Journal of Chromatography, 84 (1973) 396–401

CHROM. 6838

Note

Quantitative determination of corticosteroids from plasma by high-pressure liquid chromatography*

W. WORTMANN**, C. SCHNABEL and J. C. TOUCHSTONE

Department of Obstetrics and Gynecology, University of Pennsylvania, School of Medicine, Philadelphia, Pa. 19104 (U.S.A.)

(Received May 25th, 1973)

Many methods for the determination of corticosteroids from plasma have been published[1–3], but many of them lack specificity or are too time consuming for routine use. This paper describes a method involving the use of high-pressure liquid chromatography (LC) for the separation and analysis of corticosteroids in plasma. Little has been reported on the separation or analysis of steroid hormones by LC[4–6]. LC has been very effective for the separation of corticosteroids, either single or as a group[5]. The practical application for the routine determination of cortisol (F), cortisone (E) and aldosterone (Aldo), separated as a group, is described in this paper. The determination of E, F and Aldo together seemed acceptable, as all three hormones are important secretory products of the adrenal cortex and represent a good parameter for adrenal activity. The method is fast, sensitive, easy to perform and well reproducible.

INSTRUMENTATION AND EQUIPMENT

A Perkin-Elmer analytical liquid chromatograph (Model 1240) was used, equipped with a positive displacement pump capable of developing a pressure of 1500 p.s.i. An automatic pressure shut-off at 1000 p.s.i. provided protection of the detector cells. A 3 mm × 0.5 m stainless-steel column fitted with an on-column injection port was used. The column and solvent could be heated separately. The solvent passed through the reference cell of the detector before entering the column and returning to a separate "sample cell", the volume of which was 12 μl. At sensitivities up to 0.01 O.D., accurate results were obtained. The ultraviolet (UV) detector was equipped with a mercury line source of 254 nm peak emission. The column packing material in the present study was Sil-X-RP (reversed phase, obtained from Perkin-Elmer), and the particles were coated with octadecyl dichlorosilane as the stationary phase. Packing of the column was performed by repeated tapping of the column after adding a small amount of Sil-X-RP powder. Injections were made through a PTFE septum at the head of the column using a 5-μl high-pressure Hamilton syringe. The

* Supported in part by NIH Grant HD-01199 and a grant from the Ford Foundation.
** Ford Foundation Fellow in Reproductive Biology. Present address: Universitäts Frauenklinik, 65 Mainz, G.F.R.

column was equilibrated at 40° with the selected solvent system for about 72 h using a very slow flow-rate of 0.1 ml/min.

EXTRACTION METHOD

The method used for the extraction of the plasma is shown in Fig. 1. Human serum (5 ml) was washed with ice-cold isooctane and extracted with methylene chloride. The methylene chloride phase was washed with 0.1 *N* NaOH solution (prepared freshly before use) and with water. Small amounts of water in the methylene chloride

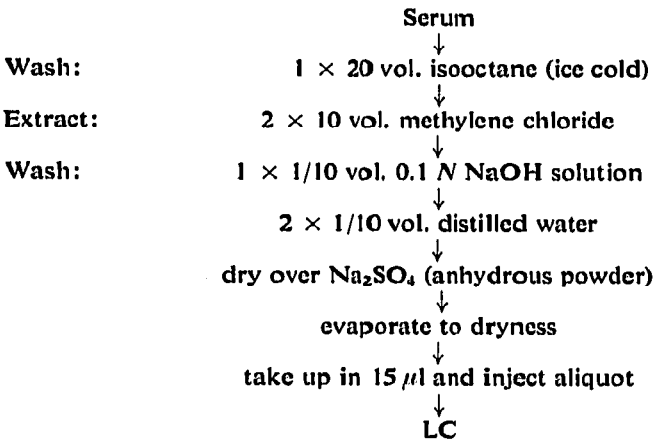

Fig. 1. Method for extraction of corticosteroids.

phase were removed with anhydrous sodium sulfate and thereafter the methylene chloride was evaporated at 40° with a rotary evaporator until about 4 ml remained. The residue was transferred into a 5-ml centrifuge tube and dried completely under nitrogen. For the determination of the precision, two plasma extracts were combined each time and diluted with 15 µl of chloroform. Portions of 5 µl of the combined extract were injected three times into the chromatograph.

RESULTS

Table I shows the separation of some corticosteroids on Sil-X-RP using methanol–water (40:60). The column was heated to 40° and the solvent mixture was at room temperature. The best results were obtained at a flow-rate of 1 ml/min; with a sensitivity of 0.05 O.D., 0.20 µg of steroid standard could be detected. The steroids were separated in groups; F, E and Aldo showed almost the same retention time and were separated from corticosterone (B) and substance S (S) and progesterone (P). The separation of F, E and Aldo isolated from a plasma extract is shown in Fig. 2. The less polar material remained near the solvent front and a clean peak for F, E and Aldo together was obtained.

For the calculation of recovery during the extraction procedure, radioactive F (20,000 dpm) was added to the blood before extraction and an aliquot counted in a

TABLE I

SEPARATION OF STEROIDS ON SIL-X-RP

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

liquid scintillation counter before injection into the chromatograph. An average recovery of 86% resulted. For the determination of the recovery from the liquid column, radioactive cortisol was injected with and without the biological material and the portion representing the cortisol peak was collected. An average recovery of 93% was obtained. Proof of identity was provided by spotting the radioactive and non-radioactive standards after collection from the column on thin-layer and paper chromatograms followed by chromatography in different systems. The systems used for thin-layer chromatography were chloroform–acetone–ethanol (2:1:0.3 and 1:1:0.1)

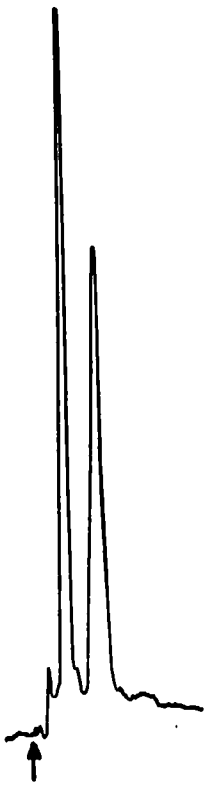

Fig. 2. Separation of 10 ml of plasma extract on Sil-X-RP. Second peak represents F, E and Aldo.

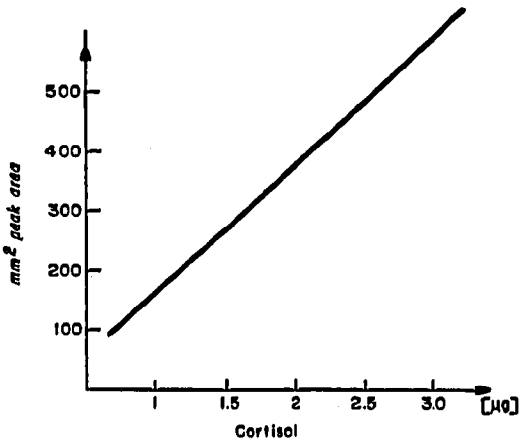

Fig. 3. Standard curve for serial amounts of cortisol.

and ethyl acetate–ethanol (7:1), and in paper chromatography propylene glycol–toluene (partition). Only F, E and Aldo were found together in one fraction after injection of several corticosteroids. For quantitation, a standard curve with serial amounts of cortisol was prepared (Fig. 3). The equation $C = V_1/V_2$ for the correction of the peak area was used[7], where V_1 is the peak area of the standard curve and V_2 that of the internal standard. A 1-μg amount of F, serving as an internal standard, was injected separately each time after the injection and separation of the biological material. Thirty plasma samples of healthy persons were analyzed, and quantitated by LC using the system and conditions described above. The results are shown in Table II.

The average value for F, E and Aldo was 16.31 μg per 100 ml of plasma with a standard deviation of 2.10 and a standard error of 0.38. The precision was obtained by combining two plasma extracts and dividing each sample into three equal portions which were injected separately into the chromatograph. As seen in Table II, the average value representing one third from 10 ml of plasma was 0.54 μg. The average standard deviation of all the triplicate determinations was 0.05, which indicates good reproducibility and precision considering the thermal and chemical lability of the compounds.

DISCUSSION

The extraction procedure, as well as the chromatography and quantitation of the corticosteroids with the presented method, is fast. Twenty to thirty determinations can easily be performed in one day. No derivatives are formed, as described by some authors[8,9].

High-pressure liquid chromatography was used with the advantage of a fast and mild separation of corticosteroids. The reversed-phase column packing material proved to be more stable and more reproducible compared with the liquid adsorption material used in other studies[4,5]. The specificity for cortisol, cortisone and aldosterone was tested by thin-layer and paper chromatography in the different systems mentioned

TABLE II

TRIPLICATE DETERMINATIONS FROM 10 ml OF PLASMA EXTRACTS

Person	*Triplicate determination* of 10 ml of plasma extract*	*Mean (μg)*	*μg per 100 ml of plasma*	*Standard deviation*	*Standard error*
1	0.55, 0.61, 0.66	0.60	18.2	0.05	0.03
2	0.62, 0.39, 0.51	0.50	15.2	0.11	0.06
3	0.49, 0.58, 0.60	0.55	16.7	0.05	0.03
4	0.44, 0.49, 0.46	0.46	13.9	0.02	0.01
5	0.72, 0.46, 0.59	0.59	17.7	0.13	0.07
6	0.48, 0.48, 0.50	0.48	14.6	0.01	0.0
7	0.63, 0.49, 0.56	0.56	16.8	0.07	0.04
8	0.40, 0.45, 0.51	0.45	13.6	0.05	0.03
9	0.46, 0.58, 0.49	0.51	15.3	0.06	0.03
10	0.54, 0.52, 0.60	0.55	16.6	0.04	0.02
11	0.53, 0.63, 0.57	0.57	17.3	0.05	0.02
12	0.67, 0.62, 0.74	0.67	20.3	0.06	0.03
13	0.62, 0.58, 0.54	0.58	17.4	0.04	0.02
14	0.46, 0.48, 0.52	0.48	14.6	0.03	0.01
15	0.44, 0.36, 0.40	0.40	12.0	0.04	0.02
16	0.77, 0.65, 0.71	0.71	21.0	0.06	0.03
17	0.56, 0.54, 0.52	0.54	16.2	0.02	0.01
18	0.50, 0.56, 0.61	0.55	16.7	0.05	0.03
19	0.60, 0.62, 0.58	0.60	18.0	0.02	0.01
20	0.47, 0.53, 0.60	0.53	16.0	0.06	0.03
21	0.43, 0.51, 0.63	0.52	15.7	0.1	0.05
22	0.51, 0.61, 0.64	0.58	17.6	0.06	0.03
23	0.57, 0.49, 0.52	0.52	15.8	0.04	0.02
24	0.62, 0.58, 0.53	0.57	17.3	0.04	0.02
25	0.49, 0.44, 0.45	0.46	13.8	0.02	0.01
26	0.56, 0.62, 0.67	0.61	18.5	0.05	0.03
27	0.61, 0.58, 0.62	0.60	18.1	0.02	0.01
28	0.42, 0.39, 0.47	0.42	12.8	0.04	0.02
29	0.51, 0.46, 0.41	0.46	13.8	0.05	0.02
30	0.64, 0.57, 0.59	0.6	18.0	0.03	0.02
	All samples:	0.54	16.3	0.05	0.03

* Each sample (10 ml of plasma extract) was divided into three equal portions before injection into the chromatograph.

above. No other standard was found in the zone corresponding to F, E and Aldo. The interference of drugs with the measured corticosteroids has been described using fluorimetric detection methods[8], but those methods did not use a final separation by thin-layer, paper or liquid chromatography. In the present study, drug interference was not studied as no drugs were taken by the group analyzed. The separation on the liquid column, however, should diminish drug contamination. This point has to be taken into consideration when the method is used on a routine basis.

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