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QUALITATIVE DETECTION OF CORTICOSTEROIDS IN EQUINE BIOLOGICAL FLUIDS AND THE COMPARISON OF RELATIVE DEXAMETHASONE METABOLITE/DEXAMETHASONE CONCENTRATION IN EQUINE URINE BY MICRO-LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Several important corticosteroids were qualitatively determined in the plasma and urine of horses by micro-liquid chromatography–mass spectrometry (micro-LC–MS). The sensitivity and specificity of micro-LC–MS are demonstrated as is the ability of micro-LC–MS to deal with endogenous interferences. In turn, the relative amount of dexamethasone and its major unconjugated metabolite were determined in equine urine by micro-LC–MS; the conclusions drawn are reported.

INTRODUCTION

Drug determinations from biological fluids can be formidable tasks for the analytical chemist. Associated difficulties are further amplified in the case of corticosteroid determination. This group of drugs attains therapeutic effectiveness at very low plasma concentrations[1] while endogenous steroidal components as well as other interferences can mask the presence of synthetic analogues. Many forensic studies such as the drug testing of race horses require sensitive, specific determination yet the various historical methods used in the determination of corticosteroids are not applicable to race horse drug testing. Indirect methods such as radioimmunoassay[2,3], competitive protein binding[3,4] and enzyme immunoassay[5] are very sensitive but due to cross-reactivity may only indicate the presence of "a" corticosteroid. The techniques cannot unequivocally distinguish similar synthetic or endogenous corticosteroids and false determinations are frequently encountered.

Direct methods which utilize ultraviolet, electron-capture or other non-specific detectors cannot unequivocally confirm the identification of particular drugs. Mass spectrometry (MS) affords specificity and sensitivity to drug detection yet when coupled to gas chromatography (GC–MS) this technique is not amenable to underivatized corticosteroids due to their low volatility and thermal instability[6]. Although suitable derivatives can be synthesized which allow GC or GC–MS determination

of the corticosteroids, these off-line derivatization procedures often require considerable preparation and time. In addition, the successive derivatization procedures form multiple products due to incomplete reactions which in turn produce mass spectra with low abundance molecular ions and fragmentation patterns that are dominated by the type of derivative rather than the drug's structure[7].

The above problems are negated when MS is coupled to on-line micro high-performance liquid chromatography (micro-LC–MS). Micro-LC is compatible and nondegrading to corticosteroids and provides high chromatographic resolution that compliments the mass spectrometer's sensitivity and specificity. Micro-LC–MS has been implemented into several limited corticosteroid determinations and proven successful[8–10]. These preliminary studies have indicated the usefulness of micro-LC–MS although the technique's full potential in rapid drug determinations has not been demonstrated.

Due to their therapeutic effectiveness, corticosteroids are usually present at low concentrations in biological fluids. Extracts of these complex biological matrices include numerous endogenous components and necessitate extensive chromatography to isolate individual drugs for identification. Consequently, both quantitative and qualitative work may suffer due to drug losses. Alternatively, micro-LC–MS affords unequivocal determination of corticosteroids in biological fluids in a direct fashion. Micro-LC resolves the corticosteroids while the mass spectrometer and modern computers allow sensitive, selective determinations devoid of background interference.

The above attributes of micro-LC–MS enable one to confirm the presence of various corticosteroids and metabolites once authentic comparative spectra are available. In doing so, other areas of drug determination have opened up to micro-LC–MS. One area which is of special interest to forensic laboratories is the relative comparison of parent drug and metabolite concentrations in plasma or urine over time. Knowledge of this comparison gives insight to metabolic time tables and is imperative when pondering drugs to be targeted for screening and determinations in equine drug testing. Relative comparison differs from quantitative determination in that there is no quantitated internal standard added to the sample as in the latter. The approach becomes viable when quantitation is not possible due to inaccessability of pure drug or metabolite in amounts condusive to proper quantitative measurement. Such scarcity is the case for many drug metabolites including equine dexamethasone metabolite[11]. Described herein are the qualitative determinations of several important corticosteroids in equine urine and plasma that illustrate the sensitivity and specificity of micro-LC–MS. In addition, the application of micro-LC–MS to determining relative dexamethasone and dexamethasone metabolite concentrations in equine urine over time is presented. The metabolite has previously been identified[11] and its micro-LC–MS spectrum is shown within this report.

MATERIALS AND METHODS

Drug administration and chemicals

Betamethasone sodium phospohate (Schering, Bloomfield, NJ, U.S.A.), dexamethasone sodium phosphate (Chromalloy Pharmaceutical, Oakland, CA, U.S.A.) and methylprednisolone acetate (Upjohn, Kalamazoo, MI, U.S.A.) were adminis-

tered intramuscularly (63, 60 and 300 mg, respectively) to healthy Standardbred horses (*ca.* 500 kg ea.). Reference betamethasone and dexamethasone were purchased from Sigma (St. Louis, MO, U.S.A.). Schering generously donated 6β-hydroxybetamethasone (U.S. Patent No. 4,201,778) and 6α-methylprednisolone was obtained from Steraloids (Wilton, NH, U.S.A.).

Chemicals and solvents used in this work were of analytical reagent grade if not otherwise indicated. Micro-LC methanol was distilled-in-glass (Burdick and Jackson, Muskegon, MI, U.S.A.) while the water was HPLC grade (J. T. Baker, Phillipsburg, NJ, U.S.A.). All 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.

Drug extraction and thin-layer chromatography (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, v/v/v) and only the TLC system chloroform–ethyl acetate–light petroleum (5:3:2, v/v/v) was used. Final separation of TLC prepared samples was afforded by micro-LC–MS.

Micro-liquid chromatography

A modified Waters M-660 solvent programmer[13] and M-6000A pump (Waters Assoc., Morristown, NY, U.S.A.) were equipped with a Rheodyne Model 7410 microloop (0.5 μl) injector (Rheodyne, Cotati, CA, U.S.A.) and either a 25 cm × 1 mm I.D. or 30 cm × 1 mm I.D. Whatman Partisil 10 ODS-3 microbore column (Whatman Chemical Separations, Clifton, NJ, U.S.A.). The microcell for this work was similar to that reported by Hermansson[14] and generously provided by Waters Associates. Micro-LC separations were accomplished with either 70:30 (v/v) or 65:35 (v/v) methanol–water at 40 μl/min.

Micro-liquid chromatography-mass spectrometry

An unmodified Hewlett-Packard Model 5985 GC/MS instrument equipped with option 01 (Hewlett-Packard, Palo Alto, CA, U.S.A.) was utilized with the above micro-LC system 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: ionization energy, 230 eV; emission current, 300 μA; ion source pressure, 0.5–0.6 Torr (66.5–80 Pa) as measured at the CI GC–MS interface thermocouple; repeller, 5 V; and electron multiplier, 2400 V. The micro-LC and MS systems were interfaced via an on-line direct liquid introduction (DLI) micro-LC–MS probe as previously described[8].

RESULTS AND DISCUSSION

Extraction methods utilized for the determination of corticosteroids in biolog-

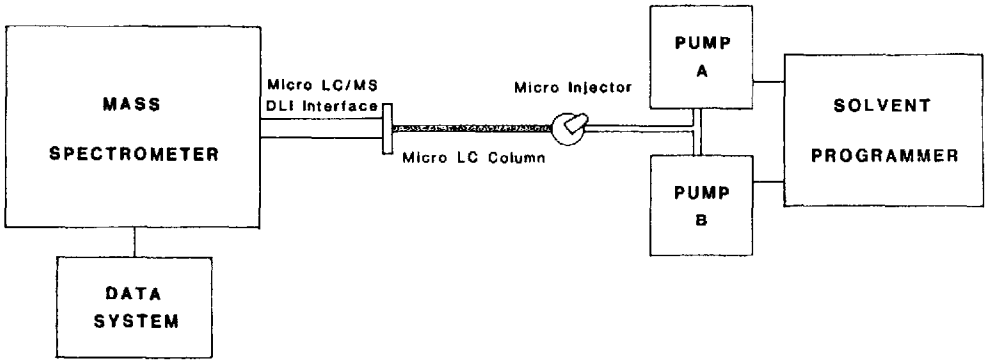
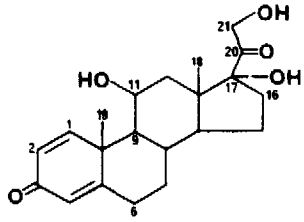


Fig. 1. Schematic of the micro-LC–MS system.

ical fluids inevitably recover endogenous products which can interfere with drugs of interest. Various chromatographic techniques may solve the individual problem at hand yet similar interference problems can arise as either the drug, subject or subject's diet changes.

Fig. 1 shows a schematic of the micro-LC–MS system used in this work. The structures of those corticosteroids discussed in this research are shown in Fig. 2 while their thin-layer chromatography data are given in Table I. Their mass spectrometry data are given in Table II. Figs. 3 and 4 illustrate the UV absorbing interferences encountered with micro-LC determinations of exogenous corticosteroids in equine



COMPOUND	MW	C-1 and C-2	C-6	C-9	C-16
Betamethasone	392			F	$CH_3(\beta)$
6β-Hydroxybetamethasone	408		OH	F	$CH_3(\beta)$
Cortisol	362	No double bond at C1-C2			
Dexamethasone	392			F	$CH_3(\alpha)$
6α-Methylprednisolone	374		CH_3		

Fig. 2. Structures of the corticosteroids under study.

TABLE I

THIN-LAYER CHROMATOGRAPHIC CHARACTERISTICS OF THE CORTICOSTEROIDS UNDER STUDY

Solvent system A = chloroform ethyl acetate–light petroleum (5:3:2, v/v/v); solvent system B = chloroform–ethanol (9:1, v/v); solvent system C = ethyl acetate–acetic acid (39:1, v/v).

Compound	R_F *in solvent system*		
	A	*B*	*C*
Betamethasone	0.02	0.32	0.68
6β-Hydroxybetamethasone	0.01	0.22	0.56
Cortisol	0.02	0.37	0.54
Dexamethasone	0.03	0.34	0.70
6α-Methylprednisolone	0.02	0.33	0.59

TABLE II

CHARACTERISTIC NCI MICRO-LC–MS FRAGMENT IONS (*m/z*) OF THE CORTICOSTEROIDS UNDER STUDY

Obtained with methanol–water (70:30) as micro-LC eluent and NCI reagent gas. Mass range scanned was *m/z* 250 to *m/z* 450.

Compound	*MW*	*m/z* *(relative abundance in percentage)*
Betamethasone	392	297 (100); 312 (36); 298 (24); 374 (15); 332 (11)
6β-Hydroxybetamethasone	408	313 (100); 333 (45); 314 (22); 343 (14); 390 (3); 297 (2)
Cortisol	362	344 (100); 342 (28); 345 (25); 301 (16)
Dexamethasone	392	297 (100); 298 (22); 312 (12); 332 (8); 374 (3)
6α-Methylprednisolone	374	356 (100); 314 (67); 297 (36); 299 (35); 357 (24); 313 (20); 354 (17); 312 (16)

urine and plasma extracts respectively using betamethasone and its metabolite, 6β-hydroxybetamethasone as relative examples.

The pre-administration (control) urine extract shown in Fig. 3 demonstrates endogenous interference at the same retention time as 6β-hydroxybetamethasone in post-administration urine extract. Such interferences are common during micro-LC determinations due to the non-specific response of UV detectors. Fig. 4 reveals endogenous UV absorbing interferences in control plasma at retention times corresponding to both betamethasone and 6β-hydroxybetamethasone. Although these particular interferences appear quantitatively small their significance increases as plasma levels of the respective drugs diminish. (Note: Endogenous plasma cortisol levels are suppressed with the administration of exogenous corticosteroids[1].)

Figs. 5 and 6 demonstrate mass spectrometry's ability to circumvent interfer-

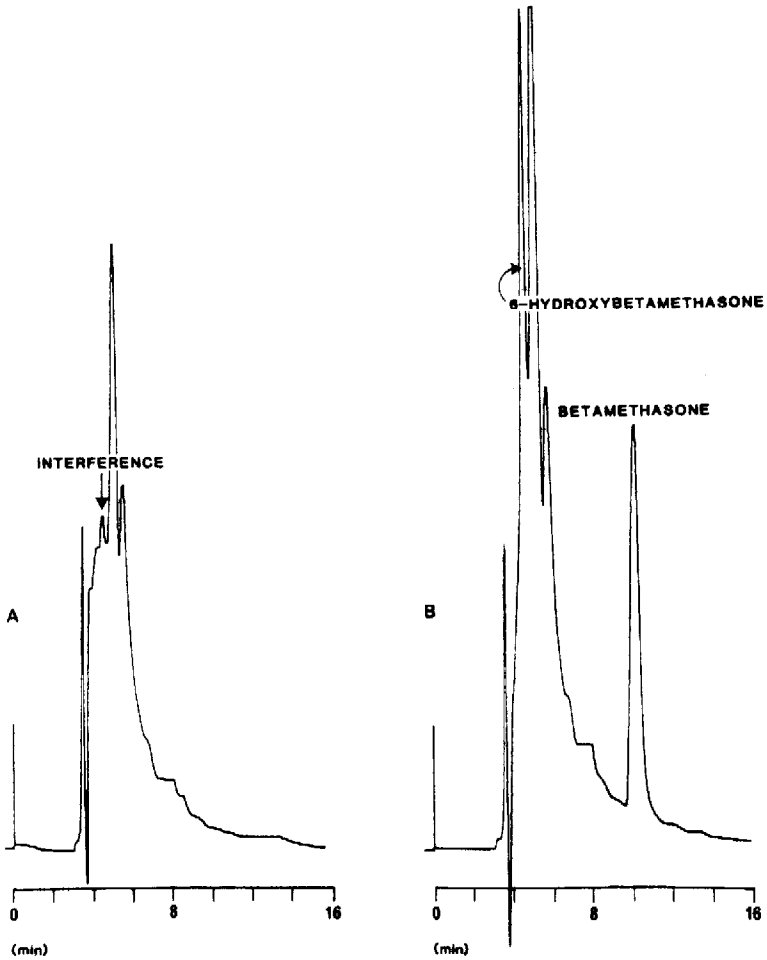


Fig. 3. Micro-LC–UV chromatograms of (A) control urine extract and (B) betamethasone administration 2 h urine extract obtained with a Whatman Partisil 10 ODS-3 microbore column (25 cm × 1 mm I.D.) and methanol water (70:30) at 40 μl/min.

ence in either of two ways. First, since the mass spectrometer only detects ionizable components in the mass range indicated, many interferences can be avoided by choosing a mass range or "window" applicable to the drugs of interest but still above the majority of ionizable endogenous material. Fig. 5A shows the negative ion chemical ionization–total ion current (NCI–TIC) profile of control plasma extract. Evident in this chromatogram is a single chromatographic peak which corresponds to endogenous cortisol present in equine plasma. The rest of the chromatogram is free of interference and appears as a somewhat noisy baseline since the ion current plot is normalized to the small cortisol peak. Fig. 5B illustrates 6β-hydroxybetamethasone and betamethasone in equine plasma extract following betamethasone administration. The ion current profile in this plot is less noisy since the plot is normalized to the more abundant betamethasone component rather than the trace endogenous cortisol.

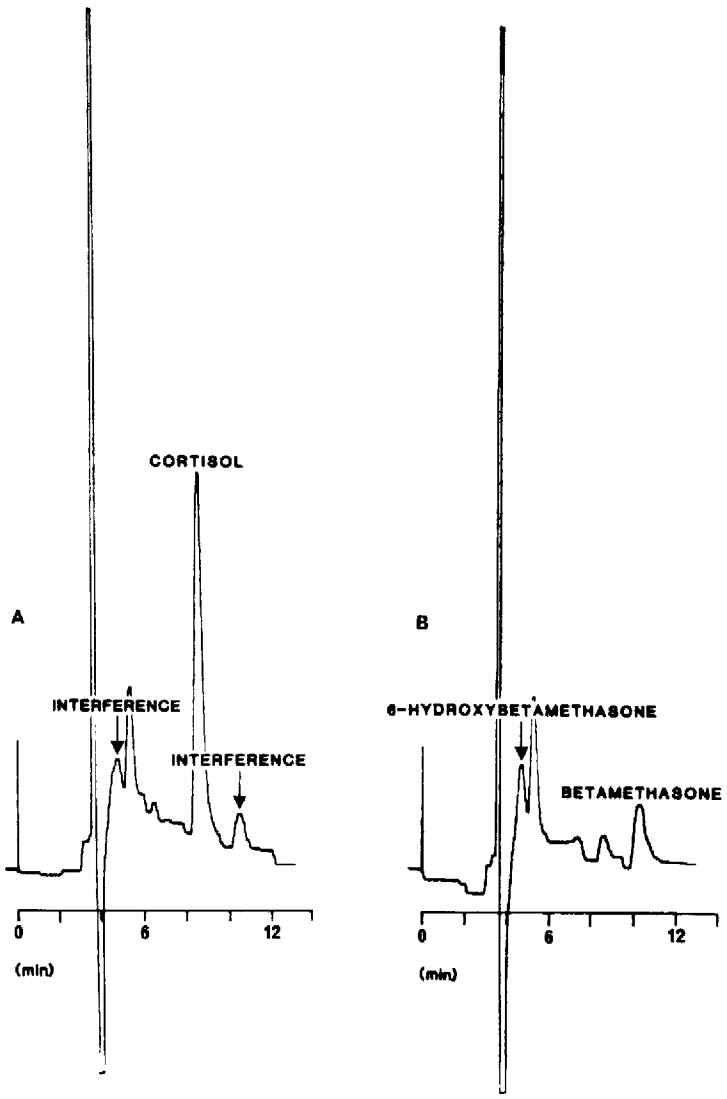


Fig. 4. Micro-LC–UV chromatograms of (A) control plasma extract and (B) betamethasone administration 2 h plasma extract obtained with a Whatman Partisil 10 ODS-3 microbore column (25 cm × 1 mm I.D.) and methanol–water (70:30) at 40 μl/min.

Nevertheless, the chosen mass range and NCI mode circumvent interference while still producing useful mass spectra.

Fig. 5C is the NCI–TIC profile of dexamethasone in equine plasma. There are no detectable interferences; endogenous cortisol is detected as a small component just prior to dexamethasone.

The second way in which the mass spectrometer can circumvent interference from unresolved components by micro-LC alone is selected ion profiling. This approach becomes useful when an extract contains endogenous products which interfere

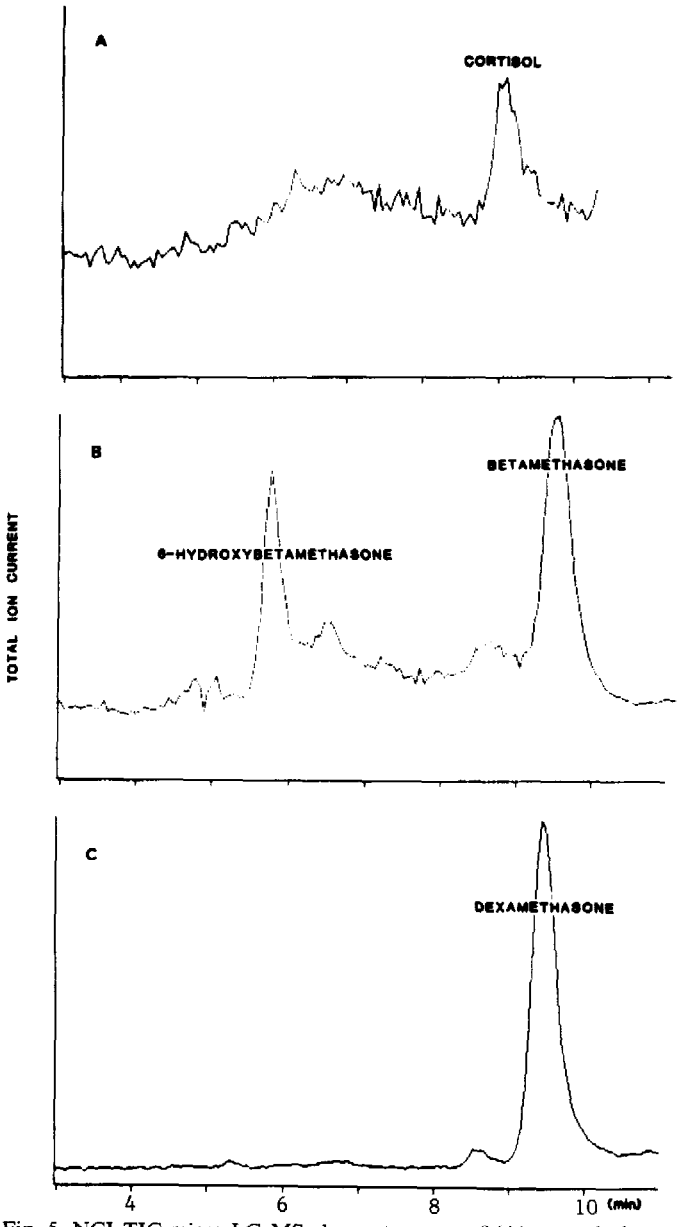


Fig. 5. NCI-TIC micro-LC–MS chromatograms of (A) control plasma extract, (B) betamethasone administration 2 h plasma extract and (C) dexamethasone administration 0.5 h plasma extract obtained with a Whatman Partisil 10 ODS-3 microbore column (25 cm × 1 mm I.D.) and methanol–water (70:30) at 40 μl/min.

with the drugs of interest within the scanned mass range. Raw extracts obtained from equine urine are such samples that necessitate selected ion profiling. Figure 6A is the NCI–TIC of a raw post-betamethasone administration urine extract. In this chromatogram the corresponding peak for 6β-hydroxybetamethasone is masked by large

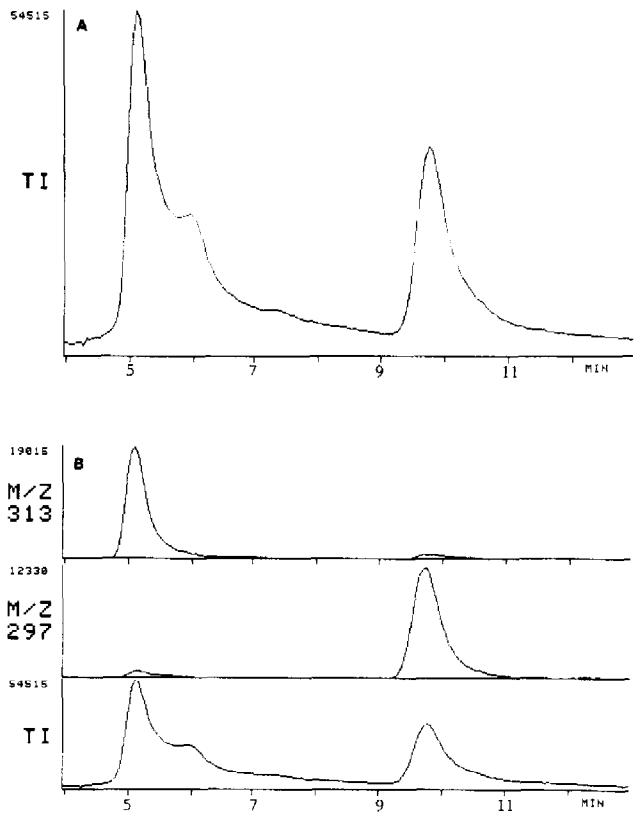


Fig. 6. NCI–TIC (A) and NCI–selected ion profile (B) micro-LC–MS chromatograms of betamethasone administration 2 h urine extract obtained with a Whatman Partisil 10 ODS-3 microbore column (25 cm × 1 mm I.D.) and methanol–water (70:30) at 40 μl/min. 6β-Hydroxybetamethasone = *m/z* 313; betamethasone = *m/z* 297.

amounts of endogenous material, yet through selected ion profiling (Fig. 6B) of the corresponding base peaks, both betamethasone (*m/z* 297) and 6β-hydroxybetamethasone (*m/z* 313) peaks are easily detected in a situation where conventional UV or fluorescence micro-LC detectors would not provide such information. When the background substraction computer program is utilized one can often obtain a satisfactory micro-LC–MS mass spectrum of the component of interest.

A final advantage derived through micro-LC–MS and selected ion profiling is illustrated in Fig. 7. The NCI–TIC profile of pooled post-administration 6α-methylprednisolone urine extract (Fig. 7B) does not indicate the drug's presence even though endogenous interference in this area of the chromatogram is not apparent. Selected ion profiling enables us to extract the diagnostic ion, *m/z* 356 (Fig. 7A), which together with corroborating chromatographic data substantiate the determination of 6α-methylprednisolone. The mass spectrum of the chromatographic peak (Fig. 7C) confirms the drug's identity.

Above data illustrate the specificity and sensitivity of micro-LC–MS in qualitative studies yet the technique's versatility further affords its application to difficult

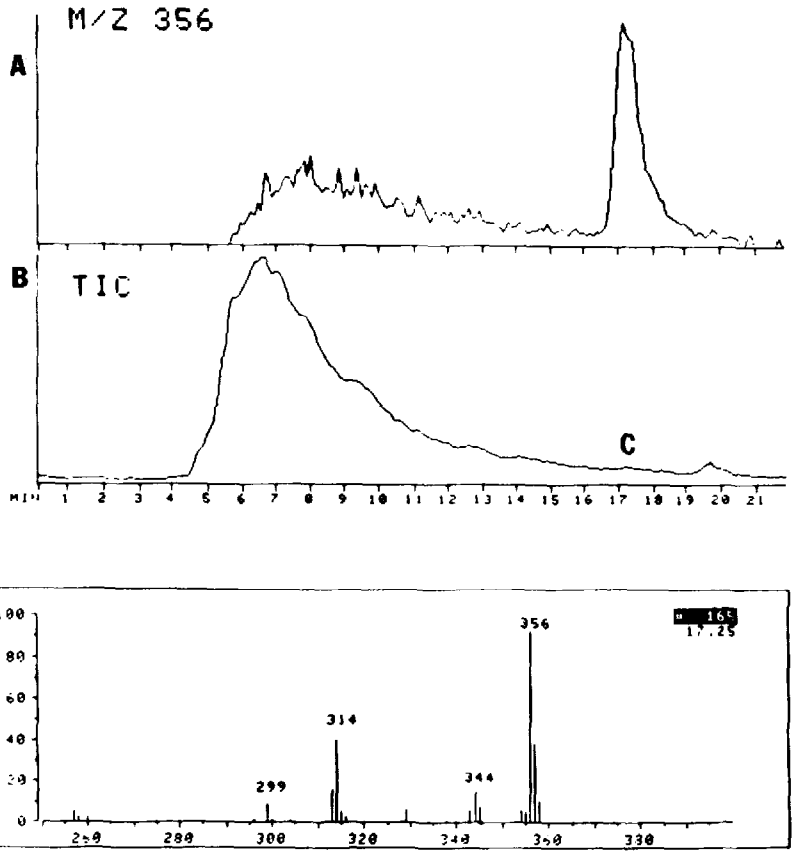


Fig. 7. NCI-selected ion profile (A) and NCI-TIC (B) micro-LC–MS chromatograms of 6α-methylprednisolone administration pooled urine extract obtained with a Whatman Partisil 10 ODS-3 microbore column (30 cm × 1 mm I.D.) methanol–water (65:35) at 40 μl/min. (C) Mass spectrum of 6α-methylprednisolone peak (*m/z* 356) in A.

studies such as determination of relative corticosteroid concentration in biological fluid. Parent drugs are the targets of most drug screens, but without knowledge of their relative concentrations to known metabolite(s) such screening directives can lack effectiveness. This is especially true when a metabolite is the predominant product within a sample and is sufficiently different from the parent drug as to produce seemingly unrelated data during sample analysis. If metabolites are overlooked while testing for a parent drug that may be below detectable levels, the drug administration can go undetected. Although it is possible to direct forensic determinations toward both drugs and their various metabolites, more efficient determinations target only the product thought predominant for a given administration.

To determine the predominant product of a drug administration one first needs to identify the products of interest. These products are then followed over time to determine and compare their abundance in the given biological fluid.

Fig. 8 shows the structures and NCI micro-LC–MS mass spectra of dexamethasone and its major equine urinary metabolite. Their TLC data are given in

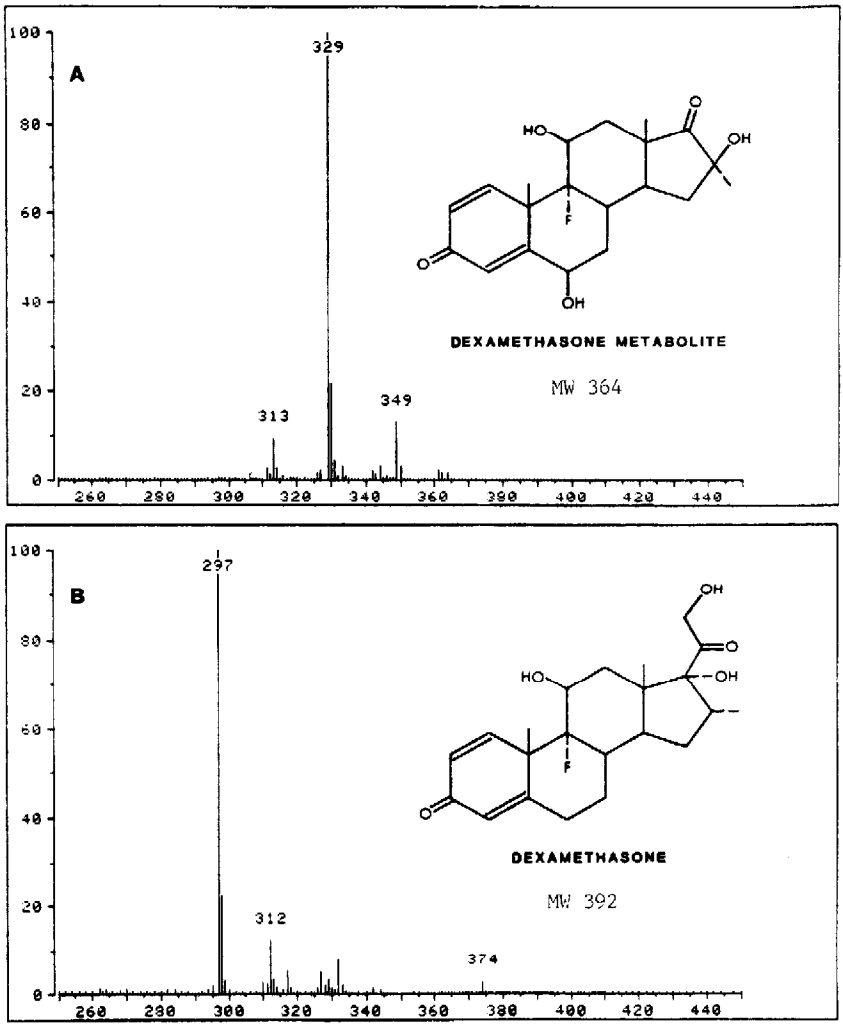


Fig. 8. Structures and NCI micro-LC–MS mass spectra of (A) dexamethasone metabolite and (B) dexamethasone obtained with water–methanol (70:30) as micro-LC eluent and NCI reagent gas.

TABLE III

THIN-LAYER CHROMATOGRAPHIC CHARACTERISTICS OF DEXAMETHASONE AND DEXAMETHASONE METABOLITE

Solvent systems as in Table I.

Compound	R_F *in solvent system*		
	A	*B*	*C*
Dexamethasone	0.03	0.34	0.70
Dexamethasone metabolite	0.01	0.27	0.47

Table III. These comparisons illustrate numerous structural and data inequities between two compounds of the same drug administration. One can see that without proper knowledge of these compounds the detection of this metabolite may not be associated with administration of the parent drug.

The NCI–TIC profile and pertinent selected ion profiles of a typical dexamethasone administration extract are shown in Fig. 9. These chromatograms illustrate the ability of micro-LC–MS to specifically target components of interest, thus affording comparison of parent drug and metabolite within a single sample.

The relative concentrations of dexamethasone and its major urinary metabolite are shown in Table IV. Data were derived from selected ion profiles of the base peaks and respective abundances for dexamethasone (*m/z* 297) and its metabolite (*m/z* 329). As expected dexamethasone is the predominant urinary product shortly following administration. This dominance is most exemplified in the first post-administration hour where the average detectable parent drug–metabolite ratio is in excess of 25:1 (*i.e.* 1.000:0.038). Nevertheless, upon carefully studying Table IV, one becomes cognizant of just how rapidly the predominance of equine urinary products change.

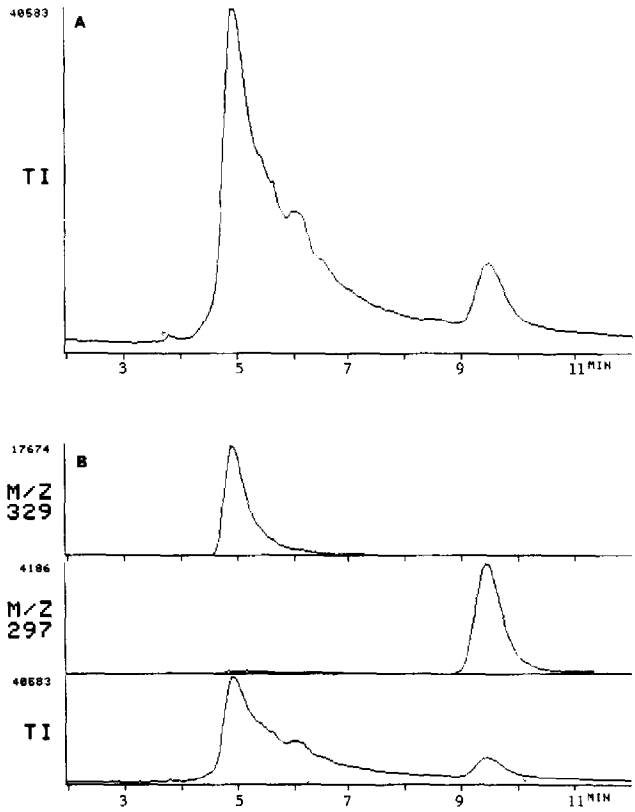


Fig. 9. NCI–TIC (A) and NCI-selected ion profile (B) micro-LC–MS chromatograms of typical dexamethasone administration (4 h) urine extract obtained with a Whatman Partisil 10 ODS-3 microbore column (25 cm × 1 mm I.D.) and methanol–water (70:30) at 40 μl/min. Dexamethasone metabolite = *m/z* 329; dexamethasone = *m/z* 297.

TABLE IV

RELATIVE CONCENTRATIONS OF DEXAMETHASONE METABOLITE/DEXAMETHASONE

$n = 3$.

Post-administration hour	*Relative concentration of dexamethasone metabolite (m/z 329)/ dexamethasone (m/z 297) ± S.E.*
1	0.038 ± 0.013
2	0.182 ± 0.072
3	0.493 ± 0.281
4	1.982 ± 1.125
5	3.380 ± 1.935
6	5.619 ± 2.684

Most striking is the change which takes place between the third and fourth hours following dexamethasone administration. During this time the dexamethasone metabolite assumes predominance and within the next two hours attains an average relative concentration to parent drug of nearly 150 times its first hour measurements (*i.e.* 5.619:0.038). By this post-administration time a clear pattern has been established and dexamethasone metabolite rather than parent drug is the major urinary constituent for determination.

CONCLUSION

Determinations of corticosteroids in biological fluids require sensitive and selective techniques capable of unequivocal identification. Many sensitive indirect techniques such as radioimmunoassay and competitive protein binding lack sufficient specificity while other direct methods utilizing non-specific detectors are unable to unequivocally identify particular drugs. Micro-LC–MS affords both sensitivity and selectivity to corticosteroid determination through combined techniques which compliment one another. Micro-LC resolves multiple corticosteroids and in turn delivers them intact to the mass spectrometer without derivatization. The mass spectrometer analyzes the samples and with its associated computer system eliminates or ignores interferences which are unavoidable with conventional liquid chromatographic analysis.

The qualitative determinations shown herein illustrate the ability of micro-LC–MS to deal with endogenous interferences as well as selectively profile diagnostic ions of interest in both plasma and urine administration extracts. Furthermore, the sensitivity of micro-LC–MS is demonstrated through its detection of endogenous plasma cortisol and minute amounts of administered 6α-methylprednisolone. Yet the sensitivity, specificity, and versatility of micro-LC–MS permits more wide ranging applications of the technique. One such application is the relative concentration determination of corticosteroids in biological fluids. By determining the relative concentrations of metabolite to parent drug over time, micro-LC–MS enables forensic chemists to better decide which component in a given biological fluid should be targeted under limiting circumstances. This affords better cost-effectiveness in drug screening and determinations.

As shown, the equine metabolism of dexamethasone is quite rapid, and consequently the relative concentration of dexamethasone metabolite rapidly increases in the urine. Predominance of the metabolite seen shortly after administration illustrates the need to target screenings and determinations toward the metabolite. The use of micro-LC–MS for the comparison of relative drug concentrations in biological fluids demonstrates the technique's versatility. And although the full capabilities of micro-LC–MS are as of yet unknown, its usefulness in difficult drug determinations, such as corticosteroids, is evident and should gain popularity with time and proven effectiveness. Inevitably the technique will find still other applications in drug determinations.

ACKNOWLEDGEMENTS

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