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Increasing thermospray response for cortisol by derivatization

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ABSTRACT

The 21-hydroxyl group of cortisol was selectively acetylated under mild conditions without affecting the more sterically hindered 11β- and 17α-hydroxyl groups. The reaction was performed with a mixture of acetic anhydride and triethylamine in acetonitrile and was complete in less than 15 min at room temperature. The thermospray mass spectrum of cortisol 21-acetate showed minimal fragmentation with the $[M + H]^+$ ion as the base peak. High sensitivity was achieved for acetylated cortisol during selected ion monitoring, the signal-to-noise ratio being increased by a factor of about 4, compared to underivatized cortisol. The limit of detection of cortisol 21-acetate was estimated at 0.24 pmol injected, making thermospray liquid chromatography–mass spectrometry competitive with gas chromatography–mass spectrometry for the determination of cortisol in biological fluids.

INTRODUCTION

Measurement of plasma, serum or urine cortisol concentration is useful in assessing adrenocortical function. Stable isotope dilution combined with gas chromatography (GC)–mass spectrometry (MS) offers the required sensitivity and selectivity and has been widely used for cortisol analysis [1–7]. These methods are based on electron ionization [1,2,4–7] or positive-ion chemical ionization [3] of the dimethoxime-tris(trimethylsilyl) derivative of cortisol. Recently, methods based on thermospray (TSP) liquid chromatography (LC)–MS have been used for serum cortisol determination [8,9], thereby obviating the lengthy, two-stage derivatization procedure required by GC–MS. Detection limits of 5 pmol [9] and 14 pmol (5 ng) [10] have been reported for cortisol by LC–TSP-MS. Gaskell *et al.* [8] found LC–MS inferior to GC–MS in terms of sensitivity and precision and concluded that the magnitude and stability of the LC–MS response for cortisol had to be improved to make this method competitive.

Detection-oriented derivatization, to increase analyte response, has been successfully applied to several areas of LC [11]. Thermospray response varies widely between different compounds [10,12,13], but although lack of response can be a serious drawback of the technique for certain applications derivatization has not been frequently used to overcome this problem. Voyksner and Bush [14] used post-column

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addition of trimethylanilinium hydroxide to achieve thermally induced methylation of carboxylic acids in the thermospray vaporizer. Voyksner *et al.* [15] also esterified carboxylic acids with diethylaminoethyl chloride, thereby increasing proton affinity by introducing a basic amino group in the derivative. Abián *et al.* [16] used diazomethane to form the carboxylic acid methyl esters of prostaglandins. They also tried methoximation of prostaglandin keto groups but obtained no improved sensitivity with this derivative [16]. We have found that acetylation of the 21-hydroxyl group of cortisol, by a simple and rapid derivatization procedure (see Fig. 1), increases thermospray response and decreases the detection limit of cortisol in biological samples.

EXPERIMENTAL

Chemicals

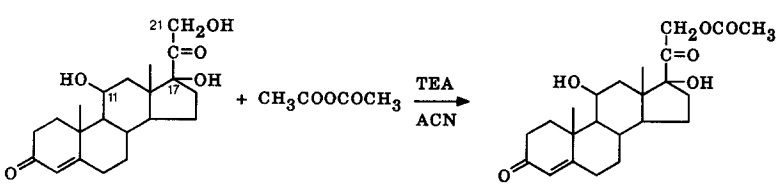
The structures of cortisol and cortisol 21-acetate are shown in Fig. 1. Cortisol (98%) was obtained from Sigma (St. Louis, MO, USA) and trideuterated cortisol ([9,12,12-$^2H_3$]cortisol) from KOR Isotopes (Cambridge, MA, USA). Acetic acid (Gold Label grade, 99+%), triethylamine (Gold Label grade, 99+%) and acetic anhydride (GC grade, 98–99%) were obtained from Aldrich-Chemie (Steinheim, Germany). Ammonium acetate (MicroSelect, >99%) was obtained from Fluka Chemie (Buchs, Switzerland) and acetonitrile and methanol (HPLC quality) from Fison (FSA Laboratory Supplies, Loughborough, UK). All water was purified in a Milli-Q system (Millipore, Molsheim, France).

Mobile phase preparation

The pH of a 1.0 *M* ammonium acetate solution was adjusted to 5.2 with 1.0 *M* acetic acid. To prepare 1 l of mobile phase, 100 ml of 1.0 *M* ammoium acetate buffer, pH 5.2, were mixed with 400 ml of water and 500 ml of methanol, giving a 0.1 *M* ammonium acetate buffer containing 50% methanol. [The pH of 0.1 *M* ammonium acetate buffer, prepared by diluting a 1.0 *M* solution (pH 5.2) with water, is approximately 5.0.]

Derivatization procedure

To mimic the eluate obtained after solid-phase extraction (the extraction will be described elsewhere), alcoholic standard solutions of cortisol were evaporated to dryness in polypropylene tubes. The residue was treated with 250 µl of a solution containing 12.5% acetic anhydride and 12.5% triethylamine in acetonitrile. After vortex-



Cortisol Cortisol 21-acetate

Fig. 1. Reaction scheme for the derivatization of cortisol with acetic anhydride in the presence of triethylamine (TEA) and acetonitrile (ACN).

ing, the tubes were allowed to stand uncapped at room temperature for 15 min, and then evaporated to dryness. The residue was dissolved in 100 μl of 30% methanol in water and 40 μl of the solution were injected into the LC–MS system.

Liquid chromatography–mass spectrometry

A Finnigan TSQ70 mass spectrometer equipped with a Finnigan thermospray interface (TSP1) (Finnigan MAT, San José, CA, USA) was used in an automated LC–MS system [17]. Mass calibration was performed with a solution of polyethyleneglycol [17]. The instrument was tuned for maximum sensitivity by direct injection of a solution of cortisol 21-acetate. Cortisol and acetylated cortisol were chromatographed on a 33 × 4.6 mm I.D. Supelcosil LC-8-DB (3 μm) cartridge. The mobile phase was 0.1 *M* ammonium acetate buffer, pH 5, containing 50% methanol, pumped at a flow-rate of 1.9 ml/min. To investigate the thermally induced fragmentation of cortisol and cortisol 21-acetate the vaporizer temperature was varied between 80 and 90°C and the jet temperature between 160 and 220°C. The repeller potential was kept at 100 V. Mass spectra were obtained by scanning from *m/z* 200 to *m/z* 500 in 1 s. For quantitative analysis the vaporizer was kept at 90°C and the jet block at 172°C. The $[M + H]^+$ ions of cortisol (*m/z* 363), cortisol 21-acetate (*m/z* 405), and $[^2H_3]$cortisol 21-acetate (*m/z* 408) were recorded by selected ion monitoring by scanning over a 0.6-a.m.u. window in 400 ms for each mass.

RESULTS AND DISCUSSION

Cortisol, as well as various synthetic corticosteroids, are known to undergo facile eliminiation of the C-20–C-21 side-chain ($[M + H - 60]^+$) under thermospray ionization conditions [8–10, 18–21]. During development work on new corticosteroid drugs we have found that the C-20–C-21 side-chain can be stabilized during thermospray ionization by acetylation of the 21-hydroxyl group. This group can be selectively acetylated under mild conditions without affecting the more sterically hindered 11β- and 17α-hydroxyl groups in cortisol (*cf.* ref. 22). The reaction was complete in less than 15 min at room temperature, and the formation of di- or triacetyl derivatives was not observed even after extended reaction time. Acetylation of the corticosteroid 21-hydroxyl group has been used previously as a derivatization reaction in GC [23] and GC–MS [24]. Fig. 2 compares thermospray mass spectra of cortisol and cortisol 21-acetate, obtained at two different interface temperature combinations. The spectra were obtained from mixtures of unlabelled and deuterium-labelled compounds. Underivatized cortisol showed extensive fragmentation, which could not be significantly improved by lowering the vaporizer and ion source temperatures. Cortisol 21-acetate, on the other hand, produced the $[M + H]^+$ ion as the base peak with minimal fragmentation even at high temperature of the thermospray interface. The formation of the $[M + H - H_2O]^+$ ion and $[M + H - 60]^+$ ion in the thermospray mass spectrum of cortisol has been suggested to be promoted by the 17α-hydroxyl group [8]. Interestingly, the corresponding fragments in the spectrum of cortisol 21-acetate were small (*m/z* 387, 5%; *m/z* 345, 20%) in spite of the presence of a free 17α-hydroxyl group.

Fig. 3 compares selected ion current profiles of three consecutive injections of 1.0 pmol of cortisol and acetylated cortisol. The results show that derivatization of

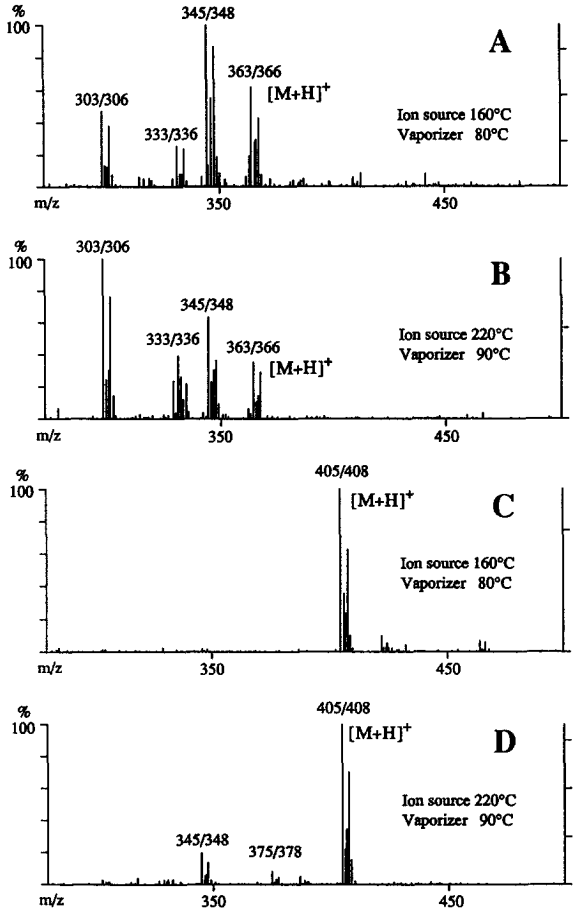


Fig. 2. Thermospray mass spectra of cortisol (A) and (B) and cortisol 21-acetate (C) and (D) at different temperatures of the thermospray interface. The spectra were obtained from mixtures of unlabelled and deuterium-labelled compounds.

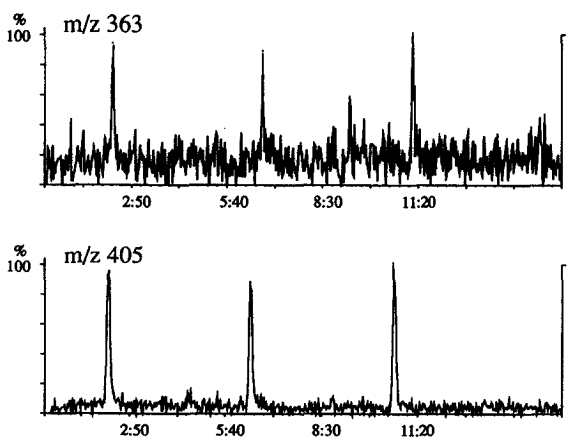


Fig. 3. Selected ion current profiles of three consecutive injections of 1.0 pmol of cortisol (m/z 363) and cortisol 21-acetate (m/z 405). Time in min.

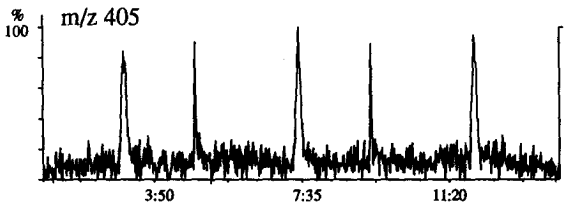


Fig. 4. Selected ion current profile of three injections of 0.24 pmol of cortisol 21-acetate. Time in min.

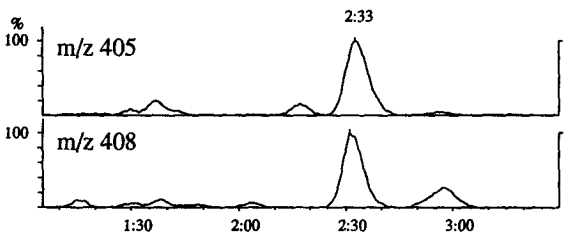


Fig. 5. Selected ion current profiles of cortisol 21-acetate (*m/z* 405) and [$^2H_3$]cortisol 21-acetate (*m/z* 408) (internal standard) obtained from a urine extract. The urine cortisol concentration was estimated at 24 nmol/l. Time in min.

cortisol increased the signal-to-noise ratio by a factor of about 4. Fig. 4 shows a selected ion current profile of three 0.24-pmol injections of cortisol 21-acetate, corresponding to the limit of detection. This limit of detection is twenty times lower than that reported by Esteban *et al.* [9], making LC–TSP-MS a viable alternative to GC–MS for the determination of cortisol in biological samples. Linear calibration curves for cortisol, analysed as the 21-acetate and by using [$^2H_3$]cortisol as internal standard, were obtained in the range 2–80 pmol injected. Fig. 5 shows a chromatogram obtained after solid-phase extraction of 1 ml of urine, with a cortisol concentration estimated at 24 nmol/l. A full report of the method will be published elsewhere.

CONCLUSIONS

Elimination of the need for derivatization is, as often stated, a great advantage of LC–TSP-MS. The present example, however, shows that derivatization can be worth considering as a means of increasing sensitivity. Acetylation of cortisol is a simple and rapid reaction making the LC–MS method less labour-intensive and less time-consuming than GC–MS.

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