DEVELOPMENT OF A SPECIFIC RADIOIMMUNOASSAY FOR CORTISOL 17-BUTYRATE

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ABSTRACT

We describe the development and validation of an assay for cortisol 17-butyrate in blood in which there is no significant cross reaction with endogenous corticosteroids at levels encountered normally in man. Preliminary data on blood levels of the drug in absorption studies are presented.

Cortisol 17-butyrate is a synthetic topical corticosteroid widely used in the treatment of skin disorders (1) such as eczema and psoriasis. It was designed to avoid the side effects of the potent fluorinated corticosteroids, but to be more effective than cortisol which is only poorly absorbed through the skin. It has been suggested (2) that esterification of the cortisol would give better skin penetration and therefore enhance the topical effect. Following absorption, plasma and tissue esterases would quickly hydrolyse the ester so that the systemic effect would be no greater than that of cortisol.

Cortisol 17-butyrate is available as a 0.1% ointment or cream (Locoid[R], Gist-Brocades). Two groups (2,3) have reported that 60g Locoid cream applied under polythene occlusion to volunteers does in fact reduce circulating cortisol and 17-oxogenic and 17-oxosteroid levels within 24 hours. They also demonstrated other indirect evidence of central effects, e.g. on circulating eosinophil counts. However, there is no direct information on the rate of release of the compound into the circulation, blood levels or clearance rate. Autoradiography has shown that dense accumulations still persist in the horny layer of skin 24 hours after application (3) but as with studies in the mini-pig

using radioactively labelled drug (4) the results do not reveal whether the radioactivity present is due to the original steroid ester or its hydrolysis or transesterification products. On chemical grounds, cortisol 17-butyrate would be expected to show relatively rapid hydrolysis and/or transesterification (to the 21 isomer) under quite mild conditions. In vitro studies of cortisol 17-butyrate incubated in human or dog plasma (2) indicated a half life of 6 hours, whereas following a very indirect in vitro study a half life of 1.3 hours was suggested in rats (2).

The development of a specific radioimmunoassay of cortisol 17-butyrate in body fluids is of course made more difficult by the relatively high normal levels of corticosteroids. This paper reports the development of an assay of sufficient specificity to measure cortisol 17-butyrate directly which permits a variety of direct studies on the absorption, circulating plasma level and clearance rates of the drug.

MATERIALS AND METHODS

Solvents and Solutions

All organic solvents were of analytical grade (BDH Chemicals Ltd.) and all except diethyl ether were redistilled before use. The liquid scintillant used contained 0.6g 2,5-diphenyloxazole (PPO) in 1 litre toluene and 0.5% Triton X-100. Sodium phosphate buffer (0.13 M) pH 7.2 containing 0.1% gelatine and 0.01% sodium azide was used for the assays. Dextran coated charcoal suspension was used to separate the antibody-bound from free fraction and was prepared by suspending 0.25g Dextran T 70 and 2.5g Norit A charcoal in 1 litre of phosphate buffer.

Steroids

Cortisol 17-butyrate and (4-^{14}C) cortisol 17-butyrate (0.7 Ci/mol), were gifts from Gist-Brocades, Delft, Holland. Other non-radioactive steroids were obtained from Sigma Chemical Co., Poole, England. (1,2,3,7-^{3}H) - cortisol (87 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, U.K.

Melting points were determined on a Kofler hot-stage microscope. UV spectra were recorded on a Perkin Elmer 402 ultraviolet-visible

spectrophotometer; IR spectra on a Perkin-Elmer 237 grating infrared spectrophotometer; NMR spectra were obtained on a Perkin-Elmer R12B (60 MHZ) NMR spectrometer or a Perkin-Elmer R32 (90 MHZ) instrument and mass spectra were recorded on an A.E.1. MS 12.

GLC was performed on a Perkin-Elmer analytical gas chromatograph F11. Analytical TLC was carried out on Merck precoated silica gel 60 F 254 plates; (ethyl acetate hexane 2:1 (x2)); HPLC was performed with a Waters Associates M6000 or Dosapro pump using either a Perkin-Elmer LC55 or an Altex analytical UV detector model 153. Radiochromatograms were recorded on a Packard 7201 Radiochromatogram Scanner.

Preparation of steroid conjugate

Cortisol 17-butyrate-3-(O-carboxymethyl)-oxime was prepared essentially by the method of Erlanger et al (5). The (4-^{14}C)-cortisol 17-butyrate (0.7 Ci/mol) was diluted with carrier (288 mg) and reacted with carboxymethoxyloxime hemihydrochloride (CBMO, 165 mg) in aqueous dioxane (50%) at pH 9. After 60 min, further CBMO (165 mg) was added and the pH lowered to 5.6 with sodium acetate in methanol. The reaction was followed by HPLC on aliquots of the mixture and reached completion in 2 hours. After initial concentration and addition of water (20 ml) the crude product was extracted with ethyl acetate. The geometric isomers (anti:syn approximately 2:1) were separated by TLC. Identifications of the separated syn and anti isomers were made from the correspondences of critical UV and NMR measurements with those for the corresponding isomers in the cortisol series (6).

Anti-isomer (Rf. 0.6-0.8)

After elution with ethyl acetate containing 0.5% acetic acid, the isomer was recrystallised from ethyl acetate to give a first crop of crystals, m.p. 186-8° (decomp), yield 160 mg (48%), specific activity 8 Ci/mmol {uvλ_m 252 nm, ε_m 2.16 x 10^4; nmr ($CDCl_3$) $C_{(4)}$-H at τ 3.6 (s); ms, M^+/e 519 ($C_{28}H_{41}O_8N$), ^{m}/$_e$ 431 (loss of butyrate)}. The syn-isomer (Rf 0.3-0.5) failed to crystallize; yield 85 mg (25%) {uv λ_m 249 nm; nmr ($CDCl_3$) C(4)-H at τ 4.3 (s); ms as anti-}.

Preparation of the Hapten

The pure anti-oxime (21 mg) was then coupled to bovine serum albumin using the mixed anhydride method (8) at pH 8 and 5°C. The cold mixed anhydride solution (540 µl) was rapidly added to cold swirled bovine serum albumin solution (10 ml, lysine equivalent 0.023 mmol); reaction was virtually complete in 5 min. Separation of the hapten was effected by ultrafiltration (Amicon cell, PM10 membrane) over 25 hr at 4°C, washing through with seven volumes of distilled water. Analysis of the eluates showed only the presence of the cortisol 17-butyrate, with less than 0.1% of the major impurity, which was cortisol 21-butyrate. The final concentrate (5 ml) was lyophilized to give cortisol 17-butyrate-3-anti-(O-carboxymethyl)-oxime bovine serum albumin conjugate (34.8 mg) {u.v. λ_m 250 nm (Tris buffer 0.05 M, pH 8.4)} with a calculated molecular ratio of hapten to protein of 27.5:1, based on radioactivity.

Synthesis of ^{3}H Cortisol 17-Butyrate

^{3}H Cortisol (4) in acetonitrile (CH_3CN) and its selective hydrolysis under mildly acidic conditions, control of the synthesis being achieved using an external standard method.

(1,2,6,7,^{3}H)-Cortisol (sp act 85 Ci/mmol) as supplied had 2.12 µg unlabelled cortisol for every 0.5 mCi. Three standards were prepared simultaneously and in an identical manner in silanized reaction vials containing 10 µl acetonitrile. Two, A and B, contained 2.12 µg unlabelled cortisol each. The third C, held 2.12 µg radioactive cortisol. Freshly prepared catalyst, (p-toluene sulphonic acid in acetonitrile, 1 µl, 6.5 mg/ml) was transferred to each vial with 5 µl CH_3CN as transferring solvent in the order A,B,C at 10 min intervals. One minute after catalyst addition, trimethyl orthobutyrate (5 µl) was added to each vial. Using HPLC monitoring of aliquots from A, formation of the butyrate was shown to be complete in 13 min; at the corresponding time of 2 µl 0.006 N HCl was added to B and C. Using aliquots of B to monitor in the same way, complete hydrolysis of the orthobutyrate ester was shown to occur in 14 min, and the reaction products in C were worked up at the corresponding time. The radioactive hydrolysis products formed in C were separated by TLC. The three uv absorbing bands, corresponding to cortisol, cortisol 17-butyrate and 21-butyrate were carefully removed and eluted with CH_3CN/toluene (1:1). Small aliquots (1 µl) of each fraction were (a) measured for radioactivity and (b) rechromatographed and scanned for radioactive purity. This procedure gave ca 2 µg of cortisol 17-butyrate (yield 77.3%) and ca 0.5 µg of cortisol 21-butyrate (yield 19%). The specific activities of the products were unchanged at 87 Ci/mmol.

Stability Studies

1. Organic Solvents : Solutions of cortisol 17-butyrate (1 ml/ml) in dry toluene, dry acetonitrile and dry (absolute) ethyl alcohol were prepared and allowed to stand for 0.5 - 14 days at temperatures from -20°C to 60°C; aliquots were then analysed by TLC on SiO_2.

2. Storage and extraction of blood samples : Serum, plasma and blood samples were treated as shown in Table 1. The extraction products were analysed by TLC on SiO_2. Recoveries and transformation products are shown in Table 1.

Immunological Procedures

Immunisation : Antisera against the cortisol 17-butyrate-3-(O-carboxymethyl)oxime were obtained from rabbits. The hapten (1 mg) was injected in 1:1 v/v 0.9% NaCl : Freunds complete adjuvant (in stable emulsion) at multiple intradermal sites. The injection of hapten (0.2 mg) with Freunds' incomplete adjuvant was repeated four weeks later and blood samples drawn from the marginal ear vein seven days later. Monthly booster injections with Freunds' incomplete adjuvant and bleeding schedules were maintained for nine months.

Characterisation of Antisera

Radioimmunoassay (RIA) : A conventional RIA procedure was followed except that stock label and stock standard solutions were stored in toluene/acetonitrile (1:1 v/v) and acetonitrile respectively and diluted immediately prior to assay. The buffer used in the assays was 0.13M sodium phosphate buffer pH 7.2 containing 0.1% NaCl, 0.01% sodium azide and 0.1% gelatine.

In each assay tube 100 µl of antiserum (diluted to give 50% binding of the tritiated cortisol 17-butyrate (75 fmol/100 µl) was incubated with 100 µl buffer solution containing various amounts of unlabelled homologous or heterologous steroid. After mixing, incubation was at room temperature for 90 minutes and 30 minutes at 4°. Bound and free steroids were separated by dextran coated charcoal at 4°C; 1 ml phosphate buffer containing 0.25% w/v Norit charcoal and 0.025% dextran was added to each tube, mixed thoroughly and centrifuged at 1000 x g (4°C) ten minutes after addition of charcoal to the last tube. The supernatant was decanted into a counting vial and counted for 10 minutes.

All determinations were made in duplicate. Association constants were determined from Scatchard plots.

Specificity Studies : The degree of cross reactivity was expressed on the basis of the mass of each steroid required to produce 50% displacement of the labelled steroid. Percent cross reaction equals 100 x/y where x = the mass of hormone required to displace 50% of the bound homologous ^{3}H hormone, y = the mass of cross reacting steroid required to displace 50% of the ^{3}H steroid.

Sample Preparation

The procedure used was based on the results shown in stability tests (Table 1) and was as follows:

Acetonitrile (2 ml) was added to each blood sample (1 ml) immediately after collection, well shaken and stored at -20°C in tightly capped tubes until required when an aliquot of the supernatant (2 ml) was directly extracted with diethyl ether (5 ml), with simultaneous addition of sodium chloride solution (1 ml). Recoveries were determined by adding 500 cpm ^{3}H cortisol 17-butyrate label per sample. The ether extract was quickly evaporated at RT under a stream of N_2, redissolved in 0.6 ml of buffer, mixed thoroughly and after 30 minutes the ester was assayed as described above using 100 µl of the extract.

Cortisol levels in the acetonitrile supernatants were measured by direct radioimmunoassay of the ether extract. The results from samples prepared as above were correlated with those obtained by the direct assay of serum from duplicate blood samples which had been allowed to clot. Data on cortisol levels measured directly and on the acetonitrile supernatants from 78 samples showed a correlation coefficient of 0.967 ($p < 0.001$) (7).

Clinical Study

A short clinical study was performed to evaluate the percutaneous absorption of cortisol 17-butyrate in six healthy adult volunteers. Treatment comprised twice-daily applications of 30g of 0.1% Locoid^{R} cream to 80% of the body surface; the cream was rubbed in for 5 minutes and polythene suits covering the treated surface were worn for 12 hours daily. Blood samples were taken at 09.00h and 09.30 for three consecutive days before treatment, for six consecutive days during treatment, and for three days thereafter. Detailed results of this and other clinical studies will be published elsewhere.

RESULTS

Stability Studies

1. In organic solvents : Cortisol 17-butyrate was found to be chemically and radioactively stable in toluene, acetonitrile or toluene/acetonitrile (1:1) when stored at either -20°C for 14 days or 60° for three days. Only 1-2% was hydrolysed to cortisol and < 1% was transesterified to the 21-butyrate. However, in ethanol nearly complete conversion to the 21-butyrate occurred during 3 days at 60°.

2. Storage and extraction of blood samples : The results of nine experiments are shown in Table 1. Total recoveries ranged from 70-100%. The considerable transformation and hydrolysis seen in samples C and D are probably ascribable to the action of esterase enzymes in the heparinized blood, whereas the decomposition seen in samples A and B is due to the instability of cortisol 17-butyrate in ethanol. When acetonitrile was used as the solvent (E and F) both transesterification (≅ 5%) and hydrolysis (1%) were minimised even at room temperature for 4 days. Extraction and recovery were equally satisfactory when high specific activity label (87 Ci/mmol) and nanogram amounts of steroid were used (samples G, H and I).

Assay Validation

A typical standard curve with serum 39/5 for cortisol 17-butyrate

is included in Figure 1. 50% inhibition of binding of the labelled cortisol 17-butyrate was produced by 130 pg of cortisol 17-butyrate. The equilibrium constant at 4°C was 8.8 x $10^9 M^{-1}$. Antisera from other

Table 1

Transesterification and hydrolysis of cortisol 17-butyrate in blood samples under varying conditions and in different solvents

Condition*	17-butyrate added		Percentage Recovery+ as:		
	(µg)	(dpm x 10^4)	Cortisol	17-butyrate	21-butyrate
A	2660	0.9	2	66.4	6.0
B	2660	1.02	12	57.8	4.6
C	2660	1.0	45	19.6	5.6
D	2660	0.8	61	29.0	8.3
E	245	4.0	0.5	86.7	0.7
F	245	15.0	0.6	87.5	0.2
G	0.100	100	0.4	83.2	0.5
H	0.010	100	0.6	76.7	1.0
I	0.001	100	1.4	71.4	5.0

*Conditions were:

A - Fresh blood (10 ml) added to ethanol (20 ml); centrifuged.
B - Heparinized bloods (10 ml) 2h at 15°; ethanol (20 ml) added, centrifuged.
C - Heparinized blood (10 ml) 2h at 15°; centrifuged.
D - Heparinized blood (10 ml) 2h at 15°; 2 weeks at -20°; then ethanol (20 ml) added; centrifuged.
E - Fresh blood (10 ml) added to acetonitrile (20 ml), 4 days at 15°; centrifuged.
F - Fresh blood (0.5 ml) added to acetonitrile (1 ml); centrifuged and supernatant stored 15 days at -20°.
G,H,I - Fresh blood (10 ml) added to acetonitrile (20 ml), 4 days at 15°; centrifuged.

+ - All supernatants were extracted with ether (2 vols) after adding saturated sodium chloride (0.2 vols).

rabbits (see Table 2) showed similar specificity but a slightly lower titre. The sensitivity of the assay (defined as the minimal concentration detectable with 95% certainty) was approximately 30 fmol, giving a working sensitivity of approximately 30 pmol/l. A small but significant blank was identified as originating from ether used in preparing the samples and this was eliminated when standards were added to tubes from

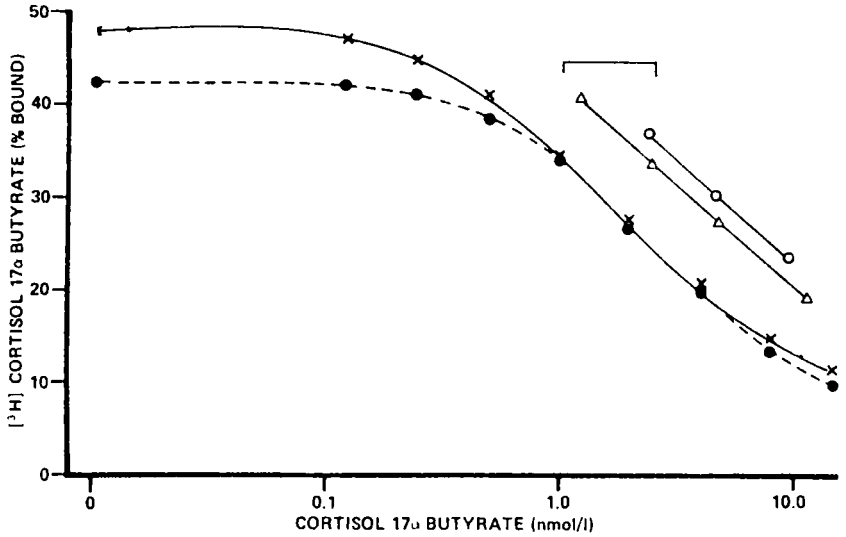

Figure 1 - Standard curve for cortisol 17-butyrate in the presence of diluent standards (x) and ether extracted diluent standards (●). o——o and Δ——Δ Serial 2-fold dilutions of two selected supernatants from acetonitrile-treated blood.

which the same volume of ether was evaporated. Serial dilutions of the plasma extract containing 17-butyrate from subjects to whom the steroid has been administered (vide infra) gave value results parallel to the standard curve (Figure 1). Mean recovery of added ^{3}H-cortisol was 90%. The coefficient of variation for duplicates within the same assay was ± 10.4% and for replicates in different assays ± 15.2%.

Clinical Studies

The results from a single "occlusion study" on a normal volunteer are shown in Table 3. Cortisol 17-butyrate was detected in the blood the morning following the first application of the ointment and remained elevated up to 24 hours after treatment was stopped, after which it disappeared rapidly from the circulation.

Cortisol levels determined from the same acetonitrile supernatants

Table 2

Percent cross reactivity data*

Steroid	Rabbit 39/5	Rabbit 2/3
Cortisol 17-butyrate	100	100
Cortisone 17-butyrate	<5	100
11-Deoxycortisol 17-butyrate	>5	83
Prednisone 17-butyrate	50	42
Cortisone 21-butyrate	<0.01	<3.5
11-Deoxycortisol 21-butyrate	5	14
Prednisone 21-butyrate	<0.001	<0.001
Dexamethasone	<1%	<1%
Cortisol	<0.001	<0.001
Prednisone	<0.001	<0.001
17-hydroxyprogesterone	<0.001	<0.001
Pregnenolone	<0.001	<0.001
17-hydroxypregnenolone	<0.001	<0.001

* Read at 50% inhibition of binding relative to cortisol 17-butyrate. Final dilutions of the antibodies were : 39/5, 1:3000; 2/3, 1:2400.

correlated well with those from serum samples from blood collected at the same time, and in this subject the cortisol levels declined during the occlusion study.

Table 4 shows serum cortisol and cortisol 17-butyrate levels of six normal volunteers who ostensibly applied Locoid^{R} under occlusion for 6 days. Subjects 1-4 all showed significant absorption of cortisol 17-butyrate and in 3 of these cases there was a significant fall in serum cortisol. Subjects 5 and 6 showed no apparent absorption; since we have subsequently investigated 40 patients in similar trials (7), as well as 6 normal volunteers without occlusion and all have shown significant absorption (less in the volunteers without occlusion but still significant) we conclude that subjects 5 and 6 in Table 4 did not in fact apply the ointment as instructed.

DISCUSSION

The radioimmunoassay described in this paper has made possible the

Table 4

Serum cortisol and cortisol 17-butyrate levels before and during treatment under occlusion (n = no. of duplicate assays).

Volunteer no.	Pre-treatment			Per-treatment						Difference test A/B	Correlation Coefficient B/C
	A : Cortisol (nmol/l)			B : Cortisol (nmol/l)			C : Cortisol 17-butyrate			t	r
	n	Mean	(SD)	n	Mean	(SD)	n	Mean	(SD)		
Apparent absorbers											
1	6	271	(52)	12	212	(54)	10	4.1	(1.0)	2.22**	0.13
2	6	327	(74)	12	184	(86)	7	5.7	(8)	4.05***	0.08
3	4	179	(52)	11	170	(66)	11	1.2	(0.6)	0.25	-0.56*
4	5	343	(71)	11	235	(55)	11	3.3	(1.0)	3.33**	-0.61*
Apparent non-absorbers											
5	6	202	(19)	12	205	(68)	not detected			-0.12	-
6	8	357	(68)	10	323	(60)	not detected			1.18	-

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 3

The effect of external applications of cortisol 17-butyrate to the skin of a healthy volunteer on cortisol 17-butyrate and cortisol levels in the blood

Day		Cortisol 17-butyrate	Cortisol+ Blood	Cortisol+ Serum
Pre-treatment	1	<0.04	396	
" "	2	<0.04	374	
" "	3	<0.04	420	400
Treatment	1	3.0	327	
"	2	3.6	362	
"	3	3.5	240	
"	4	3.0	300	
"	5	4.6	215	
"	6	5.0	228	225
Post-treatment	1	4.8	174	170
" "	2	0.2	588	
" "	3	<0.04	441	430

Results, in nmol/l are means for two samples, taken at 09.00h and 09.30 h on the indicated day, assayed in duplicate with ^{3}H-cortisol 17-butyrate (500 cpm/ml) added to each, and corrected for losses during work-up. Serum samples were assayed directly; whole-blood assays were carried out on the acetonitrile supernatants and are corrected for the resultant volume change.

first direct pharmacological studies of cortisol 17-butyrate.

Preparation of the hapten, required for raising the specific antibody, needed close chemical control to ensure that the steroid eventually bound to the hapten was the one intended. Cortisol is the major corticosteroid in human blood and any hydrolysis of the butyrate ester at any stage in the synthesis of the hapten or production of the antibody would have seriously reduced the probability of obtaining an antibody with the necessary specificity. Furthermore, since the formation of cortisol from the 17-ester occurs via transformation of the acyl group from C-17 to C-21, this isomerization must be avoided, since the presence of the 21-butyrate in the hapten would similarly interfere with the specificity. A quantitative yield of product was obtained

during the selective oximation at C-3, so that no hydrolysis or transesterification had occured at this stage; the ratio of syn and anti geometric isomers was about two to one. Preparation of the bovine serum albumin conjugate from only one (anti) of the separated isomers should also lead to higher specificity. This was achieved by working in buffer solution at pH 8 instead of the usual more alkaline solution, in which considerable interconversion of isomers would have been expected. Use of the buffer solution also reduced precipitation of protein by the carbodiimide in the Erlanger method. The introduction of ultrafiltration as a method for separating the conjugate from unbound steroid is an innovation which offers several advantages in that (a) the separation time is shorter than in the conventional dialysis method, (b) the free steroids could easily be recovered and checked directly (by HPLC) for any change in the hapten and (c) the conjugate could be lyophilised directly without further purification.

Synthesis of the tritiated cortisol butyrate used in the actual assay procedures required work on a very small scale (3 μg) and with as few steps as possible to avoid losses. HPLC and TLC were used to monitor the reactions involved, optimise conditions and isolate the product. The route finally chosen involved the cyclic ortho ester route in which commercially available ^{3}H-cortisol (sp act 87 Ci/mmol) is converted to methyl orthobutyrate and then hydrolysed.

The suspected ease of transesterification and hydrolysis of the cortisol 17-butyrate required studies of the stability of the steroid both in organic solvents and in blood. The results obtained showed that transesterification and hydrolysis both occur at room temperature and even during storage at -20°C. However, whereas the hydrolysis of

cortisol acetate in whole blood has been found to be inhibited by collection of blood samples in ethanol (9), cortisol 17-butyrate underwent a significant degree of transesterification in this solvent. Acetonitrile proved to be the solvent of choice for inhibiting such reactions after collection of blood.

The critical cross reactions of the antibody with the main plasma corticosteroid, cortisol, which is also the hydrolysis product of cortisol 17-butyrate, and with its transesterification product, cortisol 21-butyrate, were both < 0.001%. Although the antisera produced showed some high cross reactions with other 17-butyrates or 21-butyrates, such esters are not normally present in the body and hence produce no problems in the assay. The specificity of the antiserum is indeed sufficiently high for assays to be performed directly after ether extraction without any chromatographic separation from cortisol, and the assay gives acceptable intra (10.4%) and interassay (15%) precision. It has been shown previously that normal plasma corticosteroid levels were suppressed during Locoid^{R} treatment and returned to normal within two days after treatment was stopped (3,5). In our preliminary study we confirm this finding and have been able to correlate it inversely with the levels of blood cortisol 17-butyrate in two of the six volunteers studied. The relatively low circulating levels of cortisol 17-butyrate account for the failure to detect the steroid by gas chromatography-mass spectrometric analysis (4).

The availability of this assay has made possible a series of clinical studies on the absorption, circulation and clearance rate of this topically-applied drug and their correlation with effects on the pituitary adrenal axis.

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APPENDIX

IUPAC Nomenclature:

11-deoxycortisol: 17,21-dihydroxy-4-pregnene-3,20-dione
prednisone: 17,21-dihydroxy-1,4-pregnadiene-3,11,20-trione
dexamethasone: 9-fluoro-11β,17,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione
17-hydroxyprogesterone: 17-hydroxy-4-pregnene-3,20-dione
pregnenolone: 3β-hydroxy-5-pregnen-20-one
17-hydroxypregnenolone: 3β,17-dihydroxy-5-pregnen-20-one.