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Determination of dexamethasone in bovine tissues by coupled-column normal-phase high-performance liquid chromatography and capillary gas chromatography–mass spectrometry

LEE G. McLAUGHLIN and JACK D. HENION*

Drug Testing and Toxicology, NYS College of Veterinary Medicine, Cornell University, 925 Warren Drive, Ithaca, NY 14850 (U.S.A.)

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ABSTRACT

A new method for the determination of dexamethasone in bovine liver and muscle tissues has been developed. Crude tissue extracts were obtained by means of a three-phase liquid–liquid extraction scheme. The resulting residue was subjected to coupled-column normal-phase high-performance liquid chromatography which served to isolate the drug for the purposes of screening and quantification. Sample was injected onto the first column of the system, a phenyl column, from which a heart-cut was diverted to a short silica column which retained dexamethasone. The contents of this column were backflushed onto a cyanopropyl column which isolated dexamethasone. Mobile phases consisted of hexane modified with 2-propanol, acetic acid, and water. Analysis of each sample was completed in 15 min. Quantitation was performed by external standard calibration of ultraviolet response at 239 nm. Limits of detection were estimated to be 4 and 6 ppb in muscle and liver, respectively. In addition to screening and quantitation, the coupled-column system purified tissue extracts for gas chromatographic–mass spectrometric analysis which, in the selected-ion monitoring mode, confirmed the identity of the trimethylsilyl-enol-trimethylsilyl derivative of dexamethasone.

INTRODUCTION

Dexamethasone (9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione, Fig. 1) is a synthetic glucocorticosteroid used therapeutically

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in cattle to treat inflammation and primary ketosis, to induce parturition, and in conjunction with antimicrobial therapy to alleviate stress [1]. Since 1975 it has been approved for use in cattle intended for human consumption when prescribed by a licensed veterinarian [2]. Approved daily doses range from 5 to 20 mg for intravenous or intramuscular injection and 5 to 10 mg for oral administration [1]. No tolerance levels have yet been established for dexamethasone and no federal monitoring program exists. The purpose of this investigation was two-fold. Firstly, information was needed on bovine tissue levels following therapeutic administration. Secondly, the United States Department of Agriculture (USDA) sought a potential regulatory method capable of screening and quantifying dexamethasone in bovine liver and muscle below 100 ppb with confirmation by gas chromatography–mass spectrometry (GC–MS). This method is believed to be the first for determining dexamethasone in bovine liver and muscle tissues.

The approach to this work was based on a coupled-column normal-phase high-performance liquid chromatographic (HPLC) method developed to screen and quantify the anabolic steroid, melengestrol acetate (MGA), also in edible bovine tissues [3]. The method employed three HPLC columns coupled together for sample clean-up, concentration of analyte, and analytical separation prior to ultraviolet (UV) detection. Analyte, along with co-eluting matrix components, was heart-cut from the first, phenyl-silica column and collected on a second, silica column. The second column was then backflushed with a stronger eluent onto the third, silica column where the analyte was finally resolved from matrix components. This method proved to be a rugged, automated means of isolating MGA from these complex matrices. Many other examples of the utility of coupled-column chromatography for trace analysis of complex biological matrices have been noted in ref. 3. Alternative to this coupled-column approach to isolating dexamethasone, preliminary work showed that two solid-phase extraction (SPE) steps followed by separation on a single HPLC column were needed to isolate dexamethasone. The coupled-column approach replaces the SPE steps with automated, on-line sample clean-up. It is, therefore, more attractive for analyses involving many samples because it minimizes manual procedures and the accompanying sample handling losses [4]. However, the

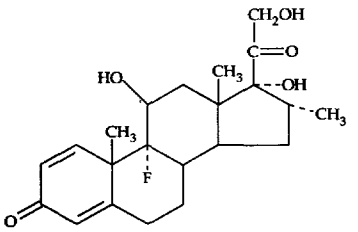

Fig. 1. Structure of dexamethasone.

coupled-column technique is limited to serial analyses instead of the parallel, multi-sample analyses possible by SPE.

Although matrices in this work are the same as those in the MGA study, the analytes exhibit very different chemical properties, dexamethasone being considerably more polar and having UV absorption at a shorter and less favorable wavelength compared with MGA. It was thus apparent that identical chromatographic conditions could not be used, precluding simultaneous determination of dexamethasone and MGA by the MGA method. Initially, the use of stronger mobile phases with the same three columns used for MGA was investigated, but the silica analytical column did not have the requisite selectivity for dexamethasone relative to other compounds in the phenyl column heart-cut. However, for regulatory convenience the dexamethasone method was kept as similar as possible to the MGA method so that a laboratory might easily switch between analyses. In this work, coupled-column HPLC conditions were developed to isolate dexamethasone from bovine tissue matrices for quantitation based on external standard calibration of UV response and for collection for GC–MS confirmation.

EXPERIMENTAL

Materials

Analytical standard of dexamethasone was a product of Sigma (St. Louis, MO, U.S.A.), as was the β-glucuronidase Type H-2S. Dichloromethane was purchased from EM Science (Gibbstown, NJ, U.S.A.). Glacial acetic acid was reagent grade from Fisher Scientific (Rochester, NY, U.S.A.). Acetone and pyridine were Fisher Scientific ACS-certified; the pyridine was redistilled at 760 Torr from potassium hydroxide pellets in-house. All other solvents and the sodium acetate trihydrate were Fisher Scientific HPLC grade, N,O-Bis(trimethylsilyl)acetamide (BSA) was obtained from Pierce (Rockford, IL, U.S.A.). [1,2,4-^{3}H]Dexamethasone used to determine extraction recovery was purchased from Amersham (Arlington Heights, IL, U.S.A.). The ScintiVerse II scintillation fluid was a product of Fisher Scientific.

Administration

Animal dosing was performed by the Food and Drug Administration Center for Veterinary Medicine. Two steers weighing between 227 and 454 kg received intravenous doses of 5 and 20 mg per day, respectively, for a total of seven days and were slaughtered 24 h after the last dose was administered. The resulting incurred liver and hindquarter muscle tissues were collected, homogenized, and stored at $-20\,^{\circ}$C for subsequent analysis.

Tissue homogenization and enzymatic hydrolysis

Control liver and hindquarter muscle tissues were purchased locally and homogenized in 500-g batches for 2 min at high speed in a Waring Blendor (VWR Scientific, Rochester, NY, U.S.A.). In 50-ml polypropylene centrifuge tubes, 5.0 g of tissue homogenate were vortex-mixed with 10 ml of a 0.04 *M* aqueous sodium acetate solution for 30 s. The pH of the mixture was brought to between 4.2 and 4.7 by adding approximately 75 μl of glacial acetic acid. Fortification of control tissues used for external standard calibration was done at this point. Then, 100 μl of β-glucuronidase (133 000 U/ml glucuronidase activity and 1000–5000 U/ml sulfatase activity) were added and the tube contents mixed by rotation (7 rotations per min) for 3 min and incubated with gentle shaking for 8 h in a 37°C water bath.

Tissue extraction

Following 8 h of hydrolysis, 20 ml of acetonitrile were added to each sample. Liver samples were then mixed by rotation (7 rotations per min) for 10 min and centrifuged at 5320 *g* for 10 min. Muscle samples were mixed by rotation (35 rotations per min) for 30 min and vortex-mixed for 30 s followed by centrifugation at 6570 *g* for 30 min. Resulting supernatant (29 ml) was decanted into glass screw-cap test-tubes (150 mm × 25 mm I.D.). To this, 8 ml of hexane and 2 ml of dichloromethane were added, mixed by rotation (7 rotations per min) for 3 min, and centrifuged at 1780 *g* for 4 min. This procedure produced a three-phase liquid system in which dexamethasone partitioned into the middle layer, composed mainly of acetonitrile [5]. From this middle layer with a volume of 17 ml, 15 ml were removed and placed in a 20-ml scintillation vial. Evaporation under nitrogen in a 60°C heater block yielded an oily residue weighing approximately 40 mg from liver and approximately 20 mg from muscle.

These liver and muscle extracts were dissolved in 500 and 250 μl of column 1 mobile phase, respectively, sonicated for 2 min to ensure dissolution, and filtered through 0.45-μm Microfilterfuge™ tubes (Rainin, Woburn, MA, U.S.A.). The filtrates (50 μl) were then subjected to HPLC analysis.

Determination of recovery

Recovery of dexamethasone from tissue homogenate was determined using a radiolabelled isotope of dexamethasone: [1,2,4-^{3}H]dexamethasone. Purity of the radioisotope standard was found to be 85% with a Vanguard radiochromatogram scanner (Digital Diagnostics, Hamden, CT, U.S.A.). Control tissues were spiked with [1,2,4-^{3}H]dexamethasone standard with a specific activity of $1.87 \cdot 10^5$ disintegrations per min (dpm) and the amount of unlabelled dexamethasone needed to obtain tissue concentrations of 8, 50, and 10 ppb before undergoing the tissue extraction procedure. Extract of control tissue spiked with $1.87 \cdot 10^5$ dpm [1,2,4-^{3}H]dexamethasone just prior to liquid scintillation counting served as a standard. Extracts were dissolved in 2 ml of

methanol and 18 ml of ScintiVerse II fluid, and activity was measured by quench-corrected liquid scintillation counting in a Beckman 6800 liquid scintillation counter (Irvine, CA, U.S.A.). The standard and the 8-, 50- and 100-ppb samples were prepared in triplicate in both liver and muscle. Activity counts (dpm) thus obtained were compared to counts from standard to find percentage remaining after extraction procedure. Appropriate controls to assess chemiluminescence and quench errors were also analyzed.

Chromatographic system

The system hardware and configuration were the same as that described previously [3]. System configuration is shown in Fig. 2. Samples were injected onto the first HPLC column using a Model ISS-100 autosampler with a 50-μl sample loop (Perkin Elmer, Norwalk, CT, U.S.A.). Column switching between three columns was accomplished with two Waters pneumatic switching valves (Waters Assoc., Milford, MA, U.S.A.). The three HPLC pumps which delivered mobile phases for the separations on columns 1 and 3 and for the regeneration of column 1 were Waters Models 510 and 6000A. A Waters Model 440

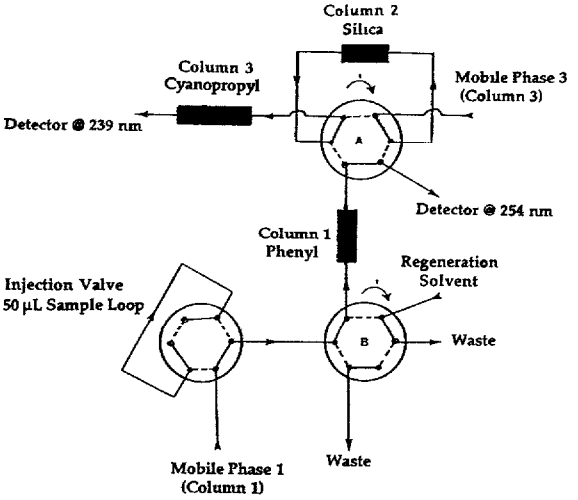

Fig. 2. Diagram of coupled-column system. With valves A and B in position 2, as shown above, sample is injected onto the phenyl column (column 1) where the separation using mobile phase 1 begins. When valve A is switched to position 1, phenyl column effluent is re-routed to the silica column (column 2) where dexamethasone in the effluent is retained. When collection of dexamethasone is complete, valve A returns to position 2 causing the contents of column 2 to be backflushed onto the cyanopropyl column (column 3) by mobile phase 3, for the final separation. While the separation on column 3 proceeds, valve B switches to position 1 and the regeneration solvent elutes retained matrix components from the phenyl column. After 7 min, valve B returns to position 2 to allow the phenyl column to equilibrate in mobile phase 1 for 4 min before the next injection.

UV absorbance detector operated at 254 nm was used to monitor the retention time of dexamethasone on column 1. Detection of dexamethasone isolated on the third column was accomplished with a variable-wavelength UV detector operated at 239 nm (Kratos Analytical Model 783, Ramsey, NJ, U.S.A.). Columns 1 and 3 were housed in a Waters temperature control module at 30°C. The integrator was a Hewlett-Packard Model 3390A (Avondale, PA, U.S.A.). A Waters Model 680 automated gradient controller was used to program pump, switching valve, and integrator functions.

Columns used were as follows: column 1 was a 50 mm×4.6 mm I.D., 3-μm Spherisorb phenyl-silica of Lot No. 23/163 packed by Keystone Scientific (State College, PA, U.S.A.); column 2 was a 12.5 mm×4 mm I.D., 5-μm silica packed by Mac-Mod Analytical (Chadds Ford, PA, U.S.A.); column 3 was a 100 mm×4.6 mm, 3-μm Spherisorb cyanopropyl-silica packed in-house. A column packed commercially by Keystone with 3-μm Spherisorb cyanopropyl-silica provided the same separation with slight adjustment of mobile phase strength. Note that columns 1 and 2, phenyl and silica, are the same types used in the MGA method.

Mobile phases were necessarily stronger normal-phase eluents than those used to elute MGA from the phenyl and silica columns. The compositions of mobile phase for column 1 (mobile phase 1) and column 3 (mobile phase 3) were water–acetic acid–2-propanol–hexane, 0.1:0.1:5.8:94 and 0.1:0.1:12.8:87 (v/v), respectively. The flow-rate for columns 1 and 3 was 1.5 ml/min. Mobile phase for regeneration of column 1 was methanol–dichloromethane–water, 49:49:2 (v/v) used at a flow-rate of 2.0 ml/min. All mobile phases were kept in capped reservoirs and sparged with helium for several minutes before use.

The retention time of dexamethasone on column 1 was checked at the beginning of each day by injecting 10–20 ng of dexamethasone standard dissolved

TABLE I

COLUMN-SWITCHING TIMETABLE

Time (min)	Switching valve A	Switching valve B	Event
0	Position 2	Position 2	Sample injection
3.2	Position 1		Begin collection of dexamethasone on column 2
4.1	Position 2		End collection; begin separation on column 3; start integrator monitoring of column 3
4.2		Position 1	Begin regeneration of column 1
11.2		Position 2	End regeneration and begin equilibration of column 1
15.2	Position 2	Position 2	End equilibration; inject next sample

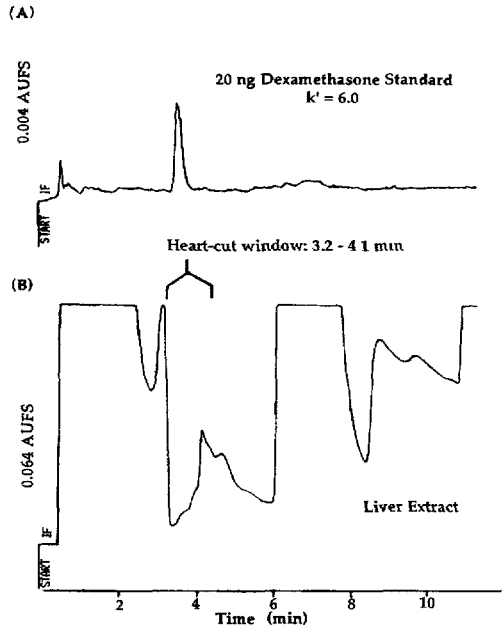

Fig. 3. HPLC–UV profiles from the phenyl column (column 1) using mobile phase 1 at a flow-rate of 1.5 ml/min, monitored at 254 nm. (A) Injection of 20 ng of dexamethasone standard: performed at the start of each analysis to determine heart-cut window needed for quantitative transfer of dexamethasone, usually 3.2–4.1 min. (B) Injection of liver extract sample: depicts heart-cut window where phenyl column eluate, containing any dexamethasone along with co-eluting matrix components, was transferred to column 2.

in mobile phase 1. The switching valve timing was then programmed to ensure quantitative collection of dexamethasone. The heart-cut window was made slightly wider than the baseline peak width of dexamethasone standard to accommodate effects matrix might have on retention time and peak width of dexamethasone, as well as any slight variation in retention time between samples. The k' of dexamethasone on column 1 was typically 6.0, and the heart-cut window was 0.9 min wide. A smaller k' on column 1 did not provide enough separation of dexamethasone from matrix to enable resolution of dexamethasone on the final column. Table I outlines the column-switching program and Fig. 3 illustrates the heart-cutting process.

Quantitation

Quantitation was accomplished by generating an external standard calibration curve using control tissue fortified with 100 μl of appropriate solutions of dexamethasone standard in ethyl acetate to obtain desired concentrations for the standard curve, just prior to addition of β-glucuronidase. These samples were processed along with the incurred samples to be analyzed, all by the pro-

cedure described above. For quantitation of incurred liver tissues, fortified levels used as external standards were 0, 20, 40, 80, and 100 ppb. Quantitation of incurred muscle below 10 ppb was attempted using fortified levels of 0, 3, 5, and 10 ppb. Integrator peak heights from duplicate injections of fortified samples were averaged to establish the calibration curve. Linear least-squares regression was then used to predict the concentration of dexamethasone in incurred samples from peak-height data.

Preparation of trimethylsilyl (TMS)-enol-TMS derivative of dexamethasone

Dexamethasone purified by coupled-column HPLC was collected in a conical 5-ml screw-cap test tube for 0.4 min before and after its retention time on the cyanopropyl column and stored at −20°C for no longer than 24 h before undergoing the following derivatization procedure [6]. The collected eluate was evaporated to dryness under nitrogen in a 60°C heater block, and 10 μl of a sodium acetate–methanol solution (1 μg/μl) were added, vortex-mixed for 10 s, and again evaporated to dryness. Next, 50 μl of acetone were added, vortex-mixed, and evaporated to dryness. BSA (5 μl) and 10 μl of pyridine were added and vortex-mixed. Vials were then capped and heated in a 90°C heater block for 100 min. Following the reaction the tubes were allowed to cool to room temperature, condensation inside tubes was rinsed with approximately 50 μl of cyclohexane, and the liquid was evaporated. Cyclohexane (10 μl) was then added, vortex-mixed, and evaporated to dryness. For storage at −20°C, 100 μl of cyclohexane were added to each tube. For GC–MS analysis, samples were evaporated and redissolved in 3 μl of cyclohexane, all of which was injected. The derivative was found to be stable for at least six months of storage at −20°C. Confirmatory results, however, were obtained no longer than four days after derivatization.

Parameters for selected-ion monitoring (SIM)

A full-scan electron ioniation (EI) mass spectrum of dexamethasone TMS-enol-TMS obtained by GC–MS is shown in Fig. 4. The ions chosen for SIM were the molecular ion at 680 daltons (Da) and three characteristic fragment ions at 345, 332, and 305 Da. For confirmation by GC–MS, these four ions were monitored for an 8-min period of the temperature program using a 100-ms dwell time for each ion. Table II lists the exact masses and elemental composition of the four ions obtained by full-scan high-resolution MS on a Kratos MS890 at a resolution of 10 000.

GC–MS instrumentation and operating conditions

GC–MS analysis was done using a Carlo Erba Model 5160 Mega Series gas chromatograph directly interfaced to a Hewlett-Packard 5970 Series mass-selective detector. The data system was a Hewlett-Packard 59970C Chem-Station. The injector was an on-column Model OC1-3 from SGE (Austin, TX,

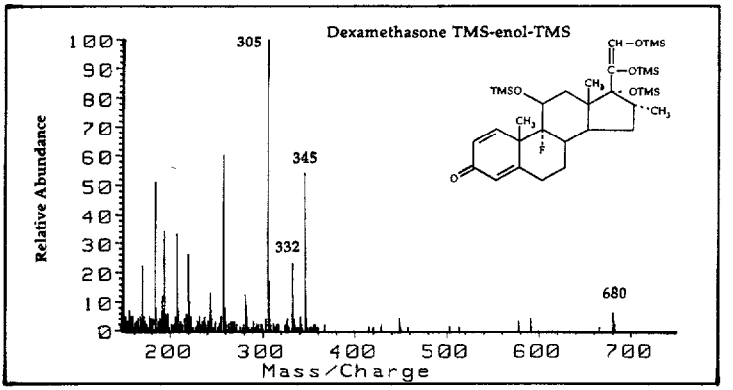

Fig. 4. Full-scan electron-impact spectrum of dexamethasone TMS-enol-TMS (40 ng, 150–170 a.m.u. in 0.7 s). The four ions used for confirmation by SIM are labelled.

TABLE II

HIGH-RESOLUTION MS ACCURATE MASS MEASUREMENT OF DEXAMETHASONE TMS-ENOL-TMS IONS MONITORED BY SIM

m/z	Measured mass	Calculated mass	Elemental composition	Error (m.m.u.)
305	305.1433	305.1425	$C_{12}H_{29}O_3Si_3$	0.89
332	332.1648	332.1659	$C_{14}H_{32}O_3Si_3$	−1.14
345	345.1732	345.1738	$C_{15}H_{33}O_3Si_3$	−0.57
680	680.3579	680.3580	$C_{34}H_{61}FO_5Si_4$	−0.11

U.S.A.). The GC capillary column was a J&W Scientific (Folsom, CA, U.S.A.) DB-1, 15 m×0.25 mm I.D., with 0.1 μm film thickness. The interface temperature was held at 280°C. On-column injections were made with the oven at 60°C. After injection, the oven temperature was held at 60°C for 3 min, then increased to 285°C at 40°C/min and held for 10 min until dexamethasone TMS-enol-TMS eluted at approximately 17.5 min. The helium flow-rate was 47 cm/s, measured at 285°C. Mass spectrometer parameters were adjusted each day to meet decafluorotriphenylphosphine (DFTPP) specifications using 15 ng of DFTPP in 1 μl of ethyl acetate [7]. Electron energy was 70 eV.

RESULTS AND DISCUSSION

Tissue extraction

Since its inception, the three-phase extraction procedure has been used successfully to affect a fast, crude fractionation of tissue components and xenobiotic drugs extracted from tissue homogenate into the aqueous acetonitrile supernatant [3,5,8,9]. Non-polar components partition into the hexane layer

(8 ml), polar and ionic components partition into the bottom aqueous layer (14 ml), and dexamethasone along with other components of the tissue partition into the middle acetonitrile layer (17 ml) [5]. As Chichila et al. [3] note, the residue remaining after evaporation of the acetonitrile layer is readily soluble in normal-phase HPLC eluents. Normal-phase HPLC also has been shown to exhibit selectivity for corticosteroid separations superior to that of reversed-phase methods [10].

Recovery of dexamethasone from tissue homogenates was determined using a radiolabelled isotope of dexamethasone. Recovery from liver tissue was found to be 67.4%, the average result of the 8, 50, and 100 ppb concentrations tested. When muscle tissue was processed with the same shaking and centrifugation parameters used for liver, recovery of [1,2,4-^{3}H]dexamethasone was unacceptably low, approximately 30%. When longer and faster shaking and centrifugation were used, recovery of [1,2,4-^{3}H]dexamethasone from muscle was increased to an average value of 73.6% over the three concentrations studied. Neither liver nor muscle showed a significant difference in recovery between the 8, 50 and 100 ppb concentrations tested. Recovery results are shown in Table III. These values characterize absolute recovery of tissue extraction and the three-phase liquid extraction uncompensated for phase transfer loss, and only to the point in the analysis of obtaining residue in the scintillation vial. Losses during filtration prior to HPLC injection were checked separately and found to be insignificant. Losses to the coupled-column chromatographic system before isolation of dexamethasone on the last column were negligible.

Possible discrepancies arising from the quantitation of endogenous dexamethasone with standards consisting of dexamethasone spiked into tissue include the cellular location of endogenous drug and the efficacy of the 2-min

TABLE III

EXTRACTION RECOVERY OF [1,2,4-^{3}H]DEXAMETHASONE FROM BOVINE TISSUES

Concentration (ppb)	n	Recovery[a] (mean ± S.D.) (%)	C.V. (%)
Bovine muscle			
8	3	72.1 ± 0.6	0.8
50	3	73.7 ± 2.3	3.2
100	3	74.9 ± 3.6	4.9
Bovine liver			
8	3	68.2 ± 2.2	3.2
50	3	67.7 ± 2.1	3.1
100	3	66.2 ± 3.8	5.8

[a]Not compensated for phase transfer loss during extraction; 15 ml of the 17-ml middle layer were used.

homogenization of tissue, the potential inability of the enzyme to hydrolyze conjugates other than glucuronides and sulfates, and the efficiency of enzyme hydrolysis of the glucuronide and sulfate conjugates. Efficiency of enzyme hydrolysis was not assessed here. However, Covey [11] has found efficiency of β-glucuronidase hydrolysis to exceed 90% for several different steroid-glucuronide substrates in several different tissue matrices and thus concluded that tissue matrix does not significantly inhibit the reaction.

Liquid chromatography

As previously indicated, the system used to isolate another steroid was modified for the determination of dexamethasone in the same matrices, bovine liver and muscle. The final silica column which selectively isolated MGA was ineffective for isolation of dexamethasone using the 2-propanol–hexane mobile phases and the same first and second columns. Instead, a cyanopropyl-silica column was found to exhibit the necessary selectivity for dexamethasone

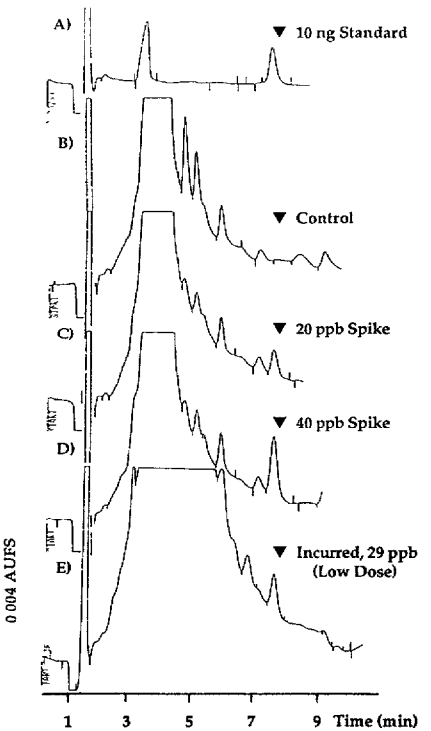

Fig. 5. LC–LC–UV profiles of dexamethasone standard (A) and liver extracts (B–E) on the cyanopropyl column (column 3) using mobile phase 3 at a flow-rate of 1.5 ml/min, monitored at 239 nm. Incurred liver (E) is from an animal administered the low dose of 5 mg per day for seven days. The peak at 3.5 min in (A) is a contaminant from the coupled-column system which did not interfere with the assay.

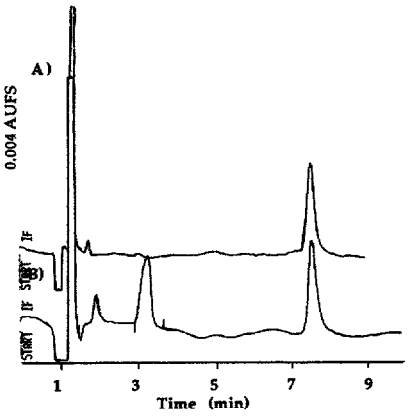

Fig. 6. HPLC–UV profiles of dexamethasone standard (20 ng) on the cyanopropyl column (column 3) comparing peak widths without column-switching (A) and with column-switching (B). Both chromatograms were obtained using mobile phase 3 at a flow-rate of 1.5 ml/min and monitored at 239 nm. The peak in (B) at 3.5 min is a contaminant from the coupled-column system.

relative to matrix. The selectivity of the final column should differ from that of the first column so that components collected along with dexamethasone in the heart-cut, because they have similar retention on column 1, may be separated from dexamethasone on the third column. The cyanopropyl stationary phase provides this useful difference in selectivity seen by comparing Fig. 3B and Fig. 5C–E.

In addition, columns must be chosen so that mobile phase 1 is a weaker eluent than mobile phase 3. This is required because the enrichment of analyte (peak compression) is achieved by eluent switching on column 2 [4]. The enrichment factor of the system, defined as k'_1/k'_2 where k'_1 and k'_2 are the analyte capacity factors on column 2 using mobile phase 1 and mobile phase 3, respectively [4], was found to be 5.8 for this system ($k'_1 = 29$ and $k'_2 = 5$). Column 2 performs several important functions. It allows analyte to be collected from column 1 in 1.35 ml of mobile phase 1, yet only 0.12 ml of mobile phase 1 is transferred to column 3. In this way, perturbations to the separation on column 3 by a foreign eluent are minimized [4]. With a k'_1 of 29, 3.15 ml of mobile phase 1 could be trapped on column 2 before breakthrough losses of dexamethasone would occur. Also, the small k' of dexamethasone on column 2 in mobile phase 3 ensures that the analyte is eluted onto column 3 as a sharp peak.

Regeneration of column 1 with a polar eluent was done here as in ref. 3 to rapidly elute highly retained components which will otherwise alter the column's efficiency and retention behavior. The retention time of analyte on column 1 must be reproducible so that it does not migrate outside the heart-cut window during an analysis. This retention time was checked every 2 h during

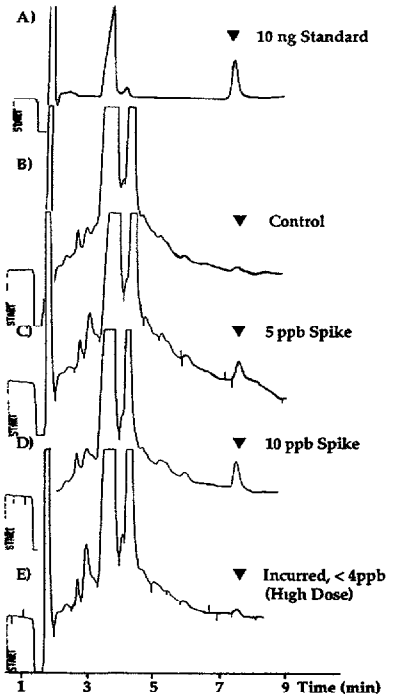

Fig. 7. LC–LC–UV profiles of dexamethasone standard (A) and muscle extracts (B–E) on the cyanopropyl column (column 3) using mobile phase 3 at a flow-rate of 1.5 ml/min, monitored at 239 nm. The concentration found in incurred muscle (E) resulted from the high dose of 20 mg per day for seven days as was below the limit of detection. The peak at 3.5 min in (A) is a contaminant from the coupled-column system which did not interfere with the assay.

an analysis by injecting dexamethasone standard, at which point the window may be reprogrammed. With a typical retention time reproducibility on column 1 of 3.56 ± 0.04 min (coefficient of variation, C.V. $= 1.1\%$, $n = 6$), reprogramming was not necessary. Over several weeks, the retention time of dexamethasone on column 1 gradually increased. Mobile phase strength was then increased as needed to maintain dexamethasone at approximately the same k'. Regeneration of the cyanopropyl column was done only occasionally when disturbances from chemical background or a decrease in column efficiency were noticed. The same eluent for regeneration of column 1 was used. Retention time reproducibility on column 3 was excellent. Typical for a day was an average retention time of 7.58 ± 0.02 min (C.V. $= 0.3\%$, $n = 22$).

Band-broadening due to column-switching was assessed as in ref. 3. The ratio of peak widths of dexamethasone on the third column with and without switching was found to be 1.008. In good agreement with this value is the slight 4.2% drop in theoretical plates per meter (from 53 969 to 51 705) with switch-

ing. Fig. 6 compares peak widths on column 3 with and without column switching.

Sample analysis

The incurred liver extracts were quantified by coupled-column HPLC on three different days. The average levels and standard deviations ($n=3$) found in liver from low- and high-dose animals were 29.2 ± 2.6 and 69.5 ± 3.1 ppb, respectively. Representative chromatograms are shown in Fig. 5. Excess background seen in the low-dose incurred sample is attributed to frozen incurred tissues having been stored for over a year. The same excess background was

TABLE IV

BLIND ANALYSIS STUDY: ACCURACY OF DEXAMETHASONE DETERMINATION IN BOVINE LIVER (SPIKED AND INCURRED)

Spiked/incurred concentration (ppb)	Concentration found (ppb)		Error (%)
Spiked			
0	N.D.[a]		0
0	N.D.[a]		0
0	N.D.[a]		0
30	37.6		25.3
30	36.0		20.0
30	31.3		4.3
60	64.5		7.5
60	64.1		6.8
60	66.1		10.2
90	88.5		−1.7
90	86.6		−3.8
90	84.1		−6.6
		Mean	6.9
		S.D.	10.6
		$n=9$	
Incurred			
29.2	59.8[b]		104.8[b]
29.2	30.3		3.8
29.2	29.0		−0.7
69.5	73.0		5.0
69.5	68.4		−1.6
69.5	62.3		−10.4
		Mean	−0.8
		S.D.	6.1
		$n=5$	

[a]N.D. = not detected; limit of detection is 6 ppb.

[b]Outlying value attributed to abnormally small volume of supernatant decanted prior to three-phase extraction for this sample; error excluded from mean error calculation.

TABLE V

ION PEAK-HEIGHT RATIO STATISTICS FOR DEXAMETHASONE TMS-ENOL-TMS

Sample	n	Ion peak-height ratio (mean ± S.D.)		
		m/z 305/680	m/z 332/680	m/z 345/680
Liver confirmation				
Standard, 7.5 ng	6[a]	6.102 ± 0.281	2.509 ± 0.213	3.914 ± 0.132
Extracts (incurred and spiked)	8[b]	6.371 ± 0.355	3.243 ± 1.054	4.002 ± 0.413
Muscle confirmation				
Standard, 10 ng	8[a]	5.466 ± 0.323	1.647 ± 0.371	3.635 ± 0.328
Extracts (incurred and spiked)	8[b]	5.444 ± 0.394	2.474 ± 0.900	3.815 ± 0.412

[a]Number of injections.
[b]Number of samples injected which met confirmation criteria.

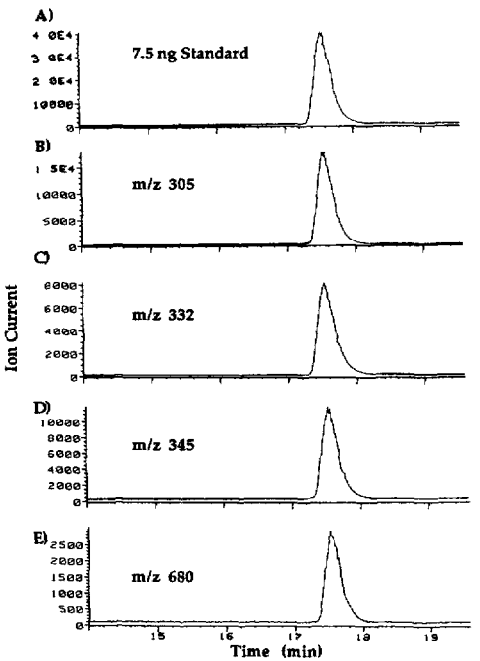

Fig. 8. GC–MS ion current profiles of 7.5 ng dexamethasone TMS-enol-TMS standard showing the total selected-ion current profile (A), and the selected-ion current profiles of each of the four ions monitored for confirmation (B–E).

seen for incurred liver from the high-dose animal. The average of linear regression correlation coefficients for the three days was 0.995 ± 0.001 and the average standard error of estimate for the three calibration curves was

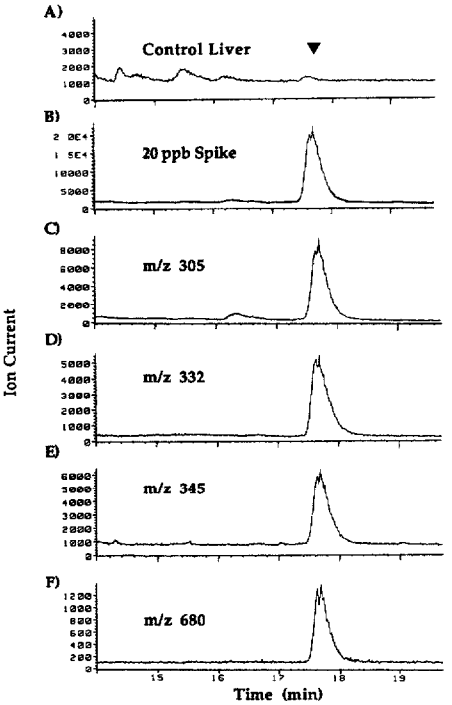

Fig. 9. GC–MS ion current profiles of control liver extract (A) and a 20-ppb liver spike (B–F). (A) and (B) are total selected-ion current profiles of dexamethasone TMS-enol-TMS and (C–F) are selected-ion current profiles of each of the four dexamethasone TMS-enol-TMS ions monitored for confirmation.

4.718 ± 0.280. Using the method of Glaser et al. [12], the limit of detection (LOD) was found to be 6 ppb in bovine liver by coupled-column HPLC.

Accurate quantitation of dexamethasone in muscle below 10 ppb was unsuccessful. The LOD in muscle was estimated to be 4 ppb. Both high- and low-dose incurred samples were below this level, but the presence of dexamethasone in the high-dose muscle extract was later confirmed by GC–MS. Fig. 7 shows representative HPLC patterns.

Blind study for method validation

As part of USDA protocol for regulatory method validation, a blind analysis was conducted for each tissue. For liver, three sets of six samples were analyzed on three different days. The identities of the six samples were unknown to the analyst. The six samples included the two incurred liver samples, a control liver, and liver fortified to 30, 60 and 90 ppb. Samples were analyzed as described above to assess method accuracy and precision. All samples were correctly identified and the results are given in Table IV. The same was done for

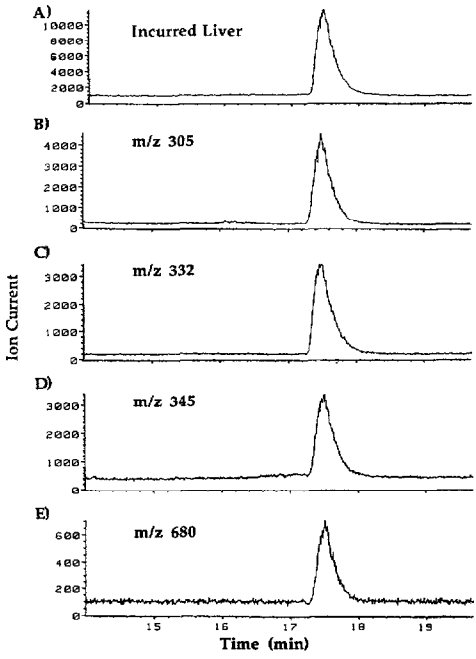

Fig. 10. GC–MS ion current profiles of incurred liver from low-dose animal, determined at the 29-ppb level by coupled-column HPLC. The total selected-ion current profile of dexamethasone TMS-enol-TMS is shown in (A). Selected-ion current profiles of the four dexamethasone TMS-enol-TMS ions monitored for confirmation are shown in (B–E).

muscle using two sets of five samples on two different days. Accuracy and precision of quantitation below 10 ppb were unsatisfactory and levels of 4, 5 and 8 ppb and were not correctly identified.

GC–MS confirmation of dexamethasone

Derivatization of dexamethasone yields a product with superior thermal stability, chromatographic behavior, and specificity of higher-mass ions compared to dexamethasone. Minagawa et al. [6] have found the TMS-enol-TMS derivatization procedure advantageous because, in addition to yielding a single, stable product, it does not require protection of ketone groups. Presence of dexamethasone TMS-enol-TMS in a sample was considered confirmed if its GC retention time was within ± 20 s of that of standard and if two out of three ion abundance ratios were within 20% of those of standard. Dexamethasone was confirmed in low-dose incurred liver extract from each of the three days of analysis, but not in the control samples, as expected. Table V lists ion ratio statistics of confirmed liver samples. Figs. 8–10 display representative ion chromatograms of a dexamethasone TMS-enol-TMS standard, control and spiked liver samples, and an incurred liver sample, respectively.

Although levels of dexamethasone in incurred muscle were below the LOD

of the coupled-column HPLC method, dexamethasone was confirmed in the high-dose muscle tissue from each of the three days of sample analysis. The presence of dexamethasone was also confirmed in control muscle. The origin of this dexamethasone in unknown. Table V includes ion ratio statistics from muscle confirmation.

CONCLUSION

The risk of false positives using this method should be low as a false positive must have a retention time on column 1 within 0.45 min of dexamethasone, must co-elute with dexamethasone on column 3, and after the derivatization reaction must have a GC retention time within 20 s as well as meeting ion ratio criteria. One known false positive is betamethasone, a configurational isomer of dexamethasone at the 16-methyl position. It co-elutes with dexamethasone on columns 1 and 3 and on the GC column and is indistinguishable by the MS procedure. It does, however, have the same pharmacological activity as dexamethasone.

The higher LODs in this methods compared with the MGA method are the result of several factors. The most significant is that the wavelength of maximum absorbance for dexamethasone (239 nm; $\epsilon = 13\,934$ l mol^{-1} cm^{-1} in methanol) is less sensitive and less selective to matrix interferences than that of MGA (287 nm, $\epsilon = 22\,387$ l mol^{-1} cm^{-1} in ethanol). Any attempt to increase dexamethasone recovery to the 85% level obtained for MGA would cause more matrix components to be recovered, further increasing background interferences. Also, the MGA method does not require the enzyme hydrolysis procedure necessary in this method, which increases the complexity of the matrices.

In summary, this coupled-column HPLC method provides for screening of dexamethasone in bovine muscle with an LOD of 4 ppb and in bovine liver with an LOD of 6 ppb. If a sample has a detectable amount of dexamethasone, quantitation is possible above 20 ppb in liver and above 10 ppb in muscle. The coupled-column HPLC procedure also purifies tissue extracts for GC–MS confirmation. Confirmation is accomplished using the TMS-enol-TMS derivative of dexamethasone.

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