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CORTISOL METABOLITES IN DOG PLASMA

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SUMMARY

[1,2-^{3}H]-Cortisol was administered i.v. to two male dogs, blood was obtained 2·5h later, and the plasma metabolites were characterized and quantified. The unconjugated fraction contained 20–30% of the plasma radioactivity, while 60 to 70% was found in the glucuronide fraction and 8–9% in the sulfate fraction. Cortols and cortolones were the most abundant metabolites in all fractions. 20-Dihydrocortisol and 20-dihydrocortisone were predominant over cortisol and cortisone in the "free" as well as in the sulfate fractions. All fractions contained substantial amounts of 6β-hydroxy, C-20-reduced metabolites. The major one was assumed to be 6β-hydroxycortol or 6β-hydroxycortolone. Others were identified as 6β-hydroxy-20β-dihydrocortisol and 6β-hydroxy-20β-dihydrocortisone. No 6β-hydroxycortisol or 6β-hydroxycortisone was detected. When compared with our analogous study on human plasma, the present results indicate that more extensive C-20 ketone reduction and 6β-hydroxylation occur in the dog.

INTRODUCTION

The metabolism of cortisol in the dog remains poorly documented. Cortol-20ξ* (3α,5β and 3β,5α), cortolone-20ξ, tetrahydrocortisone (3β,5α and 3α,5β), 20β-dihydrocortisol and 20ξ-dehydrocortisone have been characterized in dog urine after injection of radioactive cortisol[1, 2]. The former three-ring A-reduced steroids were liberated after β-glucuronidase hydrolysis of the intact dog urine, while the latter two steroids existed as "unconjugated" metabolites in the urine of intact as well as hepatectomized dogs. In a previous report[3], we investigated the biotransformation of cortisol by a variety of dog tissues *in vitro*. The present study was undertaken to obtain a profile of cortisol metabolites in peripheral plasma of the dog and to compare it with that of human plasma[3, 4].

MATERIALS AND METHODS

Materials. [1,2-^{3}H]-Cortisol (S.A. 43·9 Ci/mmol) was obtained from New England Nuclear Co., Boston, Mass. Its purity was checked by paper chromatography in the B-5 system. Most of the unlabelled reference steroids were purchased from Sigma Chemical Co., St. Louis, Mo. 6β-Hydroxycortisol and 6β-hydroxycortisone were obtained from Steraloids, N.Y. 6β-Hydroxy-20β-dihydrocortisol and 6β-hydroxy-20-β-dihydrocortisone were synthesized from 6β-hydroxycortisol and 6β-hydroxycortisone as described previously[3]. 5α-Androstane-3,6,11,17-tetrone was prepared as follows: One mg of 6β-hydroxycortisone was converted to 17,21-dihydroxy-5α-pregnane-3,6,11,20-tetrone by the method of Kornel[6]. The product was purified in the BP system, eluted and oxidized with chromic acid. The oxidation product was chromatographed in the B-3 system. The compound was located by Zimmerman reaction in a portion of the chromatogram and the corresponding area was eluted. 4-Androstene-3,6,11,17-tetrone was prepared by chromic oxidation of 6β-hydroxycortisone and purified by paper chromatography in the B-5 system. All solvents were analytical grade.

Paper chromatography. Paper chromatography was performed on Whatman No. 1 paper using the following solvent systems: Y, ethyl acetate–chloroform–methanol–water (25:75:50:50 by vol.); S-I, benzene–ethyl acetate–methanol–water (50:40:50:50 by vol.); BP, benzene–chloroform–methanol–water (50:50:50:50 by vol.); B-5, benzene–methanol–water (1000:525:475 by vol.); SL_{10}, toluene–tert-butanol–ethanol–0·02 M boric buffer (pH 9·0) (170:40:30:100 by vol.); B-1, petroleum ether–toluene–methanol–water (25:25:35:15 by vol.); and B-3, petroleum ether–benzene–methanol–water (33:17:40:10 by vol.).

Experimental. Two adult male dogs (13 and 15 kg body wt.) were kept without food for 15 h prior to the study. Anesthesia was induced with ketamin hydrochloride (3 mg/kg body wt.). [1,2-^{3}H]-Cortisol

The following trivial names and abbreviations are used: cortisol (F_K); cortisone (E_K); 20α-dihydrocortisol (20α-DHF); 20β-dihydrocortisol (20β-DHF); 20β-dihydrocortisone (20β-DHE); 6β-hydroxycortisol (6β-OH-F); 6β-hydroxycortisone (6β-OH-E); 6β-hydroxy-20β-dihydrocortisol (6β-OH-20β-DHF); 6β-hydroxy-20β-dihydrocortisone (6β-OH-20β-DHE); Cortol-20ξ; 5β-pregnane-3α,11β,17,20α or 20β,21-pentol; Cortolone-20ξ; 3α,17,20α or 20β,21-tetrahydroxy-5β-pregnan-11-one; tetrahydroxycortisol (THF); 5α-tetrahydroxycortisol (5α-THF); tetrahydrocortisone (THE); 11β-hydroxyaetiocholanolone (11-OH-Etio); 11β-hydroxyandrosterone (11-OH-Andro); 11β-hydroxyandrostenedione (11-OH-AD); 11-oxo-aetiocholanolone (11-oxo-Etio); 11-oxo-androsterone (11-oxo-Andro); 6β,11β-dihydroxyandrostenedione (6,11-diOH-AD); 6β-hydroxy-adrenosterone (6-OH-Adreno): 6β-hydroxy-4-androstene-3,11,17-trione; -sulfate: (steroid)-yl-sulfate; -glucuronide: (steroid)-yl-β-D-glucopyranosiduronide.

Table 1. Identification of steroid moieties of individually separated corticosteroids

Postulated steroid[1]	Chromatography with carrier[2]	Specific activity d.p.m./μmol	Chemical reaction applied	Derivative formed[1]	Chromatography of derivative[2]	Specific activity d.p.m./μmol
X[3]	Y (5% boric) (×1)	—*	CrO_3 oxid	—*	B-3 (×1)	*
6β-OH-20β-DHF	S-I (5% boric) (×2)	1180	KIO_4 oxid	6,11-diOH-Ad	B-5 (×1)	1151
6β-OH-20β-DHE	S-I (5% boric) (×1)	786	KIO_4 oxid	6-OH-Adreno	B-5 (×1)	751
Cortol-20α	SL_{10} (×3)	2650	KIO_4 oxid	11-OH-Etio	B-3 (×2)	2531
Cortol-20β	SL_{10} (×3)	5967	KIO_4 oxid	11-OH-Etio	B-3 (×2)	5711
5α-Cortol[4]	SL_{10} (×2)	*	KIO_4 oxid	11-OH-Andro	B-3 (×2)	*
Cortolone-20α	B-5 (5% boric) (×4)	976	KIO_4 oxid	11-oxo-Etio	B-3 (×1)	991
Cortolone-20β	B-5 (5% boric) (×4)	3712	KIO_4 oxid	11-oxo-Etio	B-3 (×1)	3694
5α-Cortolone[4]	B-5 (5% boric) (×4)	—*	KIO_4 oxid	11-oxo-Andro	B-3 (×1)	*
THF	B-5 (×5)	1815	$NaBiO_3$ oxid	11-OH-Etio	B-3 (×1)	1804
THE	B-5 (×3)	526	$NaBiO_3$ oxid	11-oxo-Etio	B-3 (×1)	537
20β-DHF	B-5 (5% boric) (×2)	2011	KIO_4 oxid	11-OH-AD	B-3 (×1)	2037
F_A	B-5 (×3)	986	$NaBiO_3$ oxid	11-OH-AD	B-3 (×1)	962
E_A	B-5 (×1)	712	$NaBiO_3$ oxid	11-oxo-AD	B-3 (×1)	681

[1] For steroid abbreviations and nomenclature see footnote in the text.
[2] For designation of chromatographic systems, see text. (5% boric) indicates the paper was pretreated in 5% boric acid. (×3) indicates 3 times single-length run.
[3] X—a metabolite with extremely high polarity, presumed to be 6β-hydroxycortol(one), see text.
[4] Separation of 20α- and 20β-isomers was not carried out.
* Carrier steroid not available for determination of specific activity.

(1 mCi) was administered in a rapid i.v. injection and 2·5 h later the blood was withdrawn into heparinized tubes through a polyvinyl catheter placed in a femoral artery. Plasma, about 200 ml from each animal, was separated and kept frozen until processed.

Processing of plasma. The procedures have been described elsewhere[4]. Two vol. methanol and 4 vol. carbon tetrachloride were added to plasma and the mixture was shaken and centrifuged. The upper alcoholic layer was saved and the precipitated protein was re-extracted once with 1 vol. butanol. Both extracts were combined and evaporated *in vacuo*. The residue was partitioned between 100 ml 75% aq. methanol and 400 ml hexane to remove lipid material and the methanolic layer was evaporated to dryness. The dried extract was redissolved in 100 ml water and "free" metabolites were extracted twice with 2 vol. ethyl acetate. The conjugated metabolites remaining in the aqueous phase were extracted on an Amberlite XAD-2 column and separated into glucuronide, sulfate and other conjugates by means of high-voltage electrophoresis[7]. The glucuronide conjugate, eluted from paper, was hydrolyzed with β-glucuronidase (Sigma, type B-1) in 10 ml water buffered with acetate buffer (pH 4·5). The amount of enzyme used was 5000 U/ml and the time of hydrolysis was 48 h. The liberated steroids were extracted twice with 3 vol. ethyl acetate. The sulfate conjugates from the two plasma specimens were combined and subjected to a modified solvolysis procedure[8] for 40 h.

Separation and identification of individual steroids. The "free" metabolites and the steroids liberated from conjugates were separated by several consecutive paper chromatographs. The sequence of systems used was the same as described previously[3]. Authentic standards were run along with the unknowns. All chromatographically identical compounds were pooled and rechromatographed in an appropriate system. Confirmation of the identity of separated radioactive steroid was done by reverse isotope dilution and derivative formation[3, 4, 7]. Briefly, an appropriate non-radioactive carrier was mixed with the radioactive steroid and the mixture was rechromatographed on paper. The S.A. (d.p.m./μmol) was determined by radioactivity measurement and chemical quantitation of an aliquot of the eluate. The remaining steroid material was oxidized either by potassium periodate or by sodium bismuthate. After chromatography, the S.A. of the derivative was determined and compared with that of the parent compound (Table 1). Procedures for radioactivity counting, correction for quenching, and correction for recovery of steroids have been described previously[3].

RESULTS

Of total radioactivity in the plasma of dog No. 1, 20·8% was extracted in the "free" fraction, 69·3% in the glucuronide fraction, and 7·9% in the sulfate fraction. Small amount (2%) of radioactivity was associated with a conjugate fraction of "unknown" type. In dog No. 2, the values were: "free", 30·2%; glucuronide, 58·9%; sulfate, 9·2%; and "unknown", 1·7%.

Analyses of individual steroids in the "free" and conjugated fractions are shown in Table 2. In the "free" metabolites, cortols and cortolones accounted for more than 40% of radioactivity. Cortolone-20β was found in the largest quantity followed by cortol-20β. Other steroids characterized were 20β-dihydrocortisol, tetrahydrocortisol, tetrahydrocortisone, cortisol, cortisone and 11-oxygenated 17-ketosteroids. Reduction of the C-20 ketone to 20α-isomers was comparatively minor and ring A reduction to 5α-configuration occurred inconsistently. Of special interest is the presence of substantial amounts of metabolites with polarity greater than that of 6β-hydroxycortisol. At least three radioactivity peaks were isolated chromatographically. The two less polar compounds were

Table 2. Individual cortisol metabolites in free and conjugated fractions

Steroid [1]	Free*		Glucuronide*		Sulfate[2]*
Dog	No. 1	No. 2	No. 1	No. 2	
X[3]	8·6	17·2	20·1	14·7	5·6
6β-OH-20β-DHF	3·7	10·6	5·6	—	7·4
6β-OH-20β-DHE	1·3	2·0	2·0	—	3·4
Cortol-20α	2·0	1·6	11·6	11·7	17·4[4]
Cortol-20β	16·0	11·8	34·5	44·2	
5α-Cortol[4]	—	2·1	3·0	6·4	—
Cortolone-20α	8·5	4·6	0·9	1·6	
Cortolone-20β	27·6	25·3	12·1	8·0	20·8[4]
5α-Cortolone[4]	2·0	—	2·9	—	—
THF	3·6	4·7	3·1	3·9	4·1
5α-THF	1·0	—	—	—	
THE	1·9	2·0	—	2·5	1·3
20α-DHF	1·4	—	—	—	17·5[4]
20β-DHF	9·1	5·9	—	—	
20β-DHE	—	—	—	—	2·0
F_K	3·8	2·2	—	—	1·9
E_K	2·3	1·9	—	—	1·8
11-OH-Andro/Etio[5]	2·9	4·2	3·0	4·2	9·7[5]
11-oxo-Andro/Etio[5]	3·1	1·8	2·2	2·8	
Y[6]	—	2·1	—	—	—

* Results are expressed as per cent of radioactivity recovered in each fraction and corrected for losses during chromatographic separation.

[1] For steroid abbreviations and nomenclature see footnote in the text.

[2] Sulfate fractions from two plasma specimens were combined and analyzed.

[3] X—a metabolite with extremely high polarity, presumed to be 6β-hydroxycortol(one). See text.

[4] Separation of 20α- and 20β-isomers was not carried out.

[5] No further separation was carried out.

[6] Y—an unidentified C-19 metabolite. See text.

identified as 6β-hydroxy-20β-dihydrocortisol and 6β-hydroxy-20β-dihydrocortisone. The most polar compound had R_F 0·15 in the system Y, which was less than half of that of 6β-hydroxy-20β-dihydrocortisol (R_F 0·33). Periodate oxidation yielded the same compound as bismuthate oxidation, suggesting a steroid with glycerol type of side chain. Chromic acid oxidation gave a product which migrated almost identically with 5α-androstane-3,6,11,17-tetrone in the systems B-3 (R_F 0·41) and B-1 (R_F 0·65). Thus, the compound was tentatively referred to as 6β-hydroxycortol or 6β-hydroxycortolone but, due to the lack of authentic standard of original compound, definite identification could not be made. In one specimen, a metabolite with R_F 0·16 in the B-5 system, which is more polar than 6β,11β-dihydroxy-4-androstene-3,17-dione (R_F 0·25), was isolated. It remained unchanged after bismuthate oxidation, and chromic oxidation formed a compound with chromatographic mobility similar to that of 5α-androstane-3,6,11,17-tetrone, indicating a ring A reduced 6-hydroxylated C-19 steroid. No further attempt was made at characterization.

In the glucuronide fraction, cortols and cortolones constituted the main bulk of the metabolites. In contrast to the "free" fraction, cortols were predominant over cortolones. 20-Dihydrocortisol, cortisol and cortisone could not be detected in any significant amounts. The 6β-hydroxy, C-20 reduced metabolites were also found in large quantities, most of which were presumed to be the ring A reduced compound.

In view of the scarcity of sulfate conjugates, the materials from both specimens were pooled and separation of isomers at C-20 and C-5 was not undertaken. In addition to cortols and cortolones, a relatively large amount of 20-dihydrocortisol was present. Among the 6β-hydroxy, C-20-reduced metabolites, the ring A intact compounds were predominant.

DISCUSSION

This paper describes a whole spectrum of cortisol metabolites in dog plasma. It should be noted, however, that the results obtained here represent a cross-section of metabolism of exogenously administered tracer cortisol at a definite time.

In the dog plasma the concentration of "free" metabolites was less than 30%, while conjugated metabolites, the bulk of which was glucuronide, constituted more than 70% of the total radioactivity. Our study of human plasma in a comparable condition[3, 5] showed more free and less conjugated metabolites. The existence of sulfate conjugated metabolites of cortisol in human plasma has been established[3], and the present study demonstrates that sulfation occurs in the dog to a slightly higher extent than in the human. Small amount of "unknown" conjugate with similar electrophoretic mobility was also noticed in human plasma[4], but the exact nature of this conjugate remains to be elucidated. No disulfate and glucurosulfate conjugates, which were found in human plasma[4], were present.

Analysis of individual steroid moieties revealed that cortols and cortolones, both ring A and C-20-ketone reduced steroids, constituted the largest group of metabolites in all fractions. It is also noteworthy that 20-dihydrocortisol and 20-dihydrocortisone were present in larger amounts than cortisol and cortisone in the "free" as well as sulfate conjugated fractions. These results contrast with our observations on human plasma[5], in which steroids with dihydroxyacetone side chain (tetrahydrocortisol, tetrahydrocortisone and their 5α-isomers, cortisol and cortisone) were predominant over their respective counterparts with glycerol side chain, suggesting that extensive reduction at the C-20 ketone takes place in the dog. This deduction is also supported by the fact that the major cortisol metabolites in dog urine were cortols and cortolones[1], while those in human urine were tetrahydrocortisone, tetrahydrocortisol and 5α-tetrahydrocortisol[9, 10].

The presence of relatively large amounts of 6β-hydroxy, C-20 reduced metabolites, deserved special comment. 6β-Hydroxy-20β-dihydrocortisol and 6β-hydroxy-20β-dihydrocortisone were first demonstrated in human urine[11] and amniotic fluid[12] as "free" metabolites. Recently the glucuronide and sulfate conjugates of these compounds were partially characterized in human plasma[5] and urine[10]. Other species may also synthesize these compounds. It has already

been demonstrated that the dog liver and adrenal can convert cortisol and 20β-dihydrocortisol to these compounds *in vitro*[3, 13]. The polar metabolite(s) of cortisol found in baboon urine[14] is reported to have a chromatographic mobility similar to that of 6β-hydroxy-20β-dihydrocortisol in the system Y. In this study, in addition to these metabolites, a compound, not yet fully identified but presumed to be a ring A reduced, 6β-hydroxylated, 20-dihydro metabolite of cortisol, was isolated. A similar compound has been isolated in human urine[10]. It is interesting that, despite active bio-oxidation at the C-6 position in the dog, no 6β-hydroxycortisol or 6β-hydroxycortisone, which have been found consistently in human plasma[5] and urine[15], could be isolated from dog plasma or urine[1]. This would suggest that, in the dog, 6β-hydroxylation of C-20 reduced metabolites of cortisol could take place more readily than 6β-hydroxylation of C-20 keto-analogues or, alternatively, 6β-hydroxylated compounds, once formed, could be quickly reduced at the C-20 position. The metabolic sequences from cortisol to these metabolites are now under investigation.

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