodi terhadap glukuronida adalah 358.06% dan 227.26% untuk kedua-dua antibodi MA dan BDDE, masing-masing. Walau bagaimanapun, kereaktifan-silang antibodi-antibodi tersebut terhadap metabolit sulfat adalah jauh lebih rendah iaitu sebanyak 20.72% dan 23.81% bagi kedua-dua antibodi MA dan BDDE. Penemuan mengenai metabolit ini menimbulkan pertanyaan tentang kajian farmakologi dan analitikal sebelumnya yang mengandaikan bahawa metabolit sulfat salbutamol dihidrolisiskan sepenuhnya oleh enzim β-glukuronidase/arilsulfatase dan kereaktifan-silang metabolit-metabolit adalah 100% dengan antibodi poliklonal.

ENANTIOSPECIFICITY OF POLYCLONAL ANTIBODIES RAISED AGAINST RACEMIC AND PURE ENANTIOMERS OF SALBUTAMOL AND THE IMPLICATIONS OF THEIR USE IN ENZYME IMMUNOASSAY

ABSTRACT

Salbutamol (albuterol) is a β_2-adrenergic agonist popularly used in the treatment of asthma and chronic obstructive pulmonary disorder (COPD), and is also commonly abused as a performance enhancer in sports as well as an effective growth promoter in livestock. The monitoring of salbutamol abuse relies on the successful implementation of urinary screening methods to detect the substance of which enzyme immunoassay plays an important role. With the trend to switch to single enantiomers as safer and more effective therapeutic agents, (*R*)-salbutamol has been introduced for use in human therapeutics as well as in veterinary medicine as a growth promoter. This switch calls into question the usefulness of traditional salbutamol immunoassays which use antibodies based on a racemic (*RS*)-salbutamol. The cross-reactivity and enantioselectivity of antibodies using immunogens synthesized via the mixed anhydride (MA) and epoxy activation (BDDE) methods in rabbits were investigated. The antibodies showed enantioselectivity either towards (*R*)-salbutamol or (*S*)-salbutamol depending on the individual animal and these antibodies are not ideal to monitor (*R*)-salbutamol levels. Three types of enantiospecific antibodies using (*R*)- and (*S*)-salbutamol as hapten were also raised. The (*R*)-salbutamol antibodies displayed cross-reactivity of 3.94-7.13% towards the antipode. The (*S*)-salbutamol antibodies showed 3.28-5.25% cross-reactivity towards the (*R*)- enantiomer. These antibodies would be potentially useful to monitor the individual enantiomer levels in urine. Porcine urine samples analysed using the ELISA method demonstrated 17.71% false positives and 0% false negative when compared to a validated chiral LC-MS/MS method. The positive porcine urine samples showed predominance of (*S*)-salbutamol concentration suggesting that the free drug clearance is stereoselective for (*S*)-salbutamol. Four known positive equine and two known human

positive samples were also analysed. The results revealed that equine metabolism is principally via glucuronidation and that the human is mainly via sulphation. The glucuronide and sulphate metabolites were isolated from equine and human urine respectively. The sulphate metabolite was extremely resistant towards hydrolysis by β-glucuronidase/arylsulphatase and standard acid hydrolysis conditions. Quantifiable amounts of the sulphate metabolite were isolated from HepG2 cells incubated with salbutamol. The isolated glucuronide and sulphate metabolites were then used to characterize the cross-reactivity of the racemic salbutamol antibodies. The cross-reactivities against the glucuronide were 358.06% and 227.26% for the MA and BDDE antibodies respectively. However, the cross-reactivity for the sulphate was much lower at 20.72% and 23.81% for the MA and BDDE. These findings on the metabolites raises questions on previous pharmacological, and analytical studies which had assumed that the sulphate metabolite to be fully hydrolysed using β-glucuronidase/arylsulphatase and the cross-reactivities of the metabolites to be 100% with the polyclonal antibodies.

CHAPTER 1:

INTRODUCTION

1.1 A brief history of salbutamol

Salbutamol (Albuterol) is chemically known as 4-[2-(tert-butylamino)-1-hydroxyethyl]-2-(hydroxymethyl)phenol (Figure 1.1). To date, it is the most popular bronchodilator drug used for the treatment of asthma and better known by its brand name, Ventolin.

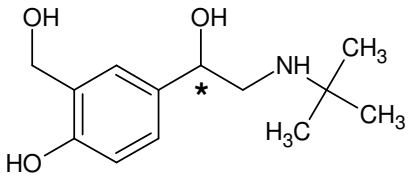
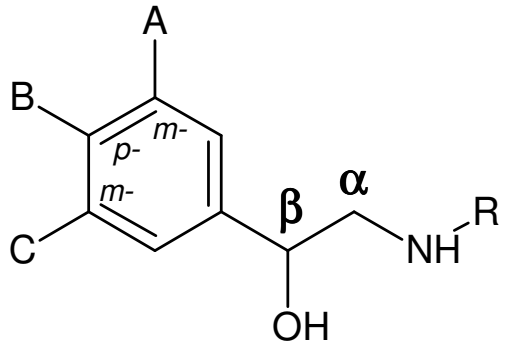


Figure 1.1: The molecular structure of salbutamol (albuterol) or chemically known as 4-[2-(tert-butylamino)-1-hydroxyethyl]-2-(hydroxymethyl)phenol. The chiral centre of the molecule is marked with *.

Salbutamol was first invented by David Jack and his team of researchers in 1966 and patented by Allen and Hansburys (Glaxo Group Research) in 1969 (Marasco, 2005) in response to finding a solution for the increasing mortality rate by isoprenaline users in the 1960s. The introduction of isoprenaline in aerosol form then, caused an estimated 3,000 deaths among asthmatic teenagers in the United Kingdom (Sneader, 2005). Their objective of producing a stable, safer, and long-acting analogue of isoprenaline led to the development of saligenin and then salbutamol. Salbutamol fulfilled their objectives as a longer-acting bronchodilator that acted selectively on bronchial muscle with minimal cardiovascular adverse effects (Cullum *et al.*, 1969; and Kennedy and Simpson, 1969).

1.2 Chemistry

Salbutamol is one of the bronchodilators grouped as β-agonists or phenethanolamine β-adrenergic agonists. These group of drugs conform to the general structure of a six-membered aromatic ring with hydroxyl group(s) bound to the β-carbon, nitrogen that is positively charged at physiological or acidic pH found in the ethylamine side chain, and bulky substituent (marked as R; Figure 1.2) on the aliphatic nitrogen (Figure 1.4). The specificity of the drugs for the β-adrenoceptor is marked by the bulky substituent (Weiner, 1980). As such this bulky substituent is not only to β-agonists but also the endogenous adrenergic neurotransmitters epinephrine and norepinephrine (Figure 1.3; Smith, 1998).



Aromatic Substitution			Category	Examples
A	**B**	**C**		
-H	-OH	-H	Phenol	Ractopamine Ritodrine
-OH	-H	-OH	Resorcinol	Fenoterol Terbutaline
-OH	-OH	-H	Catechol	Isoproterenol Dobutamine
$-CH_2OH$	-OH	-H	Saligenin	Salbutamol Salmeterol

Figure 1.2: The general structure of a phenethanolamine β-agonist and a list of the common substitution groups found on the aromatic ring and the bulky substituent (R) on the aliphatic nitrogen. The R group is usually a t-butyl group, isopropyl group, alkylphenyl, or alkylphenol. The *para*- (*p*-) and *meta* (*m*-) positions on the aromatic ring relative to the phenethanolamine β-carbon are marked (adapted from Smith, 1998).

i) ii)

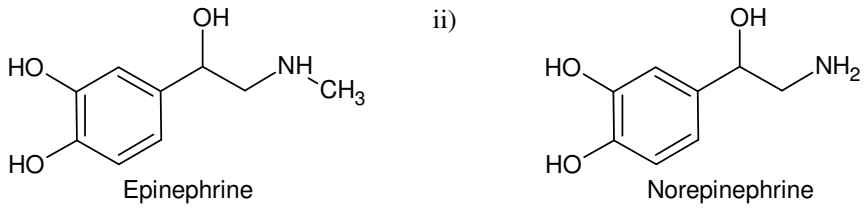


Figure 1.3: The molecular structures of the endogenous adrenergic neurotransmitters: i) epinephrine and ii) norepinephrine.

Table 1.1: The analogues of phenethanolamine β-adrenergic agonists grouped according to their categories.

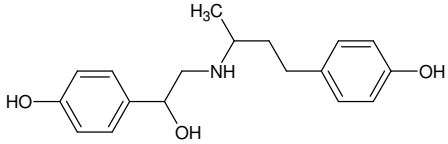
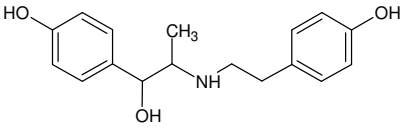
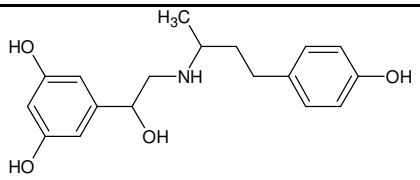
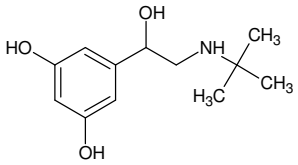
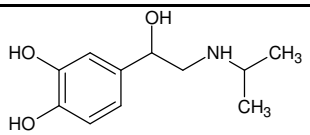
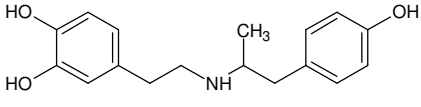
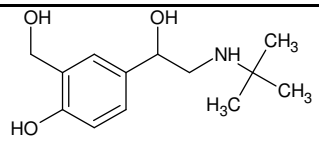
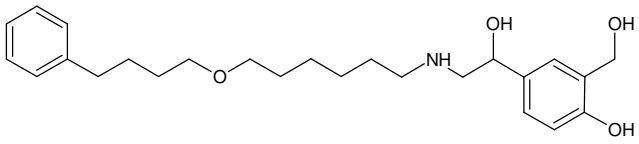
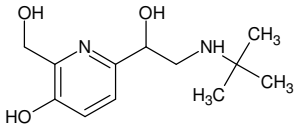
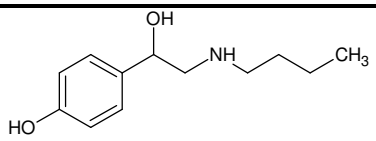
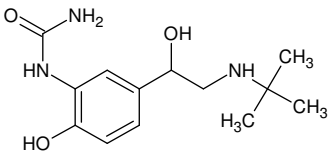
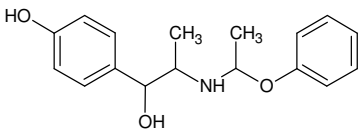
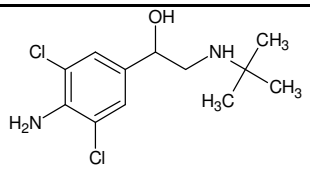
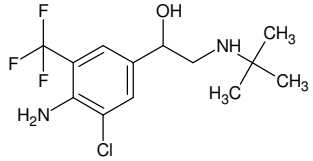
Category	Drug name	Structure
Phenol	Ractopamine	
	Ritodrine	
Resorcinol	Fenoterol	
	Terbutaline	
Catechol	Isoproterenol	
	Dobutamine	

Table 1.1: Continued.

Category	Drug name	Structure
Saligenin	Salbutamol	
	Salmeterol	
	Pirbuterol	
Monophenols	Bamethan	
	Carbuterol	
	Isoxsuprine	
Miscellaneous	Clenbuterol	
	Mabuterol	

Salbutamol (Figure 1.1) with a molecular weight of 239.31, is usually prepared in the form of a sulphate salt named salbutamol sulphate (molecular weight 576.70). It is approximately 11 Å long (Smith, 1998) with almost white crystalline powder that is freely soluble in water but not methylene chloride or ethanol (British Pharmacopoeia, 2012). The aliphatic amine present gives it pK_a values of 9.3 and 10.3 (Smith, 1998; and Shen *et al.*, 2012). A single chiral centre gives rise to two enantiomers: (*R*)-salbutamol and (*S*)-salbutamol (Figure 1.4). Planar polarised light rotation found that (*R*)-salbutamol had the (-)- configuration and (*S*)-salbutamol the (+)- orientation (Hartley and Middlemiss, 1971). However, current therapeutic drug preparations are racemates (equal proportion of both enantiomers), with the exception of Xopenex® which constitutes only (*R*)-salbutamol as its active ingredient.

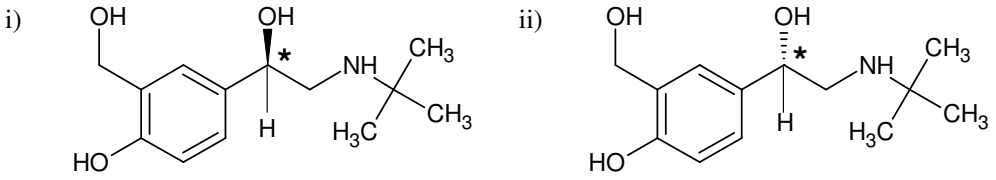


Figure 1.4: Molecular structure of i) (*R*)-salbutamol and ii) (*S*)-salbutamol. The chiral centre is marked with *.

1.3 Pharmacology

The bronchodilator group of drugs called β-agonists is made up of salbutamol, clenbuterol, terbutaline, salmeterol, formoterol, ractopamine, cimaterol, and many others. They act upon the β_2-adrenoceptors in the smooth muscles of the bronchial airways to bring about bronchodilation. These drugs are grouped into short- and long-acting β-agonists. Short-acting β-agonists exert their effects immediately within 3-5 minutes and last for 4-6 hours, whereas long-acting β-agonists are maintenance drugs lasting 12 hours. The long-acting β-agonists wield their effects 20 minutes after administration and its 12 hour protection allow

people with chronic obstructive pulmonary disease (COPD) to sleep better at night albeit less frequent usage (Table 1.2; American Thoracic Society).

Bronchodilators mimic the actions of sympathetic adrenergic stimulation acting through β-adrenoceptors. Activation of these receptors relaxes the bronchial smooth muscles, stimulates glycogenolysis in the liver, release rennin from the kidneys, and increases heart-rate. The β-adrenoceptor embedded in the cell plasma membrane is a single polypeptide glycoprotein moiety. It exists as three subtypes: β_1 (cardiac tissues), β_2 (respiratory tract), and β_3 (found on adipose tissues) (Fernandes *et al.*, 2004).

Beta-agonists interact with the β_2-adrenoceptors on the smooth muscle tissues and subsequently activate the intracellular signalling cascade through the membrane bound adenylyl cyclase enzyme (Johnson, 2001). Upon activation of the receptor, adenylyl cyclase through the guanine nucleotide regulatory protein (G_s) converts adenosine-5'-triphosphate (ATP) to cyclic adenosine-3',5'-monophosphate (cAMP). The cAMP is an intracellular messenger that regulates cellular functions such as muscle relaxation or contraction by modifying cAMP-dependent protein kinases. Phosphorylation of the myosin light chain kinase prevents interaction of the contractile protein myosin resulting in smooth muscle relaxation. Moreover, cAMP also decreases muscle contraction by inhibiting the influx of calcium via the voltage dependent calcium channels (Fernandes *et al*., 2004).

The β-agonists aromatic ring substituted with hydroxyl groups, halogens, amines, hydroxymethyl groups, and cyano groups, is the key to elicit receptor binding for executing their biological activity. The substitution groups then dictate the compound half-life and efficacy at the receptors (Smith, 1998). Eason and Stedman (1933) proposed that β-adrenoceptors interact with β-agonists at three sites on the molecule: the β-hydroxyl group, the aliphatic nitrogen, and the aromatic ring. The hypothesis was validated and it was found that β_2-adrenoceptors cloned from human, mouse, and rat are 87-93% similar in amino acid

make-up (Hieble *et al*., 1995). Hydrophilic and hydrophobic amino acids distribution in the β_2-adrenoceptor primary sequence is similar to that of rhodopsin. Rhodopsin, a visual seven-transmembrane protein, has the amino and carboxyl groups extended into the extracellular space and cytoplasm respectively. Amino acids spanning the cell membrane are arranged in α-helical orientation around a 'pore' where receptor ligands bind (Hieble *et al*., 1995).

At physiological pH values, salbutamol gets protonated at the aliphatic amine. This enables the molecule to interact with the carboxyl extensions of the β_2-adrenoceptor in a ligand-receptor interaction. The aromatic ring and cathecholhydroxyl groups form hydrogen bonding with serine^{204} (Ser^{204}) and Ser^{207} on the fifth transmembrane helix, thus increasing the binding affinity of ligand-receptor (Hieble *et al*., 1995). Consequently, salbutamol if not ionized at the receptor will fail to exert its activity without the ligand-receptor interaction.

1.3.1 Pharmacokinetics

Pharmacokinetics describes the time course of drug concentrations in the body and it involves the processes of absorption (method of drug administration), distribution (disbursement of drug to body tissues), metabolism (biotransformation of drugs into metabolites), and excretion (removal of drug and metabolites from the body system) of primarily drug substance or abbreviated as ADME.

1.3.1(a) Absorption

Beta-agonists are usually administered by inhalation, oral, and intravenous methods. In humans, salbutamol is more commonly prescribed in the form of inhalation. This method of drug administration allows limited access to the lungs but produces immediate and effective bronchodilation effect (Morgan, 1990). A high proportion of inhaled drugs is swallowed and gets metabolized in the gut. They are rapidly absorbed through the lungs and gastrointestinal tract (small intestine). Most of the β-agonists reach maximum plasma concentration within

1-3 hours post oral administration in humans (Morgan, 1990) with the exception of halogen-substituted aromatic ring β-agonists such as clenbuterol and mabuterol. They reach peak plasma concentration 4 hours after oral dosing due to drug accumulation (Meyer and Rinke, 1991).

Table 1.2: A list of short- and long-acting β-agonists with their respective brand names, method of administration, and dosage form. Dosages may vary with generic products. [MDI (Metered dose inhaler) in the form of aerosol/spray]. [DPI (Dry powder inhaler) the number of 'puffs' needed depends on the success of entire dose inhalation by the person]. Adapted from American Thoracic Society [Online]. Date accessed: 7 May 2014. (https://www.thoracic.org/clinical/copd-guidelines/for-patients/what-kind-of-medications-are-there-for-copd/what-are-beta-agonists.php).

Drug name	Brand name	Method of administration	Dosage
Short-acting β-agonists			
Salbutamol (Albuterol)	Airolin®	MDI	1-2 puffs every 4-6 hours
	Airomir®	MDI	1-2 puffs every 4-6 hours
	Asmasal®	DPI	1-2 puffs every 4-6 hours
	Buventol®	DPI	4-8 mg every 12 hours
	Inspiryl®	DPI	1-2 puffs every 4-6 hours
	Proventil®	MDI	1-2 puffs every 4-6 hours
		DPI	1 puff every 4-6 hours
		Tablets	2-4 mg every 6-8 hours
		Liquid for nebulizer	0.25-0.5 mL of 0.5% solution in nebulizer every 4-6 hours
	Salamol®	MDI	1-2 puffs every 4-6 hours
	Salbulin®	MDI	1-2 puffs every 4-6 hours
	Salbutamol®	MDI	1-2 puffs every 4-6 hours
	Ventodisk®	DPI	1-2 puffs every 4-6 hours
	Ventolin®	MDI	1-2 puffs every 4-6 hours
		DPI	1 puff every 4-6 hours
		Tablets	2-4 mg every 6-8 hours
		Liquid for nebulizer	0.25-0.5 mL of 0.5% solution in nebulizer every 4-6 hours
	Ventolin Evohaler®	MDI	1-2 puffs every 4-6 hours
Bambuterol	Bambec®	Tablets	10-20 mg every night
Fenoterol	Berotec®	MDI	1-2 puffs 2-3 times daily
		DPI	1 puff 2-3 times daily
		Liquid for nebulizer	0.2-0.4 mL with normal saline every 4-6 hours
Isoetherine	Bronkosol®	Liquid for nebulizer	0.25-0.5 mL in nebulizer with 2 mL normal saline
	Bronkometer®	MDI	2 puffs every 4 hours

Table 1.2: Continued.

Drug name	Brand name	Method of administration	Dosage
Isoproterenol (Isoprenaline)	Isuprel®	MDI	1-2 puffs every 4-6 hours
		Liquid for nebulizer	0.25-0.5 mL with 2 mL normal saline
Levalbuterol [(*R*)-salbutamol]	Xopenex®	Liquid for nebulizer	0.63-1.25 mg every 6-8 hours
Metaproterenol	Alupent®	MDI	1-2 puffs every 4 hours
		Tablets	20 mg every 6-8 hours
		Liquid for nebulizer	0.2-0.3 mL 5% solution in nebulizer 3-4 times daily
	Metaprel® ProMeta®	Liquid for nebulizer	0.2-0.3 mL 5% solution in nebulizer 3-4 times daily
		MDI	1-2 puffs every 4 hours
		Liquid for nebulizer	0.2-0.3 mL of 5% solution 3-4 times daily
Pirbuterol	Maxair®	MDI/autoinhaler	1-2 puffs every 4-6 hours
Terbutaline	Breathaire®	Tablets	2.5-5 mg every 8 hours
	Brethine®	MDI	1-2 puffs every 4-6 hours
		DPI	1 puff every 4-6 hours
		Tablets	2.5-5 mg every 8 hours
		Liquid for nebulizer	5 mg up to 4 times daily
	Bricanyl®	MDI	1-2 puffs every 6-8 hours
		DPI	1 puff every 6 hours
		Tablets	2.5-5 mg every 8 hours
		Liquid for nebulizer	5 mg every 6-8 hours
Tornalate	Bitolerol®	MDI	1-2 puffs every 8 hours
		Liquid for nebulizer	0.5-1 mL 3-4 times daily
Long-acting β-agonists			
Formoterol	Foradil®	DPI	1 puff every 12 hours
	Oxis®	DPI	1-2 puffs every 12 hours
Salmeterol	Serevent®	MDI	2 puffs every 12 hours
		DPI	1 puff every 12 hours

1.3.1(b) Distribution

Drugs absorbed into the bloodstream are reversibly distributed to the body tissues through the physicochemical interaction of the drug molecules with the tissue. Drug distribution is indicated by the volume of distribution (V_d). A high V_d value shows that the drugs are widely distributed throughout body tissues resulting in low plasma concentrations and vice versa. Drug distribution is characteristic of the individual drug molecule itself that is usually reflective of the physicochemical property of the molecule. More lipid soluble drugs tend to have higher volume of distributions e.g. steroids have large V_d values whereas drugs like caffeine and ibuprofen have small V_d values (Lombardo *et al.*, 2004; and Ghafourian *et al.*, 2006).

Salbutamol has total plasma clearance of 480 $\pm$ 123 mL/min and V_d of 156 $\pm$ 38 L (Morgan *et al.*, 1986). Salbutamol with a pK_a of 9.3 gets protonated at physiological pH of 7.4. Thus, this ensures that this molecule remains in circulation (tissue extracellular compartment) and does not partition into the adipose tissues. Moreover, the length of the aliphatic side chain is insufficient to incorporate lipid-soluble characteristics (Johnson, 1995; and Smith, 1998). This feature allows for less residue accumulation in adipose tissues. Thus, it maybe safer for use in livestock growth enhancement compared with the more lipophilic β-agonist, clenbuterol. The halogen substitution on the aromatic ring improves the lipophilicity of clenbuterol (Smith, 1998).

1.3.1(c) Metabolism

Metabolism of a drug is a biotransformation process to enable drug clearance from the system in the presence of an enzyme, thus producing a more hydrophilic product (metabolite). This process involves modifications via oxidation, reduction, hydrolysis, hydration, conjugation, and condensation reactions which ultimately determine the pharmacological and/or toxicological output (Gibson and Skett, 2001). Drug metabolism is divided into two phases: i) phase I (functionalisation reactions) and ii) phase II (conjugative

reactions). Phase I metabolism is usually assumed to 'functionalise' the parent drug in preparation for phase II conjugation metabolism by activation, addition or removal of functional group suitable for phase II conjugation (Table 1.3).

Table 1.3: The categorization of reactions involved in phase I and phase II metabolism. Adapted from Gibson and Skett (2001).

Phase I metabolism reactions	**Phase II metabolism reactions**
Oxidation	Glucuronidation/glucosidation
Reduction	Sulphation
Hydrolysis	Methylation
Hydration	Acetylation
Dethioacetylation	Amino acid conjugation
Isomerisation	Glutathione conjugation
	Fatty acid conjugation
	Condensation

Phase I metabolism

The phase I metabolism takes place mainly in the endoplasmic reticulum where reduction, hydration, and two types of oxidation can occur:

i. Hydroxylation (incorporation of oxygen into the drug molecule)
ii. Oxidative, deamination, and dealkylation (loss of functional groups)

The sole purpose of oxidation is to insert an oxygen atom into the complex substrate molecule for the next phase metabolism or excretion. Reductive mechanism catalyses azo-compounds, nitro-compounds, epoxides, heterocyclic ring compounds, and halogenated hydrocarbons in the presence of hepatic microsomal enzymes. Hydrazide and carbamate hydrolysis are some examples of reduction metabolism. Hydration is a reaction that adds water to a compound without causing dissociation of the compound. This is achieved with the assistance of epoxide hydrolase enzyme where epoxides are hydrated to form dihydrodiol.

The most important oxidation reaction occurs with the microsomal cytochrome P450 system (mixed-function oxidases). It facilitates reactions such as aromatic hydroxylation, aliphatic hydroxylation, epoxidation, *N*-dealkylation, *O*-dealkylation, *S*-dealkylation, oxidative deamination, *N*-oxidation, *S*-oxidation, phosphotionate oxidation, dehalogenation, and alcohol oxidation. Other non-mixed-function oxidase enzymes also involved with phase I metabolism are alcohol dehydrogenase, aldehyde dehydrogenase, xanthine oxidase, amine oxidases, aromatases, and alkylhydrazine oxidase (Gibson and Skett, 2001).

Phase II metabolism

Phase II metabolism utilizes a wide array of enzymes for conjugation reactions that produces more water-soluble products which can be excreted in bile or urine (Table 1.4). Glucuronidation is the main route of conjugation due to the abundant availability of the reaction co-factor, uridine-5'-diphospho (UDP)-glucuronic acid and the enzyme, UDP-glucuronosyltransferase. This conjugation is suitable for compounds containing functional groups like alcohols, phenols, hydroxylamines, carboxylic acids, amines, sulphonamides, and thiols. There are two types of glucuronides produced: *O*-glucuronide and *N*-glucuronide. Compounds with groups like phenols, alcohols, and carboxylic acids form the 'ester' or 'ether' *O*-glucuronides. There is a common observation that inversion takes place in compound reaction with the α-glucuronic acid resulting in the formation of a β-glucuronide metabolite. They are excreted into hepatic bile and can be hydrolysed by endogenous β-glucuronidase enzyme to its parent compound which will then be reabsorbed through the intestinal mucosa. This recycling of drugs is termed 'enterohepatic circulation'. *N*-glucuronides on the other hand reacts mainly with aromatic amines, amides, and sulphonamides. Other less common forms of glucuronides are *S*-glucuronides (from thiol groups) and *C*-glucuronides (direct attachment of glucuronic acid to the carbon skeleton) (Gibson and Skett, 2001).

Table 1.4: Conjugation reactions and the relevant enzymes involved in the cytoplasm phase II metabolism (adapted from Gibson and Skett, 2001).

Reaction	**Enzyme**	**Functional group**
Glucuronidation	UDP-Glucuronosyltransferase	-OH; -COOH; -NH_2; -SH
Glycosidation	UDP-Glycosyltransferase	-OH; -COOH; -SH
Sulphation	Sulphotransferase	-NH_2; -SO_2NH_2; -OH
Methylation	Methyltransferase	-OH; -NH_2
Acetylation	Acetyltransferase	-NH_2; -SO_2NH_2; -OH
Amino acid conjugation		-COOH
Glutathione conjugation	Glutathione *S*-transferase	Epoxide; organic halide
Fatty acid conjugation		-OH
Condensation		Various

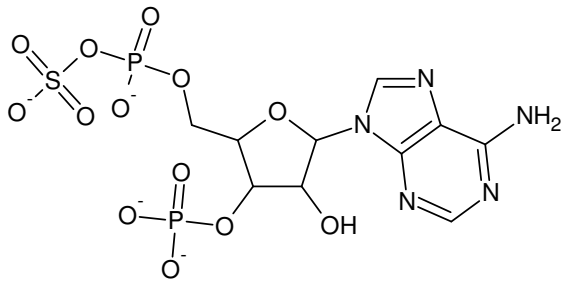


Figure 1.5: The structure of 3'-phosphoadenosine-5'-phosphosulphate (PAPS).

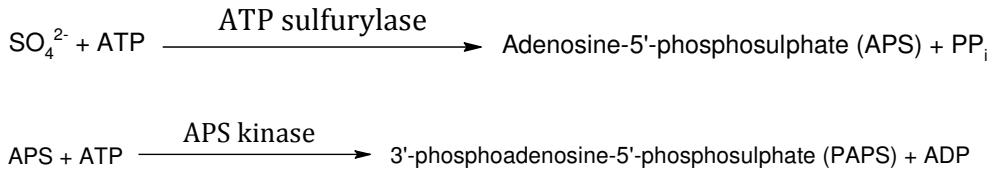


Figure 1.6: A two stage adenosine triphosphate (ATP) reaction with sulphate to form energy rich 3'-phosphoadenosine-5'-phosphosulphate (PAPS) for phase II sulphation metabolism (adapted from Gibson and Skett, 2001).

Sulphation is another major metabolic reaction for phenols taking place in the cytosol. It can also occur for alcohols, amines, although less for thiols. In this reaction, 3'-phosphoadenosine-5'-phosphosulphate (PAPS) (Figure 1.5) which acts as an energy rich donor is obtained through a two-stage reaction from adenosine-triphosphate (ATP) and sulphate (Figure 1.6) (Gibson and Skett, 2001). Interaction of PAPS and cytosolic sulphotransferase (SULT) enzyme produces sulphate conjugated metabolites.

Although salbutamol is well-absorbed orally in humans, it has low bioavailability as a result of extensive first-pass-metabolism. Salbutamol undergoes phase II metabolism to form two main metabolites namely salbutamol-3-*O*-glucuronide and salbutamol-4-*O*-sulphate (Walle *et al*., 1996; and Mareck *et al*., 2011) with the metabolites formed varying across species.

Glucuronidation of the salbutamol aromatic hydroxyl group inhibits pharmacological exertion, thus rendering the resultant metabolite inactive (Morgan, 1990; and Smith, 1998). In the presence of microsomal enzyme, UDP-glucuronosyltransferase, salbutamol is conjugated to UDP-glucuronic acid to form salbutamol-3-*O*-glucuronide (Figure 1.7). Angus *et al*., (1989) proved through perfused rat liver that extreme hypoxia is needed to produce significant impairment of the glucuronidation-dependent elimination of salbutamol.

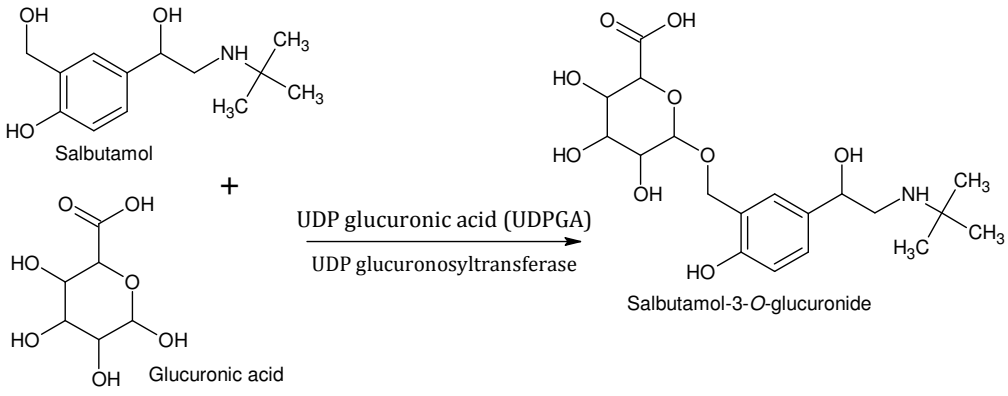


Figure 1.7: Phase II glucuronidation metabolism of salbutamol catalyzed by UDP-glucuronosyltransferase with UDP-glucuronic acid (UDPGA) as a high energy donor to form salbutamol-3-*O*-glucuronide.

Sulphotransferases (SULT) in the liver, small intestine, stomach, kidneys, and colon catalyzes sulphate conjugation of endogenous compounds and drugs to render them biologically inactive (Lin *et al*., 2011). The principal salbutamol metabolite in human is salbutamol-4-*O*-sulphate formed by sulphate conjugation to the phenolic moiety, is carried out by the active M form of human cytosolic phenolsulphotransferase (PST) enzyme (Walle *et al*., 1993a; and Dong *et al*., 2011) or more specifically the SULT1A3 (Ko *et al*., 2012) using the nucleophilic substitution reaction (Figure 1.8). Its sulphotransferase-mediated metabolism is most effective at pH 9.0.

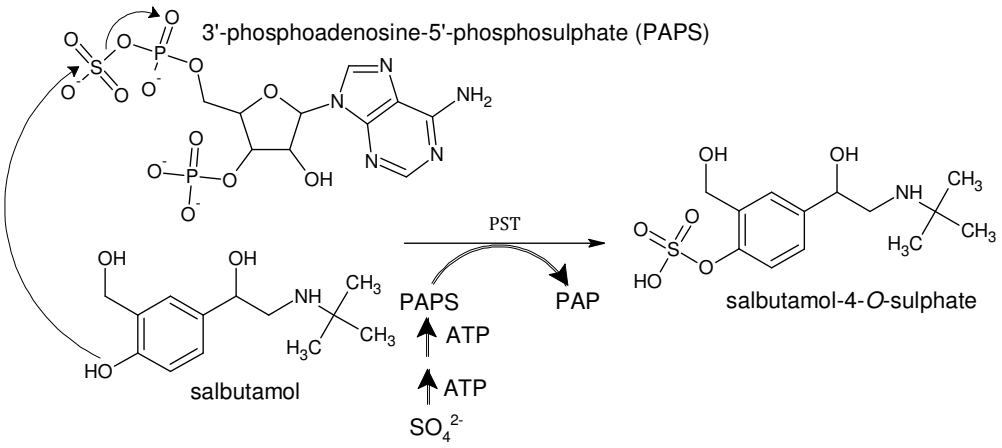


Figure 1.8: In a nucleophilic substitution reaction, the phenolic moiety of salbutamol is catalyzed to form the biologically inactive metabolite salbutamol-4-*O*-sulphate. The reaction is aided by the cytosolic phenolsulphotransferase (PST) enzyme found in the liver, small intestine, kidneys, stomach, and colon (adapted from Walle *et al*., 1996; and Dong *et al*., 2011).

Salbutamol clearance from the human body depends primarily on its sulphate conjugation (Eaton *et al*., 1996). The insignificant protein binding of salbutamol helps to facilitate its excretion from the kidneys. For intravenous salbutamol administration, 64.2% is excreted in the unconjugated form whilst 12% is excreted as the sulphate conjugate. Oral administration differs from the intravenous administration where 31.8% is excreted in the unchanged form whereas 48.2% is excreted as sulphated metabolites (Morgan *et al*., 1986).

1.3.1(d) Excretion

Excretion is the final pharmacokinetic phase that deals with the removal of drug and its metabolite(s) from the body system. Unconjugated drug and its metabolites are excreted from the body system mainly via the liver and kidneys where hydrophilic substances are eliminated faster than the lipophilic ones (Dong *et al*., 2011). Total renal excretion involves glomerular filtration, active tubular excretion, and passive tubular re-absorption. The glomerular filtration allows unconjugated drug and its metabolites to filter through its pores to be eliminated depending on the efficiency of renal blood flow. Active transporters (P-glycoprotein, multidrug resistance-associated proteins, and organic anion and cation transporters) aid ionised compounds such as salbutamol-4-*O*-sulphate and salbutamol-3-*O*-glucuronide to be excreted through the renal tubules (Morgan *et al.*, 1986; Moaddel *et al.*, 2005; and Dong *et al*., 2011). This process is influenced by the plasma pH values that affect the ionization of the compounds to be transported. The passive tubular re-absorption involves the re-uptake of non-ionized compounds by way of concentration gradient that is also pH dependent (Dong *et al*., 2011).

1.4 Therapeutic uses of salbutamol

Asthma is an age old respiratory disease characterized by inflamed and swelling of the airways leading to bronchoconstriction that is sensitive to irritants and susceptible to allergic reactions. The victims perpetually experience bouts of coughing spells at night or in the early mornings. They show symptoms such as wheezing, chest tightness, difficulty in breathing, and coughing. An estimated 315 million people worldwide are asthmatics (To *et al*., 2012) and there is no known cure (Marasco, 2005) although it can be managed with the use of β-agonists. Beta-agonists are bronchodilators developed to combat asthma attacks and bring quick relief to the victim. It is usually administered through inhalation for immediate relief or in the form of oral tablets and even via intravenous injections.

1.5 Adverse effects of salbutamol

The toxicity of salbutamol/β-agonists is marked by clinical symptoms like headache, nausea, dizziness, palpitation, tachycardia, peripheral vasodilatation, nervousness, tremors, fever, chills, and breathing irregularities in acute cases (Sheu *et al*., 2009). Non-β_2-bronchoconstriction that counter the therapeutic effects have been attributed to be due to the (*S*)-salbutamol (Lipworth *et al*., 1997; and Pesola and D'Costa, 2003).

1.6 Stereochemistry and stereoselective pharmacology

1.6.1 Chirality

Recent advances in analytical and synthetic chemistry have led to a better understanding of the differences in biological activities of enantiomers. Chiral molecules are usually asymmetrical with reference to the tetrahedral carbon atom that has four different atoms/groups bound to it (Figure 1.9 i). This carbon atom is called centre of asymmetry or chiral centre. Atoms such as nitrogen, sulphur, and phosphorus are also able to form pharmacologically important chiral molecules with four different groups bound to them in tetrahedral configuration (Figure 1.9 ii) (Allenmark, 1988).

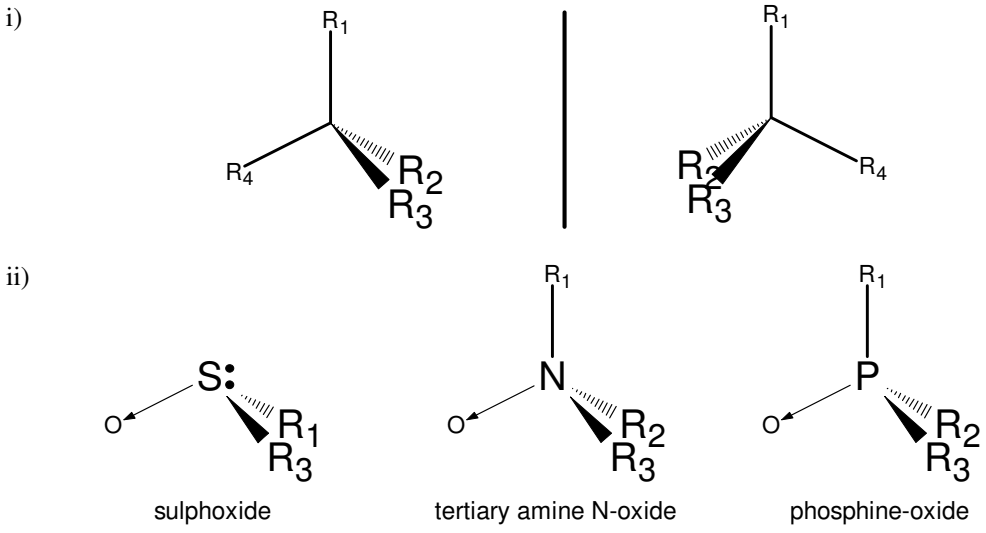


Figure 1.9: Structures in i) are a pair of enantiomers showing an asymmetric tetrahedral carbon and ii) examples of other atoms (sulphur, nitrogen, and phosphorus) that constitute a chiral centre.

Chirality derived from the Greek word '*kheir*' (*χειρ*) meaning 'hand', refers to a subject in chemistry relating to structures that is non-superimposable with its own mirror image (Eliel and Wilen, 1994). Molecule without this unique feature is termed 'achiral'. This led to the three dimensional study on atoms and/or groups absolute configuration/orientation in space within a molecule termed stereochemistry (Lin *et al*., 2011). Interest in stereochemistry began with the discovery of plane polarised light rotation in quartz and later tartaric acid by Malus in 1809 (Nagendrappa, 2007; and Lin *et al.*, 2011).

The study of chirality progressed with Louis Pasteur's observation of chirality (tartaric acid found in the sediments of fermenting wine) in both crystalline and solution form. It was an era where optical activity was characteristic of crystal (i.e. sodium chlorate and sodium bromate) but not solutions. Under the microscope, Pasteur observed that the tartaric acid crystals formed were almost identical and non-superimposable mirror images of each other. Pasteur separated the crystals manually and dissolved them in separate solutions. He found that one of the tartaric acid solutions polarised light to the left [(-)-tartrate] and another to the right [(+)-tartrate]. Thus, he concluded that optical activity was characteristic of the individual (-)- and (+)- molecules and not superimposable with their mirror images (Tan, 1996). They were termed enantiomorphs or enantiomers (Eliel and Wilen, 1994). Subsequently, Irish physicist William Thomson (Lord Kelvin) coined the name 'chirality' following Louis Pasteur's discoveries. Although chirality is found also across an axis or a plane, but few such chiral compounds are of pharmacological importance.

1.6.2 Stereoselectivity in pharmacology

Natural biological building blocks (i.e. proteins, enzymes, and receptors) are invariably chiral molecules derived from L-amino and D-carbohydrates. As such, interactions between a chiral biological macromolecule and an enantiomeric pair are essentially different and they are viewed as two different chemical entities. The resultant observation is usually an

enhanced biological/pharmacological activity residing in one of the enantiomers due to their affinity and contact with endogenous proteins, enzymes, and receptors. Thus, chirality is important to gauge drug efficacy. According to the Easson and Steadman model (Patil *et al*., 2008) the more potent compound possesses the stronger interaction or better fitting with receptors. Consequently, the enantiomer that illicit better affinity or pharmacological activity is termed the eutomer whereas its lower affinity counterpart the distomer. Both enantiomers show differences in pharmacodynamics and pharmacokinetics. However, it is known that drug molecules and their complementary receptor are able to undergo conformation changes when in contact (Albert, 1985). Their eudismic ratios measure their stereoselective receptor-mediated activation (Patil *et al*., 2008).

The importance of chiral drugs has gained more recognition with better understanding of the individual enantiomer biological activities. Studies conducted prove that the pharmacological and toxicological properties of a racemic drug are not equivalent to the simple sum of contribution from both enantiomers. Their activities are complex and their pharmacological inputs vary according to their chemical entity. Utilising both enantiomers in the dosage form without understanding the pharmacological and toxicological implications of each enantiomer is risky. A variety of differential enantiomer contributions to pharmacological effects are known and are outlined below.

1.6.2(a) Pharmacological activity residing in a single enantiomer

The ideal situation is to have the drug pharmacological activity residing in a single enantiomer while its antipode is invariably inactive. This is a rare condition seen with the anti-hypertensive drug, α-methyldopa where only the (*S*)- enantiomer is the pharmacological active entity (Gillespie *et al*., 1962).

1.6.2(b) Pharmacological activity residing in both enantiomers

Drugs like promethazine (Powell *et al*., 1988) and flecainide (Kroemer *et al*., 1989) display similar pharmacological and toxicological properties in both enantiomers. Thus treatment with either the racemate or single enantiomer does not offer any advantages.

1.6.2(c) Pharmacological activities of the enantiomers are qualitatively different

Dextromethorphan and its enantiomer levomethorphan are chiral drugs that exhibit different pharmacological activities. Dextromethorphan does not wield analgesic, sedative, and opiod-like effects but levomethorphan exerts potent opiod-like activity (Drayer, 1986).

1.6.2(d) Different potency for pharmacologically similar enantiomers

Most chiral drugs fall within this category like warfarin and verapamil. The more potent enantiomer is (*S*)-warfarin. It is 2-5 times more potent in its anti-coagulant effect than (*R*)-warfarin (O'Reilly *et al*., 1974). (*S*)-Verapamil is comparatively eight times more active in lowering cardiac activity (Echizen *et al*., 1985).

1.6.2(e) Equally active enantiomers with toxicity in one enantiomer

Ketamine, an anaesthetic with analgesic properties, does not cause circulatory or respiratory depression but elicit addiction, hallucination, and agitation. (*S*)-ketamine is a 3.4 times more effective anaesthetic but its (*R*)- antipode brings on adverse effects like psychic emergence reactions and post-operative agitated behaviour (White *et al*., 1980).

1.6.2(f) Opposite or contrary pharmacological activities with each enantiomer

Indacrinone is a loop diuretic that exhibits diuretic activity with the (-)-indacrinone but causes uricosuric activity with the (+)- antipode. The risk factor for hypertensive patients is increased and therefore, it is a more useful diuretic if the (+)- enantiomer proportion is increased (Tobert *et al.*, 1981).

1.6.2(g) Antagonism of antipode at the same receptor site

This is evident with picenadol (a phenylpiperidine derivative) that exerts analgesic properties with the (+)- enantiomer but is antagonised by the (-)- enantiomer (Powell *et al.,* 1988).

1.6.3 Stereoselectivity in pharmacokinetics

Chiral drugs not only display stereoselectivity in their pharmacology and pharmacodynamics, but also in pharmacokinetics. In view of the possible enhancing and contradictory features of drug enantiomers, it is important to study stereoselectivity in pharmacokinetics with regards to the ADME process, as well as stereoselective drug-drug interactions (Dong *et al*., 2011).

1.6.3(a) Stereoselective absorption

Absorption of drugs occurs either by passive diffusion or aided by active transport across cellular membranes. Passive diffusion is influenced by lipophilicity, molecular size, and the pK_a values. The movement of drug molecules is dictated by the concentration across the cell membrane, whereby drug molecules move from higher concentration regions to lower concentration regions. This method of absorption is spontaneous and not stereoselective. Active transportation requires energy to transport drug molecules against concentration gradient across a biological membrane and thus introduces stereoselectivity in absorption. Good synergy between the drug enantiomer and the active transporter improves absorption (Dong *et al*., 2011). For example L-dopa reacts better with the naturally occurring intestinal L-amino acid transporter and thus increases the rate of absorption (Wade *et al*., 1973).

1.6.3(b) Stereoselective distribution

Chiral drugs are usually distributed differently from each other because of stereoselectivity binding of these drugs to the naturally occurring receptors that are chiral. Distribution of chiral drugs from the plasma to cellular compartment is dependent upon the stereoselective

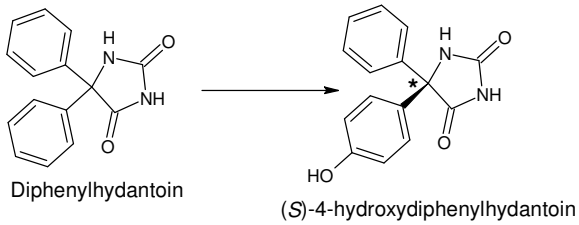
binding to receptors and protein transporters (Dong *et al*., 2011). In addition, the binding capacity of the drug to plasma and tissue proteins dictates the pharmacodynamics and pharmacokinetics. It is widely accepted that the free or unbound drug is responsible for the pharmacological activity and subject to clearance. Highly protein bound drugs may exhibit significant pharmacological effects although there is minor drug-receptor interaction. Conversely, low protein binding drugs have increased availability for receptor binding resulting in strong pharmacodynamics and pharmacokinetics properties. Competition for plasma protein binding sites gives rise to enantioselective drug interactions. α_1-Acid glycoprotein has only one binding site that preferentially recognises basic drugs. For instance, propranolol binding towards α_1-acid glycoprotein is predominantly with the (*S*)-enantiomer but its (*R*)- enantiomer shows remarkable binding capacity with human serum albumin (Hutt *et al*., 1989). Therefore, the extensive (*R*)-propranolol binding to human serum albumin makes binding of (*S*)-propranolol of greater significance.

1.6.3(c) Stereoselective metabolism

Stereoselectivity happens in both phase I and II metabolism to yield different products, at varying rates to form unique enantiomeric metabolites. Enantioselective metabolism involving various stereochemical transformations are summarized in Table 1.5.

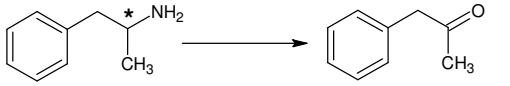
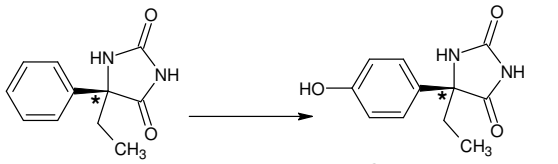
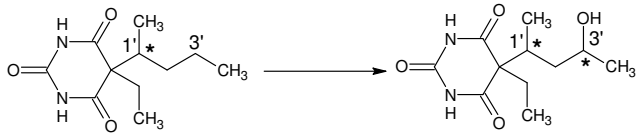
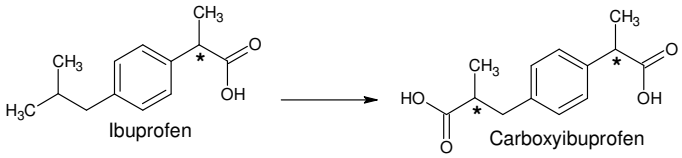
Table 1.5: Summary of various stereochemical transformations involved in stereoselective metabolism and examples of each transformation. * marks the chiral centre of the molecules.

	Stereochemical transformation	Examples
i)	Achiral to chiral transformation	An achiral molecule that undergoes metabolic transformation to produce a chiral molecule. E.g. diphenylhydantoin (phenytoin) to (*S*)-4-hydroxydiphenylhydantoin (Poupaert *et al*., 1975).



Diphenylhydantoin (*S*)-4-hydroxydiphenylhydantoin

Table 1.5: Continued.

	Stereochemical transformation	Examples
ii)	Chiral to achiral transformation	A rare metabolism transformation at the chiral centre rendering the molecule achiral. E.g. amphetamine to phenylacetone (Wright *et al*., 1977).  Amphetamine Phenylacetone
iii)	Chiral to chiral transformation	Metabolism transformation that retains the chirality of the molecule. E.g. (*S*)-nirvanol to (*S*)-4-hydroxynirvanol (Küpfer *et al.*, 1984).  (*S*)-nirvanol (*S*)-4-hydroxynirvanol
iv)	Chiral to diastereomer transformation	Introduction of a new chiral centre to produce varying degrees of diastereomerism. E.g. (*R*)-pentobarbitone to (1'*R*,3'*S*)- and (1'*R*,3'*R*)- diastereomers and (*S*)- enantiomer gives rise to (1'*S*,3'*R*)- and (1'*S*,3'*S*)-diastereomers (Palmer *et al*., 1970).  Pentobarbitone 5-ethyl-5-(pentan-2-yl)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione 5-ethyl-5-(4-hydroxypentan-2-yl)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione
v)	Chiral inversion	Metabolism transformation of a chiral molecule to be converted to its antipode. E.g. (*R*)-ibuprofen to (2'*S*,2*R*)- and ((2'*R*,2*R*)-carboxyibuprofen, and (*S*)-ibuprofen gives rise to (2'*R*,2*S*)-(2'*S*,2*S*)-carboxyibuprofen (Hutt and Caldwell, 1983).  Ibuprofen Carboxyibuprofen

1.6.3(d) Stereoselective excretion

Glomerular filtration and passive re-uptake in renal excretion are non-stereoselective. However, stereoselective binding of drugs to plasma protein influences the process of glomerular filtration and passive re-absorption. Stereoselective renal tubular re-absorption is believed to be responsible for the stereoselective excretion of (*S*)-terbutaline (Borgstorm *et al.*, 1989). Differences in stereoselective renal excretion are relatively small in comparison with the non-renal clearance processes.

Stereoselectivity in renal excretion is directly linked to the active tubular excretion where drug-receptor interaction and protein binding is prevalent. Enantiomer administration profiles are usually different from the racemate administration. It is noteworthy that drugs and their metabolites have multiple elimination sites and sometimes may not reflect the stereoselective secretion process itself. Thus, an estimation of pharmacokinetic parameters and concentration-effect relationships of total drug concentrations may be of limited value and potentially misleading (Ariens, 1984).

1.6.4 Stereoselective pharmacokinetics of salbutamol

Salbutamol exerts its desirable bronchodilating property in (*R*)-salbutamol (eutomer) (Brittain *et al.*, 1973) whereas (*S*)-salbutamol (distomer) causes bronchial contraction and hyperresponsiveness towards allergens (Templeton *et al.*, 1998). Both enantiomers have high selectivity for β-adrenoceptors in the bronchial smooth muscle cells compared with cardiac muscle cells.

Commercial preparations of salbutamol are introduced via inhalation, oral, and intravenous routes (Ward *et al.*, 2000). In the course of inhalation, salbutamol is usually deposited in small portions in the lungs for immediate activity onset and the balance swallowed (Newman