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Review

Methods for urinary testosterone analysis

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Abstract

Urinary testosterone analysis requires a multistep procedure to achieve a good degree of sensitivity and specificity in the dosage. Hydrolysis, extraction, purification and quantification are usually performed in sequence, and several options can be chosen for each of them. After introductory remarks on the applications of urinary testosterone measurement and a short description of the metabolic pathway of the hormone, an overview of the techniques most commonly used in each step is presented. Advantages and disadvantages of each of them are outlined, and a procedure for urinary testosterone analysis is suggested. The procedure consists of: enzymatic hydrolysis with *Helix pomatia* juice, followed by solid-phase extraction of hydrolyzed urine by a C_{18} cartridge coupled with an NH_2 cartridge and high-performance liquid chromatography cleanup of the extract. Then, quantification can be achieved by gas chromatography or radioimmunoassay.

Contents

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List of abbreviations

C_{18}	Octadecyl-substituted silica
ECD	Electron capture detection
FID	Flame ionization detection
GC	Gas chromatography
GC–MS	Gas chromatography–mass spectrometry
HFB	Heptafluorobutyrate
3β-HDS	3β-Hydroxysteroid dehydrogenase
HPLC	High-performance liquid chromatography
IAC	Immunoaffinity chromatography
I.D.	Internal diameter
MS	Mass spectrometry
NH_2	Amino-substituted silica
PTV	Programmed-temperature vaporization
RIA	Radioimmunoassay
SIM	Selective ion monitoring
SPE	Solid-phase extraction
TLC	Thin-layer chromatography
TMS	Trimethylsilyl

Hormone abbreviations: 5α-androstandiol: 5α-androstan-3α,17β-diol; 5β-androstandiol: 5β-androstan-3α,17β-diol; androstenediol: 5-androsten-3β,17β-diol; androstenedione: 4-androsten-3,17-dione; DHEA, dehydroepiandrosterone: 5-androsten-3β-ol-17-one; dihydrotestosterone: 5α-androstan-17β-ol-3-one; epitestosterone: 4-androsten-17α-ol-3-one; estradiol: 1,3,5(10)-estratrien-3,17α-diol; estrone: 1,3,5(10)-estratrien-3-ol-17-one; LH: luteinizing hormone; pregnenolone: 5-pregnen-3β-ol-20-one; progesterone: 4-pregnen-3,20-dione; SHBG: sex hormone-binding globulin; testosterone: 4-androsten-17β-ol-3-one.

1. Introduction

The measurement of testosterone levels in body fluids is widely employed to evaluate the androgenic status of the subject under investigation. Reproductive disorders in men are mainly represented by hypogonadism, which is generally associated with abnormally low levels of androgens. The opposite occurs in females, in whom androgen excess is responsible for a large number of abnormalities, which variously concur to the development of "hyperandrogenic syndromes".

In clinical practice, testosterone is usually assessed in blood rather than urine owing to the availability of much easier and faster methodologies. However, a single serum measurement may be misleading as a consequence of the episodical secretion of the hormone [1–3]. In contrast, testosterone glucuronide levels in urine are essentially independent of short-term fluctuations in blood and are commonly used as an index of androgen production. Data from our laboratory have shown that whereas measurement of urinary testosterone levels is useful to select breast cancer patients likely to benefit from endocrine therapy [4,5], estimation in blood is ineffective for this scope [6].

In the few last years, measurement of urinary testosterone has gained great relevance in the field of sports to investigate steroid abuse by athletes or animals engaged in sport races. For this purpose, the testosterone/epitestosterone

ratio and the testosterone/LH ratio is measured in untimed urine collections [7].

Urinary testosterone measurement is not trivial. Multistep procedures are necessary to achieve a high degree of sensitivity and specificity in the dosage. Several methods are available. All follow the same procedural steps and differ in the techniques used in each step.

In this paper, we review the most widely employed techniques and outline the advantages and disadvantages of each of them. Before going into detail about the methodologies, we give a short description of the testosterone metabolic pathway to better understand the meaning of the measurement in urine of testosterone and related steroids.

2. Testosterone metabolic pathway

Testosterone synthesis occurs either in the gonads or in extra-endocrine tissues from pro-hormones mainly of adrenal origin. Direct adrenal production of testosterone is minimal. The testosterone metabolic pathway is reported in Fig. 1. In the endocrine glands, cleavage of the side chain of cholesterol is the first and rate-limiting step in steroidogenesis. LH regulates the rate of the reaction, which is catalyzed by the mitochondrial enzyme cytochrome P-450$_{scc}$ and leads to pregnenolone formation [8]. Starting from pregnenolone, the Δ4 pathway (progesterone → androstenedione → testosterone) and the Δ5 pathway (DHEA → androstenediol → testosterone) can be followed. The Δ4 pathway is pre-eminent in the human corpus luteum, whereas the Δ5 pathway is preferred in the human testis and ovarian follicles. Extraglandular synthesis of testosterone occurs in target tissues from pro-hormones with low androgenic activity (DHEA, androstenediol, androstenedione).

Extragonadal production of testosterone is quantitatively more important in women than in men. Peripheral conversion from androstenedione is the main source of testosterone in

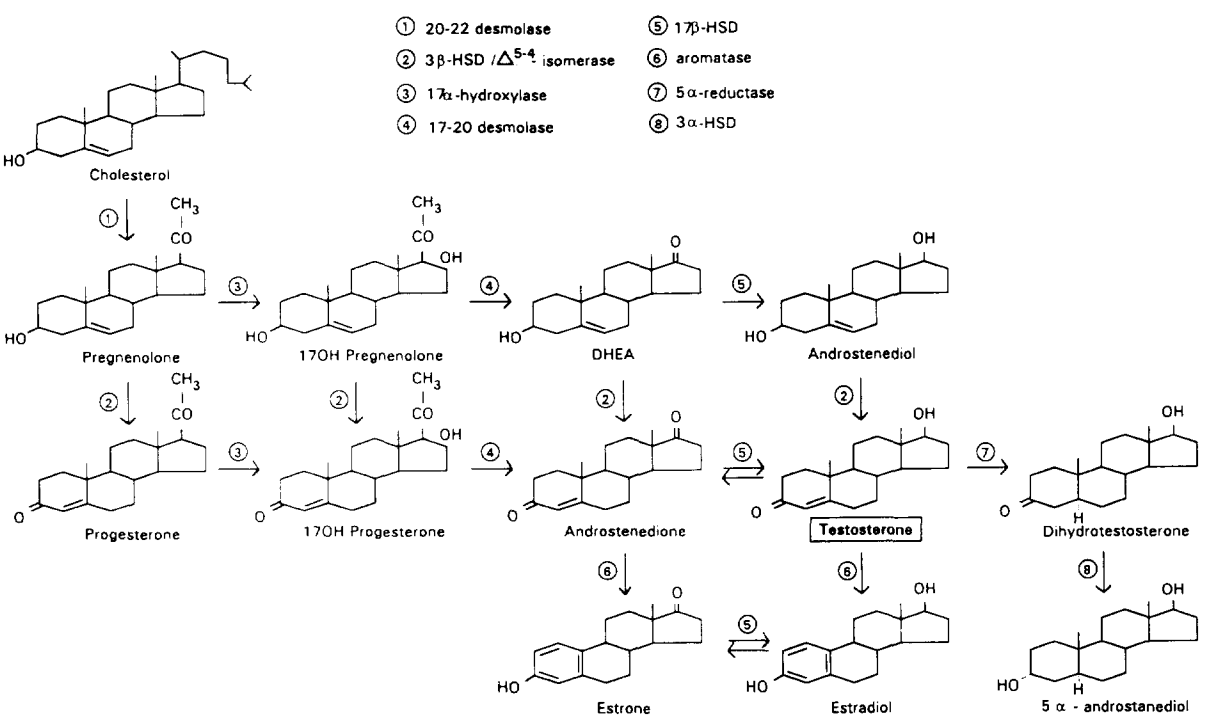

Fig. 1. Testosterone metabolic pathway.

women, and approximately one third of the conversion occurs in adipose tissue [9].

Testosterone is carried in blood largely bound to plasma proteins: aspecifically to albumin and specifically to SHBG. A small amount of unbound (free) testosterone circulates in dynamic equilibrium with the bound fraction of the hormone. It is now generally assumed that the free and the albumin-bound fractions of testosterone represent the biologically active portion of the circulating hormone [10]. At target tissues, testosterone can be converted to dihydrotestosterone by the enzyme 5α-reductase and to estradiol by the aromatase complex. These active metabolites may act locally or may re-enter the plasma. Alternatively, circulating testosterone is broken down by the liver and inactivated. The main routes of inactivation are: (1) reduction of the 3-oxo-4-ene structure in ring A, which gives rise to formation of 17-ketosteroids, and (2) conjugation with glucuronic and sulphuric acid, which favours renal clearance.

Although the excretion of testosterone glucuronide represents only about 1% of the metabolism of endogenous testosterone [11], it is widely recognized that the concentration of testosterone glucuronide in a 24-h urine collection satisfactorily reflects the daily production of testosterone and pro-androgens.

3. Sampling and storage

Urinary testosterone measurement is usually performed on a specimen of the 24-h urine. A mistake in urine collection is one of the most frequent sources of erroneous results, even if the collection procedure has been carefully explained to the patient. An accurate inquiry at the moment of urine delivery to the laboratory can reveal most procedural mistakes. The problem of appropriate collection is particularly felt in epidemiological studies, in which a large number of non-specifically motivated subjects is involved. A simplification in the collection procedure may be highly desirable, and a nocturnal 12-h urine sample may be preferable for this kind of investigation. A study set up in our laboratory showed a fairly good comparison between the 12-h and the 24-h testosterone excretion values in 50 healthy women ($r = 0.93$; $P < 0.0001$) [12].

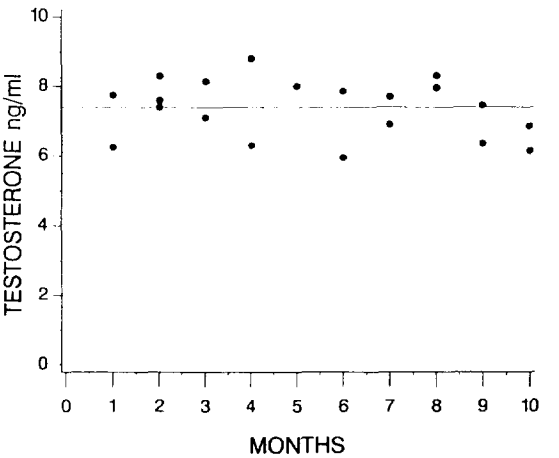

Fig. 2. Effect of time of storage on urinary testosterone measurement in 21 specimens of pooled urine stored at −25°C for 10 months. Single values are plotted around the mean.

Although it is widely accepted that high stability of steroid molecules makes it possible to freeze and store the urine sample at −20°C or less until analysis [13], to our knowledge there is no report in the literature specifically addressed to evaluate the effect of storage on urinary testosterone measurement. Results of a small study from our laboratory can give some indications. A total of 21 specimens from a pool of urine were stored at −25°C, and the amount of testosterone was measured in one of them every 2 weeks for 10 months. The mean value (± S.D.) of 21 determinations was 7.39 (± 0.82) ng/ml. The plot of single values is shown in Fig. 2. We concluded that storage time did not interfere with the assay.

4. Hydrolysis

Testosterone is excreted in urine as glucuronide and, to a much lesser extent, as sulphate. Quantification is usually performed after hydrolysis of conjugates, since the most common methods for testosterone measurement are specific for

the free hormone. Hydrolysis can be done before or after the extraction procedure.

4.1. Acid hydrolysis

A mineral acid is added to urine to cleave steroids from the conjugation acid. The yield of hydrolysis is strongly influenced by several parameters: the acid employed (hydrochloric or sulphuric), the acid molarity, and temperature and time of reaction. Complete hydrolysis is achieved by boiling the urine sample under reflux for 1 h after addition of hydrochloric acid (15%, v/v) [14]. The procedure has been described as disruptive for some steroids, such as DHEA or 17-hydroxycorticosteroids [13], but, in our experience, significant disruption of testosterone does not occur. Alternatively, Kjeld et al. [15] suggested that the optimal conditions for complete hydrolysis are 3 *M* sulphuric acid at 37°C for 24 h.

4.2. Enzymatic hydrolysis

Steroid conjugates are hydrolysed by specific lytic enzymes derived from bacteria, beef liver and molluscs. The bacterial (*Escherichia coli*) and beef liver extracts are devoid of sulphatase activity. They contain β-glucuronidase activity that yields the best hydrolysis at pH 6.8–7.0 for *E. coli* extract [16] and at pH 5.0 for beef liver extract [16,17].

Mollusc preparations are derived from limpet (*Patella vulgata*) and from snail (*Helix pomatia*). In addition to β-glucuronidase activity, they contain sulphatase activity, weak for *P. vulgata* and substantial for *H. pomatia* [16,18]. These preparations require a pH of 4.5–5.5 for optimal activity.

Androgens sulphated at position 17 are resistant to *H. pomatia* hydrolysis [18,19], and the *P. vulgata* sulphatase activity is highly specific for 5α-3β-hydroxy and Δ5-3β-hydroxy steroids [18]. Consequently, neither of these extracts is able to hydrolyse testosterone-sulphate.

In our laboratory, β-glucuronidase activity of extracts from *H. pomatia* (Sepracor, Villeneuve-la-Garenne, France), *P. vulgata* (Sigma, St. Louis, MO, USA) and *E. coli* (Sigma) was checked by adding the enzyme to a sample of stripped urine spiked with known amounts of testosterone glucuronide. After hydrolysis, the urine samples were processed by SPE, HPLC and GC quantification of unconjugated testosterone, according to the method of our laboratory [20]. The experiment was done in triplicate. In agreement with a previously published report [17], we concluded that there is no obvious preferred β-glucuronidase extract for enzymatic hydrolysis of testosterone (Table 1).

Table 1
β-Glucuronidase activity of different enzymatic preparations calculated by recovery of testosterone from stripped urine spiked with known amounts of testosterone-glucuronide

Testosterone-glucuronide added (nmol)	Testosterone recovered (mean of triplicates)					
	H. pomatia[a]		*P. vulgata*[b]		*E. coli*[b]	
	nmol	%	nmol	%	nmol	%
0.00	0.00	00	0.00	00	0.00	00
0.87	0.81	93	0.78	90	0.73	84
1.74	1.58	91	1.64	94	1.52	87
2.60	2.20	85	2.25	87	2.31	89

The experiment was performed in triplicate. Analytical conditions are given in the text.
[a] *Helix pomatia* (Sepracor, Villeneuve-la-Garenne, France).
[b] *Patella vulgata* and *Escherichia coli* (Sigma, St. Louis, MO, USA).

It is noteworthy that *H. pomatia* juice can contain 3β-HSD/Δ^{5-4} isomerase activity in addition to β-glucuronidase and sulphatase activities. The 3β-HSD/Δ^{5-4} isomerase activity is able to convert androstenediol into testosterone: the extent of conversion depends on the source of the preparation and greatly differs from batch to batch [19,21–23]. Androstenediol is excreted in urine in a large amount [24] and even a small percentage of conversion into testosterone can heavily affect the measurement of the steroid. The presence of 3β-HSD/Δ^{5-4} isomerase activity in *H. pomatia* extract is not usually checked by the producer and is not reported in the instruction sheet, thus *H. pomatia* juice must be systematically tested by the user before its use.

In a study from our laboratory, 3β-HSD/Δ^{5-4} isomerase activity was checked in several enzymatic preparations by evaluating conversion of androstenediol to testosterone. The enzymes were added to 50 ml of stripped urine spiked with 50 μg of androstenediol, and conversion to testosterone was measured by GC after hydrolysis, SPE and HPLC. Testosterone identification was confirmed by GC–MS [20]. Each enzymatic preparation was checked in triplicate. 3β-HSD/Δ^{5-4} isomerase activity was found only in the *H. pomatia* extracts from Sepracor (Table 2).

For enzymatic hydrolysis, urine is buffered to the pH optimal for the chosen preparation; the enzyme is added and the specimen incubated under appropriate conditions. Several conditions, varying from 1 to 48 h at 37–55°C, have been described in the literature. Although the best hydrolysis is achieved after a prolonged incubation, the procedure can be accelerated by increasing the incubation temperature in order to save time [16,17,23]. A good compromise is obtained by incubating the sample at 45°C for 22 h.

Enzymatic hydrolysis is effective only for testosterone glucuronide, but it is often preferred to acid hydrolysis owing to the negligible excretion of testosterone sulphate. Complete hydrolysis of conjugated testosterone is achieved by enzymatic hydrolysis of glucuronides followed by solvolysis of sulphates. Solvolysis is a mild method of hydrolysis for sulphate steroids. Several modifications of the original method of Burstein and Lieberman [25] have been proposed and are currently used [16,17,26,27].

5. Extraction procedures

Preliminary extraction of testosterone is required for further purification steps. The procedure allows sample concentration and induces a partial cleanup.

Table 2
3β-HSD/Δ^{5-4} isomerase activity of different enzymatic preparations calculated by recovery of testosterone from stripped urine spiked with 50 μg of androstenediol

Enzymatic preparations	Testosterone recovered from androstenediol (50 μg)	
	Mean (μg)	%
Helix pomatia[a]		
Lot no. 1299	14.6	29.2
Lot no. 8526	4.9	9.8
Escherichia coli[b]	n.d.[c]	0.0
Patella vulgata[b]	n.d.	0.0
Helix pomatia Type H-1[b]	n.d.	0.0

The experiment was performed in triplicate. Analytical conditions are given in the text.
[a] Purchased from Sepracor.
[b] Purchased from Sigma.
[c] n.d. = not detectable.

5.1. Liquid–liquid extraction

The principle of the extraction is based on partition of the hormone between two immiscible solvents. The method is usually employed to recover unconjugated testosterone, which is only slightly soluble in an aqueous matrix and can be extracted from urine into organic solvents, usually, diethyl ether and dichloromethane. After washing with an alkaline solution and distilled water, the sample may be submitted to further analytical steps [28–30]. This procedure is simple and fast but requires a high ratio of solvent to urine matrix.

5.2. Solid-phase extraction

In SPE, testosterone is retained on an adsorbent material and is subsequently recovered by polar solvents.

5.2.1. Amberlite XAD-2

Amberlite XAD-2 is a neutral cross-linked polystyrene polymer that has been successfully used in the past [31]. The procedure consists of percolating the urine sample through a column filled with the resin. After washing with water, the steroids are recovered by methanol, ethanol and tetrahydrofuran [26,31–33]. The procedure is time-consuming (flow-rate about 0.2 ml/cm^2/min), resin capacity is low (0.5 g/ml of urine), and a large volume of solvent is required [23,34,35]. These disadvantages are overcome by the use of C_{18}.

5.2.2. Octadecyl-substituted silica (C_{18})

C_{18} sorbent, packed on a small polystyrene cartridge, has taken the place of Amberlite XAD-2. The C_{18} sorbent efficiently retains free and conjugated testosterone and has a much higher capacity than Amberlite (about 300 mg per 100 ml of urine), the androgen is recovered in a small volume of solvent (3–5 ml), and a flow-rate as fast as 30 ml/min can be applied with a vacuum chamber [35,36].

Just before use, the C_{18} cartridge is primed with an organic solvent to activate the adsorbent and with water to remove residual solvent. The sample is applied to the column and washed with an aqueous solution, then testosterone is eluted with methanol or acetonitrile [20,35–37].

As mentioned before, the extraction step effects a partial cleanup. Schmidt et al. [38] suggested that coupling the C_{18} cartridge with an NH_2-substituted silica cartridge improves sample purification and makes the extract suitable for measurement of some steroids by GC. However, the authors did not analyze testosterone [38]. In agreement with Schmidt et al. [38], we found that C_{18} plus NH_2 cartridges are more effective than C_{18} alone to purify the sample. However, an additional cleanup with HPLC is necessary for GC analysis of testosterone (Fig. 3).

6. Additional cleanup procedures

Partial purification of urinary testosterone is obtained with the extraction procedure, and further cleanup can be achieved with Girard's reaction, which separates ketonic from non-ketonic compounds and involves subsequent extraction of the testosterone fraction [14]. However, such procedures do not allow a suitable cleanup of the sample, and chromatographic techniques such as column chromatography, TLC and HPLC are used as further purification steps.

6.1. Conventional methods of column chromatography

Column chromatography is a time-consuming technique which requires great operative care. Sephadex LH-20, Sephadex LH-20 derivatives and Celite are the most frequently used materials for steroid cleanup. Immunoaffinity chromatography has been recently proposed for steroid purification [39], but materials for this technique are not commercially available.

6.1.1. Sephadex LH-20 and Sephadex LH-20 derivatives

Sephadex LH-20 is used to separate unconjugated steroids by adsorption chromatography [16]. A method for urinary testosterone purifica-

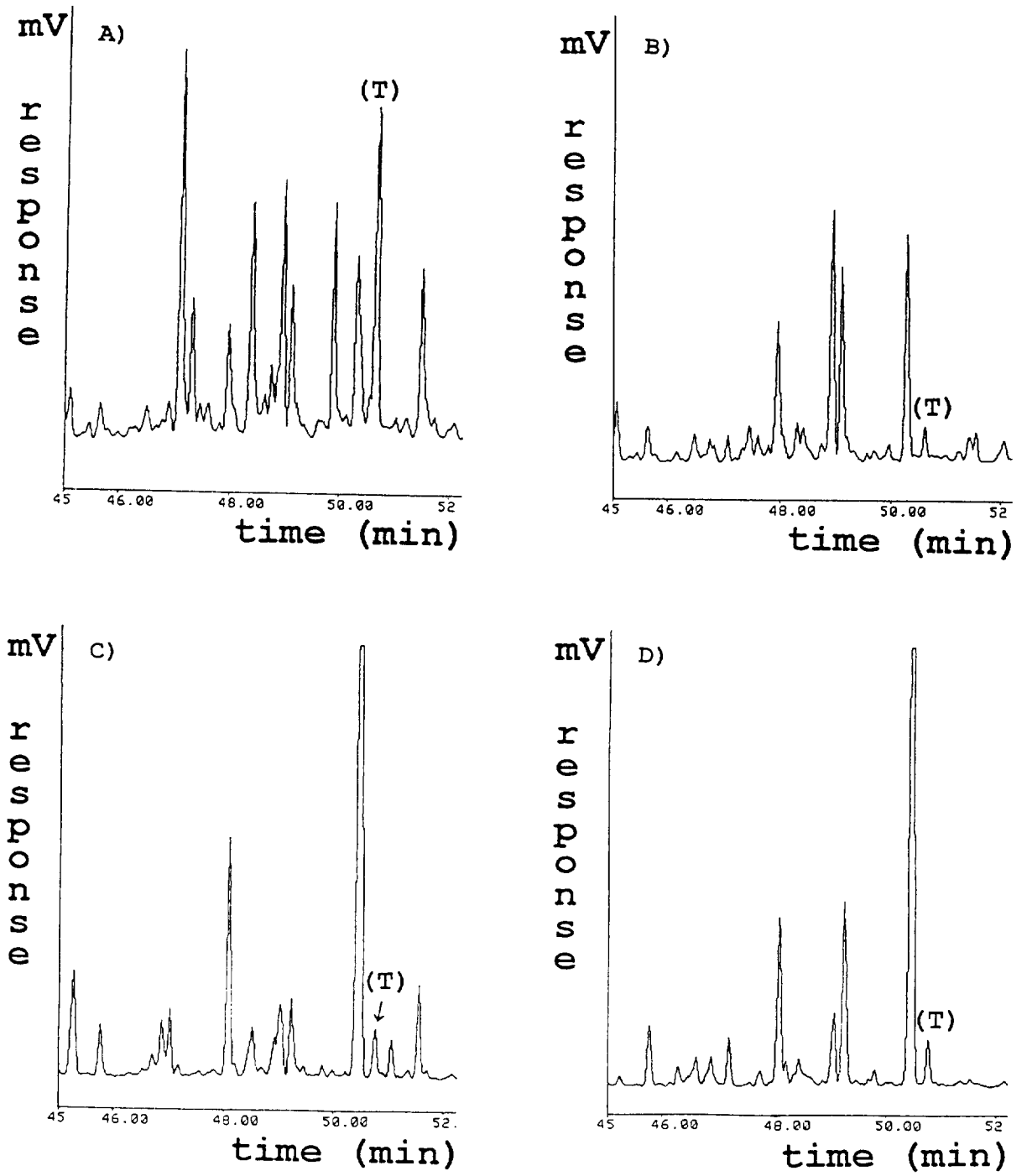

Fig. 3. Gas chromatographic analysis of testosterone in two urine samples: A and C, solid-phase extraction with C_{18} and HPLC cleanup; B and D, solid-phase extraction with $C_{18} + NH_2$ and HPLC cleanup. In A the testosterone peak is spiked with an unknown compound. Analytical conditions are reported in the flowchart of Fig. 6. T = testosterone.

tion has been recently reported [27]: the extract from 0.2 ml of hydrolyzed urine is applied to a Sephadex LH-20 column (200 mm length; 5 mm I.D.) and eluted with a mixture of dichloromethane–methanol (95:5, v/v). Testosterone is quantified by GC–MS.

Lipophilic ion exchanger derivatives of Sephadex were introduced to separate groups of conjugated steroids. Methods for testosterone glucuronide purification by these derivatives have been described [32,40].

6.1.2. Celite

Partition chromatography of unconjugated steroids can be achieved using Celite impregnated with ethylene glycol, propylene glycol and a mixture of these compounds as the stationary phase. Steroids are usually eluted with a mixture of isooctane–benzene or isooctane–ethyl acetate, under gravity flow [41–43].

Our experience in testosterone purification with Celite column chromatography is reported in Table 3. Chromatographic columns were prepared using glass pipettes (30 cm in length; 0.5 cm I.D.) filled with 0.8 g of Celite impregnated with the stationary phase in the ratio 2:1 (w/v). Elution was performed under gravity flow at a rate of 0.85 ml/min. Three methods, differing from each other for the stationary phase and elution mixture, were compared, and all guaranteed testosterone cleanup suitable for GC and RIA determination. We concluded that the choice of the chromatographic system depends on the other steroids, in addition to testosterone, to be measured.

6.1.3. Immunoaffinity chromatography

With this technique, hormones are extracted and purified by linkage to specific antibodies applied to the matrix packing the column. An exhaustive review on the use of IAC in the separation of drugs and steroids, including testosterone, has been recently published [39]. According to the author, IAC is superior to most other techniques for sample extraction and cleanup, but its diffusion is actually limited by nonavailability of materials and high costs. Future expansion of IAC will largely depend on the interest of commercial firms.

Table 3
Methods for sex steroid separation by Celite column chromatography

Method A PG (100%)			Method B PG–EG (50:50, v/v)			Method C PG–EG (20:80, v/v)		
Iso–Benz (v/v)	ml	Horm	Iso–Et.ac (v/v)	ml	Horm	Iso–Et.ac (v/v)	ml	Horm
100:0	3							
90:10	3	A	100:0	5	A			
	1			1.5		100:0	2	
65:35	3	DHT	94:6	6	DHT	97:3	3.5	DHT
	1			1.5		1		
40:60	3	T	80:20	6	T	82:18	3.5	T
	1						1	
25:75	5							
	1							
0:100	14	E2				70:30	3.5	E2

Stationary phases: (A) propylene glycol (100%); (B) propylene glycol–ethylene glycol (50:50, v/v); (C) propylene glycol–ethylene glycol (20:80, v/v). Elution mixtures: (A) isooctane–benzene (Iso–Benz); (B) and (C) isooctane–ethyl acetate (Iso–Et.ac). Analytical conditions are given in the text. PG = propylene glycol; EG = ethylene glycol; Horm = hormone; A = androstenedione; DHT = dihydrotestosterone; T = testosterone; E2 = estradiol

6.2. Thin-layer chromatography

TLC is a fast, simple and versatile technique. Exhaustive textbooks on the theory and application of the technique are available [44,45].

In our laboratory, we perform TLC using 20 × 20 cm glass plates coated with silica gel to form a 0.25 mm thin layer. Chromatography is developed by a binary mixture of chloroform–ethyl acetate (4:1, v/v) to obtain a testosterone fraction suitable for GC determination of the hormone without further purification. Liquid–liquid extraction of the urine sample followed by Girard's reaction was performed before TLC [46].

TLC purification is usually applied for unconjugated testosterone. However, TLC separation of testosterone glucuronide and sulphate has been reported by Puah et al. [47].

6.3. High-performance liquid chromatography

Great power of resolution and high repeatability of analytical conditions characterize the technique. The HPLC procedure can be made completely automatic from sample injection to eluate collection [48].

Normal-phase and reversed-phase columns are used for steroid separation [13,49]. The most frequently employed is the C_{18} type, in which octadecyl groups are bound to the silica support to give a hydrophobic surface. The coverage grade of the silica support varies from one supplier to another, thereby affecting steroid selectivity. A comparison of column efficiency by different suppliers was reported by Nice and O'Hare [50]. Stainless-steel columns of 250–300 mm in length, 4–5 mm I.D. and 5 μm particle size are commonly used. The use of "microbore" columns (1 mm I.D.) has been recently proposed to accelerate analysis and to allow interfacing with an MS detector [51,52]. A guard column is generally adopted to increase column life and working efficiency.

Steroids are eluted with a mixture of water and polar solvents, such as methanol, acetonitrile and tetrahydrofuran. A linear gradient elution is most frequently employed, but separation of testosterone has also been reported by isocratic elution with acetonitrile–water (1:1, v/v) [53]. HPLC purification is usually employed for unconjugated steroids. However, HPLC separation of testosterone glucuronide has been reported [54].

Reversed-phase columns (250 × 4 mm; 5-μm particles) coupled with a guard column (10 × 4 mm) are used in our laboratory for cleanup of deconjugated testosterone after SPE. Linear gradient elution from acetonitrile–water (10:90, v/v) to 100% acetonitrile in 25 min at a flow-rate of 1 ml/min is applied. Acetonitrile is maintained at 100% for 5 min. The collection window of testosterone is set, on the basis of retention time of the standard compound monitored by a UV detector, at 254 nm [20]. Typical GC traces of urine extracts after HPLC cleanup are reported in Fig. 3B–D.

7. Determination of testosterone

GC, GC–MS and RIA are the most widely used techniques for urinary testosterone quantification. Accurate measurement of the hormone occurs only if a sufficiently purified sample is processed. Non-radiolabelled immunoassays have been conceived to eliminate the isotope management problems inherent in RIA [55], but their use is still limited for testosterone determination.

7.1. Gas chromatography and gas chromatography–mass spectrometry

Measurement of urinary steroids by GC dates back to the early sixties [56]. Since then, several modifications have been introduced to improve performance of the technique [57]. As a consequence of continuous technical evolution, GC is still widely used in steroid analysis and is often chosen as the reference method. The method is precise and accurate. A requirement of highly experienced technical staff represents the main limitation of the technique, particularly when GC–MS is applied.

Separation of a compound mixture by GC

occurs through a partition of the analytes between the liquid stationary phase and the gas mobile phase. Preliminary derivatization of the purified sample strongly improves the yield of GC analysis by enhancing separation and detection of analytes. Silyl derivatives, oxime-silyl derivatives and acyl derivatives are commonly used for testosterone analysis [58]. Silylation of the 17-hydroxyl group is the simplest reaction, and many reagents and methods are available. 17-Silylation of testosterone is often associated with enolization of the 3-keto group, resulting in an undesirable mixture of 17-silyl- and 3,17-disilyltestosterone. To prevent multiple derivative formation, two general approaches can be followed: (1) force the reaction conditions to obtain 100% of the disilyl derivative, and (2) introduce oxime derivatization of the keto group before silylation to avoid keto enolysis thus obtaining 3-oxime-17-silyltestosterone. However, formation of isomeric forms of derivatives is observed with both procedures: for the disilyl derivative, 2,4-diene and 3,5-diene forms [58,59]; for the oxime-silyl derivative, the *syn* and *anti* forms with respect to the nitrogen of the oxime group [60–63]. The 2,4-diene and 3,5-diene isomeric forms have also been observed in the acyl derivative of testosterone [58,63].

Multiple derivative formation is the main problem with the derivatization procedures, and many expedients have been proposed to minimize this phenomenon [58,59,62–67]. Satisfactory results can be obtained with the method in use in our laboratory [20], which gives rise to negligible amounts of 3,17-di-TMS-testosterone (Fig. 4A). Interestingly, three peaks are detected when the sample is injected by the split–splitless system (Fig. 4B). We surmise that injection in a hot chamber pushes the reaction towards the formation of 3,17-di-TMS-testosterone isomeric forms.

The derivatized mixture is applied to the instrument through the injector system. Split-splitless, PTV and on-column injector systems are commonly used. Exhaustive reviews on the theory and problems inherent to the different systems have been reported by Grob [68,69]. A PTV injector is preferable for analysis of complex mixtures from urinary extracts [60]. The injector system makes it possible to charge the sample on the chromatographic column, which is within an oven. Commonly, the oven temperature is gradually increased during the run to enhance analyte separation.

Several columns are available for steroid analysis [13]. Fused-silica micro-columns (25–30 m in length; 0.2–0.32 mm I.D.) covered with 0.2–0.5 μm thick film of apolar and low polar stationary phase (methylsilica; phenylmethylsilica) are commonly employed. Good column efficiency (>3000 theoretical plates per meter) is needed for good chromatographic separation of testosterone. A total of 578 hypothetical urinary metabolites has been calculated for the hormone [70]. Continuous check of column efficiency is obtained in our laboratory by injection of a mixture of androstenedione- and 5β-androstanediol-TMS derivatives at regular time intervals. Efficiency is satisfactory when the corresponding peaks are base resolved.

After separation during the chromatographic run, compounds flow out from the column into the detection system. FID, ECD and MS systems are available. FID is used in combination with TMS derivatives and oxime-TMS derivatives. Sensitivity is in the nanogram range. Low cost and simple management make FID the most widely employed detection system.

ECD is highly sensitive for testosterone derivatized with halogenated anhydrides. HFB derivatives are usually injected, and as little as 50 pg of HFB-testosterone can be detected. Great operative care and high sensitivity to nonspecific background debris are the main limitations to the use of ECD [71].

GC–MS currently has a prominent role in steroid analysis, and extensive reviews on MS principles and applications are available [72,73]. GC–MS is suitable to obtain highly accurate and sensitive analyses by two MS operating modalities adopted in sequence: SCAN mode and SIM mode. The entire spectrum of the analyte is recorded by the SCAN mode, which is used to verify the accuracy of analytical methods and to identify unknown compounds [60,74]. In the SIM mode, only a few ions specific for the

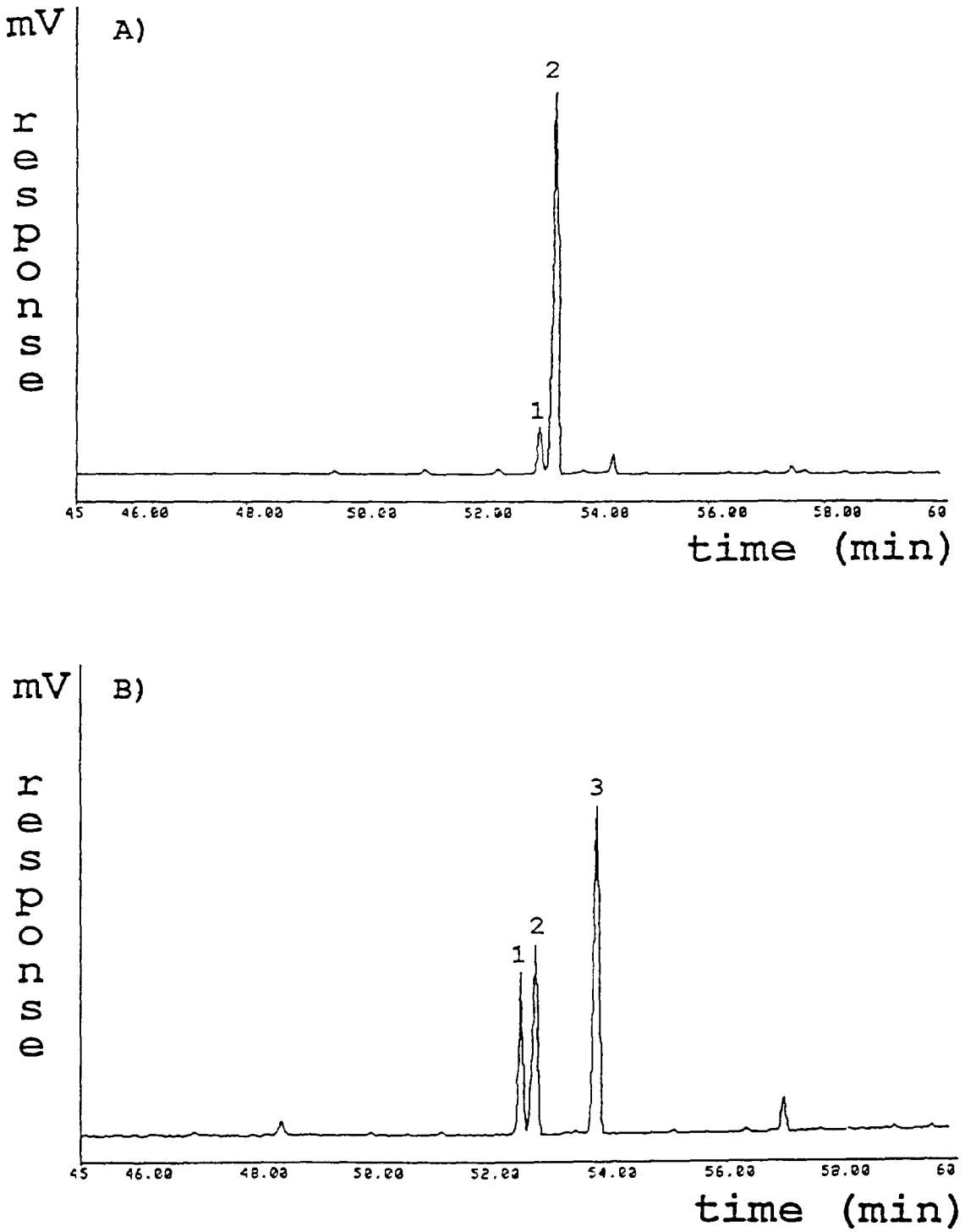

Fig. 4. GC trace of TMS-testosterone injected by: (A) programmed-temperature vaporization (PTV), (B) split-splitless. 1 and 3 = 3,17-di-TMS-testosterone isomeric forms (molecular ion: $m/z = 432$); 2 = 17-TMS-testosterone (molecular ion: $m/z = 360$).

analyte are recorded. The SIM mode is used in quantitative analysis to reach sensitivities in the picogram range [63,75–78].

7.2. Radioimmunoassay

RIA is largely used for routine analysis of steroids. Sensitivity allows detection of hormones in the picogram range, and many samples can be processed simultaneously. The principle of RIA consists of the competition between labelled and unlabelled steroid for a limited number of binding sites on the antibody molecules. Antibodies to steroids are obtained by immunization against the steroid coupled to a large antigenic molecule, such as albumin, and bear a certain degree of cross reactivity with interferers in the sample [79].

Problems of RIA analysis have been extensively studied for measurement of steroids in blood, whereas little attention has been given to urinary steroid determination. The main limitations to RIA of testosterone in urine are: (1) commercial kits are available for measurement in blood and cannot be used for direct measurement of testosterone in urine; (2) substances in the urinary matrix evoke immunological responses similar to that of testosterone.

Use of Celite column chromatography and HPLC has been reported as effective for sample cleanup [43,53]. In our laboratory, HPLC cleanup was employed to compare GC and RIA urinary testosterone determination. Urine specimens (50 ml) from 50 subjects were hydrolyzed with *H. pomatia* juice, extracted in the solid-phase $C_{18} + NH_2$ cartridges, and purified by HPLC. An aliquot of HPLC eluate corresponding to 1 ml of urine was removed, dried and redissolved in 1.2 ml of Tris buffer. An aliquot of 10 μl was then diluted in 125 μl of standard zero reagent for RIA determination (DIRIA-TESTOK Sorin, Saluggia, Italy). The remaining eluate was submitted to GC analysis, and testosterone identification was confirmed by GC–MS, as previously reported [20]. The values obtained with the two methods were quite superimposable and are shown in Fig. 5 ($r = 0.99$; $y = 1.11x - 0.51$).

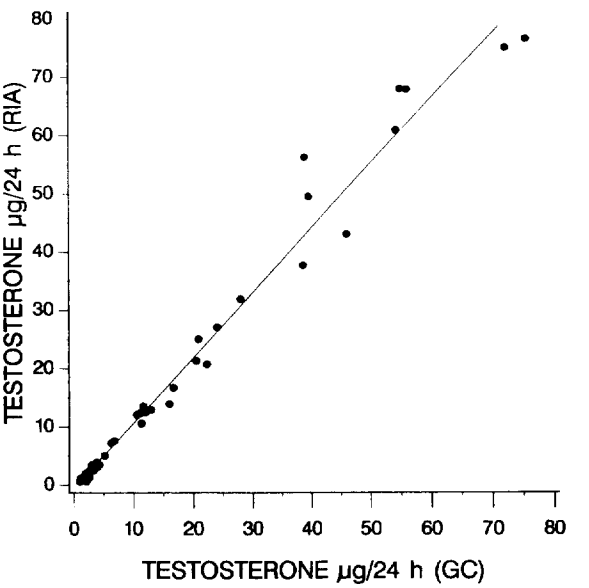

Fig. 5. Correlation between values of urinary testosterone obtained by RIA (y) and by GC (x). Computed regression line: $y = 1.11x - 0.51$, $r = 0.99$; number of subjects = 50.

8. Recovery procedures

Knowledge of losses occurring during manipulation of the sample is essential to improve the accuracy of the analytical procedure, and calculation of recovery is necessary to verify a method. Loss estimation is performed by adding an internal standard to the sample under examination as early as possible during the analytical steps. The best internal standard for a steroid is the steroid itself labelled with a stable isotope of hydrogen (^2H) and carbon (^{13}C) or with a radioactive isotope of the same elements (^3H, ^{14}C).

Use of stable isotopes requires GC–MS equipment, whereas use of radioactive isotopes may be responsible for environmental contamination. If neither of these options can be employed, unlabelled steroids can be used, but evaluation of recovery is less precise [13]. Calculation of recovery can be used to correct the final value of the analysis for the losses of each sample. This correction is possible only with GC–MS and RIA determinations. Using GC equipped with FID and ECD, a good analytical accuracy can be

attained by correcting for the mean recovery of the entire procedure.

Choice of the best internal standard is still a matter of debate. The problem is far from being resolved, and even the use of labelled hormones has been criticized [72]. The opportunity of

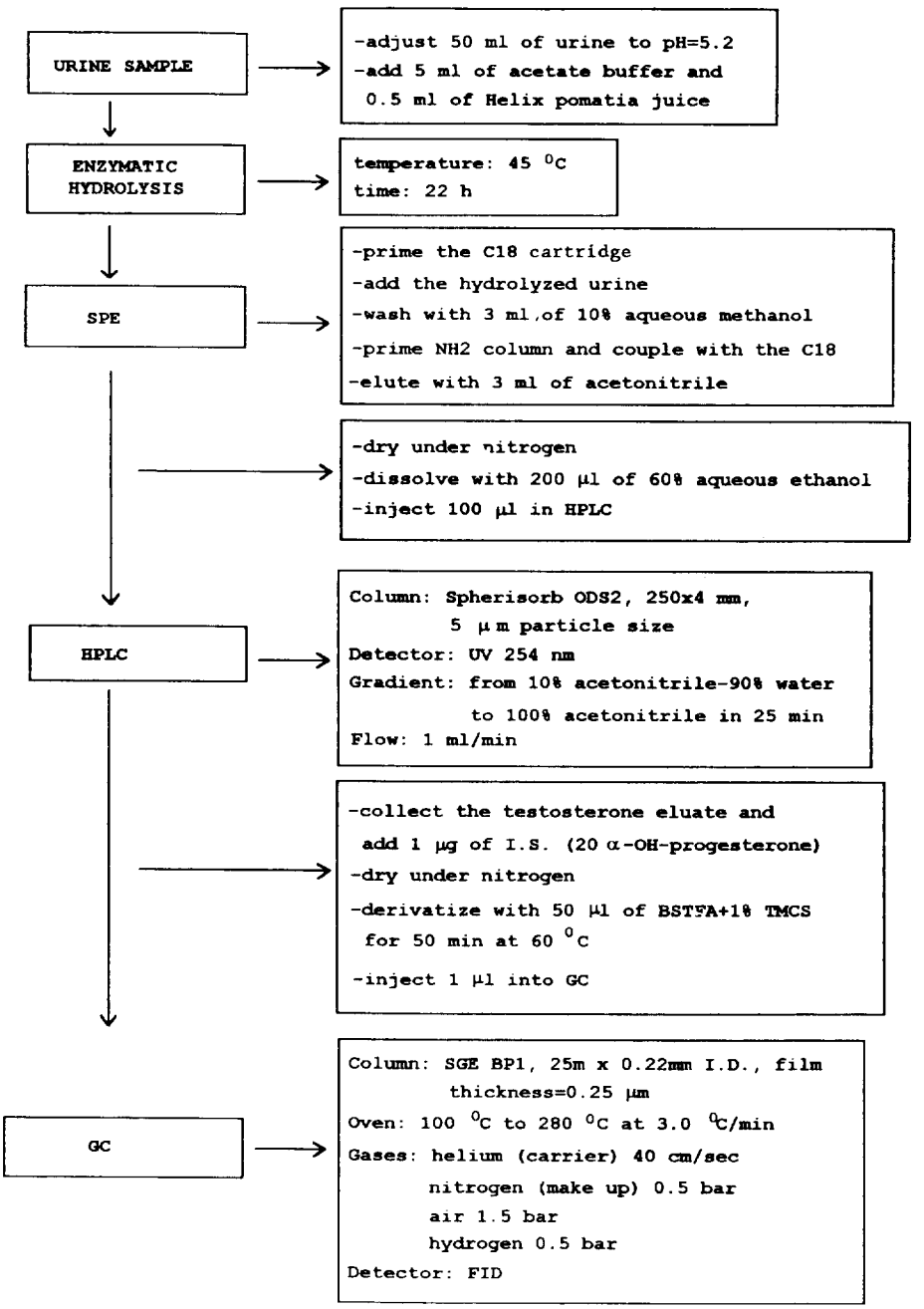

Fig. 6. Flowchart of the method used in the authors' laboratory.

Table 4
Collection windows of sex steroids in HPLC

Sex steroids	Collection windows (min)
Estradiol	15.2–15.8
Testosterone and estrone	15.9–16.8
Androstenedione and 5β-androstanediol	16.8–17.4
5α-Androstanediol	17.4–18.0
Dihydrotestosterone	18.0–18.7

Analytical conditions are given in the text.

correcting for losses should be carefully evaluated, and correction can be avoided when recovery of the entire procedure is satisfactory.

9. Method used in the authors' laboratory

A 50-ml urine specimen is processed. Enzymatic hydrolysis followed by SPE and HPLC cleanup are the preliminary steps to GC quantification of testosterone. The method was recently published in detail [20], and the procedural steps are summarized in the flowchart of Fig. 6. As reported in section 7, determination by GC can be effectively substituted by RIA, for which only 1-ml urine specimens need to be processed.

The HPLC procedure used for testosterone cleanup is suitable to fractionate other sex steroids. 5α-Androstanediol, 5β-androstanediol, androstenedione, dihydrotestosterone, estrone, and estradiol, in addition to testosterone, can be separated in the same analytical run (Table 4). Testosterone and estrone are eluted in the same fraction, and 5β-androstanediol is eluted together with androstenedione. HPLC purification is suitable for GC analysis of all these steroids. In particular, good resolution of 5β-androstanediol from androstenedione and of estrone from testosterone is achieved by GC (Fig. 7).

Recovery for testosterone, androstenedione, dihydrotestosterone, estrone and estradiol was checked by the addition of radiolabelled hormones to urine samples. Recovery for 5α-androstanediol and 5β-androstanediol was calcu-

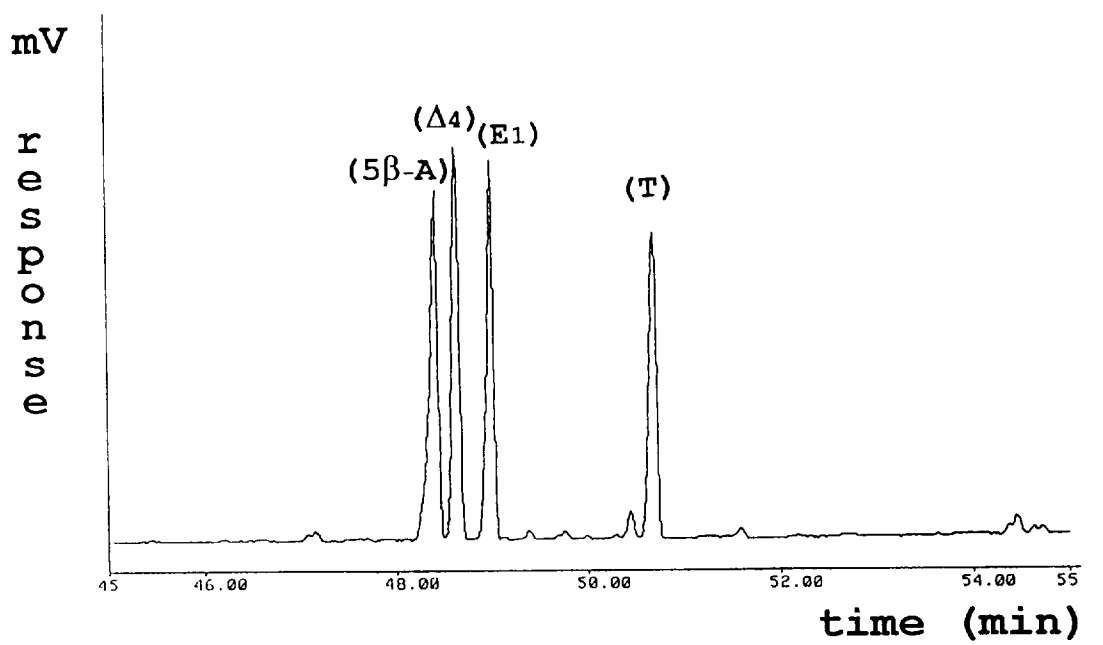

Fig. 7. Gas chromatogram of 5β-androstanediol (5β-A), androstenedione (Δ4), estrone (E1) and testosterone (T) standard compounds. Analytical conditions are given in the text.

Table 5
Recovery of sex steroids after SPE and HPLC cleanup

Sex steroids	Mean recovery (%) of triplicates
Estradiol[a]	80.35
Testosterone[a]	82.00
Estrone[a]	87.85
Androstenedione[a]	86.09
5β-Androstanediol[b]	87.77
5α-Androstanediol[b]	89.78
Dihydrotestosterone[a]	70.00

The experiment was performed in triplicate. Analytical conditions are given in the text.
[a] Calculated by the addition of radiolabelled hormone to urine.
[b] Calculated by addition of unlabelled hormone to stripped urine.

lated by adding a known amount of unlabelled hormones to stripped urine. Mean recoveries from triplicates ranged from 70% for dihydrotestosterone to 89% for 5α-androstanediol (Table 5).

Profiling urinary sex steroids may be of clinical interest in some circumstances, and the method reported can be usefully employed for this purpose.

10. Trends in analytical methods of urinary testosterone measurement

Search for simpler and faster procedures and improvement of accuracy in quantitative analysis are the main objectives pursued in the development of analytical methods. Interesting technical innovations are in progress, and we will list below those which could be favourably applied to urinary testosterone analysis.

Use of IAC has already been mentioned in this review [39]. IAC allows a very selective extraction of the analyte through binding to specific antibody, and makes further purification steps unnecessary [39,80].

A completely automated HPLC procedure for sequential extraction, purification and determination of some drugs in urine has been reported by Mück and Henion [81]. The actual limitation to applicability of the method to testosterone measurement consists in the small amount of urine that can be injected in the HPLC equipment, but work is in progress to overcome this hindrance.

The development of HPLC coupled with MS made this technique suitable for steroid analysis [82]. HPLC–MS allows direct measurement of testosterone glucuronide in urine, thus avoiding problems inherent to yield of hydrolysis and sample derivatization [54,83].

Substantial improvement in accuracy of analysis is obtained by tandem MS coupled with GC [84] or HPLC [73]. High costs and requirement of specialized technical staff are the main limitations to diffusion of these techniques.

In the last years, great interest has been addressed to the development of enzyme immunoassay with the aim to take the place of RIA [55,85], thus eliminating the problems inherent to the management of radioactive substances. However, performance of this method for urinary testosterone measurement must be further consolidated.

Each of the aforementioned methods pursues different finalities and is directed towards different categories of users. For example, doping analysis in sport needs best accuracy, and the cost of some hundred-thousand dollars for technical equipment is not expensive for analysis in a field which moves a hundred-million dollars a year. However, development of an enzyme immunoassay may be of interest for routine analysis laboratories, whereas IAC and some HPLC applications could be employed in research.

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