Journal of Chromatography, 430 (1988) 233–239
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4248

IMPROVED METHOD FOR THE DETERMINATION OF THE CORTISOL PRODUCTION RATE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND LIQUID SCINTILLATION COUNTING

HUUB E. VAN INGEN and ERIK ENDERT*

Department of Clinical Chemistry, Laboratory of Endocrinology, Academic Medical Centre, Meibergdreef 9, 1105 AZ Amsterdam Z.O. (The Netherlands)

(First received January 19th, 1988; revised manuscript received March 31st, 1988)

SUMMARY

Two new methods for the determination of the cortisol production rate using reversed-phase high-performance liquid chromatography are described. One uses ultraviolet detection at 205 nm, the other on-line post-column derivatization with benzamidine, followed by fluorimetric detection. The specific activity of tetrahydrocortisol and tetrahydrocortisone in urine from patients who had received tritium-labelled cortisol was determined by the indicated methods, followed by fraction collection and liquid scintillation counting. The post-column reaction detection procedure was superior to ultraviolet detection, both in selectivity and analysis time. Intra- and inter-assay variance of the post-column reaction detection procedure were 3.7 and 4.7%, respectively. A good correlation ($r=0.99$) was obtained between values determined by this procedure and by a thin-layer chromatographic procedure.

INTRODUCTION

Cushing's syndrome/disease involves an excessive production of cortisol. Beside the determination of urinary free cortisol and function tests (e.g. the dexamethasone suppression test) the cortisol production rate (CPR) is a valuable diagnostic parameter, which is a direct measure of adrenocortical activity, independent of the concentration of cortisol-binding globulin [1]. Measurement of the CPR also allows differentiation of Cushing's syndrome from exogeneous obesity [2]. However, owing to the complicated procedure involved, the CPR can only be measured in cases in which a strong indication of Cushing's syndrome/disease exists.

The CPR is determined via oral or intravenous administration to the patient of a dose of tritium-labelled cortisol. Subsequently, the two major urinary metabolites of cortisol, tetrahydrocortisol (THF, 5β-pregnan-3α,11β,17α,21-tetrol-20-

0378-4347/88/$03.50

one) and tetrahydrocortisone (THE, 5β-pregnan-3α,17α,21-triol-11,20-dione) are isolated by thin-layer chromatography (TLC) of an extract from a hydrolysed portion of a 24-h urine collection [3]. THF and THE are eluted from the plates and quantitatively determined by the reaction of Silber and Porter [4]. In another aliquot of the eluate, the amount of tritium-labelled THF and THE is determined by liquid scintillation counting (LSC). From these parameters the specific activity of THF and THE can be calculated. The CPR is calculated by dividing the administered activity expressed as disintegrations per minute (dpm) by the specific activity of THF and THE expressed as dpm/μmol, respectively. The mean of these values is accepted as a measure of the amount of cortisol produced in the 24-h period. The laborious chromatographic procedures involved prompted us to investigate the possibility of a simpler method for separation and quantification of labelled and unlabelled urinary metabolites of cortisol, using high-performance liquid chromatography (HPLC).

A variety of methods have been published for determination of THF and THE in urine, including radioimmunoassay [5, 6], HPLC with pre-column derivatization with dansyl hydrazine [7] or benzoyl chloride [8], and HPLC with on-line post-column derivatization with benzamidine [9]. None of these methods has been used to determine the CPR. We chose to test an HPLC procedure with UV detection because of its simplicity and an HPLC procedure with on-line post-column reaction detection with benzamidine because of its excellent selectivity for compounds carrying a dihydroxyacetone side-chain.

EXPERIMENTAL

Chemicals

THE and THF were obtained from Koch-Light Labs. (Colnbrook, U.K.). β-Glucuronidase (type H-2, 96500 U/ml) was from Sigma (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile was from Merck (Amsterdam, The Netherlands). Insta-Gel scintillation cocktail was from Packard Instrument (Groningen, The Netherlands). Water was distilled. All other reagents were analytical-reagent grade from Merck. The mobile phase was filtered and degassed in an ultrasonic bath before use. Stock solutions of THF and THE were made by weighing and dissolving in methanol; dilutions were made in the mobile phase.

Chromatography

The mobile phase [28% (v/v) acetonitrile in water] was delivered by an M45 pump (Waters Assoc., Milford, MA, U.S.A.) at a flow-rate of 0.6 ml/min. Samples were injected with an injection valve (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl loop. The column consisted of two 100 mm × 3 mm I.D. cartridges packed with 5-μm ChromSpher C18 particles, with a guard cartridge (Chrompack, Middelburg, The Netherlands) coupled to an SF770 absorbance detector (Schoeffel Instruments, Westwood, NJ, U.S.A.) operated at 205 nm, or one such cartridge coupled to a post-column reaction detector (see below). Chromatograms were recorded on a BD12 recorder (Kipp & Zonen, Delft, The Netherlands) and pro-

cessed manually. Tritium radioactivity was measured by LSC in a Tri-Carb 460c (Packard Instrument).

Post-column reaction detection

A modification of a reaction detector developed by Seki and Yamaguchi [9] was used. Solutions of 32 m*M* benzamidine hydrochloride and 0.3 *M* sodium hydroxide, both in the mobile phase, were mixed using a Model A pump (Eldex Labs., Menlo Park, CA, U.S.A.) and a 6000A pump (Waters Assoc.), respectively, at a flow-rate of 0.2 ml/min in each case. For the mixing, a T-piece (Waters Assoc.) was used. This reagent stream was mixed with the column effluent in a second T-piece. A knitted PTFE capillary (15 m×0.5 mm I.D.) in a water-bath (90°C) was used as reaction coil. The fluorescence intensity was monitored with an FS 970 fluorimeter (Schoeffel Instruments) operated at 370 nm excitation wavelength with a 470-nm cut-off emission filter.

Sample preparation

Urine samples and CPR values for these samples were obtained from the Academic Hospital Utrecht (The Netherlands). Patients were orally administered a dose of $2 \cdot 10^6$ dpm tritium-labelled cortisol. Subsequently, a 24-h urine collection was made, of which an aliquot was stored at −20°C.

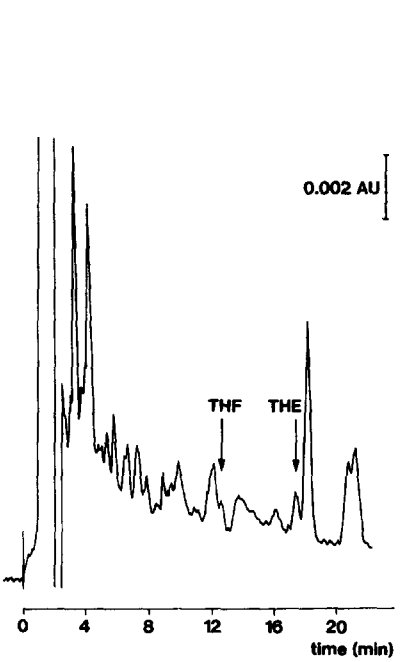
For analysis, 1 ml of urine was adjusted to pH 5.2, and 50 μl of 2 *M* sodium acetate buffer (pH 5.2) and 25 μl of β-glucuronidase solution were added. After it had been vortexed briefly, the mixture was incubated overnight at 37°C. These conditions were expected to yield optimal hydrolysis of THE and THF glucuronides [10]. The resulting solution was extracted with 10 ml of methylene chloride. The extract was washed successively with 2 ml of 0.1 *M* sodium hydroxide, 2 ml of 0.1 *M* hydrochloric acid and 2 ml of water. After centrifugal separation, the extract was transferred to a test-tube and evaporated at 40°C under a gentle stream of nitrogen. To the dried residue, 30 μl of mobile phase were added, of which a 20-μl aliquot was injected. The THE and THF fractions were collected, 10 ml of Insta-Gel were added and the tritium radioactivity was counted for 30 min.

RESULTS AND DISCUSSION

UV detection

Since THF and THE have no conjugation in the A-ring, UV detection must be performed at the relatively non-specific wavelength of 205 nm [11]. The peak height was related to the amount of compound injected via linear regression. The extraction efficiency from hydrolysed urine was 43% for THF and 69% for THE.

Using this technique, rather complex chromatograms were obtained. In most cases, adequate resolution between THF, THE and interfering substances could not be obtained (Fig. 1). In some samples this could be realised, permitting calculation of the amount of cortisol metabolites in these samples (Fig. 2 and Table I). However, in the majority of samples analysed, reliable quantification of the compounds of interest was impossible; also, owing to the presence of late-eluting



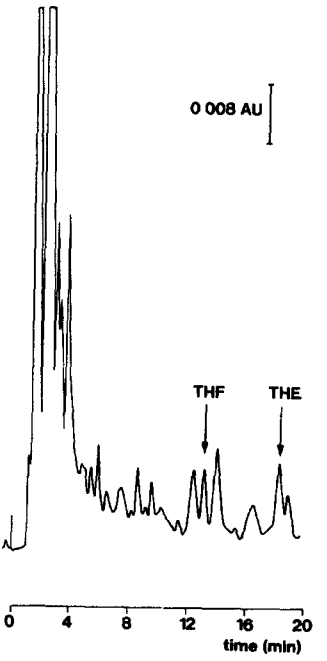


Fig. 1. Chromatogram of a 1-ml urine sample analysed according to the procedure in *Sample preparation*. Detection at 205 nm.

Fig. 2. Chromatogram of a 1-ml urine sample analysed according to the procedure in *Sample preparation*. Detection at 205 nm. The sample contained 230 ng of THF and 290 ng of THE.

TABLE I

COMPARISON OF CPR VALUES DETERMINED BY TLC AND HPLC-UV

Values were determined in duplicate.

Sample	CPR (μmol per 24 h)			
	THF		THE	
	TLC	HPLC-UV	TLC	HPLC-UV
1	73	58	69	52
2	73	72	81	69
3	29	36	61	18
4	61	63	72	35

UV-absorbing components in some samples, the chromatographic analysis time was too long to obtain an acceptable sample throughput. For these reasons we decided to test the reaction detection system using benzamidine for selective detection of compounds carrying a dihydroxyacetone side-chain.

Post-column reaction detection

In order to decrease the analysis time and increase the sample throughput, several changes were made to the reaction detection system described by Seki

and Yamaguchi [9]. The use of acetonitrile instead of methanol as organic modifier in the mobile phase resulted in a considerable increase in resolution between THF and THE, and the use of a more efficient column meant that the volume of the reaction coil could be reduced from 5.9 to 2.9 ml. These changes reduced the analysis time from 30 to 20 min (Fig. 3A).

The reproducibility of the reaction detector was tested by repeated injections of 2 μg of THF and THE. This resulted in a coefficient of variation (C.V.) of 3.1% for THF and 3.9% for THE ($n=10$). When the amount of compound injected (in duplicate) was correlated with the peak height, a good regression was obtained: THF, $r=0.996$; THE, $r=0.998$ (range 0.1–4 μg).

Very selective detection of THF and THE in hydrolysed urine samples was possible with this set-up. Thus far, no interfering substances have been found in any urine sample (see Fig. 3B and C). Cortisone eluted with a retention time of 12 min, well separated from both cortisol and THF. Determination of the specific activity of THF and THE, and subsequent calculation of CPR values, was straightforward.

The analytical performance of the procedure was tested with a pooled urine sample. The specific activity of THF and THE could be determined with an intra-assay C.V. of 2.9 and 3.7% ($n=5$), respectively, and an inter-assay C.V. of 4.7 and 4.2% ($n=5$), respectively. The amounts of compound injected were 1.1 μg (93 dpm/μg) of THF and 1.5 μg (56 dpm/μg) of THE. When the statistical errors in the reaction detection (3.1%) and the LSC (5%) are combined, a minimum

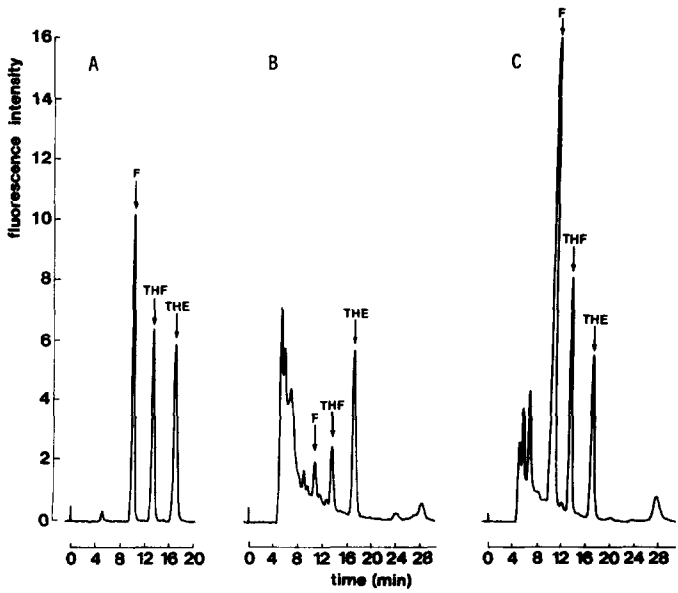


Fig. 3. Chromatograms of (A) a standard solution containing 1 μg each of cortisol (F), THF and THE, (B) a 1-ml urine sample from a healthy young subject containing 0.11 μg of F, 0.31 μg of THF and 0.95 μg of THE and (C) a 0.5-ml urine sample of a patient suffering from Cushing's syndrome containing 1.51 μg of F, 1.22 μg of THF and 0.93 μg of THE. Experimental conditions as described in *Sample preparation* and *Post-column reaction detection*.

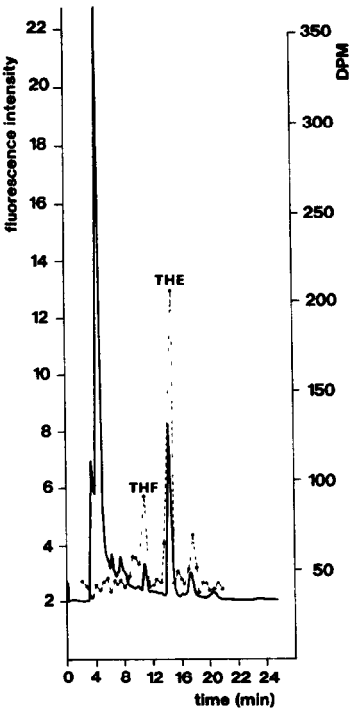


Fig. 4. Distribution of tritium radioactivity in the chromatogram of a 1-ml urine sample from a patient orally dosed with $2 \cdot 10^6$ dpm tritium-labelled cortisol. Experimental conditions as described in *Sample preparation* and *Post-column reaction detection*. The detector effluent was collected at 0.5-min intervals.

C.V. of 6% can be expected. The actual reproducibility of ca. 4% is therefore not surprising. The C.V. of the LSC can be reduced by extracting more urine or increasing the counting time.

The distribution of tritium radioactivity in the chromatogram of a urine sample is given in Fig. 4. As can be seen, almost all the radioactivity was confined to four peaks, which eluted at the positions of THF, THE and two unidentified components.

A good correlation was found between CPR values determined by our method and the values provided with the samples, which were determined by a TLC procedure [12]. The values obtained by HPLC were slightly lower than those determined by TLC (CPR values based on specific activity of THF: $y=0.882x-3.3$, $r=0.99$, $n=11$; CPR values based on specific activity of THE: $y=0.757x$, $r=0.99$, $n=11$; y represents HPLC values, x represents TLC values). This difference is possibly caused by a minor interference in the Silber–Porter reaction, which is used for quantification of THF and THE in the TLC procedure. It has been shown that several steroids and drugs interfere in this rather non-specific colorimetric reaction [13].

CONCLUSIONS

The post-column reaction detection system described here is a reliable and fast method for determination of the CPR. Separation and quantification of THF and THE in an extract from a hydrolysed urine sample is performed on-line in 20 min. This step takes at least two days in the TLC procedure [3]. Also, we regard our method as faster and more selective than a recently described HPLC procedure for determination of the CPR, using either pre-column derivatization to 11β-hydroxyaetiocholanolone and labelling with 2,4-dinitrophenylhydrazine, or off-line quantitation of THE and THF [14]. The described procedure is therefore an improvement in the determination of the CPR.

ACKNOWLEDGEMENT

We thank Professor Dr. J.H.H Thijssen (Academic Hospital Utrecht, The Netherlands) for providing the CPR samples.

REFERENCES

1 D.C. Anderson and J.S.D. Winter, Adrenal Cortex, Butterworths, London, 1985, p. 191.
2 F.S. Greenspan and P.H. Forsham. Basic and Clinical Endocrinology, Lange, Los Altos, CA, 2nd ed., 1983, p. 285.
3 C.L. Cope and E. Black, Br. Med. J., 1 (1958) 1020.
4 R.H. Silber and E. Porter, J. Biol. Chem., 210 (1954) 923.
5 A. Kambegawa and S. Honma, Folia Endocrinol. Jpn., 55 (Suppl.) (1979) 1138.
6 H. Will, R. Aderjan, T. Winkler, B. Penke and P. Vecei, Acta Endocrinol., 86 (1977) 369.
7 T. Kawasaki, M. Maeda and A. Tsuji, J. Chromatogr., 232 (1982) 1.
8 M. Novotny, K.-E. Karlsson, M. Konishi and M. Alasandro, J. Chromatogr., 292 (1984) 159.
9 T. Seki and Y. Yamaguchi, J. Chromatogr., 305 (1984) 188.
10 C.H.L. Shackleton, J. Chromatogr., 379 (1986) 91.
11 L.L. Engel, Physical Properties of the Steroid Hormones, Pergamon, Oxford, 1963, p. 79.
12 J.H.H. Thijssen, F. de Waard and A. Wiersinga, Lancet, i (1967) 814.
13 D.S. Young, D.W. Thomas, R.B Friedman and L.C. Pestner, Clin. Chem., 18 (1972) 104.
14 B.J. Passingham and R.N. Barton, J. Chromatogr., 416 (1987) 25.