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AN IMPROVED METHOD FOR THE GAS-LIQUID CHROMATOGRAPHIC DETERMINATION OF ALDOSTERONE IN URINE

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SUMMARY

An improved method for the gas–liquid chromatographic determination of aldosterone in urine has been developed. The method involves the following steps: (1) Hydrolysis of the aldosterone conjugate (pH 1-labile metabolite) by cold hydrochloric acid. (2) Addition of [1,2-$^3H_2$]aldosterone as a tracer. (3) Purification of aldosterone by adsorption chromatography on Amberlite XAD-2, followed by distribution between solvents and thin-layer chromatography on silica gel. (4) Oxidation of aldosterone by periodic acid to aldosterone-γ-lactone. (5) Purification of the γ-lactone by thin-layer chromatography. (6) Gas–liquid chromatography of the purified sample by using a solid injection system and a flame ionisation detector. (7) Measurement of radioactivity to correct for losses during the working-up procedures.

By using a time-controlled automatic solid injection system, the practicability of the method is considerably enhanced. At the same time, the sensitivity of the method is improved, since larger portions of the samples can be utilised for gas chromatographic determination as compared to the liquid injection system.

INTRODUCTION

THE DOUBLE isotope derivative method for aldosterone estimation which was described in 1960 by Kliman and Peterson[1] has a high precision, accuracy, sensitivity and specificity. However, a wider application of this excellent method is somewhat limited, since it is time-consuming and costly. Several gas–liquid chromatography methods have been developed recently for the determination of aldosterone in urine[2–4]. These methods do not require as much time as the double isotope derivative method but they are, unfortunately, less reliable. This is partly due to the fact that the detecting systems of gas chromatographs are unspecific and that the extracts, used for gas-liquid chromatography, have often not been adequately purified.

In the present paper, a gaschromatographic method for the determination of aldosterone in urine is reported which is as reliable as the double isotope derivative method but is more practicable.

MATERIALS

Urine. If possible, urine samples should be worked up immediately. Otherwise they should be stored in a deep freezer at − 15°C.

Solvents. They were of reagent grade (Merck, Darmstadt, Germany), and used after redistillation.

Resin. Amberlite XAD-2, in the form of hydrated beads with an effective size of 0·5–1·0 mm, was obtained from Serva Entwicklungslabor, Heidelberg (Germany). The resin was washed with distilled water to remove the fines. 50 g

of the resin was filled into a column (internal diameter 2·5 cm) with a coarse fritted disc and layered with glass wool and glass beads. The resin was regenerated by washing with water and used repeatedly.

Steroids. Aldosterone (18, 11-hemiacetal of 11β, 21-dihydroxy-3,20-dioxo-4-pregnen-18-al) was obtained from Merck, Darmstadt (Germany). Aldosterone-γ-lactone [18,11-hemiacetal of 11β-hydroxy-3-oxo-4-androsten-18-al-17-carbolactone (20,18)] was purchased from Ikapharm, Ramat-Gan (Israel). [1,2-$^3H_2$] Aldosterone (S.A. 43,8 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. All steroids were checked for purity by thin-layer chromatography.

Periodic acid solution. Crystalline periodic acid was dissolved in dioxane/water (1 : 1, v/v); final concentration 10 mg/ml.

METHODS

Thin-layer chromatography. Aluminium sheets (sizes 20 × 20 cm), coated with silica gel F_{254} (thickness of the layer 0·25 mm), were obtained from Merck, Darmstadt (Germany). They were prewashed for 24 h with chloroform/methanol (97 : 3, v/v) using the technique described by Truter [5].

Gas–liquid chromatography. A MicroTek MT 220 gas chromatograph (Techmation, Düsseldorf, Germany) with a double flame ionisation detector was used; it contained a siliconized glass U-tube (1·8 m × 2·2 mm i.d.), packed with 3% OV-3 or 3% OV-1 or 3% OV-7, on Chromosorb WHP 100–120 mesh (Supelco, Bellefonte, Pa.). Column temperatures were 227°C for OV-3, 210°C for OV-1 and 245°C for OV-7. Purged nitrogen was used as a carrier gas at a flow rate of 40 ml/min. The flame ionisation detector was kept at 20°C above column temperature and the flow of hydrogen (50 ml/min) and air (200 ml/min) adjusted for maximal sensitivity. The inlet temperature was 340°C. The solid injection system was obtained from Packard Instruments, Downers Grove, Ill. The capillaries used for gas–liquid chromatography were Mikrocaps of 5, 10 and 20 μl capacity (Drummonds Scientific, Broomall, Penna.).

Liquid scintillation counting. A Nuclear Chicago liquid scintillation spectrometer (Mark II) was used for the measurement of radioactivity. The scintillation fluid contained 4 g of 2,5-diphenyl-oxazol (PPO) in 1 l of dry sulfur-free toluene.

Gas chromatography and mass spectrometry. A combined instrument (LKB 9000) was used.

RESULTS

Method in detail

Hydrolysis. The sample, 200 ml of a 24 h-urine, is adjusted to pH 1 by the addition of 5–6 ml of conc. hydrochloric acid and kept in the dark for 24 h at room temperature. After hydrolysis, 250 nCi of [1,2-$^3H_2$]-aldosterone (S.A. 43·8 Ci/mmol) is added.

Chromatography on Amberlite XAD-2. The hydrolysate is neutralised to pH 7 by the addition of 8–10 ml of 6 N sodium hydroxide. Chromatography on 50 g of Amberlite XAD-2 is carried out according to the following elution scheme: (1) neutralised urine sample, (2) 100 ml of water, (3) 200 ml of water/methanol (1 : 1, v/v), (4) 250 ml of methanol. The last fraction, which contains the aldosterone, is collected and evaporated under vacuum.

Solvent distribution. The dry residue is dissolved in 0·5–0·8 ml of methanol and the solution transferred with water (3 × 5 ml) into a stoppered glass tube. The

aqueous solution is extracted with 5 ml of benzene and the organic phase discarded. The remaining aqueous phase is extracted with ethyl acetate (3 × 5 ml); the combined extracts are evaporated under vacuum.

Thin-layer chromatography. The dry residue is dissolved in a small volume of chloroform/methanol (9:1, v/v) and the solution applied to the plate as a 5 cm-line. The chromatogram is developed with chloroform/methanol (94:6, v/v). The zone containing aldosterone is located (R_F = 0·23) with the help of reference material run on a separate lane. In some experiments, radioactive aldosterone is located by using a thin-layer chromatogram scanner. The zone corresponding in mobility to aldosterone is scraped off and transferred to a funnel closed with a sealed-in sintered glass support (porosity G4). Aldosterone is eluted from the silica gel with chloroform/methanol (3 × 5 ml, 97:3, v/v) and the eluate evaporated under vacuum.

Oxidation. The dry residue is taken up in 0·5 ml of the periodic acid solution, and this is kept in the dark for 3 h at room temperature. 1·0 ml of water is added and the aqueous phase extracted with ethyl acetate (3 × 5 ml). The combined extracts are first washed with 1 ml of saturated sodium bicarbonate solution and then with 1 ml-portions of water, until the last washing is neutral. The extracts are evaporated under vacuum.

Two-dimensional thin layer chromatography. The dry residue is dissolved in a small volume of chloroform/methanol (9:1, v/v) and applied to a pre-washed plate on a 2 cm-line. The chromatogram is developed with ethyl acetate/cyclohexane (8:2, v/v); under these conditions, the R_F values are 0·05 for aldosterone and 0·20 for aldosterone-γ-lactone (Fig. 1, A). The latter compound is located by comparison with reference material which is removed by cutting off the appropriate lane. After turning the thin-layer plate around 90°, authentic aldosterone-γ-lactone is applied as a vertical 2 cm-line at a distance of 2·5 cm from the aldosterone-γ-lactone fraction from urine (Fig. 1, B). The thin-layer chromatogram (aluminium sheet) is put between two glass plates which are in a horizontal position and cover a glass jar, containing chloroform/methanol (97:3, v/v). About 4/5th of the thin-layer sheet are above the two glass plates and exposed to air. The solvent mixture evaporates as soon as it passes the glass plates. By using this technique, the aldosterone-γ-lactone fraction and the reference material, applied as 2 cm-lines, are concentrated and yield distinct small spots with a diameter less than 1 mm within 15 min of development. The thin-layer aluminium plate is allowed to dry and the chromatogram developed with chloroform/methanol (97:3, v/v) at right angles to the first direction (Fig. 1, C). The R_F values for aldosterone and aldosterone-γ-lactone are 0·1 and 0·4, respectively. The zone containing aldosterone-γ-lactone is cut off and put into a glass tube with a sealed-in sintered glass support (G 4) at the bottom. Elution is carried out with 3 ml of chloroform/methanol (99:1, v/v) and the eluate evaporated under vacuum.

Measurement of radioactivity. The residue is dissolved in 50 μl of benzene/ethanol (9:1, v/v). Using Mikrocaps, 5 μl of the solution is pipetted into a counting vial, containing 10 ml of scintillation fluid.

Gaschromatographic determination. Mikrocaps are filled with 20 μl and 10 μl, respectively, of each sample and are kept in a slanting position during evaporation of the solvent. The upper ends of the Mikrocaps are dipped into ethyl acetate to pick up a small volume (2 μl) of the solvent. After turning the Mikrocaps to the former position the ethyl acetate is evaporated; thereby the non-volatile material

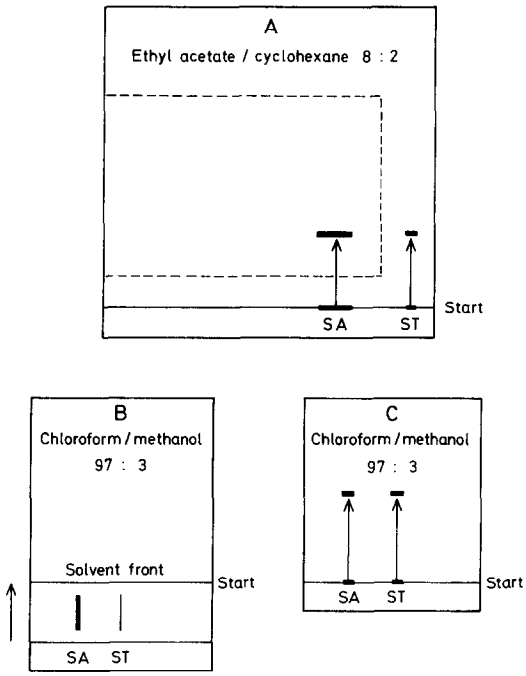

Fig. 1. Two dimensional thin layer chromatography of the aldosterone-γ-lactone fraction from urine (SA) and of authentic material (ST). The broken lines indicate the area which is cut out from the first chromatogram (A) and used for the second chromatography (B).

is concentrated at the lower end of the capillary. The lower ends of the capillaries are cut off at a length of 1 cm and put into the magazine of the solid injection system. Linen gloves and forceps are used for handling the capillaries to avoid impurities. At a preset time, a capillary falls into the GLC column, the upper end of which has an internal diameter of 6 mm. This part of the column is kept at 340°C to allow immediate evaporation of the samples to be investigated. The upper part of the column has room enough for 24 capillaries so that the various samples can be measured without any interruption. The gaschromatographic measurement was done on OV-3 columns, if not otherwise stated. Figure 2 shows a typical gas chromatogram of aldosterone-γ-lactone obtained from urine of a male subject.

Calculation of the results. The amount of aldosterone was calculated from the gas chromatographic tracings by measuring the peak heights. The samples to be investigated were chromatographed in duplicates; a standard was run after each duplicate. The reproducibility of the peak heights made it unnecessary to use an internal standard. The amount of aldosterone excreted in urine within 24 h is calculated according to the following formula

$$\text{A } (\mu\text{g}/24\text{ h}) = \frac{M \times f \times V}{R \times 2}$$

where:

M = amount of aldosterone in μg as determined by GLC; the amount of aldosterone is calculated by multiplying the value of aldosterone-γ-lactone by 1·1

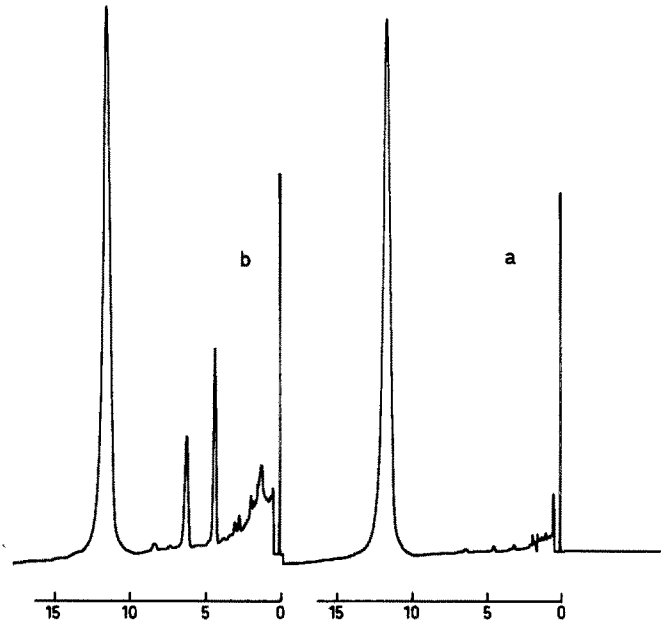

Fig. 2. Gas chromatogram of aldosterone-γ-lactone. (a) 200 ng standard and (b) fraction obtained from the urine (80 ml) of a male subject. Experimental conditions: 3% OV-3 on Chromosorb WHP; column: 1·8 m, i.d. 2·2 mm; inlet temperature: 340°C; column temperature: 227°C; detector temperature: 250°C. The gas chromatograms were recorded at the same sensitivity.

f = factor by which the injected volume (10 and 20 μl, respectively) must be multiplied to obtain the total volume (50 μl) of the sample solution
V = volume of the 24-urine in ml
R = recovery as determined from measurement of the radioactivity of the endogenous standard in %

Reliability of the method

Accuracy. The mean recovery of added [1,2-$^3H_2$] aldosterone (500000 d.p.m. = 2 ng) was found to be 35 ± 5% (60 experiments). This value agrees well with estimates of added non-radioactive aldosterone (range 0·25–5 μg). After correction for experimental losses, a linear relationship between aldosterone added and aldosterone found was obtained. The losses occurring during the various steps are given in Table 1.; the coefficient of regression was found to be $r = 0{\cdot}9997$ and the regression equation $y = 0{\cdot}007 + 0{\cdot}977 \cdot x$.

Precision. Precision of the method was estimated by performing replicate analyses on urinary samples with different concentrations of aldosterone (Table 2). In the normal range of aldosterone excretion (5–25 μg/24 h), the coefficients of variation were between 3·0 and 6·8%.

Sensitivity. When 0·25 μg of cold aldosterone was added to 200 ml urine of an adrenalectomized patient, the corrected recovery was 0·23 ± 0·03 μg. The qualitative sensitivity is defined as the lowest amount which can be distinguished from zero. In the present method, the sensitivity for the gaschromatographic deter-

Table 1. Losses occurring during the various steps of the gas–liquid chromatographic method. [1,2-$^3H_2$] Aldosterone (500,000 d.p.m.) was added to 200 ml-urine samples after hydrolysis. The figures are mean values of triplicates and include losses by transfer or by incomplete application of material to the thin-layer plates

Step	Recovery of [1,2-$^3H_2$] aldosterone in % of total	Absolute loss in %
Chromatography on Amberlite XAD-2	95	5
Distribution between benzene and water	78	18
First thin-layer chromatography	62	20
Oxidation with periodic acid	50	20
Second thin-layer chromatography (two dimensional)	35	30

Table 2. Precision of the gas–liquid chromatographic method for the determination of aldosterone in urine

Urine	Excretion of aldosterone (μg/24h)		Standard deviation ($\pm$ S)	Number of analyses	Coefficient of variation (%)
	Mean values	Range			
1	7·2	6·3 – 7·9	0·4	12	6·1
2	14·7	13·0 – 16·2	1·0	11	6·8
3	16·7	15·8 – 17·6	0·7	12	4·1
4	19·6	18·2 – 21·3	1·2	8	6·0
5	19·8	18·9 – 20·8	0·6	10	3·0
6	24·8	23·4 – 26·8	1·0	11	3·9

mination of aldosterone was calculated according to the formula $\pm ts/\sqrt{n}$ [6] and found to be 0·73 μg/24 h. At this value, the maximal error is 100%.

Specificity. Preliminary evidence for the specificity of the method was obtained by comparison of the chromatographic behaviour in various systems of authentic aldosterone (and its γ-lactone) and of the aldosterone fraction extracted from urine. The following systems were used: Chromatography on Amberlite XAD-2, one-dimensional and two-dimensional thin-layer chromatography, gas–liquid chromatography. Further evidence was obtained by the following experiments.

1. No peak with the retention time of aldosterone-γ-lactone was found in extracts from urine of an adrenalectomized patient.

2. When aldosterone-γ-lactone was added to extracts from urines of normal subjects the shape of the peak did not change, and the peak height increased proportionally to the added amount of aldosterone.

3. When the aldosterone-γ-lactone fraction was chromatographed on *different* liquid phases (OV-1, OV-3, OV-7), the amounts determined remained constant (Table 3); furthermore, the retention times were identical with those of authentic aldosterone-γ-lactone.

4. The mass spectrum of the aldosterone-γ-lactone fraction from urine, as recorded in a combined gas chromatograph/mass spectrometer, showed only small amounts of masses of nonsteroidal origin in the lower mass region. The main peaks of the mass spectrum of the urinary aldosterone-γ-lactone fraction were identical with those of authentic aldosterone-γ-lactone (Fig. 3).

Table 3. Determination of aldosterone in urine by the gas-liquid chromatographic method, using different liquid phases. The figures are mean values of duplicates

Urine	Phase	Excretion of aldosterone (μg/24h)
1	OV-1	2·7
	OV-3	2·9
2	OV-3	7·0
	OV-7	6·3
3	OV-1	14·5
	OV-3	13·2
	OV-7	15·0

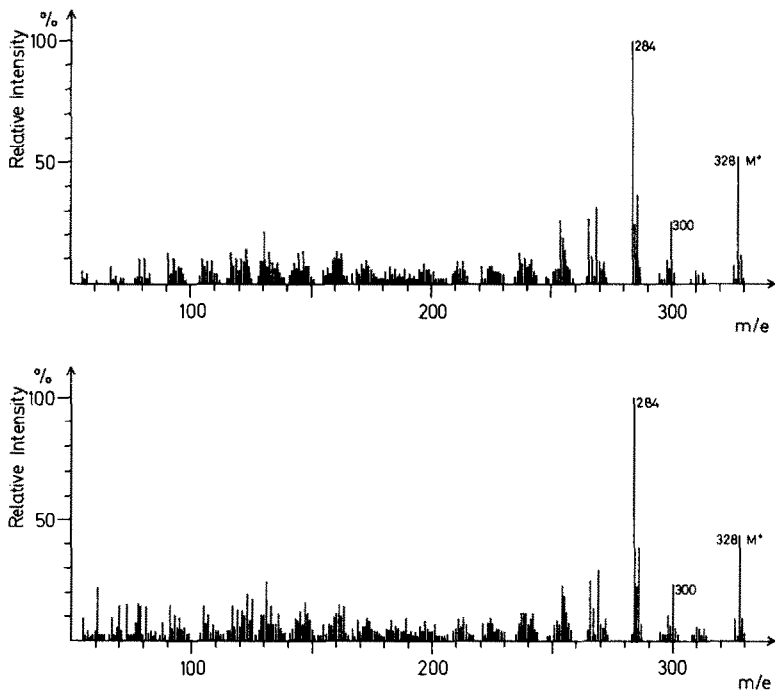

Fig. 3. Mass spectra of aldosterone-γ-lactone (top panel) and of the gaschromatographic peak of the aldosterone-γ-lactone fraction from a urinary sample. The spectra were obtained with a LKB 9000 gas chromatograph/mass spectrometer after gas chromatography on OV-3 (3%); temperature 230°C; ionization energy 35 eV.

5. The values obtained by the gas-liquid chromatographic method agreed well with those determined by the double isotope derivative method [1]; as shown in Table 4, the differences varied between −0·5 and +1·9 μg/24 h.

Practicability

Excluding hydrolysis, ten analyses (single determinations) can be carried out

Table 4. Excretion of aldosterone in urine. Comparison between the gas–liquid chromatographic method and the double isotope derivative method. The figures are mean values of duplicates

Excretion of aldosterone in urine (μg/24 h)		Difference A − B	
Gas–liquid chromatographic method (A)	Double isotope derivative method (B)	in μg	in %
2·4	1·9	+0·5	+26
2·9	3·4	−0·5	−15
6·3	6·0	+0·3	+5
7·0	6·0	+1·0	+17
13·2	12·6	+0·6	+5
13·5	14·0	−0·5	−4
16·0	14·1	+1·9	+14

within 32 working hours (approx. 4 days). The solid injection system facilitates considerably the gaschromatographic procedure, saves time and does not require maintenance during the actual gas-liquid chromatography.

DISCUSSION

The gas-chromatographic method presented here is different from those described previously in three analytical steps: First, the urine sample is chromatographed on Amberlite XAD-2[7] immediately after hydrolysis; by this fractionated chromatography, the acid-labile conjugate of aldosterone is separated from unhydrolysed steroid conjugates and also from a considerable amount of urinary pigments. Furthermore, the volume of urine sample is reduced, and no emulsions are formed; the recovery of added aldosterone approaches 100%.

Second, the introduction of a special two-dimensional thin layer chromatography leads to a concentration of the aldosterone-γ-lactone fraction which means that only tiny spots have to be eluted from the thin layer plates. This reduces the amount of nonspecific material which may otherwise interfere with the gas-chromatographic determination of aldosterone-γ-lactone. It should be noted that the technique of two-dimensional thin layer chromatography, as illustrated in Fig. 1, allows the application of the sample as a line, resulting in an improved resolution. In contrast to thin layer glass plates, the fractions to be investigated can easily be cut off from the aluminium sheets and eluted *in situ* without spoiling the eluates with silica gel.

Third, the use of a solid injection system facilitates the application of the samples to the gas chromatograph. The introduction of the solid injection system for the gaschromatographic determination of aldosterone-γ-lactone was only possible because glass capillaries were chosen as carriers for aldosterone-γ-lactone. The use of metal gauzes led to unreliable results, as has also been reported by Aakvag [8]; probably, aldosterone-γ-lactone is destroyed at the high temperature (340°C) of the inlet in the presence of metal gauzes. Taking calibrated glass capillaries makes it unnecessary to use a syringe for pipetting the sample quantitatively onto the carrier.

The present method has now been in use for more than two years. The criteria of reliability, such as precision, accuracy and specificity, are comparable to those of the double isotope derivative method. As far as those criteria are concerned,

the gaschromatographic determination of aldosterone in urine appears to be the method of choice for clinical investigations.

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