Journal of Chromatography, 341 (1985) 261—269
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2562

QUANTITATIVE DETERMINATION OF BETAMETHASONE AND ITS MAJOR METABOLITE IN EQUINE URINE BY MICRO-LIQUID CHROMATOGRAPHY—MASS SPECTROMETRY

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(First received August 22nd, 1984; revised manuscript received January 22nd, 1985)

SUMMARY

Micro-liquid chromatography—mass spectrometry (micro-LC—MS) was utilized to quantitatively determine betamethasone and its major unconjugated metabolite, 6β-hydroxybetamethasone, in equine plasma and urine. The advantage of micro-LC—MS over conventional gas chromatography—mass spectrometry in corticosteroid determination is illustrated and the reliable, steadfast nature of micro-LC—MS is demonstrated through example.

INTRODUCTION

Micro-liquid chromatography—mass spectrometry (micro-LC—MS) has continued to find new and novel applications in both qualitative [1—3] and comparative [4] determinations. The power of this analytical tool affords difficult drug determinations and although proven sensitive and specific, micro-LC—MS utilization in precision studies such as quantitative determination has found very limited application [5, 6].

Quantitative determination can profile drug concentrations over time and in turn be used to estimate pharmacokinetic parameters such as drug half-life, volume of distribution, body clearance, elimination rate and drug compartmentalization. To do this, determinations must discern between administered drugs and endogenous material as well as between parent drugs and their metabolites. Many techniques utilized for quantitative determination are unable to make both distinctions. Radioimmunoassay [7, 8], competitive protein binding [8, 9] and enzyme immunoassay [10] fail to consistently distinguish parent drugs from metabolites, while thin-layer chromatography (TLC), gas chroma-

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tography (GC) or liquid chromatography (LC) alone can not confirm analyte identities.

MS, on the other hand, when coupled to these chromatographic techniques can afford sensitve, unequivocal identification of parent drugs and metabolites. Still, the polar, non-volatile, heat-labile nature of the corticosteroids [11] makes analysis by TLC—direct inlet probe-MS or GC—MS difficult while micro-LC—MS remains a viable, non-destructive alternative.

In this study we illustrate how the sensitive, specific capabilities of micro-LC—MS can be applied to the quantitative determination of corticosteroids in biological fluids. In doing so, we demonstrate the stable, reliable nature of direct liquid introduction (DLI) micro-LC—MS in precision studies over extended periods and illustrate the advantage of micro-LC—MS over conventional GC—MS in corticosteroid determination.

MATERIALS AND METHODS

Drug administration and chemicals

Betamethasone sodium phosphate (Shering, Kenilworth, NJ, U.S.A.) was administered intravenously (60 mg) to healthy Standardbred horses (ca. 500 kg each). Reference betamethasone was purchased from Sigma (St. Louis, MO, U.S.A.) and 6α-methylprednisolone from Steraloids (Wilton, NH, U.S.A.). Dr. R. Draper of Shering (Bloomfield, NJ, U.S.A.) generously donated 6β-hydroxybetamethasone (U.S. Patent No. 4,201,778).

Micro-LC methanol was distilled-in-glass (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) while the water was HPLC grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Eluents were suction-filtered through a 0.45-μm pore-size filter (Millipore, Bedford, MA, U.S.A.) and then continuously purged with helium during micro-LC operation to eliminate dissolved gases. All sample solutions injected into the micro-LC system were filtered through a 0.45-μm pore-size Millipore filter. Derivatizing agents, methoxyamine hydrochloride and trimethylsilylimidazole, were purchased from Pierce (Rockford, IL, U.S.A.).

Drug extraction and TLC clean-up

Plasma and urine were extracted by the method of Skrabalak and Maylin [12] with the following modifications. The extraction solvent was diethyl ether—methylene chloride—isopropanol (2:1:1) and only the initial TLC system was used. Further separation of TLC-prepared samples was afforded by micro-LC—MS.

Micro-LC

A modified Waters M660 solvent programmer [13] and M6000A pump were equipped with a Rheodyne Model 7410 micro-loop (0.5 μl) injector (Rheodyne, Cotati, CA, U.S.A.) and a 25 cm × 1 mm I.D. Whatman Partisil 10 ODS-3 microbore column (Whatman, Clifton, NJ, U.S.A.). Micro-LC separations were accomplished with methanol—water (70:30) at a flow-rate of 40 μl/min.

Micro-LC—MS and GC—MS

An unmodified Hewlett-Packard Model 5985B GC—MS instrument equipped with option 01 (Hewlett-Packard, Palo Alto, CA, U.S.A.) was utilized for micro-LC—MS operation. The liquid nitrogen cooled cryopump was used in all chemical ionization (CI) experiments as recommended by the manufacturer.

The normal CI operating parameters of the mass spectrometer for all micro-LC—MS experiments were as follows: electron energy, 230 eV; emission current, 300 μA; ion source pressure; 0.5—0.6 Torr (66.5—80 Pa) as measured at the GC—CI-MS interface thermocouple; repeller, 5 V; electron multiplier, 2400 V. The micro-LC and MS systems were interfaced via an on-line DLI micro-LC—MS probe as previously described [1].

A Hewlett-Packard Model 5992 GC—MS system (Hewlett-Packard) equipped with a silicone membrane separator was utilized for electron-impact ionization (EI) GC—MS acquisitions. EI spectra were obtained with 220 μA emission current and 70 eV ionization energy. GC was carried out on a 1 m × 2 mm I.D. packed column of 3% OV-101, temperature programmed from 220°C (isothermal for 2 min) to 260°C at 10°C/min and purged with helium carrier gas at a flow-rate of 30 ml/min. Urine extracts for GC—EI-MS were derivatized as previously described [14].

Quantitation

All plasma and urine samples were spiked with known amounts (100 ng/ml) of 6α-methylprednisolone before being extracted and analyzed as described. Immediately before and after sample analysis, a mixture containing equal amounts of 6β-hydroxybetamethasone, betamethasone and 6α-methylprednisolone were determined by micro-LC—MS. Individual drug levels within these mixtures ranged from 75 to 150 ng each; their determinations afforded

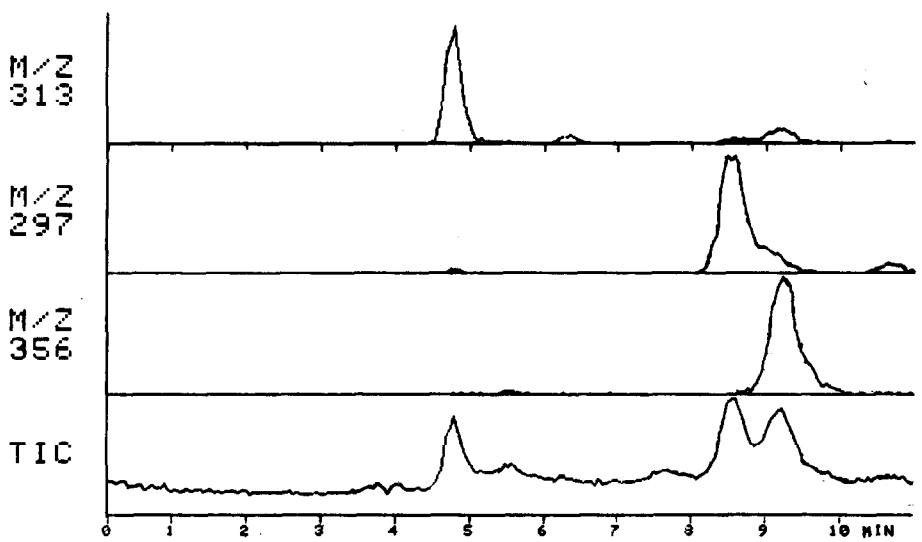

Fig. 1. Selected-ion profiles of 6β-hydroxybetamethasone (*m/z* 313), betamethasone (*m/z* 297) and internal standard, 6α-methylprednisolone (*m/z* 356) and NICI—TIC micro-LC—MS chromatograms of a representative betamethasone administration 2-h plasma extract.

comparison of the system's responses to these drugs under similar conditions over a wide range. Selected-ion abundances of 6β-hydroxybetamethasone (*m/z* 313) and betamethasone (*m/z* 297) were compared to those of 6α-methylprednisolone (*m/z* 356) and the respective standard ratios calculated (n = 4; 6β-hydroxybetamethasone/6α-methylprednisolone = 10.78 ± 2.49, x ± S.E.; betamethasone/6α-methylprednisolone = 4.89 ± 0.89, $\bar{x}$ ± S.E.).

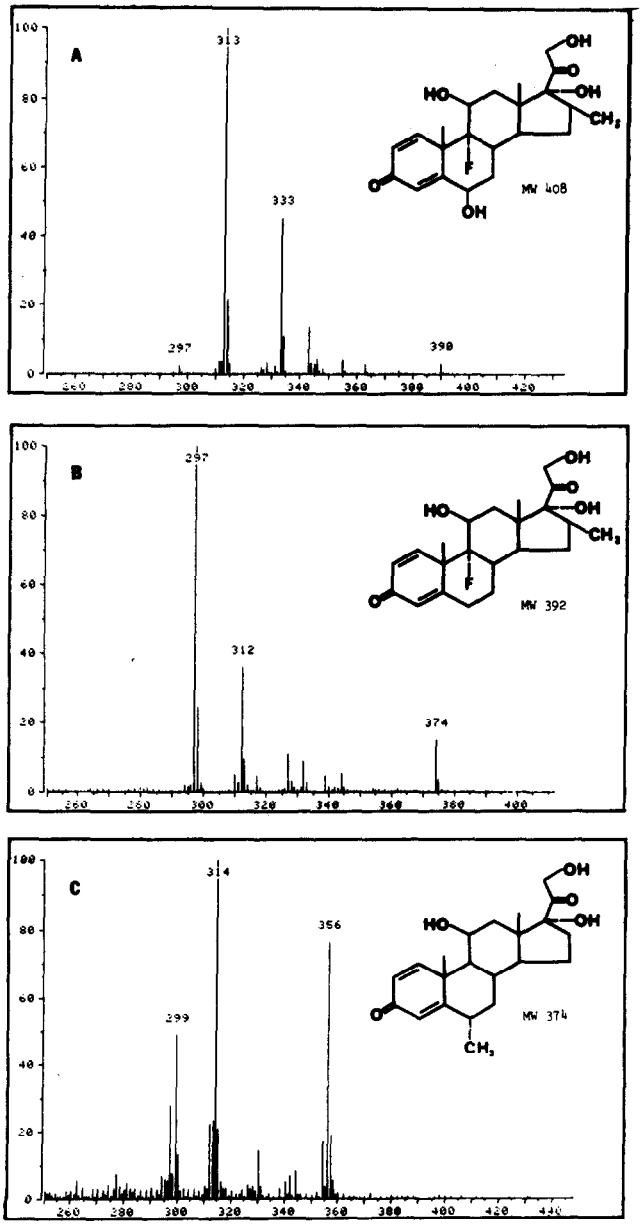

Fig. 2. Structures and mass spectra of (A) 6β-hydroxybetamethasone, (B) betamethasone and (C) 6α-methylprednisolone.

Corresponding sample ratios were later compared to respective standard ratios and multiplied by the spike concentration to estimate drug concentrations in plasma and urine. Recoveries of the three drugs from both plasma and urine were shown by re-isolation procedures and high-performance liquid chromatography (HPLC) to be in the ratio 1:1:1; extraction efficiency was 40%.

RESULTS AND DISCUSSION

The selected-ion profiles of 6β-hydroxybetamethasone (*m/z* 313); betamethasone (*m/z* 297) and 6α-methylprednisolone (*m/z* 356) as well as the negative-ion chemical ionization—total-ion current (NICI—TIC) chromatogram of a representative betamethasone administration plasma extract are shown in Fig. 1. The structures and mass spectra of these corticosteroids are given in Fig. 2. Under the conditions used, each sample was analyzed in less than 12 min. This relatively short run time affords efficient use of the micro-LC—MS system and allows numerous samples to be run within a given work period. The ability of the mass spectrometer and its associated computer system to profile selected-ions enables one to distinguish peaks of interest from both endogenous interferences as well as other analyte peaks. This ability is most evident near the end of the chromatogram where betamethasone and 6α-methylprednisolone peaks are not baseline resolved. Selected-ion profiles of betamethasone (*m/z* 297) and 6α-methylprednisolone (*m/z* 356) produce baseline resolution, thereby affording quantitation of their respective peaks.

Contrary to the results afforded by micro-LC—MS, corticosteroid determination by GC—MS usually requires derivatization of the drugs. Inevitably the derivatizing procedures command considerable preparation and time while producing multiple products that can hinder quantitative

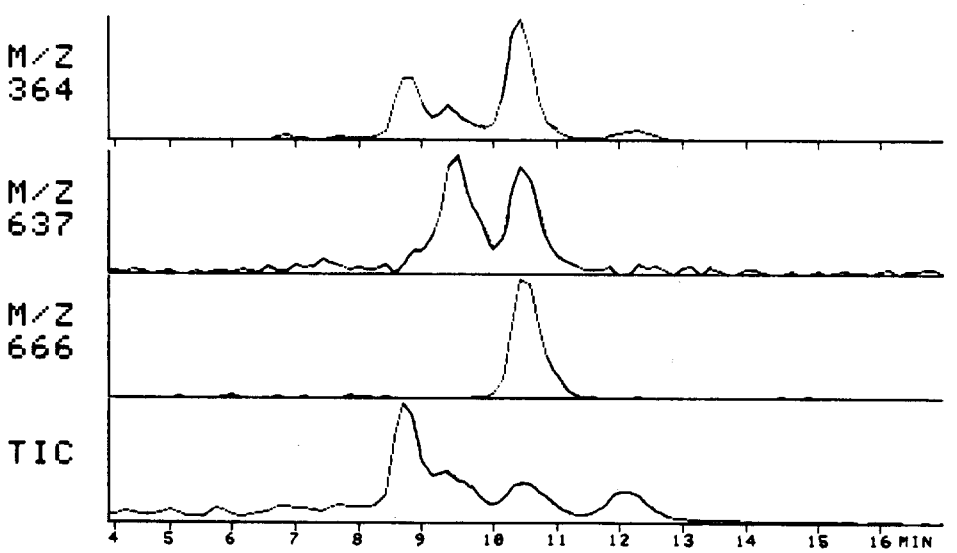

Fig. 3. GC—MS selected-ion profiles and total-ion current of spiked equine urine extract derivatized with methoxyamine hydrochloride and trimethylsilylimidazole. Urine was spiked with 6β-hydroxybetamethasone, betamethasone and 6α-methylprednisolone at 10, 10 and 0.1 μg/ml, respectively, before extraction.

measurement [14, 15]. Fig. 3 shows GC—MS selected-ion profiles and TIC of a methoxime-trimethylsilyl (MO-TMS) derivatized equine urine extract. The urine was spiked with 6β-hydroxybetamethasone, betamethasone and 6α-methylprednisolone at 10, 10 and 0.1 μg/ml, respectively, before extraction to approximate or exceed peak urine concentrations of betamethasone products quantitatively analyzed in this work by micro-LC—MS. The 6α-methylprednisolone concentrations used for standardization remained constant in all GC—MS and micro-LC—MS samples.

Betamethasone was chosen as a representative component to examine derivatized corticosteroid determination by conventional GC—MS while the scanned mass range encompassed all expected derivatization products (*m/z* 35—*m/z* 770). The TIC in Fig. 3 exhibits multiple peaks which are inconsistent with a single derivatization product for each spiked component. In fact, by inspecting the selected-ion profiles indicative of MO-TMS derivatized betamethasone, *m/z* 364 [15], we see major chromatographic peaks corresponding to both the mono-MO-tri-TMS (*m/z* 637) and bi-MO-tri-TMS (*m/z* 666) derivative. This clearly demonstrates multiple products of betamethasone derivatization and thus the incompatibility of GC—MS with quantitative corticosteroid determination.

One aspect of DLI micro-LC—MS addressed in this work is reliability over extended periods. The continually acquired NICI—TIC chromatogram of multiple injected samples is shown in Fig. 4. During these 185 min of acquisition, methanol—water (70:30) eluent continuously jetted into the mass spectrometer at 40 μl/min. Nevertheless, source ionization pressure remained at 0.6 Torr without adjustment due to the excellent performance of the liquid nitrogen cooled cryopump and the DLI micro-LC—MS probe which delivered a fine, uninterrupted stream of eluent. The entire system responded well to a

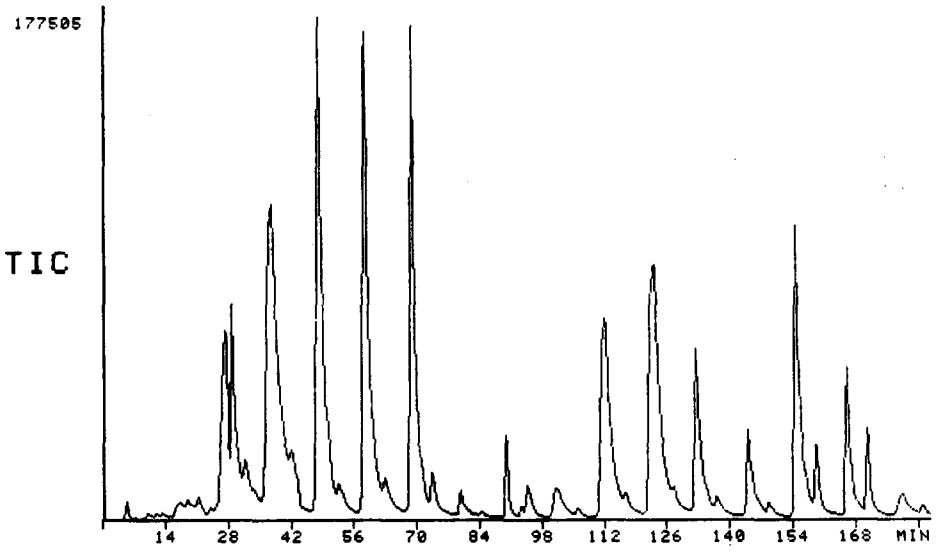

Fig. 4. Continually acquired NICI—TIC micro-LC—MS chromatogram of multiple injected samples obtained with a Whatman Partisil 10 ODS-3 microbore column (25 cm × 1 mm I.D.) and methanol—water (70:30, v/v) at a flow-rate of 40 μl/min.

TABLE I

PLASMA CONCENTRATIONS OF BETAMETHASONE AND 6β-HYDROXYBETAMETHASONE IN THE HORSE ($n = 2$), DETERMINED BY MICRO-LC—MS, FOLLOWING A SINGLE INTRAVENOUS DOSE OF BETAMETHASONE SODIUM PHOSPHATE (0.12 mg/kg)

Time (h)	Concentration ± S.E. (ng/ml)	
	Betamethasone	6β-Hydroxybetamethasone
2	61.05 ± 2.97	14.19 ± 4.74
4	40.08 ± 10.05	13.69 ± 0.41
6	18.31 ± 6.05	6.17 ± 0.79
8	11.86 ± 4.10	5.52 ± 0.32

TABLE II

URINARY EXCRETION OF BETAMETHASONE AND 6β-HYDROXYBETAMETHASONE IN THE HORSE ($n = 2$), DETERMINED BY MICRO-LC—MS, FOLLOWING A SINGLE INTRAVENOUS DOSE OF BETAMETHASONE SODIUM PHOSPHATE (0.12 mg/kg)

Time (h)	Total amount ± S.E. (μg)	
	Betamethasone	6β-Hydroxybetamethasone
1	1335.16 ± 441.02	692.46 ± 66.27
3	656.46 ± 254.27	980.77 ± 279.48
5	295.35 ± 179.06	641.35 ± 333.45
7	105.87 ± 35.28	305.89 ± 40.87
25.5	8.09 ± 1.89	54.72 ± 15.67
29	3.95 ± 0.65	27.34 ± 1.38
47.5	3.91 ± 1.77	2.38 ± 0.40

wide range of sample quantity while retaining a steady reference baseline. Such performance, essential to quantiative analysis, was achieved on multiple sample series of variable durations and thus substantiates DLI micro-LC—MS as a reliable, steadfast system.

Table I gives the plasma concentration data of bethamethasone and 6β-hydroxybetamethasone. The data illustrate relative amounts of parent drug versus metabolite over time following a single intravenous injection of bethamethasone sodium phosphate (0.12 mg/kg).

Table II gives the urinary excretion data of betamethasone and 6β-hydroxybetamethasone. These data along with those for plasma not only demonstrate the expedient excretion of drug but also demonstrate the sensitivity of micro-LC—MS in multiple samples.

Fig. 5 shows the selected-ion profiles and NICI—TIC chromatograms of a 48-h post-administration urine sample extract. The chromatograms illustrate the sensitive, selective nature of micro-LC—MS. In this extract both 6β-hydroxybetamethasone (m/z 313) and its parent, betamethasone (m/z 297) were present at very low concentrations within a complex matrix of interference. Nevertheless, micro-LC—MS was able to quantitatively determine the drugs under circumstances not amenable to conventional LC detectors.

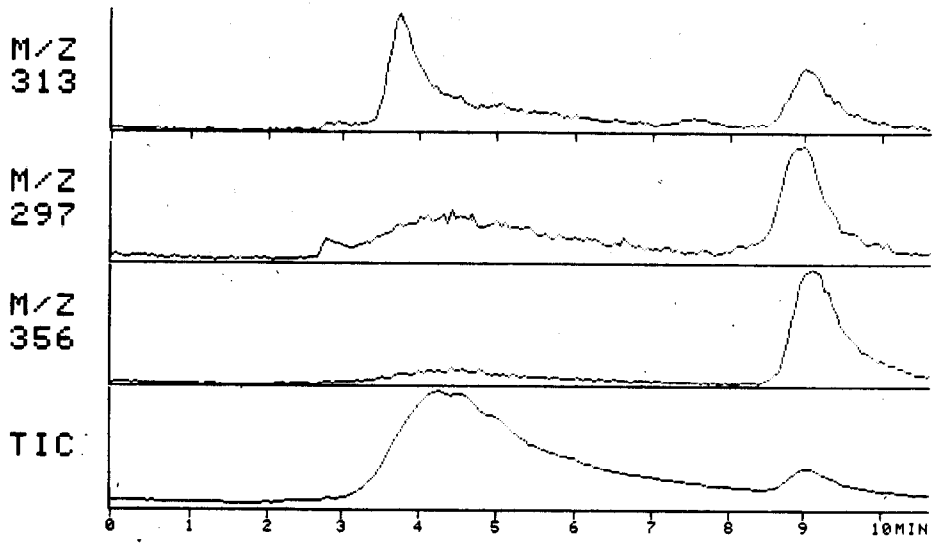

Fig. 5. Selected-ion profiles of 6β-hydroxybetamethasone (*m/z* 313), betamethasone (*m/z* 297) and internal standard, 6α-methylprednisolone (*m/z* 356) and NICI—TIC micro-LC—MS chromatograms of a 48-h post-betamethasone administration urine extract.

CONCLUSION

As shown, DLI micro-LC—MS is a reliable, stable technique which can be utilized over extended periods of time. The additional sensitivity and specificity of DLI micro-LC—MS affords its application to such precision work as quantitative determination of underivatized corticosteroids in a manner superior to that of conventional GC—MS.

ACKNOWLEDGEMENTS

We thank the New York State Racing and Wagering Board and the Harry M. Zweig Memorial Fund for financial support of this work. We also thank Whatman for their generous supply of micro-LC columns and G.A. Maylin, director of the Equine Drug Testing and Toxicology program, for his continued support of this work.

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