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MASS SPECTROMETRIC ANALYSIS FOR TETRAHYDROALDOSTERONE

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SUMMARY

Trimethylsilyl ether and methyloxime-trimethylsilyl ether derivatives of 3α,11β,21-trihydroxy-5β-pregnan-20-on-18-al (tetrahydroaldosterone) and its etiolactone were analysed by gas chromatography. The structures were established from the fragmentation pattern during mass spectrometry. A quantitative method for measuring urinary tetrahydroaldosterone is described based on selected-ion-monitoring gas chromatography–mass spectrometry (GC–MS). Urine samples were hydrolysed by β-glucuronidase, 3β-*allo*-tetrahydroaldosterone was added as internal standard, the steroids were extracted on Amberlite XAD-2 resin then purified by Sephadex LH-20 chromatography. Methyloxime-trimethylsilyl ethers were prepared and the ions of *m/e* 638 (M) and 607 (M-31) simultaneously monitored by GC–MS. Both the analyte and internal standard were resolved on OV-1 columns and the peak heights were determined. The ratio of peak height of analyte to internal standard was constant up to 40 ng per injection and the lower limit of sensitivity giving acceptable signal-to-noise ratio was around 200 pg.

INTRODUCTION

Tetrahydroaldosterone* glucuronide is the principal metabolite of aldosterone. This metabolite was first described by Ulick and Lieberman[1] and subsequent investigators have shown that about 30% of injected aldosterone is excreted in this form [2, 3]. Published methods for its quantitative analysis [4–12] have attained the required sensitivity by processing large volumes of urine, whilst specificity has been achieved at the expense of lengthy purification. A method has been established in this laboratory for the determination of tetrahydroaldosterone by selected monitoring of two specific ions in the mass spectrum of the O-methyloxime-trimethylsilyl ether. A preliminary report of this method was presented at the 1st European Congress on Clinical Chemistry, Munich [13].

MATERIALS AND METHODS

Materials. 3β-*allo*-Tetrahydroaldosterone was purchased from Ikapharm (Ramat-Ghan, Israel); tetrahydroaldosterone was donated by Dr. S. Ulick; Ketodase (β-glucuronidase) was obtained from Warner-Chilcott Laboratories, Morris Plains, New Jersey; Sephadex LH-20 from Pharmacia AB, Uppsala, Sweden; Amberlite XAD-2, periodic acid and Analar solvents were purchased from BDH, Poole, Dorset; cyclohexane was purified by passing through a column of activated charcoal (30 cm × 10 cm), followed by double redistillation; pyridine was refluxed over sodium hydroxide, redistilled and stored over sodium hydroxide; Applied Science Laboratories, P.O. Box 440, State College, Pennsylvania, supplied the hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) reagents; Eastman Organic Chemicals, Rochester 3, New York, the methoxyamine hydrochloride.

Gas chromatography. A Becker gas chromatograph fitted with a flame ionisation detector was used for the analytical gas chromatography. Open-tubular capillary columns 20 m × 0.5 mm coated with OV-101 (H & G Jaeggi, 9043 Trogen, Zurich, Switzerland) were temperature programmed at 2.5°/min from 160°–260° with helium carrier gas flow of approximately 2 ml/min. The coupling of the column to the instrument which has an automatic solid injection device has been described previously [14]. Retention vols of the steroid derivatives were determined relative to aliphatic hydrocarbons.

Gas chromatography–mass spectrometry (GC–MS). GC–MS analysis was carried out on a Varian Aerograph 2700 gas chromatograph, using a 2 m column packed with 3% OV-1 on Gas-Chrom Q (80–100 mesh). Column temperature was 270°, injection temperature 280° and helium flow rate 20 ml/min. The gas chromatograph was coupled to a Varian MAT-731 double focussing mass spectrometer via a Watson–Biemann separator. Carrier gas separator and transfer lines were maintained at 250°. The mass spectrometer settings for routine use were: ionising voltage, 70 eV; accelerating voltage, 8 kV; and resolution 1000.

When the mass spectrometer was used in the

* The following non-standard abbreviations have been used: Tetrahydroaldosterone, 3α,11β,21-trihydroxy-5β-pregnan-20-on-18-al; 3β-*allo*-Tetrahydroaldosterone, 3β, 11β,21-trihydroxy-5α-pregnan-20-on-18-al; Silyl ether, trimethylsilyl ether; Oxime-silyl ether, methyloxime-trimethylsilyl ether.

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selected ion mode the required masses (*m/e* 638 and 607) were focussed by using a multichannel switching unit. Perfluorokerosene standard was used for preliminary focussing of the masses and final adjustment made by injecting the derivatised reference steroid. Generally the samples to be analysed were dissolved in 50 μl cyclohexane and 2 μl was injected into the gas chromatograph. This amount is equivalent to about 40 ng of internal standard. Under the conditions used both analyte and internal standard are eluted from the gas chromatograph within 10 min.

Derivative formation. Trimethylsilyl ether derivatives were prepared according to the method of Makita and Wells[15]. Dried samples were redissolved in pyridine (200 μl) to which was added 200 μl of a mixture of hexamethyldisilazane–trimethylchlorosilane (HMDS–TMCS) 30:1 and the tube then sonicated. The reagents were evaporated in a stream of nitrogen at 60° to terminate the reaction. Following the addition of cyclohexane to the tube and resuspension of the solid by sonication, the precipitate was removed by centrifugation.

Methyloxime formation prior to silylation was achieved by reaction of the dry material with 2% methoxyamine hydrochloride in pyridine (200 μl) as described by Fales and Luukainen[16]. The time required for satisfactory condensation will be discussed later in the text.

Periodate oxidation. The sample was dissolved in 0.5 ml of dioxan to which was added 0.5 ml of periodic acid (approx. 50% aqueous solution) and the reaction allowed to proceed for 2 h in the dark. The solution was diluted with 5 ml of water and the oxidation products extracted on XAD-2 (bed size 12 cm × 0·8 cm). The steroids were eluted with ethanol and trimethylsilyl ethers prepared as described above.

Processing of urine samples. Adult urine samples (approx. 1/50, 24 h specimen) were extracted on Amberlite XAD-2 columns (bed size 12 cm × 0.8 cm). The resin was washed with a vol. of water equal to the sample size and the steroids eluted with two vols of ethanol. The dried extract was dissolved in 0.5 M acetate buffer at pH 4.8 and 1 μg 3β,5α-tetrahydroaldosterone added as internal standard. Steroid conjugates were hydrolysed by addition of 5,000 units of β-glucuronidase with incubation for 24 h at 37°. Urine samples from infants were hydrolysed directly after appropriate buffering. The free and hydrolysed steroids were re-extracted by XAD-2 then fractionated on 1 g columns of Sephadex LH-20 using cyclohexane:ethanol (4:1 v/v) as solvent system [14]. The fraction between 8 and 13 ml of eluate was collected.

RESULTS

Gas chromatography and mass spectrometry of tetrahydroaldosterone. Analytical GC of the silyl ether of 3α,5β-tetrahydroaldosterone showed that a single derivative was formed quantitatively with respect to cholesteryl butyrate. The mass spectrum (Fig. 2a) will

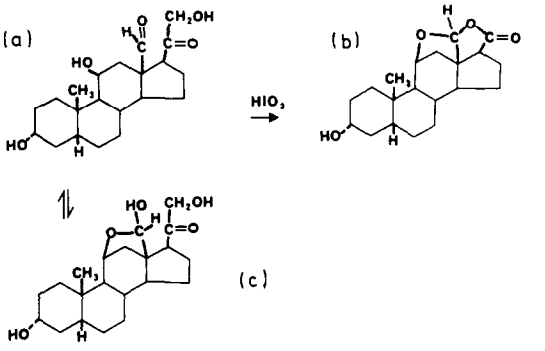

Fig. 1. Chemistry of the equilibrium of tetrahydroaldosterone (a and c) and the etiolactone formed by periodic acid oxidation (b).

be discussed in terms of the chemical forms of tetrahydroaldosterone depicted in Fig. 1. The silyl ether derivative showed no fragmentation characteristic of the free C-18 aldehyde. The molecular ion at *m/e* 580 was weak but strong ions were found at *m/e* 490 and 477 (i.e. M-90 and M-103). The ion at *m/e* 462 provides important information on the structure, since a fragment ion at M-118 was characterised by Horning[17] in the TMS derivative of the hemiacetal form of aldosterone. The derivative of tetrahydroaldosterone is therefore considered to be the hemiacetal (Fig. 1c). The fragmentation of 103 units indicates the loss of a primary trimethylsilanol group, whilst losses of two other trimethylsilanol groups at *m/e* 387 (M-103-90) and *m/e* 297 are shown. The ion at *m/e* 241 probably represents the 18 carbon nucleus of tetrahydro-aldosterone and the ions at 251 and 269, intermediate fragmentation of the nucleus and the fully derivatised compound after loss of the silanol groups.

The series of chromatograms illustrated in Fig. 3 depicts the effect of time on the condensation of tetrahydroaldosterone with methoxyamine hydrochloride prior to an 8 h silylation. A capillary column is used in the GC analysis of these samples. Peaks 1 and 2 give identical mass spectra (Fig. 2b). Few fragmentations are observed in these spectra besides the intense base peak at *m/e* 506 which is formed by loss of 103 mass units from the molecular ion at *m/e* 609. These peaks must be of the hemiacetal form (Fig. 1c) with one derivatised ketone and three silylated hydroxyls. Since the cyclisation is initiated by activation of the aldehyde group, the *d* and *l* configuration of the hydroxyl group on C-18 is feasible. The *syn-* and *anti*-oxime derivatives at C-20 are also possible. After a 72 h condensation reaction prior to silylation, only peaks 3 and 4 are observed. The mass spectra of these two peaks are indistinguishable (Fig. 2c). Tetrahydroaldosterone with free aldehyde (Fig. 1a) is the only form to give a fully derivatised bis-methoxime, tris-trimethylsilyl ether derivative with a molecular weight of 638.

Periodate oxidation of tetrahydroaldosterone gives rise to an etiolactone (Fig. 1b) which formed a silyl ether. This derivative gives a single gas chromato-

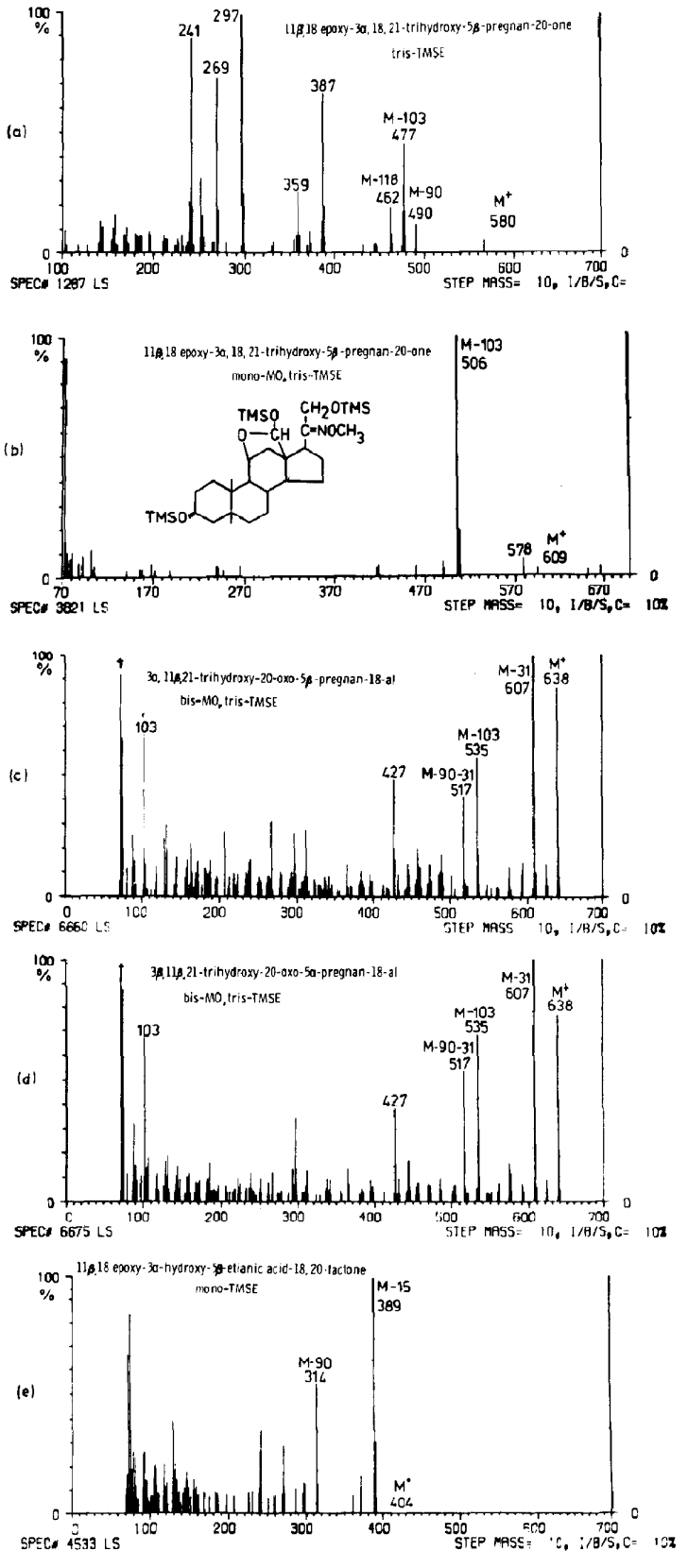

Fig. 2. Mass spectra of derivatives of tetrahydroaldosterone.

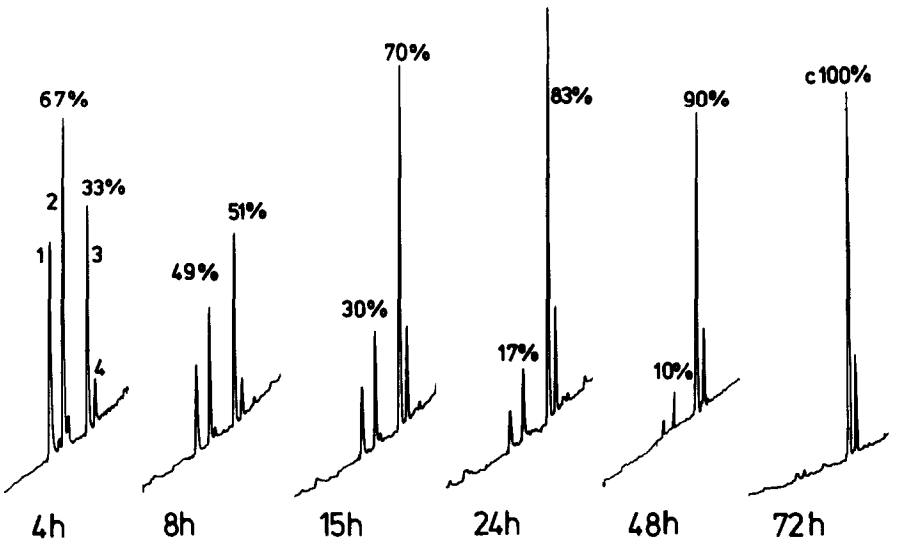

Fig. 3. Partial gas chromatograms of methyloxime-trimethylsilyl ether derivatives of tetrahydroaldosterone. The time course of methyloxime formation prior to an eight h silylation is illustrated. The percentage figures represent the sum of peak heights of 1 plus 2 and 3 plus 4.

graphic peak and mass spectrum with base peak at *m/e* 389 (Fig. 2e). This ion represents loss of 15 mass units from the parent ion at *m/e* 404, which was not observed. Other fragmentation characteristics of the compound are the M-44 due to loss of CO_2 from the lactone and an ion 90 mass units below this at *m/e* 270, due to the further loss of a trimethylsilanol group. The etiolactone did not form an oxime.

Choice of internal standard for quantitative analysis. 3β-*allo*-tetrahydroaldosterone was considered as an internal standard as it is commercially available and it is not a naturally occurring metabolite of aldosterone.

The derivatives of this isomer of tetrahydroaldosterone have longer retention times than the equivalent derivatives of the urinary metabolite when analysed by gas chromatography with OV-101 and OV-1 stationary phases. The time course for condensation of 3β,5α-tetrahydroaldosterone with methyloxime hydrochloride was similar to the urinary metabolite indicating that the equilibrium between the cyclic and free aldehyde forms were comparable. The mass spectrum of the MO-TMS derivative of the 3β,5α-tetrahydroaldosterone is illustrated in Fig. 2d and as with the other derivatives it is virtually indistinguishable from the MO-TMS derivative of the 3α,5β-tetrahydroaldosterone.

It seemed feasible to develop a quantitative method for urinary tetrahydroaldosterone based on monitoring the intensity of selected ions derived from both the analyte and the added internal standard. The minimal pre-purification of the tetrahydroaldosterone fraction meant that the mass spectrometric determination had to be highly specific. The bis-methyloxime, tris-trimethylsilyl ether derivatives was almost ideal. The parent ions at *m/e* 638 and the fragments formed by loss of one of the methoxy groups (M-31, *m/e* 607) carry a high proportion of the total ion current in each peak and are not fragments given by any other major urinary steroid. Interference from instrument background and column bleed is also at a minimum at this high mass position on the spectrum. These ions can be monitored simultaneously on the Varian 731 mass spectrometer although with this instrument it is only possible to monitor two ions and the ion of higher mass must be within 10% of the mass of the lighter ion determined.

Validation of method. Fig. 4 illustrates a typical twin pen recording of the output from the mass spectrometer. Since OV-1 packed columns are used in the GC–MS system, the *syn*- and *anti*-isomers are not resolved and each of the peaks has a shoulder. For quantitative purposes only the height of the major ion in each peak was determined.

The validity of measuring on the ion current chromatograms the ratio of the peak height of the *m/e* 607 ion from the urinary isomer to that from the internal standard as the basis for the quantitative determination of tetrahydroaldosterone was tested. Solutions with different concentrations of the reference urinary isomer of tetrahydroaldosterone were added to 1 μg amount of 3β,5α-tetrahydroaldosterone. These samples were treated as method blanks and derivatives prepared as for urinary samples. The results are shown in Fig. 5. Over the working range the slope is linear and in duplicate determinations the standard error of the slope is 0.015. When the ratio of urinary metabolite to internal standard exceeds 1, adsorption losses probably account for the deviation from this line. In repeat determinations of this calibration curve the slope varied within the expected limits considering all possible variations in the GC–MS system.

Sensitivity. Generally around 50 ng of analyte and

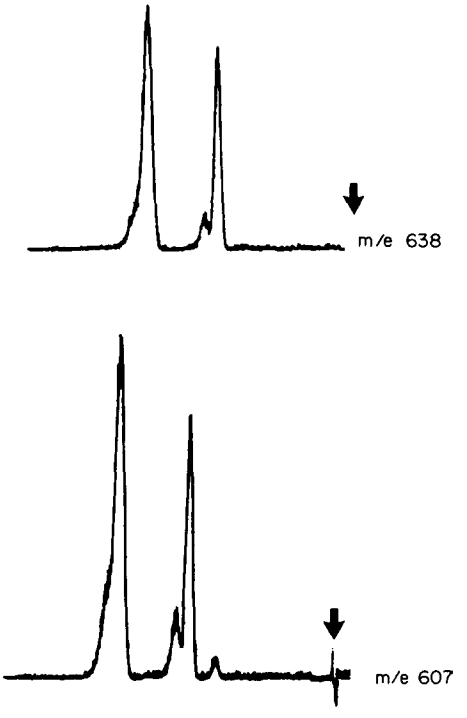

Fig. 4. Selected ion responses for the ions *m/e* 638 (M) and *m/e* 607 (M-31) in a typical determination of tetrahydroaldosterone in urine. The arrow indicates the injection point. The height of the peak produced by each ion from the tetrahydroaldosterone is related to the peak height of the corresponding ion from the added internal standard, 3β-allo-tetrahydroaldosterone.

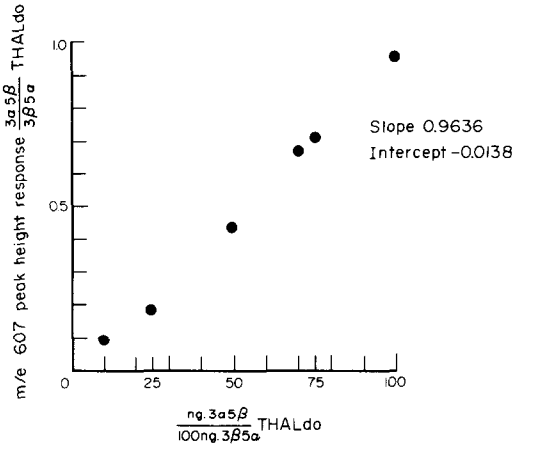

Fig. 5. Standard curve used for the quantitative determination of tetrahydroaldosterone in urine.

internal standard were injected into the mass spectrometer. However, the sensitivity can be increased considerably since 200 pg can be analysed with acceptable signal-to-noise ratio.

Precision. The precision of the method was determined by performing ten replicate analyses of a urine specimen from a normal adult. The mean excretion was calculated at 40 μg per day (coefficient of variation 8.8%).

Specificity. When reference 3α,5β-tetrahydroaldosterone (100–750 ng), along with 1000 ng of the 3β,5α isomer, was added to portions of a urine specimen, the ratio of the peak heights (*m/e* 607) in the two peaks increased proportionately to the amount of added material. The ratio of the intensities of the two ions monitored in each peak was not altered.

Practicability. Although the manipulations are few and not time-consuming the overall period required for optimum analysis is considerable, due principally to the 24 h required for enzymatic hydrolysis and 80 h for the quantitative formation of derivatives. However, the period of derivatisation can be shortened if some loss of sensitivity is acceptable. With an 8 h reaction only about 50% of the steroid has condensed to the 18,20-bis-methoxime but the ratio of peak heights of analyte and standard remain constant, so a short derivatisation may be acceptable when a result is required quickly. The GC–MS analysis is very rapid, no more than 10 min being required for each sample.

Results were calculated according to the formula:

$$X = \frac{H_x}{H_s} \times R \times \frac{V}{U} \times S$$

Symbols:

X = Urinary tetrahydroaldosterone excretion (μg/24 h).

H_x = Peak height urinary tetrahydroaldosterone.

H_s = Peak height 3β-*allo*-tetrahydroaldosterone.

S = Weight of internal standard (μg).

R = Response factor = $\frac{H_s 1}{H_x 1}$.

$H_x 1; H_s 1$ = Calibrating standards, Peak heights of identical weights of reference tetrahydroaldosterone and 3β-*allo*-tetrahydroaldosterone respectively.

V = 24 h urine vol. (ml).

U = Vol. of portion analysed (ml).

A standard curve is incorporated with each series of investigations so that a correction can be made for the difference in fragment ion intensities caused by any slight variation in temperature or pressure at any point in the GC–MS system.

DISCUSSION

A method was required for establishing the aldosterone status of hyponatraemic preterm infants. Since these infants receive constant nursing attention, collection of 24 h urine specimens was more practical than repeated blood sampling.

The technique of urinary steroid profile analysis has proved valuable in examining the overall steroid excretion of neonates with diverse pathological conditions [14]. However, the wide range of excretion rates

of all the steroids in a 24 h specimen makes this method unsuitable for studying changes in excretion rates of metabolites present in low concentration.

Tetrahydroaldosterone is the principal metabolite of aldosterone excreted in urine [2] and several methods have been reported for its measurement as an index of aldosterone secretion. A double isotope derivative analysis was first described by Kliman and Peterson[4] and used by New *et al.*[5] and Horton[6]. Several workers have utilised chromatographic purification of tetrahydroaldosterone after enzymic hydrolyis and acetylation followed by measurement of the formazan complex with blue tetrazolium in strong base. Deoxycorticosterone acetate is used as the internal standard in this method [7]. Paper chromatography in several solvent systems is necessary to separate adequately the tetrahydroaldosterone from other metabolites, although Legrand *et al.* [8] was able to limit the purification to a single chromatographic separation using celite as the supporting phase. The detection limit for the colorimetric end point is such that large vols of urine need to be processed.

A gas chromatographic method was devised by Carr and Wotiz[9]. Steroids were extracted with chloroform from 250 ml or urine, then hydrolysed with *Helix pomatia* digestive juice. The dry extract was acetylated and purified by paper chromatography prior to gas chromatography. The homogeneity of the GC peaks in the published chromatogram is questionable and indeed the excretion of tetrahydroaldosterone by an adult on normal sodium intake would be difficult to quantify from that data. These workers, however, were able to demonstrate a significant response to sodium deprivation.

New chromatographic stationary phases and derivatives designed for specific detectors, were incorporated in the method described by Nicolis *et al.* [10]. These workers measured tetrahydroaldosterone as the γ-lactone monochlorodifluoroacetate by electron capture. They later improved the sensitivity by using heptafluorobutyrate derivatives [11]. This technique has also been used by Solc and Knorr[12] to determine the excretion of tetrahydroaldosterone by infants. Although in the formation of the γ-lactone by periodic acid oxidation the glucuronide conjugate is released, the overall recoveries are low.

The methods reported to date either lack specificity or involve extensive and laborious purification. Gas chromatography–mass spectrometry with selected-ion-monitoring has been used in recent years for the measurement of several steroids and has the advantage of high sensitivity and specificity with relatively rapid throughput. The response of a selected ion, derived from the fragmentation of the analyte, is related to the response of a known amount of internal standard added at the earliest possible stage of the analysis. The nature of the internal standard has varied between three possibilities. Kelly[18] added a synthetic oestrogen, 4-methyl-oestra-1,3,5(10)-triene-1, 15α,16α,17β-tetrol, to pregnancy urine and monitored the *m/e* 191 ion derived from this standard and from the steroid to be measured—oestra-1,3,5(10)-triene-3,15α,16α,17β-tetrol. Determination of plasma cortisol [19] and aldosterone [20] have been achieved by the use of internal standards labelled with C-14 and tritium respectively. Material labelled with a stable isotope forms the ideal internal standard since then the analyte and internal standard might be most expected to behave identically, from the stage at which the standard is added. Testosterone has been measured in human male plasma after addition of deuterium-labelled testosterone as internal standard [21]. Few such standards are available commercially. On theoretical grounds, the molecular weight of the internal standard would need to be increased by at least 3 mass units (i.e. substitution by three deuterium ions in the molecule) to avoid any interference with the isotopic peaks of the natural compound [22].

Kelly *et al.*[2] found that 30% of a large oral dose of labelled aldosterone was excreted as 3α,5β-tetrahydroaldosterone. Several other minor metabolites were isolated, of which some remain unidentified [2, 24]; conversion to two other epimers of tetrahydroaldosterone with 3α,5α and 3β,5β configurations was calculated at 0.5 and 1.0% respectively [23]. This very low excretion may not reflect true catabolism since several hundred milligrams of aldosterone were initially administered. 3β,5α-Tetrahydroaldosterone (the epimer used as internal standard) has only been isolated from biological samples after incubation of aldosterone with rat liver preparations *in vitro* [3]. Performance during extraction with Amberlite XAD-2 and chromatography on Sephadex LH-20 is unlikely to differ from the urinary isomer. The retention time of the internal standard is longer than the analyte, and since the gas chromatograph is used isothermally the peak base is fractionally wider. The linearity of the peak height response of the analyte over the range 5–300 ng relative to 100 ng of internal standard confirms that this is no limitation to the use of this epimer. Analyses of urine without the incorporation of internal standard have demonstrated no peak at the expected retention time.

The ions at *m/e* 638 and 607 have proved to be unique to tetrahydroaldosterone, although the combination of retention time and ratio of intensity of two selected ions would immediately discriminate against measuring any interfering steroid. The fraction from Sephadex LH-20 selected for analysis contains principally cortisol metabolites—tetrahydrocortisone and cortolones. This type of analysis has the advantage that, by monitoring different ions, the analytical procedure may readily be used for the determination of other steroid hormones and their metabolites in the same extract. Indeed by using a mass spectrometer capable of monitoring the intensities of six ions simultaneously (Varian MAT-112) we are now able to measure tetrahydroaldosterone, 3α,

18,21-trihydroxy-5β-pregnane-11,20-dione and tetrahydrocortisone in a single chromatogram (Shackleton and Honour, unpublished).

We have evaluated a modification of the method using the trimethylsilyl ether derivative of tetrahydroaldosterone rather than the oximesilyl ether. In principal, this would be advantageous since only a single derivative is formed and the reaction is complete in 8 h. However, the mass spectrum of tetrahydroaldosterone trimethylsilyl ether is similar to that of its isomer 3α,18,21-trihydroxy-5β-pregnane-11,20-dione. The same high mass ions are given by both compounds (M, 580; M-90, 490; M-103, 477) and both steroids have similar retention times on OV-1 columns. Thus, quantitation of tetrahydroaldosterone using this derivative is less satisfactory.

Etiolactone formation has provided a convenient method for analysis of tetrahydroaldosterone, particularly when derivatives with electron capture properties have been used [10]. The lactone structure seems to stabilise the molecules which are then ideal for gas chromatography. Reaction with periodic acid has the advantage of releasing conjugates and oxidising cortisol metabolites to acidic products easily removed from the extract by alkali washing. The addition of the extra chemical steps make the method less desirable particularly if recoveries and artifact formation are considered. 3β-*allo*-Tetrahydroaldosterone was added to the urine prior to the periodate oxidation. Trimethylsilyl ether derivatives were prepared prior to the GC–MS analysis with selective ion monitoring. The ions at *m/e* 404 (M) and 389 (M-15) were not specific for the analyte and internal standard. The etiolactone derivatives of 3α,18,21-trihydroxy-5β-pregnane-11,20-dione were identified within several unresolved peaks. This method was not pursued.

In conclusion, it would appear that the method involving GC–MS analysis of methyloxime-trimethylsilyl ether derivatives is satisfactory for the quantitative analysis of urinary tetrahydroaldosterone. Clinical studies, using this technique have already been reported [25, 26, 27, 28], and therefore discussion here has been restricted to the method itself.

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