BBA 3910

THE MEASUREMENT OF URINARY TETRAHYDROALDOSTERONE BY GAS–LIQUID PARTITION CHROMATOGRAPHY

H. F. CARR JR. AND H. H. WOTIZ

Departments of Psychiatry and Biochemistry, Boston University School of Medicine, Boston, Mass. (U.S.A.)

(Received August 8th, 1962)

SUMMARY

A gas-chromatographic method for the analysis of tetrahydroaldosterone in urine has been devised. The mean basal excretion of 46.7 μg/24 h was determined in five normal subjects on normal sodium intake. This metabolite was observed to increase in response to sodium deprivation and to corticotrophin administration. Two other, more mobile components were also observed. One of these was found to be consistently increased by sodium restriction and was thought to represent an additional mineralocorticoid metabolite. The advantages of this method are those of efficiency, speed, accuracy and replicability.

INTRODUCTION

The isolation and characterization of tetrahydroaldosterone (3α,11β,21-trihydroxy-20-oxo-5β-pregnan-18-al) from human urine by ULICK AND LIEBERMAN[1] has indicated that Ring-A reduction occurs as a major catabolic step in the *in vivo* metabolism of aldosterone (11β,21-dihydroxy-3,20-dioxopregn-4-en-18-al). This is analogous to the metabolism of cortisol (11β,17α,21-trihydroxypregn-4-ene-3,20-dione) and has the further similarity of elaborating a product which, in the reduced form is excreted in higher concentration than its unsaturated precursor. Previous experience in the concurrent measurement of urinary aldosterone and tetrahydroaldosterone has shown the latter to be in general a more sensitive index of mineralocorticoid secretion and metabolism[2].

The need for greater analytical simplicity and directness that prompted an earlier investigation of the application of gas–liquid partition chromatography to aldosterone[3] also suggested the extension of these activities to tetrahydroaldosterone with a view toward the ultimate development of a technique for the measurement of this metabolite in urine. This research was facilitated by the results of these previous investigations which demonstrated that pure, crystalline aldosterone diacetate could be separated and measured by gas–liquid partition chromatography and that thermal decomposition of this relatively heat-labile steroid did not take place under the conditions described. In subsequent recovery studies in which tetrahydrocortisone diacetate was used as a model of a Ring-A reduced corticosteroid, it has also been possible to demonstrate thermal stability for a member of this group of corticosteroids[4].

MATERIALS AND METHODS

A gas chromatograph equipped with a micro-dipper injector and a beta-ionization detector was used. Commercial argon gas was employed as the mobile phase. The stationary phase was 3 % SE-30 elastomer adsorbed on diatomaceous earth (Anakrom AB 70–80 mesh, available from Analabs, Hamden, Conn.). Two different columns were used, one was a four foot, 1/8 in outer diameter and the other a six foot, 3/16 in outer diameter copper tube. Chromatography was carried out with a column temperature of 250° and a gas pressure of 30 lbs/in^2. Steroids were obtained from Elite Chemical Company and were of supposed homogeneity as shown by paper partition chromatography. The tetrahydroaldosterone was donated by Dr. J. C. MELBY and had been prepared by enzymic reduction. All steroids were employed as the acetylated derivatives. Acetylation was carried out by the addition of 1 ml each of pyridine and acetic anhydride following which the reaction was allowed to progress overnight at room temperature. The reagents were then evaporated and the residues dissolved in 0.5 ml of acetone. In order to demonstrate the efficacy of gas–liquid partition chromatography for the analysis of urinary tetrahydroaldosterone, it was decided to investigate the endogenous responses of aldosterone secretion to such stimuli as sodium deprivation and corticotrophin administration. The urinary excretion of tetrahydroaldosterone of three young males and of one young normal female was studied before and during a three-day period of sodium deprivation. The low sodium diet consisted of a commercial liquid diet containing no more than 700 mg of sodium chloride in a daily ration. Another normal male subject was studied before and during two days of corticotrophin administration. 80 units of ACTH (Acthar gel, Armour) were injected intramuscularly into the subject on two successive days. In all cases twenty-four hour urine samples were collected and stored in the cold without preservative.

Urine extracts were prepared by treating 250- and 500-ml portions of 24-h collections with 2000 units/ml urine of a *Helix pomatia* preparation (Glusulase, Endo Labs., Richmond Hill, N.Y.) at pH 4.8 for 48 h followed by extraction with freshly distilled chloroform (3 × 175 ml). These extracts were washed with distilled water and then taken to dryness *in vacuo*. All contact with alkali was carefully avoided. The residues were acetylated with 1 ml each of acetic anhydride and pyridine overnight and were then subjected to preliminary paper partition chromatography using a non-polar modified BUSH system[5] in which toluene–*n*-tetradecane (1:2) was employed as the mobile phase and methanol–water (7:1) as the stationary phase. Development was carried out at 20° for 10 h. The mobilities (R_F values) for the major reduced Ring-A corticosteroid were; tetrahydrocortisol diacetate, 0.043; tetrahydrocortisone diacetate, 0.319; tetrahydroaldosterone triacetate, 0.625. Following paper chromatography, areas expected to contain tetrahydroaldosterone triacetate, as compared to the standard, were cut with generous margins and eluted with ethanol (Fig. 1). These eluents were taken to dryness and the residues redissolved in 100 µl of acetone. 5 µl of this solution were then injected into the gas chromatograph. Measurement was accomplished by using the mean peak heights of duplicate analyses against the mean peak response of an accurately weighed sample of authentic tetrahydroaldosterone triacetate.

The technique employed for comparative measurement of tetrahydroaldosterone was a dual liquid–liquid partition chromatography technique which exploited the

anomalous mobilities of aldosterone and tetrahydroaldosterone under reversed phase conditions[6]. A second conventional chromatogram of the acetates of the eluents from the first chromatogram was introduced to promote solute homogeneity. Quantitative measurement was done by application of the blue tetrazolium reaction using the

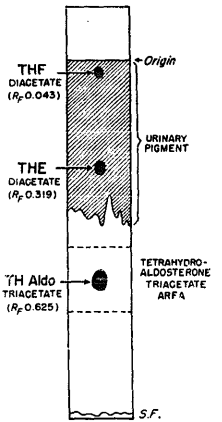

Fig. 1. Preliminary liquid–liquid partition chromatography of an acetylated, crude urine extract, showing the separation of tetrahydroaldosterone-3α,18,21-triacetate from the diacetates of the major reduced Ring-A metabolites of cortisol and from the interfering urinary chromogen. Stationary phase, methanol–water (7:1); Mobile phase, *n*-tetradecane–toluene (2:1). Developed at 20° for 10 h.

method of NOWACZYNSKI *et al.*[7]. Tetrahydroaldosterone triacetate was used as the standard. Comparison of the gas-chromatographic data was made with that previously obtained by the liquid–liquid partition technique.

RESULTS

Separation of ring-A reduced corticosteroids by gas chromatography

Under the conditions described above using a four foot long, 1/8 in outer diameter, 3 % SE-30 column, the retention times of five pure, crystalline steroids were as follows: tetrahydroaldosterone triacetate, 8 min; β-cortol triacetate, 13 min; β-cortolone triacetate, 16 min; tetrahydrocortisone diacetate, 19.5 min; tetrahydrocortisol diacetate, 26 min. Adequate separation of these steroids was achieved when they were chromatographed as a mixture (Fig. 2). On a six foot long, 3/16 in outer diameter, 3 % SE-30 column the retention time of tetrahydroaldosterone triacetate was 9 min, and the retention times of the remaining steroids correspondingly increased. It should be noted that the acetates of tetrahydrocortisone, β-cortolone and tetrahydrocortisol used for these studies displayed several smaller peaks in addition to the primary peaks when chromatographed individually. These additional components were thought to be

steroidal impurities which were not discernible by conventional chromatographic techniques. Only the derivatives of β-cortol and tetrahydroaldosterone were entirely homogeneous. In any case, none of the minor components of the first group interfered with the characteristic retention time observed for tetrahydroaldosterone triacetate.

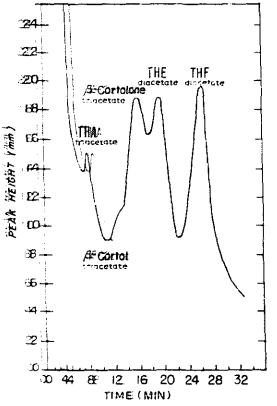

Fig. 2. The separation of a mixture of pure, crystalline steroids as the respective acetates on a four foot long, 1/8 inch outer diameter column containing 3% SE-30 elastomer. THA triacetate, tetrahydroaldosterone-3α,18,21-triacetate; β-cortol triacetate, cortol-3α,20β,21-triacetate; β-cortolone triacetate, cortolone-3α,20β,21-triacetate; THE diacetate, tetrahydrocortisone-3α,21-diacetate; THF diacetate, tetrahydrocortisol-3α,21-diacetate.

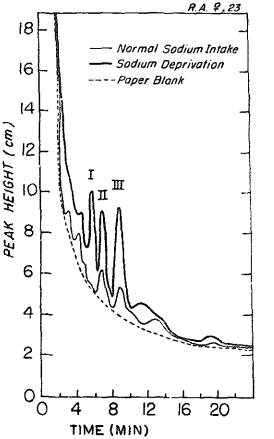

Fig. 3. Reproduction of typical gas–liquid partition chromatograms of acetylated and purified urine extracts chromatographed on a six foot long, 3/16 inch outer diameter column containing 3% SE-30 elastomer. Chromatogram of an extract of urine collected during a period of sodium deprivation (coarse line) superimposed on that of urine collected during a control period (fine line); eluent of a suitable "paper blank" also shown. Peak III, tetrahydroaldosterone-3α, 18, 21-triacetate. Endogenous response of this component to sodium deprivation used as further evidence of its mineralocorticoid identity. See text for explanation of Peaks I + II.

Preliminary paper partition chromatography of urine extracts

Initial gas chromatography of crude urine extracts indicated the need for additional purification, inasmuch as these crude residues were bulky and moderately mobile components appearing during the first 15 min of chromatography interfered with the identification and measurement of tetrahydroaldosterone. Investigation of alternative, more rapid, liquid–solid partition and elution systems disclosed these as relatively inefficient since the interfering impurities and steroid-containing fractions had identical mobilities. Recourse to a non-polar liquid–liquid partition system was taken as a result of earlier experience[2] which demonstrated that by this means tetrahydroaldosterone triacetate could be easily separated from the acetylated derivatives of the major metabolites of cortisol and from most of the interfering urinary impurities (Fig. 1).

Measurement of tetrahydroaldosterone in urine

Gas chromatography of eluents from the preliminary paper chromatograms demonstrated the presence of a peak having the same retention time as pure tetrahydroaldosterone triacetate. This view is strengthened by its increased excretion in response to sodium deprivation in four normal subjects and to corticotrophin in a normal male subject. Basal excretion and that induced by physiological stimuli in these subjects is presented in Table I. Reproductions of typical gas-chromatograms during the period of normal sodium ingestion and during sodium deprivation in a normal subject are superimposed in Fig. 3. The presence of two components (Peaks II and III in Fig. 3) preceding the tetrahydroaldosterone triacetate peak (peak IIII in Fig. 3) was regularly observed in these chromatograms. These had retention times of 6.5 min and 8 min respectively. Peak II was consistently responsive to sodium restriction while peak I increased in only one-half the subjects tested. This peak was, however, most dramatically stimulated by corticotrophin administration and by psychological stress.

TABLE I

TETRAHYDROALDOSTERONE EXCRETION IN NORMAL SUBJECTS BEFORE AND DURING ADRENOCORTICAL STIMULATION

Analyses performed on the urine collected on the last day of stimulation.

Nature of stimulus	*Subject*	*Tetrahydroaldosterone (Peak III) mcg/24 h*	
		Basal	*Stimulation*[a]
Corticotrophin 80 U I.M. × 2 days	EG	58.6	197.1
Sodium deprivation 700 mg NaCl intake × 4 days	KF	48.6	64.2
	RG	17.0	108.6
	RA	30.7	139.3
	JH	78.6	114.4
	M	43.7 ± 26.0	106.6 ± 31.4

TABLE II

A COMPARISON OF TETRAHYDROALDOSTERONE MEASUREMENT DONE BY TWO DIFFERENT TECHNIQUES

Subject	*Period*	*Tetrahydroaldosterone excretion (mcg/24 h)*	
		Liquid–liquid partition chromatography[a]	*Gas–liquid partition chromatography*
EG	Basal	70.0	58.6
	Corticotrophin stimulation	310.3	197.1
KF	Basal	54.0	48.6
	Sodium deprivation	78.3	64.2

[a] Reversed phase partition chromatography[2].

A comparative estimation of tetrahydroaldosterone excretion by two different partition chromatographic techniques is given in Table II. The present method was notable for yielding somewhat lowere excretion values while preserving good parallelism of measurable physiological responsiveness.

DISCUSSION

In an application of gas chromatography to the final separation and quantitative measurement of Ring-A reduced mineralocorticoids in urine, it has been consistently possible to discern a component having the retention time of pure tetrahydroaldosterone triacetate and which, in all subjects tested, increased in response to physiologic stimuli known to affect endogenous aldosterone biosynthesis[8,9]. In five normal subjects the mean basal excretion was found to be 46.7 ± 26.0 mcg/24 h. During sodium deprivation the mean excretion of this metabolite rose to 106.6 ± 31.4 mcg/24 h; during corticotrophin administration a value of 197.1 mcg/24 h was demonstrated. The basal excretion of tetrahydroaldosterone obtained by gas chromatography was consistent with existing knowledge of the secretory rate and metabolism of aldosterone[10]. Reproducibility of the method was implicit in a comparison of individual peak responses obtained following duplicate injections of acetylated and purified urinary extracts. A statistical analysis of this data indicated that there was essentially no difference in the peak heights obtained for the duplicate injections ($t = 0.403$ for the basal period and $t = 0.103$ for the stimulated period).

When contrasted with a series of determinations done by a liquid–liquid chromatographic technique, it is to be noted that tetrahydroaldosterone excretion measured by the present technique was lower although the magnitudes of the increments on physiological stimulation as measured by both techniques were comparable. We feel that this difference can be explained by the high resolving power of the gas-chromatographic column which made it possible to resolve tetrahydroaldosterone triacetate from a close-running, but, somewhat more mobile peak (peak II in Fig. 3) which also appeared responsive to sodium deprivation. The inference is that this latter compound would be separated with difficulty in most liquid–liquid systems and would contribute additional α-ketolic material to the final quantification. Because of the similarities of physiologic behavior and of gas-chromatographic properties it may be suggested that this component is 18-hydroxytetrahydro "A" (Kendall) described by ULICK *et al.*[10] or it may be an isomeric Ring-A reduced metabolite of aldosterone. A third peak (I in Fig. 3) was also observed in these chromatograms. This peak, however, was variable in its responsiveness to sodium deprivation and appeared to be most sensitive to psychological stress and to corticotrophin stimulation.

It was concluded that the analytical specificity of gas chromatography in the present application was augmented by the preliminary conventional paper chromatography. While this was designed as a means of preparing a less bulky extract, this technique in itself localized an area containing tetrahydroaldosterone triacetate well-separated from the slower-running diacetates of tetrahydrocortisone and tetrahydrocortisol.

The greatest advantage of this method, however, appeared to be its greater speed and efficiency. The average time for a complete analysis was 2.5 days following

hydrolysis as compared with 15 days by the contrasting technique. Since the final separation and measurement by gas chromatography required only 15 min, the method's potential for multiple consecutive analyses was evident.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the invaluable technical assistance of Mrs. V. GOODHUE and Miss I. NAUKKARINEN. We wish to thank Dr. J. MELBY for his kind gift of tetrahydroaldosterone.

This investigation was supported by Research Grants (H-7041 and A-4633) from the National Institutes of Health, Bethesda, Md. and from a Research Grant from the Smith, Kline and French Company, Philadelphia, Pa. (U.S.A.). One of us (H.H.W.) is a Research Career Development Fellow (GM-K3-15, 369-C2) of the United States Public Health Service.

REFERENCES

[1] S. ULICK AND S. LIEBERMAN, *J. Am. Chem. Soc.*, 79 (1957) 6567.
[2] H. E. CARR, JR. AND I. NAUKKARINEN, *Nature*, 197 (1963) 696.
[3] H. H. WOTIZ, I. NAUKKARINEN AND H. E. CARR, JR., *Biochim. Biophys. Acta*, 53 (1961) 449.
[4] H. E. CARR, JR., unpublished data.
[5] I. E. BUSH, *Biochem. J.*, 50 (1952) 370.
[6] H. E. CARR AND W. J. REDDY, *Anal. Biochem.*, 2 (1961) 152.
[7] W. NOWACZYNSKI, M. GOLDNER AND J. GENEST, *J. Lab. Clin. Med.*, 45 (1955) 818.
[8] J. A. LUETSCHER AND B. J. AXELROD, *Proc. Soc. Exptl. Biol. Med.*, 87 (1954) 650.
[9] G. W. LIDDLE, L. E. DUNCAN AND F. C. BARTTER, *Am. J. Med.*, 21 (1956) 380.
[10] P. J. AYRES, J. BARLOW, O. GARROD, A. E. KELLIE, S. A. S. TAIT, J. F. TAIT AND G. WALKER in A. F. MULLER AND C. M. O'CONNOR, *International Symposium on Aldosterone*, Little, Brown and Co., Boston, 1958.
[11] S. ULICK AND K. K. VETTER, *Program of the Endocrine Society, Abstracts of Endocrine Society*, Chicago, 1962, p. 39.