Journal of Chromatography, 227 (1982) 485—491
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1084

Note

Determination of urinary dehydroepiandrosterone sulphate by combined high-performance liquid chromatography and radioimmunoassay

ROGER C. CAUSON*, SUSAN L. COLLINS and DENYS E. FRY*

Epsom Hospital Laboratories, West Park Hospital, Epsom, Surrey KT19 8PB (Great Britain)

(Received June 16th, 1981)

Recent work has focussed on the regulation of adrenal dehydroepiandrosterone sulphate (DHEAS) biosynthesis by factors other than adrenocorticotrophin [1, 2]. Several lines of evidence suggest that prolactin might be such a factor [3—5] although conflicting evidence has also been presented [6, 7]. Only plasma DHEAS was measured in these studies, so a method to measure urinary DHEAS may further the understanding of DHEAS metabolism and its regulation.

Previous reports of DHEAS excretion in urine have relied on hydrolysis of the conjugate and measurement of dehydroepiandrosterone (DHEA) by gas chromatography [8]. Since DHEAS is thermolabile, its direct measurement by gas—liquid chromatography is not possible unless a derivative is made [9]. However, high-performance liquid chromatography (HPLC) enables DHEAS to be separated without hydrolysis and the sample to be recovered for further characterisation. The chromatography system was based on Wahlund and Beijersten's separation of acids using pentan-1-ol saturated buffer [10]. Partition studies with tritiated DHEAS were used to optimise the chromatography conditions.

This paper reports a method for the measurement of DHEAS in urine using HPLC and radioimmunoassay (RIA) of the pooled UV absorbing fractions.

EXPERIMENTAL

All chemicals were of reagent grade. DHEAS and prednisone were obtained from Steraloids (Croydon, Great Britain) and the other steroids from Sigma

*Present address: Department of Clinical Pharmacology, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 0HS, Great Britain.

0378-4347/82/0000—0000/$02.75 © 1982 Elsevier Scientific Publishing Company

TABLE I

PERCENTAGE CROSS REACTION OF STEROIDS WITH DEHYDROEPIANDROSTERONE ANTISERUM

Steroid	Percentage*
Dehydroepiandrosterone	100.0
Dehydroepiandrosterone sulphate	141.0
Androsterone sulphate	1.0
Aetiocholanolone sulphate	0.1
Androsterone	4.4
Aetiocholanolone	<0.4
Δ^4-Androstenedione	3.3
Oestrone	<0.4
Progesterone	<0.4
Testosterone	<0.4

*at 50% displacement of tracer.

Chemicals (Poole, Great Britain). Dehydro[7-(*n*)^{3}H]epiandrosterone sulphate (specific activity 4.6 Ci/mmol) and dehydro[1,2,6,7-^{3}H]epiandrosterone (specific activity 64 Ci/mmol) were from the Radiochemical Centre (Amersham, Great Britain). The DHEA antiserum Guildhay HP/S/48-1A was raised in sheep at the University of Surrey from an antigen, produced in this laboratory, of dehydroepiandrosterone-3-hemisuccinate-ovalbumin. The cross reaction of the antiserum is given in Table I.

Chromatography system

The apparatus consisted of a solvent delivery system (Waters Model 6000) and a Cecil CE 2012A variable-wavelength UV monitor (Cecil Instruments, Cambridge, Great Britain). A stainless-steel column (180 mm × 4.1 mm I.D.) was slurry-packed with Hypersil SAS, particle size 5 μm (Shandon Southern Products, Runcorn, Great Britain) and connected to a guard column (50 mm × 2.1 mm I.D.) dry-packed with Co:Pell ODS (Whatman LabSales, Maidstone, Great Britain). The eluent was 0.025 *M* borate buffer, pH 7, saturated with pentan-1-ol, pumped at a flow-rate of 1 ml/min. The UV monitor was set at 190 nm, 0.05 a.u.f.s.

Procedure

Five ml of urine or standard were pipetted into a 20-ml glass-stoppered tube to which were added 500 μl of a saturated solution of ammonium chloride titrated to pH 9.5 with 0.88 S.G. ammonia. Powdered sodium chloride was added to saturation followed by thorough mixing. The mixture was transferred to the top of a glass chromatography column (145 × 10 mm) containing 2.24 g Extrelut (BDH, Poole, Great Britain), allowed to distribute for 15 min and then eluted with 12 ml ethyl acetate. The extracts were evaporated under air at 45°C to about 4 ml and finally to dryness in a vortex evaporator at 50°C. An internal standard of 100 μl 0.2 mmol/l prednisone in ethanol was added and taken to dryness. The residue was dissolved in 20 μl glass distilled water and 5 μl injected onto the HPLC column. Half-minute timed

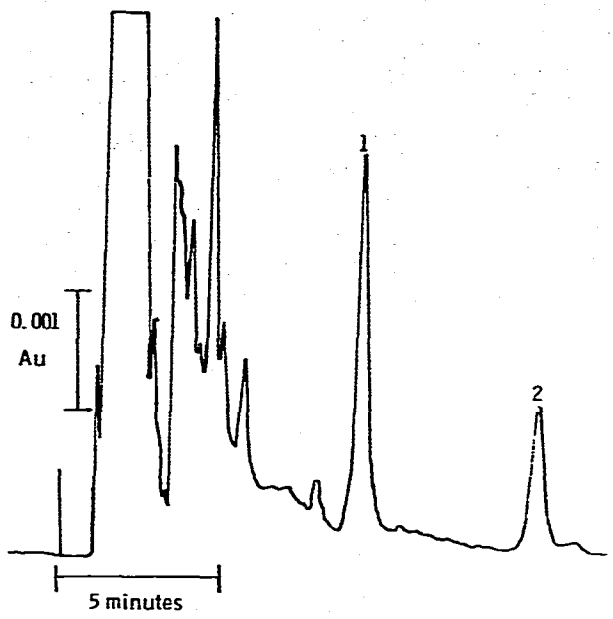

Fig. 1. Chromatogram of an extract of human urine. Peaks: 1 = DHEAS; 2 = prednisone, internal standard.

fractions were collected from the detector effluent for 15 min. A typical chromatogram of a urine extract is shown in Fig. 1. The five fractions corresponding to the UV peak for DHEAS were pooled, diluted to 5 ml with mobile phase and then further diluted with 0.1% gelatin phosphate buffer 0.1 *M*, pH 7.0, to at least 1/40. This gelatin phosphate buffer solution was used for all reagents in the RIA procedure.

One hundred microlitres of the diluted eluate and the standards were pipetted into 75 × 12 mm glass tubes and 100 µl phosphate buffer for the totals, water blanks and charcoal blanks. To all tubes was added 100 µl [^{3}H]-DHEA (1 pmol/ml) in phosphate buffer. Two hundred µl of antiserum diluted 1 : 64,000 in phosphate buffer were then pipetted into all the tubes except for the totals and charcoal blanks to which 200 µl phosphate buffer were added. All tubes were mixed and incubated at 37°C for 1 h, then placed in an ice bath for 1 h. One hundred µl of a cold well-mixed charcoal suspension of 0.7% Norit A in phosphate buffer were added to all tubes except totals which received 100 µl of phosphate buffer instead. After mixing, the tubes were incubated in an ice bath for 10 min, then centrifuged at 8°C and 1500 *g* for 15 min. The supernatant (400 µl) was transferred into a counting vial and 4.2 ml of 0.4% 2,5-diphenyloxazole in toluene—Triton X-100 (3 : 1) scintillant added, the vials shaken and counted.

RESULTS AND DISCUSSION

It was not found possible to devise an extraction and chromatographic procedure which consistently resolved DHEAS and the prednisone internal standard from other urine components with absorption at 190 nm. The RIA of the HPLC fractions reported here overcame this problem.

Chromatography

The separation of compounds in chromatographic systems using pentan-1-ol as the stationary phase and a mobile phase saturated with pentan-1-ol depends largely on partition between the two phases. Partition ratios (K) of [^{3}H]DHEAS, between equal-phase volumes of pentan-1-ol and selected aqueous solvents were determined using a procedure designed to ensure a matched scintillation counting system (Table II). The low partition ratios given by borate—pentan-1-ol systems were associated with good chromatographic separation of DHEAS. The 0.025 M borate system was chosen because of the better UV sensitivity at this molarity.

DHEAS has a considerably higher specific extinction coefficient than the other important urinary steroids and is resolved from androsterone sulphate and aetiocholanolone sulphate which cross-react with the DHEA antibody, as well as from the other UV-absorbing steroids (Table III).

TABLE II

PARTITION RATIOS (K) OF [^{3}H]DHEAS BETWEEN EQUAL-PHASE VOLUMES OF PENTAN-1-OL AND VARIOUS AQUEOUS BUFFER SOLUTIONS OF DIFFERENT pH VALUES

Buffer solution	pH		
	2.5	7.4	11.0
No counter-ion	22	10	8
Tetrabutylammonium phosphate, 0.01 M	1098	608	1200
Cetrimide, 0.01 M	258	333	311
Borate, 0.1 M	—	25	—
Borate, 0.075 M	—	24	—
Borate, 0.050 M	—	20	—
Borate, 0.025 M	—	16	—

TABLE III

RETENTION TIMES AND SPECIFIC EXTINCTION COEFFICIENTS FOR SOME STEROIDS

Steroid	Retention time (min)	$E^{1\%}_{1\ cm}$
DHEAS	10.5	246
Androsterone sulphate	17.5	3.92
Aetiocholanolone sulphate	21.5	2.85
Cortisol-21-sulphate	3.0	—
Prednisone	15.0	—
Cortisone	16.0	—
Cortisol	19.0	—
Prednisolone	22.0	—
Dexamethasone	35.0	—

Characterisation of urine DHEAS peak

A 5-ml sample of urine was spiked with 555,000 dpm [^{3}H]DHEAS and 0.5-min timed fractions of eluate collected from the HPLC column and the [^{3}H]DHEAS peak located by scintillation counting. Fractions were also collected from another 5-ml sample of the same urine, but these were analysed by RIA for DHEAS. The RIA profile and scintillation peak were both coincident with the UV peak of DHEAS.

Specificity

Six doubling dilutions of 1 mmol/l DHEAS were prepared and 100 μl of each added to the urine of a post-menopausal woman. These were assayed using the UV monitor and a statistical comparison of μmol/l DHEAS added (x) with μmol/l DHEAS detected (y) was made. A straight line of equation $y = 0.95x + 0.18$ was obtained, indicating a 95% recovery. Using normal 24-h urines a comparison was made between the UV-monitor result (y) and that obtained by RIA of the pooled peak fractions (x). A linear relationship displaced from the origin with equation $y = 1.098x + 2.034$ ($n = 85$) was found. This suggests that there is some UV-absorbing substance in the chromatographic fractions that is not measured by RIA.

The pooled HPLC fractions from an extracted urine (with marked UV interference) were serially diluted and assayed by RIA. A linear relationship (with a correlation coefficient of 0.99) was found between dilution of pooled fractions and concentration of DHEAS. The mean concentration of DHEAS after allowing for dilution was 4.5 μmol/l ($n = 10$) with a coefficient of variation of 11.5%. These results suggest that there is little interference in the RIA method from other substances that might be present in the HPLC fraction.

Precision

Within-assay precision was calculated by replicate analyses of a low, medium and high urine control. Between-assay precision was calculated from a medium urine control assayed in 20 separate analyses (Table IV).

Human values

The excretion of urinary DHEAS in eighteen normal human subjects was not normally distributed. A square root transform normalised the data which

TABLE IV

PRECISION

Precision	Control	Mean (μmol/l)	Standard deviation	Coefficient of variation (%)
Within-assay ($n = 15$)	Low	2.02	0.353	17.5
	Medium	6.06	0.318	5.2
	High	18.01	0.881	4.9
Between-assay ($n = 20$)	Medium	6.20	0.444	7.2

TABLE V

URINE LEVELS OF DHEAS

Sex	Number	DHEAS (μmol/24 h)	
		Mean	Range
Male	8	5.22	0.4 —22.46
Female	10	1.78	0.06—13.14

are presented as mean and range (Table V). Concentrations of urinary DHEAS in males were significantly higher than in females ($p < 0.001$), agreeing with the established gender differences in plasma DHEAS and in urinary 17-oxosteroids.

Predictive nature of the partition ratio, K

The solvent systems investigated show that the partition ratio has a predictive value in reversed-phase partition chromatography. Solvent combinations resolving DHEAS were associated with low ratios, while the phosphate buffer—pentan-1-ol system employed by Wahlund and Beijersten [10] to separate organic acids, was unsuccessful for DHEAS as predicted by large partition ratios.

Addition of cetrimide and tetrabutylammonium phosphate counter-ions was accompanied by a considerable increase in [^{3}H]DHEAS partition ratios, indicating the preferential partitioning of DHEAS into the organic phase. On the other hand, increasing concentration of borate buffer gave a graded response in the partition ratio. By analogy with cetrimide and tetrabutylammonium phosphate which are known counter-ions, borate may be acting similarly.

The good correlation between RIA and UV quantitation suggests that further development may lead to the establishment of a simpler HPLC—UV detection method for urinary DHEAS.

ACKNOWLEDGEMENTS

We are grateful for the help of Brian Morris of the University of Surrey in raising the antiserum and Mrs. E. Grimshaw in the preparation of this manuscript. This work was submitted by R.C.C. as part of a thesis for the M.Sc. (University of Surrey).

REFERENCES

1 E.J. Lamb, W.J. Dignam, R.J. Pion and H.H. Simmer, Acta Endocrinol., 45 (1964) 243.
2 J.L. Vaitukaitis, S.L. Dale and J.C. Melby, J. Clin. Endocrinol. Metab., 29 (1969) 1443.
3 F. Bassi, G. Guisti, L. Boisi, S. Cattaneo, P. Giannotti, G. Forti, M. Pazzagli, C. Vigrani and M. Serio, Clin. Endocrinol., 6 (1977) 5.
4 J.N. Carter, J.E. Tyson, G.L. Warne, A.S. McNeilly, C. Faiman and H.G. Friesen, J. Clin. Endocrinol. Metab., 45 (1977) 973.

5 A. Vermeulen and S. Ando, Clin. Endocrinol., 8 (1978) 295.
6 L.N. Parker, S. Chang and W.D. Odell, Clin. Endocrinol., 8 (1978) 1.
7 M.G. Metcalf, E.A. Espiner and R.A. Donald, Clin. Endocrinol., 10 (1979) 539.
8 H.-J. Egger, J. Reiner, G. Spiteller and R. Häffele, J. Chromatogr., 145 (1978) 359.
9 J.C. Touchstone and M.F. Dobbins, J. Steroid Biochem., 6 (1975) 1389.
10 K.-G. Wahlund and I. Beijersten, J. Chromatogr., 149 (1978) 313.