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SEPARATION OF SOME NATURAL AND SYNTHETIC CORTICOSTEROIDS IN BIOLOGICAL FLUIDS AND TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

A high-performance liquid chromatography (HPLC) technique was developed for the determination of radiolabeled triamcinolone acetonide (TAC), cortisol and their metabolites in rhesus monkey plasma, urine and tissue samples. After protein precipitation, the parent compounds and metabolites were simultaneously resolved using a single-column reversed-phase HPLC system. TAC was subsequently verified by mass spectrometry and TAC glucuronide was tentatively identified by enzymatic hydrolysis and mass spectrometry of the hydrolysis product. The endogenous hormones, cortisol and cortisone were presumptively identified by cochromatography with authentic standards on two different HPLC systems and positively identified by reverse-isotope recrystallization. Other metabolites of both compounds were detected by selective enzymatic hydrolysis and HPLC. This method is rapid and reproducible with a total recovery >80%.

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INTRODUCTION

Cleft palate has been produced in the offspring of a number of species by administration of either cortisol or triamcinolone acetonide (TAC) to the pregnant female [1—9]. In addition, a characteristic pattern of craniofacial malformation results from maternal TAC treatment during early gestation plus thymic involution and growth retardation from treatment during middle and late gestation in nonhuman primates [9—11]. The mechanisms by which these teratogens manifest themselves are unknown. It has been suggested that the unmetabolized drug may be the actual teratogen [12] and that embryo exposure to parent glucocorticoid may be altered due to maternal metabolism and detoxification as demonstrated in corticosteroid-resistant mouse strains [13].

To gain a better understanding of the mechanism of action of this class of hormones, metabolic studies were initiated in order to elucidate the complete metabolic profile of the compounds in question. Sensitive, specific and reproducible analytical techniques are a prerequisite to solving these problems.

Recently, high-performance liquid chromatography (HPLC) techniques have been developed for corticosteroid determination in biological samples [14—20]. However, these methods are neither specific nor useful for separation of the more polar metabolites along with the non-polar compounds. This report describes an HPLC method for separation of cortisol, cortisol glucuronide, 6β-hydroxycortisol, cortisone, cortexolone, triamcinolone (TA) and triamcinolone acetonide (TAC) and demonstrates the use of this method for the simultaneous assay of radiolabeled conjugated and non-conjugated corticosteroids in urine, plasma, and tissue.

EXPERIMENTAL

Instrumentation

The Waters Assoc. (Milford, MA, U.S.A.) HPLC system consisted of a continuous flow, constant volume Model 6000A solvent delivery system equipped with a U6K universal loop injector governed by a Model 660 solvent programmer. UV-absorbing materials passed through the 8-μl flow-cell of a Model 440 UV spectrophotometer set at a fixed wavelength of 254 nm and absorption was traced with a Fisher Recordall Series 5000 (Fisher Scientific, Springfield, NJ, U.S.A.). Fractions were collected with an ISCO Golden Retriever (Instrumentation Specialties, Lincoln, NE, U.S.A.). All radioactivity was counted in a Tracor Analytic Mark III liquid scintillation system Model 6881 (Tracor Analytic, Atlanta, GA, U.S.A.).

Chemicals

Radioisotopes [4-^{14}C]cortisol (2032.04 MBq or 54.92 mCi/m*M*) and [6,7-^{3}H(N)]triamcinolone acetonide (1339.4 GBq or 36.20 Ci/m*M*) were purchased from New England Nuclear (Boston, MA, U.S.A.) and purified on a 5-μm LiChrosorb RP_{18} column using the system described in Methods. After purification they were considered to be > 98% pure based on radioactivity.

The unlabeled triamcinolone acetonide (Kenalog®) for injection was ob-

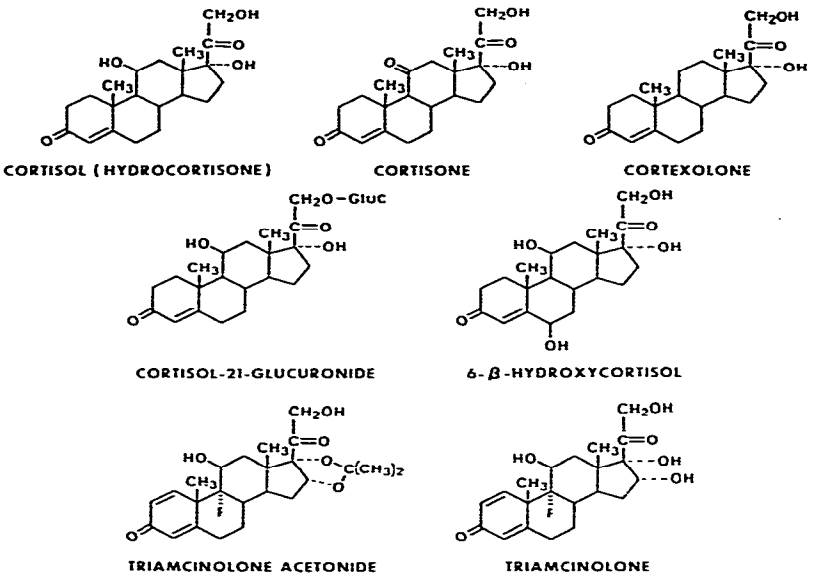

Fig. 1. Structures of cortisol, triamcinolone acetonide and analogues separated by HPLC.

tained from Squibb (Princeton, NJ, U.S.A.) and used as received. No UV (254 nm) absorbing contaminants were found with HPLC analysis.

The other unlabeled derivatives were purchased as listed: triamcinolone acetonide (TAC), triamcinolone (TA) and cortisol (hydrocortisone) from Sigma (St. Louis, MO, U.S.A.); cortisone and cortexolone (11-desoxycortisol) from Research Plus Steroid Labs. (Denville, NJ, U.S.A.) and cortisol-21-glucuronide and 6β-hydroxycortisol from Steraloids (Wilton, NH, U.S.A.). Conformation was verified by mass spectrometry. The structures of these compounds are shown in Fig. 1.

Bovine liver β-glucuronidase Type I and *Helix pomatia* β-glucuronidase with sulfatase activity Type H-1 were obtained from Sigma.

HPLC systems

HPLC System A consisted of a 250 × 10 mm I.D. stainless-steel column commercially packed with 5 μm LiChrosorb RP-18 purchased from Chrompac (Whittier, CA, U.S.A.). A 70 × 6 mm stainless-steel guard column packed with 35—50 μm Bondapak C_{18} Corasil (Waters Assoc.) was connected between the injector and the column. The guard column did not diminish the efficiency or resolution of the main column. At ambient temperature, a convex gradient (No. 5 Waters 660 programmer) was set at a flow-rate of 1.5 ml/min. The initial conditions were methanol—0.01 *M* ammonium acetate, pH 6.9 (10:90) and the final conditions were 100% methanol. Pump pressure never exceeded 136.05 bar.

HPLC System B consisted of two μBondapak C_{18} columns (Waters Assoc.) connected in series. They were each 300 × 3.9 mm, 10 μm particle size, and run isocratically with methanol—water (45:55) at 1 ml/min.

HPLC solvents and chemicals

The organic solvents used were distilled-in-glass quality from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Water was purified through a Millipore Milli-Q-system (Bedford, MA, U.S.A.) fed by a deionized water source. Ultrapure ammonium acetate (Mallinckrodt, St. Louis, MO, U.S.A.) was used as a solvent buffer. All aqueous solvents were filtered through a Millipore BDWP 0.6-μm filter and degassed prior to use.

METHODS

Urine collection and preparation

A 23 μg/kg dose of [4-^{14}C]cortisol (740 kBq) and a 0.085 μg/kg dose of [6,7-^{3}H]TAC (1480 kBq) plus 10 mg/kg unlabeled TAC in suspension was administered intramuscularly to a pregnant rhesus monkey gestational age 134 days. Urine was collected via a Foley catheter for 4 h after drug administration. Proteins and salts were precipitated by the addition of 1 volume of methanol—ethanol (1:1) to 1-ml aliquots of urine. The samples were centrifuged for 10 min at 4000 *g*. The supernatant was transferred to a 5-ml conical test tube and reduced under a stream of nitrogen at 37°C to approximately 0.2 ml for subsequent HPLC analysis.

Blood collection and preparation

Blood samples were collected through a femoral artery catheter and immediately centrifuged to separate the plasma from the red blood cells. The plasma

TABLE I

RETENTION TIME AND RECOVERY OF STANDARDS ON HPLC SYSTEM A

Standard	Amount injected* (μg)	Retention time (min)	Recovery (%)
6β-Hydroxycortisol	5 (5 μl)	17.98	96.6
Cortisol glucuronide	20 (20 μl)	23.31	100.0
TA	10 (20 μl)	25.95	80.35
Cortisone	5 (10 μl)	29.39	92.8
Cortisol	10 (10 μl)	31.07	100.0
TAC	10 (20 μl)	33.99	93.1
Cortexolone	5 (10 μl)	34.75	84.8

*The same amounts were added to urine, plasma and tissue samples.

was transferred to a 10-ml graduated test tube and the standards (Table I) were added. An equal volume of methanol—ethanol (1:1) was introduced to precipitate the protein and the sample was then refrigerated (−20°C) for 30 min or overnight. After refrigeration, the samples were centrifuged in a table top centrifuge for 10 min at 1500 *g*. The precipitate was washed two more times with an equal volume of methanol—ethanol (1:1). The pooled supernatants were transferred to a 5-ml conical test tube and reduced to dryness under nitrogen at 37°C. The residue was resuspended in 0.2 ml methanol—water (65:35) for HPLC analysis.

Tissue extraction and preparation

Fetal tissue samples were collected 5 h after administration of the dose to the maternal monkey. Tissue samples were weighed and placed in wide mouthed screw top plastic bottles; to these, 10—15 ml methanol—dimethoxymethane (1:1) plus standards (Table I) were added. The samples were then homogenized with a Polytron (Brinkman, Westbury, NY, U.S.A.) for 1 min or until breakup was complete. They were then placed in a 37°C shaker bath and the extraction allowed to proceed overnight. The suspension was then centrifuged for 5 min at 4000 *g* in a Damon IEC Model PR6000 centrifuge (Curtin Matheson, Houston, TX, U.S.A.) with a four place horizontal rotor IEC 284 pin type. The supernatant was then filtered using Whatman No. 1 filter paper. The tissue precipitate was resuspended with methanol—dimethoxymethane (1:1) and refiltered. Washing of the precipitate was done on the filter at this time with methanol. The collected filtrate was measured and 10% of the volume removed to a scintillation vial, taken to dryness and counted to determine the amount of radioactive compounds present. The remaining filtrate was taken to dryness under nitrogen and the residue resuspended with up to 0.5 ml methanol—water (65:35). The precipitate, if any, was removed by centrifugation and the resulting supernatant was ready for HPLC analysis. The tissue residue left on the filter paper was allowed to dry. A portion was later combusted in an Oxymat (Intertechnique, Plaisir, France) and the amount of non-extractable radioactivity left in the tissue residue was determined to be between 2 and 4%.

Chemical identification

Mass spectrometric identification of underivatized components separated by HPLC was performed on a Finnigan 4023 mass spectrometer combined with an Incos data system. Samples were deposited into glass sample cups (5 μl volume), evaporated to dryness with a stream of dry helium, and introduced into the mass spectrometer via the solid probe. The probe was ballistically heated to 400°C. Data were collected throughout the heating cycle. For greater sensitivity, the mass spectrometer was operated in the multiple specific ion mode. Ionization was accomplished in conventional electron-impact (EI) mode.

Sample compounds to be recrystallized were brought to constant specific activity with 50 mg of authentic carrier steroid using the solvent pair methylene chloride—isooctane. After the consecutive crystallizations, the specific activity of the crystals was ±5% of the average of the three final values [21].

The peaks considered to be metabolites of cortisol and TAC were resuspended in aqueous 0.2 *M* sodium acetate buffer, pH 5. They were incubated with bovine enzyme and *Helix pomatia* (HP) by dividing the peaks into three aliquots (20% for control, i.e. no enzyme, 40% bovine and 40% HP). One ml of enzyme solution (10,000 units/ml 0.2 *M* sodium acetate buffer, pH 5) was added to the appropriate aliquot and the samples were incubated at 37°C in a shaker bath for 4 h. An equal volume of methanol—ethanol (1:1) was introduced to stop the reaction and the samples were immediately frozen. After thawing, any precipitate was centrifuged out and the supernatant taken to dryness under nitrogen. The residue was resuspended in 400 μl methanol—water (65:35) and analyzed on the described HPLC system A.

RESULTS

Corticosteroid standards

Reversed-phase chromatography on HPLC system A provided a high degree of resolution and separation for the corticosteroid standards (cortisol, cortisone, cortexolone, cortisol glucuronide, 6β-hydroxycortisol, TAC and TA) even though they encompass a wide polarity range. The standards were initially injected individually to establish characteristic retention times. A chromatogram of the standards injected simultaneously is shown in Fig. 2. In order to determine column efficiency, the standards were injected on the column, collected, reduced in volume and reinjected on the same column. The percent recovery was determined by comparing the UV peak areas for each standard. The retention times and percent recovery on the LiChrosorb column are shown in Table I for the standards.

To remove highly pigmented material from samples such as urine or tissue, the sample was injected onto the column at initial conditions but the program was not initiated for a period of 20 min, thus allowing much of the water-soluble pigments to be eluted even before starting the program. This method provided cleaner peak fractions and allowed peak character to be demonstrated on the UV trace.

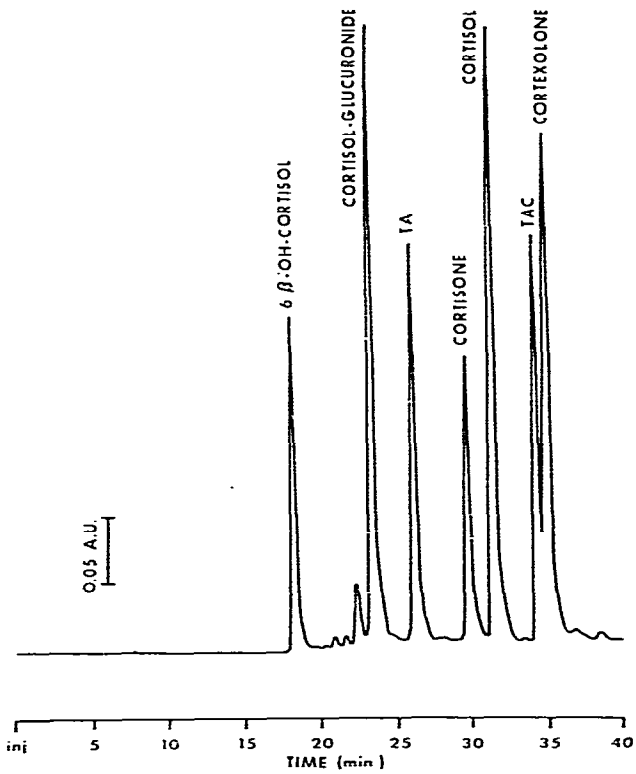

Fig. 2. HPLC profile of corticosteroid standards (HPLC system A). Chromatographic conditions: column, LiChrosorb RP-18, 5 μm, 250 × 10 mm. Mobile phase: methanol—water (0.01 *M* ammonium acetate) (10:90) to 100% methanol, convex gradient elution (Waters program No. 5) in 50 min; flow-rate 1.5 ml/min; sample size, as in Table I.

It was noticed that continued application of highly pigmented, lipid-containing samples to the column resulted in poor resolution of standards. It was discovered that if chloroform was pumped through the column at 0.3 ml/min overnight or preferably, over a weekend, the ability of the column to resolve the standards returned. Transition to and from chloroform was made directly from final conditions (100% methanol).

Urinary metabolites

The urine collected from the maternal monkey at the 60 min time point was used for analysis because of the high concentration of radioactivity. Five aliquots of 1 ml each were processed as described under Methods and applied to HPLC system A. The five separate injections were made in order to prevent overloading the column and to estimate the reproducibility of the method. Multiple injections were necessary to collect enough compound per peak for further analysis. Aliquots (50 μl) from the 0.75-ml fractions were counted by liquid scintillation and as Fig. 3 shows, the parent compounds and metabolites are designated according to retention time and radioactivity. These peaks were pooled individually and the percent radioactivity in each peak as compared to total radioactivity in all peaks was calculated (Table II).

The peaks which cochromatographed with cortisone, cortisol and TAC were resuspended in 400 μl methanol—water (65:35) and rechromatographed on HPLC system A for further cleanup. Finally they were collected, pooled and taken to dryness. The identity of TAC was verified by mass spectrometric com-

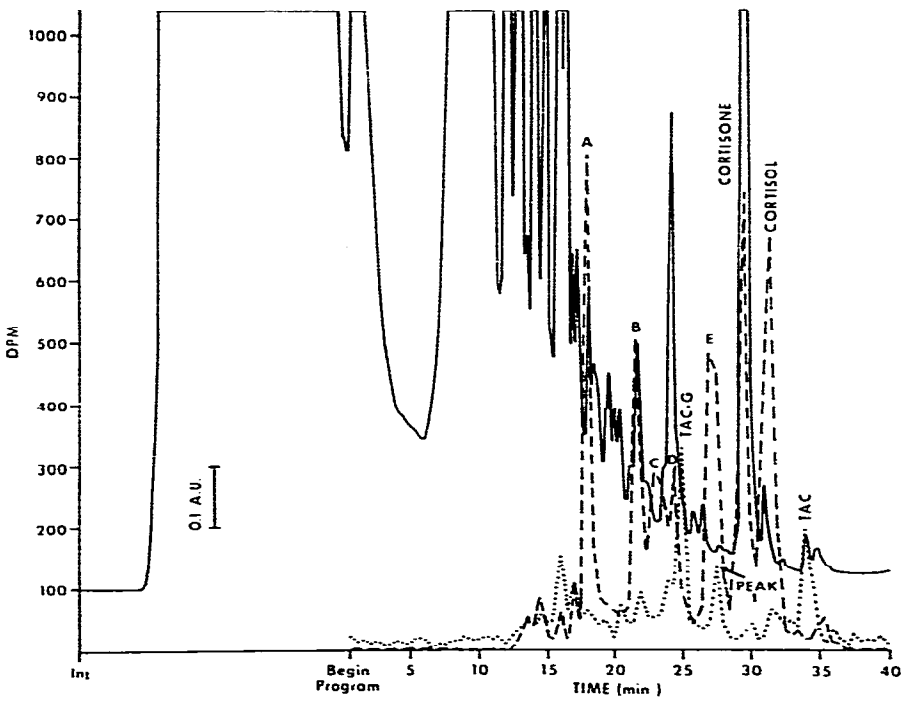

Fig. 3. HPLC profile of urinary metabolites. Chromatographic conditions: same as in Fig. 2, except that standards were not added. · · ·, ^{3}H; — — —, ^{14}C; ——, UV at 254 nm.

TABLE II

COMPARISON OF RETENTION TIMES AND RECOVERY OF INDIVIDUAL HPLC-RESOLVED RADIOLABELED COMPOUNDS

Data compiled from five separate aliquots of urine at 60 min. Values are means ± S.E.

Compound	Retention time (min)	Percentage*	Coefficient of variation
^3H			
TAC-G	25.1 ± 0.10	52.98 ± 0.94	3.96
TAC-peak 1	27.5 ± 0.16	12.97 ± 0.38	6.55
TAC	34.2 ± 0.12	34.05 ± 0.88	5.78
^{14}C			
Peak A	18.2 ± 0.12	15.71 ± 0.57	8.21
Peak B	21.6 ± 0.19	11.47 ± 0.70	13.68
Peak C	23.2 ± 0.12	6.37 ± 1.08	37.99
Peak D	24.3 ± 0.12	9.54 ± 1.40	32.91
Peak E	27.4 ± 0.19	17.54 ± 0.99	12.65
Cortisone	29.7 ± 0.12	18.12 ± 0.44	5.40
Cortisol	31.2 ± 0.12	21.23 ± 0.60	6.35

*Percentage compound of total compounds resolved.

parison to a TAC authentic standard, both having ions at 434 (molecular ion), 413 (M–HF), and 375 (base peak).

Because cortisol and cortisone are endogenous compounds, mass spectrometry was not used as a means for further verification. Alternatively, they were chromatographed on HPLC system B. One milliliter fractions were collected and aliquots counted. Radiolabeled compound thought to be cortisol comigrated with the standard. Radioactivity thought to be cortisone clearly showed two peaks, one of which comigrated with the cortisone standard. These peaks were collected and subjected to reverse-isotope recrystallization. Over 95% of the cortisol was identified as such by this method. Sixty percent of the initial HPLC resolved cortisone peak was identified as cortisone coinciding with the results obtained from HPLC system B which indicated two peaks. Therefore, the original peak on HPLC system A, which comigrated with the cortisone standard contained two compounds, cortisone and an unknown metabolite.

The polar metabolite peaks were subjected to enzyme hydrolysis as described. Although the results varied between peaks, the profiles for each peak by both enzymes were the same suggesting that none of the metabolites were sulfates. The evidence for only glucuronidation was strengthened by the percent hydrolysis data shown in Table III. The percent conversion for each enzyme is almost the same indicating that the sulfatase in the *Helix pomatia* enzyme provided no additional liberation of free steroid over what was liberated by β-glucuronidase alone.

The peak labeled TAC-G was presumed to be a glucuronide of TAC because it had the same retention time as the TAC-G produced and isolated from a liver microsomal enzyme preparation using [^{3}H]TAC and UDPGA [22]. TAC-G was predominantly hydrolyzed to TAC (Table III), and the liberated TAC was identified by mass spectrometry. TAC peak 1 exhibited virtually no conversion back to TAC. Its relative retention time is consistent with the TAC metabolite,

TABLE III

COMPARISON OF THE HYDROLYZING CAPABILITY OF TWO DIFFERENT ENZYMES ON URINARY METABOLITES

Percent conversion to less polar metabolites.

Metabolite peak	Treatment		
	Control	Bovine enzyme	*Helix pomatia*
TAC-G	0	66.9	76
TAC-peak 1	0	12.5	11
Peak A	67	–	67
Peak B	0	84.5	83.7
Peak C	0	62.2	62.9
Peak D	0	46.1	48.7
Peak E	0	100	94.5

6β-hydroxy-TAC (6β-OH-TAC) which has been cited as a major metabolite of TAC [23—27]. Mass spectrometric analysis revealed the base peak at 391 *m/z* (375+16) which is consistent with the addition of one hydroxyl group to TAC.

The enzymatic hydrolysis products of cortisol-derived material were analysed by HPLC. The controls except for peak A, showed no conversion and appeared as well defined parent peaks. Peak A control had the same profile as with enzyme added, indicating that spontaneous breakdown had occurred in the buffer system alone. Neither peak A nor its degradation products comigrated with any standard. Among the substances liberated from peaks B and C were compounds comigrating with cortisone and cortisol. Intact B and C did not migrate with available standards. Peak D was tentatively identified as cortisol-21-glucuronide because it comigrated with the authentic standard in HPLC System A and upon β-glucuronidase hydrolysis, liberated a compound comigrating with cortisol. Peak E was converted to a much less polar metabolite which chromatographed in the same region as cortexolone.

From these data, it can be said that the major urinary metabolites of TAC and cortisol are glucuronides with one of the cortisol metabolites tentatively identified as cortisol-21-glucuronide. TAC metabolism, although limited, produces a glucuronide of TAC (TAC-21-glucuronide) and probably 6β-OH-TAC.

Plasma metabolites

A 10-min plasma sample was processed as described and its HPLC metabolite profile was compared with that of a 60-min urine sample. Fig. 4 shows that the plasma profile compared favorably with the urinary profile. It consisted of radiolabeled peaks comigrating with cortisol, cortisone, TAC and several conjugates. The cortisone peak produced only one definitive peak which comigrated. with the cortisone standard in both HPLC systems. A new peak was noted having a retention time of 35 min, the same as cortexolone. This nonpolar ^{14}C-peak was collected and chromatographed on HPLC system B, but failed to migrate with either cortexolone or corticosterone thereby eliminating the possibility of it being either of those compounds. Insufficient material was available for further analysis.

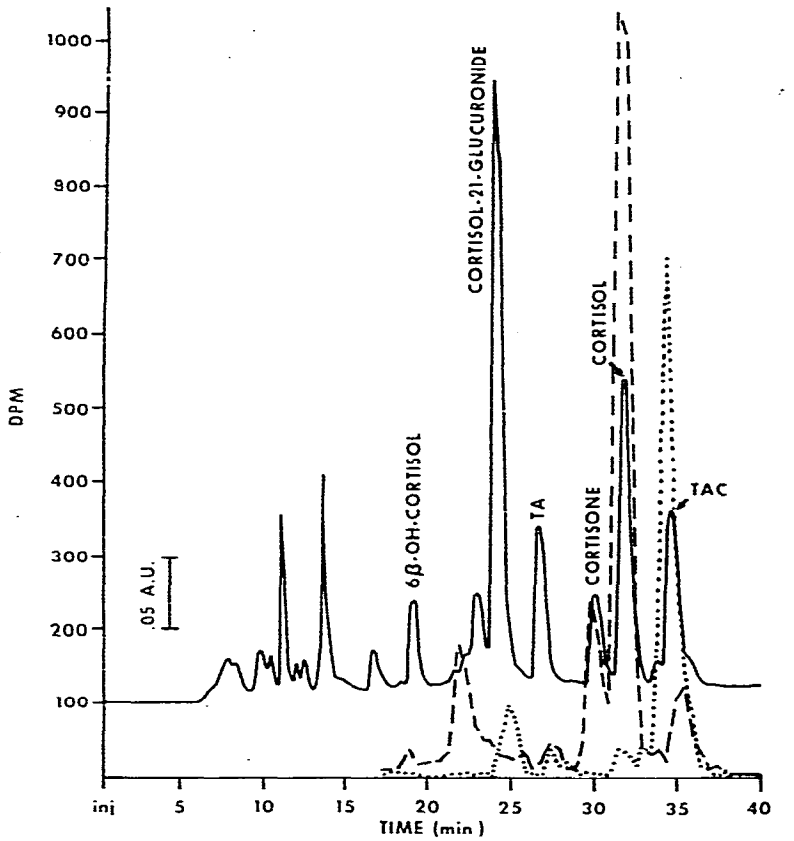

Fig. 4. HPLC profile of plasma metabolites. Chromatographic conditions: as in Fig. 2. · · ·, ^{3}H; — — —, ^{14}C; ——, UV at 254 nm.

Tissue metabolites

Five hours after the [^{14}C]-cortisol—[^{3}H]-triamcinolone acetonide dose was administered, the fetal liver tissue sample was collected and analyzed as described. The resulting chromatogram, Fig. 5, defines the TAC peak and at the same time indicates that cortisol has been metabolized to a variety of products, some of which chromatograph in the same areas as the urinary peaks B, C, E and cortisone. As with the plasma samples, there was not sufficient compound present for further analysis.

DISCUSSION

These results demonstrate that this HPLC methodology is capable of reproducibly separating both natural and synthetic corticosteroids and their metabolites from urine, plasma, and tissue. TAC was definitively identified via mass spectrometry as were cortisol and cortisone by reverse-isotope recrystallization. Evidence was obtained for the tentative identification of TAC glucuronide, 6β-OH-TAC, cortisol-21-glucuronide and several other cortisol derived glucuronide metabolites. Previous methods [23,28] utilized various extraction

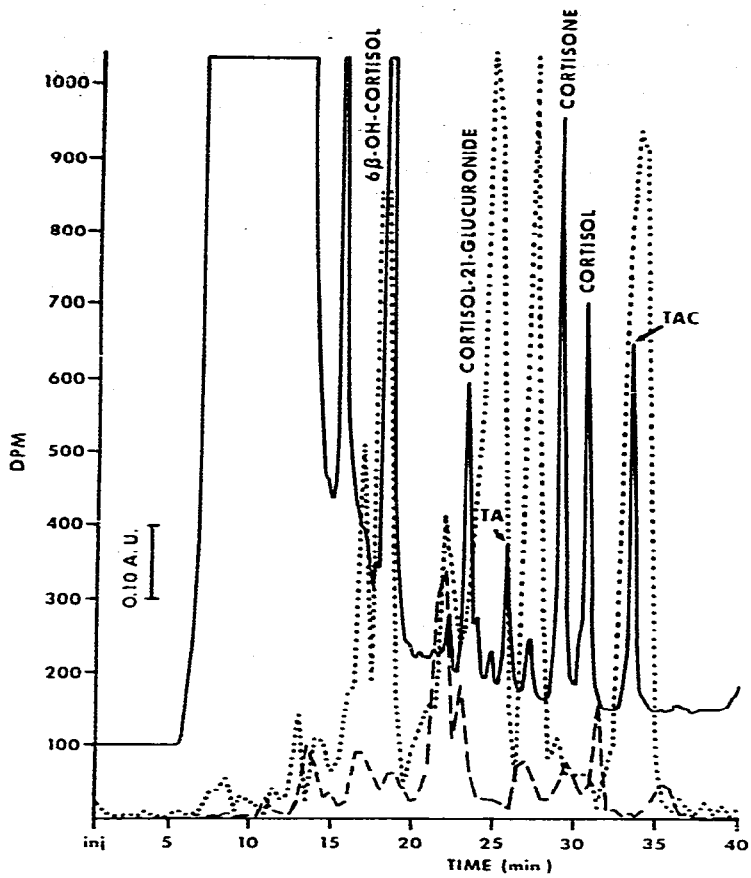

Fig. 5. HPLC profile of fetal liver metabolites 5 h after maternal administration of dose. Chromatographic conditions: as in Fig. 2. · · · , ^{3}H; — — —, ^{14}C; ——, UV at 254 nm.

procedures and several chromatography systems (column, paper, and thin-layer). In this study, a single HPLC system (A) is reported for biological samples which is fast, easy and reproducible with the added advantage that this preparative technique resolves conjugated and nonconjugated metabolites simultaneously. We have previously reported that this single-column HPLC approach provides excellent resolution of synthetic [29] and endogenous [30] estrogens.

In the present report, quantification of the various corticosteroid metabolites is based on radioactivity. An advantage of radioisotope studies is that the sensitivity of the method is determined by the specific activity of the radioisotope in question. In the case of [^{14}C]-cortisol (the lowest specific activity isotope used in this study), the isotope is available commercially and has a specific activity of over 1850 MBq/mmole or 50 mCi/mmole. Therefore, based on the ability to quantify 200 dps of radiolabeled corticosteroid (signal-to-noise ratio of 8:1) the sensitivity of the present radioisotope method is greater than 1.0 ng per 0.5 ml biological fluid (2.0 ppb).

Previous studies [23,24,27] indicate 6β-OH-TAC as being the major metabolite of TAC with little or no conjugation. Kriplani et al. [23] found gluc-

uronide conjugates accounted for 21, 6, and 4% of the radioactivity in the urine of dogs, monkeys and rats respectively. Sulfate conjugates were mentioned only with regard to dog urine and accounted for 11% of the radioactivity. Florini et al. [24] found other metabolites in the water-soluble urine constituents but could not identify them. It has been suggested [23] that the Δ^1, 9α-fluoro and the 16,17-acetonide substituents may block normal reduction reactions prerequisite to conjugation. Our studies agree with this hypothesis to the extent that a major portion of TAC radioactivity excreted is the parent compound. However, these preliminary studies indicate that a glucuronide conjugate of TAC (probably TAC-21-glucuronide) is the major metabolite with 6β-OH-TAC and other more polar metabolites constituting the remainder of the metabolic profile. The large percentage of TAC glucuronide identified in this study may be due to the mild clean-up procedure and the ability of the HPLC system to separate the more polar compounds. Unlike TAC, cortisol is largely metabolized to cortisone and other polar compounds, most of which are glucuronide conjugates as evidenced by their recovery after hydrolysis with β-glucuronidase. This is in agreement with previous findings [31—33] regarding cortisol metabolism in primates. Glucuronidation, rather than sulfation, appears to be the predominant metabolic pathway for the subhuman primate.

In conclusion, the described sample preparation and HPLC procedures are capable of an efficient and rapid separation of free and conjugated metabolites of radiolabeled corticosteroids from biological fluids and tissues.

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