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Thermospray liquid chromatography–mass spectrometry of corticosteroids

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ABSTRACT

A high-performance liquid chromatographic method was developed for thermospray mass spectrometric analysis of steroidal hormones. Using a Nova-Pak C_{18} reversed-phase column and isocratic elution with a solvent comprised of 25 m*M* ammonium formate in 30% acetonitrile, corticosteroids were separated within 10 min. This solvent also permitted ultraviolet absorbance detection down to 220 nm with low-nanogram sensitivity. The use of acetonitrile was favourable for thermospray mass spectrometric analysis because mass spectra were obtained with a pseudomolecular ion as the base peak. A combination of liquid chromatography, ultraviolet absorbance detection and thermospray mass spectrometry provided a sensitive and reliable method for unequivocal confirmation of the presence of steroidal drugs in equine urine.

INTRODUCTION

Methods for gas chromatographic–mass spectrometric (GC–MS) analysis of steroids in biological specimens have been developed over the last twenty years. Many early efforts were directed towards derivatization of functional groups for improved GC performance. Important developments include conversion of hydroxyl groups to trimethylsilyl (TMS) ethers, ketones to methyloximes and preparation of enol-TMS derivatives by base-catalyzed silylation [1–5]. In combination with capillary GC, steroid analysis is now routine in many laboratories.

Sample preparation prior to GC–MS analysis, however, is still quite extensive because of derivatization procedures and necessity for an initial enzymatic hydrolysis to release conjugated steroids. Thus, an important objective for further development of steroid analysis is the analysis of intact steroid conjugates as well as free steroids with a minimum of sample manipulation. This has been accomplished in recent years by on-line coupling of liquid chromatography with thermospray mass spectrometry (TSP LC–MS) [6–10] and atmospheric pressure ionization mass spectrometry (API LC–MS) [11–14].

TSP LC–MS is particularly suitable for corticosteroid analysis because several diagnostic ions are obtained besides the pseudomolecular ion. Coupled with an LC column, capable of complete and rapid separation of steroids, the identity of such compounds in biological specimens can be unequivocally established.

As a consequence, one objective of this study was to establish the conditions for rapid and efficient separation of corticosteroids. Another objective was the development of a TSP buffer that allows the pseudomolecular ion to constitute the base peak in the mass spectrum.

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EXPERIMENTAL

Materials

Corticosteroids were purchased from Alltech Assoc. (Deerfield, IL, USA). All solvents and ammonium formate were of HPLC quality and obtained from Fisher Scientific (Springfield, NJ, USA). Glucuronidase (*Patella vulgata*) was purchased from Sigma (St. Louis, MO, USA).

Enzymatic hydrolysis and extraction

Urine samples were mixed with 0.2 *M* potassium acetate buffer, pH 5.0 and treated with 10 000 U of glucuronidase for 2 h at 65°C. Following cooling to room temperature, saturated sodium carbonate solution, pH 10, was added and steroids were extracted into ethyl acetate. The organic layer was washed with 15% sodium sulphate in 1 *M* sodium hydroxide (w/v) and ethyl acetate finally removed under a stream of nitrogen at 50°C as described [15]. Samples were reconstituted in methanol prior to high-performance liquid chromatographic (HPLC) analysis.

High-performance liquid chromatography

Corticosteroid separations were performed on a Nova-Pak C_{18} radial compression column, 100 mm × 8.0 mm I.D., 4 μm particle size, from Waters Assoc. (Milford, MA, USA). A mobile phase containing 25 m*M* ammoniun formate in 30% (v/v) acetonitrile was delivered by a Waters 510 pump at a flow-rate of 1.3 or 1.5 ml/min. The column effluent was passed through a Waters 484 ultraviolet (UV) absorbance detector before entering the TSP vaporizer.

Thermospray mass spectrometry

The TSP instrument was a Vestec Model 201 (Houston, TX, USA) fitted with a liquid nitrogen cooled Labconco (Kansas City, MO, USA) vapour trap in the solvent vapour pumping line. A Vestec modular vaporizer fitted with a 75-μm sapphire aperture was used. Typical temperatures were as follows: vaporizer stem, 130°C; vaporizer tip, 240°C; ion source block, 250°C; and vapour (jet), 230°C. Mass calibrations were performed by injections of polyethylene glycol 600 dissolved in 50 m*M* ammonium formate using an ion source block temperature of 280°C.

The TSP buffer for flow injection experiments in negative-ion mode was 25 m*M* triethylammonium formate in 25% (v/v) acetonitrile.

RESULTS AND DISCUSSION

In view of a recent report on fragmentation by ammonolysis in the vaporizer or ion source in the presence of an ammonium salt [16], the effects of the buffer concentration were investigated. In our investigation it was observed that a lower buffer concentration resulted in a significantly decreased fragmentation (Fig. 1). The use of 10 m*M* ammonium formate gave a significant decrease of the fragment ions *m/z* 375 ($MH^+ - 18$) and *m/z* 333 ($MH^+ - 60$) as compared to 50 m*M* ammonium formate. The response of the pseudomolecular ion, however, only shows a minor decrease. The net result is an increase of the relative response of the molecular ion. Similar results were obtained with prednisone (Fig. 2). The effect of the buffer concentration was supported by further experiments featured in Figs. 3 and 4, dexamethasone and prednisone, respectively. It is noteworthy that an increase of the buffer concentration from 25 to 50 m*M* significantly enhances

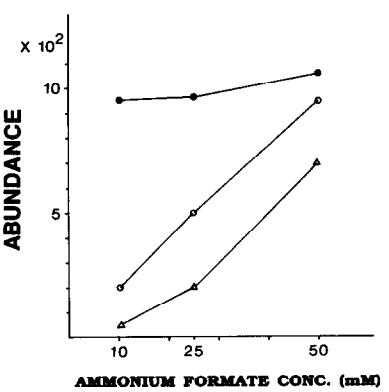

Fig. 1. Effect of ammonium formate concentration on pseudomolecular ion intensity and fragmentation of dexamethasone performed by flow injection analysis. The mobile phase was 30% acetonitrile containing different concentrations of ammonium formate. The selected ions were *m/z* 393 (●), *m/z* 375 (○) and *m/z* 333 (△) corresponding to MH^+, $MH^+ - 18$ and $MH^+ - 60$.

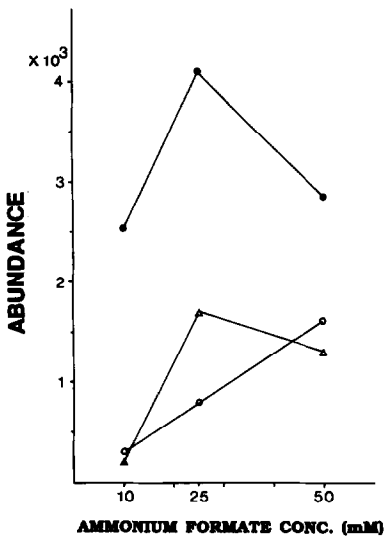

Fig. 2. Effect of ammonium formate concentration on pseudomolecular ion intensity and fragmentation of prednisone performed by flow injection analysis. The mobile phase was 30% acetonitrile containing different concentrations of ammonium formate. The selected ions were *m/z* 359 (●), *m/z* 341 (○) and *m/z* 299 (△) corresponding to MH^+, $MH^+ - 18$ and $MH^+ - 60$.

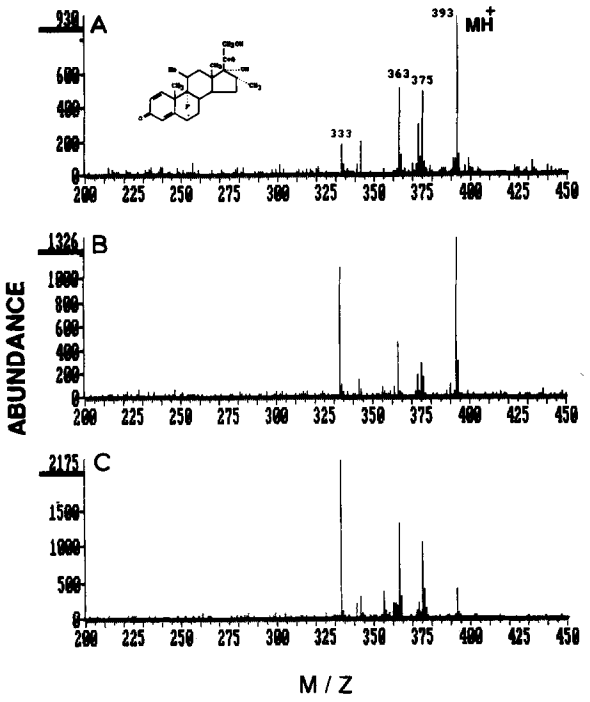

Fig. 3. Effect of ammonium formate concentration and organic solvent on the TSP spectrum of dexamethasone obtained by flow injection analysis. (A) 25 m*M* ammonium formate in 30% acetonitrile; (B) 50 m*M* ammonium formate in 30% acetonitrile; (C) 50 m*M* ammonium formate in 30% methanol.

the fragmentation, particularly *m/z* 333 and *m/z* 299 representing side-chain cleavage. A dramatic change of the appearance of the mass spectra occurs, however, when methanol is substituted for acetonitrile at a buffer concentration of 50 m*M*. The ions at *m/z* 333 and 299, Fig. 3C and 4C, respectively, now constitute the base peak in respective spectrum. These spectra resemble TSP spectra obtained by other investigators using methanol and ammonium acetate [8–10]. Furthermore, it can be seen in Fig. 4 that an increase of the buffer concentration from 25 to 50 m*M* causes a significant drop of the pseudomolecular ion intensity. This finding corroborates the similar change displayed in Fig. 2. The benefit of using acetonitrile instead of methanol with respect to pseudomolecular ion intensity has been demonstrated recently for thermolabile nitrogen mustards and glutathione conjugates [17,18].

Using 25 m*M* ammonium formate in 30% acetonitrile rapid separation of corticosteroids was achieved on a Nova-Pak C_{18} column (Fig. 5). Although partial separation was obtained between hydrocortisone and prednisone, peaks 2 and 3, respectively, the corresponding mass spectra are completely different as shown in Fig. 6. Moreover, by monitoring their pseudomolecular ions, both of these compounds are easily distinguished in a mixture as demonstrated by Fig. 6A. Each of the compounds injected onto the column represents a quantity of 30 ng. Similar conditions were used to separate and identify prednisolone and prednisone present in an equine urine extract following hydrolysis and extraction. In Fig. 7 is shown the rapid and complete separation of these corticosteroids, incurred in an equine urine extract, within 8 min. The pseudomolecular ion traces and TSP spectra are shown in Fig. 8. Peak 1 represents prednisolone while peak 2 was prednisone as depicted earlier in Figs. 4 and 6. The full advantage of using a lower concentration of ionizing additive and acetonitrile is evident in as much as the pseudomolecular ion constitutes the base peak and the abscence of ammonium adducts. On the contrary, when the pseudomolec-

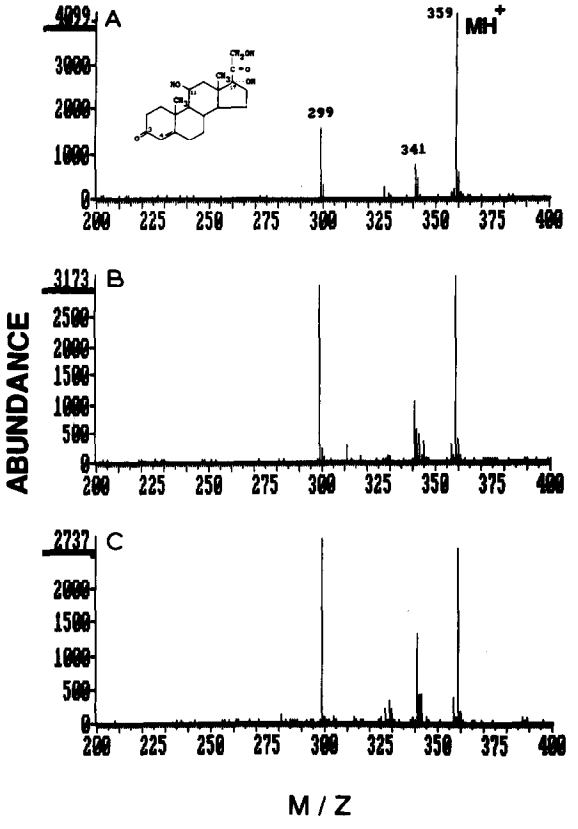

Fig. 4. Effect of ammonium formate concentration and organic solvent on the TSP spectrum of prednisone obtained by flow injection analysis. (A) 25 m*M* ammonium formate in 30% acetonitrile; (B) 50 m*M* ammonium formate in 30% acetonitrile; (C) 50 m*M* ammonium formate in 30% methanol.

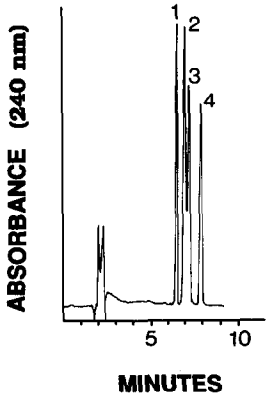

Fig. 5. Separation of corticosteroids on Nova-Pak C_{18}, 100 mm × 8 mm I.D., 4 μm using 25 m*M* ammonium formate in 30% acetonitrile at a flow-rate of 1.5 ml/min. Peaks: 1 = prednisolone; 2 = hydrocortisone; 3 = prednisone; 4 = cortisone.

ular ion is minor and with ammonium adducts present as found earlier [8,10], interpretation of an unknown spectrum might be difficult.

In our present investigation of the conditions for rapid and sensitive TSP MS of steroids the total amount of corticosteroids in urine was analyzed following conventional hydrolysis of conjugates and subsequent extraction. This method was adopted to obtain sufficient material for confirmed presence of steroidal drugs in equine urine. It would be desirable, however, to analyze conjugates simultaneously with free steroids without any hydrolysis. Sensitive analysis of steroid glucuronides and sulphates have earlier been performed by negative-ion TSP MS [6,7]. Thus, a simultaneous analysis of conjugated and unconjugated steroids would require negative-ion TSP MS of the same steroids investigated in our study. Consequently, negative-ion TSP MS of prednisone and dexamethasone was performed using triethylammonium formate as described earlier [19]. A negative-ion TSP spectrum of 10 ng of prednisone by flow injection is depicted in Fig. 9. In this mode the formate adduct at *m/z* 403 $(M + 45)^-$ constitutes the base peak while the pseudomolecular ion $(M - H)^-$ is about 30% thereof. Similar results were obtained when 10 ng of dexamethasone were subjected to negative-ion TSP MS. This mass spectrum exhibited only the formate adduct ion at *m/z* 437 $(M + 45)^-$. Thus, it was found that in negative-ion mode the sensitivity was similar or slightly improved as compared to positive ion mode. However, the number of diagnostic ions was insufficient for structural confirmation using our conditions. On the contrary, the minor fragmentation would be an advantage for TSP MS–MS where the ion current should be carried by as few ions as possible prior to collision analysis.

CONCLUSION

With respect to fragmentation and intensity of the pseudomolecular ion, this investigation demonstrates the advantage of using a lower buffer concentration than the commonly used 50–100 m*M*. Furthermore, use of acetonitrile instead of

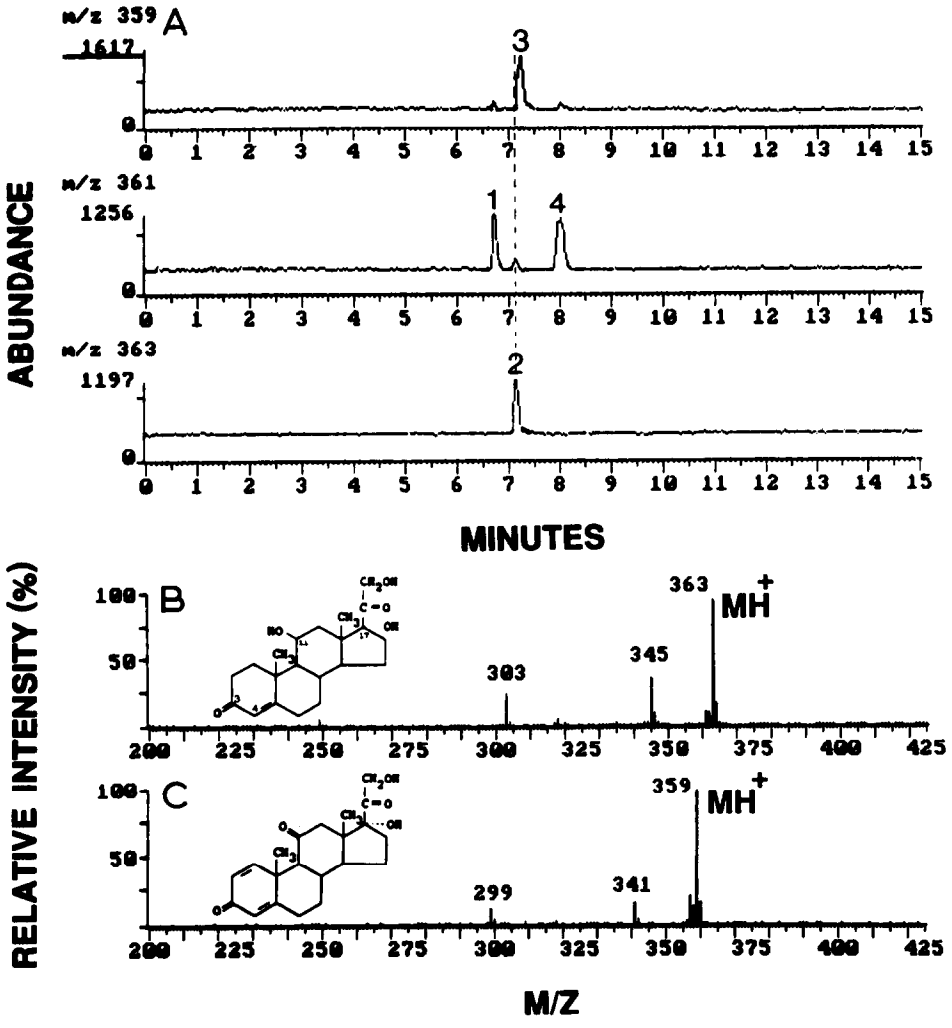

Fig. 6. (A) Pseudomolecular ion traces of corticosteroids separated on Nova-Pak C_{18}, 100 mm × 8 mm I.D. using 25 m*M* ammonium formate in 30% acetonitrile. Peaks: 1 = prednisolone; 2 = hydrocortisone; 3 = prednisone; 4 = cortisone. (B) TSP spectrum of hydrocortisone. (C) TSP spectrum of prednisone.

methanol contributed to enhanced pseudomolecular ion intensity and rapid separation of corticosteroids. In the negative-ion mode, it was possible to obtain a full-scan TSP spectrum from 10 ng injected. However, the number of diagnostic ions obtained was too small to permit an unequivocal structural confirmation. Since several reports describe the use of a repeller for collision-induced dissociation (CID) [20–25], the effects of different repeller voltages on the fragmentation of steroids were examined. Unfortunately, the use of the repeller resulted in a significant decrease of the sensitivity. This effect might be dependent on either the compound analyzed or instrument configuration. Another alternative would be the use of a different analytical technique to obtain more structural information. The use of diode-array UV spectrometry for structure analysis of steroids has been reported [10,26]. A high-quality diode-array spectrum is obtained from 10 ng or less. Since it is a non-destructive technique, it allows on-line coupling with a TSP instrument. Our results obtained with on-line diode array UV spectrometry TSP MS will be

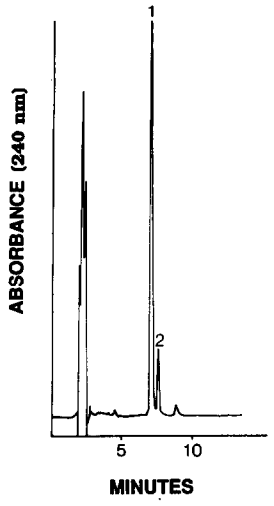

Fig. 7. Separation of corticosteroids from an equine urine extract on Nova-Pak C_{18}, 100 mm × 8 mm I.D., using 25 m*M* ammonium formate in 30% acetonitrile at a flow-rate of 1.3 ml/min. Peaks: 1 = Prednisolone; 2 = prednisone.

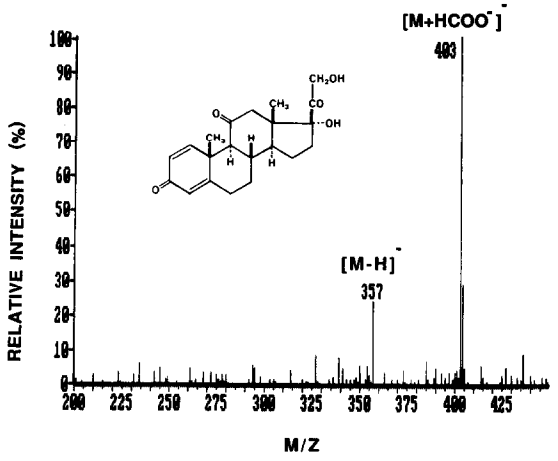

Fig. 9. Negative-ion TSP spectrum of 10 ng of prednisone obtained by flow injection analysis in 25% acetonitrile containing 25 m*M* triethylammonium formate.

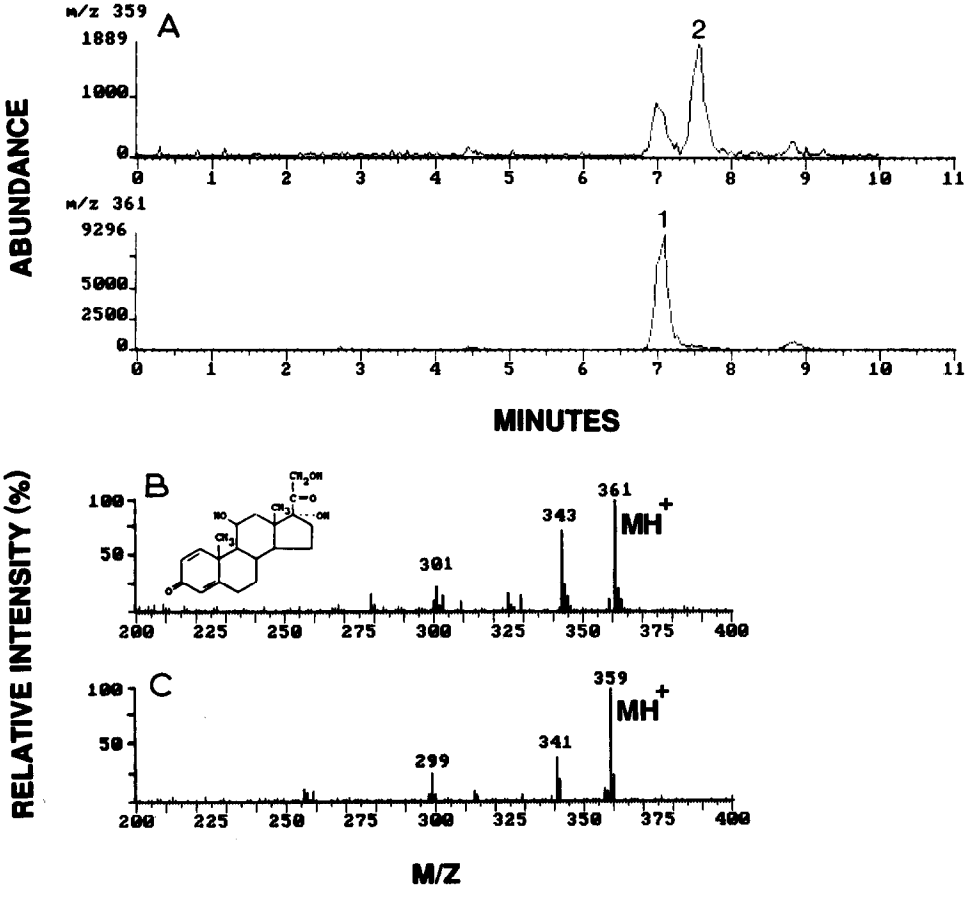

Fig. 8. (A) Pseudomolecular ion traces of corticosteroids from an equine urine extract separated on Nova-Pak C_{18}, 100 mm × 8 mm I.D., using 25 m*M* ammonium formate in 30% acetonitrile. Peaks: 1 = prednisolone; 2 = prednisone. (B) TSP spectrum of prednisolone. (C) TSP spectrum of prednisone.

reported in a forthcoming paper.

Thus, further developments in steroid analysis by LC–MS will be directed towards separation of corticosteroids and their conjugates followed by on-line diode-array UV spectrometry and negative-ion TSP MS. Alternatively, negative-ion TSP could be used as an interfacing technique for MS–MS for structure analysis of steroids.

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REFERENCES

1 T. Luukainen, W. J. A. VanDenHeuvel, E. O. A. Haati and E. C. Horning, *Biochim. Biophys. Acta*, 52 (1961) 599.
2 H. Fales and T. Luukainen, *Anal. Chem.*, 37 (1965) 955.
3 W. L. Gardiner and E. C. Horning, *Biochim. Biophys. Acta*, 115 (1966) 524.
4 E. M. Chambaz, G. Defaye and C. Madani, *Anal. Chem.*, 45 (1973) 1090.
5 S. Zaraga Nicosia, G. Galli, A. Fiecchi and A. Ros, *J. Steroid Biochem.*, 4 (1973) 417.
6 D. Watson, G. W. Taylor and S. Murray, *Biomed. Mass Spectrom.*, 12 (1985) 610
7 D. Watson, G. W. Taylor and S. Murray, *Biomed. Environ. Mass Spectrom.*, 13 (1986) 65.
8 D. J. Liberato, A. L. Yergey, N. Esteban, C. E. Gomez-Sanchez and C. H. L. Shackleton, *J. Steroid Biochem.*, 27 (1987) 61.
9 S. J. Gaskell, K. Rollins, R. W. Smith and C. E. Parker, *Biomed. Environ. Mass Spectrom.*, 14 (1987) 17.
10 Song-Ja Park, Yun-Je Kim, Hee-Soo Pyo and Jongsei Park, *J. Anal. Toxicol.*, 14 (1990) 102.
11 L. O. G. Weidolf, E. D. Lee and J. D. Henion, *Biomed. Environ. Mass Spectrom.*, 15 (1988) 283.
12 P. O. Edlund, L. Bowers and J. Henion, *J. Chromatogr.*, 487 (1989) 341.
13 W. M. Muck and J. D. Henion, *Biomed. Environ. Mass Spectrom.*, 19 (1990) 37.
14 E. C. Huang, T. Wachs, J. J. Conboy and J. D. Henion, *Anal. Chem.*, 62 (1990) 713A.
15 A. K. Singh, B. Gordon, D. Hewetson, K. Granley, M. Ashraf, U. Mishra and D. Dombrovskis, *J. Chromatogr.*, 479 (1989) 233.
16 K. J. Volk, R. A. Yost and A. Brajter-Toth, *J. Chromatogr.*, 474 (1989) 231.
17 M. F. Bean, S. L. Pallante-Morell and C. Fenselau, *Biomed. Environ. Mass Spectrom.*, 18 (1989) 219.
18 M. F. Bean, S. Pallante-Morell and C. Fenselau, *Anal. Chem.*, 62 (1990) 121.
19 S. Steffenrud and G. Maylin, *Rapid Commun. Mass Spectrom.*, 4 (1990) 463.
20 C. Lindberg and J. Paulson, *J. Chromatogr.*, 394 (1987) 117.
21 W. H. McFadden and S. A. Lammert, *J. Chromatogr.*, 385 (1987) 201.
22 F. Aladar Bencsath and F. H. Field, *Anal. Chem.*, 60 (1988) 1323.
23 M. E. Harrison, G. J. Langley and M. A. Baldwin, *J. Chromatogr.*, 474 (1989) 139.
24 C. E. M. Heeremans, R. A. van der Hoeven, W. M. A. Niessen, U. R. Tjaden and J. van der Greef, *J. Chromatogr.*, 474 (1989) 149.
25 M. A. McLean and R. B. Freas, *Anal. Chem.*, 61 (1989) 2054.
26 J. O. De Beer, *J. Chromatogr.*, 489 (1989) 139.