SIMPLE, EFFICIENT AND SIMULTANEOUS DETERMINATION OF ANABOLIC STEROIDS IN HUMAN URINE

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ABSTRACT

A high performance liquid chromatographic method for the determination of free anabolic drug "sustanon 250" (Organon) is developed. Four testosterone esters, propionate, phenylpropionate, isocaproate and decanoate were detected simultaneously and quantitatively in only 15 minutes. The percentage extraction in dichloromethane of the four testosterone esters from doped urine was 88, 87, 89 and 94% respectively. The separation recorded at 254 nm using analytical column ODS-C18 and methanol as an eluent, showed no interference of naturally occurring steroids. This method can be used for pharmacokinetic studies, routine analysis in pharmaceutical industry, routine therapeutic surveillance and in drug abuse by sportsmen (JPMA 42: 216,1992).

INTRODUCTION

Testosterone is the natural androgen secreted by interstitial cells of the testis, for the promotion and growth of sex characteristics[1]. Another important property of testosterone is its ability to stimulate protein synthesis. This is associated with the retention of nitrogen, potassium and calcium leading to additional build up of muscle[1]. Steroids with anabolic effects are normally used as therapeutic drugs to improve the nitrogen retention in human beings suffering from protein consuming diseases[2-4]. They are also used by athletes for additional power to muscles and hence these are not allowed by olympic committee[5]. In male, normally about 4-8 mg of testosterone is secreted daily giving a plasma level of 0.6-1 ug/100 ml after puberty and do not vary significantly with age[1]. Testosterone is not effective if swallowed because of extensive hepatic first pss metabolism, therefore, it must be administered by intramuscular injection in the form of esters. Esters of testosterone (Figure 1)

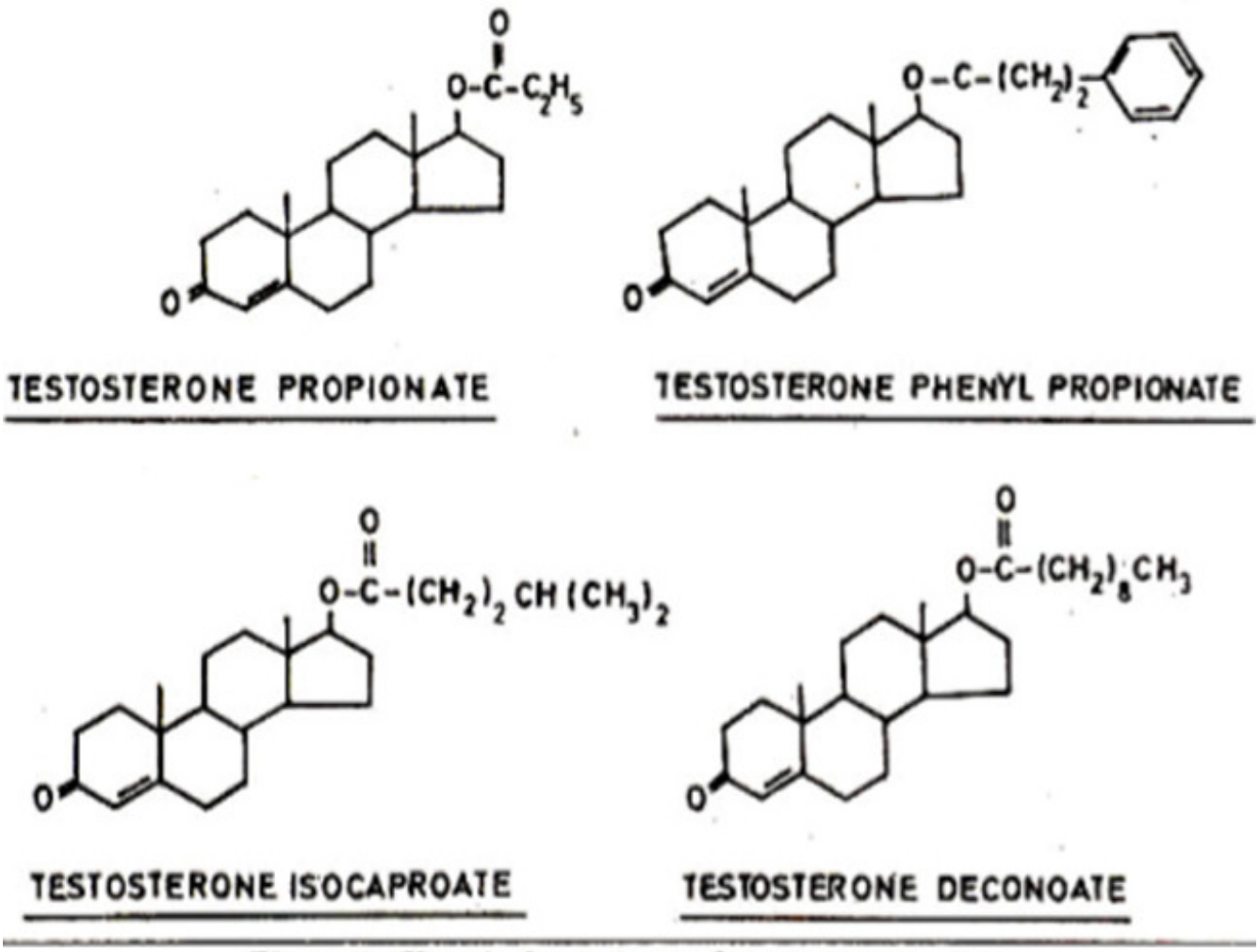

Figure 1. Chemical structure of testosterone esters.

have a more rapid prolonged action than the parent compound[6]. Testosterone propionate has a rapid onset and short duration of action, testosterone phenylpropionate and isocaproate have a less rapid onset and long duration whereas testosterone decanoate has a slow onset and long duration of action[6]. The thug is a combination of these four testosterone esters. The therapeutic use of these anabolic steroids can produce toxic effects. They can also cause jaundice, damage to liver and foetus[1]. Continuous treatment with anabolic steroids may lead to sodium and water retention which result in oedema. Several methods for the analysis of urinary steroids and steroidal thugs have been developed[3,7,8]. We report here a high efficiency HPLC method for simultaneous detection of mixture of four testosterone esters which is fast, sensitive and quantitative. This method can be used for routine analysis in pharmaceutical industry and clinical studies as well as in drug abuse in the presence of natural excreted steroids in urine.

EXPERIMENTAL

Reagents

Pure esters were obtained in the form of sustanon (Organon) intramuscular injection Hormone Laboratories Karachi, Pakistan. The following lichrosolv-grade solvents of E. Merck were used in all the procedures: chloroform, diethylether, dichloromethane, ethanol and methanol. Demineralized, double distilled and sterilized water was used in the experiment.

Instrumentation

Perkin Elmer liquid chromatograph (USA) series 10, solvent delivery system fined with a Rheodyne 7120 (USA) and sample injector having a 6 ul loop was used. The detector was U.V. Spectrophotometer Dupont Instruments (USA). Chromatograms were recorded on a 4270 series integrator of Varian (USA). The analytical column was ODS-ET 250/8/4 Nucleosil 5 C,8 (25 cmx 4.6 mm), Macherey-Nagel (FRG).

Preparation of stock solution

The "sustanon 250" injection was completely soluble in diethylether. Thus injection was dissolved in 30 ml of diethylether and 70 ml of methanol. The total standard volume was 100 ml, giving a final concentration of 300 ug mr' testosterone propionate, 600 ug mr' each of testosterone phenylpropionate and testosterone isocaproate and 1000 ug mf' testosterone decanoate. The working solution of the four testosterone esters of concentration (1-36 ng) was prepared by adding 3-30 ul of stock solution in 5 ml of methanol and used as standard to find out detection, limits, standard recovery times as well, as amount vs peak height relationships (Table I and Figure 2).

TABLE I. Retention times and detection limits of four testosterone esters.

Name of esters	Retention time (min)	*Detection limits (ng)
Testosterone propionate	8.82	1.1 (±0.05)
Testosterone phenylpropionate	9.59	2.2 (±0.1)
Testosterone isocaproate	10.09	2.2 (±0.1)
Testosterone decanoate	14.14	3.6 (±0.1)

*Average of three determinations

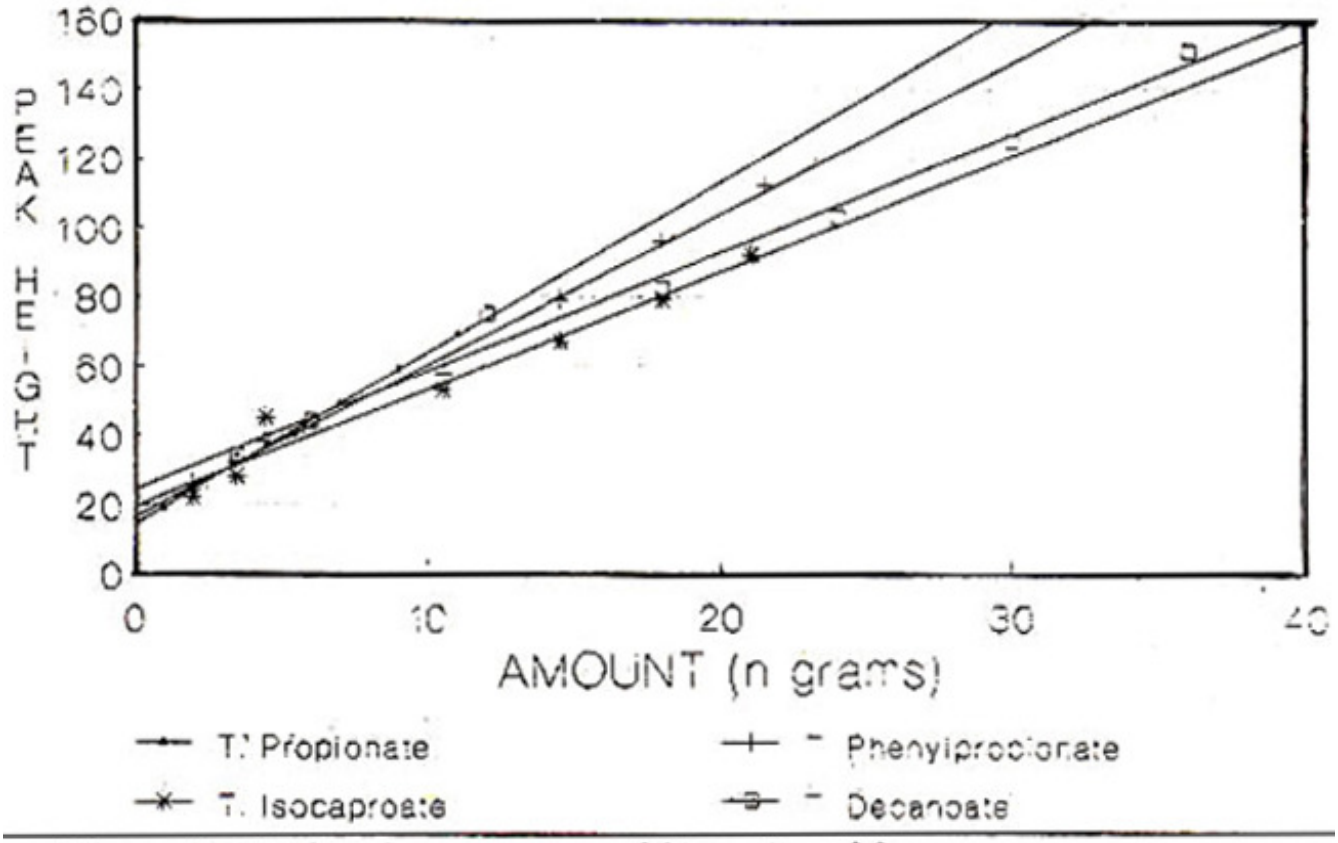

Figure 2. Calibration curves and linearity of four testosterone esters.

Extraction and recovery from urine:

The solvents chloroform, diethylether and dichloromethane were used as extractants for the recovery of testosterone esters from urine. To check the extraction efficiency 5 ml of blank urine was doped with known amount of 25 ul of stock solution per 5 ml of methanol. It was then extracted with 5 ml of organic solvents respectively. The organic layer was separated, evaporated under vacuum at 25°C. The esters were then recovered in 5 ml of mobile phase and out of this 6 ul was injected onto the column giving the final concentration as mentioned in Table II. The extraction efficiency of testosterone esters was poor in chloroform (36, 42, 42 and 48%) and diethylether (46, 46, 54 and 58%) respectively. The best % recoveries of testosterone esters were obtained in DCM, these with standard deviation are also given in Table II.

TABLE II. Percentage recoveries of testosterone esters from doped urine in dichloromethane.

Name of esters	*Amount added (ng)	*Amount detected (ng)	Recovery (%)
Testosterone propionate	9.0	7.9 (±0.4)	87.8
Testosterone phenylpropionate	18.0	15.6 (±0.7)	86.7
Testosterone isocaproate	18.0	16.0 (±0.8)	88.9
Testosterone decanoate	30.0	28.1 (±0.9)	93.7

*Average of three determinations

RESULTS

The standard prepared as well as doped urine sample extracts were eluted by methanol at a flow rate-of 0.4 ml/min at 259C and detected at 254 nm[9,10] (Figure 3 and 4).

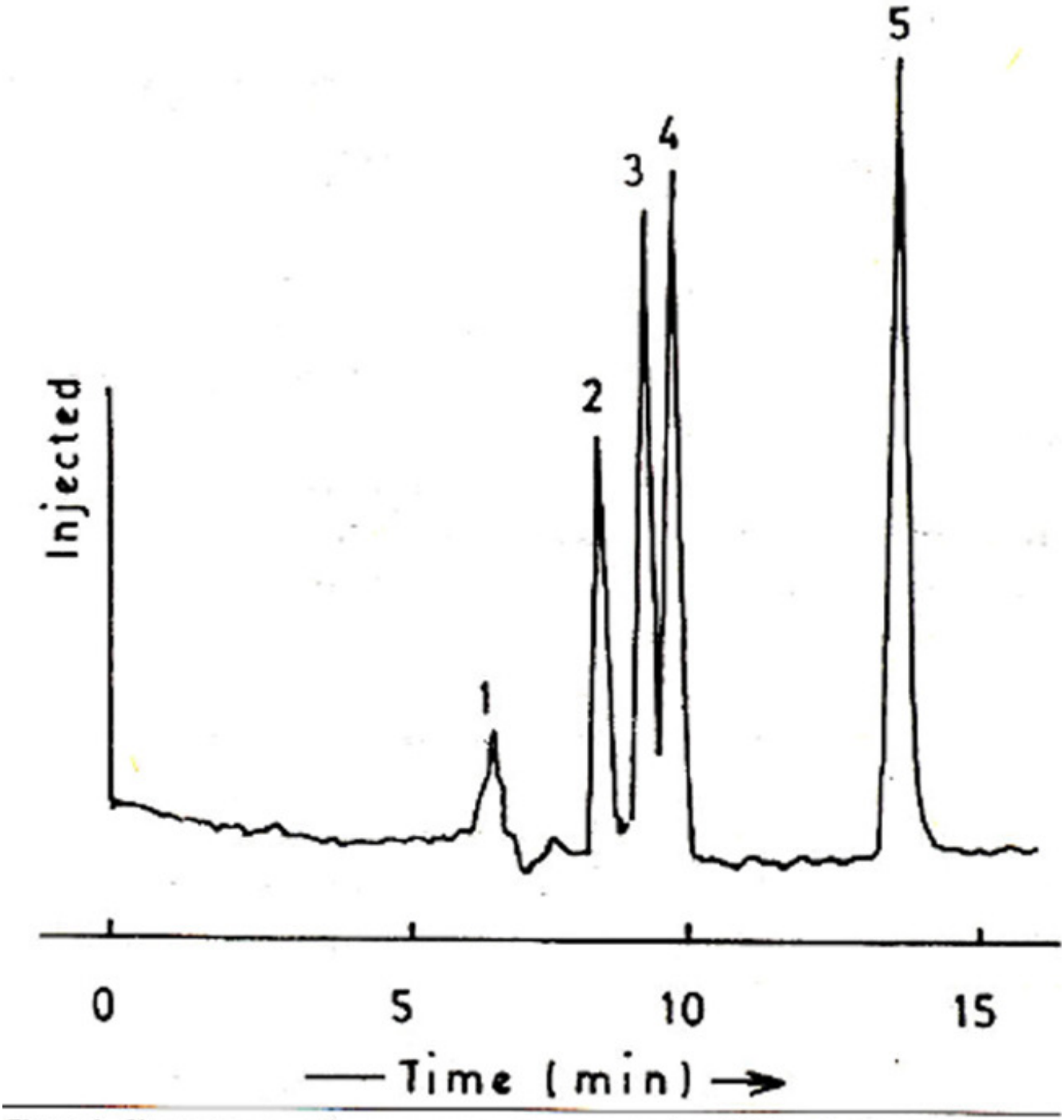

Figure 3. Chromatogram of testosterone esters standard peak identification 1 = solvent peak, 2= T. propionate (3.6 ng), 3= T. isocaproate (7.2 ng), 4= T. phenylpropionate (7.2 n), 5= T. decanoate (12 ng). Column ODS - Nucleosil C18, detector UV= 254 nm; flow rate= 0.4 ml. min^{-1}, eluent pure methanol. Temperature= ambient (25°C).

Figure 4. Chromatogram of dichloromethane extract of urine doped with testosterone esters standard peak identification (1, 2, 3, 4 = natural urine components), 5 = T. propionate, 6 = T. isocaproate, 7 = T. phenyl propionate, 8 = T. decanoate chromatographic conditions same as in Figure 3.

The detection limits achieved from standard testosterone solution was 1.1 ng for testosterone propionate, 2.2 ng each for testosterone phenylpropionate and testosterone isocaproate and 3.6 ng for testosterone decanoate utilizing a total analysis time of 15 minutes (Table I). The mean standard deviations are 0.05-0.1 and coefficients of variance are 2-4.5% (Table I). External caliberation method

was used for quantification. The best % recoveries achieved from doped urine using dichloromethane were 88% for testosterone propionate, 87% for testosterone phenylpropionate, 89% for isocaproate and 94% for testosterone decanoate (Table II). The urine extracts when injected showed a clear separation of testosterone esters at the same specified time as those of standard (Figure 4). The mean standard deviations of doped urine samples each carried out in triplicate was only 0.4-0.9 with coefficients of variation of only 3-5% (Table JI). This indicates good accuracy and precision of results considering the difficult analysis of steroid esters.

DISCUSSION

The ester derivatives of steroids are present in urine in un conjugated form. As demonstrated in this study, these can be easily extracted and detected in presence of natural urinary steroids. The natural steroids do not interfere with the synthetic esters of testosterone as they are normally excreted in free form in very small amounts (testosterone 1.0-1.1 ug/24 hour) that they are not detectable as such in urine without further concentration. Thus the method outlined for detection of testosterone esters can be used to detect these in presence of each other as well as other natural urinary steroids.

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