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QUANTITATIVE DETERMINATION OF CORTISOL IN HUMAN PLASMA BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

FRIEDRICH K. TREFZ, DENNIS J. BYRD and WALTER KOCHEN
Radioisotope Division, Universitäts-Kinderklinik, Im Neuenheimer Feld 150, 6900 Heidelberg (G.F.R.)
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SUMMARY

A method is described for the determination of cortisol in human plasma by high-pressure liquid chromatography. The simplified extraction procedure makes the method applicable to routine clinical assays. Partition chromatography is carried out on a Zorbax-Sil column with the eluent system dichloromethane–ethanol–water. A 78% recovery was obtained for cortisol. The detection limit is 1 μg per 100 ml in 1 ml of plasma. Cortisol values were determined in samples from a random selection of patients.

INTRODUCTION

It has recently been demonstrated that high-pressure liquid chromatography (HPLC) is suitable for the analysis of synthetic mixtures of steroids[1–6]. HPLC has also been used to assay cortisol and corticosterone in biological materials[1,7,8]. This paper reports experiences with the quantitative determination of cortisol in human plasma by HPLC.

EXPERIMENTAL

Materials and equipment

All reagents (p.a. grade) were purchased from Merck, Darmstadt, G.F.R., and were not further purified.

Reagents. Dichloromethane, absolute ethanol, anhydrous sodium sulphate, 0.05 *N* sodium hydroxide saturated with sodium sulphate; standard solutions of cortisol and prednisolone in dichloromethane (20 ng/μl).

Standard steroid mixture. 17-Hydroxyprogesterone, deoxycortisol, corticosterone, cortisone, prednisone, cortisol and prednisolone in dichloromethane (20 ng/μl).

Equipment. Centrifuge test-tubes, 90 × 40 mm; 50-ml round-bottomed flask; 100-ml separating funnel; reaction vials (Serva, Heidelberg, G.F.R.).

Instruments. DuPont Model 820 high-pressure liquid chromatograph (DuPont, Wilmington, Del., U.S.A.); Chromatronix 200 (254 nm) and Schoeffel SF 770 (variable

wavelength) UV detectors; Honeywell two-channel compensation recorder. Samples were injected with a sample loop (DuPont) with an effective volume of 52 μl. In addition, we used a Siemens S 200 liquid chromatograph (Siemens, Karlsruhe, G.F.R.) with a PM 4 spectrophotometer (Zeiss, Oberkochen, G.F.R.).

Column. Zorbax-Sil, 250 × 2.1 mm (DuPont). The eluent system dichloromethane–ethanol–water was used according to Hesse and Hövermann[6]. The eluent was prepared as follows: 12 ml of double-distilled water and 20 ml of absolute ethanol were made up to 1000 ml with dichloromethane. The mixture was stirred for 2 h at 20° with a magnetic stirrer and then transferred to a separating funnel and allowed to stand until the emulsion had cleared. After separation, the organic phase was ready for use.

Preparation of blood samples

Fresh venous blood is centrifuged (3000 rpm, 10 min) in heparinized tubes, then 1.0 ml of plasma is removed with an Eppendorf pipette and transferred into a 90 × 40-mm centrifuge tube together with 0.5 ml of ethanol, 1.0 ml of double-distilled water an 10 μl of the internal standard (200 ng of prednisolone) with a Hamilton syringe. The mixture is stirred for 3 min with a magnetic stirrer (2 cm). A 10-ml volume of dichloromethane and 0.25 g of anhydrous sodium sulphate are added and stirring is continued for 15 min. The organic phase is separated by centrifugation, removed with a 20-ml glass syringe, transferred into a separating funnel and collected in a 50-ml round-bottomed flask. The aqueous phase is washed from the separating funnel back into the centrifuge tube with 10 ml of dichloromethane, and the previous 15-min stirring and separation step is repeated. The total organic phase is washed in a separating funnel with 1.0 ml of 0.05 *N* sodium hydroxide saturated with sodium sulphate, transferred into a round-bottomed flask and evaporated to dryness in a rotary evaporator at 40°. The residue is dissolved in *ca.* 5 ml of dichloromethane, transferred into the reaction vial and evaporated to dryness in a stream of nitrogen. The reaction vial is then closed with a septum-fitted screw-cap. The residue is dissolved in 200 μl of dichloromethane introduced through the septum with a Hamilton syringe. Samples (*ca.* 160–180 μl) for chromatography are withdrawn with a 0.5-ml glass syringe, which is fitted with a 6-cm needle.

Chromatography

After equilibrating the column until the base-line is stable, the sample is injected into the sample loop with the syringe. The column flow-rate at 20° is 0.45 ml/min, which corresponds to a pressure drop of 1500 p.s.i. in the column used.

RESULTS

A standard mixture of seven steroids was used to test the efficiency of the column. The chromatogram recorded at 239 and 254 nm presented in Fig. 1 shows that a good separation of the compounds was achieved. The ratio between the peak areas at 239 and 254 nm is constant; the ratio for cortisol is 1.54 (see Table III).

Figs. 2A and 2B show the chromatograms of the same blood sample from a child with a normal cortisol level, before and after addition of 113 ng of authentic cortisol. The other peaks in the chromatogram have not yet been identified. They

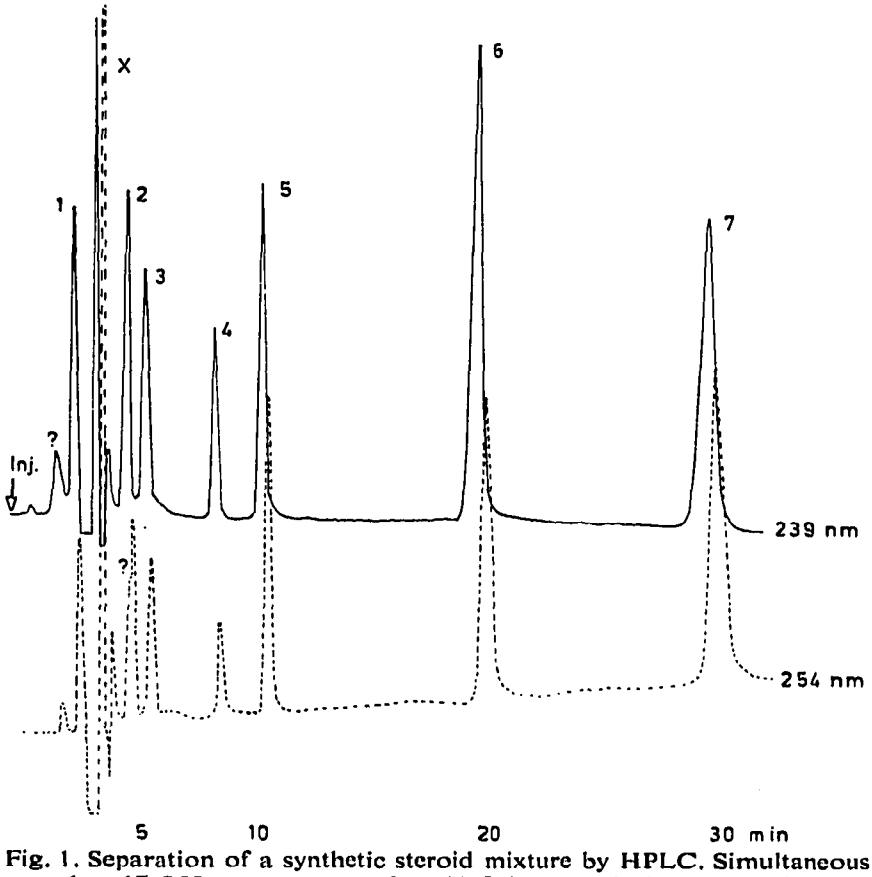

Fig. 1. Separation of a synthetic steroid mixture by HPLC. Simultaneous recording at 239 and 254 nm. 1 = 17-OH-progesterone; 2 = 11-β-deoxycortisol; 3 = corticosterone; 4 = cortisone; 5 = prednisone; 6 = cortisol; 7 = prednisolone; X = artefact. Column, Zorbax-Sil, 250 × 2.1 mm; eluent, dichlormethane–ethanol–water (see text); flow-rate, 0.45 ml/min; temperature, 20°; sample injection, sample valve with 52-μl sample loop.

appear to represent acidic compounds present in blood as their appearance and intensity are influenced by washing the organic phase with sodium hydroxide.

Quantitation of cortisol

The linearity and sensitivity of the detector response (Schoeffel detector) were determined for cortisol and the internal standard prednisolone. The standard calibration curve is shown in Fig. 3. Fig. 4 shows that under the conditions employed, 0.5 ng of cortisol can be quantitated unequivocally. The limit of detection of cortisol in plasma is 1 μg per 100 ml with an error of ±5%. The favourable signal-to-noise ratio of 32:1 (found when 52 μl or 2 ng of the 200-μl extract of 1 ml of serum are injected through the sample loop) allows quantitation at this low level.

Cortisol in plasma was quantitated as follows. The peak area was determined by calculating the product of the height and the width at half the peak height. The amount of cortisol was read from the standard calibration curve and corrected against the internal standard.

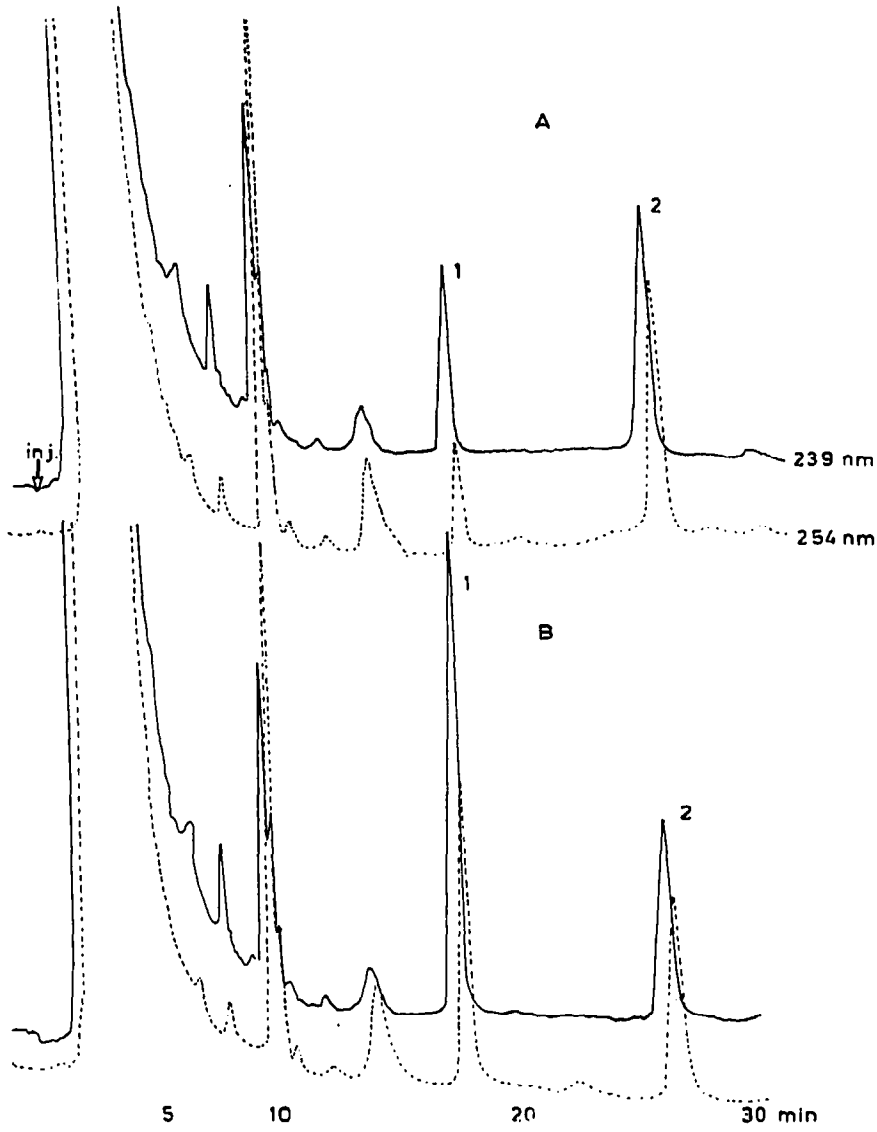

Fig. 2. A, Chromatogram of the plasma extract of a healthy child. B, Chromatogram of the same sample as in A after addition of 113 ng of cortisol prior to extraction. 1 = Cortisol; 2 = internal standard (prednisolone). Chromatography as in Fig. 1. Recording at 239 nm, Schoeffel SF 770 detector, 0.02 A.U.; Recording at 254 nm, Chromatronix 200 detector, 0.01 A.U.

Recovery

The results of recovery experiments on the internal standard prednisolone (78.8%) and cortisol from plasma (78.0%) are given in Tables I and II.

Table III presents the results of the determination of cortisol in plasma from randomly selected patients at this hospital and from healthy controls. Included for comparison are cortisol values determined on the same samples by the radio-

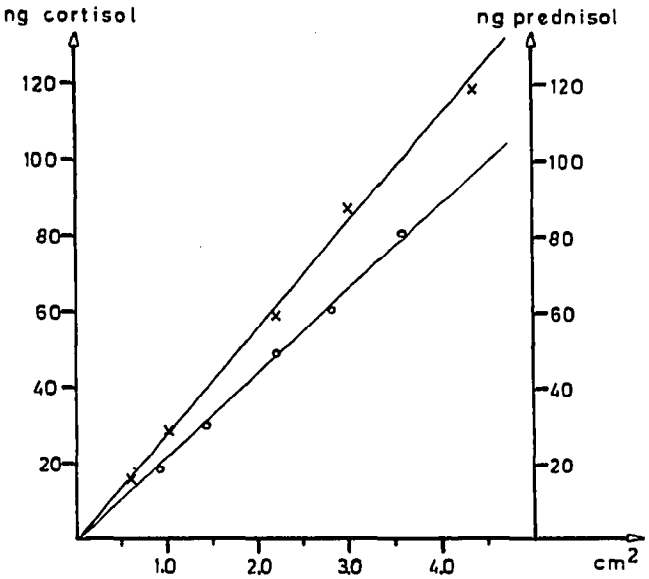

Fig. 3. Calibration curves for cortisol (○) and prednisolone (×) at 239 nm.

immunological method. In general, the values obtained by HPLC are slightly higher than those from the radioimmunological method: the average value for cortisol by HPLC is 11.1 μg per 100 ml, whereas the radioimmunological value is 7.1 μg per 100 ml. There is one noteworthy exception: in the case of patient No. 15, who was receiving 5 mg of decortin per day, no cortisol could be detected by HPLC, as was to be expected, but the radioimmunological assay gave a value of 6.8 μg per 100 ml.

Fig. 5 shows the HPLC chromatograms of cortisol in patient No. 15's blood

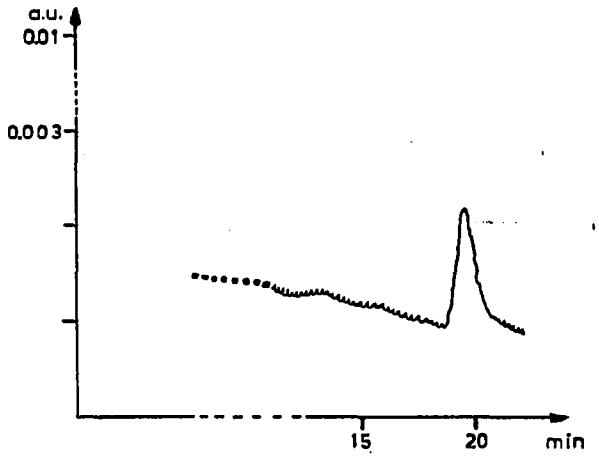

Fig. 4. Detection of 0.52 ng of cortisol; signal-to-noise ratio = 16:1 at 239 nm, 0.01 A.U. (Schoeffel SF 770 detector).

TABLE I

RECOVERY OF THE INTERNAL STANDARD (PREDNISOLONE) AFTER ADDITION OF 201.6 ng PRIOR TO EXTRACTION

Standard deviation $= \left[\frac{\Sigma(\bar{x} - x)^2}{n - 1}\right]^{\frac{1}{2}}$

Sample No.	*Prednisolone*		*Recovery (%)*
	cm²	*ng*	
15	1.42	154.8	76.7
16a	1.43	155.9	77.2
16b	1.56	170.0	84.3
16c	1.50	163.5	81.0
17	1.44	156.9	77.8
18	1.43	155.9	77.2
19	1.62	176.5	87.5
20	1.45	158.0	78.3
21	1.46	159.1	78.9
22	1.44	156.9	77.8
23	1.43	155.9	77.2
24	1.41	153.6	76.2
25	1.43	155.9	77.2
27	1.42	154.7	76.7
Mean			78.8
Standard deviation			±3.2

TABLE II

RECOVERY OF VARIOUS AMOUNTS OF AUTHENTIC CORTISOL ADDED TO PLASMA PRIOR TO EXTRACTION

Standard deviation (see Table I).

Sample No.	*Cortisol added (ng)*	*Value before spiking (ng)*	*Value after spiking (ng)*		*Recovery (%)*
			Actual	*Theoretical*	
9	53	93.8	120.0	146.8	81.7
17	103	10.0	89.0	113.0	78.7
18	113	61.3	138.6	174.3	79.5
27	71	72.2	113.4	143.6	78.9
27	113	72.2	135.5	185.2	72.0
27	119	72.2	142.8	191.2	74.6
27	190	72.2	212.5	262.4	80.9
Mean					78.0
Standard deviation					±3.6

during the therapy with prednisolone and after the therapy had been discontinued by gradually decreasing the doses.

The profiles of cortisol in two healthy persons during the day are presented in Table IV; they exhibit a typical decrease towards the evening.

TABLE III

PLASMA CORTISOL VALUES OBSERVED IN ENDOCRINOLOGICALLY HEALTHY (E.H.) CONTROLS AND IN SOME RANDOMLY SELECTED PATIENTS

Values are compared with results of a radioimmunological assay.

Subject	*Age (years)*	*Plasma-cortisol (μg per 100 ml)*		*Ratio of cortisol peak areas at 239 and 254 nm*	*Remarks*
		HPLC	*Immunoassay*		
18	11	7.9	5.3	1.68	E.H.
19	0.33	18.2	7.3	1.52	E.H.
20	17	8.9	—	1.52	E.H.
21	13	11.1	12.5	1.52	E.H.
22	10	13.7	6.5	1.60	E.H.
23	8	10.0	7.5	1.54	E.H.
24	8	11.7	8.1	1.60	E.H.
25	9	24.0	9.0	1.58	Adiposis
27	24	9.5	—	1.53	E.H.
11a	11	17.3	10.8	1.52	E.H.
11b		17.1	—	1.67	
13	9	10.0	6.8	1.54	E.H.
14	11	10.6	5.9	1.54	E.H.
17	9	1.1	0.1	—	E.H. Sample stored 3 days at 5°
12	0.67	4.3	2.5	1.47	PKU and hypothyreosis
15	2	0	6.8	—	Decortin, 5 mg/day
15	2	13.8	8.1	1.58	After decortin therapy
16a	29	5.1	2.1	1.47	E.H. Taken at 18.00 hrs
16b		4.3	—	1.44	
16c		4.3	—	1.50	
Mean		11.1 ($n = 20$)	7.1 ($n = 15$)	1.54 ($n = 18$)	

DISCUSSION

Several methods have been described for the determination of cortisol in human plasma. They can be subdivided into three general types: photometry after formation of derivatives[9], fluorimetry in sulphuric acid solutions[10] and protein binding reactions[11]. Pirke and Stamm[10,12] critically examined these three methods and found that only three out of nine fluorimetric procedures possessed sufficient specificity for cortisol. The competitive protein binding method of Murphy[11] enjoys widespread popularity[13] in routine clinical analysis because of its greater specificity in comparison to fluorimetric methods. The disadvantage of this method is that several naturally occurring steroids possess a high affinity for the corticosteroid-binding globulin. At low concentrations, these steroids do not disturb the determination. However, the protein-binding method is of only limited use in the case of newborns, pregnancy, adrenogenital syndrome and in the metopiron tolerance test[12]. In addition, the methodology of the protein-binding procedure is difficult and time consuming in some respects[13]. These same drawbacks are also inherent in the recently developed radioimmunological procedure for the determination of cortisol[14]. In view of these

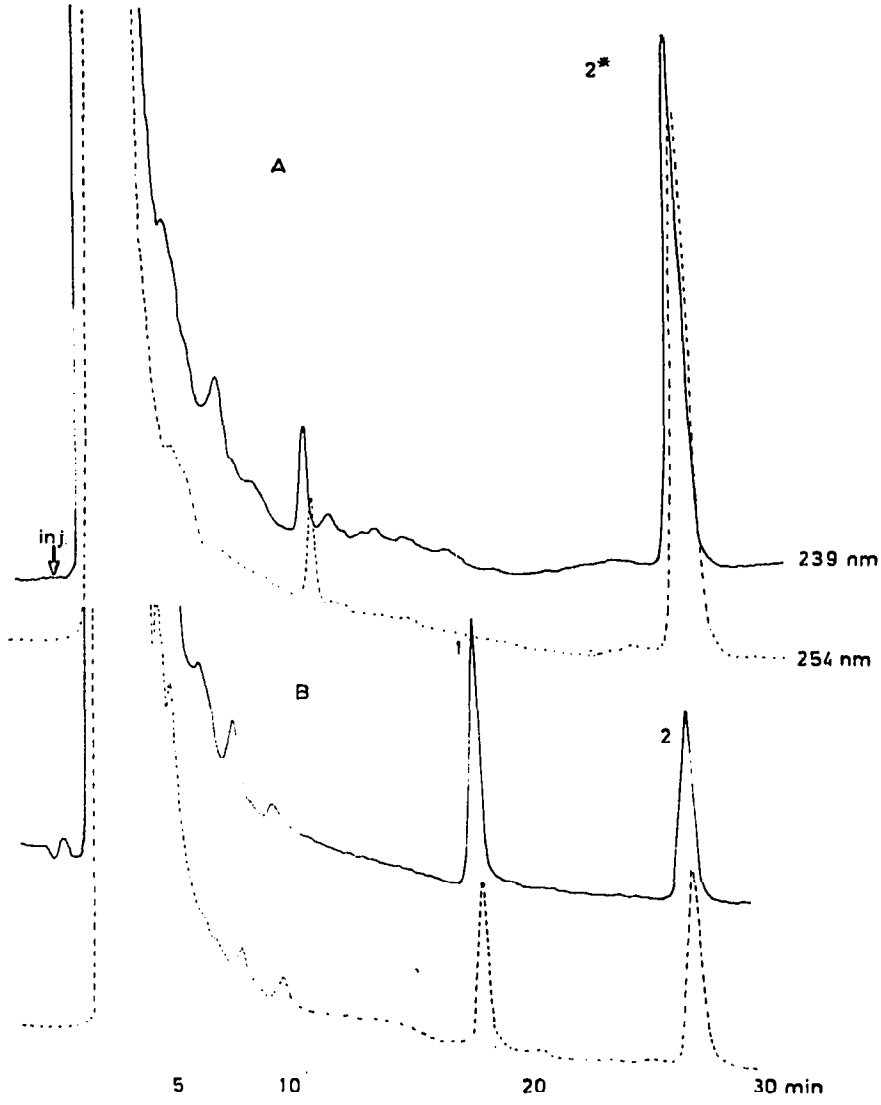

Fig. 5. A. Chromatogram of plasma extract from patient No. 15 during Decortin therapy (5 mg/day). B. Same patient 3 weeks later after gradual discontinuation of the Decortin therapy. 1 = Cortisol; 2 = standard prednisolone; 2* = standard prednisolone and prednisolone from plasma.

TABLE IV

EIGHT-HOUR PROFILE OF PLASMA CORTISOL IN TWO HEALTHY CONTROLS
Values determined by HPLC.

Time	*Cortisol (μg per 100 ml)*	
	Subject 1	*Subject 2*
9.00	15.5	21.0
11.30	10.8	11.7
14.30	9.0	9.6
17.00	6.1	8.2

difficulties, a search for a simpler but specific method of determination of cortisol in plasma appeared to be justified.

This example of the determination of cortisol in human plasma demonstrates that HPLC, despite the time required for an analysis, is suitable for clinical analysis in those cases where the isotope dilution methods are not applicable. Such a situation arose in the case of patient No. 15 (Table III), where obviously a reaction of the antibody with prednisolone led to false results[15]. The HPLC chromatogram reveals that only prednisolone and no cortisol is present in the patient's blood.

The sensitivity of the HPLC method is dependent on the capacity of the ultraviolet detector and the resolution of the column. Under the experimental conditions used, a serum cortisol concentration of 1 μg per 100 ml is easily quantitated (see Fig. 4). Sample loss is correctable with the internal standard. The disadvantage when using prednisolone is that the chromatography time is increased by 10 min.

In quantitative work, the introduction of the sample onto an HPLC column raises problems, especially when highly volatile solvents are used. The most accurate and convenient method of introduction is the sample loop, but it has the disadvantages of a relatively large dead volume and the fact that only a fraction of the sample volume actually enters the column.

The elution system dichloromethane–ethanol–water proved to be suitable when used with the Zorbax-Sil column. This is demonstrated by the efficiency of 5000 theoretical plates for cortisol at a pressure drop of 1500 p.s.i. and a flow-rate of 0.45 ml/min.

The resolution capacity of the column was not reduced even after the chromatography of more than 100 samples of test mixtures and plasma extracts.

The extraction of the plasma presents no problems. The formation of emulsions is easily prevented by the addition of ethanol and sodium sulphate to the sample prior to extraction. The addition of ethanol also had a positive influence on the recovery of cortisol. Extracting the sample twice proved to be sufficient.

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