Journal of Chromatography, 308 (1984) 295—300
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2064

Note

Application of reversed-phase high-performance liquid chromatography for radioimmunoassay of plasma 18-hydroxycorticosterone

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(First received October 20th, 1983; revised manuscript received January 4th, 1984)

Marked elevation of 18-hydroxycorticosterone (18-OHB), a major by-product of adrenal aldosterone biosynthesis [1, 2], has been found in primary aldosteronism due to an adrenal adenoma [3—7] and with 17α-hydroxylase deficiency [8], but not in patients with idiopathic aldosteronism due to adrenal hyperplasia [5, 6] and with normal and low renin essential hypertension [6]. Thus, measurement of plasma or urinary 18-OHB together with aldosterone has proved very useful in the differential diagnosis of these hypertensive disorders [1—8]. High-performance liquid chromatography (HPLC) has recently been proposed for the isolation of multiple adrenal steroids including 18-OHB in serum [9], urine [10] and adrenal tissue extract [11].

We herein describe a method for the separation of plasma 18-OHB from cross-reacting steroids by reversed-phase HPLC prior to its radioimmunoassay (RIA).

EXPERIMENTAL

Materials and reagents

Methanol was of HPLC grade and all other organic solvents were of analytical grade. Water was distilled and then purified by a Millipore filter assembly including a charcoal and ion-exchange tube. Unlabelled 18-OHB was purchased from Fluka (Buchs, Switzerland) and its labelled form ([1,2-^{3}H]18-OHB, specific activity 42 Ci/mmol) from the Radiochemical Centre, Amersham, U.K. The antiserum of 18-OHB raised against 3-(O-carboxymethyl)-

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oxime—bovine serum albumin conjugate (18-OHB-3-CMO-BSA) was supplied by the Teikoku Hormone Mfg. Co. (Kawasaki, Japan). The characteristic of this 18-OHB-3-CMO-BSA antibody was reported by Ojima and Kambegawa [12], who found it to be highly specific. Other unlabelled steroids for examination of cross-reactivity were obtained from Merck (Darmstadt, F.R.G.). The purity of all unlabelled and labelled steroids was checked by HPLC under the conditions described below.

Instruments

The HPLC system used here was constructed with a Kyowa Model KHP-010 liquid chromatograph (Kyowa Co., Tokyo, Japan) and a Oyo-Bunko Model UVILOG 5 III-A variable-wavelength ultraviolet (UV) spectrophotometer (Oyo-Bunko Co., Tokyo, Japan).

Methods

The total assay procedure consisted of three steps: extraction of steroids, HPLC and RIA.

Extraction. Half a millilitre of plasma was extracted with 20 ml of dichloromethane after adding 1.0 ml of 1 *M* sodium hydroxide. After removal of the upper layer, the dichloromethane layer was washed with 1.0 ml of water and evaporated at 37°C under a stream of nitrogen. The resulting residue was dissolved in methanol—water (2:1, v/v) and then injected for HPLC.

HPLC. Conditions of HPLC are as follows: separation column, Finepack SIL $C_{18\text{-}5}$ (250 mm × 4 mm I.D., particle size 5 µm); precolumn, Fine SIL $C_{18\text{-}10}$ (5 mm × 4 mm I.D., particle size 10 µm); eluent, 0.005 *M* hydrochloric acid—methanol (35:65, v/v); flow-rate, 1.0 ml/min; column temperature, 37°C; monitoring wavelength, 245 nm. The separation of 18-OHB from other UV-absorbing steroids was checked photometrically by the injection of a steroid mixture containing 0.6 nmol of each steroid standard, and the retention time of 18-OHB was measured by the injection of 0.6 nmol of standard 18-OHB alone prior to the analysis of plasma samples. The 18-OHB-containing fraction of plasma extract eluted by HPLC was collected in a tube, neutralized with ammonium hydroxide and evaporated at 37°C under a stream of nitrogen. The resulting residue was subjected to RIA.

RIA. RIA of 18-OHB was performed by the method of Ojima and Kambegawa [12]. Total recovery of 18-OHB throughout the procedure was determined by the addition of about 5000 dpm of the labelled steroid to plasma.

RESULTS AND DISCUSSION

We used a reversed-phase chromatographic system to elute 18-OHB alone by a non-gradient method at a relatively early retention time compared with that using a normal-phase chromatographic system. The mean recoveries (± S.D.) of 18-OHB in five plasma samples before and after HPLC in this method were 89.7 ± 5.3% (range, 83.6—94.7%) and 69.9 ± 5.1% (range, 63.4—75.7%), respectively. The mean recovery after HPLC in our method was slightly higher than that (55.3 ± 4.4%) reported by Schöneshöfer et al. [9], who used a

normal-phase chromatographic system. A chromatogram with UV detection of steroid standards is shown in Fig. 1a. The separation of 18-OHB from other steroids such as aldosterone, cortisol, corticosterone, deoxycorticosterone, 18-hydroxydeoxycorticosterone (18-OHDOC) and progesterone was satisfactory. A chromatogram with UV detection of the extract of a normal plasma sample is shown in Fig. 1b. 18-OHB was eluted at the retention time of 6.53

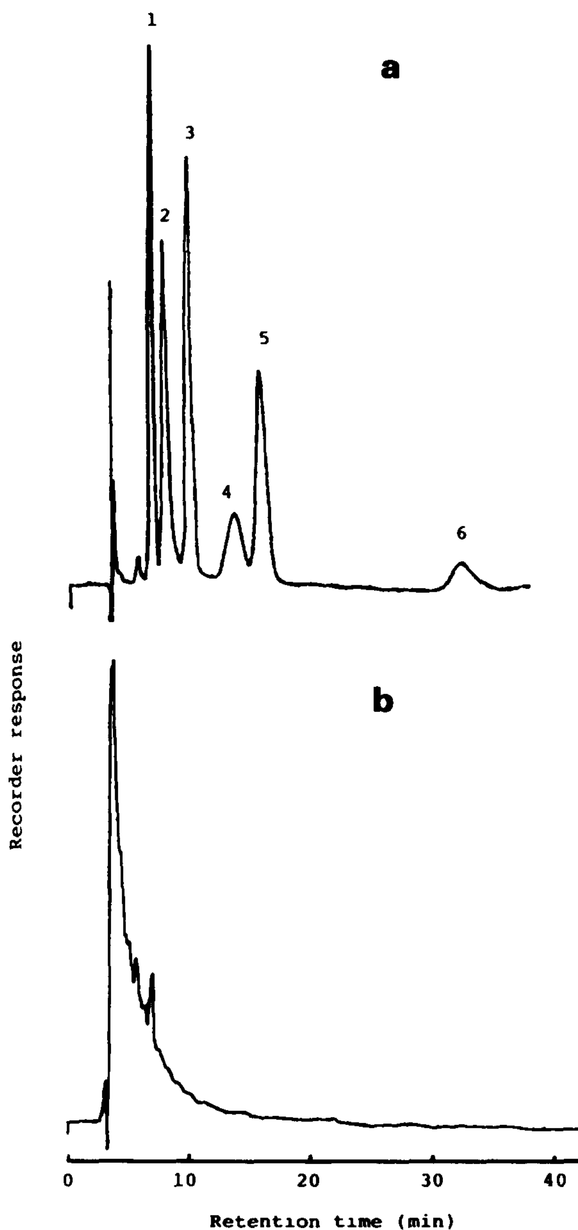

Fig. 1. UV-detected chromatograms of a mixture of steroid standards (a) and of the dichloromethane extract of a normal plasma sample (b). Peaks: 1 = aldosterone and cortisol, 2 = 18-OHB, 3 = corticosterone, 4 = 18-OHDOC, 5 = deoxycorticosterone, 6 = progesterone.

TABLE I

PRECISION OF THE RIA OF 18-OHB

	Pooled plasma	Plasma 18-OHB (nmol/l)					C.V. (%)
		1	2	3	4	Mean ± S.D.	
Intra-assay	A	0.35	0.29	0.26	0.25	0.29 ± 0.04	13.6
	B	1 10	1.23	1.31	1.00	1.16 ± 0.12	10 3
	C	1.99	1 80	2.25	1.89	1.98 ± 0 17	8.6
Inter-assay	A	0.35	0.38	0.25		0.33 ± 0.05	16.5
	B	1.09	1.30	0.97		1.12 ± 0.14	12.2
	C	1.99	1.85	2.36		2.07 ± 0.22	10.4

min after its injection. The coefficient of variation for the retention time of 18-OHB ranged from 0.2 to 0.9%.

The detection limit of the RIA of 18-OHB, defined as the mean blank measurement plus 2.5 S.D. (2.5 × standard deviation of zero bound) [13], was 0.016 pmol. Intra- and inter-assay coefficients of variation are summarized in Table I. The intra-assay coefficient of variation ranged from 8.6 to 13.6% and the inter-assay coefficient of variation ranged from 10.4 to 16.5%. The residue of neutralized eluate did not affect the RIA of 18-OHB (Fig. 2). The pattern of immunoreactivities of standard 18-OHB and plasma extract arising against the 18-OHB antiserum is shown in Fig. 3. The portion showing the maximum immunoreactivity of the plasma extract corresponded with that of the standard 18-OHB. Although the 18-OHB antiserum used here has been reported to be highly specific [12], the multiple non-specific immunoreactive

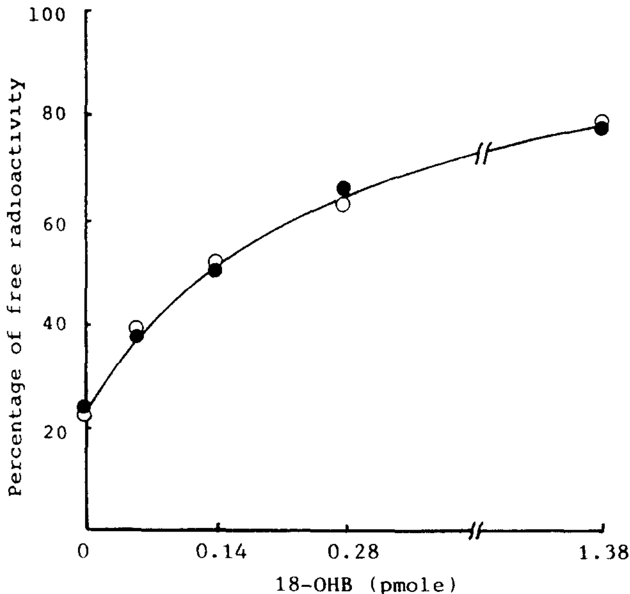

Fig. 2. Calibration curves for the standard 18-OHB alone (•) and for the standard 18-OHB added to the dried residue of 1.5 ml of neutralized eluent (○).

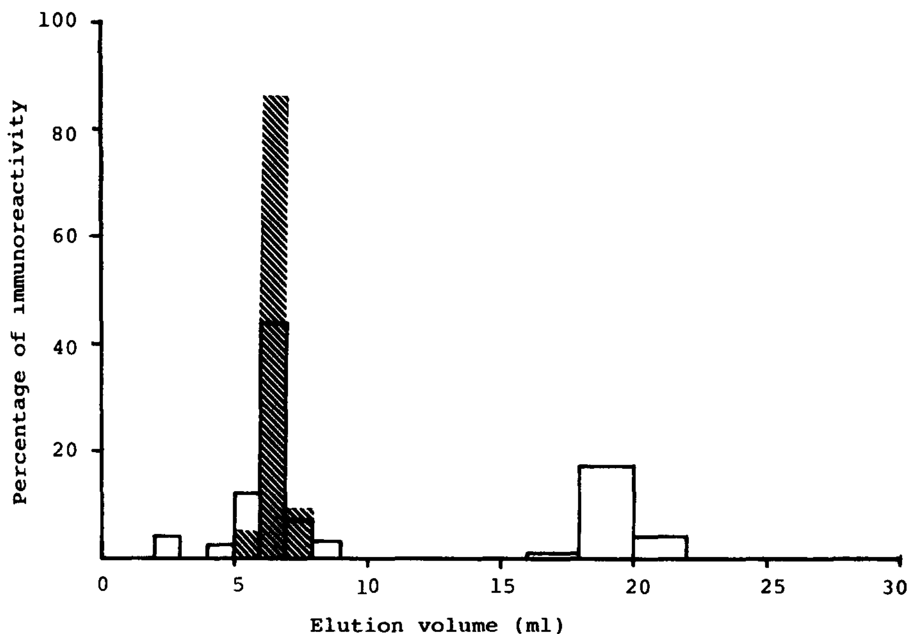

Fig. 3. Patterns of immunoreactivity arising in the HPLC eluates of the standard 18-OHB (hatched area) and of the dichloromethane extract of a normal plasma sample (open area). Values are plotted as the percentage of total immunoreactivity. Fractions of the eluate were collected every 1.0 ml for the first 10 ml and every 2.0 ml thereafter.

portions including the presumed 18-OHDOC one also were found in normal plasma as reported by Schöneshöfer et al. [9]. When interference of the steroids eluted around 18-OHB was tested in the assay system by adding 0.28 μmol of aldosterone, cortisol or corticosterone to 0.28 nmol of 18-OHB, no interference of these steroids was observed.

Plasma 18-OHB levels were measured at 08:00 h after overnight recumbency in fourteen normal subjects and at 12:00 h after 2-h recumbency in the six normal subjects while they were on an ad libitum sodium intake. The plasma 18-OHB level decreased from 0.59 ± 0.24 (S.D.) nmol/l (range, 0.26—0.88 nmol/l) to 0.42 ± 0.14 (S.D.) nmol/l (range, 0.22—0.63 nmol/l), suggesting some circadian rhythm. These normal levels of 18-OHB measured by the present method closely resembled those reported previously in the literature [9, 12, 14, 15]. Plasma 18-OHB levels at 08:000 h after overnight recumbency in five patients with primary aldosteronism due to an adrenal adenoma ranged from 2.62 to 5.83 nmol/l and were similar to those reported by Biglieri and Schambelan [5].

In conclusion, the method described here presents a simple, rapid and reliable isolation of plasma 18-OHB prior to its RIA. The preceding chromatographic isolation of plasma 18-OHB makes its RIA highly appropriate for clinical use.

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