Journal of Chromatography, 416 (1987) 25–35
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3536

APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE MEASUREMENT OF CORTISOL SECRETION RATE

B.J. PASSINGHAM* and R.N. BARTON*

MRC Trauma Unit, Stopford Building, University of Manchester, Oxford Road, Manchester M13 9PT (U.K.)

(First received July 22nd, 1986; revised manuscript received November 28th, 1986)

SUMMARY

After administration of [^{3}H]cortisol, the specific radioactivity of the urinary cortisol metabolites is determined by chemical conversion of the 17-hydroxycorticosteroid conjugates to 17-ketosteroids, extraction with Sep-Pak C_{18} cartridges, derivatisation with 2,4-dinitrophenylhydrazine and isocratic reversed-phase high-performance liquid chromatography. This allows on-line measurement of ultraviolet absorbance; fractions are collected and ^{3}H is measured by scintillation counting. Alternatively extraction and chromatography are done after enzymic hydrolysis of the steroid conjugates, and the major metabolites are determined colorimetrically in the fractions. The results from the two methods were consistent with each other and with published data.

INTRODUCTION

Among the tests available for investigating the hypothalamus–pituitary–adrenal axis, studies with isotopically labelled cortisol are essential if disturbances of cortisol clearance are to be assessed. The most convenient method is to measure the specific radioactivity of urinary cortisol metabolites after oral or intravenous administration of the labelled steroid [1,2]. Although undemanding on the patient, this procedure is technically exacting in that it requires separation of the metabolites unique to cortisol from other urine constituents. This is normally done by one or more paper chromatographic steps, sometimes in conjunction with column or thin-layer chromatography [1,3–5]. However, high-performance liquid chromatography (HPLC) is inherently more suitable than any of these techniques because of the ease of collecting the eluate for scintillation

*Present address: MRC Toxicology Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K.

0378-4347/87/$03.50

counting, the only accurate way of determining tritium. Additional advantages of HPLC are the ability to measure the absorbance of the effluent, the ease of sample application, good resolution and high sample capacity, so that the dose of label given can be minimised. This paper describes the use of HPLC for measuring the specific radioactivity of cortisol metabolites.

The classical approach is to isolate either or both of the two predominant metabolites, tetrahydrocortisol (THF) and tetrahydrocortisone (THE), in the urine after enzymic hydrolysis of their glucuronic acid conjugates. However, even after exhaustive purification the specific radioactivity of these and other supposedly unique metabolites of cortisol may differ [4–6]. Several workers have sought to minimise this problem and simplify the methods by converting most of the major metabolites to the 17-ketosteroid, 11β-hydroxyaetiocholanolone (OH-aetio), so that the specific radioactivity determined is in effect a weighted mean [3,7,8]. This conversion is done by the reactions used for the determination of 17-ketogenic steroids [9]: reduction of all carbonyl groups with sodium borohydride followed by oxidative cleavage of steroid side-chains with sodium periodate.

We have applied HPLC to both these approaches. We have used an octadecylsilane column in reversed phase, run isocratically with methanol–water mixtures. Since the 3-keto,$\Delta^{4,5}$ chromophore of cortisol is reduced during metabolism the metabolites cannot be measured directly by UV absorbance. For the derived 17-ketosteroids, therefore, we have formed adducts of the 17-keto group with 2,4-dinitrophenylhydrazine (DNPH), which absorbs strongly in the UV and visible ranges, before chromatography [10,11]. For the unchanged THE and THF, however, we have not attempted to monitor the effluent spectrophotometrically but have measured the quantities of 17-hydroxycorticosteroids in the column fractions colorimetrically [12]. For this purpose we have modified the phenylhydrazine reagent so that it can be added directly to the fractions without any need for evaporation. We have also modified the standard techniques by substituting Sep-Pak cartridges [13] for organic solvents as a means of extracting the steroids from urine.

EXPERIMENTAL

Apparatus

HPLC analyses were performed with an LDC Model III system (Laboratory Data Control, Stone, U.K.) equipped with a 25 cm$\times$4.6 mm Spherisorb 5 ODS column (Technicol, Stockport, U.K. or Phase Separations, Queensferry, U.K.). Sep-Pak C_{18} cartridges were supplied by Waters Assoc. (Hartford, U.K.).

Chemicals

[1,2,6,7-^{3}H]Cortisol [stated purity 96–98%; at the time of use 90–95% eluted as a single peak on HPLC with methanol–water (1:1) as eluent] was bought from Amersham International (Amersham, U.K.); unlabelled steroids and phenylhydrazine from Sigma (London) (Poole, U.K.); methanol (AR grade) from Koch-Light (Haverhill, U.K.); benzene and trichloroacetic acid from Fisons (Loughborough, U.K.); Rialuma scintillant from LKB (South Croydon, U.K.); β-glu-

curonidase (from ox liver; arylsulphatase activity <0.2% β-glucuronidase activity) and all other reagents from BDH Chemicals (Poole, U.K.).

Direct determination of specific radioactivity of THE and THF

Steroid conjugates are first hydrolysed enzymically [14]. A 100-ml volume of urine, or about one tenth of the 24-h volume if this is below 1000 ml, is mixed with 50 000 Fishman units β-glucuronidase and 10 ml of 2.5 mol/l acetate buffer, pH 4.7. After it has been left for 48–72 h at 37 °C, it is filtered through Whatman No. 1 paper; this is necessary to prevent clogging of the Sep-Pak cartridge. The filtrate is passed through a Sep-Pak C_{18} cartridge, which has been primed with 2 ml methanol followed by 5 ml water; this is conveniently done by suction or under pressure from a syringe equipped with a valve to allow refilling. The cartridge is washed successively with 5 ml water and 2 ml methanol–water (1:4, v:v) as recommended by the manufacturer, and then eluted with 2 ml methanol from a syringe. The extract is evaporated to 0.3–0.5 ml on a water bath and centrifuged at about 1700 *g* if turbid. A 100-μl aliquot is injected into the HPLC system. Elution is with methanol–water (2:3, v:v) at a flow-rate of 1 ml/min at room temperature, and fractions of 2.5 ml are collected. Half of each fraction is taken for scintillation counting. To the other half is carefully added 1.7 ml modified Porter–Silber reagent (300 mg phenylhydrazine freshly dissolved in a mixture of 100 ml concentrated sulphuric acid with 30 ml methanol). After gentle mixing the tubes are left at room temperature overnight and the absorbance is then read at 410 nm. The concentrations of THF and THE are calculated with reference to standards (5–10 μg) made up in eluent at the same time.

Determination of specific radioactivity after conversion to 17-ketosteroids

Urine is first subjected to the procedure of Few [9] which also hydrolyses the steroid conjugates. A 100-ml volume of urine, diluted if necessary as above, is mixed with 25 ml of 16% (w/v) sodium borohydride in 0.1 mol/l sodium hydroxide and left for 2 h or overnight at room temperature. A 25-ml volume of 25% (w/v) acetic acid is gradually added to decompose the excess borohydride and the pH is suitably adjusted if not within the range 6–7. (Sometimes no effervescence was observed if the smaller quantity of borohydride recommended by Few [9] was used, indicating that this was insufficient.) After evolution of hydrogen has stopped, 40 ml of 10% (w/v) sodium periodate is added and the mixture is left for a further 60 min at 40 °C. Between 5 and 10 ml of 10 mol/l sodium hydroxide is added as required to bring the pH above 11 and the mixture is left for at least 20 min more.

The mixture is filtered and the steroids are extracted with a Sep-Pak C_{18} cartridge as described above. Derivatisation is then carried out by the method of Treiber and Oertel [15]. A suitable volume of the extract (0.4–0.8 ml, equivalent to 1–2% of the 24-h urine output) is mixed with 2 ml of 0.1% (w/v) DNPH in methanol and blown dry on a water bath at 30–40 °C. As much of the residue as possible is redissolved in 5 ml of 0.03% trichloroacetic acid in benzene, left for 1 h at room temperature and then evaporated to dryness as before. The residue is thoroughly mixed with 0.5 ml methanol and the mixture is centrifuged at about

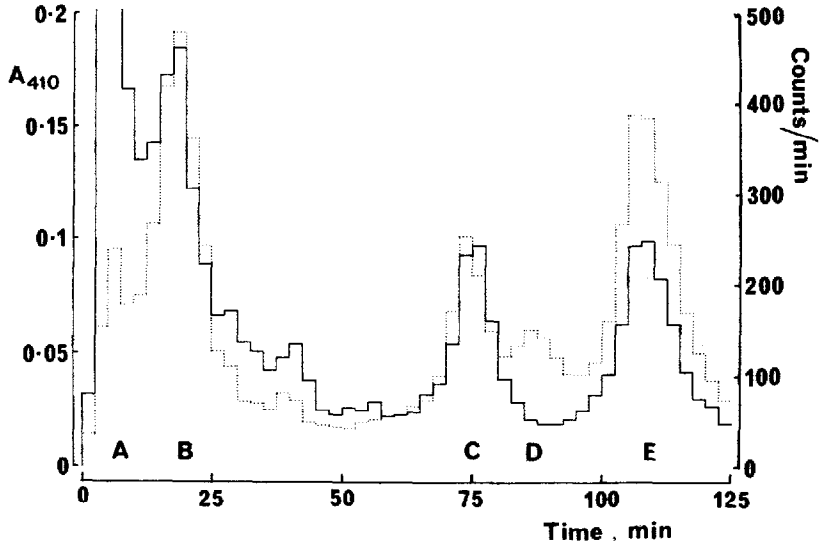

Fig. 1. Chromatogram of extract of urine treated with β-glucuronidase. Urine was from 24-h pool after ingestion of 2 μCi [1,2,6,7-^{3}H] cortisol by normal male (subject 762 in Table I). (—) Absorbance at 410 nm after treatment with acid phenylhydrazine reagent; (----) radioactivity determined by scintillation counting. For explanation of letters see text.

1700 *g*. Supernatant (0.1 ml) is chromatographed as described above but with methanol–water (2:1, v:v) as eluent. The absorbance of the effluent is read at 365 nm and the areas under the relevant peaks are determined by automatic integration or by cutting and weighing the chart recorder paper. The quantities of the steroid DNPH derivatives are calculated from the areas under the absorbance–time curves by using standard values for the molar absorptivities (see Results). Fractions of 2.5 ml are collected and all or part of them are taken for scintillation counting.

RESULTS

Chromatography of urine extracts after hydrolysis with β-glucuronidase

Fig. 1 shows a typical chromatogram of urine after digestion with β-glucuronidase and extraction with Sep-Pak cartridges as described above. The urine was part of a 24-h collection from a normal male who had ingested 2 μCi [^{3}H] cortisol. The major peaks of both radioactivity and Porter–Silber chromogen were C and E. Chromatography of pure steroid solutions under similar conditions showed that peak E contained THE and peak C contained both THF and *allo*-THF which were not separated from each other in this system. In some analyses, especially if a slightly more aqueous eluent was used so that retention times were longer, the peaks of radioactivity were slightly displaced from those of chromogen; this was probably an isotope effect. Peak D represented radioactivity not associated with Porter–Silber chromogen. At least part of this material consisted of the cortols. If the fractions comprising peak E (THE) were pooled, partially evaporated,

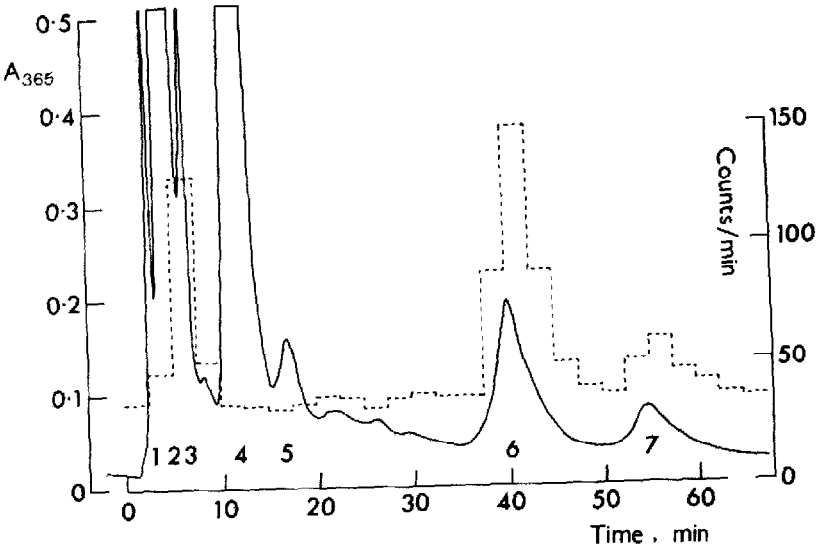

Fig. 2. Chromatogram of extract of urine after conversion of 17-hydroxycorticosteroids to 17-ketosteroids and derivatisation with DNPH. Urine was from same pool as in Fig. 1. (——) Absorbance at 365 nm; (----) radioactivity determined by scintillation counting. For explanation of numbers see text.

reduced with an excess of sodium borohydride, extracted with a Sep-Pak cartridge and rechromatographed with the same eluent, nearly all the radioactivity emerged in a peak corresponding to peak D.

Although the early peaks A and B contained both colour and radioactivity, this association was probably fortuitous as they were coloured by extracted urinary pigments even before addition of Porter–Silber reagent. The amount of radioactivity in peak B was very variable and it may partially have represented unhydrolysed glucuronic acid conjugates.

Chromatography after conversion to 17-ketosteroids and derivatisation

Fig. 2 shows the chromatogram obtained when the same urine was subjected to the procedure of Few [9], extracted and treated with DNPH before chromatography. Derivatisation of the pure steroids showed that peaks 6 and 7, which were always associated with both absorbance and radioactivity, contained the derivatives of OH-aetio and 11β-hydroxyandrosterone (OH-andro, derived from *allo*-THF), respectively. Radioactivity not emerging as either of these peaks was always eluted early as a single peak. Presumably with this less aqueous eluent material not derivatised with DNPH was scarcely retained by the column. The early peaks of absorbance showed a consistent pattern, apart from the variable solvent front (peak 1). Peaks 2 and 5 were present when free DNPH was chromatographed and presumably represented, respectively, the pure material and an impurity. Experiments in which the amount of trichloroacetic acid used for derivatisation was varied showed that peak 4 was an adduct of trichloroacetic acid with DNPH. The identity of peak 3 was uncertain but it was substantial only if the derivatised

material (either urine or pure steroid) had first been treated by the procedure of Few [9].

Molar absorptivities

Pure standards made up in column eluent were included with each set of fractions determined by the Porter–Silber colour reaction. Molar absorptivities varied from 15 000 to 21 000 for THF and 20 000 to 26 000 for THE. Absorbance was linear with THE concentration up to a final concentration of at least 0.01 mmol/l, corresponding to a concentration in the eluate of 0.022 mmol/l, which was well in excess of those encountered.

For the derivatised 17-ketosteroids molar absorptivities had to be determined separately. To find that for OH-aetio, three chromatograms were run: unchanged DNPH; DNPH put through the derivatisation procedure without steroid; and an excess of OH-aetio derivatised with a limited quantity of DNPH. From the first two chromatograms the molar absorptivity of DNPH and its adduct with trichloroacetic acid could be determined; hence from the areas under the relevant peaks in the third chromatogram the quantity of DNPH not used for forming the steroid derivative could be calculated. By subtracting this from the quantity added, a molar absorptivity of the OH-aetio derivative of 23 300 was obtained, in good agreement with the value of 23 600 found for the derivative of androsterone by Treiber and Oertel [15]. A molar absorptivity of 23 300 has therefore been taken for the derivatives of both OH-aetio and OH-andro.

Recovery of radioactivity during procedures

After enzymic hydrolysis, extraction and chromatography the overall recovery from the column of radioactivity originally present in the urine ranged from 56 to 89% (median 74%). Most of the loss occurred during the extraction stage. The proportion of the eluted label in the THF plus THE peaks was usually around 40%.

After treatment by the procedure of Few [9], extraction, derivatisation and chromatography, the overall recovery of urine radioactivity ranged from 52 to 84% (median 59%). About half the loss generally took place during extraction, with a smaller loss during treatment with borohydride and periodate; losses during derivatisation and chromatography were smaller still (around 5%). Of the radioactivity recovered from the column, generally around 50–60% was in the peaks corresponding to the DNPH derivatives of OH-aetio and OH-andro. This proportion variably decreased if less DNPH was used for derivatisation.

Administration of unlabelled cortisol after suppression with dexamethasone

As a test of the methods a normal subject was given dexamethasone to suppress the hypothalamus–pituitary–adrenal axis and then ingested labelled and unlabelled cortisol simultaneously. Under these circumstances, the specific radioactivities of the urinary metabolites should agree with each other and should be only slightly lower than the specific radioactivity of the ingested cortisol.

As a preliminary, dexamethasone was administered orally in 1-mg doses at 8-h intervals starting at midnight. The third dose was accompanied by 2 μCi

TABLE I

SPECIFIC RADIOACTIVITY OF URINARY CORTISOL METABOLITES IN NORMAL MALES

All were given 2 μCi [1,2,6,7-^{3}H] cortisol orally and urine was collected for the next 24 h. The cortisol secretion rate was calculated from the weighted mean specific radioactivity of OH-aetio and OH-andro and was corrected for label not excreted during this period [16]

Subject No.	Age	Specific radioactivity (μCi/μmol)				Cortisol secretion rate	
		THF+ *allo*-THF	THE	OH-aetio	OH-andro	μmol per 24 h	mg per 24 h
754	41	0.0197	0.0257	0.0226	0.0237	81.5	29.5
759	35	0.0477	0.0685	0.0692	0.0794	25.1	9.1
760	32	0.0480	0.0555	0.0461	0.0343	44.3	16.1
762	37	0.0246*	0.0387**	0.0406	0.0316	48.3	17.5
865	62	0.0186	0.0307	0.0277	0.0281	68.1	24.7
1064	65	0.0265	0.0406	0.0375	0.0276	46.4	16.8
2190	22	0.0386	0.0462	0.0456	0.0442	42.8	15.5

*After isolation of peak, conversion to 17-ketosteroids and derivatisation, specific radioactivity in OH-aetio was 0.0297 μCi/μmol and in OH-andro 0.0275 μCi/μmol.
**After isolation of peak, conversion to 17-ketosteroids and derivatisation, specific radioactivity in OH-aetio was 0.0401 μCi/μmol.

[^{3}H] cortisol, after which urine was collected for 24 h. Calculation of the cortisol secretion rate from the specific radioactivity of OH-aetio gave a value of 4.6 μmol per 24 h (1.7 mg per 24 h).

A few days later the subject was given the same treatment with dexamethasone but now ingested 55.2 μmol (20 mg) unlabelled cortisol at the same time as the [^{3}H] cortisol and urine was collected for only 8 h afterwards. Recovery of the ingested radioactivity in this urine was 35%. Analysis as described above gave specific radioactivities as follows: after enzymic hydrolysis, THF+*allo*-THF 0.0235 μCi/μmol, THE 0.0228 μCi/μmol; after treatment by the procedure of Few [9] and derivatisation with DNPH, OH-aetio 0.0253 μCi/μmol, OH-andro 0.0252 μCi/μmol. These values are in reasonable agreement with each other, but are lower than the specific radioactivity of the ingested cortisol, 0.0362 μCi/μmol. This discrepancy cannot all be explained by dilution with endogenous cortisol which, if secreted constantly at the rate determined previously, would only lower the specific radioactivity by about 8%.

Results in normal subjects

The results of using these methods in seven normal subjects are given in Table I. In all subjects the specific radioactivity of THE exceeded that of THF, and the value for OH-aetio was usually somewhere in between them. There was no consistent difference between the specific radioactivities of OH-aetio and OH-andro, the latter being in any case subject to considerable experimental error because of its size.

As a further check, after chromatography of the urine from one subject the

fractions comprising the THF + *allo*-THF and THE peaks were separately pooled, converted to 17-ketosteroids, derivatised with DNPH and rechromatographed as described above for urine. The specific radioactivities of the resulting OH-aetio and OH-andro peaks were in reasonable agreement with those of the precursor steroids (Table I). The peak containing the cortols (peak D in Fig. 1) was also subjected to this procedure and gave a specific radioactivity in OH-aetio of 0.0365 μCi/μmol. As expected, no OH-andro was formed from either this or the THE peak.

Precision

The within-subject variability of cortisol secretion rate could not be determined because we were not permitted to administer [^{3}H]cortisol repeatedly to the same subject, despite the negligible radiation hazard. However, 24-h urine collections from three subjects with widely differing cortisol secretion rates were used to estimate analytical precision. The urinary cortisol metabolites were converted to 17-ketosteroids, derivatised with DNPH and chromatographed by the usual procedure, but chromatography was repeated several times. For each chromatogram the cortisol secretion rate was calculated from the specific radioactivity of OH-aetio (cf. Table I, where the weighted mean for OH-aetio and OH-andro was used). The results were as follows (mean $\pm$ S.D.). Subject treated with dexamethasone (see above): 3.3 $\pm$ 1.5 μmol per 24 h (six replicates). The large coefficient of variation was due to one value of the specific radioactivity being just over half the next highest value. Normal subject (No. 2190 in Table I): 43.5 $\pm$ 1.9 μmol per 24 h (six replicates). Subject suffering from nausea (no vomiting after receiving label): 124.7 $\pm$ 6.2 μmol per 24 h (five replicates). Thus the coefficient of variation of the cortisol secretion rate was 4–5%, except when the rate was extremely low.

DISCUSSION

In applying HPLC to the measurement of cortisol secretion rate two approaches have been used. The first is that of Kelly et al. [7] who simplified the technique by converting most of the major metabolites to OH-aetio, an approach validated by Zumoff et al. [8]. Besides minimising the problems caused by discrepancies in specific radioactivity between metabolites, this approach has practical benefits when applying HPLC. The conversion procedure, involving first the reduction of all existing carbonyl groups with borohydride and then the rather specific oxidation of the reduced 17-hydroxycorticosteroids with periodate [9], ensures that few carbonyl groups will be present in compounds other than OH-aetio and OH-andro. Thus DNPH, a non-specific carbonyl reagent, can be used for derivatisation with a small chance that the compounds of interest will be swamped by derivatives of other urinary constituents. Chromatography of the DNPH derivatives allows the facility for on-line UV detection to be exploited and greatly simplifies the technique. Furthermore, derivatisation increases the retention of the steroids on the column, minimising the likelihood of label present originally as com-

pounds other than 17-hydroxycorticosteroids co-eluting with these peaks and spuriously increasing their specific radioactivity.

The main problem encountered with this technique was in obtaining an adequate yield in the derivatisation reaction. It must be stressed that no correction is necessary for losses here or elsewhere in the procedure as it is intended for measuring only the specific radioactivity of cortisol metabolites and not their total quantity. Nevertheless, excessive losses would reduce the precision of the final measurements of radioactivity and absorbance, and hence would increase the dose of labelled cortisol required. The standard conditions recommended previously for derivatisation of steroids [10,11] gave poor yields, in keeping with early observations that the 17-keto group was relatively resistant to derivatisation with DNPH [17]. The method of Treiber and Oertel [15] in a completely non-aqueous medium has been found satisfactory provided enough DNPH is used. The quantity recommended here achieves a reasonable yield while avoiding the problem of a continuously declining baseline following an excessive initial peak of free DNPH, as seen if much larger quantities of DNPH are used. At no stage has solubility proved a problem.

This method is therefore recommended as the procedure of choice for the routine determination of cortisol secretion rate. It gives a value for the specific radioactivity of OH-andro, derived from *allo*-THF, as well as that of OH-aetio derived from the other major metabolites, and presumably the best final estimate of cortisol secretion rate will be obtained from the weighted mean specific activity of the two 17-ketosteroids. At least 0.1% of the administered radioactivity can be finally recovered from the column in these two peaks, making it possible to limit the dose of [^{3}H]cortisol to 1–2 μCi.

We have also applied HPLC to the classical approach of determining the specific radioactivity of the individual cortisol metabolites, THE and THF. In common with most other workers, we have not attempted to measure routinely the specific radioactivity of the cortols and cortolones which lack any reactive groups. Because there are likely to be many carbonyl compounds in urine we thought it unwise to rely on derivatisation of the 20-keto group and monitoring of absorbance for measuring the mass of the peaks. Instead we have applied the rather specific Porter–Silber reaction to the column fractions. (Measurement by the blue tetrazolium method [18] was also tried but was discarded because of its lack of specificity, high background colour and intolerance to water, making it necessary to evaporate the samples to dryness.) This technique is not as convenient to use as that involving conversion to 17-ketosteroids and derivatisation. Adding the Porter–Silber reagent to each fraction and measuring its absorbance need to be done with care and are clearly much more time-consuming than on-line measurement. The retention of steroids is rather sensitive to small changes in eluent composition and incompleteness of elution with a new batch of eluent would not be noticed until the fractions had been worked up. The failure to separate THF and *allo*-THF could also be a disadvantage. We have not looked at the possibility of rectifying this by using a different solvent system.

Clearly it is essential in this procedure that metabolites that contain label but not Porter–Silber chromogen are not co-eluted with THE or THF and spuriously

increase their specific radioactivity. We have used reduction of labelled THE with borohydride to demonstrate that the cortols are eluted in between these two peaks. To eliminate the possibility of interference by other metabolites we have had to rely on two further checks. First, in urine from a normal subject, the specific radioactivities of the THF + *allo*-THF and THE peaks did not change substantially when they were converted to 17-ketosteroids and rechromatographed (Table I). For the second check we examined urine from a subject given cortisol of known specific radioactivity after suppression with dexamethasone. It was expected that all the unique cortisol metabolites, both unchanged and after conversion to 17-ketosteroids, would have specific radioactivities similar both to each other and to the administered cortisol. Within experimental error the first of these expectations was met, making it unlikely that the specific radioactivity of any peak was seriously overestimated by co-elution of label not associated with UV absorbance or Porter–Silber chromogenicity. However, all the specific radioactivities were lower than that of the administered cortisol, the discrepancy being greater than attributable to dilution with residual endogenously secreted cortisol. It is possible that co-elution of unlabelled material reacting like the steroids of interest led to overestimation of the mass of the peaks, but this seems unlikely to have happened equally for all four of them, especially in view of the specificity of the procedures involved. We do not yet have an alternative explanation.

The results obtained with these methods are in keeping with published data. The cortisol secretion rates in normal subjects (Table I) are within published ranges [1,2,4,8] and the rate in the subject given dexamethasone is similar to that after administration of other synthetic steroids [1]. The tendency of the specific radioactivity of THE to exceed that of THF is also in agreement with previous results [4–6]. The distribution of label between the various steroid peaks is compatible with that found in normal subjects by Zumoff et al. [19]. In addition the data in Table I are internally consistent in that in most subjects the specific radioactivity of OH-aetio is bracketed by those of THE and THF which are its major precursors.

The present studies show that the advantages of HPLC can be exploited to simplify the measurement of cortisol secretion rate. It is hoped that this will lead to a renewal of interest in this powerful but currently neglected method of assessing adrenal function.

REFERENCES

1 C.L. Cope and E. Black, Br. Med. J., 1 (1958) 1020–1024.
2 C.L. Cope and J. Pearson, J. Clin. Pathol., 18 (1965) 82–87.
3 H.L.J. Makin, R.W.H. Edwards and B.E. Clayton, Clin. Sci., 34 (1968) 549–558.
4 D.K. Fukushima, H.L. Bradlow, L. Hellman and T.F. Gallagher, J. Clin. Endocrinol. Metab., 28 (1968) 1618–1622.
5 R. Libert and R. De Hertogh, Steroids, 16 (1970) 539–552.
6 A. Kowarski, F.M. Kenny and C.J. Migeon, J. Clin. Endocrinol. Metab., 30 (1970) 114–116.
7 W.G. Kelly, S.R. Ranucci and J.C. Shaver, Steroids, 11 (1968) 429–452.
8 B. Zumoff, D.K. Fukushima and L. Hellman, J. Clin. Endocrinol. Metab., 38 (1974) 169–175.
9 J.D. Few, J. Endocrinol., 22 (1961) 31–46.
10 R.A. Henry, J.A. Schmit and J.F. Dieckman, J. Chromatogr. Sci., 9 (1971) 513–520.

11 F.A. Fitzpatrick, S. Siggia and J. Dingman, Sr., Anal. Chem., 44 (1972) 2211-2216.
12 R.H. Silber and C.C. Porter, J. Biol. Chem., 210 (1954) 923-932.
13 C.H.L. Shackleton and J.O. Whitney, Clin. Chim. Acta, 107 (1980) 231-243.
14 S.L. Cohen, J. Biol. Chem., 192 (1951) 147-160.
15 L. Treiber and G.W. Oertel, Z. Klin. Chem. Klin. Biochem., 5 (1967) 83-86.
16 C.L. Cope and J. Pearson, Clin. Sci., 25 (1963) 331-341.
17 A.G. Gornall and M.P. MacDonald, J. Biol. Chem., 201 (1953) 279-297.
18 R.O. Recknagel and M. Litteria, J. Lab. Clin. Med., 48 (1956) 463-468.
19 B. Zumoff, H.L. Bradlow, T.F. Gallagher and L. Hellman, J. Clin. Invest., 46 (1967) 1735-1743.