SOLID PHASE AND IMMUNOAFFINITY EXTRACTION METHODS IN THE ANALYSIS OF 17α-METHYL STEROIDS

By

YONG KOOI LING

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ABBREVIATIONS

IOC	International Olympic Committee
GC-MS	Gas Chromagraphy-Mass Spectrometry
HRMS	High Resolution Mass Spectrometry
DHT	Dihydrotestosterone
DHA	Dehydroandrosterone
UDPGA	Uridinediphosphate glucuronic acid
PAPS	Phosphoadenosine phosphosulphate
IAE	Immunoaffinity extraction
Ag	Antibody
Ag	Antigen
AgAb	Antibody-antigen complex
K_a	Affinity constant
SPE	Solid phase extraction
SFE	Super critical fluid extraction
TLC	Thin layer chromatography
GC	Gas chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
HRMS	High Resolution Mass Spectrometry
UV	Ultra violet
MSD	Mass Selective Detector
MS	Mass Spectrometry
SIM	Selected ion monitoring
EI	Electron ionisation
TMS	Trimethylsilyl
t-BDMS	tert-Butyldimethylsilyl
MSTFA	N-Methyl-N-trimethylsilytrifluoroacetamide
SAX	Trimethylaminopropyl
SCX	Benzesulfonic acid
IAC	Immunoaffinity chromatography

ml	Mililitre
mg	Miligram
ng	Nanogram
L	Litre
g	Gram
IU	International Uint
m/z	Mass to charge
µl	Microlitre
min	Minute
MS-MS	Ion Trap Tandem Mass Spectrometry
LLE	Liquid-liquid extraction
CBA	Carboxypropyl
PRS	Propylsulfonic acid
SD	Standard deviation
CV	Coefficient of variation
Crit. Val	Critical value
TIC	Total Ion Chromatogram
EIC	Extracted Ion Chromatogram
kDa	kiloDalton
Ig	Immunoglobulin
ELISA	Enzyme ImmunoSorbent Assay
SC	Subcutaneous
ID	Intradermal
IM	Intramuscular
IV	Intravenous
IS	Intrasplemic
IN	Intranodal
IP	Intraparticular
QA	Quality Assurance
q_m	Theoretical binding capacity
λmax	Absorbance maximum

BT	Breakthrough
MeOH	Methanol
H_2O	Water

ABSTRACT

The analysis of anabolic steroids at low concentrations presents a major problem in many analytical laboratories. The need for a better detection limit requires improvements in currently used analytical methods. Although sensitive instruments e.g. HRMS are available for the trace analysis of steroids, improvement in sample preparation has not received much attention. In the present study, two sample preparation methods i.e. direct hydrolysis and immunoaffinity extraction (IAE) are evaluated in order to improve analytical performance. Various SPE cartridges were studied for direct hydrolysis and mixed mode cartridges (C_8/SAX) showed comparable recoveries for clenbuterol, norandrosterone, 17α-methylandrostanediol and 3-hydroxystanozolol to that obtained from indirect hydrolysis, i.e. 61.6 %, 75.3 %, 88.3 % and 90.9 % for indirect hydrolysis and 65.1 %, 74.6 %, 92.2 % and 86.1 % for direct hydrolysis respectively. However, when the urine background becomes dirtier, indirect hydrolysis was more robust.

An immunoaffinity gel was developed with antibodies raised against 17α-MT and immobilized on to Sepharose gels. The immunogen was synthesized using mixed anhydride method. The antisera having the relatively higher titre value among the antisera obtained were used for subsequent study. The antibodies were raised in rabbits and showed cross-reactivity with a few structurally similar steroids. Especially those steroids having a 17α-methyl group such as metendienone, mestanolone, 17α-methylandrostandiol and stanozolol were cross-reacted with 17α-MT (33.0 %, 22.6 %, 14.1 % and 8.3 % respectively). Those steroids that lack the 17α-methyl group had poor cross-reactivity. The

optimal solvent conditions for washing and elution of 17α-MT were studied using the immunoaffinity gel. It was found that 30 % and 70 % methanol in water was the best washing and elution solvents respectively and extraction efficiency of 98.9 % 17α-MT could be achieved. The binding capacity for the gel was determined as 800 ng 17α-MT. Subsequently, this IAE was evaluated using individual urinary samples spiked with 17α-MT, 17α-methylandrostandiol and 3-hydroxystanozolol. The gels showed relatively good recoveries towards 17α-MT (86.37 %), 17α-methylandrostandiol (62.14 %) as well as 3-hydroxystanozolol (50.25 %). Full scan mass spectra of the extracts showed relatively cleaner backgrounds, making them suitable for confirmatory analysis at concentrations as low as 5 ng/ml.

KAEDAH PENGESTRAKAN FASA PEPEJAL DAN IMMUNOAFFINITI DALAM ANALISIS STEROID-STEROID 17α-METIL

ABSTRAK

Analisis steroid-steroid anabolic pada kepekatan rendah merupakan satu masalah utama yang hadir dalam kebanyakan makmal analitikal. Keperluan untuk satu had pengesanan yang lebih baik memerlui pembaikan-pembaikan dalam kaedah analitikal yang diguna pada masa kini. Walaupun instrumen-instrumen yang sensitive seperti HRMS sedia ada untuk analisis pengesanan steroid-steroid, tetapi pembaikan dalam penyediaan sampel tidak menerima banyak perhatian. Dalam pelajaran masa sekarang, dua kaedah penyediaan sampel iaitu hidrolisis secara langsung dan pengestrakan immunoaffiniti telah dinilaikan supaya membaiki perlaksanaan analitikal. Pelbagai kartus fasa pepejal (SPE) telah dipelajari untuk hidrolisis secara langsung dan kartus mod bercampur (C_8/SAX) menunjukkan pemulihan yang setanding untuk clenbuterol, norandrosterone, 17α-methylandrostanediol dan 3-hydroxystanozolol kepada yang didapati daripada hidrolisis secara tidak langsung, iaitu 61.6 %, 75.3 %, 88.3 % dan 90.9 % untuk hidrolisi secara tidak langsung dan 65.1 % , 74.6 %, 92.2 % dan 86.1 % untuk hidrolisis secara langsung masing-masing. Akan tetapi, apabila latar belakang air kencing menjadi lebih kotor, hidrolisis secara tidak langsung adalah lebih tegap.

Satu gel immnoaffiniti telah dibinakan dengan antibodi yang dibangkit bertentangan dengan 17α-methyltestosterone dan ditetapkan pada gel-gel Sepharose. Immunogen telah

disintesiskan dengan kaedah 'anhydride' bercampur. Antisera mempunyai nilai 'titre' yang lebih tinggi berbanding dengan antisera-antisera yang diperolehi telah digunakan untuk pelajaran selanjutnya. Antibodi-antibodi ini dibangkit dalam arnab dan menunjukkan reaktivi bersilang dengan beberapa steroid yang dengan strukturalnya seakan-akan sama. Terutamanya, steroid-steroid yang mempunyai satu kumpulan 17α-metil seperti metanedienone, mestanolone, 17α-methylandrostanediol dan 3-hydroxystanozolol adalah bertindak silang dengan 17α-methyltestosterone (17α-MT) (33.0 %, 22.6 %, 14.1 % dan 8.3 % masing-masing). Steroid-steroid yang kekurangan kumpulan 17α-metil mempunyai reaktivi bersilang yang kurang. Syarat solven (pelarut) yang optimal untuk membasuh dan elusi mengenai 17α-methyltestosterone telah dipelajari menggunakan gel immunoaffiniti ini. Ini adalah didapati bahawa 30 % dan 70 % metanol dalam air masing masing adalah solven pembasuhan dan elusi yang terbaik dan 98.9 % kecekapan pengestrakan mengenai 17α-methyltestosterone boleh dicapai. Kemampuan pengikatan untuk gel ini telah ditentukan pada 800 ng 17α-methyltestosterone. Seterusnya, pengestrakan immunoaffiniti ini telah dinilaikan dengan mengguna sampel-sampel air kencing yang individu dicampur dengan 17α-methyltestosterone, 17α-methylandrostanediol dan 3-hydroxystanozolol. Gel-gel ini menunjukkan pemulihan-pemulihan yang baik terhadap 17α-methyltestosterone (86.37 %), 17α-methylandrostanediol (62.14 %) dan 3-hydroxystanozolol (50.25 %). Spektra jisim sekali lintas penuh (full scan) untuk ekstrak-esktrak ini telah menunjukkan latar belakang yang lebih bersih supaya menjadikan mereka sesuai diguna untuk tujuan pengesahan pada kepekatan yang rendah seperti 5 ng/ml.

CHAPTER 1: INTRODUCTION

1.1 Doping Control In Sport

The use of drugs to enhance performance in sport is not a new phenomenon but one that has been known since the time of the ancient Greeks. In the early 1960s, the Council of Europe banned the use of endogenous or exogenous agents which help to achieve an artificial and unfair increase in performance of the athlete in competition so that a "level playing field" can be achieved (Bowers and Segura, 1996). In 1967, the International Olympic Committee (IOC) re-established a medical commission whose major responsibility is to control drug abuse in sport. This was followed by the introduction of drug testing in 1968 (Gower *et al.*, 1995). The first drug testing activity was carried out at the 1972 Olympic Games in Munich, where gas chromatography with nitrogen-selective detectors were used to test more than 2000 urine specimens for stimulants (Bowers, 1997).

1.2 Abuse Of Anabolic Steroids In Sport

By 1968, the use of anabolic steroids among athletes was common. In that year, a decathlon athlete claimed that an estimated one third of the US track and field team had used steroids at the pre-Olympic training camp before the Mexico City Games. At that time, anabolic steroids were not included in the banned class of compounds because there was no proper testing method available (Cowan and Kicman, 1997). With the successful introduction of a radioimmunoassay screen for anabolic steroids and a GC-MS method for confirmatory purposes, a trial test was introduced at the

Commonwealth Games in New Zealand in February 1974. Out of 55 samples 9 failed the screening test and 7 samples were confirmed positive. The current groups of drugs that are banned have been expanded to include anabolic agents, stimulants, narcotics, β-blockers, diuretics, peptide hormones and theirs analogues (Cowan and Kicman, 1997). Recent statistics from the IOC accredited laboratories indicates that anabolic steroids remain the primary performance-enhancing substances detected in athletes (Bowers, 1997).

Lately, the detection of prohibited substances has become increasingly difficult. This is due to the availability of numerous potent synthetic steroids and the increasing number of dietary supplements used by athletes as well as the pharmacological sophistication of drug use (Wu, 1997).

Anabolic steroids are usually taken for a number of weeks before an important contest (Wu, 1997). Several anabolic steroids may be administered simultaneously both orally and by injection. This procedure is known as "stacking". The weekly dose is five to ten times the manufacturer's recommended therapeutic dose (Wu, 1997). Doses will often follow a "pyramid" program where maximum amounts are being administered in the middle or at the end of a cycle of steroid administration (Wilson, 1988). Therefore, the implementation of "no-notice" testing (while out of competition) in addition to competition testing of athletes is necessary in order to minimize the opportunity of getting benefit from the banned drugs (Bowers, 1997). Diuretics are also commonly used to mask the appearance of the drug from their sample through dilution and forced diuresis (Mueller *et al.*, 1995).

To catch the defaulters during any competition, very sensitive and reliable analytical methods are needed. Therefore, the desire of improvement or development in analytical methods and sample preparation is of prime importance in most IOC accredited laboratories. One of the thrust areas of this development is to achieve the detection limit of 2 ng/ml for various anabolic steroids in order to achieve better retrospecificity. Recent advances in mass spectrometry, for instance, use of HRMS have provided an opportunity to decrease the detection limits of these agents, culminating in the use of HRMS for steroid screening at the Summer Olympic Games in 1996 (Horning *et al.*, 1997).

1.3 Adverse Effects Of Anabolic Steroids

The health hazards induced by anabolic steroids administration have been extensively reviewed. The adverse effects may be manifested with pharmacologically recommended doses but is of much greater consequence for those who administer excessive amounts over long periods of time, as is done in sports.

The adverse effects of anabolic steroids abuse depend on the age, sex of the individual, the duration, total dose and the type of steroid used. These effects may be differentiated into androgenic or toxic effects. The former effects are amplification of the physiological effects of androgens (discussed in section 1.4.2.1), whereas the latter effects are regarded to have more serious consequences (Wu, 1997). The toxic effects of anabolic steroids are listed in Table 1.1.

Table 1.1. The toxic adverse effects of anabolic steroids at different target systems.

Target system	Adverse effects
1. Cardiovascular	i. Cardiomyopathy ii. Acute mycocardial infarct iii. Cerebral vascular accident iv. Pulmonary embolism
2. Liver	i. Cholestatic jaundice ii. Peliosis hepatis iii. Tumour
3. Psychological	i. Aggression increased ii. Dysphoria-rage iii. Psychosis iv. Addiction v. Withdrawal effects-depression

One of the most serious problems associated with anabolic steroids abuse is the decrease in circulating HDL-cholesterol because of the action of androgens in suppressing hepatic endothelial lipase activity. This may have long-term consequences in increasing the risk to ischemic heart disease (Wu, 1997).

Sustained suppression of hypothalamic-pituitary-gonadal axis can cause prolonged infertility, testicular atrophy and secondary amenorrhea. In women, some of the effects of virilization may be irreversible, e.g. deepening of the voice and clitoral

hypertrophy. Liver dysfunction and hepatotoxicity are associated with the use of the 17α-alkylated steroids. Sustained side effects of anabolic steroids observed in human included effects on liver, cardiovascular and reproductive system and premature epiphyseal cell closure (Gower *et al.*, 1995).

1.4 Anabolic Steroids (Androgens)

1.4.1 Structure and nomenclature

Anabolic steroids most frequently used by athletes are synthetic male sex hormones, the androgens. The androgens comprise a group of C_{19} steroids derived from cholesterol, which has the basic steroid molecular skeleton made up of four rings of carbon atoms, labeled as A-D. Their hydrocarbon structure corresponds to *androstane*. Almost all natural steroids possess either one or more, usually two methyl (CH_3) groups at positions 18 and 19 in the α-configuration. However, if only one methyl group is present at position 18, the structure is *estrane*, i.e. structure for steroidal hormone oestrogen (Kirk *et al.*, 1995). The molecular structure for the steroid skeleton is shown in Figure 1.1.

Androgens such as testosterone possess both androgenic and anabolic activities. In male athletes, enhancement in performance may be due to an indirect anabolic effect of steroids via the androgenic effects in increasing aggression and competitiveness (Brooks, 1978). Hence, the more accurate term for anabolic steroids is anabolic-androgenic steroids but for simplicity the shorter term is used in this chapter.

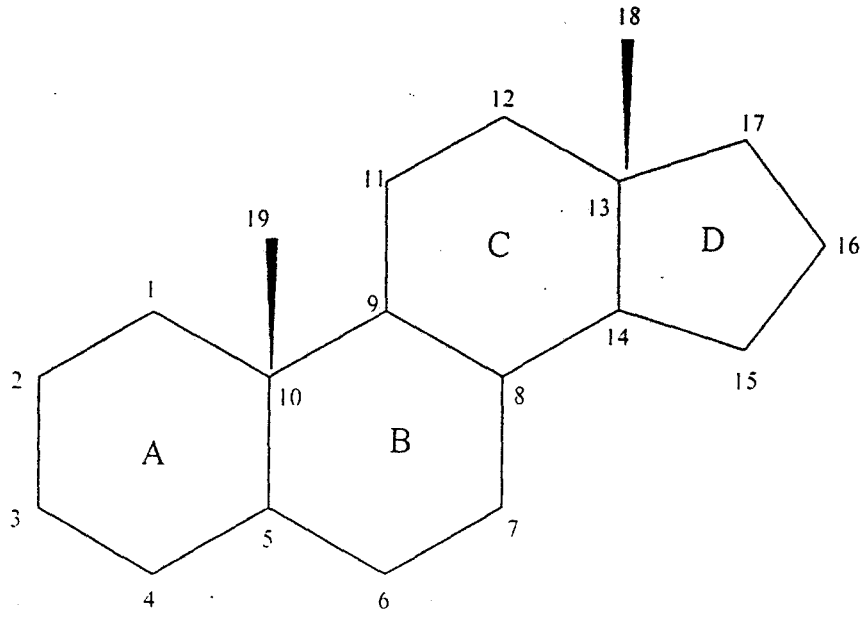


Figure 1.1. The molecular structure of the steroid skeleton.

1.4.1.1 Relationship of structure and androgenicity

For a C_{19} steroid to be an androgen, a 17-oxygen function should be present with, either the 4-en-3-oxo configuration as in testosterone or a 3-oxo-group with a saturated A ring as in 5α-dihydrotestosterone (5α-DHT) (Figure 1.2). If the 17-oxygen function is absent, as in 4,16-androstanedien-3-one, androgenic activity is completely lost (Gower, 1972). In addition, if a 17-hydroxyl group (-OH) appears in the structure, it can be presented in either α- or β- configuration. For instance, those steroids with 17β-hydroxyl group as in testosterone and 5α-DHT mainly are steroids with relatively high androgenic activity. Alternatively, if a 17α-hydroxyl group is present as in epitestosterone, the steroids concerned have little or no androgenicity. If oxidation of the 17β-hydroxyl group occurs to give a 17-oxosteroid, as in the case of testosterone being converted to 4-androstenedione or 5α-DHT to 5α-androstane-

3,17-dione, then androgenicity may be respectively much reduced or completely lost (Gower and Fotherby, 1975).

The major naturally occurring androgens are testosterone, 5α-DHT and 5α-androstane-3α,17β-diol. Besides, there are 4-androstenedione and dehydroepiandrosterone (DHA) which only have weak androgenic activity. With the purpose of maximizing the anabolic effect and minimizing the androgenic activity of anabolic steroids, many modifications on testosterone molecule have been undertaken. Some of the major structural modifications have been introduced into testosterone or 5α-DHT is as follows (Gower *et. al.*, 1995): -

(i) Introduction of a second double bond into the A-ring, e.g. boldenone.

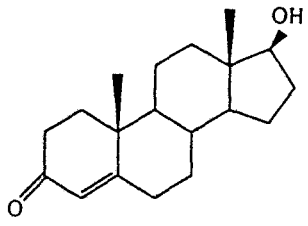
(ii) Attachment of α-alkyl substituents at the 17-position in case of orally active anabolic agents, e.g. methandienone.

(iii) Attachment of a pyrazole ring to the A-ring, e.g. stanozolol.

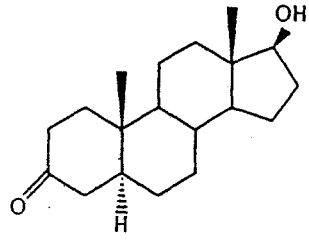
(iv) Attachment of an oxymethylene group at C-2, e.g. oxymetholone.

(v) Replacement of the C-2 carbon in the A-ring with oxygen, e.g. oxandrolone.

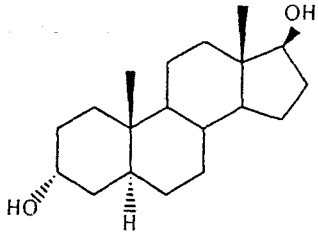
(vi) Substitution of a chlorine atom or hydroxy group at C-4, e.g. in 4-chloromethandienone, oxymesterone (Figure 1.2.).



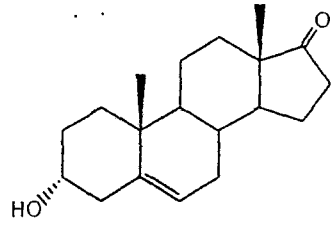
Testosterone



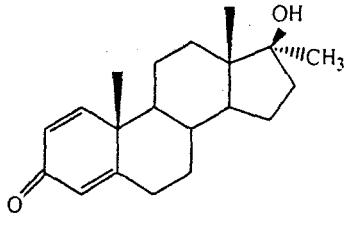
5α-Dihydrotestosterone (5α-DHT)



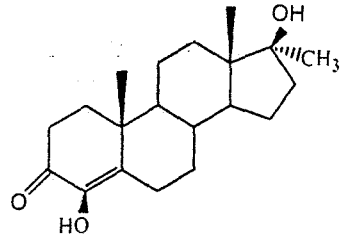
5α-Androstane-3α,17β-diol



Dehydroepiandrosterone (DHA)

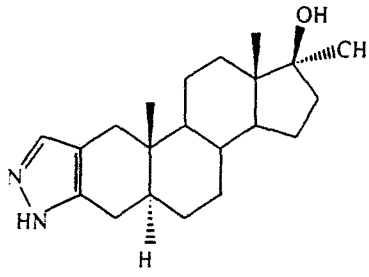


Methanedienor e

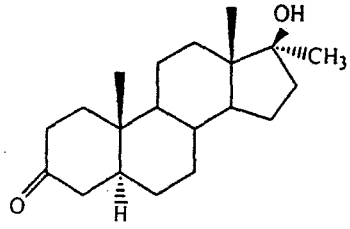


Oxymesterone

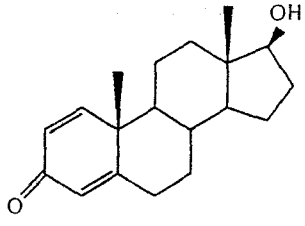
Figure 1.2. Structures of some major naturally occurring and authentic anabolic steroids.



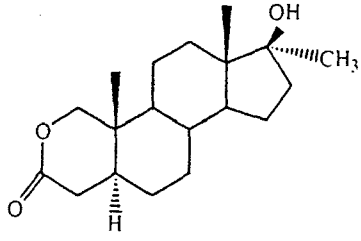
Stanczolol



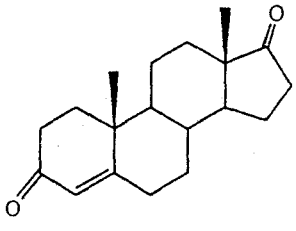
Mestanolone



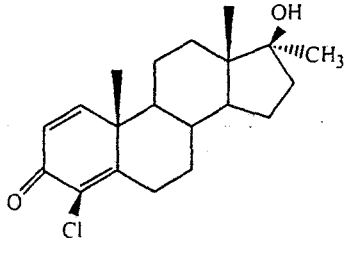
Boldenone



Oxandrolone



4-Androstenedione



4-Chloromethanedienone

Figure 1.2 (contd.). Structures of some major naturally occurring and synthetic anabolic steroids.

1.4.2 Pharmacology of androgens

1.4.2.1 Physiological actions and effects

Androgens are largely secreted by the Leydig cells of the interstitial tissue of the testes and to a lesser extent by the adrenals and ovaries. These androgens are required life long in males and are responsible for the differentiation and development of the male reproductive activities and male secondary sex characteristics, as well as the maintenance of these male qualities ("maleness").

The numerous diverse effects of the androgens manifest most clearly at puberty, such as the maintenance of the normal function and structure of the prostate gland and seminal vesicles, in particular with spermatogenesis (Sharpe *et. al.*, 1990). The androgens also possess some effects on non-sexual organs and tissues, for example, stimulated growth of pubic, facial hair and enhanced the deepening of the voice because of enlargement of larynx and thickening of the vocal cords. The anabolic effects of androgens are also noted in muscle mass and bone in giving rise to increased height and weight at puberty (Gower, 1979). In addition, androgens also demonstrate the activation of sexual behavior in the male during adolescence and young adult life (Wu, 1997).

1.4.2.2 Biosynthesis of andrc gens

Much of our knowledge on the biosynthesis pathways of androgen is obtained from incubation studies with relatively large quantities of steroid precursors such as

pregnenolone and progesterone and then analyzing the metabolites formed (Slaunwhite *et al.*, 1965; Kwan *et al.*, 1984).

As a result of a great number of *in vivo* and *in vitro* studies, it appears that there are two pathways for androgen biosynthesis from the pregnenolone. One of these involves 5-ene-3β-hydroxysteroid metabolites such as 17-hydroxypregnenolone and DHA and is called the "5-ene-3β-hydroxy" pathway. While the other involves 17-hydroxyprogesterone is called the "4-ene-3oxo" pathway. They are sometimes referred to as the Δ^5 and the Δ^4 pathways, respectively to indicate the positions of unsaturation in the relevant intermediates (Slaunwhite *et al.*, 1965). Both pathways are shown in Figure 1.3 (Gower, 1995).

1.4.2.3 Metabolism of anabolic steroids

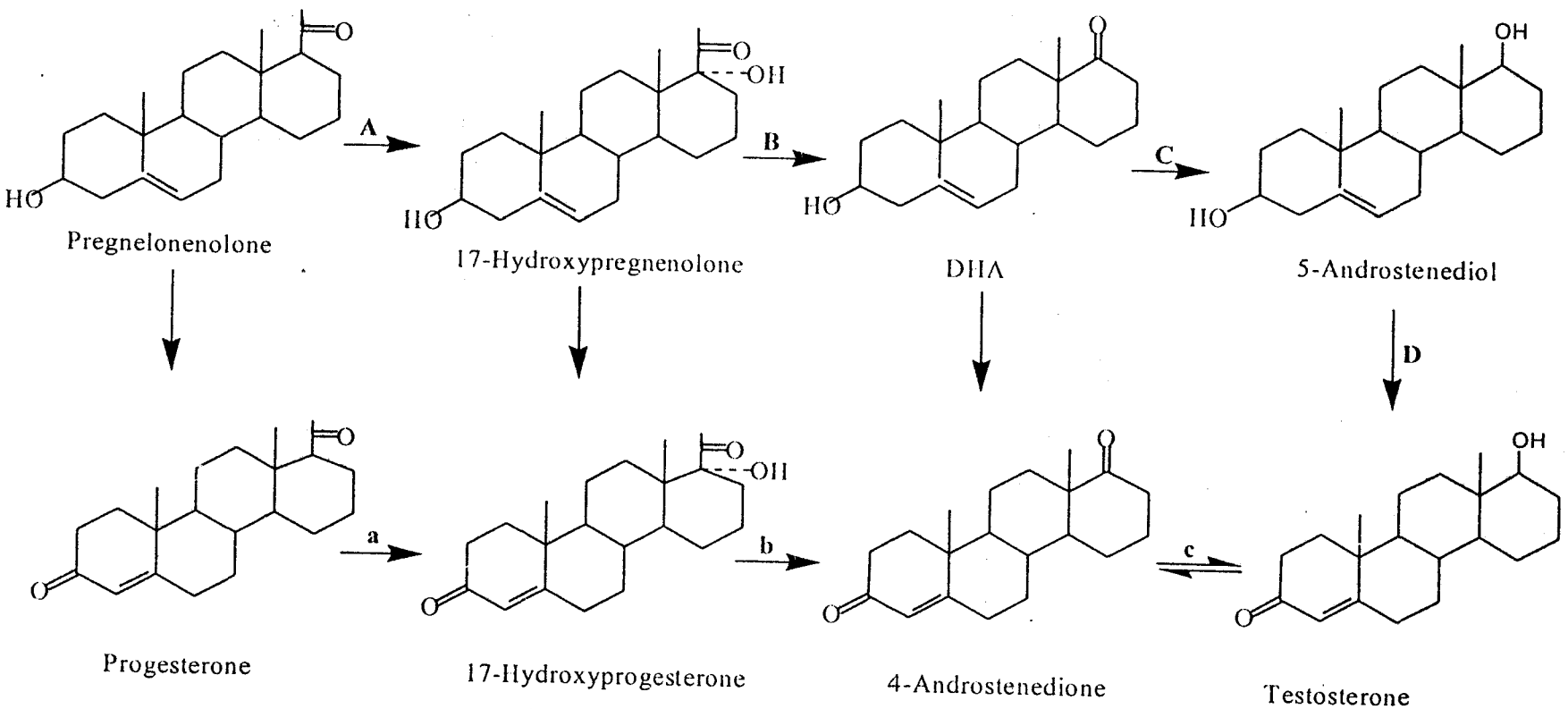
The metabolism of some of the common anabolic steroids was investigated as early as the 1950s. In humans, anabolic steroids are extensively metabolized and being excreted in the urine with little or unchanged parent drug. The pathways elucidated involve oxidation, reduction, hydroxylation and epimerization (phase 1 reactions), and conjugation reaction with uridinediphosphate glucuronic acid (UDPGA) and phosphoadenosine phosphosulphate (PAPS) (phase 2 reactions).

In humans, phase 1 reactions mainly involve reduction in the A-ring, yielding both the 5α- and 5β-isomers whereas reduction at C-3 gives predominantly the 3α-isomers. Oxidation at C-17 occurs without subsequent reduction in the case of nandrolone in man, the major metabolites obtained are 5α- and 5β- isomers of 3α-hydroxyestran-

17-one i.e. norandrosterone and noretiochlolanolone, respectively (Massé *et al.*, 1989a).

Besides natural occurring steroids, synthetic steroids like stanozolol and 17α-methyltestosterone also undergo metabolism in order to produce their major metabolites. For instance, the hydroxylation of stanozolol occurs at position C-3, C-4 and C-16 produces a series of mono- and di-hydroxy metabolites. Among them, 3-hydroxystanozolol is one of the major metabolite for stanozolol (Masse *et al.*, 1989b). In metabolism study of 17α-methyltestosterone, the analysis of metabolites revealed that with reduction of the A-ring and the 3-oxo group, 17α-methylandrostandiol (Schoene *et al.*, 1994) was produced as its major metabolite. The metabolisms of nandrolone, stanozolol, 17α-methyltestosterone and their major metabolites in human urine are shown in Figure 1.4.

The mechanisms of phase 2 reactions involve conjugation with UDPGA to yield glucuronides and with PAPS to yield sulphates. Anabolic steroids and their metabolites appear in the urine almost exclusively as water soluble glucuronide or sulphate conjugates. Steroids having a 3β-hydroxyl group are excreted mainly as sulphates, while 3α-hydoxysteroids are excreted as β-glycosidically linked glucuronides.



[A→B→C→D = Δ^5 pathway; a→b→c = Δ^4 pathway]

Figure 1.3. Biosynthesis of androgen in human.

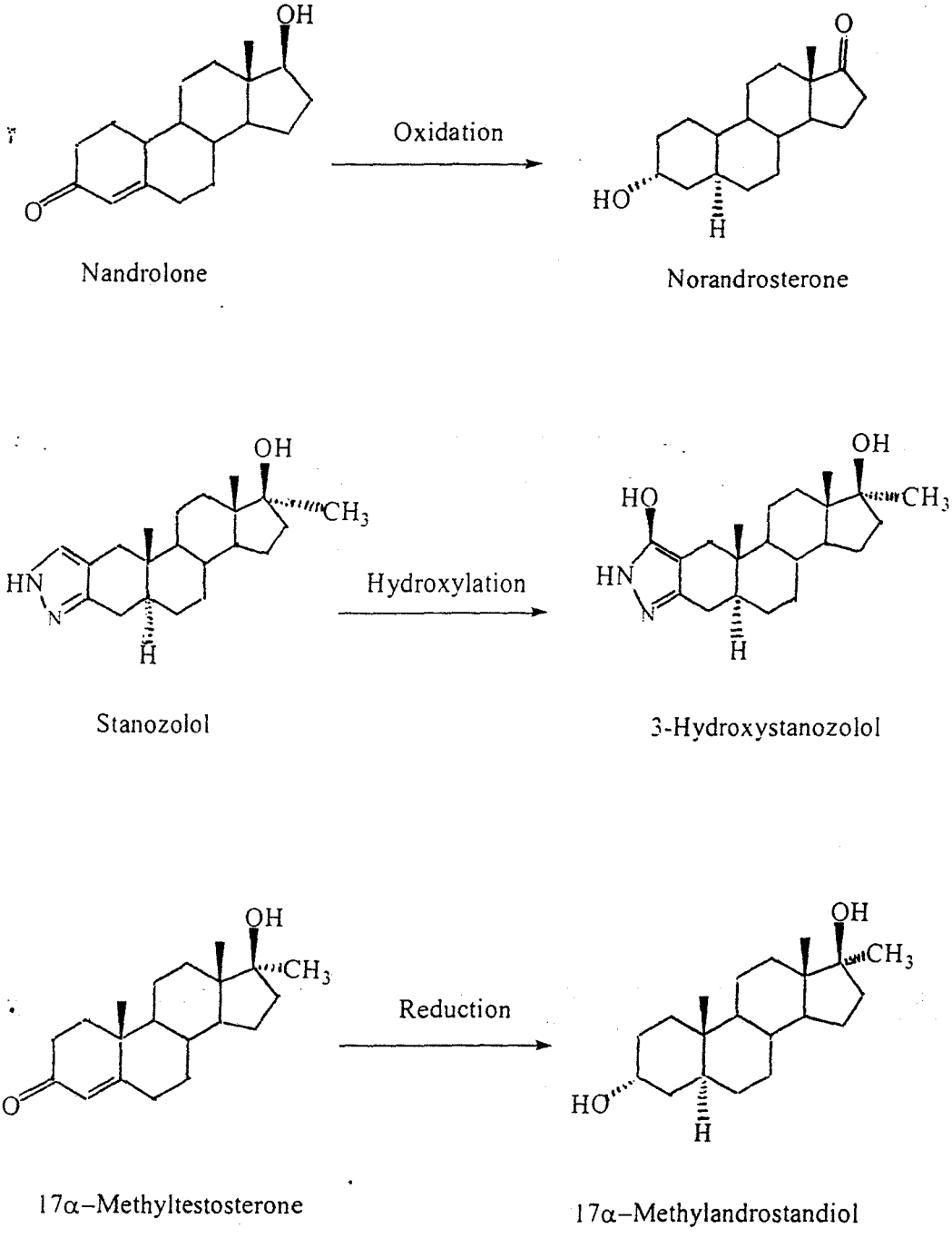


Figure 1.4. The metabolism of nandrolone, stanozolol, 17α-methyltestosterone and their major metabolites in human urine.

1.5 Analysis Of Anabolic Steroids In Urine

1.5.1 Nature of sample for steroid analysis

For the analysis of steroids, the biological fluids that can be used are urine, blood, plasma and serum. Among all these, only urine has been used in doping control by the International Sport Federations. However, blood is also the alternative choice rine samples when urine is unavailable since 2000. Untimed urine samples are collected from athletes to determine whether administration of a banned drug has occurred. Urine was selected as the biological fluid of choice for athletes for two main reasons. First, the collection is non-invasive to the individual and after administration, many banned drugs and their metabolites are more concentrated in the urine than in serum. Secondly, an untimed collection of urine gives a sufficiently large volume for both a full drug screen and a confirmatory analysis (Cowan, 1993/4).

However, the analysis of anabolic steroids in urine is fraught with challenges (Bower, 1997). These challenges are: -

(i) The low concentration of anabolic steroid metabolites, in the range of μg/L.

(ii) The large number of steroids and their metabolites appearing in the urine matrix.

(iii) The complexity of the urine matrix, which contains endogenous steroids of similar structure at concentrations of more than 1000-fold higher than the compounds of interest. In addition, urine contains large amounts of different

types of organic compounds and acids, which often cause problems during sample clean-up.

1.5.2 Sample preparation

A sample preparation step is necessary in the analysis of drugs in biological fluids to isolate the compounds of interest from a sample matrix as well as to purify and concentrate the analyte. Various sample preparation methods prior to chromatographic analysis are listed in Table 1.2. However, those commonly used for chromatography are extraction methods like liquid-liquid extraction and solid phase extraction where the latter represents the more popular method nowadays.

1.5.2.1 Solvent extraction

Solvents that are miscible or immiscible with water, such as ethyl acetate, ether, chloroform and dichloromethane have long been used to extract free (unconjugated) steroids from biological matrices. Analytes are extracted into organic solvents only if they possess sufficient lipophilic character to be attracted into a non-polar solvent (McDowall, 1989). Solvent polarity is an important consideration when planning a solvent extraction. Other factors affecting the choice of solvent for extraction include boiling points, density, toxicity and purity.

Table 1.2. Sample preparation procedures prior to chromatographic analysis (Stevenson, 1996; McDowall *et al.*, 1989).

1. Solvent extraction	12. Freeze drying
2. pH Change	13. Enzyme digestion
3. Chemical derivatization	14. Hydrolysis
4. Solid Phase Extraction (SPE)	15. Column switching
5. Steam extraction	16. Low temperature storage
6. Homogenization	17. Column chromatography
7. Protein precipitation	18. Microwave assisted extraction
8. Dialysis	19. Headspace analysis
9. Sonication	20. Ultrafiltration
10. Centrifuge	21. Supercritical Fluid Extraction (SFE)
11. Evaporation	

The choice of solvent for extraction depends on the desired degree of specificity. The most non-polar solvent that completely removes a drug will be the one least likely to remove more polar metabolites. In comparison, a more polar solvent will remove both the drug and metabolites for separation.

For an extraction to be successful, the compounds to be extracted should be un-ionized to facilitate partition into organic phase. Therefore, adjustment of the pH of the medium which the compound is to be extracted from can be used to optimize extraction. An optimal pH is selected based on the pKa value of the compound

(Schwartz and De Silva, 1979). As a rule, the pH should be 1-2 units below the pKa value for acidic drugs and 1-2 units above the pKa value for basic drugs. For example, the pKa value for an acidic compound having a carboxyl group (R-COOH) is 4.9. Thus, in order to extract this compound, the pH of the medium should be adjusted to 2.9-3.9 (Simpson, 2000).

1.5.2.2 Solid Phase Extraction (SPE)

In solid phase extraction, the biological fluid is first passed through an adsorbent (stationary phase) which is usually packed in a small cartridge or column. As the sample passes through the stationary phase, the analyte and other endogenous substances are bound to the stationary phase by mechanisms which include hydrogen bonding, dipole-dipole interactions, hydrophobic dispersion forces and electrostatic (ionic) interactions. The analyte and endogenous substances are thus separated according to the degree in which they are partitioned or absorbed by the stationary phase (McDowall, 1989). The absorbent is subsequently washed with water or a suitable solvent to selectively remove the interfering endogenous substances from the sample matrix. By further selective elution with an appropriate solvent system, the compound of interest is eluted for subsequent analysis.

The classical adsorbents used in SPE are carbon, Amberlite XAD or alumina. However, these have been largely replaced by bonded phase silica such as C_{18} or C_8 since 1980s (McDowall, 1989). With various bonded phases available, the analyst can adjust chemical selectivity to maximize extract cleanliness. The more common phases are classified as non-polar, polar and ion exchange phases such as cation and anion

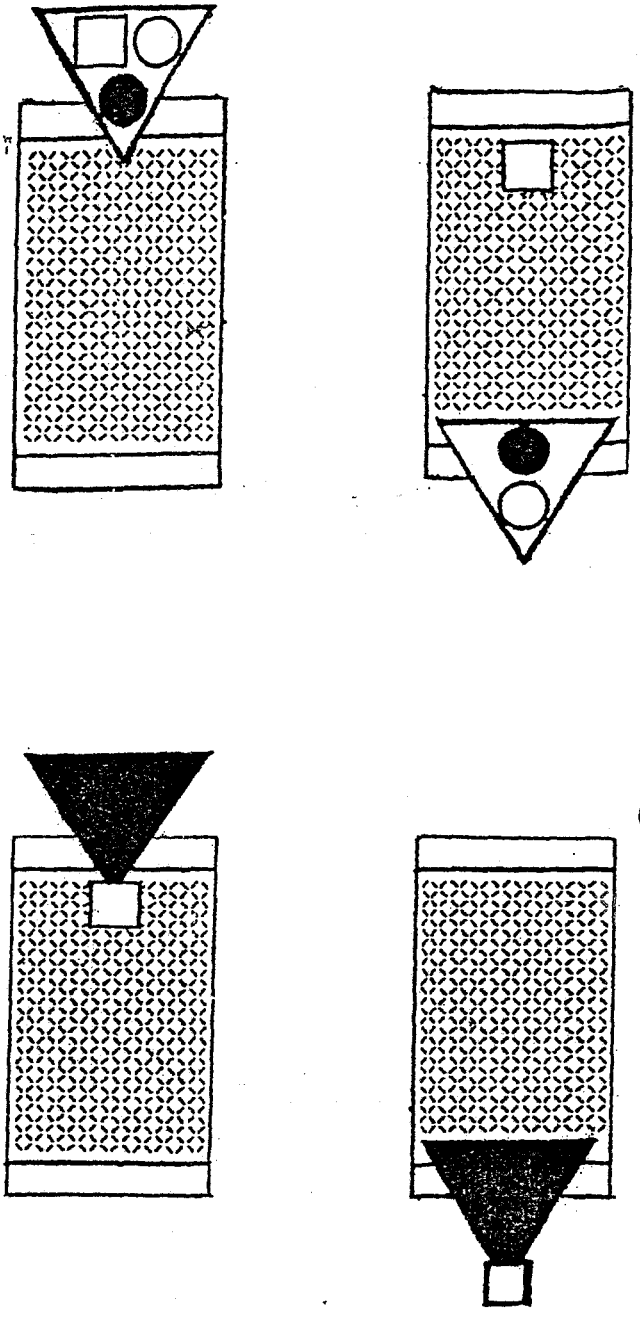
exchange with mixed retention mechanisms. Usually, mixed phases were prepared from mixture of non-polar and ion exchange phases and are used for specific applications, e.g. to extract more polar steroids. Affinity phase materials are also found to have a role in SPE. The mechanism of SPE is shown in Figure 1.5 whereas the schematic of different type of sorbent interactions is shown in Figure 1.6 (Simpson and Van Horne, 1993).

1.5.2.3 Hydrolysis of steroids

A method of hydrolysis is necessary in order to deconjugate the steroid conjugates that appear as glucuronides and sulphates during the sample preparation of steroid analysis (Bowers and Sanaullah, 1996). This is because of the conjugated steroids are very polar and seldom determined directly.

In general, there are two types of hydrolysis method. These are solvolysis and enzymatic hydrolysis. Solvolysis is a method that involves the use of concentrated acid such as sulphuric acid or hydrochloric acid for deconjugation of sulphate conjugates. In solvolysis, cleavage of steroid conjugates is rapid by heating the conjugates at moderate temperature within a short period of time. However, it has gradually become less important because it involves the use of concentrated acid (Gower, 1995). Enzymatic hydrolysis involves the use of β-glucuronidase and sulphatase enzyme for cleavage of glucuronides and sulphates. In this method, the enzymes are heated at moderate temperature such as 60°C for 2 to 8 hours. This method is popular in the routine analysis of steroid in most laboratories as it is safer to use. However, the shortcoming is that it is time consuming.

There are two main sources for the deconjugation enzymes, i.e. *E. coli* and *Helix pomatia*. *E.Coli* only possess glucuronidase activity as compared to *Helix pomatia* which possess both glucuronidase and sulphatase activity. *Helix pomatia* is preferred for routine work because it is cheaper as compared to *E. coli* and it has aryl sulphatase activity to hydrolyze the sulphate conjugates, thereby enabling complete hydrolysis to be achieved (Vanluchene *et al.*, 1982; Messeri *et al.*, 1984). However, *Helix pomatia* also possess dehydrogenase enzymes which can convert 5-androstenediol and DHA that are present in urine sample to testosterone and 4-androstenedione, respectively. This may lead to problems in the interpretation of the endogenous urine profiles of endogenously excreted steroids. On the other hand, hydrolysis with *E. coli* will produce cleaner extracts than *Helix pomatia* because there is no sulphatase and other oxidase enzyme activity. Therefore, it is preferred for confirmatory analysis.



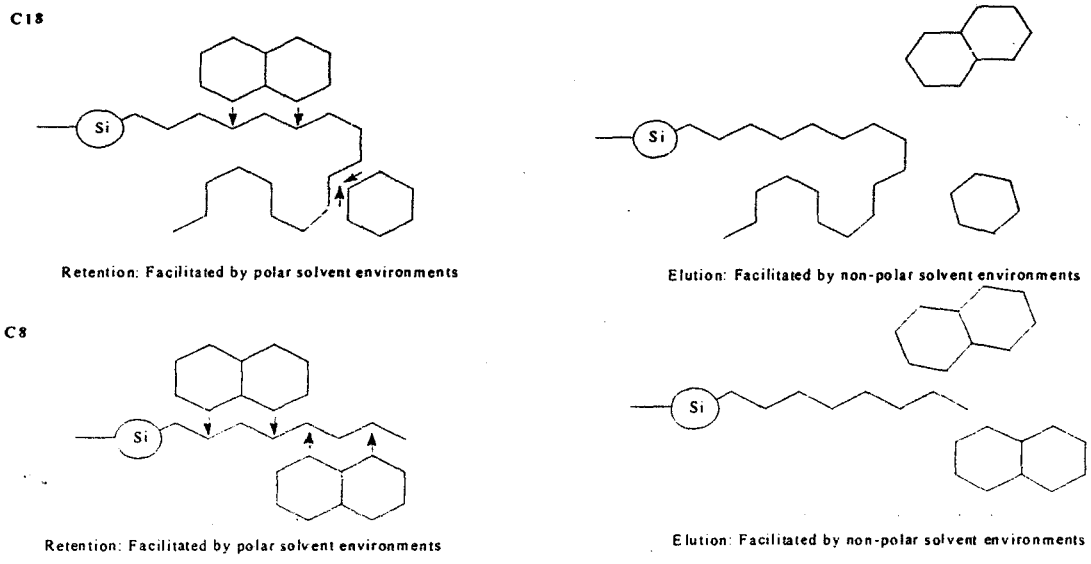
(a) Compound of interest retained.

Interfering substances pass through absorbent or are subsequently washed off.

(b) Compound of interest eluted from absorbent.

Figure 1.5. The mechanism of solid phase extraction (Simpson and Van Horne, 1993).

"Non-Polar Interaction" using C_8 (Octylsilane) or C_{18} (Octadecylsilane) sorbent



"Polar Interaction" using CN (Cyanopropyl)or NH_2 (Aminopropyl) sorbent

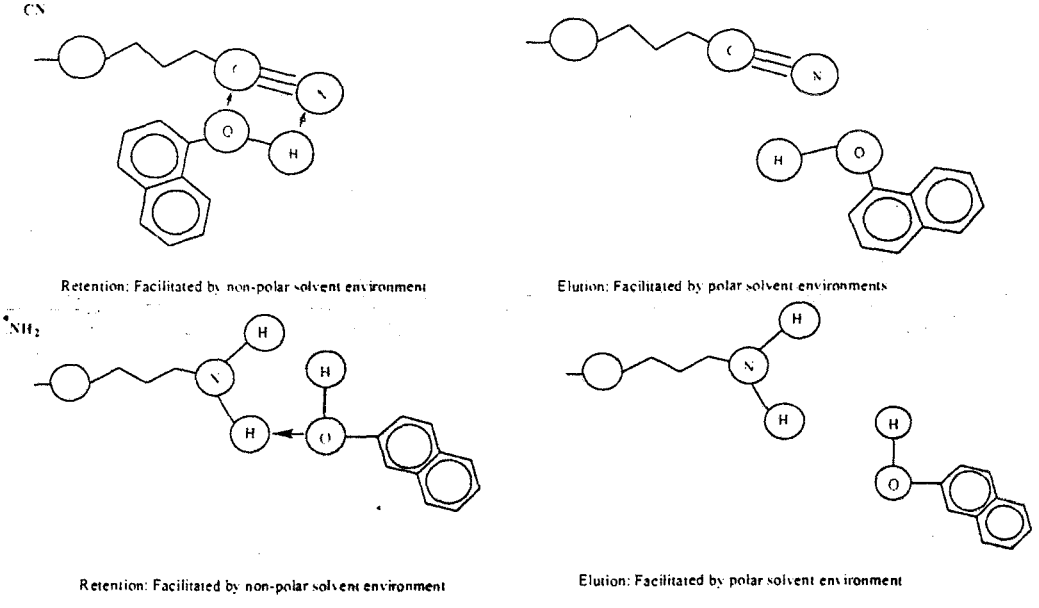
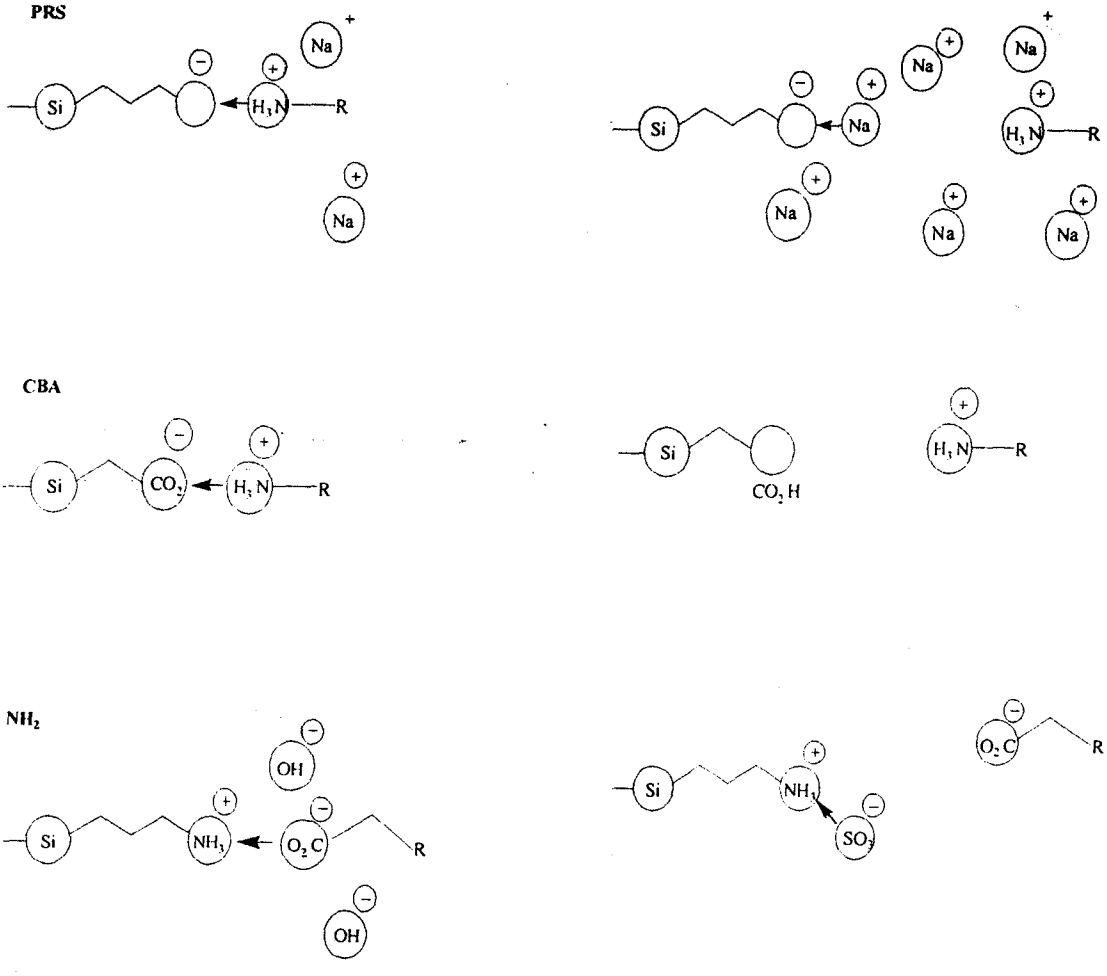


Figure 1.6. The schematic of different types of sorbent interactions (Simpson and Van Horne, 1993).

"Ionic Interaction" using PRS (Sulfonylpropyl), CBA (Carboxymethyl) or NH_2 (Aminopropyl) sorbent



- Retention: Facilitated by solvents: -
  (a) having low ionic strength
  (b) containing low selectivity counter-ions
  (c) at a pH where both sorbent and isolate are charged

Elution: Facilitated by solvents: -
(a) having high ionic strength
(b) containing high selectivity counter-ions
(c) at a pH where either the sorbent or isolate is neutral

Figure 1.6. (contd.). The schematic of different type of sorbent interactions (Simpson and Van Horne, 1993)

1.5.2.4 Immunoassay

Immunoassay is basically a competitive binding assay utilizing specific binding proteins or antibodies. In this assay, the analyte of interest is labeled with a radioactive tracer or enzyme and competes with the unlabeled analytes or antigens for the binding sites of antibodies or binding proteins. The binding that occurs between analytes and proteins is based on antigen-antibody interaction. The percentage of radioactivity or enzyme activity contained in the antibody or protein bound fraction is inversely related to the concentration of unlabelled analyte or antigen in the standard or sample (Butt, 1984).

If a radioactive tracer is used, the immunoassay is called radioimmunoassay (RIA). Alternatively, if an enzyme is used, the immunoassay is then named as an enzyme immunoassay (EIA). In RIA, the radioactive tracers (radioisotope) that is usually used in many laboratories are ^{125}I and ^{3}H. The use of radioisotopes in RIA provides a convenient and sensitive immunoassay. However, they produce a health and environmental hazard. Another type of RIA is immunoradiometric assay (IRMA). In this assay, instead of using a labeled antigen, a labeled antibody is used. This method is very convenient to carry out but it is less sensitive than RIA. Newer enzyme immunoassays, e.g. Enzyme Linked Immunosorbent Assay (ELISA) work on the same principle except that an enzyme is used as a label. The theory of ELISA is as described in section 3.1.3. This method has the advantage that no radioisotope is involved, it is commercially available and comparatively sensitive to RIA.