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# QUANTITATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF $\Delta^4$-3-KETOSTEROIDS IN ADRENOCORTICAL EXTRACTS

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## SUMMARY

A high-performance liquid chromatographic method is described for the determination of seven steroids in adrenocortical extracts showing a $\Delta^4$-3-ketonic conjugated system. The seven steroids (cortisol, cortisone, 11-dehydrocorticosterone, corticosterone, 11-deoxycortisol, aldosterone and 11-deoxycorticosterone) were separated with a chloroform–methanol gradient on a 5-$\mu$m silica column and with a water–acetonitrile gradient on a 10-$\mu$m RP-8 column. Effluents were monitored by UV absorption at 242 nm. Quantitative analysis was performed by comparing peak areas, which are proportional to the amounts of the individual substances (external standard method). The method is rapid, sensitive, easy to perform and reproducible.

## INTRODUCTION

Adrenocortical extracts, prepared according to the Cartland and Kuizenga[1] procedure, are used in pharmaceutical preparations. They contain numerous natural steroids, most of which are known. We have devised a high-performance liquid chromatographic (HPLC) method for analysing seven such steroids which show UV absorbance near 240 nm because they contain a $\Delta^4$-3-ketonic conjugated system (see Table I). Previous HPLC methods for the analysis of steroids have been reviewed by Heftmann and Hunter[2].

This paper reports the results obtained in the HPLC analysis of a mixture of adrenocortical steroids by means of two columns, a 5-$\mu$m silica column and a 10-$\mu$m RP-8 column.

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## EXPERIMENTAL

### *Apparatus*

A high-pressure liquid chromatograph (Hewlett-Packard 1084 A) was equipped with two independent pumps, a variable-wavelength spectrophotometer (Hewlett-Packard 1030B), and a liquid chromatograph terminal (Hewlett-Packard 79850 A) to programme the chromatographic conditions. The stainless-steel columns (Brownlee Labs, Santa Clara, CA, U.S.A.) (250 × 4.6 mm I.D.) were packed with LiChrosorb SI-100 (Merck, Darmstadt, G.F.R.) (particle size 5 $\mu$m) for normal-phase chromatography and with LiChrosorb RP-8 (Merck) (particle size 10 $\mu$m) for reversed-phase chromatography. The detector was set at 242 nm.

### *Reagents*

Doubly distilled water was filtered through a 0.2-$\mu$m pore-size membrane filter (Sartorius Type SM 11307). Acetonitrile, ethanol-free chloroform (stabilized with amylene) and methanol for chromatography (Merck) were filtered through a 0.2-$\mu$m pore-size membrane filter (Sartorius Type SM 11607).

### *Preparation of standards*

The reference compounds used are listed in Table I.

TABLE I

REFERENCE COMPOUNDS USED

| *Trivial name* | *Abbreviation* | *Systematic name* |
|---|---|---|
| Cortisol | F | 11$\beta$,17$\alpha$,21-Trihydroxypregn-4-ene-3,20-dione |
| Cortisone | E | 17$\alpha$,21-Dihydroxypregn-4-ene-3,11,20-trione |
| 11-Dehydrocorticosterone | A | 21-Hydroxypregn-4-ene-3,11,20-trione |
| Corticosterone | B | 11$\beta$,21-Dihydroxypregn-4-ene-3,20-dione |
| 11-Deoxycortisol | S | 17$\alpha$,21-Dihydroxypregn-4-ene-3,20-dione |
| Aldosterone | ALDO | 11$\beta$,21-Dihydroxypregn-4-ene-18-al-3,20-dione |
| 11-Deoxycorticosterone | DOC | 21-Hydroxypregn-4-ene-3,20-dione |

A standard solution in chloroform for normal-phase chromatography and another in absolute ethanol for reversed-phase chromatography, containing F (1000 $\mu$g/ml), E (600 $\mu$g/ml), A (400 $\mu$g/ml), B (800 $\mu$g/ml), S (200 $\mu$g/ml), ALDO (200 $\mu$g/ml) and DOC (100 $\mu$g/ml), were prepared. From these standard solutions were prepared 1 : 2 and 1 : 4 solutions by dilution with the appropriate solvent.

### *Preparation of samples*

Commercially available adreno-cortical extracts were used. An amount containing *ca.* 1500 $\mu$g of total steroids, expressed as hydrocortisone, was diluted with 20 ml of water and extracted five times with 30 ml of chloroform. The chloroform extracts were washed with 10 ml of 0.1 *N* sodium hydroxide solution and twice with 20 ml of water. This procedure removes preservatives, such as thimerosal and esters of *p*-hydroxybenzoic acid, which would otherwise interfere with the chromatogram. The chloroform extracts were dried over anhydrous sodium sulphate, evaporated to dryness and taken up in 1 ml of chloroform for normal-phase or 1 ml of

ethanol for reversed-phase chromatography. The alkaline wash does not remove other preservative agents such as benzyl alcohol and benzaldehyde. In normal-phase chromatography these products do not interfere, because their capacity factors are very low, but in reversed-phase chromatography they coincide with the peaks of F and E. After filtration through a membrane filter (Sartorius Type SM 11607), 20 $\mu$l of sample solution were injected into the chromatograph.

*Elution*

*Normal-phase chromatography.* Gradient elution was carried out with chloroform as solvent A and chloroform containing 5% (v/v) of methanol as solvent B. Each steroid, dissolved in chloroform, was injected into the chromatograph and first assayed by isocratic elution with methanol at concentrations of 1, 2, 3 and 4% at a flow-rate of 1.2 ml/min and at 33 °C. From the retention times the capacity factors of the steroids were calculated, and are shown in Fig. 1. The capacity factors increase considerably in the sequence DOC, A, S, B, E, ALDO, F. While the first three have a $k'$ value suitable for analysis with 1% of methanol, for the others the $k'$ values are too high and they require higher percentages of methanol for elution. We therefore used the chloroform–methanol gradient shown in Table II (see also Fig. 3).

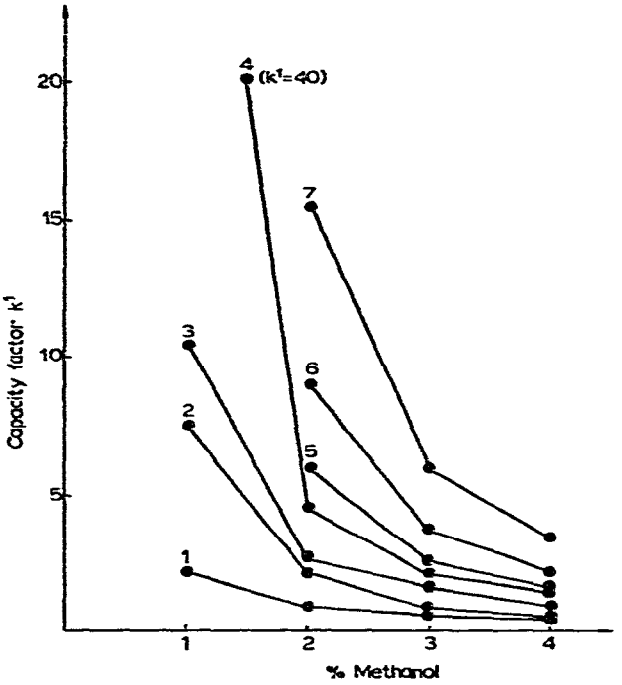


Fig. 1. Capacity factor ($k'$) *versus* concentration of methanol in chloroform. Column: silica (5 $\mu$m). Flow-rate: 1.2 ml/min. 1 = DOC; 2 = A; 3 = S; 4 = B; 5 = E; 6 = ALDO; 7 = F.

*Reversed-phase chromatography.* Each steroid, dissolved in ethanol was chromatographed under isocratic conditions using water–acetonitrile with concentrations of acetonitrile of 25, 30 and 35% at a flow-rate of 1.2 ml/min and at 33 °C. The

TABLE II

CHLOROFORM–METHANOL GRADIENT USED FOR NORMAL-PHASE CHROMATOGRAPHY

| *Time (min)* | *Solvent B (%)* | *Methanol (%)* |
|---|---|---|
| 0 | 20 | 1 |
| 5 | 20 | 1 |
| 7 | 25 | 1.25 |
| 14 | 25 | 1.25 |
| 20 | 35 | 1.75 |
| 30 | 70 | 3.5 |
| 40 | 70 | 3.5 |
| 42 | 20 | 1 |
| 50 | Stop | |

capacity factors of the steroids were determined and plotted against the percentage of acetonitrile (Fig. 2). With a decreasing percentage of acetonitrile in the mobile phase, the capacity factors increased considerably. For the steroids ALDO, F, E, A, B and S a separation under isocratic conditions with 30% of solvent B was chosen. DOC, which lacks the 17-hydroxyl group, shows anomalous behaviour compared

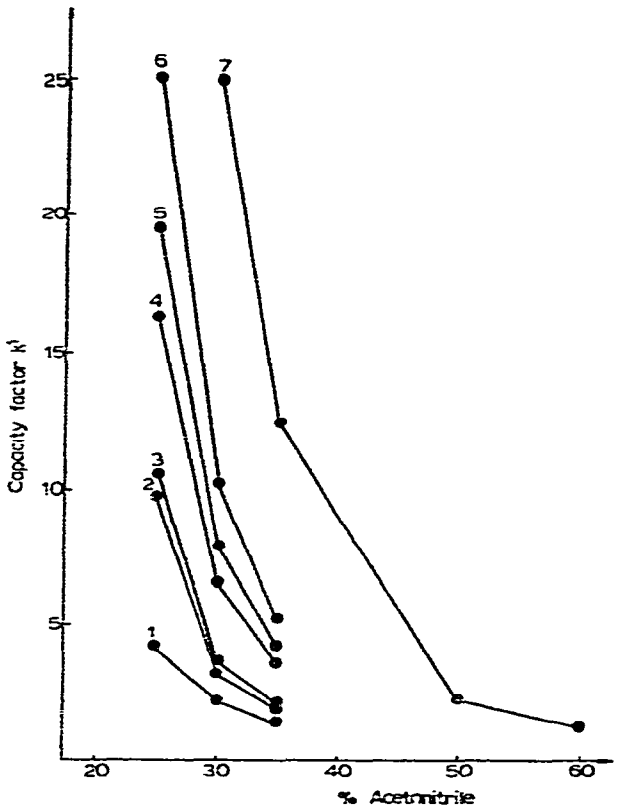


Fig. 2. Capacity factor (*k'*) *versus* concentration of acetonitrile in water. Column: RP-8 (10 μm). Flow-rate: 1.2 ml/min. 1 = ALDO; 2 = F; 3 = E; 4 = A; 5 = B; 6 = S; 7 = DOC.

TABLE III

WATER–ACETONITRILE GRADIENT USED FOR REVERSED-PHASE CHROMATOGRAPHY

| *Time (min)* | *Acetonitrile (%)* |
|---|---|
| 0 | 30 |
| 30 | 30 |
| 31 | 60 |
| 41 | 60 |
| 42 | 30 |
| 50 | Stop |

with the other steroids, and a higher percentage of acetonitrile is therefore required for its elution. The water–acetonitrile gradient shown in Table III was chosen (see also Fig. 4).

## RESULTS AND DISCUSSION

The linearity of the detector response for the steroids was ascertained by injecting 20 $\mu$l of each standard solution (for every solution six determinations were carried out). Typical chromatograms of the reference steroids are shown in Figs. 3 and 4, in which the gradients are also drawn. In Figs. 5 and 6 the resolution of pairs of steroids having neighbouring peaks is shown.

Solutions containing the pairs of steroids were chromatographed under isocratic conditions at concentrations of 1, 2, 3 and 4% of methanol for the normal-phase column and 25, 30 and 35% of acetonitrile for the reversed-phase column.

From the chromatograms it is possible to calculate the values of the resolution, $R_s$, from the following equation:

$$R_s = \frac{2(t_{r2} - t_{r1})}{W_1 + W_2}$$

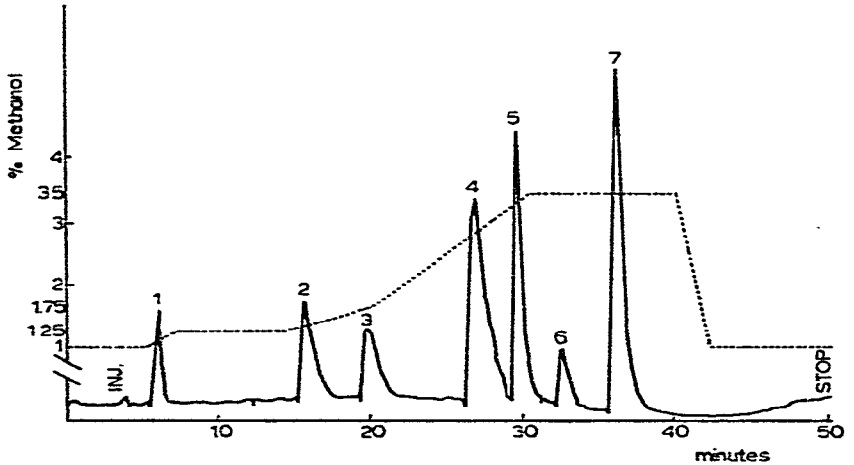


Fig. 3. Chromatogram of a mixture of the reference steroids, using a methanol–chloroform gradient (broken line) (normal phase). Attenuation: $128 \cdot 10^{-4}$ a.u.f.s. Column: silica (5 $\mu$m). Peaks: 1 = DOC; 2 = A; 3 = S; 4 = B; 5 = E; 6 = ALDO; 7 = F.

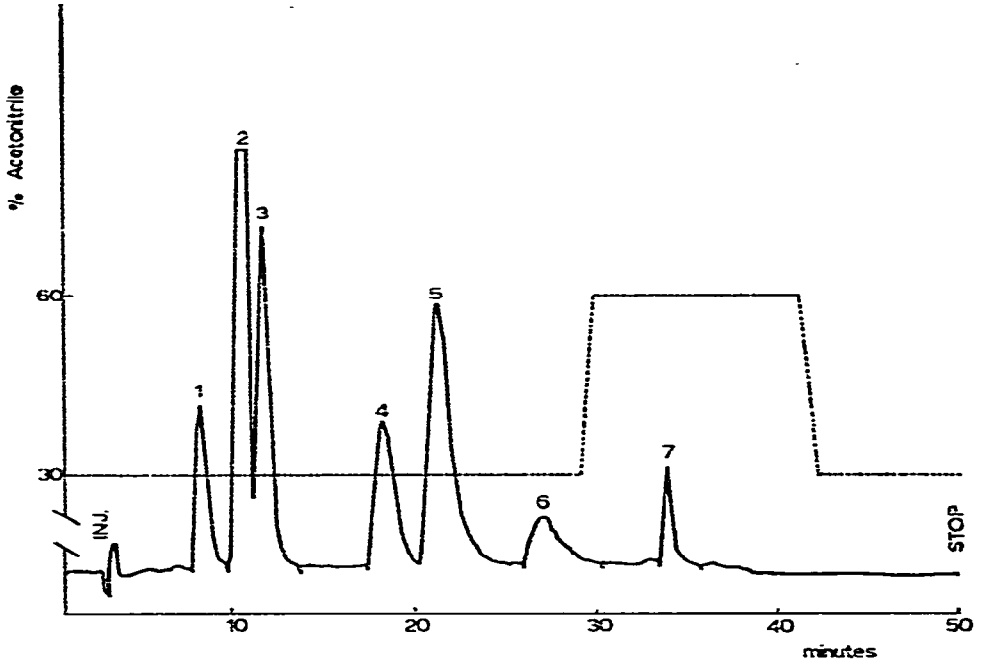


Fig. 4. Chromatogram of a mixture of the reference steroids, using an acetonitrile–water gradient (broken line) (reversed phase). Column: RP-8 (10 μm). Attenuation: $128 \cdot 10^{-4}$ a.u.f.s. Peaks: 1 = ALDO; 2 = F; 3 = E; 4 = A; 5 = B; 6 = S; 7 = DOC.

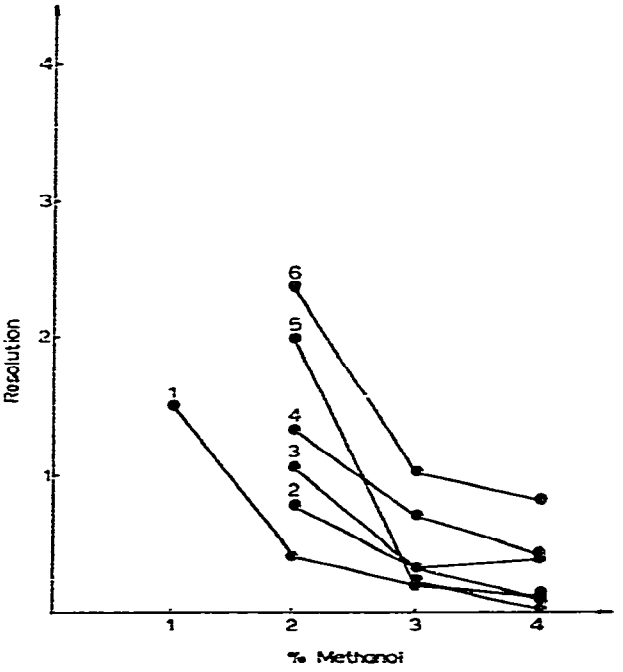


Fig. 5. Resolution *versus* concentration of methanol in chloroform. Column: silica (5 μm). Flow-rate: 1.2 ml/min. 1 = S–A; 2 = E–B; 3 = B–S; 4 = ALDO–E; 5 = A–DOC; 6 = F–ALDO.

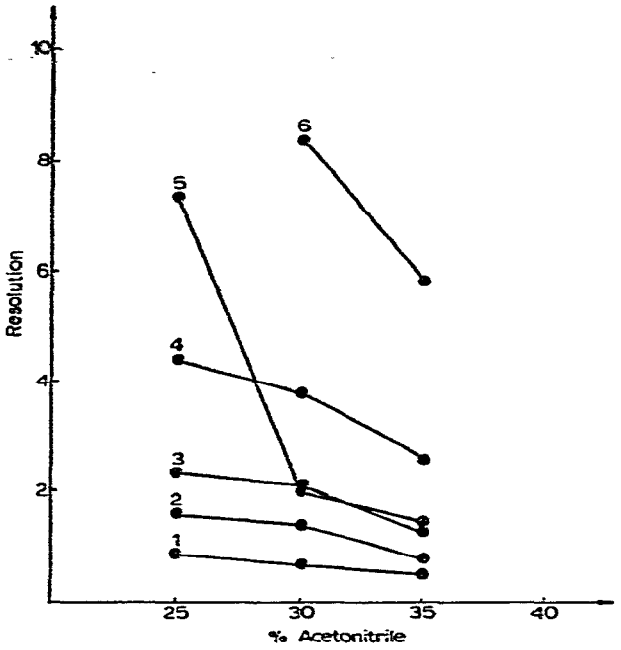


Fig. 6. Resolution *versus* concentration of acetonitrile in water. Column: RP-8 (10 $\mu$m). Flow-rate: 1.2 ml/min. 1 = E–F; 2 = B–A; 3 = S–B; 4 = A–E; 5 = F–ALDO; 6 = DOC–S.

where $t_{r2}$ and $t_{r1}$ are the retention times of the two components and $W_1$ and $W_2$ are the band widths determined by the intersection of the tangents of the inflection points of the Gaussian peaks with the baseline.

For high-speed separations $R_s = 1$ is taken as a satisfactory separation. When $R_s$ is less than 0.8, the separation is usually unsatisfactory. Figs. 5 and 6 show that the resolution is excellent for all pairs of steroids tested, but slightly poorer, although still satisfactory, for the pair E/F with the RP-8 column [$R_s$ (E/F) = 0.9 with 25% of acetonitrile] and for the pair E/B with the silica column [$R_s$ (E/B) = 0.8 with 2% of methanol]. These values for the resolution were obtained under isocratic conditions; with gradient elution the $R_s$ values for the above pairs were $R_s$ (E/F) = 0.8 and $R_s$ (E/B) = 1.2.

For the quantitative analysis of samples the test solution and the standard solution were injected alternately many times. For each steroid in the sample the mean of the peak areas was compared with the mean of the peak areas of the same steroid in the standard solution (external standard method).

The analyses carried out by using as an internal standard one of the steroids having an intermediate retention time showed an analytical error comparable to that obtained by using the external standard method. This is due to the excellent reproducibility of the automatic injector of the chromatograph and to the absence of physical or chemical transformations of the sample.

The relative standard deviations are different for each steroid and for each column. The results are summarized in Table IV.

In conclusion, the proposed method is rapid and facile and gives important

TABLE IV

RELATIVE STANDARD DEVIATIONS FOR THE DIFFERENT STEROIDS ON THE TWO COLUMNS

| *Steroid** | *Relative standard deviation (%)* | |
|---|---|---|
| | *Silica column* | *RP-8 column* |
| F | 1.2 | 2.5 |
| E | 3.3 | 4.2 |
| A | 4.7 | 3.7 |
| B | 2 | 2.5 |
| S | 6.4 | 7.1 |
| ALDO | 9.5 | 3.2 |
| DOC | 8.7 | 4.2 |

* For abbreviations, see Table I.

information on the quality of the adrenocortical extract, in addition to allowing the quantitative determination of the known steroids.

REFERENCES

1 G. P. Cartland and M. H. Kuizenga, *J. Biol. Chem.*, 116 (1936) 57.
2 E. Heftmann and I. R. Hunter, *J. Chromatogr.*, 165 (1979) 283.