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Short communication

Measurement of urinary free cortisol by stable isotope dilution mass spectrometry using a new cortisol derivative

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Abstract

The reaction of the bismethylenedioxy derivative of cortisol (cortisol-BMD) with heptafluorobutyric anhydride to give the corresponding 3,5-dienol heptafluorobutyrate (cortisol-BMD-HFB) has been shown to proceed with dehydration. Acid-promoted dehydration of either cortisol-BMD or cortisol-BMD-HFB, or concurrent dehydration of both, is the proposed reaction mechanism leading to a trienol heptafluorobutyrate, whose chromatographic properties and mass spectral data are consistent with the additional double bond in the C_9–C_{11} position. Forming the 3,5-dienol heptafluorobutyrate of cortisol-BMD in benzene rather than acetone gave a compound whose chromatographic properties and mass spectral data were different to that of the 3,5,9(11)-trienol heptafluorobutyrate but consistent with that of a cortisol-BMD-HFB. The mass fragmentometry of this new cortisol derivative was found to be more suited to the technique of isotope dilution mass spectrometry than the 3,5,9(11)-trienol heptafluorobutyrate, and thus was applied to our intended goal of measuring urinary free cortisol by gas chromatography–mass spectrometry. An efficient and convenient solid-phase extraction technique is employed in our assay to isolate cortisol from 5 ml of urine.

1. Introduction

The assay of cortisol in biological fluids by gas chromatography (GC) has traditionally involved conversion of the corticosteroid to its corresponding methyloxime-trimethylsilyl derivative [1–5]. While this derivative is a convenient one to prepare, it is however impractical for processing large numbers of sample because the reagents used to prepare the derivative are not sufficiently volatile to be conveniently removed under a stream of inert gas. As a consequence, column chromatography [6] is required to remove excess reagents. This purification step is necessary because accumulation of derivatizing reagents at the sample inlet of the gas chromatograph can lead to a loss of analyte through its chemical interaction with derivatizing reagents [7] or their decomposition products.

Recently, Shibasaki et al. [8] reported novel derivatives of cortisol, cortisone, prednisolone and prednisone that are suitable for GC–mass spectrometry (MS). The reagents employed by these workers to prepare the so-called bismethylenedioxyheptafluorobutyrate (BMD-HFB) derivatives of the four corticosteroids are sufficiently volatile that the excess reagents can be removed under a stream of nitrogen. We

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report here the application of a new BMD-HFB derivative of cortisol to the measurement of urinary free cortisol by stable isotope dilution MS, and provide evidence that the cortisol-BMD-HFB derivative obtained under the conditions described by Shibasaki et al. is a trienol heptafluorobutyrate rather than the dienol heptafluorobutyrate they suggest.

2. Experimental

2.1. Chemicals and reagents

[9,11,12,12-$^{2}H_4$]Cortisol (cortisol-d_4) was purchased from Cambridge Isotope Labs. (Woburn, MA, USA). The isotopic purity of this material was determined by a selected ion monitoring assay to be such that it contains 78% cortisol-d_4, 20% cortisol-d_3 and 2% cortisol-d_2. Cortisol and 4,9(11)-pregnadiene-17α,21-diol-3,20-dione were purchased from Steraloids (Wilton, NH, USA). Paraformaldehyde (95%) and heptafluorobutyric anhydride were purchased from Aldrich (Milwaukee, WI, USA) and Sigma (St. Louis, MO, USA), respectively. Benzene was dried and distilled from calcium hydride. All other solvents and reagents were of analytical-reagent grade, and were used without further purification.

2.2. Stock solutions

Stock solutions of cortisol (174.41 and 8.70 mg/ml) and cortisol-d_4 (3.55 μg/ml) were prepared in ethanol–water (1:1, v/v). All solutions were kept at 4°C until required.

2.3. Gas chromatography–mass spectrometry

GC was performed on a Hewlett-Packard (HP) Ultra 2 (12 m $\times$ 0.2 mm; 0.33 μm film thickness) fused-silica capillary column housed in an HP 5890 Series II gas chromatograph fitted with electronic pressure control. Helium was used as the carrier gas at a constant flow of 0.65 ml/min, and an initial column head pressure of 2 kg/cm^2. The initial oven temperature was set at 100°C and after 1 min was raised to 230°C at 30°C/min and then to 275°C at 3°C/min. The final oven temperature was maintained for 5 min. Sample injections were performed in the splitless injection mode using an HP 7673 auto-injector. The injector temperature was set at 275°C. The purge activation time was 1.5 min.

The capillary column was directly interfaced to an HP 5971 mass-selective detector, which was operated in the electron impact (EI) mode. The energy of the electron beam was 70 eV. The ion source temperature was 180°C.

2.4. Sample preparation

Urine (5 ml) containing 0.97 nmol of cortisol-d_4 was applied to a Sep-Pak Plus tC_{18} cartridge (Waters, Milford, MA, USA), which had previously been conditioned by washing with methanol (5 ml) and water (10 ml). The cartridge was then successively washed with water (5 ml), 30% aqueous methanol (3 ml) and finally heptane (5 ml). The organics were then eluted from the cartridge with methanol (3 ml) and the eluate was evaporated to dryness under a stream of nitrogen. The resulting residue was then treated in the manner described immediately below.

2.5. Derivatization of urine extract

The dried urine extract was covered with dichloromethane (250 μl) and then treated with acidified formalin (500 μl), which was freshly prepared by stirring a mixture of paraformaldehyde (1 g), water (3 ml) and concentrated hydrochloric acid (3 ml) at room temperature until the suspension became homogeneous. The resulting mixture was stirred at room temperature for 2 h, after which time the aqueous layer was separated. The organic layer was diluted with dichloromethane (500 μl) and then washed with saturated aqueous sodium bicarbonate (500 μl). The organic layer was separated and evaporated to dryness under a stream of nitrogen. The residue was covered with dry benzene (500 μl) and treated with heptafluorobutyric anhydride (25 μl) at 50°C. After 30 min, the solvent and excess reagent were removed under a stream of nitrogen, and the residue was extracted with

cyclohexane (100 μl). Aliquots of the extract were then analysed by GC–MS.

2.6. Calibration curve

A calibration curve was constructed by plotting the peak area ratios of the analyte target ion, $m/z = 600$, to that of the internal standard target ion, $m/z = 604$, against the corresponding mol ratios of the analyte to a fixed amount (355 ng) of internal standard in the range of 1:50 to 50:1. The plot was linear over this range ($y = 0.979x + 0.123$, $r = 1.000$).

3. Results and discussion

The procedure described by Shibasaki et al. [8] for the preparation of the BMD-HFB derivative of cortisol involves reacting cortisol with acidified formalin to give the spiroketal **2** (Fig. 1). This so-called cortisol-bismethylenedioxy [9] (cortisol-BMD) derivative is then subsequently treated, in a separate step, with heptafluorobutyric anhydride [10] in acetone at room temperature for 1 h to form a 3,5-dienol heptafluorobutyrate, the structure of which was assigned by these authors as that of **3** shown in Fig. 1 on the basis on its mass spectrum.

In our hands the derivatization of cortisol by this protocol afforded a product which was shown by GC–MS to contain two cortisol-BMD-HFB derivatives in a ratio of ca. 40:1. The EI mass spectrum (Fig. 2) of the major component was consistent with that reported by Shibasaki et al. [8] for their cortisol-BMD-HFB derivative, whereas the EI mass spectrum of the minor component shows an intense ion at an m/z value corresponding to that of the molecular mass of the cortisol-BMD-HFB derivative, i.e. 600, reported by Shibasaki et al. [8]. In light of this, and the report by Shibasaki et al. that an intense $[M-H_2O]^{+\cdot}$ ion, but no molecular ion was

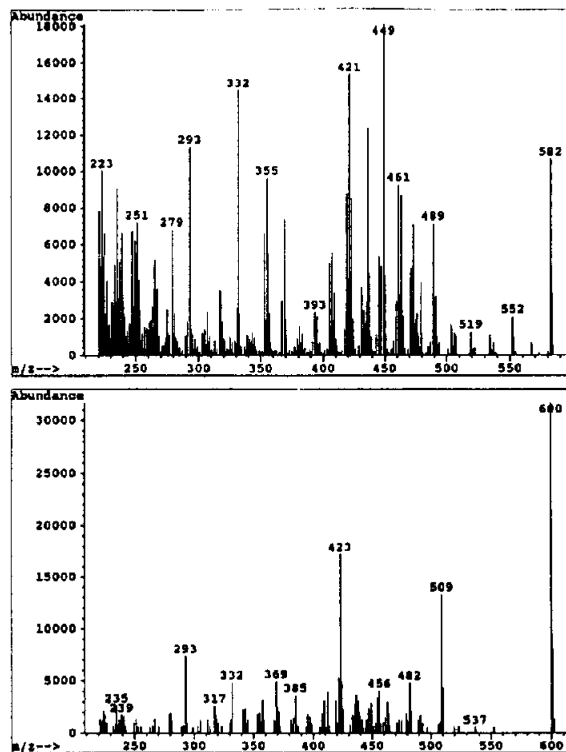

Fig. 2. Electron impact mass spectra (in the region m/z 220–650) of the two cortisol bismethylenedioxyheptafluorobutyrates obtained from the reaction of **2** with heptafluorobutyric anhydride under the conditions described by Shibasaki et al. The spectum shown at the top is for the major component and that at the bottom is the minor component.

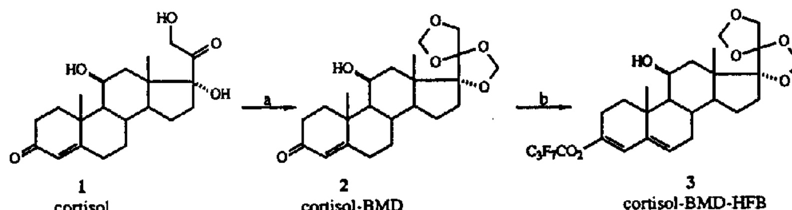

Fig. 1. Summary of the reaction sequence reported by Shibasaki et al. [8] for the preparation of the bismethylenedioxyheptafluorobutyrate (BMD-HFB) derivative of cortisol and their assigned structure to this compound. a = CH_2O, concentrated aqueous HCl; b = $(C_3F_7CO)_2$.

observed in the EI mass spectrum of their cortisol-BMD-HFB derivative, it occurred to us that the mass spectrum reported [8] by these authors for their cortisol-BMD-HFB derivative is not for the compound with the structure they show for this derivative, i.e. **3**, but rather for a dehydration adduct thereof. We reasoned that under their reaction conditions the cortisol-BMD derivative **2** and the 3,5-dienol heptafluorobutyrate **3** can either individually or concurrently (Fig. 3) eliminate water via their corresponding conjugate acids to give what appears to be one only of the trienol heptafluorobutyrates **8** or **9**. Certainly there is precedence to support this postulation: the treatment of aldosterone with heptafluorobutyric anhydride in acetone at 80°C for 30 min was reported [11] to give diheptafluorobutyryl esters of "dehydrated aldosterone, with the additional double bond in position 9,11 or 11,12". Mindful of the fact that acetone – the solvent used by Shibasaki et al. – is hydroscopic (and can also self condense in the presence of an acid to give aldol products and water), we reasoned that heptafluorobutyric anhydride undergoes significant hydrolysis in this solvent to give heptafluorobutyric acid, the presence of which catalyses the competing dehydration reaction(s). It seemed possible that if the extent of hydrolysis of heptafluorobutyric anhydride could be reduced by forming the 3,5-dienol heptafluorobutyrate of the cortisol-BMD derivative **2** in a dry inert solvent, then the competing reaction(s) might be suppressed. This proved to be the case in practice. Thus when **2** (Fig. 1) was treated with a 5% (v/v) solution of heptafluorobutyric anhydride in benzene at 50°C for 30 min we obtained a single cortisol-BMD-HFB derivative, the structure of which was assigned as that of **3** shown in Fig. 1 on the basis that the EI mass spectrum of this compound could be rationalised from **3** (see Fig. 4). When this cortisol-BMD-HFB derivative was treated with heptafluoro-

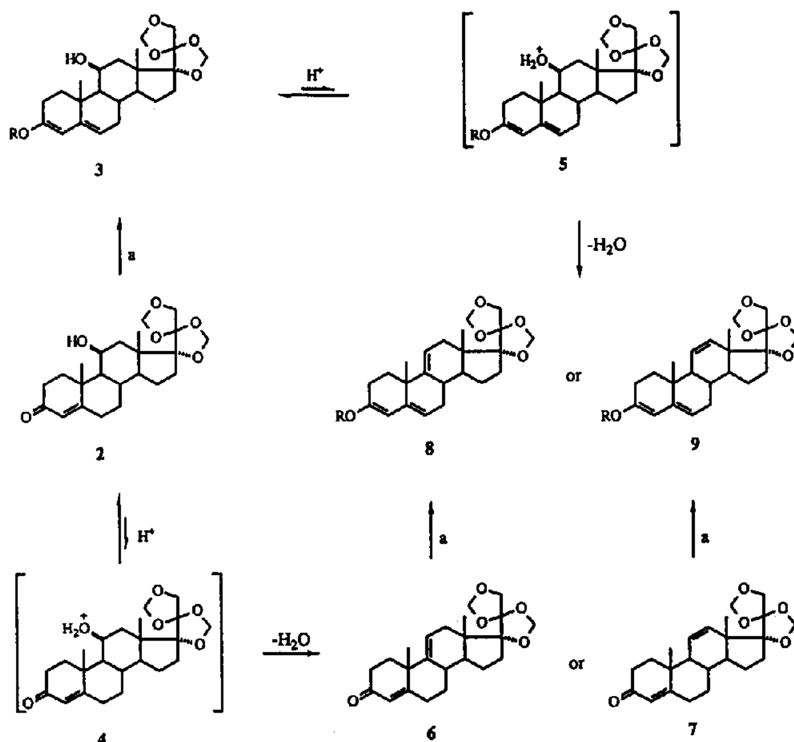

Fig. 3. Proposed reaction pathways leading to the formation of the cortisol-BMD-HFB derivative obtained under the reaction conditions described by Shibasaki et al. a = $(C_3F_7CO)_2O$; R = C_3F_7CO.

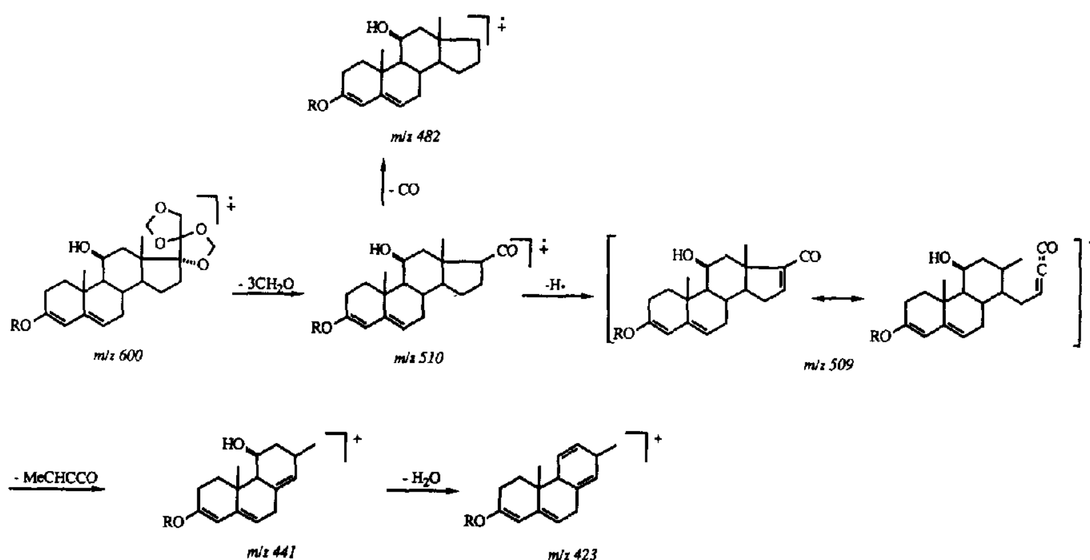

Fig. 4. Proposed EI fragmentation pathway for the cortisol-BMD-HFB derivative 3. $R = C_3F_7CO$.

butyric anhydride in acetone at room temperature for 1 h it underwent smooth conversion to a new cortisol-BMD-HFB derivative. The relative retention time index (MU) and the EI mass spectrum of this new cortisol-BMD-HFB were found to be identical to both that of the 3,5,9(11)-trienol heptafluorobutyrate 8, which was prepared from an authentic sample of 4,9(11)-pregnadiene-17α,21-diol-3,20-dione, and the cortisol-BMD-HFB derivative obtained by the method of Shibasaki et al. [8]

Our primary goal that led us to Shibasaki et al.'s report [8] was to develop a reference assay of urinary free cortisol based on the technique of isotope dilution selected ion monitoring MS [12,13]. The inherent accuracy and, perhaps most importantly, the ability to confirm both compound identity and contributions from interfering substances from the ratio(s) of the selected ions monitored are, without doubt, the strengths of this technique. Ideally, for selected ion monitoring the mass fragmentometry of a compound should be such that the majority of the ion current is carried by a single ion of high m/z value and that there are a few relatively intense ions which can be used as qualifiers. It was on this basis that we selected the cortisol-BMD-

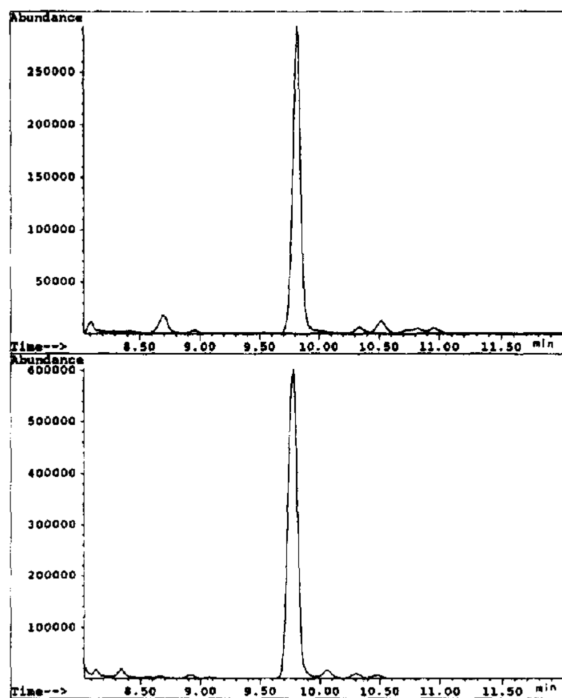

Fig. 5. Typical selected ion chromatograms [m/z 600 (top): cortisol-BMD-HFB derivative, m/z 604 (bottom): cortisol-d_4-BMD-HFB derivative] obtained for the assay of urinary free cortisol using the present GC–MS method.

Table 1
Accuracy of GC–MS assay of urinary free cortisol ($n = 5$)

Sample	Expected concentration (ng/ml)	Measured concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Recovery (%)
Unspiked urine	–	28.5 ± 0.48	1.7	–
Urine spiked with 17.4 ng cortisol	45.9	46.3 ± 0.10	2.2	102.2
Urine spiked with 34.8 ng cortisol	63.3	61.9 ± 0.17	2.7	96.5

HFB 3 over that obtained under the conditions described by Shibasaki et al. [8] to establish a GC–MS assay for the measurement of urinary free cortisol. We used cortisol-d_4 as the internal standard and monitored the ion pairs 509, 600 and 513, 604. The sensitivity of the present method was determined to be 200 pg of cortisol when a S/N ratio of 2.0 was set as the criterion. Quantitation of urinary free cortisol was performed by comparing the peak area of the analyte target ion (600) to that of the corresponding target ion (604) of the internal standard. The peak area ratios of the target ions to that of their corresponding qualifier ions were used to assess possible contributions from interfering substances. Initial work indicated there were no such contributions. Consequently, to increase sensitivity and reduce imprecision of area measurement, only the target ions of the analyte and its internal standard were monitored. In this situation, we used the shape of the chromatographic peaks as an indicator of interference. It can be seen from a typical chromatogram shown in Fig. 5 obtained using the present method that the peaks are essential Gaussian.

It would be well to emphasize that washing of the Sep-Pak cartridge with 30% aqueous methanol prior to elution of the organics removed a significant amount of polar organic material that would otherwise be sources of interference. It would also be well to mention that the heptane wash incorporated in the sample preparation greatly reduced the time required to evaporate the methanol fraction to dryness by removing interstitial water from the cartridge. The overall recovery of cortisol from urine was determined to be at least 95% using radiolabeled cortisol.

The accuracy and inter-assay precision of the present method were determined by analyzing unspiked and spiked samples of urine. The results are summarised in Table 1. Intra-assay precision was determined to be 1.0% ($n = 5$).

In summary a sensitive and highly selective assay for urinary free cortisol has been described. The sample preparation and volatility of all the reagents used in this method makes it amenable to processing large numbers of sample.

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