Journal of Chromatography, 425 (1988) 237–243
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4022

DETERMINATION OF CORTISOL IN HUMAN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY USING [$^2H_5$]CORTISOL AS AN INTERNAL STANDARD

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(First received March 31st, 1987; revised manuscript received October 19th, 1987)

SUMMARY

A capillary gas chromatographic–mass spectrometric method for the determination of cortisol in human plasma using cortisol M+5 as an internal standard is described. For calculation of plasma cortisol, peak areas were measured by selected-ion monitoring of the characteristic fragment ions of the dimethoxime–tri(trimethylsilyl) derivatives of cortisol and cortisol M+5 (*m/z* 605 and 610, respectively). The inter- and intra-assay coefficients of variation for plasma sample were 3.07 and 1.77%, respectively. The method needed no complex corrections for contributions and provides a sensitive and reliable technique with good accuracy, precision and reproducibility.

INTRODUCTION

The assay of cortisol in plasma has been greatly simplified by the introduction of the competitive protein binding assay (CPB) [1, 2], radioimmunoassay (RIA) [3, 4] and high-performance liquid chromatography (HPLC) [5–7]. RIA is highly sensitive [4], but there are serious restrictions to its use because cross-reactivity with other steroids is a serious problem in achieving acceptable specificity. CPB cannot avoid the same problem [8, 9]. HPLC offers the advantage that the corticosteroids can be analysed without derivatization, but it lacks sensitivity. Moreover, methods such as RIA, CPB and HPLC do not have the advantage of the isotope dilution technique in that corrections for losses of a particular substance in various biological samples in the extraction and purification procedure cannot be easily made.

The use of gas chromatography–mass spectrometry (GC–MS) and stable isotope-labelled substances as diluents has found broad application in pharmacological studies [10]. We have successfully used stable isotope dilution MS for the accurate measurement of natural and synthetic steroids, in which stable isotope-

0378-4347/88/$03.50

labelled analogues served as ideal internal standards to correct for losses of the sample substance in the initial isolation procedure [11–14]. We are currently interested in applying the stable isotope methodology to the selective and accurate quantitation of cortisol in human plasma and urine by GC–MS. This paper describes the accurate and sensitive determination of cortisol in plasma by using cortisol M+5 ([$^2H_5$]cortisol) as an internal standard for the GC–MS assay.

EXPERIMENTAL

Chemicals and reagents

Stable isotope-labelled cortisol, [1,1,19,19,19-$^2H_5$]cortisol (cortisol M+5), was synthesized in this laboratory [15]. Unlabelled cortisol was purchased from Sigma (St. Louis, MO, U.S.A.). [4-^{14}C]Cortisol (55.0 mCi/mmol) was purchased from New England Nuclear Research Products (Boston, MA, U.S.A.). The methoxyamine reagent (5%, w/v) was prepared by dissolving methoxyamine hydrochloride (Eastman Kodak, Rochester, NY, U.S.A.) in pyridine. N,O-Bis(trimethylsilyl)acetamide (BSA) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals and reagents were of analytical-reagent grade and were used without purification.

Gas chromatography–mass spectrometry–selected-ion monitoring (GC–MS–SIM)

GC–MS–SIM measurements were made with a Shimadzu QP1000 gas chromatograph–mass spectrometer equipped with a data processing system. An SP-2100 fused-silica capillary column (10 m×0.25 mm I.D.) with a 0.25-μm thin film (Supelco, Bellefonte, PA, U.S.A.) was connected directly to the ion source. Helium was used as the carrier gas at a flow-rate of 0.7 ml/min. The splitless injector was used with a septum purge flow-rate of 1 ml/min and a split flow-rate of 30 ml/min. The purge activation time was 2 min. The initial column temperature was set at 150°C and 2 min after the sample injection was increased at 30°C/min to 210°C, and then at 20°C/min to 280°C. Thereafter, the column temperature was maintained at 280°C. The electron energy was set at 70 eV. The ion source temperature was 285°C. The multiple-ion detector was focused on the characteristic fragment ions (M−31) at *m/z* 605 for the dimethoxime–tri(trimethylsilyl) (diMO–triTMS) derivative of unlabelled cortisol and at *m/z* 610 for the diMO–triTMS derivative of labelled cortisol.

Sample preparation for GC–MS–SIM

To 1 ml of plasma were added 162.3 ng of cortisol M+5 dissolved in 10 μl of methanol and 2 ml of 0.5 *M* orthophosphoric acid solution. The plasma sample was applied to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.). The cartridge was washed with 5 ml of distilled water and then eluted with 2 ml of methanol. After evaporation of the eluate in a 2-ml V-vial under a stream of nitrogen, 200 μl of acetone were added to the residue and the sample was transferred into a 0.3-ml V-vial. The solvent was evaporated, 100 μl of methoxyamine reagent were added to the residue and the reaction mixture was heated at 100°C for 30 min. After cooling, the excess of reagent was removed under a stream of

nitrogen. To the residue were added 50 μl of BSA and the vial was heated at 100°C for 2 h. After cooling and evaporating under a stream of nitrogen 200 μl of *n*-hexane were added to the residue. The solution was applied to a Sep-Pak silica cartridge (Waters Assoc.), which was eluted with 7 ml of *n*-hexane. The eluate was evaporated under a stream of nitrogen and the residue was dissolved in 10 μl of carbon tetrachloride. A 1-μl portion of the solution was subjected to GC–MS.

Absolute recovery

To 1.0 ml of human blank plasma were added 22 000 dpm of [4-^{14}C]cortisol in benzene–ethanol (9:1, v/v) and 2 ml of 0.5 *M* orthophosphoric acid solution. The plasma was applied to a Sep-Pak C_{18} cartridge as described above.

Preparation of calibration graph

To each of six standards containing known amounts of cortisol (5.685, 11.37, 56.85, 113.7, 284.3 and 568.5 ng) dissolved in methanol, 162.3 ng of cortisol M+5 dissolved in 10 μl of methanol were added. After evaporation of the solvent, the samples were derivatized and purified as described above. A 1-μl portion of a carbon tetrachloride solution (5–10 μl) was analysed by GC–MS and the peak-area ratios (*m/z* 605–610) were measured.

Determination of accuracy

Cortisol in amounts of 11.37 ng dissolved in 1 μl of methanol and 28.43 ng dissolved in 2.5 μl of methanol was added to 1.0-ml portions of human plasma. After preparation of the sample for GC–MS–SIM as described above, the peak-area ratio (*m/z* 605–610) was determined.

RESULTS AND DISCUSSION

In the GC analysis of corticosteroids that undergo thermal decomposition [16], the choice of an appropriate derivatization is of primary concern. Several derivatives of glucocorticoids have been introduced in the past [16–19]. It has been suggested that in order to produce stable silylated derivatives of corticosteroids it is necessary first to protect the ketone groups at C-3 and C-20 by forming the methoxime [20, 21]. From a quantitative point of view, the MO–TMS derivative has been shown to be the most satisfactory owing to its thermal stability and good GC behaviour [22] and has been used for the determination of synthetic glucocorticoids (prednisone and prednisolone) [23] and cortisol [24–29] in biological fluids using GC–MS.

Fig. 1 shows the electron-impact mass spectra of the MO–TMS derivatives of unlabelled cortisol and cortisol M+5. The relative intensities of the fragment ions (M−31) at *m/z* 605 (unlabelled) and 610 (labelled) were prominent compared with those of the molecular ions at *m/z* 636 and 641, and these fragment ions were selected as the monitored ions for mass fragmentography. In these mass spectra (Fig. 1) the fragment ions (M−31) were observed as multiplets rather than as single ions because the isotope peaks were augmented by the two major isotopes of ^{28}Si, i.e. ^{29}Si and ^{30}Si, in the TMS groups. This multiplicity of the

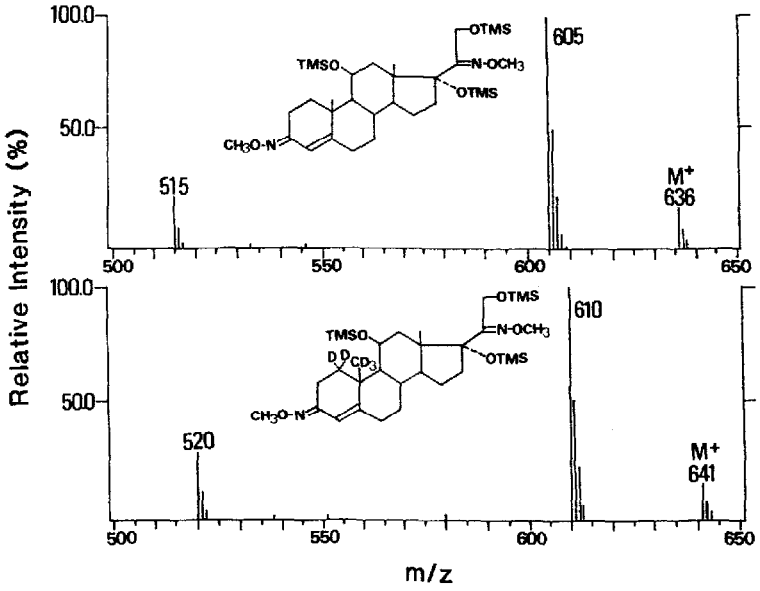

Fig. 1. Mass spectra of the diMO-triTMS derivatives of unlabelled cortisol (top) and cortisol M+5 (bottom).

monitored ions imposes certain restrictions on the choice of an internal standard for the GC–MS analysis using the isotope dilution technique. For example, when [4-^{14}C]cortisol M+2 [24–27], [$^{2}H_3$]cortisol M+3 [28] and [$^{2}H_4$]cortisol M+4 [29] were used as internal standards, the monitored ion cluster of the analyte overlapped with the monitored ion of its isotope-labelled internal standards and corrections for contributions then had to be made, which required considerable manipulation. Therefore, in the GC–MS analysis of cortisol using the MO–TMS derivative, the analyte and its isotope-labelled internal standard must differ by at least 5 mass units so that the monitored ion cluster of the unlabelled molecule does not overlap with the monitored ion of the isotope-labelled molecule and the calibration graph is rectilinear. In this study, the use of [1,1,19,19,19-$^{2}H_5$]cortisol M+5 as an internal standard made it possible to determine plasma cortisol with good accuracy and precision without complex corrections for contributions.

Sep-Pak C_{18} cartridges have been used as a simple method for extracting steroids from biological fluids prior to capillary GC or GC–MS analysis [30–34]. We have used these cartridges to extract dexamethasone, a synthetic corticosteroid, from plasma and urine and obtained good recovery [13, 14]. When the same procedure was employed for cortisol, the absolute recovery from human plasma examined by using [4-^{14}C]cortisol was found to be poor and variable (36.7–67.8%) compared with the recovery from distilled water (75.6%). It has been reported that steroid binding proteins in plasma caused poor and variable recoveries and that acid denaturation of plasma proteins, adjusting the plasma pH to about 2 prior to application to a Sep-Pak C_{18} cartridge, resulted in a marked improvement in recovery (72–100%) [35]. In the present method we used 0.5 *M* orthophos-

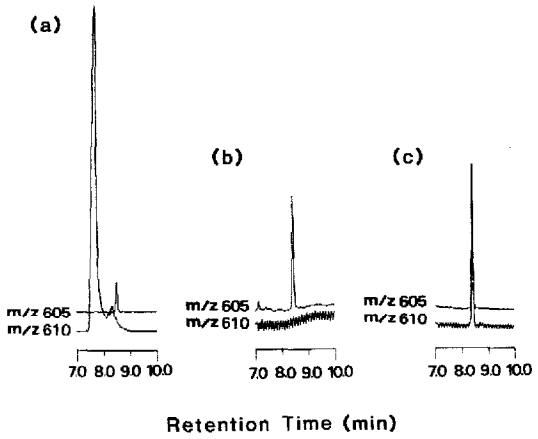

Fig. 2. Mass fragmentograms at *m/z* 605 and 610 after processing of plasma samples. (a) Plasma blank sample without application to Sep-Pak silica after derivatization; (b) plasma blank sample applied to Sep-Pak silica after derivatization; (c) plasma sample spiked with cortisol M+5 (162.3 ng).

phoric acid solution to acidify the plasma sample (pH 1.5), leading to the good absolute recovery of cortisol from human plasma (75.1–92.3%).

A 1.0-ml plasma blank sample was extracted with a Sep-Pak C_{18} cartridge, derivatized to the diMO–triTMS derivative and subjected to GC–MS analysis. The selected-ion recordings at *m/z* 605 and 610 obtained from the plasma blank sample are shown in Fig. 2a. A single peak of the diMO–triTMS derivative of endogenous cortisol appeared at *m/z* 605 in the recordings and the retention time was 8.3 min. There were, however, interfering peaks with retention times close to that of the diMO–triTMS derivative of cortisol M+5 peak on the recordings at *m/z* 610. Application of the Sep-Pak silica cartridge to the plasma blank sample after derivatization removed such interfering peaks (Fig. 2b). The elution of the derivative was completed with the use of 7 ml of *n*-hexane and hydrolysis of TMS groups did not occur during this elution procedure. The *syn–anti* isomers of the 3-methoxime in diMO–triTMS derivative of cortisol were eluted as one peak on a methylsilicone SP-2100 column (a relatively non-polar stationary phase). Fig. 2c shows the selected-ion recordings of the diMO–triTMS derivatives of cortisol (*m/z* 605) and cortisol M+5 (*m/z* 610) for a processed plasma sample spiked with 162.3 ng of cortisol M+5. Sharp and single peaks appeared and the retention times of the derivatives were the same.

A calibration graph was prepared without any correction for contributions by using cortisol M+5 as an internal standard. Each sample was analysed in duplicate, monitoring the fragment ions at *m/z* 605 for cortisol and at *m/z* 610 for cortisol M+5. The logarithms of the peak-area ratios of the recordings at *m/z* 605 and 610 were plotted against those for the molar ratios of unlabelled cortisol to cortisol M+5. The graph was rectilinear for the examined molar ratio range of 0.03551–3.551. A least-squares linear analysis of the logarithms of the observed ratio gave a regression line with a slope of 0.9851 and a correlation coefficient of 0.9998, indicating no contributions and interferences at the masses monitored.

TABLE I

ACCURACY OF CAPILLARY GC–MS DETERMINATION OF CORTISOL IN PLASMA (INTER-ASSAY)

Added (ng)	Expected (ng/ml)	Measured concentration (ng/ml)							C.V. (%)	Relative error (%)
		Individual values						Mean ± S.D.		
–	–	60.05	57.63	58.06	56.46	59.20	61.47	58.81 ± 1.80	3.07	–
11.37	70.18	65.56	68.96	63.70	68.19	73.43	68.11	67.99 ± 3.31	4.87	−3.12
28.43	87.24	87.45	83.94	83.46	84.67	85.54	–	85.01 ± 1.57	1.85	−2.56

TABLE II

ACCURACY OF CAPILLARY GC–MS ANALYSIS OF CORTISOL IN PLASMA (INTRA-ASSAY)

Added (ng)	Measured concentration (ng/ml)						C.V. (%)
	Individual values					Mean ± S.D.	
–	57.63	59.23	59.58	–	–	58.81 ± 1.04	1.77
11.37	65.56	70.65	72.96	68.71	70.42	69.66 ± 2.75	3.94
28.43	87.45	86.92	85.09	–	–	86.49 ± 1.24	1.43

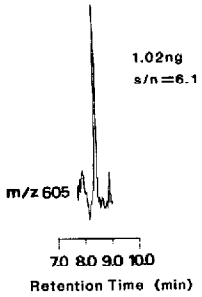

Fig. 3. Sensitivity of the method.

The accuracy of measurement was determined for cortisol added to 1.0-ml aliquots of pooled plasma. The plasma samples contained 162.3 ng of the internal standard and different amounts (11.37 and 28.43 ng) of cortisol. The cortisol content in the pooled plasma measured was 58.81 ± 1.80 ng/ml. The results presented in Table I show that the amounts of cortisol added were in good agreement with the amounts of cortisol measured, the relative error being less than 4%. The inter-assay coefficients of variation (C.V.) were 3.07% for plasma ($n=6$), 4.87% for plasma spiked with 11.37 ng ($n=6$) and 1.85% for plasma spiked with 28.43 ng ($n=5$). Table II shows that the intra-assay coefficients of variation were 1.77% for plasma ($n=3$), 3.94% for plasma spiked with 11.37 ng ($n=5$) and 1.43% for plasma spiked with 28.43 ng ($n=3$). The sensitivity of the GC–MS–SIM assay was 1.02 ng per injection with a signal-to-noise ratio of about 6 (Fig. 3).

The method provided a sensitive and reliable technique for determining plasma levels of cortisol with good accuracy and precision without complex corrections for contributions by using cortisol M+5 as an internal standard. The method can be applied to studies of detailed plasma cortisol changes, reflecting metabolism, secretion and excretion.

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