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IMPROVED GAS CHROMATOGRAPHIC—MASS FRAGMENTOGRAPHIC ASSAY FOR TETRAHYDROALDOSTERONE AND ALDOSTERONE IN URINE*

B.J. KOOPMAN*, IJ.G. LOKERSE, H. VERWEIJ, G.T. NAGEL and J.C. VAN DER MOLEN

Central Laboratory for Clinical Chemistry, University Hospital, P.O. Box 30.001, 9700 RB Groningen (The Netherlands)

N.M. DRAYER

Department of Pediatrics, University Hospital, Groningen (The Netherlands)

and

B.G. WOLTHERS

Central Laboratory for Clinical Chemistry, University Hospital, P.O. Box 30.001, 9700 RB Groningen (The Netherlands)

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SUMMARY

A newly devised procedure for a simultaneous determination of urinary tetrahydroaldosterone and aldosterone is described. The procedure is based on deconjugation and acetalization, followed by extraction and derivatization of the urinary compounds to their trimethylsilyl ethers and subsequent gas chromatographic—mass fragmentographic detection. To evaluate the assay, aliquots of a urine sample of a healthy individual were analysed in multiplicate; a mean tetrahydroaldosterone concentration of 103 nmol/l and a within-sample, within-day- and day-to-day coefficient of variation of 1.8, 3.2 and 3.4%, respectively, were found. Determination of aldosterone in the same sample yielded a mean concentration of 25.3 nmol/l and the following coefficients of variation: 2.8% (within-sample), 3.8% (within-day) and 4.3% (day-to-day). The urinary excretion of tetrahydroaldosterone and aldosterone in 24-h urine portions was determined in twenty healthy individuals, aged 23—77 years; for tetrahydroaldosterone and aldosterone, an excretion of 94 ± 66 nmol per 24 h and of 40 ± 22 nmol per 24 h was found, respectively, in accord with the literature.

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An example of the usefulness of the described assay is given by establishing the cause of severe salt-wasting in an infant; a highly elevated tetrahydroaldosterone and aldosterone excretion was demonstrated, proving that the child suffered from unresponsiveness to aldosterone (pseudohypoaldosteronism).

INTRODUCTION

The mineralocorticoid aldosterone acts on the distal tubules of the kidney to enhance the reabsorption of sodium ions from the tubular fluid into the plasma, while increasing urinary excretion of both potassium and hydrogen ions [1].

Aldosterone is synthesized from cholesterol in the outer (glomerulosa) zone of the adrenal cortex [2, 3]. From the daily produced aldosterone, 7—15% is eventually excreted into urine as aldosterone glucuronide. About 50% of the secreted aldosterone is transformed into the tetrahydro metabolite (tetrahydroaldosterone), produced by ring A reduction (more than 75% of the circulating aldosterone is normally inactivated during a single passage through the liver). Tetrahydroaldosterone is conjugated to glucuronic acid before excretion into the urine [2]. The remainder of daily endogenous synthesized aldosterone (ca. 40%) is excreted in the form of other metabolites.

The reason for developing a quantitative assay for tetrahydroaldosterone and aldosterone in urine was the following. Urine of a newborn infant with severe salt-wasting was presented to the laboratory. Combined gas chromatographic—mass spectrometric (GC—MS) analysis of methyloxime-trimethylsilyl ether derivatives of the urinary steroids [4] revealed the presence of relatively high concentrations of aldosterone precursor metabolites, including 18-OH-THB (5β-pregnan-3α,11β,18,21-tetrol-20-one) [5] and 18-OH-THA (5β-pregnan-3α,18,21-triol-11,20-dione) [6]. This indicated either an 18-OH-steroid dehydrogenase deficiency [3, 7] or an unresponsiveness to aldosterone [8—10]. Unfortunately, in normal gas chromatographic steroid profiling [4], aldosterone or its metabolites are not detectable, owing to their relatively low concentrations and the occurrence of several isomers after derivatization of only one single substance. However, to discriminate between both of the disorders, quantitative measurements of aldosterone or its metabolites in serum or urine are necessary. The serum aldosterone level, determined with radioimmunoassay (RIA) [11], was elevated. This pointed to an unresponsiveness to aldosterone, but possible interference of aldosterone precursors owing to lack of specificity of the employed RIA could not be excluded [12]. Therefore, our laboratory decided to quantify aldosterone and its main metabolite tetrahydroaldosterone in urine in a specific manner. After having tried a number of GC—MS procedures [13—15] without much success, we succeeded in establishing an improved procedure to measure, simultaneously, both steroids in urine samples. This method is based on gas chromatography—mass fragmentography using trimethylsilyl derivatives prepared after acidic hydrolysis of urine samples to which 5α-pregnan-18-al-3β,11β,21-triol-20-one was added as internal standard. Normal values of aldosterone and tetrahydroaldosterone in urine were determined with this assay in twenty healthy

volunteers, and they were in close agreement with the literature. The overall applicability of the procedure in relation to other methods for investigating the aldosterone metabolism is discussed. From the results of this study, it was concluded that the infant indeed suffered from an unresponsiveness to aldosterone, clinically known as pseudohypoaldosteronism [8—10].

EXPERIMENTAL

Materials

Aldosterone (4-pregnen-18-al-11β,21-diol-3,20-dione), tetrahydroaldosterone (5β-pregnan-18-al-3α,11β,21-triol-20-one) and 5α-pregnan-18-al-3β,11β,21-triol-20-one were purchased from Makor Chemicals (Jerusalem, Israel). Trimethylbromosilane (TMBS) was from Fluka (Buchs, Switzerland). All other chemicals were from Merck (Darmstadt, F.R.G.) and were of analytical grade. The fused-silica CP-Sil 19 CB capillary column (25 m × 0.32 mm I.D.) was from Chrompack (Middelburg, The Netherlands).

Patients and samples

Untimed urine samples from a three-month-old baby with severe salt-wasting (patient A) and also from a healthy baby of the same age (patient B) were stored at − 20°C until analysis. Untimed urine voidings from healthy controls were pooled and used for construction of the calibration curves. A urine sample from a 24-year-old healthy volunteer (control C) was used for the determination of the coefficients of variation. Samples of 24-h urine from twenty healthy volunteers, aged 23—77 years, were collected for the determination of normal daily urinary-excreted aldosterone and tetrahydroaldosterone.

Determination of urinary aldosterone and tetrahydroaldosterone

Sample preparation. To 75 μl of the internal standard solution containing 2.74 μmol/l 5α-pregnan-18-al-3β,11β,21-triol-20-one, which was evaporated to dryness under a stream of nitrogen, 2 ml of centrifuged urine were added. Then, 1 ml of a solution containing 12 mol/l hydrochloric acid was added. This mixture was incubated for 75 min at 75°C. After cooling, 5 ml of a mixture of toluene–ethyl acetate (4:1, v/v) were added. After shaking for 10 min, the two layers were separated by centrifuging for 5 min at 550 *g*. The organic layer was transferred to another tube and washed with 3 ml of a solution containing 0.1 mol/l sodium hydroxide and 0.15 mol/l sodium chloride, and subsequently with 3 ml of water. The organic layer was evaporated to dryness at 50°C under nitrogen. Silylation was performed by the addition of 100 μl of a freshly prepared mixture of BSA [bis(trimethylsilyl)acetamide], pyridine and TMBS (4:5:1) and allowing to stand overnight at 40°C. Cleaning-up was performed by the addition of 6 ml of distilled hexane and 3 ml of 0.1 mol/l hydrochloric acid solution. Following vortexing and centrifugation, the aqueous layer was removed and 3 ml of distilled water were added. After vortexing, centrifugation and removal of the water layer, the hexane solution was evaporated to dryness at 30°C under nitrogen. After the addition of 30 μl of 30% BSA in hexane, 3 μl were introduced by split injection (1:10) into the GC—MS system.

Gas chromatography—mass spectrometry. GC—MS analysis was performed

on a Varian 3700 gas chromatograph connected to a Finnigan MAT 212 mass spectrometer using a direct coupling device. Helium was used as carrier gas at a flow-rate of 1.5 ml/min. A 25-m fused-silica CP-Sil 19 capillary column was employed. The injection temperature was set at 250°C. Following injection, the oven temperature was kept at 200°C for 10 s, then programmed at a rate of 20°C/min to a final temperature of 300°C and kept for 3 min at this temperature. The ionization mode was electron impact (EI), set at 70 eV. Source and interface temperatures were at 250°C. Data were processed by an SS 200 data system and the mass spectra were retrieved on the tops of the peaks in the total-ion-current chromatogram.

Urinary aldosterone and tetrahydroaldosterone were quantitatively measured by monitoring the peak areas of their respective mass fragments, *m/z* 486 and *m/z* 490, relative to the *m/z* 490 of the internal standard. Calibration curves were constructed in a pooled urine (with low concentrations of aldosterone and tetrahydroaldosterone) by addition of internal standard and varying amounts of tetrahydroaldosterone and aldosterone. These calibration curves were used to calculate the amount of tetrahydroaldosterone and aldosterone in the analysed urine samples.

RESULTS

In Fig. 1, the mass spectra of trimethylsilyl derivatives of acetalized aldosterone, tetrahydroaldosterone and the internal standard (5α-pregnan-18-al-3β,11β,21-triol-20-one) are shown. The prominent ion for the aldosterone derivative is *m/z* 486 ($[M^+]$) and for both the tetrahydroaldosterone derivative and the internal standard derivative, *m/z* 490 ($[M]^+$). Therefore, in the mass fragmentographic determinations of aldosterone and tetrahydroaldosterone, these two ions were selected and monitored.

A typical mass fragmentogram derived from a urine sample of control C, is shown in Fig. 2. Calibration curves for aldosterone, employing the area ratios of fragments *m/z* 486/490, were found to be linear in the range 0—450 nmol/l. The equation of the calibration curve was $y = 0.00613x - 0.0016$ ($n = 7$), with a correlation coefficient of 0.9995, y being the peak area ratio and x the aldosterone concentration in nmol/l. Calibration curves for tetrahydroaldosterone, employing the area ratios of fragments *m/z* 490/490, were constructed in the range 0–1300 nmol/l and appeared also to be linear. The equation of the standard calibration curve was $y = 0.00466x + 0.0756$ ($n = 7$), with a correlation coefficient of 0.9995, y being the peak area ratio and x the tetrahydroaldosterone concentration in nmol/l.

The within-sample ($n = 10$), within-day ($n = 10$) and day-to-day ($n = 10$) coefficients of variation (C.V.) in a urine portion of patient C and the corresponding mean values were determined, being 25.3 nmol/l (C.V. 2.8%), 25.3 nmol/l (C.V. 3.8%) and 25.2 nmol/l (C.V. 4.3%), respectively, for aldosterone and 105 nmol/l (C.V. 1.8%), 101 nmol/l (C.V. 3.2%) and 104 nmol/l (C.V. 3.4%), respectively, for tetrahydroaldosterone. The results of recovery experiments are listed in Table I. Daily urinary excretions of aldosterone and tetrahydroaldosterone were determined in 24-h urine portions from twenty healthy individuals. Daily urinary excretions were normally

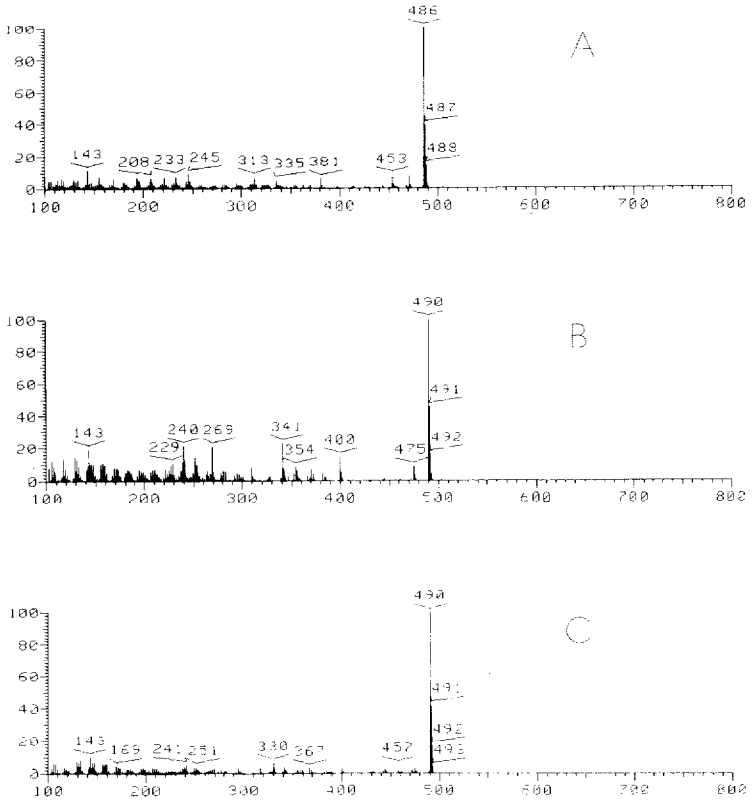

Fig. 1. Electron-impact mass spectra (trimethylsilyl derivatives) of acetalized aldosterone (A), acetalized tetrahydroaldosterone (B) and the internal standard (5α-pregnan-18-al-3β,11β,21-triol-20-one) (C).

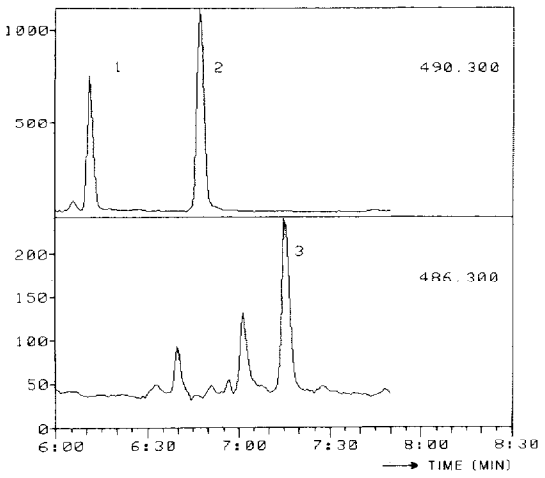

Fig. 2. Mass fragmentograms of a normal urine sample (control C) monitored on the mass fragments *m/z* 486 of acetalized trimethylsilyl aldosterone, *m/z* 490 of acetalized trimethylsilyl tetrahydroaldosterone and *m/z* 490 of the internal standard. Peaks: 1 = tetrahydroaldosterone; 2 = internal standard; 3 = aldosterone.

TABLE I

RECOVERIES OF ALDOSTERONE AND TETRAHYDROALDOSTERONE ADDED TO URINE

Values given are measured amounts (pmol per 2 ml) and recovery percentages (in parentheses).

Amount of aldosterone added to 2 ml of urine (pmol)					
0	56	111	167	222	347
132	185 (95)	248 (105)	304 (103)	352 (99)	454 (93)
175	224 (88)	268 (84)	349 (104)	417 (109)	558 (110)
43	105 (111)	157 (103)	194 (90)	251 (94)	369 (94)
56	115 (105)	163 (96)	216 (96)	261 (92)	395 (98)
62	113 (91)	162 (90)	244 (109)	279 (98)	404 (99)
Amount of tetrahydroaldosterone added to 2 ml of urine (pmol)					
0	165	329	494	659	1029
277	446 (102)	584 (93)	725 (91)	874 (91)	1287 (98)
386	531 (88)	672 (87)	912 (106)	1069 (104)	1507 (109)
189	361 (104)	475 (87)	713 (106)	871 (103)	1198 (98)
198	364 (101)	499 (91)	672 (96)	817 (94)	1203 (98)
367	540 (105)	695 (100)	876 (103)	1102 (112)	1530 (113)

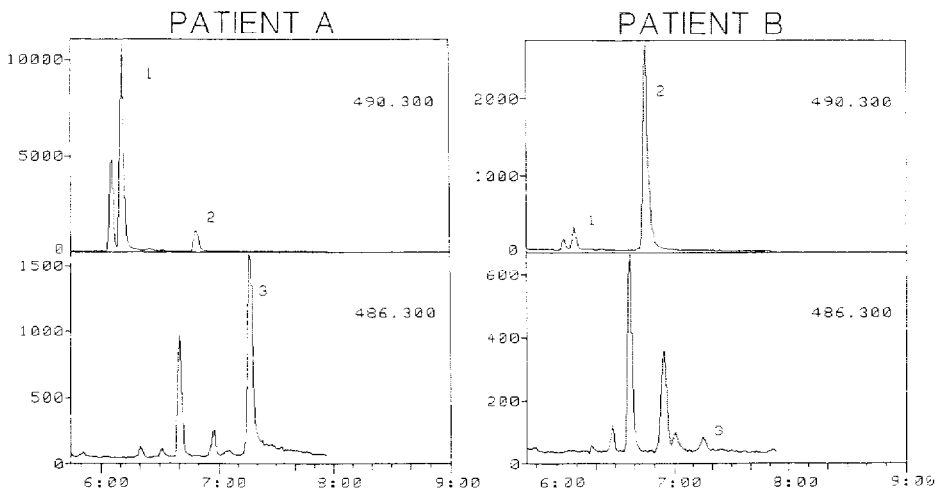

Fig. 3. Mass fragmentograms of urine samples of patients A and B monitored on the mass fragments *m/z* 486 of aldosterone, *m/z* 490 of tetrahydroaldosterone and *m/z* 490 of the internal standard. Peaks: 1 = tetrahydroaldosterone; 2 = internal standard; 3 = aldosterone.

distributed, mean ± S.D. excretions of tetrahydroaldosterone and aldosterone being 94 ± 66 and 40 ± 22 nmol, respectively.

Finally, urinary excretions of aldosterone and tetrahydroaldosterone in patients A and B were determined. Mass fragmentograms representing deter-

minations of urinary aldosterone and tetrahydroaldosterone in urine samples of patients A and B are shown in Fig. 3. In patient A, urinary excretions of aldosterone and tetrahydroaldosterone were 620 nmol/mmol of creatinine and 1400 nmol/mmol of creatinine, respectively; and in patient B, 2.8 nmol/mmol of creatinine and 22 nmol/mmol of creatinine, respectively.

DISCUSSION

A number of diseases are known in which the production or function of aldosterone is impaired. Among them are (a) primary hyperaldosteronism, caused by overproduction of aldosterone owing to an adrenal tumour; (b) secondary hyperaldosteronism caused by malfunctioning of the hormone in the kidneys; (c) Bartter syndrome; (d) deficient production owing to enzyme deficiencies as a result of inborn errors of metabolism, such as 18-hydroxylase deficiency and 18-dehydrogenase deficiency; and (e) pseudohypoaldosteronism, owing to a decreased number of receptors in the renal compartment. Apart from measuring the plasma renine activity (PRA) and the concentrations of sodium and potassium ions in serum or urine, these diseases can be detected by either assaying the hormone itself in plasma or its main metabolite in urine. Because of a general preference for determining active hormones in plasma and the availability of a RIA for aldosterone, the first option is often preferred. However, one can raise objections against rejecting the second alternative. The concentration of aldosterone in plasma is subject to large fluctuations and is dependent on time of sampling, body status (e.g. laying or standing) and stress. Such fluctuations are far less important with respect to aldosterone and tetrahydroaldosterone excretions into urine. Moreover, one cannot exclude, especially in the newborn period, cross-reactivity of the employed antibodies in the RIA with, for example, steroids similar in structure to aldosterone, like the aldosterone precursor 18-hydroxycorticosterone or its metabolites. For these reasons, it could be advantageous to resort to measurements of tetrahydroaldosterone in urine in at least some cases; those which are difficult to interpret.

Numerous procedures dealing with the measurements of aldosterone and tetrahydroaldosterone in urine have been reported, including RIA [18–20], fluorimetry [21, 22], gas chromatography with flame-ionization detection (FID) [22, 23] and with electron-capture detection (ECD) [19, 24, 25, 27], and gas chromatography—mass fragmentography [13–15]. Unfortunately, methods to determine tetrahydroaldosterone by RIA, or preferably by highly specific methods like GC—MS, are not readily available.

We were confronted with this subject when a baby was presented to the hospital who suffered from severe salt-loss. A highly elevated plasma aldosterone, determined by RIA, was found and the gas chromatographic analysis of the urinary steroid profiling [4] showed the presence of some unknown peaks. Mass spectrometric analysis of these peaks revealed the identity of 18-OH-THA [6] and 18-OH-THB [5], both 18-OH-B metabolites (4-pregnen-11β,18,21-triol-3,2-dione), normally not detected in measurable quantities. Raised levels of 18-OH-B can be seen in 18-dehydrogenase deficiency [7] or in so-called pseudohypoaldosteronism, caused by a

(sometimes transient) lack of aldosterone receptors, accompanied with elevated aldosterone levels. As it was not completely certain whether the raised plasma aldosterone level found in the baby could be ascribed to interference with aldosterone precursors as a result of non-absolute specificity of the employed RIA, our laboratory was asked to investigate whether signs of an increased aldosterone metabolism could also be demonstrated by analysis of urine.

With the required instrumentation at our disposal, existing GC—MS determinations of urinary aldosterone and tetrahydroaldosterone were evaluated. A number of described assays [13—15] were tried, but without much success. Another well tested assay [26] was considered to be laborious and did not give a detailed description for urine samples. Therefore, we devised a new procedure based on the following facts. During hydrolysis in a strong hydrochloric acid solution, aldosterone and tetrahydroaldosterone are deconjugated and converted into unique stable isomers by acetalization of the aldehyde function at C-18 with the hydroxyl functions at C-11 and C-21 [16, 17]. We found that persilylation of both of the extracted compounds yielded derivatives containing two trimethylsilyl groups, with very characteristic EI mass spectra (see Fig. 1). The yield of the $[M]^+$ for these derivatives is prominent (with minor other fragmentation), resulting in relatively high responses in mass fragmentography when monitoring the $[M]^+$ fragment. 5α-Pregnan-18-al-3β,11β,21-triol-20-one was chosen as internal standard, since its structure is very similar to tetrahydroaldosterone as well as aldosterone.

The presented procedure was evaluated in the following way. The within-sample, within-day and day-to-day variation in a urine sample of control C and the corresponding mean values were determined. In addition, aldosterone and tetrahydroaldosterone in varying amounts were added to aliquots of urine and the recovery was determined for both steroids. The overall results established the reliability of the GC—MS procedure. Subsequently, the urinary-excreted aldosterone and tetrahydroaldosterone of twenty healthy individuals were measured. Normal values of daily urinary-excreted aldosterone and tetra-

TABLE II

NORMAL DAILY URINARY EXCRETED ALDOSTERONE AND TETRAHYDROALDOSTERONE IN ADULTS

Authors	Method	Daily urinary excretion (nmol/day)	
		Aldosterone	Tetrahydroaldosterone
Von Breuer et al. [22]	GC—FID	14—70	
	Fluorimetry	0—66	
Posadas et al. [25]	GC—ECD		33—100
Whigham [21]	Fluorimetry	0—61	
Nielsen et al. [28]	RIA		85—159
Delassalle et al. [29]	RIA		47—360
Mattox and Nelson [30]	RIA	5—42	38—240
Bauknecht et al. [31]	RIA	10—50	27—180
Marks et al. [32]	RIA	14—55	106—330
Lewicka and Vecsei [20]	RIA	10—50	25—200
Our method	GC—MS	0—84	0—226

hydroaldosterone, both by our assay as well as by other techniques, are given in Table II. As can be seen, our normal values are in close agreement with those previously published.

Urinary aldosterone and tetrahydroaldosterone were measured in the infant with severe salt-wasting. The excreted amount turned out to be very high with respect to a control of about the same age. Therefore, it was concluded that the patient was not suffering from an enzyme deficiency in aldosterone synthesis. In spite of a very high aldosterone level, severe salt-wasting occurred. This means that the patient does not respond to aldosterone and thus suffers from pseudohypoaldosteronism.

In conclusion, urinary aldosterone and tetrahydroaldosterone can be determined reliably according to the present GC—MS procedure and can be successfully applied in studies concerning aldosterone metabolism. In addition, owing to its specificity, the applicability of the procedure as a reference method could be a matter of consideration. The present method will be evaluated on other patient groups with expected abnormalities in mineralocorticoid metabolism.

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