Identification of steroids in rat adrenal glands by liquid chromatography–thermospray mass spectrometry

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Liquid chromatography–thermospray mass spectrometry was applied to identify corticosterone, 11-deoxy-18-hydroxycorticosterone, 18-hydroxycorticosterone and aldosterone as the major corticosteroids in the intracellular and extracellular pools of rat adrenals.

INTRODUCTION

It is conventionally assumed that the secretion of steroid hormones by the rat adrenal cortex occurs by a process of simple diffusion, and that secretion rates consequently reflect rates of biosynthesis. Many studies from this laboratory have shown that, when adrenal tissue is incubated *in vitro*, steroids are retained within the tissue against a considerable concentration gradient (Sibley *et al.*, 1980). This suggests that the release of steroids from intracellular pools may be an important aspect of the secretory mechanism. As these studies develop, it will be particularly important to verify the identity of the steroids present in intra- and extra-cellular pools. We describe an application of l.c.–thermospray m.s. in this context.

EXPERIMENTAL

Aldosterone and corticosterone were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.), and deoxycorticosterone and progesterone from Koch–Light (Haverhill, Suffolk, U.K.). Other steroids were kindly donated by Professor D. Kirk (M.R.C. Steroids Reference Collection, Queen Mary College, London E.1, U.K.). H.p.l.c. solvents used were water (Milli-Q grade) and acetonitrile (Rathburn, Walkerburn, Borders, Scotland, U.K.); ammonium acetate was of AnalaR grade (BDH Chemicals, Poole, Dorset, U.K.).

H.p.l.c. was performed with a Waters (Harrow, Middx., U.K.) dual-pump gradient-elution high-performance liquid chromatograph. Chromatography was undertaken with a C₁₈ Nova-Pak reverse-phase column (15 cm × 0.46 cm internal diam.). The solvent composition was raised linearly from 21% to 70% (v/v) acetonitrile in 50 mM-ammonium acetate over a period of 20 min at a constant flow rate of 1.2 ml/min. The eluent was continuously monitored in series by a Finnigan (Sunnyvale, CA, U.S.A.) MAT 4500 quadrupole mass spectrometer equipped with a Finnigan thermospray interface. A mass range of *m/z* 150–450 was scanned every 2 s in the positive-ion mode. The ion-source block was maintained at a temperature of 200 °C, and the vaporizer was controlled at 130 °C. Data acquisition and reduction were performed by an Incos data system with Idos II software. Each of the ten standard steroids (1 µg

in 100 µl of 21% acetonitrile) was separately chromatographed to determine its retention time and positive-ion thermospray mass spectrum.

Fifteen female Wistar rats were killed by cervical dislocation, and their adrenals were removed and decapsulated between glass plates to yield capsule fractions (containing mainly glomerulosa cells) and inner zones containing mainly fasciculata/reticularis cells. As a routine the capsules were incubated in pairs in 5 ml of Krebs–Ringer bicarbonate buffer, pH 7.2 (containing 3.6 mM-K⁺), containing glucose (2 mg/ml) for 1 h at 37 °C in a shaking water bath under an atmosphere of O₂/CO₂ (19:1). After incubation, the medium was decanted off and the tissue was homogenized in 5 ml of 10 mM-potassium phosphate buffer, pH 7.4 containing KCl (20 mM), triethanolamine hydrochloride (15 mM), MgCl₂ (5 mM), CaCl₂ (2 mM) and sucrose (0.25 M). Incubation media and homogenates were extracted with ethyl acetate (2 × 2.5 ml). The solvent was removed under a stream of air, and the samples were analysed by g.l.c. as derivatives (Vinson *et al.*, 1978) or by radioimmunoassay (Vazir *et al.*, 1981). For the l.c.–thermospray-m.s. experiments an equivalent of tissue totalling approx. 250 mg for glomerulosa and 675 mg for inner zones was incubated separately in Krebs–Ringer bicarbonate buffer and homogenized (as above). After ethyl acetate extraction of the homogenates and media, samples were dissolved in 1 ml of 21% acetonitrile in aqueous ammonium acetate, and 100 µl portions were analysed by h.p.l.c.–m.s.

Steroids were converted into their *O*-methoxyamine derivatives by reaction with methoxyamine hydrochloride (Sigma Chemical Co.) in pyridine (10 mg/ml) for 1 h at 60 °C. The 1:1 isotopically labelled methoxyamine derivatives were prepared with a 1:1 mixture of methoxyamine hydrochloride and [²H₂]methoxyamine hydrochloride (Phase Separations, Queensferry, Clwyd, Wales, U.K.) in pyridine. Aldosterone was isomerized by incubation at 70 °C for 1 h with NaOH (67 mM) in 80% (v/v) methanol.

RESULTS AND DISCUSSION

G.l.c. has been used successfully for many years for the analysis and identification of steroids (Axelson & Sjövall, 1974; Setchell *et al.*, 1975; Kwan *et al.*, 1985).

Abbreviation used: l.c.–m.s., liquid chromatography–mass spectrometry.

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Table 1. H.p.l.c. retention data and thermospray mass spectra of ten steroids

Each steroid was chromatographed separately under identical conditions to determine relative retention times. The positive-ion thermospray mass spectrum was obtained for each steroid post-h.p.l.c. Ion intensities (relative to the peak at 100%) are given in parentheses. Ions of low intensity (< 10% base peak) were observed for most species corresponding to dehydrogenations and multiple water loss. Progesterone, a species unlikely to be present in rat adrenal extracts, was initially considered for use as an internal standard, but proved to be unsuitable.

Sample	Retention time (min)	Major ions (m/z)		
		$[M+H]^+$	$[M-H_2O+H]^+$	$[M+NH_4]^+$
3 α ,5 β -Tetrahydroaldosterone	7.5	365 (5)	347 (100)	382 (8)
18-Hydroxycorticosterone	7.6	363 (66)	345 (100)	380 (5)
Aldosterone	8.1	361 (100)	343 (35)	378 (18)
3 α ,5 α -Tetrahydroaldosterone	8.6	365 (6)	347 (100)	382 (9)
5 β -Dihydroaldosterone	9.5	363 (6)	345 (100)	—
5 α -Dihydroaldosterone	10.0	363 (12)	345 (100)	380 (9)
11-Deoxy-18-hydroxycorticosterone	10.7	347 (100)	329 (72)	—
Corticosterone	11.4	347 (100)	329 (12)	364 (8)
11-Deoxycorticosterone	15.5	331 (80)	313 (100)	—
Progesterone	20.2	315 (100)	297 (10)	—

Fig. 1. (a) Representative h.p.l.c.-thermospray-m.s. profile of 11-deoxy-18-hydroxycorticosterone and (b) thermospray mass spectrum post-h.p.l.c. of 1 μ g of 11-deoxy-18-hydroxycorticosterone showing $[M+H]^+$ at m/z 347 and $[M-H_2O+H]^+$ at m/z 329

In (a) the ion at m/z 329 was monitored. The 'spiky' appearance of the profile is a result of the ionization process.

When interfaced with a mass spectrometer, the technique is unrivalled for qualitative and quantitative analysis of these substances. For g.l.c. analysis, there is an absolute requirement for sample volatility. This is usually achieved by the formation of suitable derivatives. The formation of derivatives may, however, be accompanied

by unexpected side reactions, and is not always successful.

In contrast, there are no such requirements for volatility if h.p.l.c. is used as the inlet system for a mass spectrometer, and the recently developed l.c.-thermospray-m.s. interface (Vestal, 1984; Blakey & Vestal,

Fig. 2. H.p.l.c.–m.s. profiles of rat adrenal capsular homogenate

The h.p.l.c. eluent was monitored mass-spectrometrically. The total ion current (TIC, complicated by the high background from low-mass solvent ions) is shown together with selected ion channels for m/z 347, 361 and 363. Identification was based on h.p.l.c. retention times and thermospray mass spectra (Table 2); authentic standards were chromatographed immediately afterwards. I, 18-Hydroxycorticosterone; II, aldosterone; III, an unidentified steroid of the same mass as aldosterone; IV 11-deoxy-18-hydroxycorticosterone; V, corticosterone. Ion intensities are normalized to the m/z 347 channel. Only peaks IV and V were present in the homogenate from the adrenal inner zone (results not shown).

1984) promises to be a useful addition to the range of m.s. methods applicable in the study of steroid biochemistry. Thermospray-m.s. data have already been obtained on a variety of substances that are not normally amenable to g.l.c., including steroid conjugates (Watson *et al.*, 1985, 1986).

Thermospray m.s. is a soft form of ionization and generates molecular-ion species with little fragmentation directly post-h.p.l.c., and is comparable with other soft-ionization techniques such as fast-atom-bombardment m.s. With l.c.–thermospray-m.s., data are obtained on-line; there is no need for vacuum-drying and resuspension of the sample (as required with fast-atom-bombardment m.s.), and handling losses are minimized.

The h.p.l.c.–thermospray-m.s. behaviour of a number of steroids was examined. Each steroid chromatographed as a single peak on h.p.l.c. and generated a positive-ion thermospray mass spectrum. The data are summarized in Table 1, with a representative h.p.l.c.–m.s. profile and thermospray mass spectrum for 11-deoxy-18-hydroxycorticosterone shown in Fig. 1. For the majority of the steroids examined, the base peak in the thermospray mass spectrum corresponded to the protonated molecular-ion species $[M+H]^+$. However, for the four hydrogenated aldosterones and 18-hydroxycorticosterone, ready loss of water occurred to give $[M-H_2O+H]^+$ as

the major ion (Table 1). No other significant fragment ions were formed. Water loss probably arises through thermal degradation on vaporization, and is diagnostically useful in identifying these species. By changing the vaporizer temperature, the degree of water loss may be varied. In the present study the vaporizer temperature was set at 130 °C to optimize ionization for all the steroids studied. Standards and adrenal extracts were then analysed under identical conditions. Ammonium adduct ions $[M+NH_4]^+$ were sometimes observed (albeit with low intensities), which facilitated molecular-ion identification. As the ion source became contaminated, ions were observed 2 atomic mass units below the molecular-ion species, particularly in the mass spectra of aldosterone and its hydrogenated derivatives. These probably arise by dehydrogenation within the ion source and correspond to $[M+H-H_2]^+$, $[M+NH_4-H_2]^+$ and $[M-H_2O+H-H_2]^+$.

In each case a full mass spectrum was readily generated on 1 μ g, with good-quality data obtained on as little as 30 ng in favourable cases (e.g. with the hydrogenated aldosterones). This compares very favourably with other soft-ionization techniques such as fast-atom-bombardment m.s., where the requirement for h.p.l.c. purification before analysis (especially for samples present in complex biological matrices) limits the overall sensitivity to the microgram level. Lower limits of

Table 2. H.p.l.c. retention data and thermospray mass spectra of the major steroid components of rat adrenal capsular homogenate

Retention times and positive-ion thermospray mass spectra (ion intensities relative to the base peak in parentheses) of the major components of rat adrenal capsular homogenate are given. Authentic standards were chromatographed immediately after the adrenal extract to confirm structural assignments.

H.p.l.c. peak	Retention time (min)	Major ions (m/z)			Identification
		$[M+H]^+$	$[M-H_2O+H]^+$	$[M+NH_4]^+$	
I	7.6	363 (80)	345 (100)	380 (8)	18-Hydroxycorticosterone
II	8.3	361 (100)	343 (40)	378 (75)	Aldosterone
III	8.7	361 (100)	343 (32)	378 (8)	Not identified
IV	10.2	347 (100)	329 (32)	—	11-Deoxy-18-hydroxycorticosterone
V	11.1	347 (100)	—	364 (8)	Corticosterone

Fig. 3. H.p.l.c.-m.s. profiles of rat adrenal capsular incubation medium

The total ion current (TIC) is shown together with selected ion channels for m/z 347, 361 and 363. The same five species observed for the homogenate are also present. Ion intensities are normalized to the m/z 347 channel. The absolute response of the m/z 347 channel for the incubation medium is 3.6-fold higher than that obtained for the homogenate. Assuming unit density for the tissue, the concentration of steroids in the medium is, however, only 20% of that in the homogenate.

detection are available with capillary g.l.c.-m.s. (with full scanning spectra obtained on 50–100 ng), but, however, the formation of derivatives is a prerequisite for analysis.

The h.p.l.c.-thermospray-m.s. profile for rat adrenal capsular incubation medium is shown in Fig. 2. Little information could be obtained from the total-ion-current profile, which is complicated by high-abundance low-mass solvent ions. However, selected ion profiles for m/z 347, 361 and 363 clearly show the presence of five species. The retention times and thermospray mass spectra of these species were consistent with the presence of 18-hydroxycorticosterone, aldosterone, 11-deoxy-18-

hydroxycorticosterone, corticosterone and an unidentified steroid of the same mass as aldosterone (Table 2). There were no peaks in other ion channels corresponding to authentic steroid standards. The l.c.-m.s. profile for the capsular homogenate was similar to that obtained for the incubation medium (Fig. 3). The absolute m.s. responses of the ion channels gave an indication of gross changes in steroid content between the homogenate and medium. Assuming unit density for the tissue (see below), there is a 5-fold concentration increase for corticosterone and 11-deoxy-18-hydroxycorticosterone in the tissue compared with the medium. Although these data can

Fig. 4. L.c.–thermospray-m.s. profile of (a) aldosterone and (b) base-treated aldosterone

The ion at m/z 361 was monitored. Treatment with base (reported to produce the hemiacetal hemiketal isomer) generates two species with the same mass as aldosterone, but with shorter retention times (6.5 min and 8 min). The species eluted at 10.1 min is a dehydrated derivative of aldosterone. Steroid III (Table 2 and Fig. 2) is not a base-derived isomer of aldosterone.

only be considered semi-quantitative, the ability of the tissue to retain 11-deoxy-18-hydroxycorticosterone is strongly apparent. The role of sequestered 11-deoxy-18-hydroxycorticosterone is not fully understood, but it is believed to be a specific substrate for aldosterone biosynthesis (Vinson & Whitehouse, 1973).

Corticosterone and 11-deoxy-18-hydroxycorticosterone were also observed in the medium and homogenate from the inner zone of the adrenals; neither aldosterone, nor the unidentified steroid nor 18-hydroxycorticosterone was present.

In a separate experiment, steroids in incubation medium and homogenate of the capsular fraction were analysed by g.l.c.–electron-capture detection. Again, assuming unit density for the tissue, the steroid concentrations were determined. In broad agreement with the thermospray data, a 4-fold increase in 11-deoxy-18-hydroxycorticosterone concentration was observed in the homogenate (245 ± 28 ng of steroid per adrenal pair) compared with that in the medium (61 ± 11 ng), although the increase was not observed for corticosterone (53 ± 17 ng per adrenal pair compared with 114 ± 10 ng). Differences in distribution of steroid between tissue and medium in the two experiments are attributable to the different incubation conditions. High tissue/incubation medium ratios for corticosterone occur when more tissue is used per 5 ml incubation volume (Sibley *et al.*, 1980).

Of particular interest is the steroid eluted after aldosterone (8.7 min) but with a similar thermospray mass spectrum (Table 2). Derivatives of aldosterone have been observed in adrenal tissue (Cozza *et al.*, 1985), and in solution aldosterone is known to form the 11,18-hemiacetal and the 11,18-hemiacetal 18,21-hemiketal (De Vries & De Jong, 1980; Mattox *et al.*, 1981). When aldosterone is treated with methanolic NaOH (conditions known to cause isomerization), two species with thermospray mass spectra similar to that of aldosterone were generated ($[M+H]^+$, m/z 361; $[M-H_2O+H]^+$, m/z 343); however, both were eluted earlier than aldosterone (Fig. 4), indicating that the unknown was not a simple isomeric form of aldosterone. A less-polar dehydrated aldosterone was also generated by treatment

with base. Interestingly, this unidentified species was not observed as a peak in the g.l.c. assay. Either it does not form a derivative suitable for g.l.c. analysis, or it co-chromatographs with another steroid, leading to an overestimation of steroid concentrations.

Clearly, an assay based on thermospray ionization would reduce such problems (as both retention time and mass are used to identify the steroid); however, suitable internal standards would be required. In order to obtain quantitative data in the present study, progesterone was used as an internal standard, as it was unlikely to be present in adrenal extracts. However, it was found to be unsuitable because of differential recovery and variable h.p.l.c. elution times. Thus no accurate account was taken of differential losses on extraction. However, gross (semi-quantitative) changes in steroid concentrations were readily observed. There are, as yet, few reports of the successful application of l.c.–thermospray m.s. as an assay technique. In common with the most precise m.s. assays, the synthesis of 3H -labelled internal standards will be of prime importance; this limits the current use of l.c.–thermospray m.s. to the qualitative and structural aspects of steroid analysis.

Although l.c.–thermospray m.s. yields molecular-mass information on sub-microgram amounts of steroids, little structural information, apart from the presence of labile hydroxy groups, may be obtained. This may be partly overcome by the formation of derivatives. For example, treatment of aldosterone with methoxyamine hydrochloride results in methoximation of available carbonyl functions. The elution time of the modified steroid is markedly altered (to 11.0 and 12.3 min for the mono- and di-methoxyamine respectively); the protonated molecular ion in the thermospray mass spectrum is increased by 29 atomic mass units for each carbonyl function modified (in this case to m/z 390 and m/z 419 for one and two carbonyl functions respectively). Isotopically labelled reagents can facilitate identification of sample ions post-h.p.l.c. If aldosterone is methoximated with a 1:1 mixture of methoxyamine and $[^3H_3]$ methoxyamine hydrochloride in pyridine, the monomethoxyamine derivative generates a 1:1 doublet protonated molecular ion ($[M+H]^+$) at m/z 390/393; the dimethoxyamine

Fig. 5. Selected l.c.-m.s. ion currents (a) and thermospray mass spectra (b) for aldosterone (m/z 361; i) and the 1:1 isotopically labelled mono- (m/z 390/393; ii) and di- (m/z 419/422/425; iii) methoxyamine derivatives

Two mono- and one di-methoxyamine derivatives may be generated.

derivative generates a 1:2:1 triplet at m/z 419/422/425 (Fig. 5). Isotopic labelling is especially valuable when dealing with complex matrices, when multiplets stand clearly above an otherwise complex background (see Morris *et al.*, 1980).

L.c.-thermospray m.s. has been applied to establish the identity of the major steroids in the intra- and extra-cellular pools in the rat adrenal. Further, a species with the same mass as aldosterone has been observed in both the incubation medium and tissue. This illustrates the advantage of this technique over methods such as g.l.c., where unknown species, if observed at all, will appear as a peak on the g.l.c. trace with a non-standard retention time. The current drawback of the technique is the requirement for suitable (^2H -labelled) internal standards for quantitative analysis; this will require initiation of a synthetic programme.

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