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Quantification of corticosteroids in human plasma by liquid chromatography–thermospray mass spectrometry using stable isotope dilution

Hiromi Shibasaki, Takashi Furuta, Yasuji Kasuya*

Department of Medicinal Chemistry and Clinical Pharmacy, Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03 Japan

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Abstract

Liquid chromatography–thermospray mass spectrometry (LC–TSP-MS) using isotope dilution was investigated for quantitative analysis of cortisol, cortisone, prednisolone and prednisone in human plasma. Complete separation attained by a LiChroCART Supersupher reversed-phase column and elution with 0.05 *M* ammonium formate–tetrahydrofuran–methanol (180:53:17, v/v/v) resulted in a significantly large isotope effect of the deuterium-labeled analogs on the HPLC behavior and caused difficulty in quantification. Reduction of the isotope effect on the retention times using 0.05 *M* ammonium formate–acetonitrile (65:35, v/v) permitted accurate quantification of cortisol and cortisone by the isotope dilution LC–TSP-MS, although separation between cortisol and prednisone was incomplete.

Keywords: Corticosteroids; Cortisol; Cortisone; Prednisolone; Prednisone

1. Introduction

High-performance liquid chromatographic (HPLC) separations coupled with specific mass spectral identification offer a powerful tool for the analysis of non-volatile and thermally labile compounds present in biological samples. Without the need of sample derivatization, the liquid chromatography–mass spectrometry (LC–MS) technique provides increased specificity not available with conventional HPLC. Recently, the LC–MS analysis of free steroids, as well as steroid sulfates and glucuronides, has been reported [1–12]. There are, however, only a few examples of the use of stable isotope dilution in quantitative analyses of steroids by LC–MS [4,7–9].

In achieving precise and accurate quantification of corticosteroids, we have previously developed a capillary GC–MS method for the simultaneous assay of cortisol, cortisone, prednisolone and prednisone in human plasma using their respective deuterated analogs synthesized in this laboratory as internal standards [13]. The use of the isotopically labeled internal standard may be particularly important in LC–MS to compensate for variations in instrumental and ionization conditions encountered during the course of analysis [4,7,14].

The purpose of the present study was to evaluate an LC–MS method for the simultaneous quantitative

*Corresponding author

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determination of four corticosteroids, cortisol, cortisone, prednisolone and prednisone, in human plasma by on-line coupling of liquid chromatography with thermospray mass spectrometry (LC–TSP-MS) using deuterium-labeled internal standards.

2. Experimental

2.1. Chemicals and reagents

Cortisol, cortisone, prednisolone and prednisone were purchased from Sigma (St. Louis, MO, USA). [1,1,19,19,19-$^2H_5$]Cortisol (cortisol-d_5), [1,1,19,19,19-$^2H_5$]cortisone (cortisone-d_5), [1,19,19,19-$^2H_4$]prednisolone (prednisolone-d_4) and [1,19,19,19-$^2H_4$]prednisone (prednisone-d_4) were synthesized in this laboratory as described elsewhere [15,16]. Ammonium formate and tetrahydrofuran (THF) were of analytical reagent grade and purchased from Kanto (Tokyo, Japan). THF was redistilled before use. All other chemicals and solvents were of analytical reagent grade and were used without further purification. High-purity water for HPLC was obtained from a water purification system (Advantec, Tokyo, Japan). The mobile phase was filtered under vacuum through a 0.45 μm pore size filter (Nihon Millipore, Tokyo, Japan) and then continuously purged with helium during analysis in order to remove dissolved gasses.

2.2. Stock solutions

Stock solutions of cortisol (10.07 mg per 50 ml), cortisone (10.38 mg per 100 ml), prednisolone (10.55 mg per 100 ml), prednisone (10.75 mg per 100 ml), cortisol-d_5 (44.65 μg per 50 ml), cortisone-d_5 (37.28 μg per 50 ml), prednisolone-d_4 (14.12 μg per 25 ml), and prednisone-d_4 (12.65 μg per 25 ml) were prepared in methanol. All analyses were performed by diluting the stock solutions with methanol.

2.3. High-performance liquid chromatography

Chromatographic separations were performed on a LiChroCART Superspher 100 column, 125×4.0 mm I.D., 4 μm particle size, from Merck (Darmstadt, Germany). The mobile phases consisted of 0.05 *M* ammonium formate–THF–MeOH (180:53:17, v/v/v) and 0.05 *M* ammonium formate–acetonitrile (65:35, v/v) were delivered by a Shimadzu 6A HPLC pump (Shimadzu, Kyoto, Japan) at flow-rates of 0.4–1.0 ml min^{-1}. A second HPLC pump was used for post-column addition of the mobile phase to provide total flow-rates of 1.0–1.5 ml min^{-1}. Both pumps were coupled through a T connector (Shimadzu, Kyoto, Japan) to the thermospray interface. Samples were injected with a Model 7125 20-μl Rheodyne sample injector (Rheodyne, Cotati, CA, USA).

2.4. Thermospray mass spectrometry

A Shimadzu (Kyoto, Japan) LCMS-QP1000EX equipped with a Vestec (Houston, TX, USA) Model 750B thermospray instrument. The vacuum system was a Balzers Model 016 mechanical pump which pumps away the vapor produced by the thermospray vaporizer probe. The thermospray mass spectrometer conditions were optimized for monitoring the pseudomolecular ions (MH^+) of the corticosteroids in the positive ion mode (*m/z* 363 for cortisol, *m/z* 368 for cortisol-d_5, *m/z* 361 for cortisone, *m/z* 366 for cortisone-d_5, *m/z* 361 for prednisolone, *m/z* 365 for prednisolone-d_4, *m/z* 359 for prednisone, and *m/z* 363 for prednisone-d_4). Typical temperatures were: vaporizer control, 155°C; vaporizer tip, 195°C; vapor, 274°C, ion source block, 295°C; and tip heater, 305°C. The electron beam or electrical discharge was kept off throughout the analysis.

2.5. Sample preparation

2.5.1. Extraction.

To 1.0 ml of human plasma were added 134.0 ng of cortisol-d_5 and 74.56 ng of cortisone-d_5. The plasma sample was applied to a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA). The cartridge was washed with 8 ml of distilled water and then eluted with 4 ml of ethyl acetate into a 10-ml vial. After evaporation to dryness at 70°C under a stream of nitrogen, the residue was dissolved in 30 μl mobile phase and filtered (0.45 μm) (HLC DISK, Kanto, Tokyo, Japan).

2.6. Calibration graphs

To each of six standards containing known amounts of cortisol (10.07, 25.18, 50.35, 120.8, 181.3 and 251.8 ng) and cortisone (4.152, 10.38, 25.95, 51.90, 72.66 and 103.8 ng), prednisolone (10.55, 21.10, 105.5, 158.3, 211.0 and 316.5 ng), and prednisone (10.75, 21.50, 107.5, 161.3, 215.0 and 322.5 ng) dissolved in methanol, 134.0 ng of cortisol-d_5, 74.56 ng of cortisone-d_5, 56.48 ng of prednisolone-d_4, and 50.60 ng of prednisone-d_4 were added. After evaporation of the solvent to dryness, the samples were dissolved in 30 μl mobile phase. A 10-μl portion of the mobile phase was subjected to LC–MS. The peak-area ratios (*m/z* 363 to 368, *m/z* 361 to 366, *m/z* 361 to 365 and *m/z* 359 to 363) were determined using selected ion monitoring (SIM) in duplicate. The calibration graphs were obtained by an unweighted least-squares linear fitting of the peak-area ratios versus the mixed molar ratios of cortisol/cortisol-d_5, cortisone/cortisone-d_5, prednisolone/prednisolone-d_4, prednisone/prednisone-d_4 on each analysis of the standard mixtures.

2.7. Accuracy

Accuracy was determined by assaying six preparations of 1.0-ml portions of human plasma spiked with 40.28 ng of cortisol (134.0 ng of cortisol-d_5 as internal standard), 20.76 ng of cortisone (74.56 ng of cortisone-d_5 as internal standard). After preparation of the sample for LC–MS-SIM as described above, the peak-area ratios were measured.

3. Results and discussion

Our first objective was to establish the conditions for rapid and complete separation of cortisol, cortisone, prednisolone, and prednisone on a reversed-phase column, taking into account the effects of ammonium formate concentration on the pseudomolecular ion intensity and fragmentation on the thermospray mass spectral analysis. Using the buffer concentration ranged from 0.01–0.1 *M* and acetonitrile, methanol, ethanol, or ethyl acetate or a combination of these solvents, only partial separation of the four corticosteroids was achieved. However, the benefit of using tetrahydrofuran (THF) [17,18] with 0.05 *M* ammonium acetate and methanol was demonstrated to result in complete separation of the four corticosteroids on a LiChroCART Supersupher reversed-phase column as shown in Fig. 1.

The thermospray interface can be operated at mobile phase flow-rates of 0.5–1.5 ml min^{-1}. For a good HPLC separation of the four corticosteroids described above, the optimum flow-rate was 0.6 ml min^{-1}. This flow-rate, however, was found not to be favorable for thermospray MS analysis because mass spectra were obtained with a low ionization efficiency. When a second HPLC pump was used for post-column addition of the mobile phase to deliver a total flow-rate of 1.3 ml min^{-1}, a significant enhancement of the pseudomolecular ion intensity was observed.

After optimizing thermospray MS conditions, the mass spectra of cortisol and cortisone (400 ng each) depicted in Fig. 2A were obtained. The thermospray spectra are characterized by abundant pseudomolecular ions (MH^+) at *m/z* 363 for cortisol and *m/z* 361 for cortisone, accompanied by fragment ions at $[MH-18]^+$, $[MH-30]^+$, and $[MH-60]^+$. These fragment ions are derived by loss of water, formaldehyde, and glycoaldehyde, respectively, from the protonated parent molecules [7,8]. The thermospray mass spectra of the deuterium-labeled analogs,

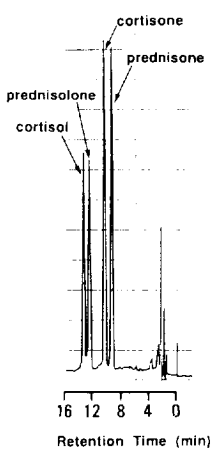

Fig. 1. HPLC separation of corticosteroids using 0.05 *M* ammonium formate–tetrahydrofuran–methanol (180:53:17, v/v/v) as mobile phase.

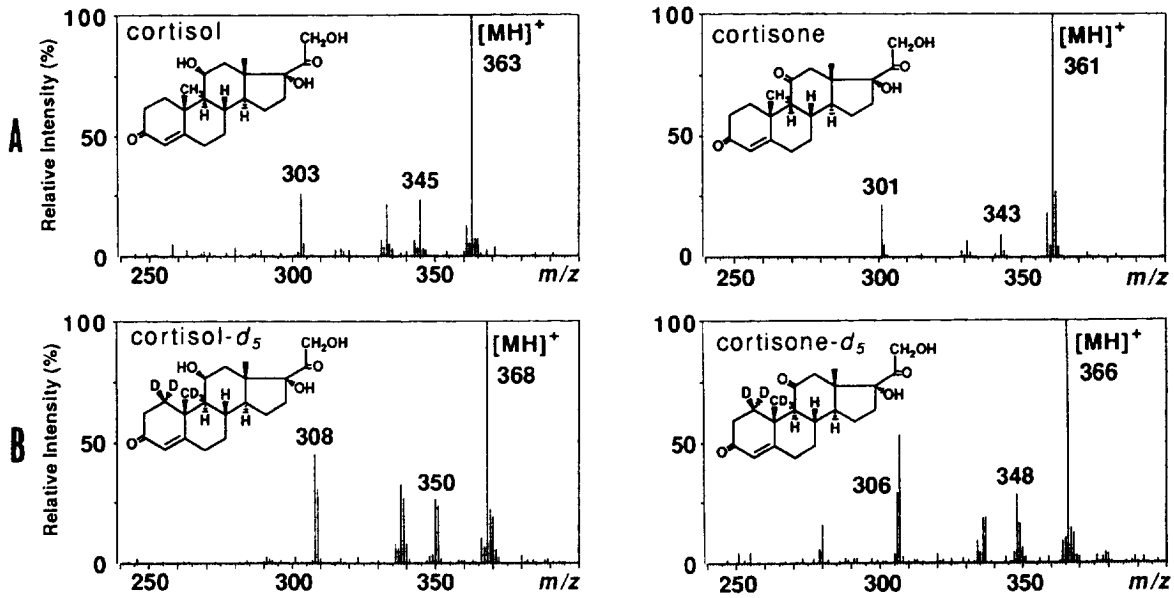

Fig. 2. Thermospray mass spectra of (A) cortisol (left) and cortisone (right) and (B) cortisol-d_5 (left) and cortisone-d_5 (right).

cortisol-d_5 and cortisone-d_5 (400 ng each), depicted in Fig. 2B demonstrate the similarity in relative intensity and fragmentation to those of unlabeled cortisol and cortisone, except that the corresponding pseudomolecular and fragment ions reside at m/z of 5 mass units higher. It is then evident that the deuterium-label at C-1 and C-19 of the cortisol or cortisone molecule is thermally and chemically stable during the thermospray MS analysis.

In Fig. 3 are shown the thermospray mass spectra

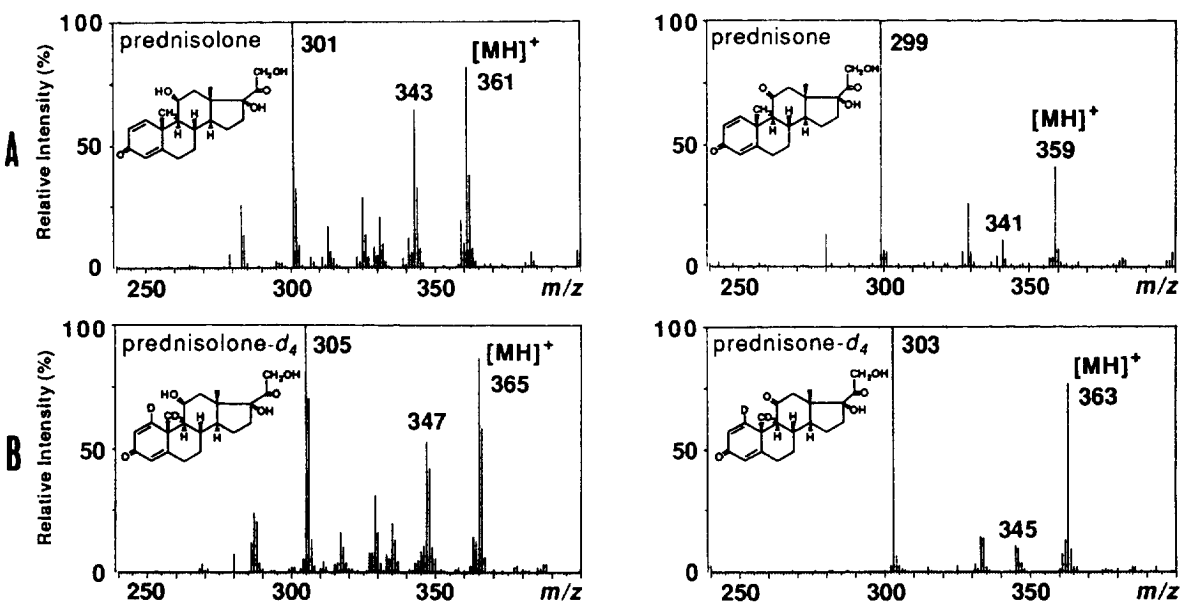

Fig. 3. Thermospray mass spectra of (A) prednisolone (left) and prednisone (right) and (B) prednisolone-d_4 (left) and prednisone-d_4 (right).

of prednisolone and prednisone and the respective deuterium-labeled analogs (400 ng each). The $[MH]^+$ ions (*m/z* 361 for prednisolone, *m/z* 365 for prednisolone-d_4, *m/z* 359 for prednisone, and *m/z* 363 for prednisone-d_4) were accompanied by the $[MH-18]^+$, $[MH-30]^+$, and $[MH-60]^+$ fragment ions, as observed in the mass spectra of cortisol and cortisone (Fig. 2). These mass spectra, however, exhibited the base peaks at the $[MH-60]^+$ fragment ions for both prednisolone and prednisone, while the base peaks for cortisol and cortisone were the protonated parent molecules (MH^+). The deuterium-label in the prednisolone and prednisone molecules was also stable during the thermospray MS analysis.

By monitoring the $[MH]^+$ ions of cortisol, cortisone, prednisolone, and prednisone at *m/z* 363, *m/z* 361, *m/z* 361, and *m/z* 359, respectively, the detection limits at ca. 2:1 signal-to-noise (*S/N*) were 250 pg for cortisol, 500 pg for cortisone, and ca. 1 ng for prednisolone and prednisone (Fig. 4). While the detection limit of cortisol was comparable to that obtained by our previous GC–MS analysis in the electron impact mode [13], the GC–MS method gave better sensitivity limits of 10 pg for cortisone, *S/N*= 2.7; 100 pg for prednisolone, *S/N*=4.4; and 250 pg for prednisone, *S/N*=2.7. With respect to the pseudomolecular ion intensity, Steffenrud and Maylin recently demonstrated the beneficial use of acetonitrile instead of methanol [12]. Furthermore, these authors found that an increase of the buffer concentration of ammonium formate from 0.025–0.05 *M* caused a significant drop of the pseudomolecular ion intensity for dexamethasone and prednisolone. As a consequence, 0.025 *M* ammonium formate in 30% acetonitrile was used for rapid separation of cortisol, cortisone, prednisolone and prednisone, although partial separation was obtained between cortisol and prednisone. Since the pseudomolecular ions of cortisol and prednisone-d_4 appear at the identical *m/z* of 363, complete separation of these compounds is required in order to easily distinguish them in a mixture, using selected ion monitoring (Fig. 3).

The selected ion recordings illustrated in Fig. 5 were obtained by monitoring the $[MH]^+$ ions of the four corticosteroids and their deuterium-labeled analogs. The pseudomolecular ion traces clearly demonstrate rapid and complete separation of the compounds without chromatographic peak distortion using the buffer concentration of 0.05 *M* ammonium formate in tetrahydrofuran and methanol. In view of the isotope effect reflected on the retention time of the chromatographic peak, it should be noted that under the HPLC conditions employed the deuterium-labeled analogs were eluted earlier than the respective unlabeled corticosteroids. The differences in the retention times were 23 s for cortisol, 14 s for cortisone, 11 s for prednisolone and 15 s for prednisone.

It has been indicated that TSP can exhibit a wide

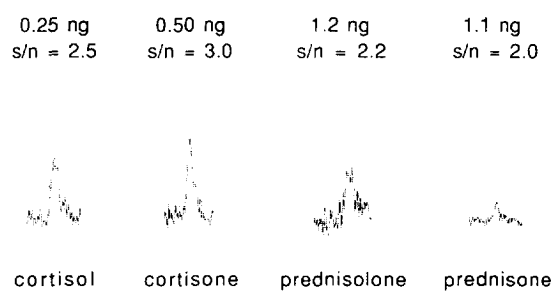

Fig. 4. Detection limits for the four corticosteroids by LC–TSP-MS.

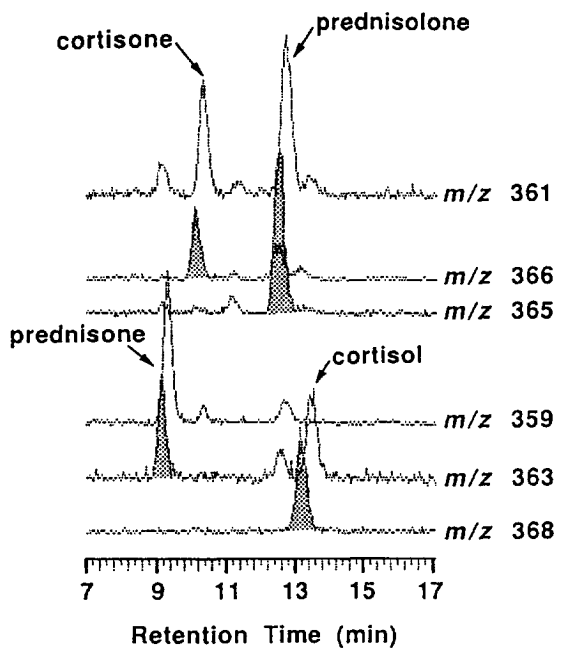

Fig. 5. Pseudomolecular ion traces of unlabeled (solid-line peaks) and deuterium-labeled (shaded peaks) corticosteroids.

fluctuating variation in the ionizing species and hence for the compound under study [7,14]. The substantial variations in ionization conditions may then render the TSP technique inapplicable for the quantitative analysis. The observed difference in the retention times of the LC–MS-SIM between the unlabeled corticosteroid and its labeled analog would perturb the relationship of the peak intensity ratio versus the molar ratio.

Using an equimolar labeled/unlabeled corticosteroid ratio, the peak area ratios of the pseudomolecular ions between labeled and unlabeled compounds were measured for d_5/d_0-cortisol and d_5/d_0-cortisone. The obtained peak area ratios were found to range from 0.5438–1.611 with a relative standard deviation (R.S.D.) of 30% ($n=6$) for a d_5/d_0-cortisol molar ratio of 1.032 and from 1.544–2.194 with an R.S.D. of 14% ($n=6$) for a d_5/d_0-cortisone molar ratio of 0.9743. Similar results were obtained when peak area ratios between the ions at $[MH-30]^+$ or $[MH-60]^+$ were considered. Attempts for simultaneous quantification of cortisol, cortisone, prednisolone, and prednisone by the LC–TSP-MS procedure using deuterium-labeled internal standards have therefore indicated unsuccessful results with deviations in excess of the acceptable analytical limits. To effectively compensate for changes in ionization efficiencies using stable isotope labeled analogs as internal standards, it is necessary that the difference in the retention time between the labeled and unlabeled analog be minimized.

In GC or GC–MS analysis, it is often found that an isotopically labeled compound can be eluted earlier than its unlabeled analog owing to the primary isotope effect on the GC behavior [19–21]. During the simultaneous assay of the four corticosteroids by GC–MS [13], we observed slight differences in the retention times between the unlabeled corticosteroids and their respective labeled internal standards (less than 6 s). The differences, however, did not cause any significant difficulty in achieving precise and accurate quantification of the corticosteroids. There are also a number of reports available dealing with the isotope effect on the HPLC behavior [22–28].

Fig. 6 (left panel) shows the selected ion recordings of LC–TSP-MS obtained by monitoring the $[MH]^+$ ions of cortisol and cortisone and their

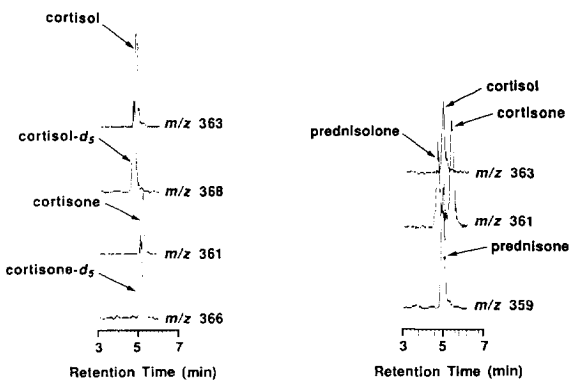

Fig. 6. (Left) LC–TSP-MS-SIM analysis of cortisol and cortisone and their deuterium-labeled analogs. (Right) HPLC separation of corticosteroids using 0.05 *M* ammonium formate–acetonitrile (65:35, v/v) as mobile phase.

deuterium-labeled analogs using 0.05 *M* ammonium formate: acetonitrile (65:35, v/v) as mobile phase. The isotope effect reflected on the differences in the retention times were 3.6 s for cortisol and 2.4 s for cortisone, which were reduced to a significant extent (about 1/6 of those shown in Fig. 5).

When the mobile phase composed of 0.05 *M* ammonium formate: acetonitrile (65:35, v/v) was used, separation was incomplete between cortisol and prednisone as shown in Fig. 6 (right panel). Since the $[MH]^+$ ions of cortisol and prednisone-d_4 appear at the identical m/z of 363 on the mass spectra as pointed above, selected ion monitoring of m/z 363 should account for the contribution of cortisol present at the $[MH]^+$ ion of prednisone-d_4 and vice versa. Therefore, adequate quantification of prednisone in human plasma is not possible by isotope dilution LC–TSP-MS using prednisone-d_4 as internal standard.

Under the HPLC conditions using the buffer concentration of 0.05 *M* ammonium formate in acetonitrile, capable of sufficient separation of cortisol and cortisone, the peak area ratios of the $[MH]^+$ ions between labeled and unlabeled compounds were measured to be 0.9690–1.034 for a d_5/d_0-cortisol molar ratio of 1.005 and 1.042–1.216 for a d_5/d_0-cortisone molar ratio of 1.149. The R.S.D.s were 3.3% ($n=6$) for cortisol and 5.6% ($n=6$) for cortisone. When the quantification of cortisol and cortisone in human plasma were performed by LC–TSP-MS-SIM using cortisol-d_5 and cortisone-d_5 as

internal standards, it was found that the isotope dilution method was successful in achieving accurate quantification. That is, a good correlation was found between the observed area ratios and the mixed molar ratios of the d_0/d_5-cortisol and d_0/d_5-cortisone, respectively, to obtain excellent calibration curves. Unweighted least-squares regression analysis gave a regression line of $y=1.001x+0.050$ ($r=0.995$) for cortisol and $y=1.079x+0.010$ ($r=1.000$) for cortisone.

Pooled plasma samples spiked with 40.28 ng of cortisol and 20.76 ng of cortisone were analysed to determine the precision and accuracy of the employed LC–MS-SIM and the results were presented in Table 1. The estimated amounts of cortisol and cortisone were in good agreement with the expected amounts, the relative error being +3.6% for cortisol and −0.85% for cortisone. The R.S.D.s were less than 6%. These results were comparable to those obtained by a capillary GC–MS method previously developed in this laboratory for the simultaneous assay of cortisol, cortisone, prednisolone and prednisone in human plasma using their respective deuterated analogs [13]. When the above plasma samples spiked with 40.28 ng of cortisol and 20.76 ng of cortisone were analysed by the GC–MS method, the relative errors were determined to be +1.3% for cortisol and +4.6% for cortisone. The R.S.D.s were less than 2% and 4% for cortisol and cortisone, respectively. Gaskell et al. have reported data on the quantification of serum cortisol by LC–TSP-MS and GC–MS, using [9,12,12-$^2H_3$]cortisol as internal standard [7]. Although satisfactory agreement between LC–TSP-MS and GC–MS data were observed, these authors have pointed out that the poorer precisions observed with the LC–MS method, compared with GC–MS, is attributable to the lower sensitivity and to the signal instability commonly observed with TSP analysis. The sensitivity obtained by the present LC–MS method was slightly inferior to that obtained by the GC–MS method. Nevertheless, the sensitivity achieved by LC–MS was adequate enough for determining cortisol and cortisone in human plasma.

The administration of prednisolone and prednisone, synthetic analogs of cortisol and cortisone, respectively, suppresses cortisol production [29] and examination of the effect of these synthetic corticosteroids on circulating cortisol or cortisone concentrations has significant therapeutic and clinical importance. In comparison with the sensitive and specific GC–MS method we previously developed [13], a stable isotope dilution LC–TSP-MS method is not suitable for the simultaneous assay of the four corticosteroids in human plasma due to the isotope effect reflected on the retention time of the chromatographic peak. Without requiring derivatization, however, the advantage of using LC–MS is evident in as much as isotope dilution is applied to accurate quantification of cortisol and cortisone in human plasma.

Acknowledgments

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Table 1
Accuracy of LC–TSP-MS-SIM determination of cortisol and cortisone in plasma

	Added (ng ml^{-1})	Expected (ng ml^{-1})	Found (ng ml^{-1})							Relative error (%)	C.V. (%)
			Individual values						Mean±S.D.		
Cortisol			185.6	196.7	166.9	182.8	177.8	180.0	181.6±9.8		5.4
	40.28	221.9	229.4	–	233.6	223.7	234.7	227.6	229.8±4.5	+3.6	2.0
Cortisone			28.03	29.02	27.28	27.75	26.22	25.06	27.23±1.40		5.2
	20.76	47.99	48.07	46.18	49.82	47.73	47.38	46.32	47.58±1.33	−0.85	2.8

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