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IDENTIFICATION AND MEASUREMENT OF 18-HYDROXYCORTICOSTERONE METABOLITES BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Combined gas chromatography–mass spectrometry was used to identify methyloxime-trimethylsilyl ether derivatives of 18-hydroxycorticosterone metabolites in a urinary extract from an infant with pseudo-hypoaldosteronism. Mass spectra are presented of derivatives of two metabolites (3α,18,21-trihydroxy-5β-pregnane-11,20-dione and 3α,11β,18,21-tetrahydroxy-5β-pregnan-20-one) and a mechanism is proposed for a fragmentation characteristic of these 18-hydroxylated C-21 steroids. A method is described for the quantitative determination of 3α,18,21-trihydroxy-5β-pregnane-11,20-dione in urine. 3β,17,21-Trihydroxy-5α-pregnane-11,20-dione was used as internal standard and the ions of mass 609 and 578 were selectively monitored by the mass spectrometer.

INTRODUCTION

The probability that 18-hydroxycorticosterone was an intermediate in aldosterone biosynthesis originated with the work of Ulick and co-workers who first identified this compound in bullfrog adrenals [1] and later demonstrated that its secretion rate in man was related to, but greater than, that of aldosterone [2]. The parallel changes of secretion in response to stimulation and suppression suggest that both steroids are under the control of the same hormonal system.

The major metabolite of 18-hydroxycorticosterone was 3α,18,21-trihydroxy-5β-pregnane-11,20-dione [3], and a high excretion of this steroid in patients with a congenital deficiency of aldosterone [4, 5] gave further support for the precursor role of 18-hydroxycorticosterone since it was best explained by a block in 18-hydroxy steroid dehydrogenation in these cases. A recent study undertaken in this laboratory on the excretion of 18-hydroxycorticosterone metabolites by an infant with 18-hydroxy steroid dehydrogenase deficiency [6] prompted us to examine the mass spectrometric properties of these steroids. In our study and the studies of Ulick and co-workers [2, 7], 3α,18,21-trihydroxy-5β-pregnane-11,20-dione was measured after periodate oxidation to an etiolactone derivative. However, to bring the assay into line with other steroid gas chromatographic methods carried out in this laboratory [8], we desired a method based on the analysis of the unchanged material. This communication describes the gas chromatographic–mass spectrometric (GC–MS) analysis of O-methyloxime-trimethylsilyl ether and trimethylsilyl ether derivatives of 3α,18,21-trihydroxy-5β-pregnane-11,20-dione and 18-hydroxytetrahydrocorticosterone.

MATERIALS AND METHODS

Materials. Gas chromatograph–mass spectrometers, Varian MAT-731 and 112 instruments, Varian GmbH, Bremen, Germany; *gas chromatograph*, Model 409, Packard–Becker, Delft, Netherlands; *open-tubular column*, H & G Jaeggi, 9043 Trogen, Zurich, Switzerland; *Sephadex LH-20*, Pharmacia AB, Uppsala, Sweden; *Lipidex 5000*, Packard–Becker, Delft, Netherlands; *Helix pomatia*, L'Industrie Biologique Française, Gennevilliers, Seine, France; *trimethylsilylimidazole*, Supelco Inc., Bellefonte, Pennsylvania, USA; *pyridine* (analar), BDH, Poole, England, distilled and stored over sodium hydroxide; *cyclohexane* (analar), BDH, Poole, England, charcoal washed and double-distilled; *ethanol*, James Burrough Ltd, London, England, distilled over sodium hydroxide; and *reference steroids* obtained from the Medical Research Council, Steroid Reference Collection, Westfield College, London.

Source of 3α,18,21-*trihydroxy*-5β-*pregnane*-11,20-*dione and* 18-*hydroxy-tetrahydrocorticosterone.* These urinary steroids have previously been identified in this laboratory by GC–MS of their etiolactone derivatives formed by periodic acid oxidation of urine extracts [6]. Because of a lack of authentic standards and insufficient material present in the urine samples it was impractical to identify and study the mass spectrometric properties of these steroids prior to oxidation. A urine sample was obtained however, from an infant with end-organ unresponsiveness to aldosterone, and in which large amounts of tetrahydroaldosterone and the compound subsequently identified as 3α,18,21-trihydroxy-5β-pregnane-11,20-dione were found. The steroid extract was obtained by enzymatic hydrolysis of urine (*Helix pomatia* and Ketodase), extraction on

a column of Amberlite XAD-2 and fractionation of the steroids on a 6-g column of Sephadex LH-20 [8]. This column was equilibrated in, and eluted with the solvent system cyclohexane–ethanol, 4:1 (v/v), and 10 ml fractions were collected when 55 ml had been eluted. The fraction eluted between 55–65 ml contained tetrahydrocortisone, tetrahydrocorticosterone and tetrahydroaldosterone, whilst the fraction between 65–75 ml contained principally the compound later identified as 3α,18,21-trihydroxy-5β-pregnane-11,20-dione with small amounts of tetrahydrocortisone and tetrahydrocorticosterone. This latter fraction was chosen for the mass spectrometric study. The fractions eluted between 85–105 ml contained a compound which was subsequently identified as 18-hydroxytetrahydrocorticosterone.

Derivative formation. Methyloxime-trimethylsilyl ether (MO-TMS) derivatives were prepared in a two-step reaction (a) addition of 0.2 ml 2% methoxyamine hydrochloride in pyridine to the dried extract. The reaction time for steroids is normally 8 h at room temperature but for the analysis of the steroids in the present study it was desirable to leave the reaction for 3 days at room temperature [9]; (b) 100 μl trimethylsilylimidazole (TSIM) was added to pyridine solution of the methyloxime derivatives and the mixture heated for 2 h at 100°C. Excess reagents were removed by chromatography on small columns of Lipidex 5000 [10].

Gas chromatographic analysis. Steroid derivatives were analysed by gas chromatography on a 20 m OV-101 open-tubular column with helium as carrier gas, according to the method described by Shackleton and Honour[8]. Solid injection was employed and the temperature was programmed between 160°C and 260°C at a rate of 2.5°C/min.

Gas chromatography–mass spectrometry. Repetitive scanning GC–MS was carried out on a Varian MAT-731 mass spectrometer coupled to an Acrograph 2700 gas chromatograph (2 m packed OV-1 columns) temperature programmed at 2°C/min between 220°C and 300°C. After injection of the samples, spectra were taken every 10 sec throughout the period of elution of the sample, and acquired onto magnetic tape. SpectroSystem 100 was used for subsequent data processing.

Multiple ion recordings for quantitation were obtained on a Varian MAT-112 mass spectrometer equipped with multiple ion selection (MIS) device. This allowed simultaneous recording of the intensities of up to eight different masses. These analyses were carried out on a 2 m OV-1 column isothermally at 260°C.

RESULTS

Identification of 3α,18,21-*trihydroxy-5β-pregnane*-11,20-*dione*

The mass spectrum of the O-methyloxime-trimethylsilyl ether of the steroid identified as 3α,18,21-trihydroxy-5β-pregnane-11,20-dione is illustrated in Fig. 2. The parent ion is at *m/e* 609 and prominent ions are seen at *m/e* 578 (M − 31) and 457 (M − 152), the presence of the latter ion confirmed the structure. Two gas chromatographic peaks gave the same mass spectrum and were almost certainly *syn-* and *anti-*forms of the oxime derivative, since all the deoxycorticosterone (DOC) and corticosterone metabolites previously studied have given two well-resolved peaks on derivatisation [8].

Although reference 3α,18,21-trihydroxy-5β-pregnane-11,20-dione and 18-hydroxytetrahydrocorticosterone were not available, the mass spectrometric properties of the O-methyloxime-trimethylsilyl ethers of other 18-hydroxylated corticosteroids have been studied by Maume and co-workers [9, 11, 12]. All of these steroids give a significant ion at (M − 152) which, with the exception of 3α,18,21-trihydroxy-5β-pregnan-20-one (18-hydroxytetrahydroDOC) these authors implied was formed by a loss of trimethylsilanol plus removal of two methoxy groups from the methyloxime derivatives [M − 90 − (2 × 31)]. In the mass spectrum of 18-hydroxytetrahydroDOC the ion at *m/e* 443 (i.e. M − 152) is of equal abundance to that at *m/e* 564 (M − 31) [12]. The interpretation of the M − 152 fragmentation implied by Prost and Maume[9] does not hold for 18-hydroxytetrahydroDOC since this compound has only one methyloxime moiety. High resolution mass measurement was carried out on the equivalent fragment from 3α,18,21-trihydroxy-5β-pregnane-11,20-dione. The measured atomic mass (457.2899) agreed within 0.3 m mass units with the elemental composition $C_{26}H_{43}NO_2Si_2$. Thus the 152 mass unit loss from the molecular ion corresponds to $C_5H_{16}O_3Si$. The mechanism of this fragmentation is complex and no metastable ions could be detected to substantiate either a multi-step process or a concerted pathway. A simplified representation of a possible concerted mechanism is shown in Fig. 3. This is envisaged as an initial rearrangement loss of the methoxy group and the C-17 hydrogen as methanol, followed by transfer of the C-18 trimethylsilanol group to the nitrogen on C-20, and release of carbon-18 along with two hydrogens and oxygen as formaldehyde. Finally, cyclisation after a loss of trimethylsilanol results in a substituted 2-azirine fragment at M − 152.

Formation of trimethylsilyl ethers alone resulted in two chromatographic peaks of approximately equal size giving identical mass spectra. These peaks could either represent the 3α,5β and 3α,5α forms of 3α,18,21-trihydroxy-5β-pregnane-11,20-dione or epimeric forms of the 18–20 cyclic ketal (20α- or 20β-hydroxyl). The mass spectrum of one of these peaks is illustrated in Fig. 2(b). The parent ion was at *m/e* 580 and prominent ions were seen at *m/e* 490 (M − 90) and *m/e* (M − 103). If the reaction time for methoxime formation during the preparation of MO-TMS derivatives was less than 3 days at room temperature, the tris-trimethylsilyl ether (Fig. 1, V) was formed along with

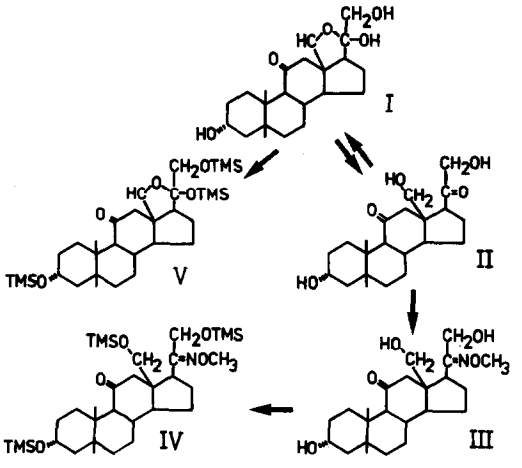

Fig. 1. Chemistry of 3α,18,21-trihydroxy-5β-pregnane-11,20-dione during derivative formation.

the mono-methoxime, tris-trimethylsilyl ether (Fig. 1, IV). 3α,18,21-Trihydroxy-5β-pregnane-11,20-dione must exist in solution predominantly as the hemiketal form (Fig. 1, I) in equilibrium with the free C-18 alcohol. Formation of a methoxime at C-20 (Fig. 1, III) pulls the equilibrium in favour of the free form, such that during a 3 day reaction complete condensation is achieved. This reaction could be accelerated by heating at 60°C for 3 h but this entailed some loss of steroid (10–15%).

The steroid extracts occasionally contained a minor component which was probably the 20-ethyl-ketal of 3α,18,21-trihydroxy-5β-pregnane-11,20-dione formed during ethanol storage. This steroid (as trimethylsilyl ether) gave the mass spectrum illustrated in Fig. 2(c). The base peak was at *m/e* 433 formed by the loss of 103 mass units (a primary trimethylsilyl group) from the parent ion. The formation in ethanol or methanol solution of 20-ethyl or methyl ketal artifacts of 18-hydroxylated steroids has been discussed at length by Roy *et al.*[13] and may be avoided by use throughout the method of alcohol containing 0.1% trimethylamine or alcohol distilled over sodium hydroxide.

Identification of 18-*hydroxytetrahydrocorticosterone*

This steroid was of comparatively minor importance compared to 3α,18,21-trihydroxy-5β-pregnane-11,20-dione. The mass spectrum of the O-methyloxime-trimethylsilyl ether (Fig. 2d) was however sufficiently unambiguous to prove identity. The parent ion was at *m/e* 683 and important fragments were at *m/e* 652 and *m/e* 531 (M − 152). This steroid was first identified in a previous study following periodic acid oxidation of urine extracts [6].

Development of a method for the semi-quantitative analysis of 3α,18,21-*trihydroxy*-5β-*pregnane*-11,20-*dione*

The mass spectrometer monitored the ions *m/e* 609 (M), *m/e* 578 (M − 31) and *m/e* 457 (M − 152) during the GC analysis and a chromatogram is illustrated in Fig. 4. The intensities of the first two ions derived from the analyte were related to the peak heights of these ions derived from the internal standard—3β, 17,21-trihydroxy-5α-pregnane-11,20-dione which was added prior to the derivatisation. The *m/e* 457 ion was monitored to prove identity of the 3α,18,21-trihydroxy-5β-pregnane-11,20-dione peak since it is specific for this compound. Three peaks (3, 4, 5) give a response for all three ions, peaks 3 and 5 represent *syn-* and *anti-* forms of the methyloxime derivative of the 3α-hydroxy-5β epimer since this is the major 18-hydroxycorticosterone metabolite (Ulick, personal communication) and peak 4 is most probably the 3α-hydroxy-5α epimer. 3β,17,21-Trihydroxy-5α-preg-

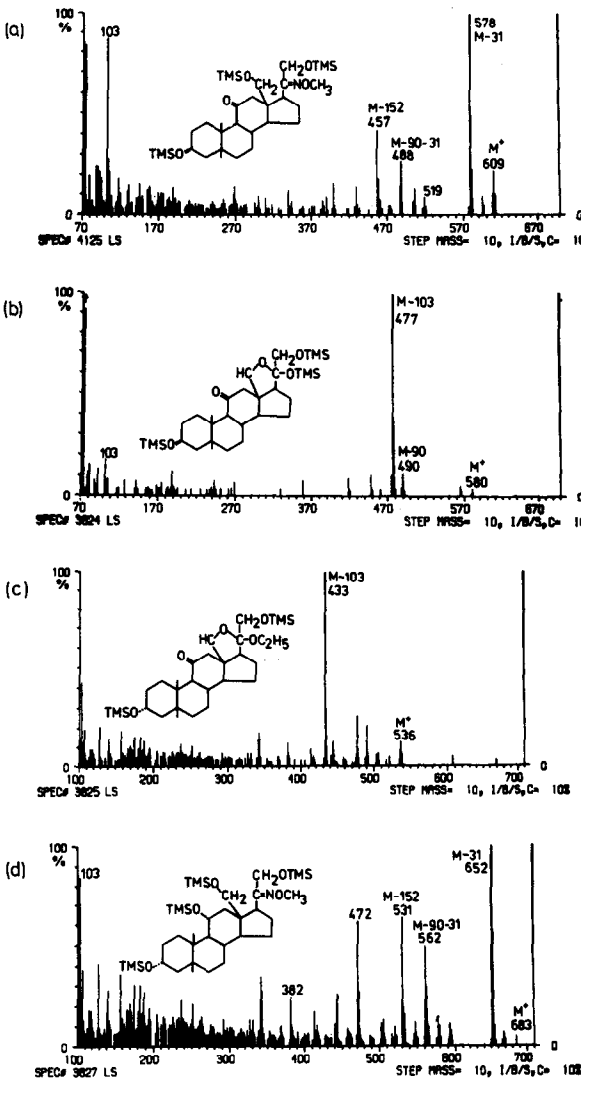

Fig. 2. Mass spectra of: (a) 3α,18,21-trihydroxy-5β-pregnane-11,20-dione, monomethyloxime, tris-trimethylsilyl-ether. (b) 3α,18,21-trihydroxy-5β-pregnane-11,20-dione, 20, 18-hemiacetal, tris-trimethylsilyl ether (c) 20β-ethyl, 3α,18, 21-trihydroxy-5β-pregnane-11,20-dione, 20,18-hemiacetal, tris-trimethylsilyl ether (d) 3α,11β,18,21-tetrahydroxy-5β-pregnan-20-one, monomethyloxime, tetra-trimethylsilyl ether.

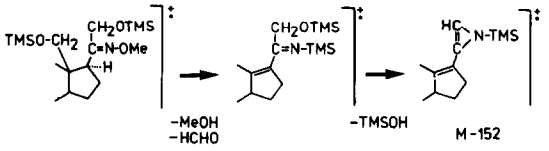

Fig. 3. Proposed mechanism for the formation of fragment ion M-152 from 18-hydroxy C-21 steroids during mass spectrometry.

nane-11,20-dione is not a naturally occurring metabolite of cortisol.

An accurate response-factor for analyte and internal standard could not be determined without a pure standard of 3α,18,21-trihydroxy-5β-pregnane-11,20-dione. An experiment was carried out to determine if the *m/e* 609 and 578 ions from the analyte and the internal standard carried comparable partial ion current. To a sample (5–10 μg) of partially purified 3α,18,21-trihydroxy-5β-pregnane-11,20-dione was added 5 μg of internal standard. This was derivatised and a portion analysed by gas chromatography on an open-tubular column with linear temperature increase between 160°–260°C. The heights of both peaks were summed and related to the peak height of 3β,17,21-trihydroxy-5α-pregnane-11,20-dione. On the assumption that both steroids gave equal gas chromatographic response the sample was found to have 8.0 μg of 3α,18,21-trihydroxy-5β-pregnane-11,20-dione. The sample was then analysed by GC-MS isothermally at 260°C and the fragments *m/e* 609 and *m/e* 578 were monitored. Peak areas were measured and the sum of the areas for the analyte peaks were related to the peak area of the internal standard. If the concentration of 3α,18,21-trihydroxy-5β-pregnane-11,20-dione was determined from the *m/e* 609 peaks the amount present was 8.50 μg and for the *m/e* 578 peak was 8.35 μg. These figures compare favourably with the 8.0 μg previously obtained by conventional gas-chromatographic analysis. From these figures it was concluded that the partial ion current given by *m/e* 609 and *m/e* 578 fragments was almost identical for 3α,18,21-trihydroxy-5β-pregnane-11,20-dione and 3β,17,21-trihydroxy-5α-pregnane-11,20-dione, suggesting the suitability of using the latter compound as internal standard, since no correction factor was necessary.

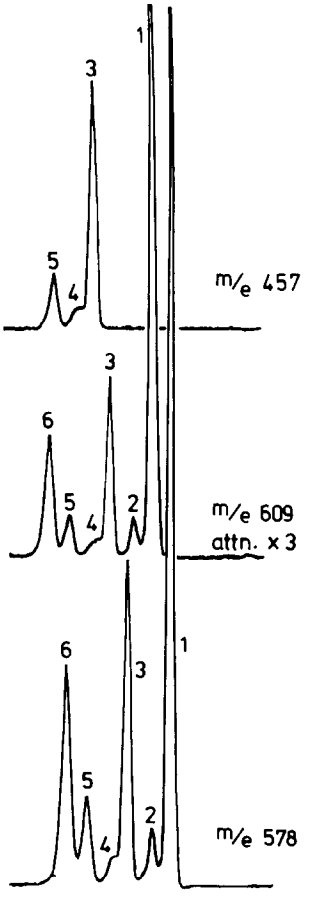

Fig. 4. Selected ion recordings of *m/e* 609, 578, 457 in the analysis of 3α,18,21-trihydroxy-5β-pregnane-11,20-dione in urine (peaks 3 and 5). Peak 1 is tetrahydrocortisone and peak 6 is from 3β,17,21-trihydroxy-5α-pregnane-11,20-dione added as internal standard.

DISCUSSION

The analysis of 18-hydroxylated metabolites of deoxycorticosterone (DOC) and corticosterone is complicated by the existence of different forms. Each may exist as cyclic hemiketals (20α- and 20β-hydroxyls), in a 'free' 18-hydroxy form, and as dimers [14]. The chemistry of the interconvertible forms of 18-hydroxy DOC and 18-hydroxycorticosterone was discussed at length by Dominguez[15] and Damasco and Lantos [16] and their results suggested that this tautomerism was an oversimplification. In addition, a recent publication by Roy *et al.*[13] demonstrated that the cyclic hemiketal forms of 18-hydroxyDOC were unstable in ethanolic or methanolic solutions containing traces of acid. These workers showed that there was a partial conversion of 18-hydroxyDOC hemiketal to C-20 ethyl or methylketals under these conditions Maume and co-workers achieved a quantitative conversion of 18-hydroxyDOC, 18-hydroxytetrahydroDOC and 18-hydroxycorticosterone to methyloxime-trimethylsilyl ether derivatives, in the open form by extending the period normally adequate for condensation of carbonyl groups in steroids with methoxyamine hydrochloride. Even so, single derivatives are not obtained, since the *syn-* and *anti-* forms of the 20-oxime are formed during the reaction and are separated by gas chromatography on OV-101 columns. 3α,18,21-Trihydroxy-5β-pregnane-11,20-dione has been shown to derivatise in a similar way under these reaction conditions. Prost and Maume[11] and Bournot *et al.*[12] reported the mass spectra of methyloxime-trimethylsilyl ethers of 18-hydroxycorticosterone and 18-hydroxytetrahydroDOC, and demonstrated that each compound gave a characteristic fragment formed by the loss of 152 mass units from the parent ion. These workers implied that this fragmentation was due to loss of trimethylsilanol (−90) and two methyoxy groups (−2 × 31). We have used the specificity of this

fragmentation to identify human urinary 3α,18,21-trihydroxy-5β-pregnane-11,20-dione and 18-hydroxytetrahydrocorticosterone. Both these steroids as methyloxime derivatives gave ions at *m/e* M − 152 but from high resolution mass measurement a new mechanism for the fragmentation is proposed. Only one methoxy group is involved in the fragmentation. A suitable internal standard has permitted a method to be established for the semi-quantitative analysis of 3α,18,21-trihydroxy-5β-pregnane-11,20-dione. This method has the advantage over previous methods that periodic acid oxidation was not necessary thus reducing by one step the GC–MS method described previously based on etiolactone formation [6]. The assay described can be combined with the tetrahydroaldosterone method previously reported [17, 18], since a long derivatisation procedure is required for both analyses. All that is required is that 3β, 11β,21-trihydroxy-5α-pregnan-20-on-18-al is included as a second internal standard. Monitoring two pairs of ions, *m/e* 638 with 607, and 609 with 578, permits determination of tetrahydroaldosterone and 3α,18,21-trihydroxy-5β-pregnane-11,20-dione respectively. The simultaneous determination of aldosterone and 18-hydroxycorticosterone metabolites is particularly useful in diagnosis of defects in late stages of aldosterone biosynthesis whether of the initial 18-hydroxylation or the subsequent transformation to the aldehyde. In the light of new evidence on the mechanism of this biosynthesis, Ulick[19] refers to these defects as corticosterone methyloxidase type 1 and 2 respectively. The present method based on analysis of urinary steroids augments the established radioimmunoassay for plasma aldosterone and the recently developed immunoassay for 18-hydroxycorticosterone [20].

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