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METABOLISM AND CONJUGATION OF 20β-DIHYDROCORTISOL BY VARIOUS DOG TISSUES *IN VITRO*

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SUMMARY

The metabolism and conjugation *in vitro* of [4-^{14}C]-20β-dihydrocortisol by a variety of dog tissues was studied. C-11 oxidation to 20β-dihydrocortisone was the most prevalent reaction occurring in the kidney, liver, adrenal and the lung. Side chain cleavage to 11β-hydroxy-androstenedione took place in the liver and the kidney to a small extent. Reduction of ring A to cortol-20β and cortolone-20β was substantial in the liver with a small amount of 6β-hydroxy-20β-dihydrocortisone also being formed in this tissue. All the tissues except the brain sulphate 20β-dihydrocortisol to considerable extents. When compared with the previous study on cortisol, 20β-dihydrocortisol seems to be metabolized less, while being conjugated with sulphate in larger quantities.

INTRODUCTION

Several C-20-dihydro metabolites of cortisol are known to occur in the blood and urine. These compounds have been thought to exist exclusively in the free (unconjugated) form or to be conjugated with glucuronic acid after the reduction of ring A to cortols and cortolones [1]. However, recently we have characterized C-21 sulphate conjugates of the C-20-reduced metabolites of cortisol in human urine and plasma [2, 3]. The present study was undertaken to elucidate the role of various dog tissues in the metabolism and conjugation of 20β-dihydrocortisol *in vitro*. Similar studies have been performed with cortisol [4, 5] so that the biotransformation of two steroids were compared.

MATERIALS AND METHODS

Materials

[4-^{14}C]-20β-Dihydrocortisol was prepared from [4-^{14}C]-cortisol (New England Nuclear Co., Boston, Mass. S.A. 55 mCi/mmol) by the borohydride reduction as described by Bradlow *et al.*[6]. The product was purified by paper chromatography in the system of B_p for 4 h and separated into the 20α- and 20β-isomers in the system B-5 for 8 h on 5% boric acid impregnated paper. The radioactive area corresponding to authentic 20β-dihydrocortisol was eluted. The purity was checked and found to be over 96% by repeated crystallization with the carrier standard. 20β-Dihydrocortisol-21-sulphate was prepared from cortisol-21-sulphate (supplied by Dr. W. Garn, Schering AG, Berlin) by the same reduction method. The product was extracted on an Amberlite XAD-2 column [7] and chromatographed on paper in the system K-5 for 16 h. One U.V.-absorbing band, the mobility relative to that of cortisol-21-sulphate 0·74, was obtained. Rechromatography in the system K-4 for 24 h gave two U.V.-absorbing bands at relative mobilities to that of cortisol-21-sulphate 0·42 and 0·55. The less polar zone was predominant and was assumed to be the 20β-epimer of C-20 reduced cortisol sulphate. The steroid sulphate eluted from the paper was converted to the sodium salt by passing through an ion exchange column (I.R.-120 cation exchange resin, Rome and Hass, in the sodium cycle) [8] and was recovered in the methanol wash of an Amberlite XAD-2 column. Recrystallization three times from methanol–ether gave the material of analytical quality; m.p. was 199·5–198·0°C, Porter–Silber colour reaction was negative, methylene blue colour reaction for organic sulphate [2, 9] was positive. I.R. spectrum in potassium bromide gave absorption bands at 3450 cm^{-1} (hydroxyl), 1650 cm^{-1}, 1610 cm^{-1} (4-ene-3-one) and at 1235 cm^{-1} (sulphate), but no band corresponding to 20-ketone at around 1700 cm^{-1}. Analysis: calculated for $C_{21}H_{32}O_8SNa$, C 53·95%, H 6·91%, S 6·87%. Found: C 54·01%, H 6·99%, S 6·47%. Solvolysis yielded one steroid, 20β-dihydrocortisol, which was identified by paper chromattographic mobilities of the compound and its acetylation and periodate oxidation products and by I.R.

The following trivial names and abbreviations are used for steroids: 20β-dihydrocortisol (20β-DHF); 11β,17α,20β,21-tetrahydroxy-4-pregnen-3-one; 20β-dihydrocortisone (20β-DHE); 17α,20β,21-trihydroxy-4-pregnene-3,11-dione; 6β-hydroxycortisol (6β-OH-F); 6β,11β,17α,21-tetrahydroxy-4-pregnene-3,20-dione; 6β-hydroxycortisone (6β-OH-E): 6β,17α,21-trihydroxy-4-pregnen-3,11,20-trione; 6β-hydroxy-20β-dihydrocortisol (6β-OH-20β-DHF): 6β,11β,17α,20β,21-pentahydroxy-4-pregnen-3-one; 6β-hydroxy-20β-dihydrocortisone (6β-OH-20β-DHE): 6β,17α,20β,21-tetrahydroxy-4-pregnen-3,11-dione; cortol-20β: 5β-pregnane-3α,11β,17α,20β,21-pentol; cortolone-20β: 3α,17α,20β,21-tetrahydroxy-5β-pregnan-11-one; 11β-hydroxy-androstenedione (11-OH-AD): 11β-hydroxy-4-androstene-3,17-dione: adrenosterone: 4-androstene-3,11,17-trione; 6β-hydroxy-adrenosterone (6β-OH-Adreno): 6β-hydroxy-4-androstene-3,11,17-trione; sulphate: (steroid)-21-yl-sulphate.

analysis. 6β-Hydroxy-20β-dihydrocortisol and 6β-hydroxy-20β-dihydrocortisone were prepared from 6β-hydroxycortisol (supplied by Dr. S. Bernstein, Lederle Laboratories, Pearl River, N.Y.) and from 6β-hydroxycortisone (Mann Research Laboratories, New York, N.Y.) as described previously [5]. All other reference steroids were purchased from Sigma Chemical Co., St. Louis, Mo.

Chromatographic systems

The following solvent systems were used for the chromatography of the free and the conjugated steroids on paper: K-4—benzene–isopropyl alcohol–water (100:70:30 by vol.); K-5—petroleum ether–ethyl acetate–isopropyl alcohol–water (70:30:85:20 by vol.); 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.); B_p—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–methanol–0·02 M boric acid buffer, pH 9·0 (170:40:30:100 by vol.); B-1—petroleum ether–toluene–methanol–water (25:25:35:15 by vol.).

Incubation and extraction

The method was the same as described in previous reports [4,5]. Adult male dogs were stunned and exsanguination. Tissue mince (1500 mg) was incubated with 1·0 μCi of [4-^{14}C]-20β-dihydrocortisol in 20 ml of Krebs–Ringer bicarbonate buffer, pH 7·4, without $CaCl_2$, supplemented with 1500 μmol of ATP disodium salt, for 3 h at 36°C under 95% O_2:5% CO_2. The tissue was homogenized in the medium and extracted twice with 2 vol. of acetone–ethanol (1:1 v/v) and once with 1 vol. of *n*-butanol. Approximately 97% of the radioactivity was extracted. The combined extracts were evaporated and the residue was redissolved in 30 ml of distilled water. The free (unconjugated) steroids were extracted twice with 3 vol. of ethyl acetate. To the aqueous phase approximately 500 μg of authentic 20β-dihydrocortisol-21-sulphate was added as a recovery standard and the conjugated steroids were extracted on a 15 g Amberlite XAD-2 column [7].

Separation of free metabolites

The ethyl acetate extract was evaporated and subjected to consecutive paper chromatographies to separate it into individual metabolites as described previously [2, 3, 5]. The first chromatography in system B_p for 4 h yielded five radioactive peaks. The two most polar peaks were found only in the liver extract. Peak 1 was close to the origin and rechromatographies in system Y for 4 h and Y on 5% boric acid-treated paper for 5 h achieved the separation of 6β-hydroxy-20β-dihydrocortisol and 6β-hydroxy-20β-dihydrocortisone. Peak 2 corresponded to cortol-20β and was further purified in systems B_p for 16 h and SL_{10} for 8 h. Peak 3, the main peak, had mobility similar to that of unchanged 20β-dihydrocortisol. Rechromatography in system B-5 on 5% boric acid-impregnated paper for 12 h separated 20β-dihydrocortisol from cortolone-20β. Both compounds were further purified in system SL_{10} for 4 h. Peak 4 corresponded to 20β-dihydrocortisone and was rechromatographed in systems B-5 on boric acid impregnated paper for 6 h and SL_{10} for 4 h. Peak 5 had the mobility of C_{19} steroids. Rechromatography in system B-1 separated 11β-hydroxy-androstenedione and adrenosterone.

Isolation of conjugated metabolites

Methanolic eluate from the Amberlite column was evaporated and subjected to high voltage electrophoresis in pyridine formic–acetic acid buffer, pH 2·2, as described by Kornel [10]. Radioactivity scanning revealed a single "steroid monosulphate" peak, which was eluted and chromatographed on paper in systems K-5 for 16 h and K-4 for 24 h. In each system, a radioactive peak which had the same mobility as that of authentic 20β-dihydrocortisol-21-sulphate was obtained.

Radioactivity measurement and quantitative determination. The radioactivity was measured in a liquid scintillation counter/Beckman (LB-222B) in toluene phosphor. Quench corrections were made by automatic external standarization. At each chromatographic step for free metabolites, the recovery of steroid was corrected to 100% in terms of loss of radioactivity in the individual metabolites. The yield of sulphate conjugate was calculated from the radioactivity eluted from the K-4 chromatogram. The results were corrected for procedural losses from the recovery of authentic 20β-dihydrocortisol-21-sulphate measured by the absorption at 240 nm and expressed as the percentage conversion of the substrate incubated.

RESULTS

Metabolic conversion of 20β-dihydrocortisol

Table 1 gives the percentage distribution of radioactivity in the free metabolites. No significant dehydrogenation or epimerization of the 20β-hydroxyl group took place. C-11 oxidation to 20β-dihydrocortisone was the most predominant reaction. The conversion rate was largest in the kidney, followed by the liver, adrenal and the lung. The diaphragm, spleen, intestine and the brain had no measurable activity. The reduction of ring A to cortol-20β was substantial in the liver and a small amount of cortolone-20β was also formed. No 5α-isomers were detected. The oxidative cleavage of the glycerol side chain to 11β-hydroxy-androstenedione occurred in the liver and the kidney to a small extent. A very low peak corresponding to adrenosterone was detected in two of five liver incubates. 6β-Hydroxylation was a minor but definite reaction demonstrated in the liver. 6β-Hydroxy-20β-dihydrocortisone was the principal product and a trace amount of 6β-hydroxy-20β-dihydrocortisol-like compound was detected in two of five liver incubates.

Synthesis of sulphate conjugate is shown in Table 2. Sulfation was most marked in the kidney followed by

Table 1. Free metabolites of [4-^{14}C]-20β-dihydrocortisol by various dog tissues*

Tissue	No. of animals	Steroids†					
		6β-OH-20β-DHE	Cortol-20β	Cortolone-20β	20β-DHF	20β-DHE	11-OH-AD
Adrenal	5	—	—	—	97·5 ± 2·3	4·3 ± 0·7	—
Liver	5	3·0 ± 0·6	7·5 ± 2·3	2·4 ± 0·8	73·5 ± 6·9	8·7 ± 2·3	3·9 ± 1·3
Kidney	5	—	—	—	86·8 ± 4·8	10·5 ± 2·8	2·7 ± 1·2
Lung	5	—	—	—	97·9 ± 3·7	2·1 ± 0·6	—
Diaphragm	5	—	—	—	100·0	—	—
Spleen	3	—	—	—	100·0	—	—
Intestine	3	—	—	—	100·0	—	—
Brain	3	—	—	—	100·0	—	—

* Results are expressed as per cent of total free radioactivity extracted by ethyl acetate and corrected for procedural losses. Mean ± S.D.

† For steroid abbreviation and nomenclature see footnote in the text.

the liver, adrenal, intestine, lung and the muscle. The brain had no measurable sulfation. Glucuronide conjugation could not be demonstrated in any tissue.

Identification of free metabolites

The details and validity of the method were fully described in previous papers [2, 3, 5]. No attempts were made to identify the minimal and inconstant products such as 6β-hydroxy-20β-dihydrocortisol and adrenosterone.

Each radioactive compound from all chromatograms was pooled, supplemented with approximately 200 μg of authentic carrier steroid and rechromatographed in a proper system: Y for 6β-hydroxy-20β-dihydrocortisone, SL_{10} for cortol-20β, cortolone-20β, 20β-dihydrocortisol and 20β-dihydrocortisone, and B-1 for 11β-hydroxy-androstenedione. The S.A. (d.p.m./μmol) was determined by radioactivity counting and chemical quantitation on an aliquot of the eluate. The remaining part was subjected to sodium periodate oxidation (all compounds except 11β-hydroxy-androstenedione) or chromic acid oxidation (11β-hydroxy-androstenedione), and their derivatives were purified by chromatography in the system B-5 (for 6β-hydroxy-adrenosterone) or B-1 (for derivatives from other steroids). The S.A. of the derivatives was in close agreement with that of the parent compounds (Table 3).

Table 2. Biosynthesis of "sulphate conjugate" from [4-^{14}C]-20β-dihydrocortisol by various dog tissues*

Tissues	No. of animals	Yields, %
Adrenal	5	6·0 ± 1·3
Liver	5	8·1 ± 1·2
Kidney	5	10·3 ± 1·5
Lung	5	2·5 ± 1·0
Diaphragm	5	2·0 ± 0·9
Spleen	3	1·6
Intestine	3	3·4
Brain	3	not detected

* Results are expressed as percent of radioactivity initially added and corrected for methodological losses. Mean ± S.D.

Table 3. Identification of metabolites of [4-^{14}C]-20β-dihydrocortisol

Postulated steroid metabolite*	S.A. (d.p.m./μmol) Parent compound†	Oxidation product†
6β-OH-20β-DHE	168	180
Cortol-20β	372	359
Cortolone-20β	112	103
20β-DHF	2092	2122
20β-DHE	552	463
11-OH-AD	212	222
20β-DHF‡	331	345

* For steroid abbreviation and nomenclature see footnote in the text.

† For details of the procedure see text.

‡ Steroid liberated from "sulphate conjugate" by means of solvolysis.

Identification of sulphate conjugate

The radioactive peak corresponding to authentic 20β-dihydrocortisol-21-sulphate was eluted and the eluates from all chromatograms were pooled. A part of the compound was mixed with 20 mg of the authentic carrier and recrystallized to a constant specific activity (Table 4). Another part was solvolyzed and the liberated steroid was identified as [4-^{14}C]-20β-dihydrocortisol by the same method as used for the free steroid (Table 3).

DISCUSSION

Few systematic studies have been reported concerning the metabolism of C-20-dihydro metabolites of

Table 4. Identification of "sulphate conjugate" by successive crystallization with authentic 20β-dihydrocortisol-21-sulphate

Solvent	Crystals (d.p.m./mg)	Mother liquors (d.p.m./mg)
Methanol	1157	1450
Methanol–benzene	1183	1211
Methanol–ether	1160	1166
Methanol–ether	1150	1141

cortisol. In humans *in vivo*, Bradlow *et al.*, have demonstrated that the two principal metabolic reactions of 20β-dihydrocortisol are oxidation of the 11β-hydroxyl group and reduction of 4-ene structure and C-3 ketone of ring A [6, 11]. The present study showed that the oxidation of 11-hydroxyl group to 20β-dihydrocortisone was the most prevailing reaction *in vitro*. According to our previous study [5], however, C-11 oxidation of cortisol takes place in all the tissues investigated to larger extents. This corresponds well with the finding of Bradlow *et al.*, that in humans *in vivo* [11], the 11-hydroxy ⇄ 11-keto redox equilibrium is shifted more to the reductive state for 20β-dihydrocortisol than for cortisol. The reduction of ring A was limited to the liver. Cortol-20β was the major, and cortolone-20β was the minor product. In contrast to cortisol [5], only the 5β-isomers were formed. The side chain cleavage of 20β-dihydrocortisol was a quantitatively minor pathway observed only in the liver and the kidney, whereas cortisol underwent the reaction more readily in most of the tissues except the brain [5].

In the previous study [5], we have demonstrated the biosynthesis of 6β-hydroxy-20β-dihydro metabolites from cortisol in the liver. The results of the present study suggest that at least a part of the compounds could be derived from the C-20 reduced metabolites of cortisol which are subsequently 6β-hydroxylated. Rather puzzling is that, either from cortisol or from its 20β-dihydro metabolite, the predominant product was 6β-hydroxy-20β-dihydrocortisone. Whether C-11 oxidation occurring at the initial step facilitates the subsequent reaction(s) or whether the 11-hydroxy ⇄ 11-keto equilibrium of the final 6β-hydroxy-20β-dihydro compounds is shifted strongly to the oxidative direction remains to be elucidated.

Sulfoconjugation of 20β-dihydrocortisol was carried out in a number of tissues to larger extents than cortisol sulfation reported in the previous reports [4, 5]. The presence of 20α- and 20β-dihydrocortisol-21-sulphate was first demonstrated by us in human urine [2], human plasma [3] as well as in dog plasma [10], constituting an appreciable portion of all sulphate conjugated metabolites of i.v. administered [4-^{14}C]-cortisol. The results of the present study seem to support the previous deduction *in vivo* [2, 3] i.e. that the steroids with glycerol side chain are better substrates for the pertinent sulphokinases than the corresponding compounds with dihydroxyacetone side chain.

No glucuronide conjugates were detected in significant amounts. *In vivo*, however, a large fraction of i.v. or orally administered 20β-dihydrocortisol is reduced at ring A and excreted as glucuronide conjugates [6, 9]. The discrepancy between *in vitro* and *in vivo* glucuronide conjugation was discussed elsewhere [5].

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