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Determination of Dextromethorphan and Dextrorphan in Human Plasma by Liquid Chromatography/Tandem Mass Spectrometry

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Rapid, sensitive and selective methods were developed for the determination of dextromethorphan and its major metabolite, dextrorphan, in human plasma using liquid chromatography/tandem mass spectrometry (LC/MS/MS). Plasma samples spiked with stable-isotope internal standards were prepared for analysis by a liquid–liquid back-extraction procedure. Dextromethorphan and dextrorphan were chromatographed on a short reversed-phase column, using separate isocratic mobile phase conditions optimized to elute each compound in ~1.1 min. For both analytes, calibration curves were obtained over four orders of magnitude and the limit of quantitation was 5 pg ml^{-1} using a 1 ml plasma sample volume. The accuracy across the entire range of spiked DEX and DOR concentrations was, in general, within 10% of the spiked value. The precision was generally better than 6% for replicate sample preparations at levels of 50 pg ml^{-1} or higher and typically better than 12% at levels below 50 pg ml^{-1}. The method was applied for the evaluation of the pharmacokinetic profiles of dextromethorphan and dextrorphan in a human volunteer following peroral administration of a commercially available cough formulation.

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INTRODUCTION

Dextromethorphan (DEX, Fig. 1) is the active ingredient in many over-the-counter cough formulations sold in the USA and Europe. In most individuals, DEX is extensively metabolized by a first-pass metabolic effect, resulting in low systemic peak plasma levels of DEX, typically in the 1–5 ng ml^{-1} range.[1–3] However, a small percentage of the population are slow metabolizers, due to a phenotypic variation and achieve DEX levels in the 10–20 ng ml^{-1} range.[4–6] Dextrorphan (DOR, Fig. 1), the major metabolite of DEX, also achieves low systemic levels owing to rapid conjugation via glucuronidation.[7] Due to the low systemic levels of DEX and DOR achieved in most individuals, highly sensitive methods are required for the determination of these analytes in plasma.

A variety of methods have been employed for the determination of DEX in plasma, urine and saliva, including direct fluorescence spectrometry,[8] high-performance liquid chromatography (HPLC) with fluorescence detection,[9–12] HPLC with ultraviolet (UV) detection,[13] gas chromatography (GC) with nitrogen–phosphorus and mass spectrometric detection,[4,6,14] capillary zone electrophoresis with UV detection[15] and radioimmunoassay (RIA).[16] The HPLC, GC and RIA methods have reported limits of detection (LOD) of 0.5, 1 and 2 ng ml^{-1}, respectively, when using 1 ml or larger volumes of plasma. Plasma and urinary quantitation of DOR has mainly been done by HPLC with fluorescence

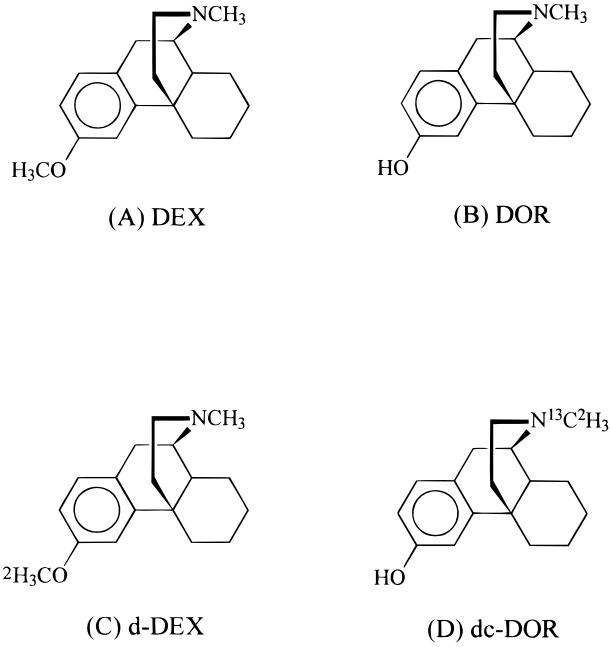

Figure 1. Structures of (A) dextromethorphan (DEX), (B) dextrorphan (DOR), (C) [$^2H_3$-*O*-methoxy]dextromethorphan (d-DEX) and (D) [$^2H_3$,^{13}C-*N*-methyl]dextrorphan (dc-DOR).

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detection, with an LOD of 1 ng ml^{-1}.[12,17] The LODs achievable with these methods are marginal for determining the pharmacokinetic profile of DEX and DOR following peroral (PO) administration. The chromatographic profiles obtained by these methods often contain extraneous matrix peaks, complicating the analysis and, in general, require 5–15 min run times.

We report here the development of stable-isotope dilution-based HPLC/electrospray ionization (ESI) tandem mass spectrometric (LC/MS/MS) methods for the determination of DEX and DOR in human plasma. Plasma samples were spiked with internal standards, [$^2H_3$-*O*-methoxy]dextromethorphan (d-DEX, Fig. 1) and [$^2H_3$,^{13}C-*N*-methyl]dextrorphan (dc-DOR, Fig. 1) and prepared for analysis by liquid–liquid extraction (LLE). The plasma extracts were then chromatographed on a short, high-resolution reversed-phase HPLC column. All samples were analyzed first for DEX and subsequently for DOR. A sequential analysis resulted in optimal LC and MS/MS conditions for each analyte. Elution occurred at ~1.1 min and injection to injection analysis times were less than 1.8 min per analyte. Internal standards and analytes were selectively detected using ESI-MS/MS. Transitions for selected reaction monitoring (SRM) schemes were optimized for both DEX and DOR to achieve maximum sensitivity and selectivity in the human plasma matrix. The LC/MS/MS methods have at least a 100-fold lower limit of quantitation (LOQ) for DEX and DOR, 5 pg ml^{-1}, in plasma than previously reported methods which specify an LOQ.

EXPERIMENTAL

Chemicals and reagents

DEX hydrobromide was obtained from the United States Pharmacopeial Convention (Rockville, MD, USA) and DOR was obtained from Research Biochemicals International (Natick, MA, USA). The stable-isotope internal standards, d-DEX and dc-DOR, were prepared at Procter & Gamble Pharmaceuticals Health Care Facility (Mason, OH, USA) and Norwich Eaton Laboratories (Norwich, NY, USA), respectively. Methanol (HPLC grade), diethyl ether (ACS Reagent grade), sodium hydrogencarbonate ($NaHCO_3$, Reagent grade) and formic acid (Reagent grade) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Blank human plasma was obtained from volunteers at Procter & Gamble Pharmaceuticals Miami Valley Laboratories (Cincinnati, OH, USA), using heparin as the anticoagulant.

Preparation of standard solutions

Combined DEX and DOR stock standard solutions, and also separate combined d-DEX and dc-DOR stock standard solutions, were prepared at various concentrations in methanol–water (50:50, v/v) and stored at 4 °C. Combined DEX and DOR plasma calibration standards, covering a concentration range from 5 to 50000 pg ml^{-1}, were prepared on the day of analysis by adding 10 µl of the appropriate combined DEX and DOR stock standard solution to 1.0 ml of human plasma already containing 2 ng of d-DEX and dc-DOR. The standards were then prepared for analysis by LLE, as described below.

Preparation of DEX/DOR plasma control samples

A series of control plasma samples were prepared by spiking 10 ml of blank human plasma with an appropriate aliquot of a combined DEX and DOR stock standard solution to yield final plasma DEX and DOR concentrations of 10, 20, 50, 200, 1000, 5000 and 20000 pg ml^{-1}. Aliquots (1.0 ml) of the spiked samples, and of control blank plasma samples, were added to Pyrex test-tubes containing 2 ng of d-DEX and dc-DOR and prepared for analysis by LLE, as described below.

LLE sample preparation

Unknown, control samples and calibration standards were prepared for analysis by LLE. Aliquots (1.0 ml) of the plasma samples, spiked with the internal standards, were added to separate screw-top test-tubes already containing 1 ml of a 0.1 M $NaHCO_3$ (pH 10.5) buffer and vortex mixed. Diethyl ether (3 ml) was added to each test-tube; the test-tubes were capped with a Teflon-lined cap and the samples extracted by hand inversion for 5 min. The ether layer was removed from each sample, placed in separate test-tubes and dried under nitrogen. The plasma samples were extracted a second time using 2 ml of diethyl ether. The ether layers were again isolated and added to the appropriate test-tubes containing the previously dried ether extracts. The combined ether extracts were then back-extracted with 0.2 ml of a 1% aqueous formic acid solution by continuous hand inversion for 5 min. The aqueous layers were then isolated, placed in small-volume autosampler vials and injected directly.

Relative recovery of DEX and DOR from LLE

A blank human plasma sample (10 ml) was prepared to contain 500 pg ml^{-1} of DEX and DOR. Replicate ($n = 5$) aliquots (1.0 ml) of this sample were then extracted by the LLE procedure described above, except that the internal standards were not added to the plasma samples. The final 1% formic acid extract for each sample was subsequently spiked with the internal standards, 2 ng each, and analyzed by the LC/MS/MS method to determine the relative recovery of DEX and DOR.

Stability of DEX and DOR—whole blood, plasma and freeze–thaw

Whole blood (50 ml) was spiked to contain 500 pg ml^{-1} DEX and DOR and replicate ($n = 5$) ali-

quots (3.0 ml) of the spiked whole blood were removed immediately. Similarly, replicate ($n = 5$) aliquots (3.0 ml) of the whole blood sample were withdrawn after incubating at ambient temperature for 0.5 and 3.5 h. All whole blood aliquots were immediately centrifuged after sampling to yield plasma. Aliquots (1.0 ml) of the plasma obtained from each sample were added to test-tubes containing 2 ng of d-DEX and dc-DOR and mixed by inversion. The samples were prepared for analysis by LLE as described above. A large pool of blank plasma was spiked to contain DEX and DOR at the 500 pg ml^{-1} level. Replicate ($n = 4$) aliquots (1.0 ml) of this sample were withdrawn, immediately and after sitting at ambient temperature for 4 h, and added to separate screw-top test-tubes already containing 2 ng of d-DEX and dc-DOR. The samples were then prepared for analysis by LLE as described above.

Additionally, a portion of the same sample was subjected to three freeze–thaw cycles. Following the third cycle, replicate ($n = 4$) aliquots (1.0 ml) were added to separate screw-top test-tubes already containing 2 ng of d-DEX and dc-DOR. The samples were prepared for analysis by LLE as described above.

Stability of DEX and DOR—LLE extract

The stability of DEX and DOR in the final LLE solvent (1% aqueous formic acid) obtained from the extraction of plasma spiked with 500 pg ml^{-1} of each analyte was examined. The samples ($n = 30$) and standard extracts were analyzed immediately and then after storage at $-20\,^{\circ}C$ for 5 days.

LC/MS/MS conditions

A Waters (Milford, MA, USA) Model 616 HPLC system, a PE-Sciex (Thornhill, Ontario, Canada) API III-Plus triple-quadrupole mass spectrometer and a Gilson (Middletown, WI, USA) Model 234 autosampler were used with a Waters Symmetry C_8 column (2.1 × 50 mm, 3.5 μm) for LC/MS/MS analysis. The mobile phases for DEX and DOR were water–methanol–formic acid (67:33:0.1, v/v/v) and water–methanol–formic acid (77:23:0.1, v/v/v), respectively. The flow rate and injection volume for each method were 300 μl min^{-1} and 20 μl, respectively. Samples and standards with DEX or DOR concentrations of $\geqslant 20$ ng ml^{-1} were diluted 3:1 with 1% formic acid prior to analysis. The entire chromatographic effluent was passed into the mass spectrometer interface for subsequent detection. Under these conditions, the HPLC retention times for DEX and DOR were each ~ 1.1 min.

The mass spectrometer was operated in the Turbo-IonSpray configuration, consisting of the articulated IonSpray inlet used in conjunction with the heated TurboProbe desolvation unit. The TurboProbe temperature and nitrogen gas flow rate were 500 °C and 8 l min^{-1}, respectively, and the nebulizer gas pressure was 54 psi (nitrogen). Protonated analyte ions were generated using ESI and orifice potentials of 4000 and 70 V, respectively. Collisionally activated dissociation (CAD) was achieved using argon as the collision gas, at a thickness of 300×10^{13} molecules cm^{-2} and a collision energy of 30 and 27 eV for DEX and DOR, respectively. The SRM transitions m/z 272 to 147 and m/z 275 to 150 were sequentially monitored for detection of DEX and d-DEX, respectively, while the SRM transitions m/z 258 to 199 and m/z 262 to 199 were sequentially monitored for detection of DOR and dc-DOR, respectively. The dwell time for each transition was 200 ms. Peak area ratios for the chromatographic peaks were determined using the PE-Sciex software package MacQuan, Version 1.4.

Quantitation of DEX and DOR

Calibration curves were constructed by plotting peak area ratios (DEX/d-DEX) for standards versus DEX concentration and fitting these data to a weighted ($1/x^2$) linear regression line within the MacQuan software package. Drug concentrations in test samples were then interpolated from this line. The concentrations of DOR in plasma samples were determined analogously.

Human dosing protocol

A healthy male volunteer, aged 39, was fasted overnight and fitted with an in-dwelling intravenous catheter for blood sampling. A blank blood sample (10 ml) was then obtained from the subject. Following the blood sampling, the subject received a single 15 ml peroral dose of commercially purchased Vicks 44 cough syrup (Procter & Gamble, Cincinnati, OH, USA) containing 30 mg of DEX hydrobromide. Blood samples (10 ml) were subsequently obtained at 0.25, 0.50, 1, 1.5, 2, 4, 6, 8 and 24 h post-dose. The blood was immediately placed on ice and then processed by centrifugation to yield the plasma. The resulting plasma samples were then stored in Pyrex tubes with Teflon-lined caps at $-70\,^{\circ}C$ until analysis. On the day of analysis, each sample tube was removed from storage, allowed to warm to room temperature and mixed by repeated gentle inversion. An aliquot (1.0 ml) of each sample was then added to a Pyrex test-tube already containing 2 ng of d-DEX and dc-DOR, mixed by gentle inversion and then prepared for LC/MS/MS analysis by LLE as described above.

RESULTS AND DISCUSSION

ESI mass spectra

The ESI mass spectra obtained for DEX, d-DEX, DOR and dc-DOR were dominated by intense pseudomolecular ions at m/z 272, 275, 258 and 262, respectively. The spectra for the analyte and stable-isotope pairs, DEX–d-DEX and DOR–dc-DOR, were similar except for the m/z shifts due to the incorporation of the stable-isotope labels. The product ion spectra obtained for DEX–d-DEX and DOR–dc-DOR pairs following CAD of their respective protonated molecular ions are

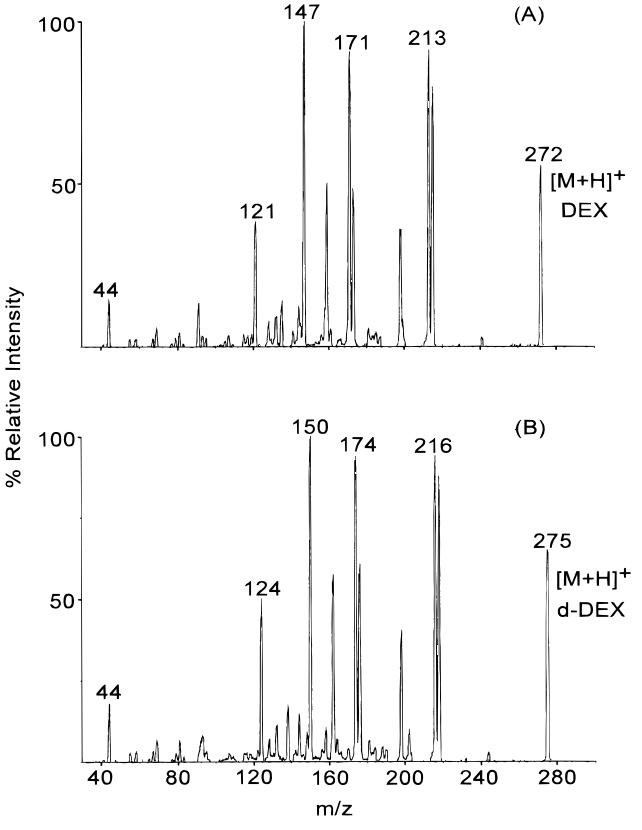

Figure 2. Electrospray ionization product ion spectra of (A) DEX and (B) d-DEX.

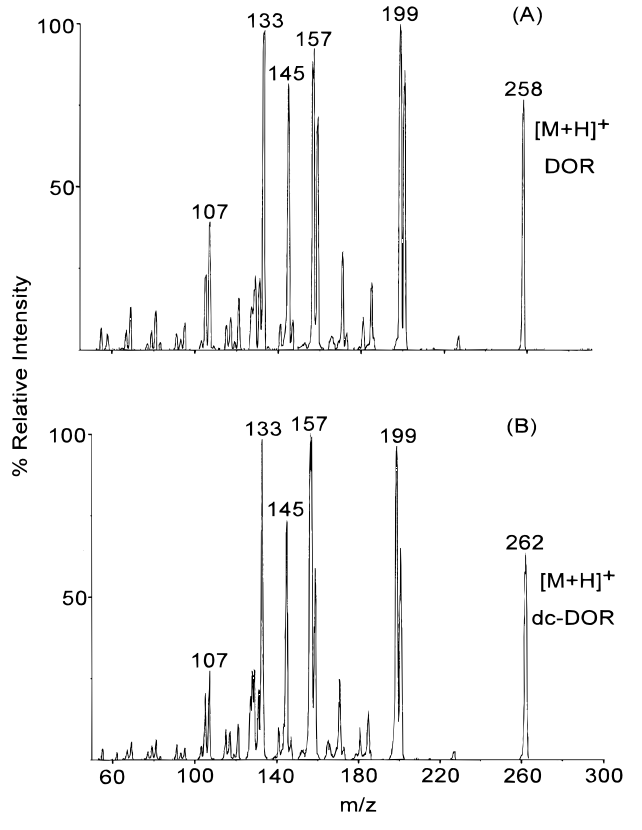

Figure 3. Electrospray ionization product ion spectra of (A) DOR and (B) dc-DOR.

shown in Figs 2 and 3, respectively. The fragment ion spectra for DEX and d-DEX contained several prominent ions retaining the stable isotope-labeled structural moiety. However, for DOR and dc-DOR, the majority of the prominent product ions did not contain the isotopic label. The SRM transition schemes chosen for DEX determination were *m/z* 272 to 147 and *m/z* 275 to 150 for DEX and d-DEX, respectively. The common product ion transitions *m/z* 258 to 199 and *m/z* 262 to 199 for were chosen for the determination of DOR and dc-DOR, respectively.

Relative recoveries of DEX and DOR from LLE

The relative recoveries of DEX and DOR from a 0.5 ng ml^{-1} spiked plasma sample using the LLE procedure were found to be 84 and 88%, respectively, with a relative standard deviation (RSD) of 10% for each analyte. Near quantitative recovery of DEX and DOR was achieved using the simple LLE with diethyl ether, followed by a back-extraction into a small volume of 1% aqueous formic acid. The back-extraction into a small volume of 1% aqueous formic acid provided a clean, pre-concentrated extract and allowed the direct injection of the extract without further manipulation that may be required with other preparation techniques such as solid-phase extraction or protein precipitation. Since the generation of the original validation data, this procedure has been modified by the elimination of the second ether extract and the addition of an automated shaker to perform the extractions. These improvements have resulted in a much faster sample preparation.

LC/MS/MS plasma profiles—DEX and DOR

The mobile phase conditions were optimized for each analyte so that DEX and DOR eluted in ~1.1 min, with k' values of ~3. LC/MS/MS profiles for DEX and DOR generated under typical analysis conditions are shown in Fig. 4 for blank human plasma and blank human plasma containing 5 pg ml^{-1} of each analyte. Sample analysis was more time efficient using two separate methods than if a single isocratic or gradient method was employed. Even under these rapid analysis conditions, blank human plasma presented no interferences in the DEX and DOR retention time regions. Similarly, chromatograms of blank plasma using conditions for d-DEX and dc-DOR analysis were free from interferences from endogenous materials (data not shown).

Typical chromatographic profiles generated during the analysis of study samples are shown in Fig. 5. The sample used to generate these chromatograms was obtained at 60 min after a 30 mg peroral DEX dose and contained 180 pg ml^{-1} of DEX and 1.2 ng ml^{-1} of DOR.

Calibration curves

The calibration curves were linear over four orders of magnitude, with the correlation coefficients for the cali-

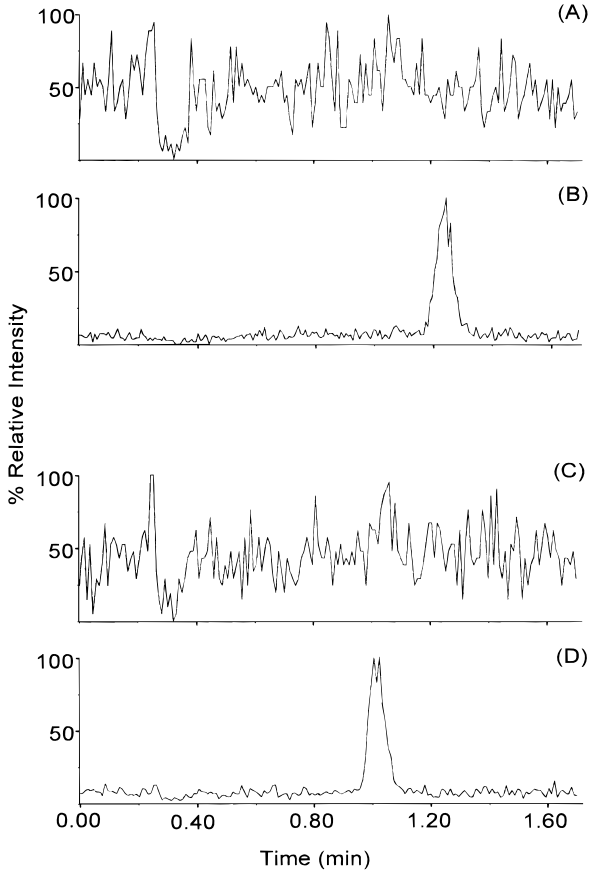

Figure 4. LC/MS/MS SRM profiles for DEX from 1 ml of (A) blank human plasma and (B) blank human plasma spiked with 5 pg ml^{-1} of DEX. Profiles are also shown for DOR from 1 ml of (C) blank human plasma and (D) blank human plasma spiked with 5 pg ml^{-1} of DOR.

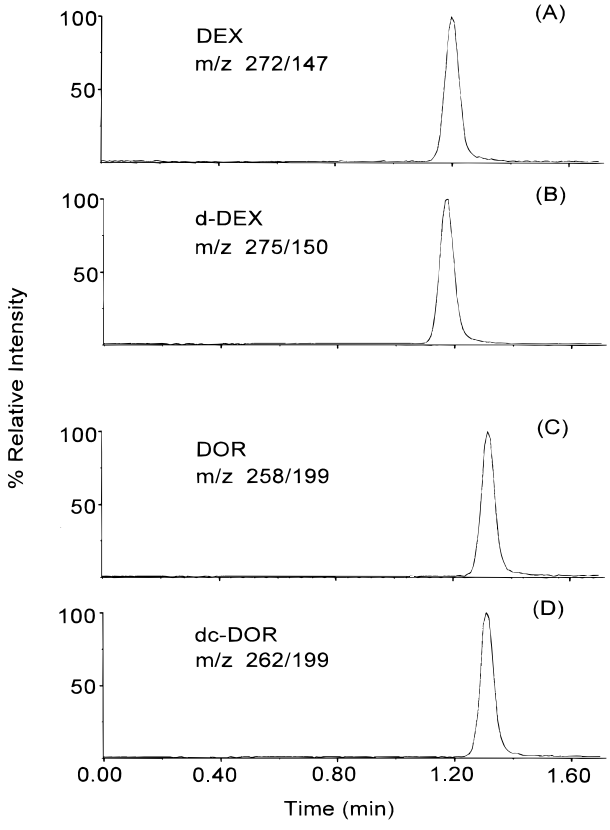

Figure 5. LC/MS/MS traces for (A) DEX, (B) d-DEX, (C) DOR and (D) dc-DOR generated by analysis of a study plasma sample collected 60 min after peroral dosing with 30 mg of DEX hydrobromide. This sample contained 180 pg ml^{-1} of DEX, 1.2 ng ml^{-1} of DOR and 2 ng ml^{-1} of d-DEX and dc-DOR.

bration regression lines being typically 0.995 or greater. The average recoveries (measured level/spiked level × 100) for the calibration standards used to generate the spike and recovery data on days 1 and 2 are summarized in Table 1. Typically, the average recoveries are 100 ± 10%. Recoveries of more than 100% may be due to spiking errors of either the analytes or the internal standards during the preparation of the samples and standards or to statistical variation. Replicate ($n = 5$) injections of the 50 pg ml^{-1} and 1 ng ml^{-1} calibration standards resulted in RSD values of <3.5% for both DEX and DOR.

Table 1. Accuracy of DEX and DOR calibration standards

Spiked level (pg ml^{-1})	DEX recovery (%)	DOR recovery (%)
5	105	101
10	90	89
20	96	117
50	99	94
100	101	100
200	102	102
500	101	106
1000	105	103
2000	99	101
5000	104	99
10000	101	97
20000	97	96
50000	88	94

Accuracy and precision—analysis of spiked control samples

The accuracy and precision data for the LC/MS/MS analysis of blank human plasma spiked with DEX and DOR at various levels ($n = 8$) are presented in Tables 2 and 3, respectively. The accuracy across the entire range of spiked DEX and DOR concentrations was, in general, within 10% of the spiked value. The precision,

Table 2. Accuracy and precision for determination of DEX in spiked plasma samples

Spiked [DEX] (pg ml^{-1})	Day 1 Recovery (%)	Day 1 RSD (%)	Day 2 Recovery (%)	Day 2 RSD (%)
10	94	19.2	107	11.6
20	111	8.7	97	11.8
50	107	7.8	91	1.0
200	110	4.2	99	4.2
1000	106	1.8	94	4.7
5000	96	4.3	92	3.2
20000	95	1.3	88	2.1

Table 3. Accuracy and precision for determination of DOR in spiked plasma samples

Spiked [DOR] (pg ml^{-1})	Day 1		Day 2	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
10	119	9.4	109	12.8
20	118	5.0	102	6.2
50	101	3.5	97	10.2
200	107	2.3	99	5.0
1000	103	2.2	97	4.9
5000	99	1.3	91	4.4
20000	95	2.9	92	2.8

as assessed by the RSD for the replicate sample preparations, was generally better than 6% at levels of 50 pg ml^{-1} or higher and typically better than 12% at levels below 50 pg ml^{-1}.

Stability of DEX and DOR—blood, plasma and freeze–thaw

The stability of DEX and DOR in whole blood, at ambient temperature, was evaluated over a 3.5 h period using whole blood samples spiked with 500 pg ml^{-1} DEX and DOR. The concentrations of DEX and DOR found in whole blood incubated for 0.5 and 3.5 h at ambient temperature were compared with the values obtained from the same whole blood with no incubation. The recoveries for DEX after the 0.5 and 3.5 h incubations were 103 $\pm$ 4% and 98 $\pm$ 3%, respectively. Similarly, recoveries found for DOR after 0.5 and 3.5 h were 97 $\pm$ 2% and 94 $\pm$ 3%, respectively. The results indicate that both analogs are stable for at least 3.5 h in whole blood stored at ambient temperature.

Additionally, the stability of DEX and DOR in the plasma matrix at ambient temperature was examined by analyzing plasma samples spiked with both analytes (500 pg ml^{-1}) immediately after spiking and 4 h after spiking. The recoveries after 4 h at ambient temperature were 101 and 100% for DEX and DOR, respectively, with RSD values ($n = 5$) of $<10\%$. Therefore, the stability of the analytes in plasma was not an issue during the LLE sample preparation step.

The stability of both analytes to repeated freeze–thaw cycles was also examined using a spiked plasma sample (500 pg ml^{-1}). After three freeze–thaw cycles, the proportion of DEX and DOR remaining, relative to the initial analysis, was 112 and 94%, respectively, with RSD values $<7\%$. The plasma samples can, therefore, tolerate at least three freeze–thaw cycles without degradation of the analytes.

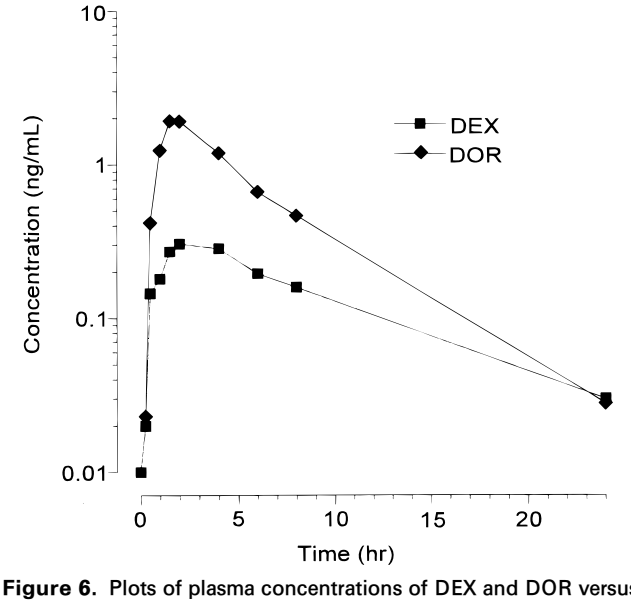

Figure 6. Plots of plasma concentrations of DEX and DOR versus post-dose collection time obtained from a human subject dosed perorally with 30 mg of a commercially available DEX hydrobromide cough formulation.

Stability of DEX and DOR in LLE extracts

The stability of DEX and DOR in the 1% formic acid back-extraction solvent was confirmed by analyzing extracts from plasma samples spiked with both analytes at the 500 pg ml^{-1} level. The samples ($n = 30$) and the corresponding standards were analyzed initially and after 5 days of storage at $-20\,^{\circ}$C. After 5 days of storage, the DEX and DOR values in the samples were found to be within 6 $\pm$ 4% and 4 $\pm$ 4% of their initial values, respectively. Once prepared for analysis by LLE, the samples may be analyzed immediately or after storage at $-20\,^{\circ}$C for up to 5 days.

Human DEX pharmacokinetic profile

Plots of the subject's plasma DEX and DOR levels versus post-dose sampling time, following a 30 mg peroral dose of DEX hydrobromide from a commercial cough formulation, are shown in Fig. 6. The subject was an extensive metabolizer, with the peak DEX plasma level being <500 pg ml^{-1}. Similarly, a low peak level of unconjugated DOR was found in the plasma. The high sensitivity of the LC/MS/MS methodology allowed the plasma levels of DEX and DOR to be followed over the entire 24 h plasma time course obtained from the subject.

CONCLUSION

The combination of a simple LLE sample preparation step with LC/MS/MS analysis on a short, high-resolution HPLC column resulted in a rapid, highly selective and sensitive method for the determination of DEX and DOR in human plasma. Injection-to-injection analysis times of <1.8 min and an LOQ of 5 pg ml^{-1}, >100-fold lower than with previous methods, were achieved. The improved LOQ enabled DEX and DOR plasma levels to be monitored for at least 24 h in plasma samples obtained from a human subject following PO dosing with a commercially available cough formulation.

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