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Routine screening and quantitation of urinary corticosteroids using bench-top gas chromatography–mass-selective detection

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ABSTRACT

A method was developed for the routine screening, confirmation and quantitation of corticosteroids in human urine using bench top capillary gas chromatography (GC)–mass-selective detection. The free and conjugated corticosteroid fractions were isolated by liquid–liquid partition. After evaporation to dryness under vacuum the corticosteroid residues were derivatized to form the methyloxime trimethylsilyl ether derivatives. Both GC retention data and characteristic spectral data based on authentic reference standards were used for the identification and quantitation of cortisol, cortisone, tetrahydrocortisol and tetrahydrocortisone in the ppb (ng/ml) concentration range. The method is simpler and more efficient than the other GC–mass spectrometric (MS) techniques. It is also more sensitive than the liquid chromatographic–MS method.

INTRODUCTION

A range of methodologies for detecting corticosteroids in biological fluids have previously been investigated. The methods include radioimmunoassay (RIA) [1,2], competitive protein binding (CPB) [3–5], high-performance liquid chromatography (HPLC) [6–8], gas chromatography–mass spectrometry (GC–MS) [9–11], GC [12–14] and liquid chromatography–mass spectrometry (LC–MS) [15–17]. Many of these methods have been reviewed and the disadvantages and advantages of the different techniques have similarly been discussed [18,19]. Apart from GC–MS and LO–MS procedures, which offer the advantage of combined screening and confirmation on the same analysis, separate confirmatory analyses are required for the other methods.

A survey of the literature reveals that nearly all the previous analyses have been directed towards the determination of cortisol in plasma. Although GC [12,13] and GC–MS [10,11] procedures have been utilised for detecting urinary and plasma cortisol, the combined clean-up and derivatization steps tend to be too lengthy for routine screening protocols that require fast turnaround times. Recently, LC–MS [17] has been employed for screening certain corticosteroids, however, the sensitivity lies in the picomolar range [15,16] compared to GC–MS which is sensitive at the sub-picomolar level.

At present, bench-top capillary gas chroma-

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tography–mass-selective detector (GC–MSD) is used for the routine screening of anabolic steroids in compliance with the requirements of the International Olympic Commission's (IOC) Drug Testing Programmes. Although the use of corticosteroids is currently restricted by the IOC, there have been only limited published analytical procedures suitable for the routine screening and profiling of urinary corticosteroids.

This report details a method for the routine screening, confirmation and quantitation of cortisol (F), cortisone (E) and metabolites tetrahydrocortisol (THF) and tetrahydrocortisone (THE) in human urine using bench-top capillary GC–MSD. The method is simple and less time-consuming than other published GC–MS methods. It is also more sensitive than the LC–MS technique.

EXPERIMENTAL

Chemicals and reagents

N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Macherey-Nagel (Düren, Germany). 1-(Trimethylsilyl)imidazole (TMS-imid) was obtained from Aldrich (Milwaukee, WI, USA) and β-glucuronidase *Escherichia coli* enzyme came from Boehringer Mannheim (Mannheim, Germany). The reagents were used as supplied. The XAD-2 resin (Serva, Heidelberg, Germany) was prewashed with demineralised water, methanol, water and used as a water-slurry. Sephadex LH-20 resin (Pharmacia, Uppsala, Sweden) was prewashed with hexane–chloroform solvent and allowed to swell in the same solvent at ambient temperature overnight. F, E, THF, THE, methyltestosterone and methoxyamine hydrochloride from Sigma (St. Louis, MO, USA) were used as supplied.

Methoxyamine hydrochloride (8% w/v) was dissolved in anhydrous pyridine (May & Baker, Dagenham, UK) and equilibrated at ambient temperature. Diethyl ether (Mallinckrodt, Paris, KY, USA) was distilled before use. All other solvents used were of analytical grade.

Gas chromatography–mass spectrometry

Selected-ion monitoring (SIM) measurements were performed with a Hewlett-Packard 5890 gas chromatograph and 5970 mass-selective detector coupled to the Unix data processing system.

The mass-selective detector was set at 70 eV and focussed on the following characteristic ions produced by electron impact: F, *m/z* 636 (M^+), 605 (M − 31), 515 (M − [31 + 90]); E, *m/z* 562 (M^+), 531 (M − 31), 441 (M − [31 + 90]); THF, *m/z* 683 (M^+), 652 (M − 31); THE, *m/z* 609 (M^+), 578 (M − 31).

GC–MSD conditions

An HP Ultra-1, capillary cross-linked methyl silicone (17 m × 0.22 mm I.D., 0.11 μm) column was used in the gas chromatograph. Ultra-high-purity helium at a flow-rate of 1 ml/min was used as the carrier gas. A splitless injection mode was used with the injector set at 250°C. The purge flow-rate was set at 2.0 ml/min and activated at 2.0 min after injection. The initial oven temperature was set at 142°C and programmed to increase at 5°C/min to 150°C (held for 2 min), after which the temperature was increased at 20°C/min to 285°C and held for 5 min. The detector temperature was set at 285°C.

Sample preparation

Steroid-free urine was initially prepared by percolating the urine through a Pasteur pipette (230 mm × 7 mm) containing a 10-mm plug of Amberlite XAD-2 resin. The eluates were pooled and used as the base matrix for corticosteroid spikes.

Combined free and conjugated corticosteroid fractions

A 2-ml aliquot of human urine was passed through a 10-mm plug of XAD-2 resin as described above. The plug was rinsed with 0.5 ml of demineralised water; three 0.5-ml volumes of methanol were added to elute the corticosteroids into a separate tube. The eluate was evaporated to dryness under vacuum at 54°C and buffered with 1 ml of Na_2PO_4–KH_2PO_4 (0.2 *M*) solution to pH 7. Methyltestosterone internal standard solution (200 ng) was added. Diethyl ether (5 ml) was added and the sample was rotary-mixed for 10 min followed by centrifugation at 800 *g* for 10 min. The ether layer containing the free corticosteroid fraction was removed and evaporated to

dryness under vacuum. The residue was derivatized to the methyloxime trimethylsilyl ether (MO-TMS) derivatives according to the derivatization procedure below.

The residual ether in the remaining aqueous layer was evaporated under nitrogen gas at ambient temperature; methyltestosterone (internal standard) solution (200 ng) and 50 μl of β-glucuronidase enzyme were added and the sample was hydrolysed at 50°C for 1 h. After hydrolysis, 200 mg of K_2CO_3–$KHCO_3$ (1:2) ground solid buffer at pH 9 were added. The conjugated corticosteroid fraction was extracted with 5 ml of diethyl ether and the dry residue derivatized to the MO-TMS derivatives as before.

Derivatization

The dry corticosteroid residue was reacted with 100 μl of methoxyamine hydrochloride–pyridine solution at 60°C for 30 min. After cooling the excess pyridine was evaporated under nitrogen gas; 50 μl of MSTFA–TMS-imid (0.2%) reagent was added and the mixture reacted at 70°C for 15 min to form the MO-TMS ether derivatives. Excess reagent was removed by percolating the derivatives through a 20-mm plug of swollen LH-20 resin packed in a capillary Pasteur pipette, based on a previous procedure [20]. The derivatives were eluted with 3 ml of hexane–chloroform (1:1) [21] and evaporated to dryness under vacuum. The dry residue was redissolved in 25–50 μl of isooctane solvent for injection into the GC–MSD system.

Preparation of calibration graph

Standard solutions (2 ml) containing 25, 50, 100 and 150 ppb of F, E, THE and THF were used to prepare the calibration graphs. Peak-area response ratios were plotted against concentration ratios. All the graphs were linear in the 0–150 ppb concentration range with correlation coefficients ranging from 0.944 to 0.979.

Determination of reproducibility and accuracy

Aliquots (2 ml) of steroid-free urine were spiked with 20- and 100-ng corticosteroid standards to produce 10- and 50-ppb sample spikes, respectively. They were subsequently worked-up according to the procedure for the free and conjugated corticosteroid fractions. The free fraction was prepared by leaving out the step for the conjugated fraction.

To determine the effect of the step for the conjugated fraction on the steroid recovery, separate aliquots (2 ml) of steroid-free urine were spiked with 20 ng of corticosteroid standard solutions to produce 10-ppb sample spikes. The samples were prepared according to the above procedure by leaving out the step for the free fraction.

The peak-area ratios of the following corticosteroid ions were used for calculating the recovery values, intra-assay and inter-assay coefficients of variation: F, *m/z* 605; E, *m/z* 531; THE, *m/z* 578; THF, *m/z* 652.

RESULTS AND DISCUSSION

Ion chromatograms and mass spectra

Figs. 1 and 2 show the ion chromatograms of the MO-TMS ethers of F, E, THF and THE, respectively. The M − 31 fragment ions were selected as the major ions for quantitation due to the higher ion abundance obtained. Both the diMO-

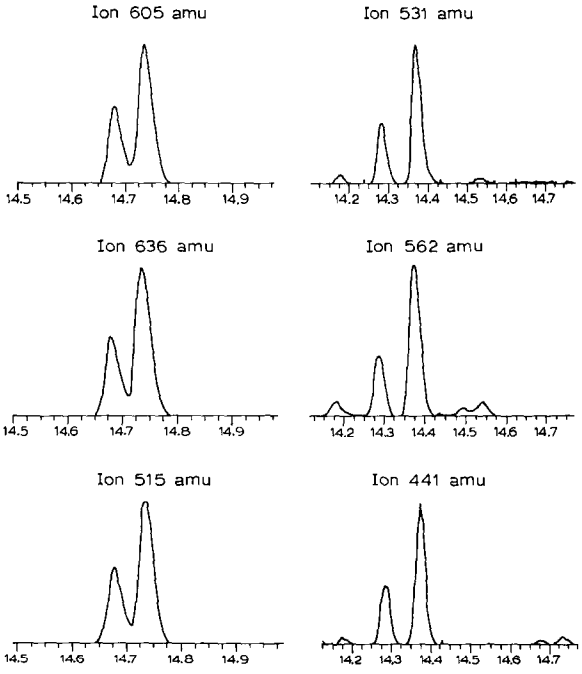

Fig. 1. Ion chromatograms of diMO-triTMS derivatives of cortisol and cortisone. Left-hand side shows diMO-triTMS cortisol. Right-hand side shows diMO-triTMS cortisone. (Both analytes equivalent to 100 ppb in urine.)

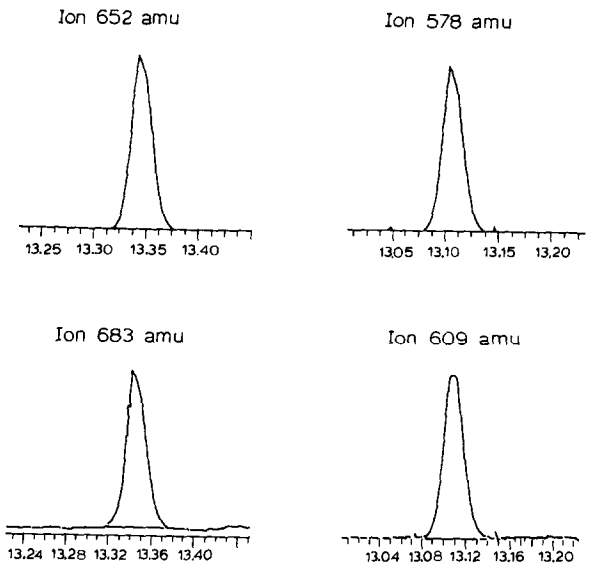

Fig. 2. Ion chromatograms of derivatives of the metabolites tetrahydrocortisol and tetrahydrocortisone. Left-hand side shows the MO-tetraTMS tetrahydrocortisol. Right-hand side shows the MO-triTMS tetrahydrocortisone. (Both analytes equivalent to 100 ppb in urine.)

triTMS derivatives of cortisol and cortisone were eluted as *syn–anti* isomer pairs, the ratio of which remained relatively unchanged in the analyses (Fig. 1). These results were consistent with previously reported observations [22]. The MO-tetraTMS THF and MO-triTMS THE derivatives and the internal standard were eluted as single peaks (Fig. 2). Fig. 3 depicts the electron-impact mass spectra of the MO-TMS derivatives of F, E, THF and THE. The multiplets observed in the M − 31 fragment ions were due to the two major silicon isotopes (^{30}Si and ^{29}Si) in the TMS groups [23].

Recovery and reproducibility

Analytical recovery was determined from a minimum of four replicate analyses of steroid-free urine spiked with 10 and 50 ppb of each corticosteroid for the free fraction. Separate samples were spiked with 10 ppb of the corticosteroids for the hydrolysis step.

The recovery values for the free corticosteroid fractions at 10 ppb were higher than the hydrolysed fractions at the same concentration. The lower values for the latter probably resulted from losses of the analyte during the solid-phase clean-up and hydrolysis steps. Intra-assay and inter-assay variability at the 10-ppb level were generally greater than those at the 50-ppb level due to the low analyte concentration used. Both recovery data and intra- and inter-assay coefficients of variation are compiled in Tables I–VI.

Limit of detection

The limit of detection of the GC–MSD–SIM analyses was 0.2 ng per injection of 1-μl volume for each corticosteroid (Fig. 4). The limit of detection was based on the signal-to-noise ratio of the M − 31 ion compared to the calculated mean abundance of the baseline noise levels. The signal-to-noise ratio was 7:1 for F, 7:1 for E, 15:1 for THF, and 12:1 for THE. This represents 2 ppb in the urine. The limit of detection obtained was higher than the previously published GC–MS [23] and LC–MS [15,16] values.

Methodology

For routine screening procedures requiring fast turnaround times the combined clean-up and derivatization steps should be minimal. In previous publications [23,24], reaction times longer than 2 h in the derivatization step had been reported whilst another method [25] required 16 h for complete reaction. The method detailed in this report required only 45 min for complete derivatization of the analyte. Similarly, the combined clean-up step, which was based on a modified procedure [26] for urinary anabolic steroids, facilitated the simultaneous work-up of both the free and conjugated corticosteroids in the same sample.

Corticosteroid profile of urine

A 5-ml aliquot of fresh urine, obtained from a subject in a rested state, was used for obtaining the urinary profile of free F, E and the metabolites THF and THE. The sample was prepared immediately after sampling to minimise hydrolysis of the steroids. Fig. 5 shows the chromatographic profile of the free corticosteroid fraction. The corticosteroid and internal standard peaks were well separated with relative retention times ranging from 1.19 for F, 1.16 for E, 1.08 for THF, to 1.06 for THE compared to the internal standard.

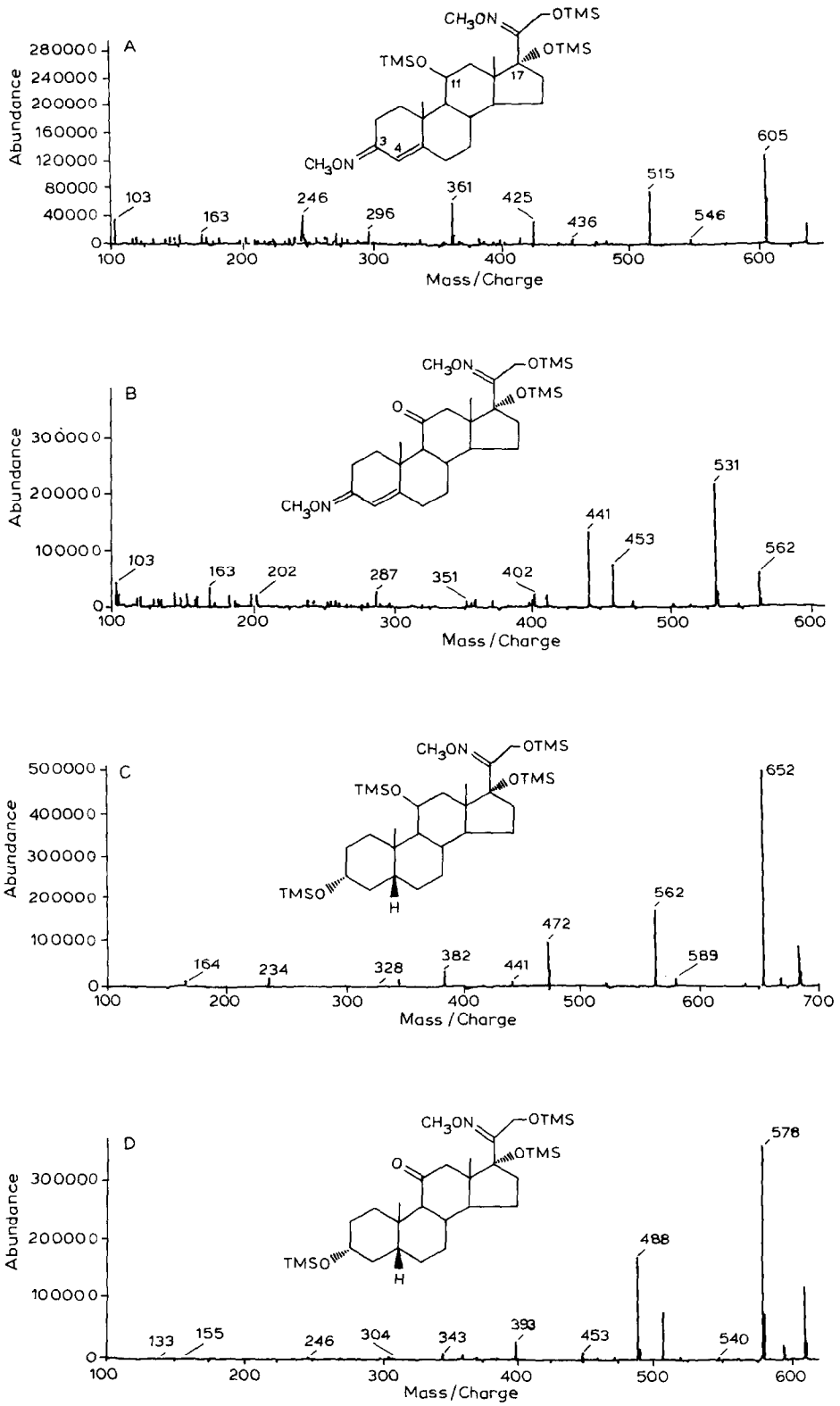

Fig. 3. Electron-impact mass spectra of the derivatives of corticosteroids and their metabolites. (A) diMO-triTMS cortisol; (B) diMO-triTMS cortisone; (C) MO-tetraTMS tetrahydrocortisol; (D) MO-triTMS tetrahydrocortisone.

TABLE I

FREE CORTICOSTEROID SPIKES AT 10 ppb IN URINE (INTRA-ASSAY)

	Measured concentration (ppb)			
	F	E	THE	THF
	6.86	6.24	6.36	5.73
	8.09	8.03	6.69	5.71
	6.95	6.12	5.42	5.42
	6.18	6.17	7.32	7.16
Mean	7.02	6.64	6.45	6.01
Recovery (%)	70.2	66.4	64.5	60.1
Inter-assay C.V. (%)	11.3	14.0	12.2	13.0

TABLE II

CORTICOSTEROID SPIKES AT 10 ppb FROM HYDROLYSED FRACTION IN URINE (INTRA-ASSAY)

	Measured concentration (ppb)			
	F	E	THE	THF
	4.38	5.22	5.47	4.63
	4.00	5.06	5.44	4.65
	4.29	3.96	5.28	4.00
	4.51	5.21	5.45	4.48
Mean	4.30	4.86	5.41	4.44
Recovery (%)	43.0	48.6	54.1	44.0
Intra-assay C.V. (%)	5.0	12.5	1.6	6.8

TABLE III

FREE CORTICOSTEROID SPIKES AT 50 ppb IN URINE (INTRA-ASSAY)

	Measured concentration (ppb)			
	F	E	THE	THF
	33.4	37.4	53.3	48.7
	36.1	35.8	44.5	47.0
	33.2	34.4	48.5	47.5
	34.4	39.0	50.1	51.4
	35.1	36.3	50.3	48.1
Mean	34.4	36.6	49.3	45.6
Recovery (%)	68.8	73.2	98.6	91.2
Intra-assay C.V. (%)	3.5	4.7	6.5	6.0

TABLE IV

INTER-ASSAY VARIATION FOR FREE CORTICOSTEROID SPIKES AT 10 ppb

	Measured concentration (ppb)			
	F	E	THE	THF
	6.16	7.29	4.23	4.00
	4.97	5.17	6.42	5.96
	5.56	5.27	6.12	5.94
	6.95	6.12	5.42	5.42
Mean	5.91	5.96	5.54	5.33
Inter-assay C.V. (%)	14.3	16.5	17.6	17.3

TABLE V

INTER-ASSAY VARIATION FOR FREE CORTICOSTEROID SPIKES AT 50 ppb

	Measured concentration (ppb)			
	F	E	THE	THF
	28.9	29.4	28.0	29.6
	33.4	34.6	32.3	33.4
	30.7	33.1	32.7	32.6
	27.5	25.2	33.8	38.7
Mean	30.1	30.6	31.7	33.6
Inter-assay C.V. (%)	8.5	13.7	8.0	11.2

TABLE VI

INTER-ASSAY VARIATION FOR CORTICOSTEROID SPIKES AT 10 ppb FROM HYDROLYSED FRACTION

	Measured concentration (ppb)			
	F	E	THE	THF
	4.49	4.59	5.36	4.22
	3.64	3.52	6.00	3.75
	4.31	4.36	4.88	4.74
Mean	4.10	4.16	5.41	4.24
Inter-assay C.V. (%)	9.8	13.5	10.4	11.1

A separate urine sample obtained from a physically stressed athlete was used for analysis. The urine, which had been frozen at −20°C initially to minimise hydrolysis, was used for determining the conjugated corticosteroid profile. Fig. 6

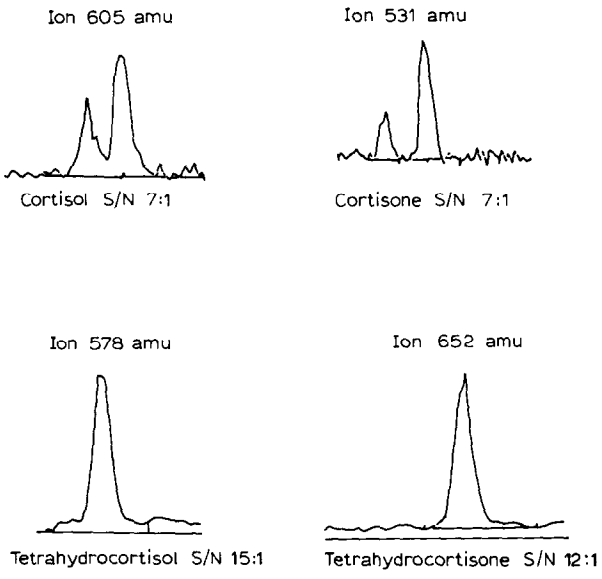

Fig. 4. Limit of detection of the MO-TMS derivatives of cortisol, cortisone, tetrahydrocortisol and tetrahydrocortisone at 0.2 ng/μl.

TABLE VII

FREE CORTICOSTEROIDS IN UNKNOWN URINE FROM PHYSICALLY STRESSED ATHLETES

Subject	Measured concentration (ppb)			
	F	E	THE	THF
1	40.38	48.23	52.45	41.37
2	8.10	21.15	6.97	7.30
3	12.58	25.95	8.82	5.02
4	13.19	26.94	11.32	17.02
5	21.05	56.89	21.98	42.46
6	15.98	29.45	39.90	55.77
7	29.84	35.63	36.14	18.81

shows that the *allo*-THF metabolite was excreted in a greater amount in the conjugated fraction whilst it was not evident in the free corticosteroid profile depicted in Fig. 5.

Further applications of the method were subsequently extended to the routine screening and quantitation of free corticosteroids in unknown urine samples obtained from a group of physically stressed athletes. The data obtained are tabulated in Table VII.

CONCLUSIONS

The method provided a reliable technique that was sensitive, fast and suitable for the routine screening, confirmation and quantitation of free and conjugated F and E and metabolites THF and THE in urine. The method is more efficient than previous GC–MS methods. It is applicable for the routine detection of corticosteroid doping in sports and human stress studies related to the profiling, secretion, metabolism and excretion of urinary corticosteroids. At present the method is being used for the analyses of corticosteroids in physically stressed athletes.

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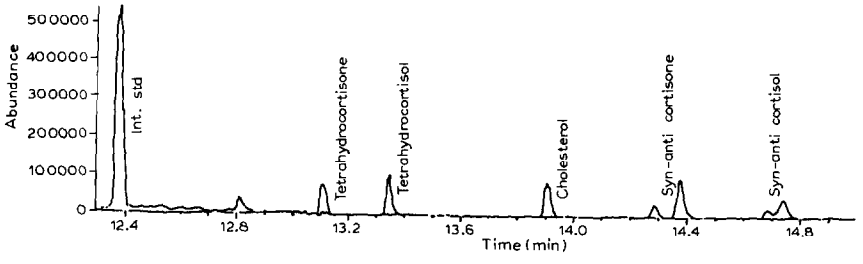

Fig. 5. Free corticosteroid profile in urine.

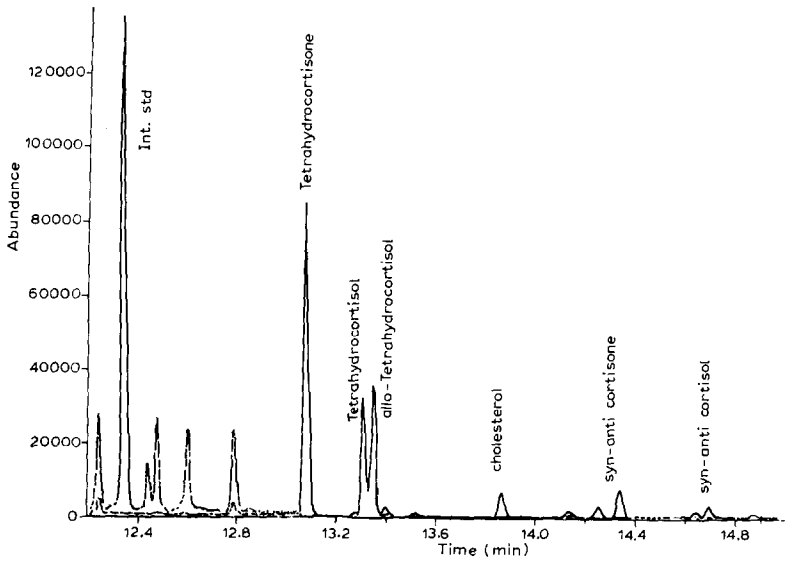

Fig. 6. Conjugated corticosteroid profile in urine sample from a physically stressed athlete.

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