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QUANTITATIVE DETERMINATION OF 18-HYDROXYDEOXYCORTICOSTERONE AND CORTICOSTERONE BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

High pressure liquid chromatography (HPLC) offers a rapid, qualitative as well as quantitative method for the determination of 18-hydroxydeoxycorticosterone and corticosterone. It is more rapid and more specific than existing methods for the estimation of these two steroids in rat adrenal incubation media. The limitation of the method lies in its sensitivity. The lowest limit of detection is 0.05 μg.

INTRODUCTION

Both 18-hydroxydeoxycorticosterone (18-OH-DOC) and corticosterone (B) are adrenal steroids native to several species including rat and man. The observations that 18-OH-DOC, at physiological dosage, can significantly increase the blood pressure of unilaterally nephrectomized saline-treated rats [1] and that implanted pellets of 18-OH-DOC raise the blood pressure of intact dogs [2] strengthen the hypothesis that 18-OH-DOC contributes to the etiology of hypertension. In addition, it has been noted that the blood pressure changes in rats developing adrenal regeneration hypertension are associated with changes in the ratios of B to 18-OH-DOC [3, 4]. In order to evaluate the many biological effects of 18-OH-DOC, it becomes necessary to develop rapid, sensitive and quantitative methods of assay for 18-OH-DOC and B in biological fluids. At present, these steroids can be separated by paper chromatography [5] followed by extraction and quantitation by spectrophotometric methods. The process is time-consuming and tedious at best. The analyses of 18-OH-DOC by the Porter–Silber reaction [6] and of B by fluorimetry [7] without chromatography are subject to interference from contaminants. As part of our program to study the chemistry and biochemistry of 18-OH-DOC, we have examined the quantitative measurement of 18-OH-DOC and B by high pressure liquid chromatography (HPLC) [8].

Separation of a number of steroids including B by HPLC has been reported by Siggia and Dishman [9], and by Henry *et al.* [10]. However, the analysis of 18-OH-DOC itself, and in tandem with B, by HPLC appears not to have been reported.

MATERIALS AND METHODS

Apparatus. A Water's Associates ALC 202/401 Liquid Chromatograph with a U6K (loop type) injector was used. The column packing was 'Bondapak Phenyl/Porasil B' (Water's Assoc.). Four columns, each 61 cm by 2 mm I.D., of stainless steel tubing were used. The solvent used for elution was acetonitrile:water (30:70 v/v) at a flow rate of 1.0 ml/min and pressure of 2000 p.s.i. The U.V. detector operated at 254 nm. Alternatively, a SF 770 spectroflow monitor operated at 244 nm was also used.

Chemicals. Water was deionized and distilled. Acetonitrile (Fisher reagent grade) was distilled from P_2O_5 and then from CaH_2. Corticosterone was obtained commercially. 18-OH-DOC and 18-hydroxyprogesterone (18-OH-P) were synthesized in this laboratory [11]. Standard solutions of 18-OH-DOC, B, and 18-OH-P were prepared in CH_3CN.

Sample preparation. The incubation medium of rat adrenal glands was washed with 0.7 vol. of petroleum ether and extracted twice with two vol. of methylene chloride. The methylene chloride extracts were evaporated to dryness under nitrogen. The residue was dissolved in 25 μl in acetonitrile which contained 0.1 mg/ml of 18-OH-P as internal standard.

For the determination of B by fluorimetry and of 18-OH-DOC by the Porter–Silber reaction the methylene chloride extracts were washed with 0.2 vol. of 0.1 N NaOH and 0.2 vol. of water before addition of the fluorescent and Porter–Silber reagents.

RESULTS AND DISCUSSION

Because of the conjugated carbonyl chromophore in both 18-OH-DOC and B, the U.V. detector is the most convenient and sensitive detector for this work. While the λ_{max} centres at 241 nm for 18-OH-DOC and at 248 nm for B, a detector of 254 nm does not suffer much loss in sensitivity. We have operated at either 254 nm or at 244 nm and found the results to be comparable.

We have used the reversed-phase method for the separation of 18-OH-DOC and B, with Bondapak

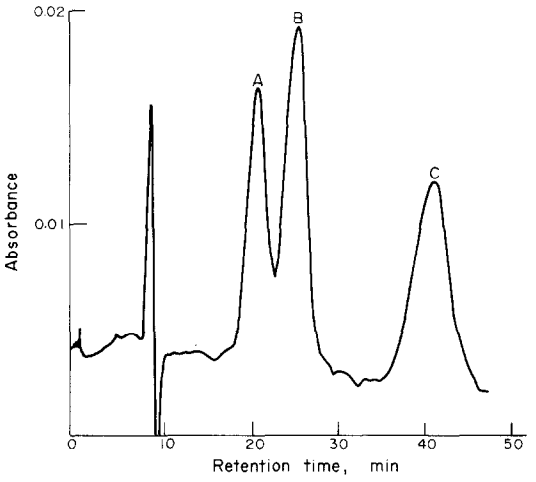

Fig. 1. Separation of standards by high pressure liquid chromatography. A = 18-hydroxydeoxycorticosterone, B = corticosterone and C = 18-hydroxyprogesterone. For experimental conditions, see Methods.

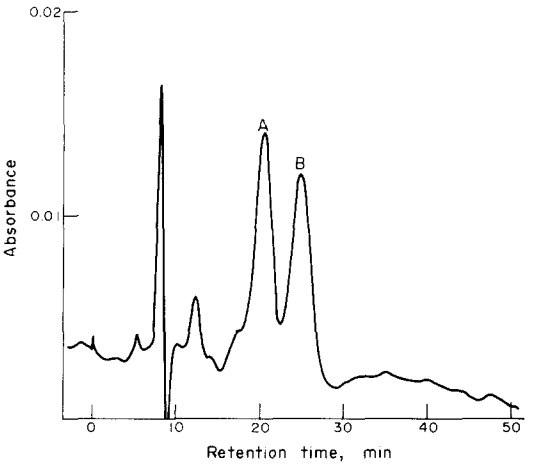

Fig. 2. Separation of a sample by high pressure liquid chromatography. A = 18-hydroxydeoxycorticosterone and B = corticosterone. For sample preparation and liquid chromatography procedure, see Methods.

Phenyl/Porasil B as the stationary phase and acetonitrile:water (30:70 v/v) as the mobile phase. The resolution [8] between 18-OH-DOC and B, has been found to depend largely on the column length. With four columns in series and a solvent flow rate of 1.0 ml/min, the resolution has been found to be 1.0 which is sufficient for the purpose of quantitative determination [8].

Under these conditions, 18-hydroxyprogesterone is completely resolved from 18-OH-DOC and B (Fig. 1) and appears at a retention time which is free from other components in the biological samples studied (Fig. 2). We have therefore used 18-hydroxyprogesterone as the internal standard for the quantitative determination of 18-OH-DOC and B.

Standard solutions of 18-OH-DOC, B and 18-OH-P were prepared in acetonitrile. Standard curves of ratios of peak areas of 18-OH-DOC or B over 18-OH-P versus the ratios of concentration were plotted and found to be linear and pass through the origin. The peak area ratios are in general quite reproducible. For example, three different runs of the 1.0 μg standard on different days gave a mean peak area ratio of 0.435 $\pm$ 0.046 (S.D.) for 18-OH-DOC/18-OH-P, and of 0.687 $\pm$ 0.036 for B/18-OH-P. The limit of detection is about 0.05 μg at which sensitivity the signal to noise level is about 2.

For a number of samples obtained from rat adrenal incubation media, the quantitative determination by HPLC of 18-OH-DOC and B with 18-OH-P as internal standard has been compared with the estimation of 18-OH-DOC by the Porter–Silber reaction and of B by fluorimetry (Table 1). The results obtained from HPLC are in general lower in absolute values than

Table 1. Comparison of the estimation of 18-OH-DOC and corticosterone by high pressure liquid chromatography and spectrophotometric methods

Sample	18-OH-DOC (μg)			Corticosterone (μg)			18-OH-DOC/corticosterone	
	PS	HPLC	PS/HPLC	FL	HPLC	FL/HPLC	PS/FL	HPLC
1.	2.88	2.62	1.10	3.54	4.05	0.87	0.81	0.65
2.	1.41	1.32	1.07	1.40	1.40	1.00	0.01	0.94
3.	1.00	0.15	6.66*	0.33	0.21	1.87*	3.03*	0.71*
4.	2.80	1.28	2.19	2.98	1.70	1.75	0.94	0.75
5.	0.47	0.05	9.40*	0.23	0.31	0.74*	2.04*	0.16*
6.	1.53	0.90	1.70	2.05	1.56	1.31	0.75	0.58
7.	1.39	0.74	1.88	2.02	1.05	1.92	0.69	0.70
8.	1.04	0.77	1.35	1.41	0.99	1.42	0.74	0.78
9.	1.44	0.96	1.50	1.47	1.17	1.26	0.98	0.82
10.	1.51	1.51	1.00	2.41	1.95	1.24	0.63	0.77
11.	1.73	1.53	1.13	3.55	2.05	1.73	0.49	0.75
12.	2.01	1.37	1.47	3.38	2.43	1.39	0.59	0.56
13.	2.82	2.76	1.02	4.19	3.10	1.35	0.67	0.89
14.	1.97	2.00	0.97	3.25	2.55	1.27	0.61	0.78
15.	5.71	3.61	1.58	3.94	2.74	1.44	0.61	0.69
			1.35 $\pm$ 0.10			1.38 $\pm$ 0.08	0.73 $\pm$ 0.04	0.74 $\pm$ 0.03

18-OH-DOC = 18-hydroxydeoxycorticosterone; PS = Porter–Silber reaction; FL = fluorimetry; HPLC = high pressure liquid chromatography.

* Sample not included in computing the average.

those obtained by spectrophotometric and fluorimetric measurements indicating that the spectrophotometric methods are open to interference, which leads to higher than actual values. A similar discrepancy has been observed in the assay of warfarin in human plasma by HPLC and by fluorimetry [12]. The ratios of 18-OH-DOC/B were, however, comparable if one excludes the two samples (Table 1, Sample No. 3 and 5) in which the steroid concentration, derived from unstimulated glands, was very low.

The speed and convenience of the method should make it one of choice for the study of adrenal function by *in vitro* procedures and by adrenal vein cannulation. Because of its low sensitivity HPLC is not suitable for the analyses of peripheral plasma, which can be performed by fluorimetry and protein binding assay in the case of corticosterone and by radioimmunoassay in the case of 18-OH-DOC [13].

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