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Note

Paper chromatographic systems for the separation of aldosterone, 18-hydroxycorticosterone, 18-hydroxydeoxycorticosterone, corticosterone and deoxycorticosterone

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Regulation of aldosterone (Aldo) biosynthesis is still open to controversy[1]. The direct sites of action of the known stimulants (*e.g.*, potassium, angiotensin II, adrenocorticotropin) on the different enzymes involved in the biochemical pathway of Aldo are not yet clearly defined[2]. We have developed a radioimmunoassay for the determination of Aldo, 18-hydroxycorticosterone (18OH-B), 18-hydroxydeoxycorticosterone (18OH-DOC), corticosterone (B) and deoxycorticosterone (DOC) in plasma. The aim was to evaluate a single chromatographic system for the separation of these five mineralocorticoids before radioimmunoassay.

EXPERIMENTAL

Chemicals

Acetone, benzene, cyclohexane, dichloromethane, ethanol, formamide, isooctane, isatin (2,3-dihydroindole-2,3-dione), isopropanol, methanol, *n*-butanol and light petroleum (b.p. 40–60°C) were of analytical-reagent grade (Merck, Darmstadt, G.F.R.).

Radioactive steroids. [1,2-^{3}H]-Cortisol (specific radioactivity 42 Ci/mmol), 18-hydroxy-11-deoxy-[1,2-^{3}H]-corticosterone (51 Ci/mmol), 18-hydroxy-[1,2(n)-^{3}H]-corticosterone (32 Ci/mmol), deoxy-[1,2(n)-^{3}H]-corticosterone (35 Ci/mmol) and [1,2,6,7(n)-^{3}H]-aldosterone (102 Ci/mmol) were obtained from Amersham Buchler (Braunschweig, G.F.R.) and [1,2,6,7-^{3}(n)]-corticosterone (82.1 Ci/mmol) and [1,2-^{3}H-(n)]-cortisone (43.6 Ci/mmol) from NEN (Langen, G.F.R.). All radioactive chemicals were purified by thin-layer chromatography before use.

Paper for chromatography

Schleicher and Schüll No. 2043 filter-paper (40 × 560 mm) was used.

Solvent systems

See Tables I and II.

Apparatus

The scanner for evaluating the radioactive paper chromatograms (Berthold LB, 280, Munich, G.F.R.) was connected to a rate-meter system.

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Extraction procedure

Radioactive Aldo, 18OH-B, 18OH-DOC, B and DOC in 50 μl of methanol (8000 cpm of each) were added to 1 ml of heparin-treated plasma. The samples were extracted by continuous shaking in glass vials for 1 h with 40 ml of dichloromethane.

Preparation of paper chromatograms

Descending chromatography was performed in closed tanks. The stationary and mobile phases were equilibrated with the atmosphere in the tank for 6 h. The temperature and humidity in the chromatographic room were kept constant by air-conditioning. The residue after evaporation of the dichloromethane extracts was dissolved in two portions of 400 μl of methanol and subsequently applied to the paper strips under a stream of warm air. Isatin (dissolved in methanol) was used as the indicator, because its chromatographic behaviour is known to be similar to that of aldosterone. Before the steroids were applied, the paper strips were impregnated by dipping them in methanol. Chromatographic development was stopped when isatin had run a distance of 12 cm from the start. Five paper chromatograms were prepared in parallel for each system. Only those chromatographic runs in which the distribution of the radioactive peaks was similar for all five paper strips were accepted for analyses. For calculating the chromatographic resolution of different solvent systems the migration distances of the steroids were determined relative to that of Aldo (R_{Aldo} value). This value was preferred to the conventionally used R_F value because it provided better comparability of the different systems, especially when the mobile phase had run past the end of the paper.

Recoveries of the steroids after extraction and chromatography were determined by eluting the bands from the paper strips with 10 ml of methanol for 1 h. The residue after evaporation of methanol was dissolved in 1 ml of ethanol and used for recovery determinations.

Radioimmunoassays

The procedure for radioimmunoassay of mineralocorticoids and the characteristics of their antisera are described in detail elsewhere[3].

Statistical analyses

Student's *t*-test was used for statistical evaluation of the results. The results are given as the means ± standard deviations (S.D.).

Experiments with human blood

Blood was drawn from healthy volunteers (mean age 39 years; seven male, three female) after 2 h in the supine position and 2 h after administering 40 mg of furosemide i.v., combined with active orthostasis under ambulatory conditions. Samples were collected in heparinized tubes. After immediate centrifugation, the plasma was stored at —20°C and analysed as described above.

RESULTS AND DISCUSSION

The chromatographic behaviour of the steroids in the different systems is shown in Tables I–III.

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF STEROIDS IN SYSTEMS 1–9 EXPRESSED AS R_{Aldo} VALUES

System 1 is system Bush BV[4].

System No.	Chromatographic system*		R_{Aldo} values				
	Mobile phase	Stationary phase	18OH-B	Aldo	18OH-DOC	B	DOC
	Benzene	*Methanol–water*					
1	2	1:1	0.6	1	1.6	1.6	1.6
	Benzene	*Ethanol–water*					
2	2	1:1	0.6	1	1.2	1.2	1.3
3	10	7:3	0.5	1	1.2	1.2	1.3
4	2	2:1	1	1	1	1	1
5	5	2:3	—	1	1.5	1.5	1.5
6	20	7:13	0.5	1	1.3	1.3	1.3
7	10	3:7	0.4	1	1.3	1.3	1.3
	Benzene	*n-Propanol–water*					
8	2	1:1	1	1	1	1	1
	Benzene	*n-Butanol–water*					
9	2	1:1	1	1	1	1	1

* Components of the mobile and stationary phases are given in italics, followed by their relative proportions in each system.

After extraction from plasma, complete separation of the five mineralocorticoids was provided by systems 12, 17 and 22 (Fig. 1 and Table II). Table III shows the recoveries obtained after extraction and chromatography in systems 12, 17 and 22. No statistical difference was found among the recoveries of any given steroid in the different systems ($p > 0.05$). The recoveries are sufficiently high for a sensitive radioimmunoassay of all the steroids in small amounts of human plasma. The following mean concentrations ($\pm$ S.D.) were found in healthy volunteers after 2 h in the supine position: Aldo 166 $\pm$ 52, 18OH-B 216 $\pm$ 49, 18OH-DOC 113 $\pm$ 48 and DOC 140 $\pm$ 78 pg/ml; the level of B was 2.40 $\pm$ 1.78 ng/ml. Two hours after administration of 40 mg of furosemide i.v. combined with active orthostasis, these concentrations increased to Aldo 361 $\pm$ 129, 18OH-B 876 $\pm$ 281, 18OH-DOC 168 $\pm$ 48 and DOC 233 $\pm$ 103 pg/ml, with B 6.36 $\pm$ 4.86 ng/ml. The major glucocorticoids, cortisol (F) and cortisone (E), did not interfere with the peaks of the mineralocorticoids. F was detected between 18OH-B and Aldo and E between Aldo and 18OH-DOC (Table II).

Table I gives R_{Aldo} values from systems that contained benzene in the mobile phase and water, mixed with an alcohol, in the stationary phase. 18OH-B and Aldo were separated from 18OH-DOC, B and DOC by systems 1–3 and 5–7. In systems 2 and 3 DOC could be separated from 18OH-DOC and B. In systems 4, 8 and 9 no separation of the steroids was possible.

The systems presented in Table II differ from those in Table I mainly by having an additional hydrophobic organic solvent in the mobile phase. The stationary phase was composed of a mixture of water and methanol or ethanol, except systems 25 and 26, which consisted of formamide and acetone. All systems in Table II separate 18OH-B and Aldo from 18OH-DOC, B and DOC.

TABLE II

CHROMATOGRAPHIC BEHAVIOUR OF STEROIDS IN SYSTEMS 10–25

System 10 is system Bush B VII[4] and system 24 was described by Zaffaroni *et al.*[5].

System	Chromatographic system*		R_{Aldo} values						
	Mobile phase	Stationary phase	18OH-B	F	Aldo	E	18OH-DOC	B	DOC
	Light petroleum–benzene	*Methanol–water*							
10	33:17	40:10	0.4	–	1	–	2.8	2.8	6.7
11	5: 5	7: 3	0.4	–	1	–	2.5	2.6	4.8
12	3: 7	6: 4	0.3	0.8	1	1.3	1.8	2.1	2.6
	Light petroleum–benzene	*Ethanol–water*							
13	1: 1	1: 1	0.6	–	1	–	1.9	1.9	2.3
14	2: 3	7: 3	0.6	–	1	–	2.7	2.7	2.7
15	3: 7	3: 7	0.7	–	1	–	1.8	1.8	2.2
16	3: 7	6: 4	0.7	–	1	–	1.2	1.2	1.4
	Isooctane–benzene	*Methanol–water*							
17	3: 7	6: 4	0.3	0.8	1	1.5	2.3	2.8	4.4
	Isooctane–benzene	*Ethanol–water*							
18	3: 7	3: 7	0.3	–	1	–	2.2	2.2	3.0
19	3: 7	6: 4	0.4	–	1	–	1.6	1.6	2.0
20	2: 8	5: 5	0.4	–	1	–	2.4	2.4	3.8
21	5: 5	3: 7	0.4	–	1	–	3.6	3.6	7.1
	Cyclohexane–benzene	*Methanol–water*							
22	3: 7	6: 4	0.4	0.8	1	1.4	2.0	2.4	3.3
	Cyclohexane–benzene	*Ethanol–water*							
23	3:17	5:10	0.4	–	1	–	2.2	2.2	2.4
	Chloroform–benzene	*Formamide–acetone*							
24	10:10	3: 7	1	–	1	–	1	1	1
25	7: 3	5: 5	1	–	1	–	1	1	1

* Components of the mobile and stationary phases are given in italics, followed by their relative proportions in each system.

TABLE III

RECOVERIES OF STEROIDS AFTER EXTRACTION AND CHROMATOGRAPHY WITH SYSTEMS 12, 17 AND 22

Values given are mean percentages ± S.D. for ten chromatograms for each system. There was no statistical difference between the three mean recoveries of each steroid ($p > 0.05$).

System No.	*Recovery (%)*				
	18OH-B	*Aldo*	*18OH-DOC*	*B*	*DOC*
12	38.7 ± 5.3	36.3 ± 4.7	38.3 ± 4.7	40.1 ± 6.1	48.3 ± 8.1
17	39.4 ± 7.0	37.1 ± 6.9	38.1 ± 5.3	40.0 ± 6.2	49.7 ± 7.9
22	35.2 ± 3.5	36.7 ± 4.3	37.6 ± 4.8	36.8 ± 3.9	50.3 ± 10.1

In all systems in which a separation could be obtained the sequence of the steroids (from the start) was 18OH-B, Aldo, 18OH-DOC, B and DOC.

The relationship between temperature and flow-rate, shown in Fig. 2 for system 17, is representative of all solvent systems. A maximum flow-rate was obtained

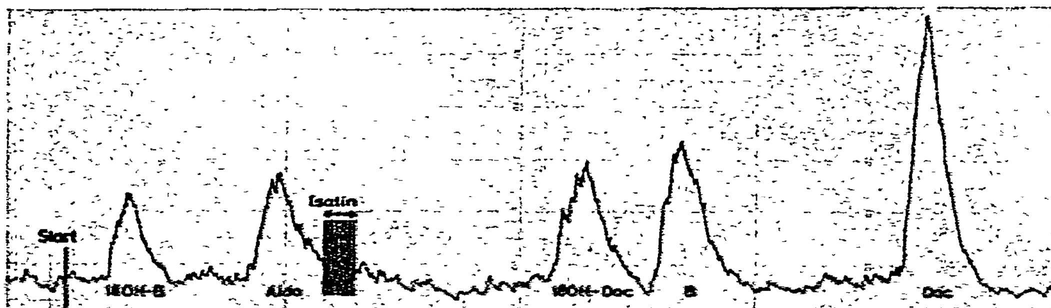

Fig. 1. Radiochromatogram of 18OH-B, Aldo, 18OH-DOC, B and DOC in system 17 (isooctane–benzene–methanol–water, 3:7:6:4) (*cf.*, Table II).

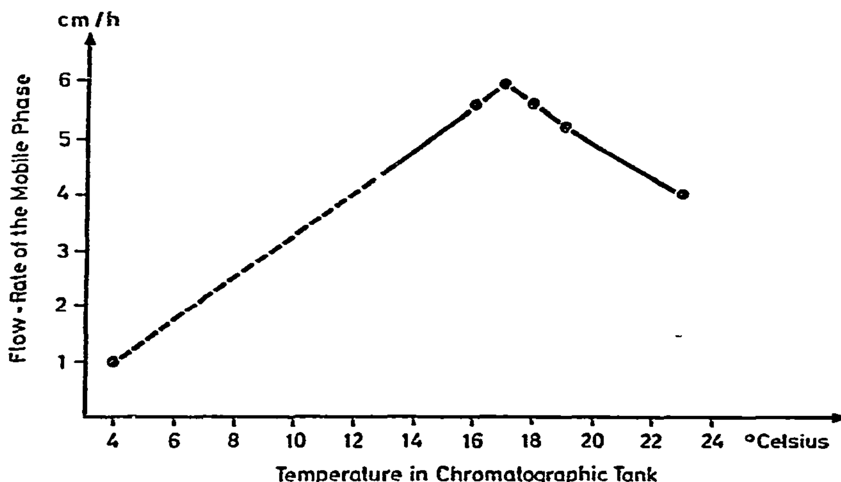

Fig. 2. Relationship between flow-rate and temperature for system 17 (isooctane–benzene–methanol–water, 3:7:6:4).

at 16–18°C. For reproducibility of the results it is therefore useful to keep the temperature constant. Our experiments were performed at 16–18°C. Under these conditions, isatin runs a distance of 12 cm in about 7–9 h. In all of the chromatographic systems tested isatin could be used as a precise marker for the localization of aldosterone (Fig. 1).

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