ELSEVIER

Journal of Chromatography B, 673 (1995) 159–164

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of epitestosterone and testosterone in urine by high-performance liquid chromatography

R. Navajas*, C. Imaz, D. Carreras, M. García, M. Pérez, C. Rodríguez, A.F. Rodríguez, R. Cortés

Laboratorio de Control de Dopaje, Consejo Superior de Deportes, c/ Greco s/n, 28040 Madrid, Spain

First received 1 March 1995; revised manuscript received 30 May 1995; accepted 13 June 1995

Abstract

This paper describes an alternative HPLC method for the determination of testosterone and epitestosterone, which is simple, rapid, selective, sensitive and reproducible. Samples were prepared using a previous enzymatic hydrolysis with liquid–liquid extraction. The determination was carried out on a Hypersil BDS-C_{18} reversed-phase column with gradient elution and UV absorbance detection (240 nm). The limits of quantification (signal-to-noise ratio = 6) were 20 ng/ml for testosterone and 30 ng/ml for epitestosterone.

1. Introduction

Epitestosterone is the inactive 17α-epimer of testosterone which is secreted by the gonads [1]. Human urine contains approximately equal concentrations of testosterone and epitestosterone and the ratio between them in normal subjects is not dependent on the extent of urine dilution [2]. The structures of testosterone (T), epitestosterone (E) and methyltestosterone (MT) are shown in Fig. 1.

In 1983, testosterone was forbidden in sports by the International Olympic Committee. This ban was expressed as the T/E ratio because the epitestosterone urinary level remains fairly constant after testosterone administration. The IOC Medical Commission established that in any case of T/E > 6, it is mandatory that the responsible authority conduct an investigation before the sample is declared positive.

Nevertheless, epitestosterone ingestion can be used as a masking agent to achieve "false-negative results" for testosterone, because of modification of the T/E ratio. For this reason, the IOC Medical Commission recommends reporting any case with epitestosterone urinary levels above 200 ng/ml [3].

Different immunological methods have been developed to determine urinary testosterone and epitestosterone concentrations. However, the immunological assays are not selective and specific enough because related structures show similar immunological responses [4,5].

The most commonly used technique for the determination of epitestosterone and testosterone is gas chromatography coupled with mass spectrometry, where multiple sample work-up procedures are needed to avoid endogenous

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(95)00260-X

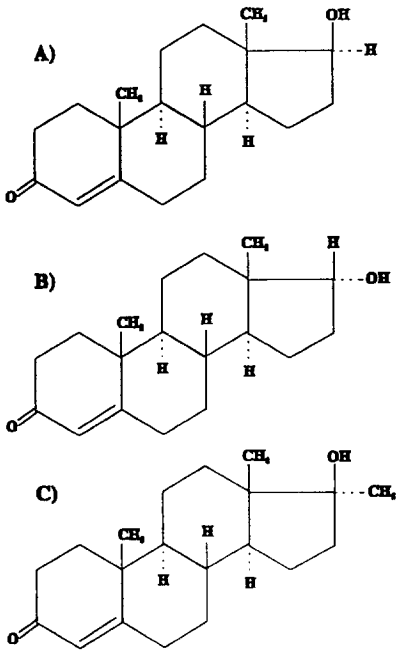

Fig. 1. Structures of (A) testosterone, (B) epitestosterone and (C) methyltestosterone (I.S.).

interferences. In addition, a previous O-trimethylsilyl derivatization must be carried out and the reproducibility obtained is not always sufficient [6–8].

Several HPLC methods with good resolution of closely related compounds such as anabolic steroids have been reported [9–15]. Moreover, HPLC prepurification assays have been developed for subsequent spectroscopic analysis of anabolic steroids [16]. This paper describes a reliable alternative HPLC method which is simple, rapid, selective, sensitive and reproducible for the determination of testosterone and epitestosterone.

2. Experimental

2.1. Reagents and chemicals

Testosterone (17β-hydroxyandrost-4-en-3-one) was obtained from Serva (Heidelberg, Germany) and epitestosterone (17α-hydroxyandrost-4-en-3-one) and methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one) from Sigma (St. Louis, MO, USA).

All reagents were of analytical-reagent grade. Acetonitrile, methanol and *n*-pentane were obtained from Scharlau (Barcelona, Spain), KH_2PO_4, H_3PO_4, NaOH, Na_2SO_4, K_2CO_3, $NaHCO_3$ and methyl *tert.*-butyl ether from Merck (Darmstadt, Germany), diethyl ether from Carlo Erba (Milan, Italy), β-glucuronidase from *Escherichia coli* from Boehringer (Mannheim, Germany) and Serdolit AD-2 from Serva. Water was doubly distilled, deionized and purified with a Milli-Q system (Millipore, Milford, MA, USA).

2.2. Stock solutions and internal standard solution

Stock solutions were prepared in methanol at a concentration of 1000 μg/ml. These solutions were diluted to 100 μg/ml, sealed and refrigerated at 4°C until use.

Methyltestosterone was used as an internal standard (I.S.). It was also dissolved in methanol to 1000 μg/ml and diluted to 100 μg/ml.

2.3. Calibration standards

Steroid-free urine was initially prepared by percolating the urine through a Pasteur pipette (230 × 7 mm) containing a 20-mm plug of Serdolit AD-2 resin. The eluates were collected and used as the matrix for steroid spiked urines.

Calibration graphs were obtained by adding known amounts of testosterone and epitestosterone from 20 to 600 ng/ml. Quantification was based on peak-area ratios of compound to I.S. versus concentration of compound spiked.

2.4. Sample preparation

Routine extraction procedure for anabolic steroids [17]

Solid-phase extraction. A 10-μl volume of I.S. solution (100 μg/ml) was added to 5 ml of urine.

The urine was passed through a Pasteur pipette (230 × 7 mm) containing a 20-mm plug of Serdolit AD-2 resin. The resin was washed with 5 ml of deionized water, eluted with 2 ml of methanol and evaporated to dryness.

Enzymatic hydrolysis. The residue was dissolved in 1 ml of 0.2 *M* sodium phosphate buffer (pH 7.0), then 50 μl of β-glucuronidase from *E. coli* K12 were added. This mixture was incubated at 55°C for 1 h.

Liquid–liquid extraction. The hydrolysate was cooled to room temperature and 250 μl of 5% (w/v) potassium carbonate solution (pH 9–10) were added. This mixture was extracted with 5 ml of diethyl ether, then the urine was saturated with 1 g of sodium sulphate and shaken for 30 s. After standing 10 min, this mixture was centrifuged at 1500 *g* for 5 min and the organic fraction was evaporated to dryness. The residue was dissolved in 50 μl of methanol and 20 μl of the solution were injected into the HPLC system.

Liquid–liquid extraction with previous enzymatic hydrolysis

Enzymatic hydrolysis. A 10-μl aliquot of I.S. solution (100 μg/ml) was added to 5 ml of urine, then 1 ml of 0.2 *M* sodium phosphate buffer (pH 7.0) and 50 μl of β-glucuronidase from *E. coli* K12 were added. This mixture was incubated at 55°C for 1 h.

Liquid–liquid extraction. The hydrolysate was cooled to room temperature and 1 g of $NaHCO_3$–K_2CO_3 (1:2) was added (pH 11). The mixture was extracted with 5 ml of *n*-pentane, then the urine was saturated with 1 g of sodium sulphate and shaken for 20 min. The mixture was centrifuged at 1500 *g* for 5 min and the organic fraction was washed with 2 ml of 1 *M* sodium hydroxide. A 1-g amount of sodium sulfate was added while shaking. The mixture was centrifuged at 1500 *g* for 5 min and the organic fraction was evaporated to dryness. The residue was dissolved in 50 μl of methanol and 20 μl of the solution were injected into the HPLC system.

2.5. Chromatographic conditions

The chromatographic system consisted of a Hewlett-Packard (Palo Alto, CA, USA) HP 1090 series, a liquid chromatograph equipped with an autosampler/autoinjector and a HP 1040A diode-array UV detector. Chromatography was performed at 40°C on a reversed-phase analytical column. The columns used were as follows: Hypersil ODS, 5 μm (100 × 2.1 mm I.D.); LiChrospher 60 RP Select B, 5 μm (250 × 4 mm I.D.); LiChrospher 100 RP-18, 5 μm (250 × 4 mm I.D.); Hypersil ODS, 5 μm (200 × 4.6 mm I.D.); Hypersil MOS, 5 μm (200 × 4.6 mm I.D.); LiChrosorb RP-18, 5 μm (200 × 4.6 mm I.D.); LiChrospher 100 RP-18, 5 μm (125 × 4 mm I.D.); and Hypersil BDS-C_{18}, 5 μm (250 × 4 mm I.D.). All the columns were obtained from Hewlett-Packard. The mobile phase was 1 m*M* phosphoric acid–acetonitrile (75:25) at a flow-rate of 1.2 ml/min. The initial acetonitrile content was increased to 30% in 10 min, to 35% in 16.5 min, maintained at 35% until 28 min and returned again to the initial conditions. The injection volume was 20 μl and the column effluent was monitored at 240 nm (band width 4 nm), where epitestosterone and testosterone exhibit maximum absorption. For data evaluation an HP 79994A Chemstation was used, which consisted of an HP 900 Series 300 computer, a 10 Mbyte Winchester disk drive and a Thinkjet printer.

3. Results and discussion

Representative chromatograms of urine samples are shown in Figs. 2 and 3, where testosterone, epitestosterone and methyltestosterone (I.S) are well resolved and no endogenous interferences are observed.

3.1. Optimization of sample extraction

Preliminary studies were performed using a routine extraction procedure for anabolic steroids, where the chromatograms obtained had a high background and resolution with endogenous compounds was not always achieved. For this

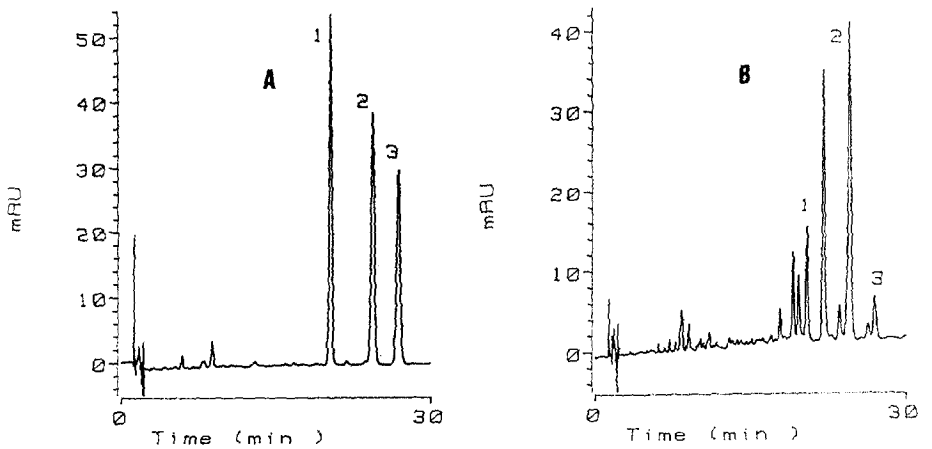

Fig. 2. Chromatograms of (A) standard spiked urine (previously percolated), with (1) testosterone, (2) methyltestosterone (I.S.) and (3) epitestosterone, each at a concentration of 200 ng/ml; (B) normal urine profile to which I.S. at a concentration of 200 ng/ml (peak 2) was added. The concentrations of testosterone and epitestosterone found were 45 and 30 ng/ml, respectively.

reason, we proceeded to optimization of the extraction. The best results were obtained using a previous enzymatic hydrolysis with liquid–liquid extraction. Three different solvents (methyl *tert.*-butyl ether, diethyl ether and *n*-pentane) were studied in the pH range 7–13. The highest recoveries (about 90%) were obtained using pH 11. There were no significant differences among the three solvents, but the cleanest chromatograms were obtained with *n*-pentane. Fig. 4 compares the two extraction procedures.

3.2. HPLC columns

Several columns with different packing materials (LiChrospher, LiChrosorb and Hypersil) were compared. The best results were achieved using Hypersil packing material. Higher resolu-

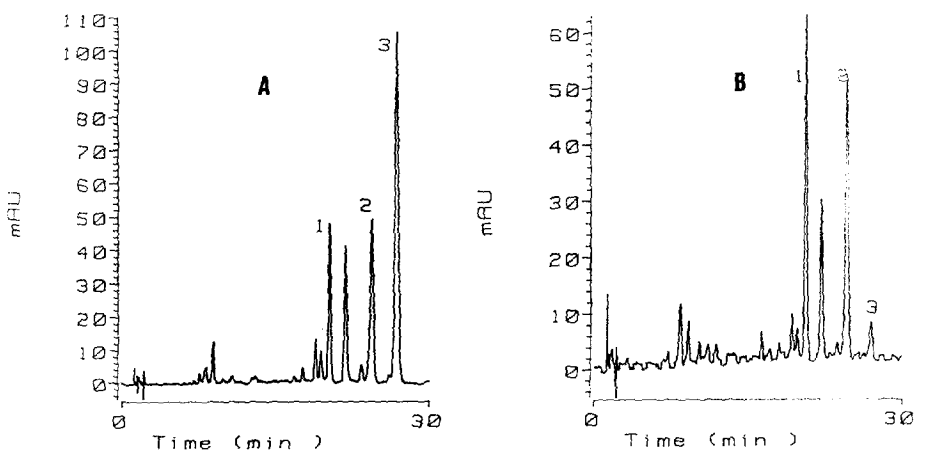

Fig. 3. Chromatograms of (A) urine sample from an athlete who had taken epitestosterone, where levels close to 500 ng/ml for epitestosterone and 130 ng/ml for testosterone were found; (B) physiological urine sample with a T/E ratio of 5.8.

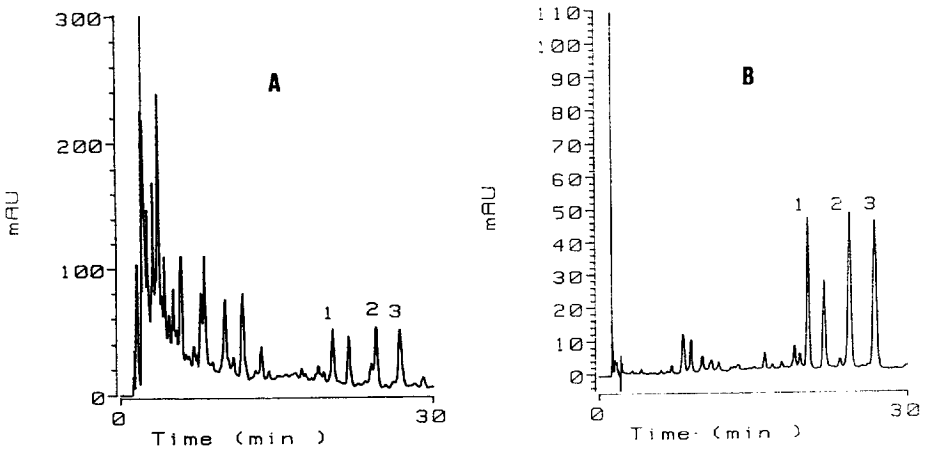

Fig. 4. Comparison between the extraction procedures: (A) routine extraction procedure for anabolic steroids; (B) liquid–liquid extraction with previous enzymatic hydrolysis.

tion from endogenous compounds and better peak shapes were obtained in all cases with Hypersil BDS-C_{18}. Therefore, more precise quantification was possible.

3.3. Precision and accuracy

The precision and accuracy were estimated using five different replicate samples containing 40, 220 and 400 ng/ml, respectively. Each concentration was calculated on the basis of the peak-area ratio with respect to the I.S. Statistical results are given in Table 1.

3.4. Calibration and linearity

The linearity was evaluated over the range of concentrations 20–600 ng/ml using duplicate samples spiked at levels of 20, 80, 140, 200, 260, 320, 400, 500 and 600 ng/ml. The linear regression equations are shown in Table 2.

Table 2
Equations of linear calibration graphs

Compound	Equation[a]	r^2
Testosterone	$y = 0.966x + 0.0259$	0.995
Epitestosterone	$y = 0.853x + 0.00259$	0.997

[a] y = Peak area (arbitrary units); x = concentration (ng/ml).

3.5. Limit of quantification

The limit of quantification was defined as an analyte signal-to-background noise (S/N) ratio of 6. The limits of quantification were 20 ng/ml for testosterone and 30 ng/ml for epitestosterone.

Table 1
Analytical accuracy and reproducibility for testosterone and epitestosterone in spiked urine ($n = 5$)

Concentration added (ng/ml)	Testosterone		Epitestosterone	
	Found (mean ± S.D.) (ng/ml)	R.S.D. (%)	Found (mean ± S.D.) (ng/ml)	R.S.D. (%)
40	43.4 ± 1.5	3.5	43.9 ± 0.5	1.1
220	228 ± 8	3.1	223 ± 9	4.1
400	412 ± 13	3.1	412 ± 10	2.5

3.6. Selectivity, specificity and interferences

Several urinary endogenous steroids which are available in our laboratory were examined to establish possible interferences. None of the following compounds interfered with testosterone, epitestosterone and methyltestosterone: 5α-androstan-3α-ol-17-one (androsterone), 5β-androstan-3α-ol-17-one (etiocholanolone), 11β-hydroxy-androsterone, 11β-hydroxyetiocholanolone, 5β-androstan-3,17-dione, 5β-androstan-3β-ol-17-one, 5-androsten-3β-ol-17-one, 5α-androstan-3,17-dione, 5α-androstan-3β-ol-17-one, 5α-androstan-17-one, 1,4-androstadien-3,17-dione, 19-nor-4-androsten-3,17-dione, 17α-methyl-5α-androsten-17β-ol-3-one, 5α-androstan-17β-ol-3-one, 5-androsten-3β, 17β-diol, 5β-androstan-3β, 17β-diol, 5α-androstan-3β, 17β-diol, 5β-androstan-3α, 17β-diol, 4-androsten-3, 17-dione, 11α-hydroxy-17α-methyltestosterone and 5-androsten-3β-ol-17-one.

4. Conclusion

Owing to the sample work-up procedures and chromatographic separation optimization, the proposed method is selective and specific enough for the determination of testosterone and epitestosterone. The relative standard deviations, which are always lower than 10%, demonstrate the precision and reproducibility of the method. As the differences between the mean value and the true value are always less than 10%, we can also confirm the accuracy of the method. We have tested this method by analysing over 30 physiological samples with different levels of testosterone and epitestosterone, and in all cases good results were obtained.

References

[1] A.C. Moffat (Editor), Clarke's Isolation and Identification of Drugs, Pharmaceutical Press, London, 2nd ed., 1986.
[2] M. Donike, K.R. Barwald, K. Klosterman, W. Schanzer and J. Zimmermann, in H. Heck, W. Hollmann and H. Liesen (Editors), Leistung und Gesundheit, Deutscher Artzte-Verlag, Cologne, 1983, pp. 293–300.
[3] IOC Definition of Doping and List of Doping Classes and Methods, IOC, Lausanne, 1994.
[4] E. Daeseleire, A. De-Guesquiere and C. Van-Peteghem, J. Chromatogr., 102 (1991) 564.
[5] A.T. Kicman, R.V. Brooks, S.C. Collyer, D.A. Cowan, M.N. Nanjee, G.J. Southan and M.J. Wheeler, Br. J. Sports Med., 24 (1990) 253–264.
[6] R.H.B. Sample and J.C. Baenziger, in Analytical Aspect of Toxicology, Wiley, New York, 1989.
[7] R. Massé, C. Ayotte and R. Dugal, J. Chromatogr., 489 (1989) 23–50.
[8] D.H. Catlin, R.C. Kammerer, C.H. Hatton, M.H. Sekera and J.L. Merdink, Clin. Chem., 33 (1987) 319.
[9] J. Wei, J. Wei, C. Lucarelli, X. Zhon, D. Wan, W. Dai and S. Li, Clin. Chem., 38 (1992) 76–82.
[10] M.J. Walters, J.A. Russell and D.J. Brown, J. Assoc. Off. Anal. Chem., 73 (1990) 904–926.
[11] A. Berthod, J.M. Asensio and J.J. Laserna, J. Liq. Chromatogr., 12 (1989) 2621–2634.
[12] K. Oka, T. Hirano and M. Noguchi, Clin. Chem., 34 (1988) 557–560.
[13] O.R. Kling and G.S. Dizerega, Steroids, 40 (1982) 381–388.
[14] P.G. Stoks and T.J. Benraad, J. Chromatogr., 276 (1983) 408–412.
[15] B.L. Lampert and J.T. Stewart, J. Liq. Chromatogr., 12 (1989) 3231–3249.
[16] E. Venturelli, A. Manzari, A. Cavalleri, M. Benzo, G. Secreto and E. Marubini, J. Chromatogr., 582 (1992) 7–12.
[17] M. Donike, J. Zimmermann, K.-R. Bärwald, W. Schänzer, V. Christ, K. Klostermann and G. Opfermann, Dtsch. Z. Sportmed., 35 (1984) 14–24.