MASS FRAGMENTOGRAPHY OF CORTISOL AND CORTISONE: PRELIMINARY STUDIES ON THE DEVELOPMENT OF A REFERENCE METHOD

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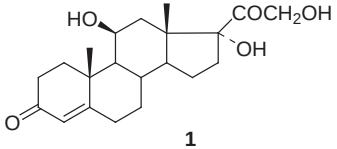
Dedicated to the memory of Dr Václav Černý.

Cortisol and cortisone are the two main glucocorticoids found in human plasma. Cortisol is commonly measured as a means of assessing adrenal function, usually by immunoassay, but such procedures must be subject to proper quality control which ideally uses analyte target values measured by isotope dilution mass fragmentography (GC-MS). Three derivatives of these steroids have been investigated for their chromatographic and mass spectral characteristics in order to develop a simple and rapid GC-MS method for this purpose. Trimethylsilyl ether-*O*-methyloxime derivatives gave poor sensitivity and the *syn*- and *anti*-isomers were resolved into two peaks. Cyclic methylboronate-*O*-methyloxime derivatives gave excellent sensitivity but were difficult to prepare, occasionally giving rise, by some unknown mechanism, to 11-oxidation of cortisol. The underivatised 11-hydroxyl of this derivative of cortisol also gave rise to chromatographic problems. Enol-trimethylsilyl ethers however were easy to prepare and gave excellent sensitivity (10–17 times that achieved with the trimethylsilyl ether-*O*-methyloxime derivatives). Straight line calibration graphs were obtained and the method gave clean traces allowing high specificity and sensitive measurement of cortisol and cortisone in human plasma using tri-deuterated cortisol as an internal standard.

Keywords: Steroids; Mass spectrometry; Glucocorticoid hormones; Analytical methods; GC-MS.

Cortisol (**1**) is the main glucocorticoid hormone circulating in human plasma. It is secreted from the adrenal cortex and converted in both peripheral and target tissues to cortisone, which is also found at low levels in human plasma. The measurement of plasma cortisol is widely used in clinical laboratories as a means of assessing adrenal and pituitary function in conjunction with suppression and stimulation tests[1]. These routine assays are

now carried out using immunoassay systems, which are convenient, simple and capable of automation. However, the use of such systems, which are not always specific, requires careful control, preferably using external quality assurance schemes. In the U.K., National Health Service Laboratories are required, for the purposes of accreditation, to belong to the National External Quality Assurance Scheme (NEQAS), which includes QA of cortisol assays[2]. Schemes such as these should have target concentrations obtained on samples circulated to participating laboratories using reference methods, usually agreed to utilise gas chromatography-mass spectrometry (GC-MS). In the EU, efforts have been made to produce certified reference material, using GC-MS for the measurement, by a number of different laboratories[3], of cortisol concentration in the material provided. Continuing monitoring of performance of routine laboratories is essential for the maintenance of high standards and the use of agreed reference methodology, usually GC-MS, is an important part of proper surveillance of performance.



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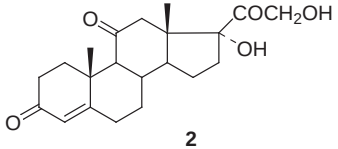

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Cortisol is a polar molecule, which cannot be subjected to gas chromatography without derivatisation, both to improve chromatographic performance and to protect the steroid side chain. In the EU study[3], all seven participating laboratories used *O*-methyloxime-per-trimethylsilyl ether derivatisation and [$^2H_4$]-cortisol as an internal standard. In this exercise only cortisol was measured, although cortisone is also present, albeit in lower concentrations, in human plasma. An examination of the literature over the last ten years has indicated a move towards LC-MS as a means of analysis for plasma cortisol and other steroids present in human plasma. In our view such systems, while more convenient in that they do not require derivatisation, are more expensive than today's simple GC-MS systems and also sacrifice a considerable degree of specificity (unless tandem systems are used) and sensitivity. Modern technology often ignores the lessons of the past and in order to provide a simple, sensitive and, perhaps most important, specific reference GC-MS procedure for cortisol (**1**) and cortisone (**2**), we investigated the chromatographic and mass spectral characteristics of enol-trimethylsilyl and cyclic boronate derivatives and compared them to those of the widely used *O*-methyloxime-trimethylsilyl ether derivatives.

EXPERIMENTAL

Materials

All chemicals were obtained in the purest form possible, usually analytical reagents from Merck Eurolab (Lutterworth, Leicestershire LE17 4XN, U.K.), Sigma–Aldrich Company (Poole, Dorset BH12 4QH, U.K.) or Perbio Science U.K. Ltd., Tattenhall, Cheshire CH3 9RJ, U.K.) and were used without further purification. [$^2H_4$]-Cortisol was a kind gift from Dr C. H. L. Shackleton (Oakland (CA), U.S.A.) and cortisol and cortisone were from Steraloids (U.K.) Ltd. (New Barnet, Hertfordshire EN5 1PR, U.K.). GC-MS was carried out using an Hewlett–Packard HP5890 gas chromatograph linked to an HP5970 mass selective detector in positive ion electron impact mode, as previously described[4,5]. Electron impact spectra were recorded across each peak and stored. Mass spectra recorded here were obtained by averaging the spectra from each peak and subtracting the background. GC was carried out using an HP1 cross-linked silicone stationary phase (15–20 m × *ca* 0.22 mm i.d.) as previously described[6]. The injection system of the GC was modified to accommodate a JADE septumless injection system, which eliminates septum bleed and allows high-temperature injection (350 °C). The advantages of this injection system have been discussed elsewhere[7].

Methods

Formation of O-methyloximes. Steroids were incubated at 40 °C for 1 h or alternatively overnight (>12 h) at room temperature with a solution of 10% methoxylamine hydrochloride in pyridine. Pyridine was removed under vacuum and the steroid derivatives dissolved in toluene.

Formation of per-trimethylsilyl ethers. This was achieved by incubation of the *O*-methyloxime derivative with (trimethylsilyl)imidazole at 50 °C for 1 h. This derivatises all hydroxy groups. The steroid derivatives are then purified on a small Lipidex 5000 column as previously described[8]. This procedure forms the trimethylsilyl (TMS) ethers on all hydroxy groups, including the sterically hindered 17α-hydroxyl.

Formation of enol-trimethylsilyl ethers. The underivatised steroid was incubated at 60 °C for 15 min with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide : iodo(trimethyl)silane : dithiothreitol (1 000 : 2 : 2, v/v/v). These enol-TMS derivatives were very rapidly formed. The reagent mixture could be injected directly into the GC without further purification.

Formation of cyclic boronate esters. These were formed by incubating the steroid, after formation of the *O*-methyloxime derivative as described above, with methylboronic acid as described previously[9].

RESULTS

O-Methyloxime-TMS ether derivatives of cortisol and cortisone gave rise to two peaks during GC (see Fig. 1). These peaks were resolved in the case of cortisone but not in the case of cortisol. Mass spectrometry of the cortisol derivative showed a low abundance molecular ion *m/z* 636 with the usual ions at *m/z* 605 $(M - 31)^+$, *m/z* 515 $(M - 90 - 31)^+$ and *m/z* 425 $(M - 90 - 90 - 31)^+$ all of roughly equal abundance.

Enol-TMS ethers also gave two peaks during GC but the late eluting peak was minor. It will be seen from Fig. 2 that the major peaks from cortisol and cortisone are resolved during GC and the use of single ion monitoring allows resolution of the major peak of the cortisol derivative from the minor peak of the cortisone derivative. The mass spectrum of the cortisol derivatives showed a high-abundance ion (base peak) at *m/z* 632 $(M - 90)^+$ with very few other major ions. The mass spectrum of the cortisone derivative showed high-abundance ions at *m/z* 630 $(M - 90)^+$ and *m/z* 615 $(M - 90 - 15)^+$ (base peak) and again very few other major ions.

The steroid side chain can be protected by the formation of a cyclic boronate esters[10] across C17–C21. The procedure used for the formation of the cyclic methylboronate esters appeared to be very destructive in the case of cortisol and cortisone. Protection of the oxo groups as *O*-methyloximes prior to formation of cyclic boronates limited this destruction but there were still major problems with quantitative conversion to these derivatives. The chromatography of the cortisol *O*-methyloxime-cyclic boronate was very poor presumably due to absorption by the underivatised 11β-hydroxy group. In addition the reagent gave rise to conversion of the cortisol deriva-

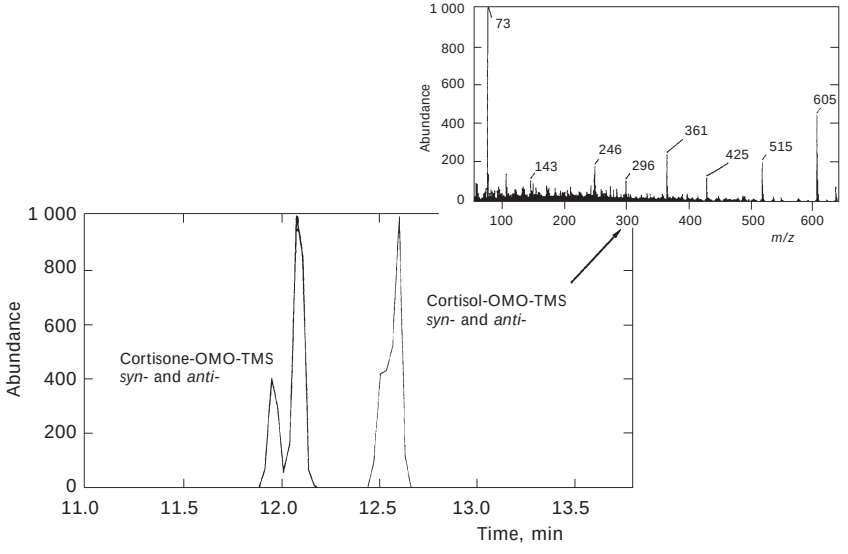

FIG. 1

GC-MS of *O*-methyloxime-TMS (OMO-TMS) derivatives of cortisol and cortisone. Ions *m/z* 531 (cortisone derivative) and *m/z* 605 (cortisol derivative) were monitored and the mass spectrum obtained from the major peak (unresolved) of the cortisol derivative is shown in the upper right hand corner

tive to the cortisone derivative. Although this conversion could be minimised by the addition of ascorbic acid to the derivatising reagent, it was not possible to obtain quantitative conversion in a reproducible fashion and chromatographic behaviour of the cortisol derivative could not be improved (see Fig. 3).

The sensitivity of detection of both cortisol and cortisone was examined using both *O*-methyloxime-TMS and enol-TMS derivatives. Standard curves for each derivative were set up over the range 0–120 ng for cortisol and for cortisone, using an internal standard of cholestane (40 ng). Figure 4 illustrates these curves showing the 10–17-fold increase in sensitivity from the *O*-methyloxime-TMS ether when using the enol-TMS derivatives.

A simple procedure using the enol-TMS derivatives for the measurement of plasma cortisol and cortisone was set up and this is summarised in Fig. 5. Standard curves for both cortisol and cortisone were linear (correlation coeficients 0.999) and the intercept on the *y* axes were not significantly different from zero (Fig. 6). Figure 7 shows the results from the use of this

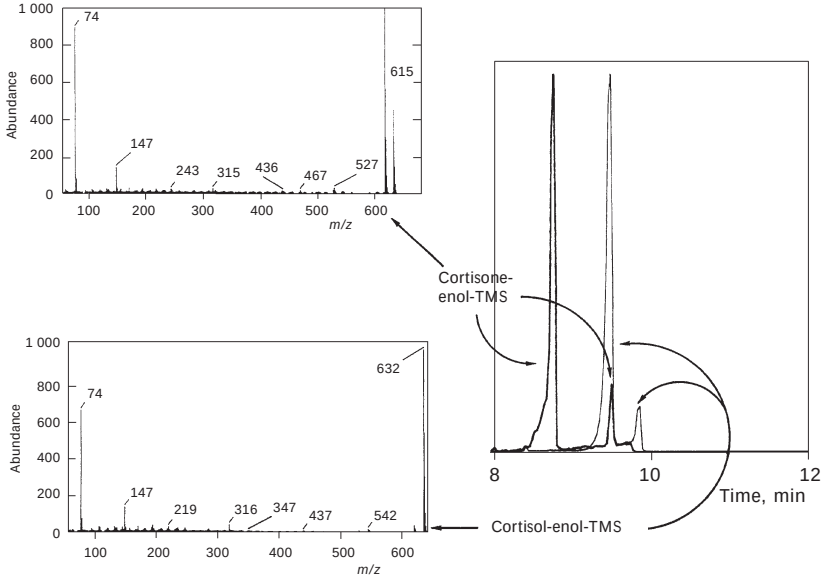

FIG. 2

GC-MS of enol-TMS derivatives of cortisol and cortisone. Ions *m/z* 615 (cortisone derivative) and *m/z* 632 (cortisol derivative) were monitored and the mass spectra of each of the major peaks are shown on the left. The ordinate of the mass fragmentogram is time in min and the abscissa is a measure of relative abundance

method for the analysis of 1 ml of human serum. While further development work is still required, it is estimated that normal levels of cortisol and cortisone could be routinely measured in 0.5 ml of serum with signal : noise ratios of >10 for cortisone and >100 for cortisol.

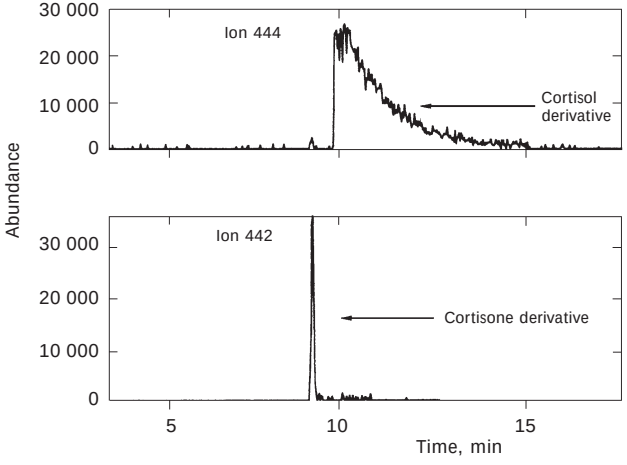

FIG. 3
GC-MS of *O*-methyloxime-methylboronate derivative of cortisol. Ions *m/z* 444 (cortisol derivative; poor peak shape due to adsorption during chromatography) and *m/z* 442 (cortisone derivative; this cortisone peak was formed from cortisol during derivatisation) were monitored

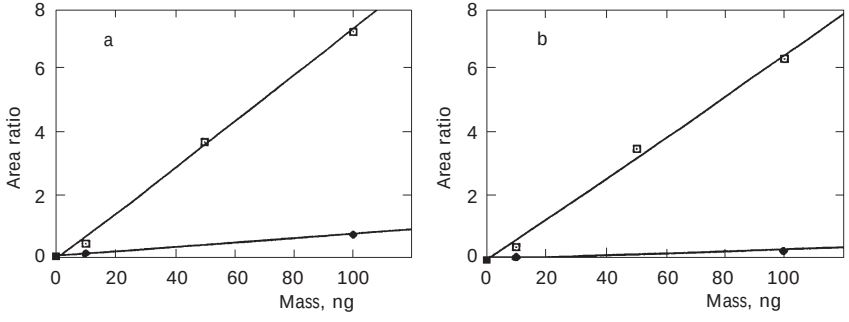

FIG. 4
Comparison of GC-MS response between *O*-methyloxime-TMS (◆) and enol-TMS (❑) derivatives of cortisol (a) and cortisone (b). The slope of the line indicates the sensitivity

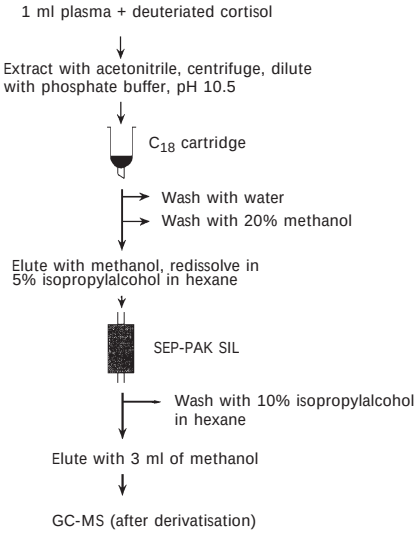

FIG. 5
Flow chart showing proposed methodology for the measurement of cortisol and cortisone in human plasma using the enol-TMS derivatisation procedure described in the text

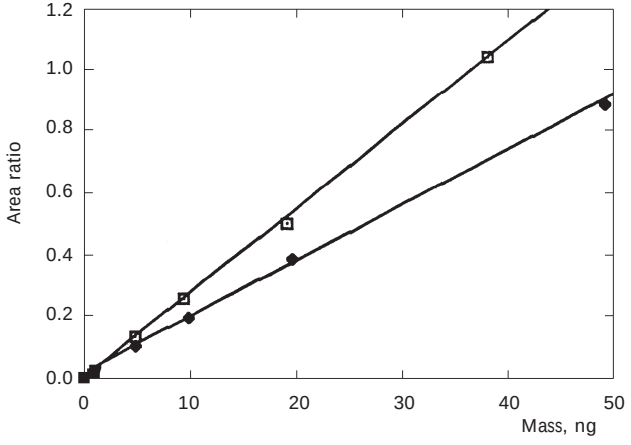

FIG. 6
Standard curves for cortisol and cortisone using the methodology outlined in Fig. 5. Enol-TMS ethers were made as described in the text and subjected to GC-MS. Monitoring ions *m/z* 632 (cortisol), *m/z* 615 (cortisone) and *m/z* 635 [$^2H_3$]-cortisol used as an internal standard. The area ratios 632/635 (❑; $y = -2.2337 \cdot 10^{-3} + 2.7104 \cdot 10^{-2}x$, $R_2 = 0.999$) and 615/635 (◆; $y = 1.2957 \cdot 10^{-2} + 1.7967 \cdot 10^{-2}x$, $R_2 = 0.999$) are plotted on the abscissa against mass of cortisol and cortisone on the ordinate

DISCUSSION

The use of *O*-methyloxime-TMS derivatives for the GC-MS of steroids is still widely used. These derivatives and procedures based thereon have proved very useful, particularly when studying urinary steroid profiles[11]. However when applying GC-MS for the measurement of the lower concentrations of cortisol and cortisone in human plasma, sensitivity assumes considerable importance. The work described here has shown that the use of enol-TMS derivatives increases the sensitivity of detection by 1–2 orders of magnitude. Use of these derivatives for the detection and measurement of anabolic steroids is widespread[12] and although a paper was published[13] in 1972 advocating the use of enol-TMS derivatives, to the best of our knowledge it has not been applied to the GC-MS of cortisol. The simple method described here needs further evaluation, including recovery experiments and assessment of within- and between-batch precision, before it could be widely adopted. This investigation has demonstrated the need fully to eval-

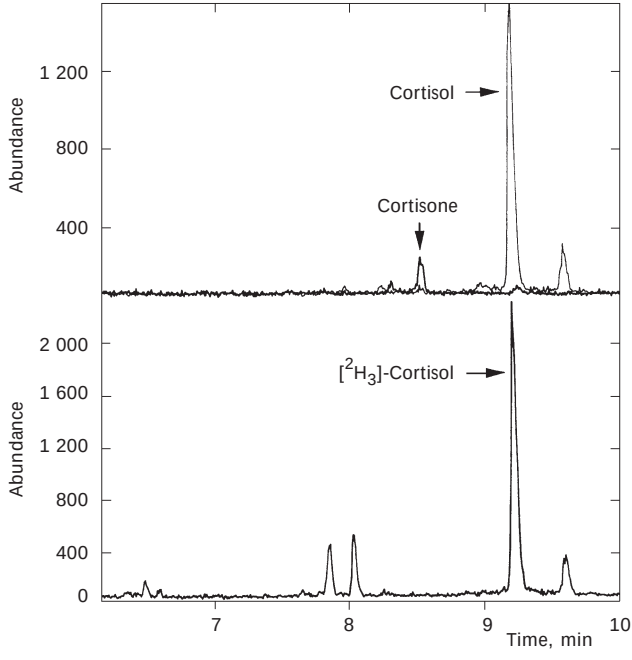

FIG. 7

GC-MS of extracts from 1 ml of human plasma processed as outlined in Fig. 5. The ions monitored during GC-MS are shown on the upper trace for cortisol (*m/z* 632) and cortisone (*m/z* 615). The ion *m/z* 635 was monitored to produce the lower trace, showing the [$^2H_3$]-cortisol

uate existing methodology before use and to consider derivatives described many years before which may provide solutions to today's problems, especially when allied to modern technology.

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