Journal of Chromatography, 76 (1973) 244–247
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CHROM. 6375

Note

High-pressure liquid chromatography of corticosteroids*,**

The development of simplified high-pressure liquid chromatographs, as well as the introduction of new column packing materials, provides a powerful technique for rapid and highly efficient separation and analysis of organic mixtures by liquid chromatography (LC)[1]. Little has been reported on the practical application of liquid–solid or liquid–liquid column chromatography for corticosteroids[2,3].

Because of this and the clinical importance of the C-21 steroids, an attempt was made to separate some of the important corticosteroids, singly or as a group using a commercially available liquid chromatograph. The present paper describes the results obtained. Cortisol, cortisone and aldosterone are readily separated and quantitated[4].

Instrumentation and equipment

A Perkin-Elmer Model 1240 analytical liquid chromatograph was used in this study. From a reservoir for continuous stirring, the solvent goes to a reciprocating, positive displacement pump capable of developing 1500 p.s.i. The pump was equipped with a dampening device to eliminate pulsations. An automatic pressure shut-off provided protection of the detector cells for pressures over 1000 p.s.i. The solvent flows first through the reference cell of the detector and from there to a 0.5 m × 3 mm stainless-steel column, fitted with an on-column injection port. The effluent from the column returns to the detector in a separate "sample cell" and leaves the instrument after passing through a flow meter. The solvent and the column can be heated separately.

The instrument was equipped with an ultraviolet (UV) detector having a mercury line source of 254 nm peak emission. The volume of the detector cells was 12 μl. Accurate results were obtained at sensitivities up to 0.01 O.D. full scale. The column packing materials were treated silica gels from Perkin-Elmer. For liquid–adsorption LC it was Sil-X; for liquid–liquid Sil-X (RP) was used. The latter used octadecyltrichlorosilane as stationary phase.

Steroids were dissolved in chloroform to give a concentration of 1 mg/ml. Injections were made with a Hamilton high-pressure microliter syringe.

Main interest was given to the separation of the following corticosteroids: aldosterone (A), cortisol (F) and cortisone (E). The difference in the chemical structure lies in additional or missing keto or hydroxyl groups. The separation of A, F, E sample as a group was emphasized, since these steroids are reliable indicators of adrenal function.

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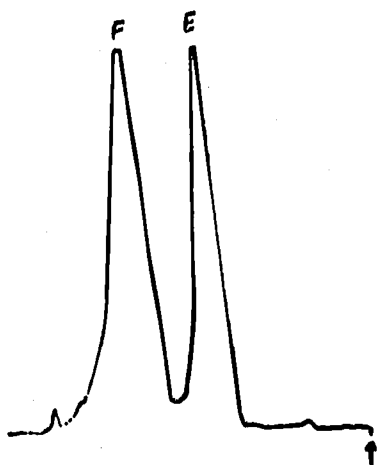
* Presented in part at the 8th International Congress of Clinical Chemistry, Copenhagen, June 20, 1972.
** Supported in part by USPHS Grants HD01199, Career Development Award AM-K-14,013 and a grant from the Ford Foundation.



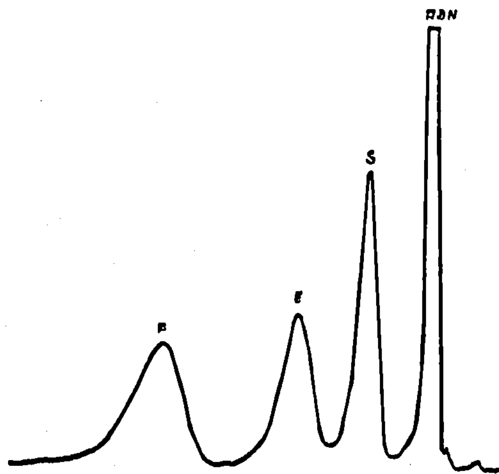


Fig. 1. Separation of cortisol (F) and cortisone (E) by high-pressure liquid–adsorption chromatography on Sil-X. Column, 3 mm × 50 cm; solvent, chloroform–dioxane (100:5); pressure, 400–500 p.s.i.; flow-rate, 1 ml/52 sec; detection, UV; attenuation, 0.05 O.D.; speed of recorder, 30/h; amount injected, 5 μg E + F.

Fig. 2. Separation of cortisol (F), cortisone (E), substance S, and androstenedione (ADN) by high-pressure liquid–adsorption chromatography on Sil-X. Column, 3 mm × 50 cm; solvent, chloroform–dioxane (100:4); pressure, 350–450 p.s.i.; flow-rate, 1 ml/55 sec; detection, UV; attenuation, 0.05 O.D.; speed of recorder, 30/h; amount injected, 4 μg each.

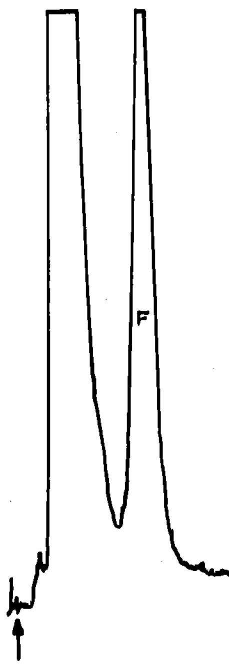


Fig. 3. Separation of cortisol (F) from an extract of 5 ml human plasma. Column, 3 mm × 50 cm; solvent, chloroform–dioxane (100:5); pressure, 500–600 p.s.i.; flow-rate, 1 ml/40 sec; detection, UV; attenuation, 0.02 O.D.; speed of recorder, 6/h; amount injected, extract of 5 ml of plasma.

Results and discussion

The separation of corticosteroids using adsorption chromatography with Sil-X is shown in Figs. 1 and 2. F and E are separated completely 20–25 min after injection as seen in Fig. 1. The solvent system used was chloroform–dioxane (100:5) and the flow-rate was 1 ml/min. The amount injected represented 4 μg of each steroid. A similar separation was obtained with chloroform–methanol (100:1) as solvent as seen in Fig. 2. A flow-rate of 1 ml/min was used and again 4 μg of each standard was injected. Finally, the practical application of this separation is given in Fig. 3, showing separation of cortisol from plasma extracts. The flow-rate in this case is faster. The result is a faster appearing cortisol peak. The peak is well separated from the less polar material which eluted first.

A different separation of corticosteroids resulted on liquid–liquid chromatography as shown in Table I. In this case the reversed phase was used. The solvent polarity was reversed. F, E and A are not sufficiently separated by this column.

TABLE I

SEPARATION OF STEROIDS BY LC ON SIL-X (RP)

Retention time of cortisol (F), cortisone (E), aldosterone (A), corticosterone (B), substance S and progesterone (Prog.) on reversed-phase high-pressure liquid chromatography on Sil-X (RP) Speed of the recorder was 0.26 cm/min.

Steroid	*Retention*	*Band width (cm)*
E	1.8 cm	0.9
F	2.0	1.0
A	1.7	1.0
B	3.1	1.6
S	3.5	0.9
Prog.	15.0	5.8

However, there was a wide separation of the first group from 17-hydroxydesoxycorticosterone and 11-hydroxydesoxycorticosterone which are less polar steroids. These steroids are not separated from each other. An even better separation of progesterone and similar non-polar compounds from the other more polar groups was obtained. This indicates that the reversed-phase packing may be more suitable for group separation rather than for separation of steroids with similar polarity. From the practical standpoint, this packing has an ease of operation and good reproducibility. F, E and A separated together. Aldosterone in the plasma is present in low quantities; this peak really represents F and E. The sharp and fast separation in 10 min makes this packing very useful for determination of F and E as a parameter for adrenal function.

For proof of identity of the compounds, the effluent representing the cortisol peak was collected and subjected to thin-layer chromatography on Silica Gel G plate. Parallel to the isolated F there is the reference F. The same was done with [^{3}H]cortisol. A recovery of radioactivity of 93.2% was obtained from the column.

A detailed report on the use of this method for determination of plasma cortisol will be published later.

One of the problems in LC of corticosteroids is the choice of a suitable solvent system. Small changes of the solvent polarity result in significantly different retention volumes. For liquid–solid chromatography on Sil-X, the system chloroform–methanol (100:1), chloroform–dioxane (100:5) used at room temperature, proved to give good separation. In liquid–liquid (reversed-phase) chromatography methanol–water (40:60) was a useful system. For better separation the column was heated to 40°. The separation of corticosteroids was usually done at a flow-rate of 1 ml/min, at which the best separation was obtained. The reproducibility and stability of the column are still problems in LC. These problems seem more pronounced in liquid–solid than in liquid–liquid chromatography. The previous equilibration of the column is important, especially in liquid–liquid chromatography. Time required to get stable column conditions varies from hours to days. However, after finding the correct conditions for the best separation, LC can prove to be very useful in separation and quantitation of corticosteroids.

The advantages of using LC separation of corticosteroids are: (1) corticosteroids are thermally and chemically unstable compounds, so working at or near room temperature results in less destruction during separation, and (2) all corticosteroids, which can be separated by thin-layer chromatography can be separated and quantitated by LC in less time.

Disadvantages are: (1) the low sensitivity of the detectors commercially available at the present time, and (2) the stability of some column packing materials over longer periods of time also presents problems.

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Received September 15th, 1972

* Ford Foundation Fellow in Reproductive Biology.