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Analysis of biological samples by gas chromatography–mass spectrometry without a reference standard: measurement of urinary 18-hydroxytetrahydro-11-dehydrocorticosterone excretion rate in human subjects

Loai A. Shakerdi[b,*], John M.C. Connell[a], Robert Fraser[a], A. Michael Wallace[b]

[a]*MRC Blood Pressure Group, Western Infirmary, Glasgow G11 6NT, Scotland, UK*
[b]*Department of Clinical Biochemistry, Macewen Building, Glasgow Royal Infirmary, Glasgow G4 0SF, Scotland, UK*

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Abstract

Reference standards for some minor urinary steroid metabolites are sometimes unavailable. We describe a novel procedure to quantitate a urinary steroid metabolite of known structure and mass spectrum, using as a standard a compound which produces ions in common with it and has a similar retention time in gas chromatography–mass spectrometry. The steroid of interest was 18-hydroxy-11-dehydrotetrahydrocorticosterone (18-OH-THA), the major urinary metabolite of 18-hydroxy-corticosterone (18-OH-B), a putative intermediate in the conversion of 11-deoxycorticosterone to aldosterone. The steroid used as an alternative to the authentic 18-OH-THA standard was β-cortol which, like 18-OH-THA, produces a fragmentation ion at m/z 457. Allo-tetrahydrodeoxycorticosterone (5α-THDOC) was used as the internal standard. β-Cortolone also has the fragmentation ion at m/z 449 (in common with β-cortol) and an authentic standard is available commercially. To validate the procedure, we quantitated β-cortolone urinary excretion rate against this alternative standard and also against authentic β-cortolone standards. Both methods produced similar results (adjusted R^2: 0.998, $P<0.001$). The method was then used to measure urinary excretion of 18-OH-THA rate in healthy volunteers. The reference range obtained was 20–204 μg/24 h ($n=32$). This is similar to the few results available by conventional assay. Method performance was also similar to other assays of urinary steroids. This procedure could be generally applicable for assays when authentic standards are not available but mass spectra are known or can be predicted.

Keywords: Aldosterone synthase; 18-Hydroxytetrahydro-11-dehydrocorticosterone

1. Introduction

In developing a method of quantitative analysis for any compound, the necessity of an authentic standard is self-evident. This frequently presents a difficulty, particularly for the analysis of biological material.

*Corresponding author. Tel.: +44-141-211-4490; fax: +44-141-553-0604.
E-mail address: loaishakerdi@yahoo.co.uk (L.A. Shakerdi).

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For example, there are innumerable steroid hormone metabolites, analysis of which might provide valuable information of normal and abnormal processes of synthesis and metabolism but for which, for many minor compounds, standards are no longer or have never been commercially available. Ideally, the solution is to carry out specific synthesis but this is time-consuming and expensive and therefore, at least for initial investigations, uneconomic.

The corticosteroid, 18-hydroxycorticosterone, is a putative intermediate in the conversion of 11-deoxycorticosterone to aldosterone. It has potential value as an index of the efficiency of the second and third mono-oxygenation reactions catalysed by aldosterone synthase in the zona glomerulosa [1,2]. It has been suggested that mutations and polymorphisms in the aldosterone synthase gene (CYP11B2) may specifically affect the efficiency of one or both of these reactions [3,4]. In population surveys, 18-hydroxycorticosterone secretion rate would be conveniently monitored by the excretion rate of its principal unique urinary metabolite, 18-hydroxy-11-dehydrotetrahydrocorticosterone (18-OH-THA). This compound is not available commercially although it has been synthesised and its mass spectrum has been published [5,6].

The most convenient and reliable method of screening urine samples for steroid metabolites is GC–MS of their trimethylsilyl methyloxime (TMSI-MO) derivatives [7]. We investigate here the possibility of using a heterologous standard (β-cortol) producing an ion in common with 18-OH-THA for quantitation of the latter compound. By way of validation, we measured urinary β-cortolone excretion rate using both this indirect approach and the homologous standard. The chemical structures of β-cortol, β-cortolone and 18-OH-THA are shown in Fig. 1.

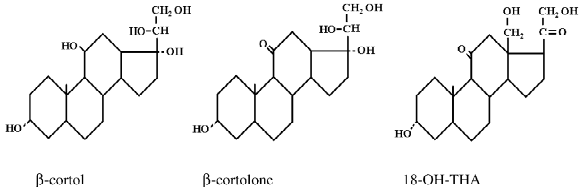


Fig. 1. Chemical structures of β-cortol, β-cortolone and 18-OH-THA.

2. Materials and methods

The sources of reagents were as follows: methoxyamine hydrochloride (Sigma, Poole, UK) trimethylsilyl imidazole (Pierce, Rockford, IL, USA); alloTHDOC, β-cortol, β-cortolone (Sigma); Lipidex (Packard, Pangbourne, UK); β-glucuronidase/sulphatase (*Helix pomatia* juice, BioSepra, France); Sep-Pak C_{18} cartridges (Waters, Chromatography Division, Millipore, Milford, MA, USA); acetic acid and sodium acetate were obtained from (BDH, Poole, UK)

2.1. Extraction and derivatisation

Urinary steroids were extracted and derivatised as described by Shackleton [8] with minor modifications. Briefly, steroid conjugates were extracted from urine (10 ml) on Sep-Pak cartridges, reconstituted in 10 ml of 0.5 *M* acetate buffer and hydrolysed by incubation with 100 μl of *Helix pomatia* juice (48 h, 37 °C). The steroid metabolites were then re-extracted, also with Sep-Pak cartridges. A 10-μl volume of (1 mg/10 ml) of the I.S. allo-tetrahydrodeoxycorticosterone (5α-THDOC) was added to each sample and the solvent then evaporated under nitrogen at 40 °C. Methoxyamine hydrochloride in pyridine (100 μl) was added and the samples incubated overnight. On the second day, 50 μl of trimethylsilylimdazole was added and samples were incubated at 100 °C for 3 h. The derivative, diluted with 1 ml Lipidex solvent (cyclohexane–hexamethyldisilazane–pyridine, 98:1:1, v/v) was purified by Lipidex 5000 chromatography. The steroid derivatives were eluted with 2 ml of Lipidex solvent, which was then evaporated under nitrogen at 40 °C. The residue was reconstituted in 50 μl Lipidex solvent and 2 μl was injected into the GC.

2.2. GC–MS

Throughout, a GCQ™Plus Benchtop Ion Trap GC–MS^n (ThermoQuest, Finnigan, USA) was used. The GC was fitted with a split/splitless injector operated in the splitless mode. The inlet temperature was held at 260 °C and the splitless time was 2 min at a constant septum purge. The right carrier was in constant pressure mode with an initial value of 40

kPa. The gas saver flow was 20 ml/min for 2 min. Following injection, the temperature programme was as follows: 100 °C for 3 min, 20 °C/min without hold for 7 min to 190 °C, 2 °C/min for 45 min to 285 °C. The temperature was held at 285 °C for 10 min, giving a total run time of 62 min. The GC column (liquid phase: DB-1. dimensions: 30 m × 0.322 mm, film thickness 0.25 μm, J&W Scientific, USA) was directly coupled, via a transfer line heated to 290 °C, to the ion source of the ion-trap mass spectrometer. Effluent steroids were fragmented and ionised by positive electron ionisation mode (EI). This was interfaced to and controlled by a data system using XCALIBUR software running under Windows NT.

2.3. Calibration curve

The standard curve for the measurement of 18-OH-THA was constructed by using five concentrations of the alternative standard ''β-cortol'' (0.35, 0.69, 1.38, 2.77 and 11.08 μg/2 μl). The internal standard (I.S.) 5α-THDOC was added to each standard (10 ng/2 μl) and was detected by selected ion monitoring (SIM) of the ion at *m/z* 476. The ion at *m/z* 457, which occurs in the mass spectrum of β-cortol was monitored by SIM. The calibration curve was constructed by calculating the peak ratios from ion signals of β-cortol and the I.S., 5α-THDOC. The ion at *m/z* 457 accounts for 0.231% of β-cortol. To make this standard curve suitable to measure 18-OH-THA, each of the five alternative standards concentration was multiplied by 0.00231. This is to correct for the difference in the abundance of the ion at *m/z* 457 in the mass spectra of β-cortol and 18-OH-THA. A similar calibration curve was constructed by using β-cortol as an alternative standard to measure β-cortolone, but in this case the ion at *m/z* 449 was monitored by SIM. β-Cortolone was also measured by using authentic standards and 5α-THDOC as an I.S. for confirmation.

3. Results

3.1. Identification of 18-OH-THA

The TMSI-MO ether derivative of 18-OH-THA

Fig. 2. TMSI-MO derivatisation of 18-OH-THA. Three (OH) groups at C-3, 18, 21 were silylated and the keto group at C-20 was methoximated.

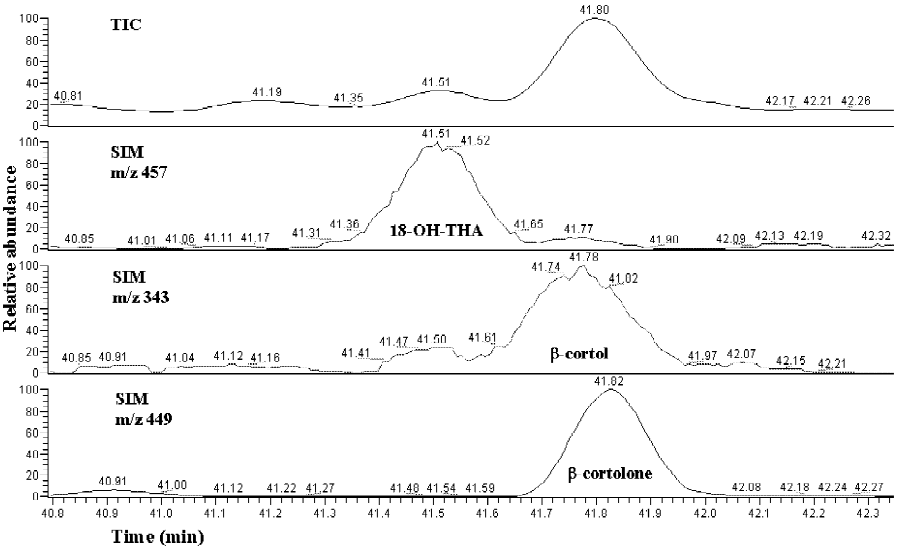
(3α,18,21-trihydroxy-5β-pregnane-11,20-dione) has a parent ion at *m/z* 609. This implies that all hydroxyl groups form trimethylsilyl ether links but only one keto group, at C-20, is methoximated (Fig. 2). The major ions in the mass spectrum of 18-OH-THA with their probable origins are listed in Table 1. Most of the ions are explained by cleavage reactions except the ion at *m/z* 457, which could be explained by cleavage and rearrangement reactions [9]. 18-OH-THA was detected in urine samples by SIM of the ions at *m/z*: 609, 608, 594, 578, 577, 518, 488, 487, 471, 458 and 457. β-Cortol and β-cortolone were detected in the same urine sample by SIM of the ions at *m/z* 343 and 449, respectively (Fig. 3A). Total ion chromatography (TIC) of the 18-OH-THA peak in these samples gave a mass spectrum which accords with that published by

Table 1
Major fragments of TMSI-MO derivatives of 18-OH-THA by GC–MS under positive electron ionisation (IE)

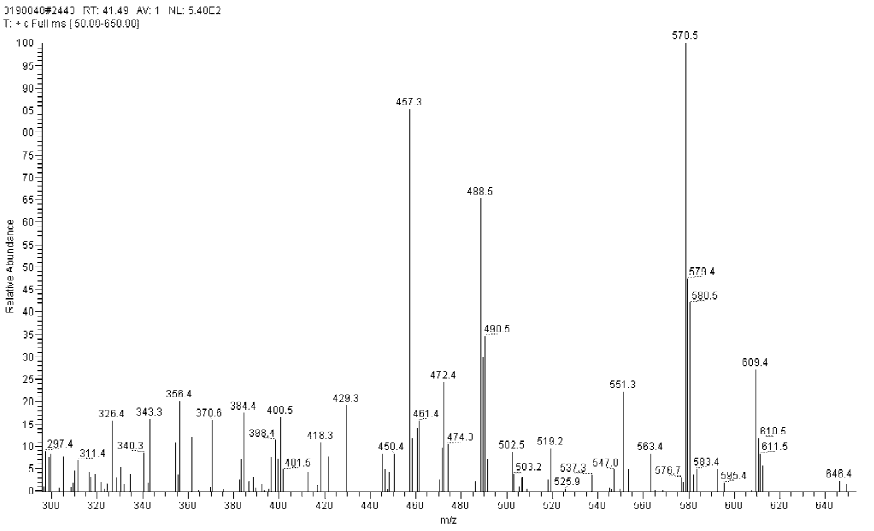
m/z	Interpretation
609[a]	$M-0.0=609$
608	$M-H^{+}=608$
594	$M-CH_3=594$
578	$M-CH_3^{\cdot}=578$
577	$M-CH_3OH=577$
519	$M-(CH_3)_3SiO^{\cdot}=519$
518	$M-(CH_3)_3SiOH=518$
488	$M-(CH_3)_3SiO^{\cdot}-CH_3O^{\cdot}=488$
487	$M-CH_3O^{\cdot}-(CH_3)_3SiOH=487$
471	$M-(CH_3)_3SiOH-CH_3OH-CH_3=471$
458	$M-CH_3O^{\cdot}-HCHO-(CH_3)_3SiOH=458$
457	$M-CH_3OH-HCHO-(CH_3)_3SiOH=457$[b]

[a] The ion at *m/z* 609 is the parent ion. This ion is formed by the silylation of three (OH) groups and methoximation of one (=O) group, M_r $(364)+(3\times72)+(29)=609$.

[b] Proposed mechanism for the formation of fragment ion at *m/z* 457 was described by Shackleton et al. [9].



(A)



(B)

Fig. 3. Chromatograms and mass spectrum of steroids in a urine sample obtained from healthy volunteers. (A) SIM chromatography of 18-OH-THA, β-cortol, and β-cortolone, the ions at m/z 457, 343 and 449 were used to monitor each of these steroids, respectively. (B) Mass spectrum of 18-OH-THA.

Shackleton et al. [10] (Fig. 3B). The relative retention time (RRT) is the ratio of the retention time of the steroid to be measured to the retention time of the internal standard i.e. 5α-THDOC in this case. The RRT for 18-OH-THA was 1.148.

3.2. Measurement of urinary 18-OH-THA

β-Cortol has a RRT of 1.153, close to that of 18-OH-THA (1.148). Standard quantities of β-cortol were derivatised as described above. This experiment

Table 2
Standard solution of β-cortol was derivatised by TMSI-MO and subjected to GC–MS. Total ion chromatography was used to monitor all fragments; only the ions with a relative intensity more the 10% and m/z more than 300 are shown here with the exception of the ions at 449 and 457

m/z	Relative abundance (%)	Standard deviation
343	99.7	0.74
433	58.7	11.64
523	56.0	15.33
535	47.1	12.28
445	42.7	6.04
398	29.5	2.23
458	27.0	3.10
355	23.3	1.85
536	23.3	4.12
434	23.0	2.53
548	21.2	2.67
446	17.3	1.14
507	11.6	1.69
546	11.0	1.41
537	10.7	1.12
449	0.45	0.048
457	0.23	0.048

was repeated ten times. The mass spectrum showed the presence of the ion at m/z 457 with a relative intensity of 0.231±0.048%. The ion occurs in the mass spectra of both reference standards and in the steroids extracted from urine samples.

3.2.1. Validation

To validate the indirect method, urinary β-cortolone concentration was measured in 19 urine samples from healthy volunteers and patients with different adrenal disorders. The ion at m/z 449 occurred in the mass spectrum of β-cortol with a relative intensity of 0.45±0.048% (Table 2), while it comprised 100% of the intensity of β-cortolone. β-Cortol and β-cortolone have similar but distinct retention times (RRTs of β-cortol and β-cortolone are 1.153 and 1.160, respectively). β-Cortolone excretion rate was measured against authentic β-cortolone standards and against β-cortol standards. The results for the two methods were highly correlated (adjusted R^2 0.998, $P<0.001$).

3.2.2. Precision and sensitivity

The precision of the method was estimated by repeated measurement of 18-OH-THA concentration in one sample of pooled urine. The sensitivity was defined as the lowest concentration producing a peak two-thirds greater than the baseline (Table 3).

3.2.3. Normal and clinical values

Twenty-four hour urine collections were obtained from thirty-two healthy adult volunteers (eighteen males and fourteen females aged 18–65 years). 18-OH-THA excretion rates are compared with available published data in (Table 4). Twenty-four hour collections were also obtained from six patients with

Table 3
Precision and sensitivity of the method

Steroid	Relative standard deviation (%)		Lower limits of detection (μg/l)
	Within batches $n=6$	Between batches $n=10$	
18-OH-THA	12.1	12.5	2.4

Table 4
Comparison of adult reference ranges between current study and available published information

	Shakerdi et al. Current study $n=32$	Ulick et al. [13] $n=9$	Shackleton [12] $n=18$
18-OH-THA (μg/24 h)	20–204	97–182	20–150

Table 5
Urinary excretion of 18-OH-THA in six patients with primary hyperaldosteronism

Patient	Urinary 18-OH-THA (μg/24 h)	Urinary aldosterone-18-glucoronide (μg/24 h)	Plasma renin (μU/ml)	Plasma aldosterone (ng/100 ml)	Pathology
1	1868	16	1	118	Adenoma
2	2463	27	0.4	1200	Hyperplasia
3	963	31	3	59	Adenoma
4	253	5	2	44	Adenoma
5	152	17	1	11	Hyperplasia
6	1266	25	4	45	Hyperplasia
Normal range	20–204	1 –25	5–50	3–25	

previously diagnosed primary hyperaldosteronism. Most patients had high plasma aldosterone concentrations and evidence of an adrenal tumour or hyperplasia. The results are listed in Table 5.

4. Discussion

Availability of appropriate reference steroid compounds is essential for steroid determination by GC–MS. If not readily available, synthesis can be laborious and expensive [11]. In the present study, we have developed a GC–MS method to measure 18-OH-THA excretion rate using the non-homologous reference standard, β-cortol. The principle is based on the observation that similar TMSI-MO-derivatised steroids often produce ions of the same m/z. Shackleton [12] used 3β,5α-18-hydroxy-THA as an I.S. for measuring 3α,5β-18-hydroxy-THA. As this steroid was unavailable to us, we used 5α-THDOC as an I.S. To validate the method, we measured urinary β-cortolone excretion rate using authentic standards and β-cortol. There was a close correlation between the two sets of results (adjusted R^2: 0.998, $P<$ 0.001).

For the assay of 18-OH-THA, within and between assay RSD values were less than 15%, at least comparable with other assays of urinary steroid metabolites of similar concentration. The lowest detectable amount was 120 pg/2 μl or 2.4 μg/l. Although few publications are available on urinary 18-OH-THA concentration, the normal values obtained were similar to the previously published data [13]. This method is vulnerable to interference from β-cortol excreted naturally in urine. The degree of interference depends on the abundance of the ion at m/z 457 in the β-cortol spectrum and on its excretion rate. Interference is less than 2% when the excretion rate of β-cortol is at the upper limit of normal range because the contribution of the ion in its mass spectrum is very low (0.23%). However, β-cortol is always quantitated as part of the urinary steroid metabolite screen. If, as might occur rarely, the concentrations are present that would cause significant interference, the 18-OH-THA can easily be corrected appropriately. Although using an ion occurring at very low intensity (i.e. 578) minimises interference from β-cortol in biological samples, very high concentrations of standard β-cortol are necessary. A better approach would be to use a synthetic compound which does not occur naturally.

18-OH-THA is the major urinary metabolite of 18-hydroxcorticosterone. Urinary excretion rate of this compound is a valuable index of the activity of the aldosterone biosynthesis pathway. For example, in pseudohypoaldosteronism, in which there is diminished renal tubule sensitivity to aldosterone, the urinary excretion rate of 18-OH-THA is ten times higher than normal [14,15]. Some salt-wasting disorders in infants are attributable to defects in either of the two mixed-function oxidation reactions, corticosterone methyloxidase types I and II, catalyzed by aldosterone synthase [16]. In corticosterone methyl oxidase type II deficiency, 18-OH-THA is significantly higher than normal and its ratio with aldosterone metabolites is diagnostic [17]. Primary

aldosteronism is not an uncommon cause of hypertension. Recent reports estimate a prevalence rate of almost 12% [18], although this estimate has been disputed [19]. The most common forms of primary aldosteronism are unilateral benign adrenal adenoma (Conn's syndrome) and bilateral hyperplasia. In a small number of patients with previously diagnosed primary aldosteronism, excretion rates of 18-OH-THA were high. Measurement of serum potassium concentration and plasma renin activity may be an inadequate screening test for primary aldosteronism because of high rates of false-positive and false-negative; this is not the case with urinary 18-OH-THA measurement [20]. Furthermore, plasma levels of 18-OH-B were found to be an effective discriminator of adenoma from hyperplasia [21]. Recent studies have shown subtle changes in the activity and genetics of aldosterone synthase, which relate to hypertension and cardiovascular risk [22,23]. Used in tandem with aldosterone assays, the measurement of 18-OH-THA excretion should provide a convenient index of activity in clinical and epidemiological studies [24]. The same principle could be applied to the measurement of other steroids for which no reference standards are available.

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