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SCREENING OF STEROIDS IN HORSE URINE AND PLASMA BY USING ELECTRON IMPACT AND CHEMICAL IONIZATION GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Gas chromatography with chemical ionization mass spectrometry and selected-ion monitoring provided a sensitive method for the screening and confirmation of steroids in horse urine and plasma. Chemical ionization mass spectrometry was more sensitive than the electron impact ionization mass spectrometry for most of the steroids except for testosterone, prednisone-metabolite-2 and prednisolone-metabolite-2. The chromatographic conditions used in this study provided clean separation of different natural and synthetic steroids. Approximately 75–85% of the steroids added to plasma and approximately 65–70% of the steroids added to urine were recovered by the extraction procedure used in this study.

INTRODUCTION

Corticosteroids play an important role in glucose and electrolyte metabolism[1,2], and produce strong anti-inflammatory effects[3,4]. Hydrocortisone (cortisol) is the major natural anti-inflammatory corticosteroid present in horse plasma and urine[5]. By selective modifications of the natural steroids, several synthetic steroids have been designed which are more potent anti-inflammatory drugs than cortisol[6]. Prednisone and prednisolone are important synthetic corticosteroids which are extensively used in horses for therapeutic purposes and for improving the performance of the racing horse[7]. Previously reported analytical procedures used for the detection of corticosteroids include radioimmuno assay (RIA), receptor binding assay (RBA) and gas chromatographic assay (GC)[8–11]. The RIA and RBA methods are sensitive but the antibodies partially crossreact with the naturally occurring corticosteroids[12]. The

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GC method lacks sensitivity and cannot be used for the detection of trace levels of compounds[11]. Gustafson and Sjovall[13] and Houghton[14] described the use of gas chromatography–mass spectrometry (GC–MS) in the electron impact (EI) ionization mode for the analysis of steroids in urine and feces. Although the method detected various steroids and their metabolites, EI ionization produced smaller and diagnostically insignificant ions which were not suitable for analyzing steroids in the selective-ion monitoring (SIM) mode. The SIM analysis has been reported to be several times more sensitive than the conventional MS[15]. Recently Matin and Amos[16] described a specific and sensitive method for the screening of prednisolone and prednisone by using GC–MS and chemical ionization (CI). They observed that CI of prednisolone and prednisone provided diagnostically significant ions at high m/z values. The objective of this investigation was to compare the EI and CI mass fragmentation patterns of various natural and synthetic steroids, and to develop a sensitive procedure for the simultaneous screening of synthetic and natural steroids and their metabolites in plasma and urine.

MATERIALS AND METHODS

Reagents

Methoxyamine hydrochloride, pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from the Aldrich. Various steroid standards and other reagents were obtained from the Sigma (St. Louis, MO, U.S.A.).

Instrument

The GC–MS system used was an HP 5980 equipped with electron impact and chemical ionization sources. Methane gas was used as the reagent gas (20 ml/min).

Synthesis of prednisone and prednisolone metabolites

Prednisone metabolite-1 (M_1) (pregna-1,4-dien-17α,20β,21-triol-3,11 dione) and prednisolone metabolite-1 (M_1) (pregna-1,4-dien-11β,17α,20β,21-tetrol-3-one) were synthesized as described by Gray *et al*[17]. Prednisolone or prednisone (100 mg) was mixed with sodium borohydride in methanol and incubated for 30 min at 0°C. After incubation, the mixture was acidified and dried at 45°C under reduced pressure. The dried residue was redissolved in 100 μl of methanol, spotted on a thin-layer chromatography (TLC) plate and developed in ethyl acetate–methanol–ammonium hydroxide (85:10:5) for 5 cm. The metabolite spot, which appeared below the parent spot, was scraped and the compound was eluted from silica with methanol as described previously[17]. Prednisone metabolite-2 (M_2) (androsta-1,4-dien-11β-ol-3,17-trione) and prednisolone metabolite-2 (M_2) (androsta-1,4-dien-11β-ol-3,17-dione) were synthesized by oxidizing the drugs as described previously[17]. 6 mg of prednisone or prednisolone solution in 3 ml of 15% acetic acid was mixed with 200 mg of sodium bismuthate and the mixture was rotoracked for 1.0 h. Thereafter, the mixture was centrifuged at 1500 g for 20 min, supernatant was transferred into a small flask and dried at 45°C under reduced pressure. The dried residue was redissolved in 100 μl of methanol, spotted on a TLC plate and developed in ethyl acetate–methanol–ammonium hydroxide (85:10:5) for 5 cm. The M_2 spot, which appeared above the parent spot, was scraped and eluted with methanol. The methanol extract was further purified by alumina chromatography as described previously[17].

Collection of urine and plasma samples from horses

Urine samples from control (undrugged) horses were collected before they were treated with steroids. The urine sample was mixed with prednisolone, prednisone and their metabolites before analysis. After the collection of a control urine sample, horses were treated with testosterone (25 μg/kg), prednisolone (1.0 mg/kg), or prednisone (1.0 mg/kg) by intramuscular injections. Blood samples were collected from the testosterone injected horse at 0.5, 1, 2, 4, 10, 24 and 48 h after injection. Urine samples were collected from all the drugged horses at 12 h after the drug injection.

Processing of the urine samples for analysis

Urine samples of 5 ml obtained from the drug-injected horses or from the control horses were mixed with glucuronidase and incubated for 2 h at 60°C as described by Singh *et al.*[18]. After incubation, the urine sample was mixed with saturated sodium borate solution (1.0 ml) and the mixture was extracted with ethyl acetate (5 ml). The ethyl acetate layer was collected and washed with 1.0 ml of 15% sodium sulfate in 1.0 *M* sodium hydroxide (w/v). The ethyl acetate layer was collected and dried at 60°C in nitrogen and mixed with 50 μl of methoxyamine (prepared by dissolving 600 mg of methoxyamine hydrochloride in 10 ml of pyridine). The mixture was heated at 110°C for 45 min, thereafter 50 μl of BSTFA was added to each sample and the sample was reheated at 120°C for 30 min. A 1-μl volume of this derivative was injected into the GC–MS system.

Processing of plasma samples for GC–MS analysis

A 2-ml volume of plasma was mixed with 5 ml of light petroleum (b.p. 34.4°C) and the mixture was shaken for 5 min. After centrifugation (1500 *g*), the organic layer was separated and dried at 60°C under nitrogen. The dried residue was derivatized as described for the urine sample. A 1-μl sample was injected into the GC–MS. For quantitative analysis cloprednol was used as an internal standard as described previously[19]. The level of testosterone in plasma was also determined by a radioimmuno assay (RIA) procedure. A 10-μl volume of plasma was incubated with [^{3}H]testosterone and the antibody obtained from the Diagnostic Products Corporation. Unbound tracer was removed from the sample by using dextran coated charcoal. The bound radioactivity was counted in a scintillation counter and the levels of testosterone were determined by using the RIA programs built into the scintillation counter.

GC–MS analysis

The following GC–MS conditions were used in this study: column used, fused-silica capillary (25 M) DB5; inlet temperature, 200°C; initial oven temperature, 150°C; rate, 15°C/min; final oven temperature, 280°C; run time, 30 min. For EI ionization, the source temperature was 200°C, the electron energy was 70 eV, and the mass spectrometer source pressure was $2.0 \cdot 10^{-4}$ Torr. For CI ionization, the ionization gas was methane, the source temperature was 150°C, and the electron energy was 200 eV.

Clean-up of urine extract by TLC

If large volume of urine sample is extracted and pooled for screening, it may be necessary to clean the extract by using TLC before GC–MS analysis. The R_F values of various steroids and their metabolites were determined in different solvents (Table III). A volume of 20–40 ml of urine sample (obtained from the prednisolone or prednisone treated horse) was extracted by using the extraction procedure described earlier. The extract was dried at 50°C under nitrogen and the dried residue was redissolved in 100 μl of ethyl acetate and spotted on a preparative TLC plate as described previously[8]. The plate was developed in ethyl acetate–methanol–ammonium hydroxide (85:10:5) solvent for 5 cm. The standards and part of the sample spot was sprayed with sulfuric acid–ethanol and the sprayed portion was heated at 60°C until the standards turned brown. Silica gel from the unsprayed sample corresponding to the standard spot(s) were scraped and transferred into another tube. Steroids were eluted from silica gel with methanol and the methanol layer was dried. The dried residue was derivatized and injected into the GC–MS system as described previously.

Recovery of steroids from urine and plasma

The extraction efficiency for different steroids were determined by adding known amounts of compounds in urine and determining the amount recovered. The concentration of each steroid was determined by both the CI and EI methods. Cloprednol was used as an internal standard for the quantitation of steroids as described previously[19].

RESULTS AND DISCUSSION

Mass fragmentation of steroids

The major ions produced by the CI or EI of steroids is listed in Table I. Similar to previous EI ionization studies, this study also indicated that ion at m/z 73 was the predominant ion produced by most of the steroids. Testosterone produced the ion at m/z 129 in highest abundance. Prednisolone-M_2 exhibited base ion at m/z 120 and another significant ion at m/z 327 (70% abundance). The testosterone metabolites 1 and 2 exhibited significant ions at m/z 180 and 309, respectively. These observations indicated that the steroid metabolites were more stable to EI fragmentation than their parent drugs. Unlike the EI fragmentation, the CI fragmentation produced larger ions at high abundances and the molecular (M^+) ions were also present in high abundance for most of the steroids (Table I). However, the CI and EI fragmentations were similar for prednisone-M_2 or prednisolone-M_2.

Selection of ions for the analysis of steroids

The ions selected for the qualitative and quantitative analysis of steroids are shown in Table II. The criterion used for the selection of ions for selected-ion monitoring (SIM) has been described by Singh *et al.*[20].

Chromatography

The capillary column and the chromatography conditions used in this study provided a clear separation of prednisone, prednisolone, their metabolites and several other natural steroids (Fig. 1). Unlike the observations of Matin and Amos[16], a clear

TABLE I

MASS FRAGMENTATION AND ABUNDANCES OF INDIVIDUAL IONS FOR VARIOUS STEROIDS SUBJECTED TO CI OR EI

	CI [m/z (abundance)]	*EI [m/z (abundance)]*
Prednisolone	635(100)[a], 636(30), 603(30)	73(100), 103(10), 149(10), 262(10)
Prednisolone-M_1	606(100), 516(80)	73(100), 205(75), 147(70)
Prednisolone-M_2	357(100), 325(25)	356(100), 91(80), 174(75)
Prednisone	561(100)[a], 589(30), 602(10)	73(100), 215(25), 309(10), 561(1), 103(10)
Prednisone-M_1	320(100), 410(90), 681(30)	73(100), 147(20), 205(10)
Prednisone-M_2	359(100), 327(90), 387(20)	120(100), 73(90), 149(70), 327(70)
Testosterone	563(100)[a], 368(50)	129(100), 73(80), 329(50), 368(30)
Testosterone-M_1	–	180(100), 73(80)
Testosterone-M_2	–	73(100), 309(70)
Hydrocortisone	141(100), 476(90), 637(80)[a], 605(50)	73(100), 103(15), 147(10), 605(1)
Tetrahydro-cortisone	520(100), 594(90), 639(10)[a]	73(100), 103(20), 147(15), 488(5)
11-β-Hydroxy-etiocholanone	464(100)[a], 300(80), 268(75), 480(50)	73(100), 448(70), 268(50), 479(2), 129(80)
α-Cortol	141(100), 459(50), 714(50)	73(100), 147(30), 253(20), 343(15)
β-Cortol	141(100), 459(50), 714(50)	73(100), 147(30), 253(15), 343(10)
α-Cortolone	475(100), 639(50), 565(30)	73(100), 147(20), 359(10), 449(10)
β-Cortolone	475(100), 639(80), 565(50)	73(100), 147(20), 243(20), 359(1), 445(5)
5-β-Pregan-3α, 11β,-17α,21-tetrol	523(100), 668(80)[a], 504(50)	73(100), 103(10), 147(15) 652(15), 653(5)

[a] M + 1 ions, M_1 and M_2: metabolites 1 and 2.

TABLE II

IONS (m/z) SELECTED FOR THE MONITORING OF VARIOUS STEROIDS

	CI (m/z)	*EI (m/z)*
5-β-Pregan-3α,-11β, 17α,21-tetrol	523, 668	147, 652
Prednisolone	635	149, 262
Prednisolone-M_1	606, 516	205, 147
Prednisolone-M_2	357	356, 91
Prednisone	561	215, 309
Prednisone-M_1	320, 410	147, 205
Prednisone-M_2	359, 327	149, 327
Testosterone	563	129, 329
Testosterone-M_1	–	180
Testosterone-M_2	–	309
Hydrocortisone	476, 637	103, 147
Tetrahydrocortisone	520, 594	103, 147

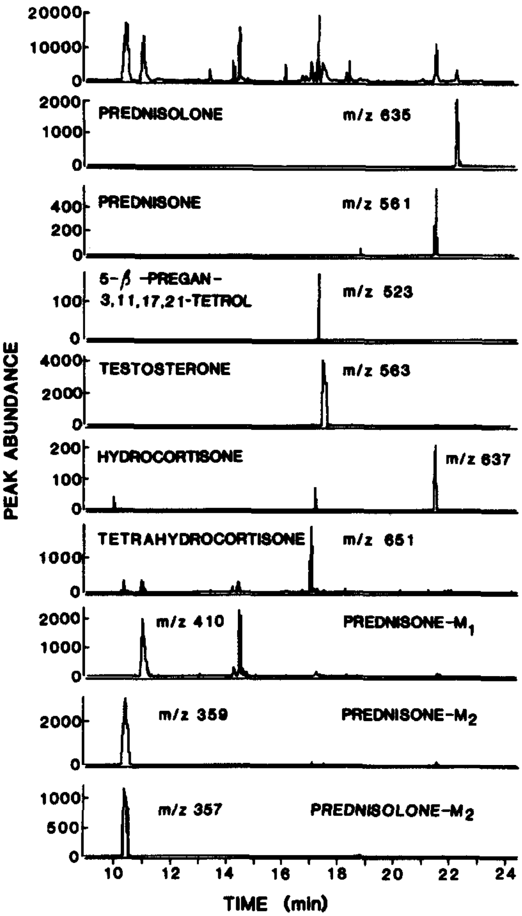
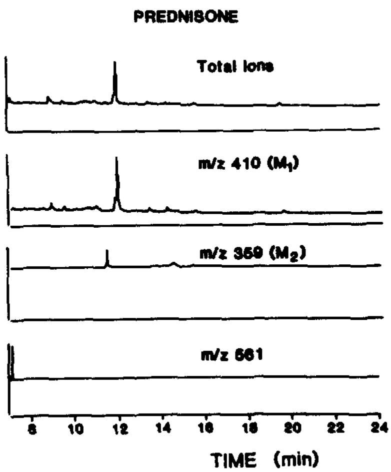


Fig. 1. Selected-ion chromatogram for various steroids present in horse urine which was analyzed by CI-GC–MS.

separation of prednisone and hydrocortisone was observed. As shown in Fig. 1, presence of testosterone, hydrocortisone, tetrahydrocortisone and 5-β-pregan-3α,11β,17α,21-tetrol were also detected in horse urine. Since these steroids were not added to the urine, they may have appeared from the endogenous source. In urine samples obtained from the prednisone-treated horse, only the metabolites M_1 and M_2 were detected and the parent drug was not detected (Fig. 2). However, in the prednisolone injected horse, trace amounts of the parent prednisolone were detected in urine at 24 h after injection (Fig. 3). Although the presence of prednisolone was detected only by the CI analysis, the presence of testosterone and its metabolites were confirmed in plasma (Fig. 4) and urine (Fig. 5) by both the EI and CI analyses.

Extraction efficiency and recovery

The recovery of various steroids from urine is shown in Fig. 6. A linear relationship was observed between the amount of steroids added and the amount of steroids



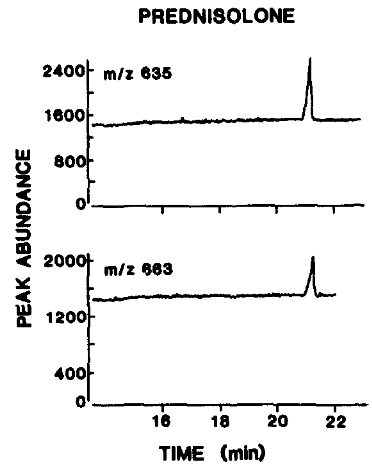


Fig. 2. Selected-ion chromatogram for prednisone and its metabolites extracted from horse urine and analyzed by CI.

Fig. 3. Selected-ion chromatogram for prednisolone extracted from horse urine and analyzed by CI-GC–MS.

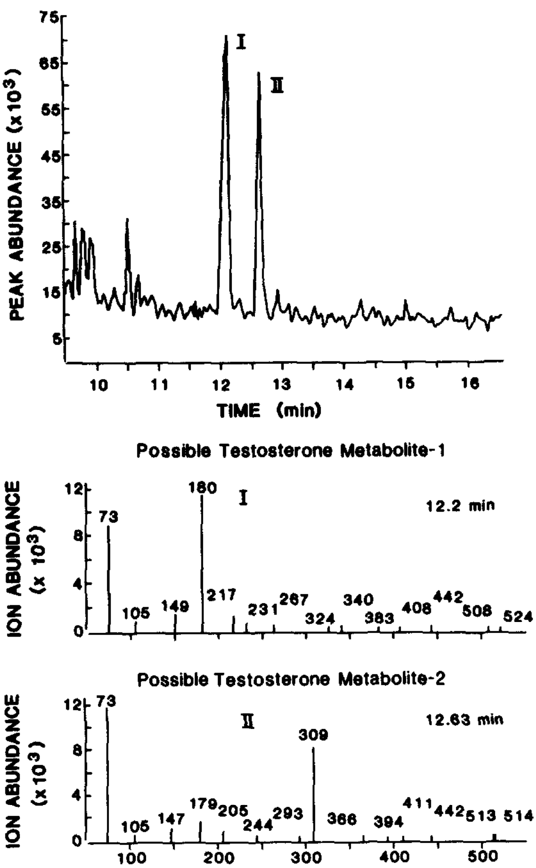


Fig. 4. Chromatographic separation and EI mass fragmentation patterns of the two possible testosterone metabolites.

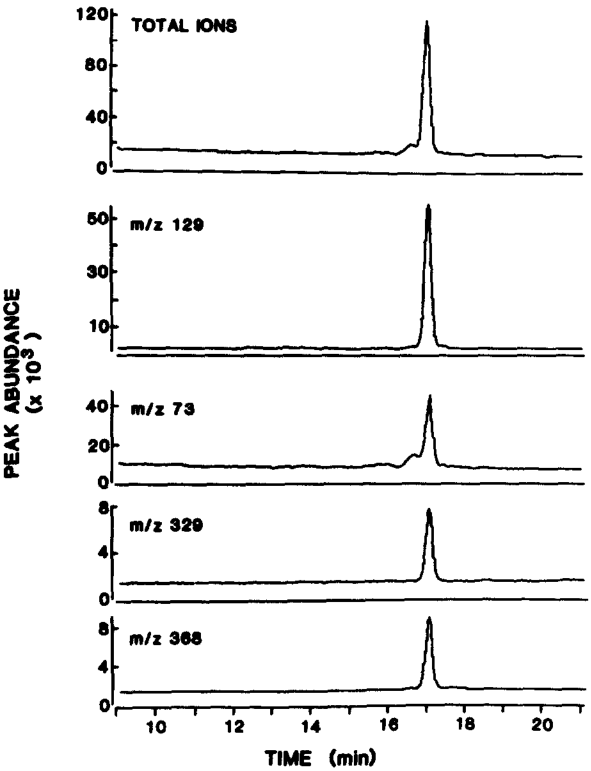


Fig. 5. Selected-ion chromatogram for testosterone extracted from plasma and analyzed by EI-GC–MS.

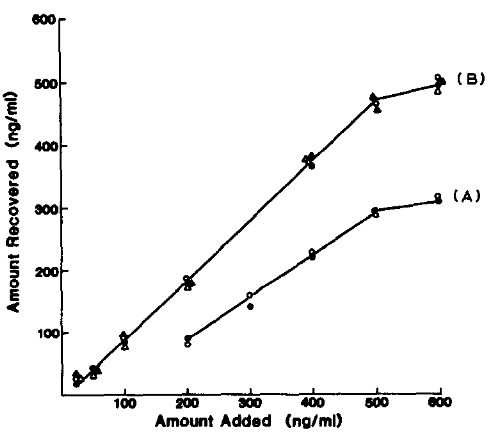


Fig. 6. Recovery of various steroids from horse urine (n = 5). (A) ○ = Recovery of prednisone determined by the EI–SIM method; ● = recovery of prednisolone determined by the EI–SIM method; (B) △ = recovery of testosterone determined by the EI–SIM method; ▲ = recovery of testosterone determined by the CI–SIM method; ● = recovery of prednisolone determined by the CI–SIM method; and ○ = recovery of prednisone determined by the CI–SIM method.

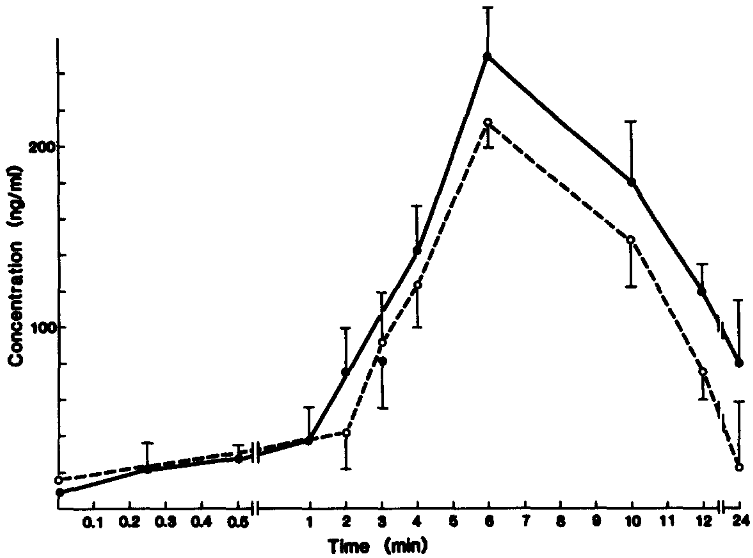


Fig. 7. Comparison of plasma testosterone levels determined by the RIA and the EI-GC–MS method described in this study. (n = 3, values are mean ±S.D.). ● = RIA; ○ = GC–MS.

TABLE III

TLC MIGRATION (R_F) IN DIFFERENT SOLVENT SYSTEMS OF VARIOUS STEROIDS

	DAV.[a]	*PA*[b]	*T-1*[c]	*9-1*[d]	*4/4/2*[e]	S_1[f]	S_2[g]
5-β-Pregan-3α,-11β,17α,21-tetrol	0.40	0.62	0.9	0.2	0.04	0.3	0.4
Prednisolone	0.44	0.70	0.9	0.26	0.04	0.4	0.5
Prednisolone-M_1	0.20	0.56	0.84	0.1	0.00	–	–
Prednisolone-M_2	0.80	0.96	0.86	0.74	0.32	–	–
Prednisone	0.50	0.82	0.9	0.4	0.1	0.4	0.5
Prednisone-M_1	0.30	0.68	0.84	0.25	0.1	–	–
Prednisone-M_2	0.80	0.94	0.9	0.25	0.28	–	–
Testosterone	0.55	0.50	0.9	0.3	0.1	–	–
Testosterone-M_1[h]	0.35	0.23	0.85	0.2	0.04	–	–
Testosterone-M_2[h]	0.76	0.13	0.9	0.4	0.1	–	–
α-Cortolone	0.18	0.45	0.85	0.04	0.04	0.1	0.2
β-Cortolone	0.18	0.45	0.85	0.04	0.04	0.1	0.2
α.Cortol	0.20	0.45	0.85	0.04	0.04	0.1	0.2
β-Cortol	0.20	0.45	0.85	0.04	0.04	–	–
11-β-Hydroxy-etiocholanolone	0.74	0.90	0.9	0.5	0.24	0.5	0.7
Tetrahydrocortisone	0.40	0.70	0.9	0.26	0.04	–	–
Hydrocortisone	0.50	0.72	0.9	0.3	0.04	–	–

[a] DAV = Ethyl acetate–methanol–concentrated ammonium hydroxide (85:10:5, v/v).
[b] PA = Chloroform–methanol–propanoic acid (72:18:10, v/v).
[c] T-1 = Methanol–concentrated ammonium hydroxide (100:1,5, v/v).
[d] 9-1 = Chloroform–ethanol (90:10, v/v).
[e] 4/4/2 = Chloroform–cyclohexane–acetic acid (40:20:20, v/v).
[f] S_1 = Ethyl acetate–acetic acid (39:1, v/v), followed by dichloromethane.
[g] S_2 = Chloroform–ethyl acetate–methanol (50:45:5, v/v) followed by DAV.
[h] Possible metabolites.

recovered from the urine. The curve lost linearity at concentrations above 500 ng/ml possibly because of the saturation of the extraction procedure. The extraction efficiency was approximately 60–70% from urine and 75–80% from plasma. The comparison of plasma testosterone levels determined from the RIA and GC–MS methods have been shown in Fig. 7. The values obtained by GC–MS were 70–80% of the values obtained by the RIA procedure. Since the RIA analysis was carried out with whole plasma (unextracted) and the GC–MS analysis with extracted plasma, the differences between the two assays may be due to the extraction efficiency of testosterone.

Cleanup of extract by TLC

The TLC procedure was necessary only if a large volume of urine was extracted to achieve high concentrations of steroids. Although the TLC cleanup provided clean mass spectra, the recovery of the procedure reduced to 45–55%. Because of the lower sensitivity, the use of TLC cleanup remains limited. For the screening of different steroids by TLC, the R_f value of steroids has been listed in Table III.

CONCLUSIONS

From the results of this study it is concluded that (1) CI–SIM provided a sensitive method for screening steroids, (2) CI–SIM was more sensitive than EI–SIM for most of the steroids except for testosterone and prednisone or prednisolone metabolites, (3) the chromatographic conditions provided clean separation of different steroids, and (4) the recovery of steroids from urine and plasma were $>70\%$.

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