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Journal of Chromatography B, 698 (1997) 147–154

JOURNAL OF CHROMATOGRAPHY B

Highly sensitive high-performance liquid chromatographic–tandem mass spectrometric method for the analysis of dextromethorphan in human plasma

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Received 7 March 1997; received in revised form 21 May 1997; accepted 22 May 1997

Abstract

A stable-isotope-dilution HPLC–tandem mass spectrometry-based method was developed for the determination of dextromethorphan in human plasma. Plasma samples were prepared for analysis by solid-phase extraction on octadecylsilane extraction cartridges. Dextromethorphan and the deuterium-labeled dextromethorphan internal standard were chromatographed on a short reversed-phase column and detected by a selected-reaction-monitoring scheme. Linear standard curves were obtained over three orders of magnitude and the limit of quantitation for dextromethorphan was 50 pg/ml, using a 1-ml plasma sample. The combination of HPLC and electrospray tandem mass spectrometry resulted in a rapid, selective and sensitive method for the analysis of dextromethorphan in plasma. The method was applied for the evaluation of the pharmacokinetic profile of dextromethorphan in human volunteers following peroral administration. © 1997 Elsevier Science B.V.

Keywords: Dextromethorphan

1. Introduction

Dextromethorphan (DEX, Fig. 1) is the active ingredient in many over-the-counter cough formulations sold in the United States and Europe. In most individuals, DEX is extensively metabolized by a first-pass metabolic effect, resulting in low systemic peak levels of DEX in plasma, typically in the 1–5-ng/ml 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 range [4–6]. The low systemic levels of DEX achieved in most individuals require a highly sensitive method for the determination of DEX plasma levels. A variety of methods have been

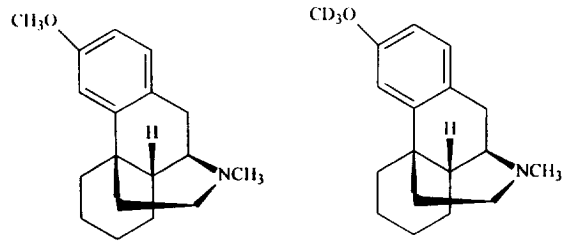

Fig. 1. The chemical structures of dextromethorphan (DEX) and $^{2}H_{3}$-methoxydextromethorphan (d-DEX).

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0378-4347/97/$17.00
PII S0378-4347(97)00308-3

employed for the analysis of DEX in plasma, urine and saliva, including direct-fluorescence spectrometry [7], HPLC with fluorescence detection [8–11], HPLC with ultraviolet (UV) detection [12], gas chromatography (GC) with nitrogen-phosphorous and mass spectrometric detection [4,6,13], capillary zone electrophoresis with UV detection [14] and radioimmunoassay (RIA, [15]). The HPLC, GC and RIA methods have reported limits of detection (LOD) of 0.5, 1 and 2 ng/ml, respectively, when using 1-ml or larger volumes of plasma. The LODs achievable with these methods are marginal for determining the pharmacokinetic profile of DEX following peroral (p.o.) administration. The use of HPLC with continuous fast-atom bombardment mass spectrometry was also investigated for the analysis of DEX in plasma. However, the 100-ng/ml LOD achieved was clearly not useful for DEX plasma analysis [16].

We report on the development of a stable-isotope-dilution-based HPLC electrospray ionization (ESI) tandem mass spectrometry (HPLC–MS–MS) method for the analysis of DEX in human plasma. DEX and the internal standard, $^{2}H_{3}$-methoxydextromethorphan (d-DEX, Fig. 1), are isolated from plasma by solid-phase extraction (SPE). DEX and d-DEX are then chromatographed on a reversed-phase column and detected using ESI, followed by a selected-reaction-monitoring (SRM) scheme. In the SRM scheme, a parent ion is isolated by the first quadrupole and undergoes collisionally-activated dissociation (CAD) in the second quadrupole. A resulting daughter ion, characteristic of the analyte of interest, is then isolated by the third quadrupole for detection. The HPLC–MS–MS method has at least a 10-fold lower limit of quantitation (LOQ) for DEX in plasma, 50 pg/ml, than previously reported methods. In addition, the MS–MS-based approach provides a rapid analysis, typically less than 2 min, with unparalleled selectivity for DEX in the human plasma matrix.

2. Experimental

2.1. Chemicals and reagents

DEX hydrobromide was obtained from the United States Pharmacopeial Convention (Rockville, MD, USA) and *N*-^{3}H-methyldextromethorphan (^{3}H-DEX, 85 Ci/mmol) was obtained from DuPont NEN (Boston, MA, USA). The d-DEX was prepared at Procter & Gamble Pharmaceuticals Health Care Research Center (Mason, OH, USA). Methanol (HPLC grade) and formic acid (reagent grade) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Triethylamine (reagent grade) was purchased from Aldrich Chemical (Milwaukee, WI, USA). Blank human plasma was obtained from volunteers at Procter & Gamble Pharmaceuticals Health Care Research Center, using EDTA as the anticoagulant.

2.2. Preparation of standards

Stock solutions of DEX and d-DEX were prepared at various concentrations in methanol and stored at −20°C. DEX plasma standards, covering a concentration range from 0.025 to 25 ng/ml, were prepared on the day of analysis by adding 10 μl of the appropriate DEX stock solution to 1.0 ml of human plasma already containing 1 ng of d-DEX. The standards were then prepared for analysis by SPE, as described below.

2.3. Preparation of DEX plasma control samples

A series of control plasma samples were prepared by spiking 10 ml of blank human plasma with the appropriate volume of a DEX stock solution to yield final plasma DEX concentrations of 0.05, 0.20, 1.0, 2.0 and 10 ng/ml. Aliquots (1.0 ml) of the spiked samples, as well as control blank plasma samples, were added to Pyrex test tubes containing 1 ng of d-DEX and then prepared for analysis by SPE, as described below.

2.4. Solid-phase extraction sample preparation

Unknown and control samples, as well as standards, were prepared for analysis by SPE using octadecylsilane (ODS) cartridges. SPE was performed on a Speed Wiz semiautomated instrument (Applied Separations, Allentown, PA, USA) using Isoelute (Jones Chromatography, Lakewood, CO, USA) ODS cartridges (3 ml, 200 mg). The ODS cartridges were conditioned with 2 ml methanol followed by 2 ml of water–methanol (97:3, v/v) at a flow-rate of 20 ml/min. The plasma samples were

then loaded at a rate of 0.5 ml/min. Following sample loading, the cartridges were washed with 2 ml of water–methanol (97:3, v/v) followed by 2 ml of water–methanol (75:25, v/v) at a flow-rate of 20 ml/min. The samples were then eluted into 16×100-mm polypropylene test tubes using 4 ml of water–methanol–triethylamine–formic acid (20:80:0.5:0.32, v/v) at a flow-rate of 4 ml/min. The elution solvent was removed in a TurboVap evaporator (Zymark, Hopkinton, PA, USA) using 172 kPa nitrogen and an evaporation temperature of 35°C. In general, the residue was reconstituted in 100 ml of water–methanol (90:10, v/v) and placed into autosampler vials.

2.5. Absolute recovery of DEX from SPE

A blank human plasma sample (10 ml) was prepared to contain 0.42 ng/ml of ^{3}H-DEX (280 000 dpm/ml) and an additional blank human plasma sample (10 ml) was prepared to contain 5 ng/ml of DEX plus 0.42 ng/ml of ^{3}H-DEX (280 000 dpm/ml). Aliquots (1.0 ml) of each sample were applied to separate SPE cartridges (n=4 each) and the extraction procedure performed as described above. Aliquots of the SPE effluents and the final reconstituted SPE samples, as well as the direct analysis of the spiked plasma samples, were analyzed by liquid scintillation counting on a Packard Model 2000CA liquid scintillation analyzer (Packard Tri-Carb, Downers Grove, IL, USA). The absolute recovery was determined by comparing the results obtained from the SPE samples to those obtained by the direct analysis of an aliquot of the spiked plasma samples.

2.6. HPLC–MS–MS conditions

A Waters 616 HPLC system (Milford, MA, USA), a PE-Sciex API III-*plus* triple quadrupole mass spectrometer (Thornhill, Ontario, Canada), and a Gilson 234 autosampler (Middletown, WI, USA) were used with a Waters Symmetry C_{18} column (2.1×50 mm, 3.5 μm) for HPLC–MS–MS analysis. The injected sample volume was typically 10 μl (10% of sample). The mobile phase was water–methanol–formic acid (40:60:0.1, v/v) and the flow-rate was 300 μl/min. The entire chromatographic effluent was passed into the mass spectrometer interface for subsequent detection. Under these conditions, the HPLC retention time for DEX was typically under 2 min.

The mass spectrometer was operated in the TurboIonSpray 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 580°C and 7.5 l/min, respectively, and the nebulizer gas pressure was 300 kPa (nitrogen). Protonated analyte ions were generated using ESI and orifice potentials of 4000 and 75 V, respectively. The MS–MS detection scheme utilized CAD and SRM. CAD was accomplished using argon as the collision gas, at a thickness of 280×10^{12} molecules/cm^{2}, and an ion energy of 25 eV. The SRM transitions, m/z 272–147 and m/z 275–150, were sequentially monitored for detection of DEX and d-DEX, respectively. Dwell time for each transition was 180 ms. Peak area ratios for the SRM chromatographic peaks were determined using the PE-Sciex software package, MacQuan.

2.7. Quantitation of DEX

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.

2.8. Human dosing protocol

Three healthy male volunteers, ages 39–45, were fasted overnight and were fitted with an in-dwelling intravenous catheter for blood sampling. A blank blood sample (10 ml) was then obtained from each subject. Following the blood sampling, each subject received a single 15-ml p.o. dose of commercially purchased Vicks 44 (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°C until analysis. On the day of analysis, each sample tube was removed from storage, allowed to warm to room

temperature and was then mixed by repeated gentle inversion. An aliquot (1.0 ml) of each sample was then added to a Pyrex test tube already containing 1 ng of d-DEX, mixed by gentle inversion and then prepared for HPLC–MS–MS analysis by SPE as described above.

2.9. Pharmacokinetic method

Pharmacokinetic parameters were calculated by non-compartmental techniques using Pharm-NCA© (Simed S.A., Créteil, France).

3. Results

3.1. Absolute recovery of DEX

The absolute recovery of DEX in the SPE effluent and the reconstituted SPE extract was determined using ^{3}H-DEX-spiked plasma. The absolute recovery of DEX in the effluent from the SPE cartridges was found to be 87±3.2 and 85±6.0% for plasma samples containing 0.42 and 5.4 ng/ml DEX, respectively. The absolute recovery of DEX in the reconstituted SPE extract was slightly lower, 72±3.0 and 67±11.5% for the 0.42- and 5.4-ng/ml DEX samples, respectively. If glass test tubes were used for the collection and drying process, the recovery of DEX was found to be even lower, suggesting DEX may stick to the glass surface.

3.2. ESI mass spectra

The ESI mass spectra obtained for DEX and d-DEX are shown in Fig. 2. These spectra are characterized by an intense protonated molecular ion at *m/z* 272 and 275 for DEX and d-DEX, respectively. The two spectra are identical except for the *m/z* shifts due to the mass differences between DEX and its stable-isotope-labeled analog. The daughter mass spectra obtained for DEX and d-DEX, following CAD of their respective protonated molecular ions, are shown in Fig. 3. The daughter spectra of both

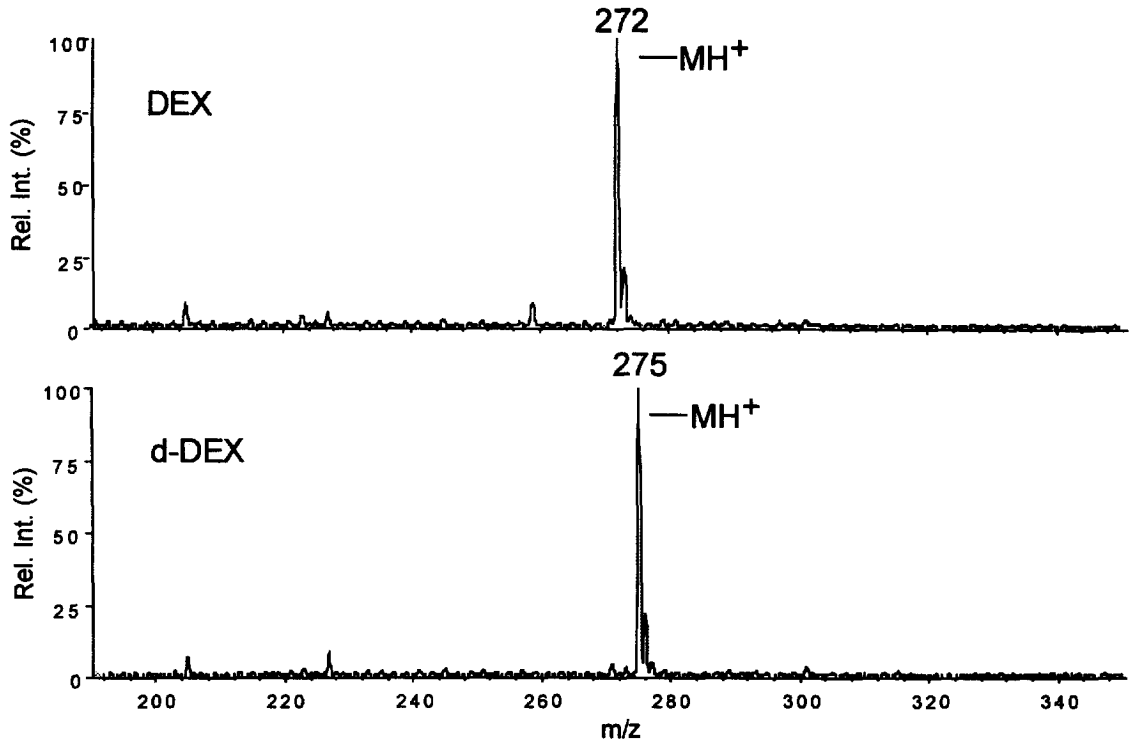

Fig. 2. Electrospray ionization mass spectra of: DEX (top) and d-DEX (bottom).

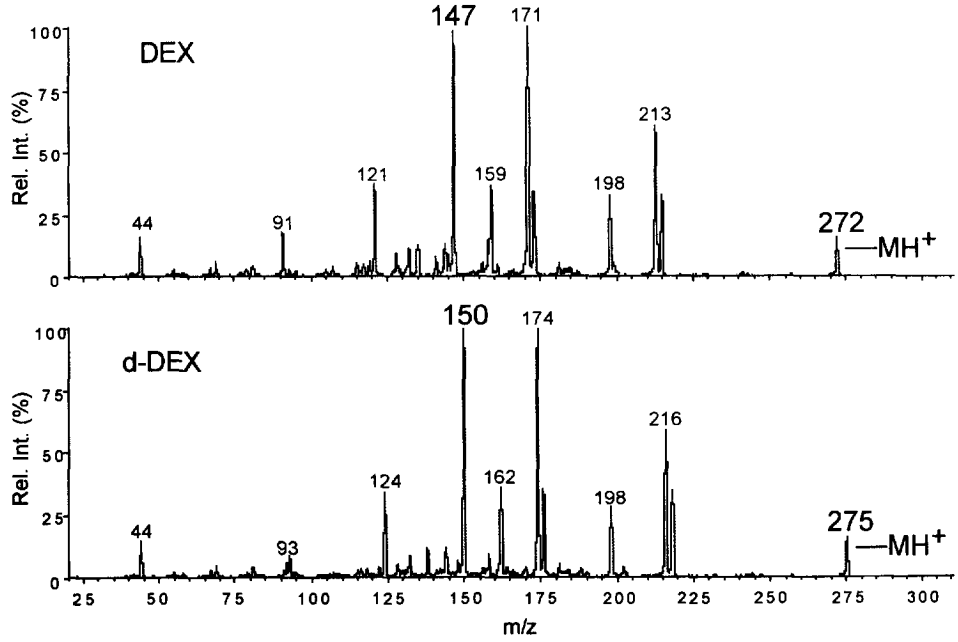

Fig. 3. Electrospray ionization tandem mass spectra (daughters-of-MH^+) of: DEX (top) and d-DEX (bottom).

DEX and d-DEX contained several prominent ions, with retention of the stable-isotope-labeled structural moiety. Several potentially useful SRM schemes could be employed based on these daughter spectra. However, the SRM transition schemes chosen for this work were *m/z* 272–147 and *m/z* 275–150 for DEX and d-DEX, respectively.

3.3. Chromatographic profiles of blank and DEX-spiked human plasma

HPLC–MS–MS chromatographic profiles obtained using the selected SRM schemes for blank plasma, blank plasma plus 1 ng/ml d-DEX, and blank plasma containing 0.1 ng/ml DEX plus 1 ng/ml d-DEX are shown in Fig. 4a–c, respectively. DEX eluted from the column in less than 2 min, with a k' value of about 3.0 and with reasonably symmetrical peak shape. Even under these rapid analysis conditions, blank human plasma was free of interferences in the DEX retention time region. Similarly, the signal for the SRM scheme of d-DEX was free of interferences from endogenous materials (Fig. 4a). The combination of MS-MS and a short, high-resolution HPLC column allowed the rapid and selective analysis of DEX in human plasma. The major DEX plasma metabolites: dextrorphan, 3-methoxymorphinan, 3-hydroxymethylmorphinan [17], did not interfere with the analysis since they were resolved from DEX by the HPLC and were not detectable using the SRM scheme for DEX (data not shown).

3.4. Standard curve

Standard curves were linear over three orders of magnitude, with the correlation coefficients for calibration regression lines being typically 0.999 or greater. Replicate ($n=5$) injections of the 0.1- and the 1.0-ng/ml standards resulted in %R.S.D. values of less than 3%. The *S/N* ratio obtained for the DEX peak from the 50-pg/ml standard was greater than 5 and represents the LOQ under the present method conditions.

3.5. Analysis of spiked control samples

The accuracy and precision data for the HPLC–MS-MS analysis of blank human plasma spiked with DEX at various levels are presented in Table 1. The

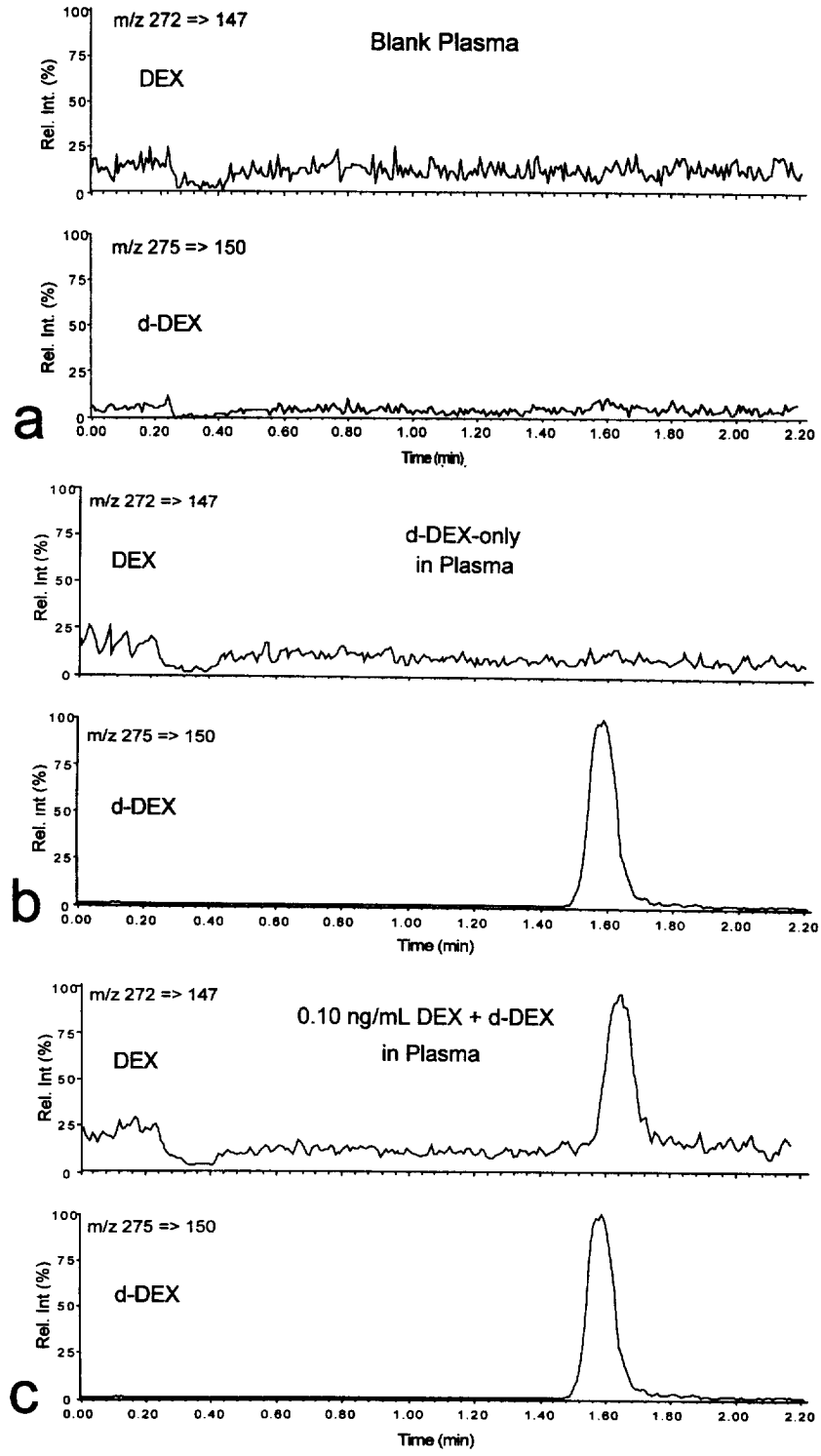

Fig. 4. HPLC–MS–MS chromatographic profiles corresponding to DEX (top) and d-DEX (bottom) for 1-ml human plasma samples containing the following: (a) blank; (b) 1 ng/ml d-DEX only; and (c) 0.1 ng/ml DEX plus 1 ng/ml d-DEX.

Table 1
Accuracy and precision of DEX plasma analysis

Spiked (DEX) (ng/ml)	*n*	Recovery (%)	R.S.D. (%)
0.05	5	98.6	9.2
0.10	5	96.5	3.8
0.20	5	102.3	6.3
1.0	7	103.6	1.8
2.0	4	102.0	1.8
5.0	3	101.5	2.1
10	4	110.8	4.3

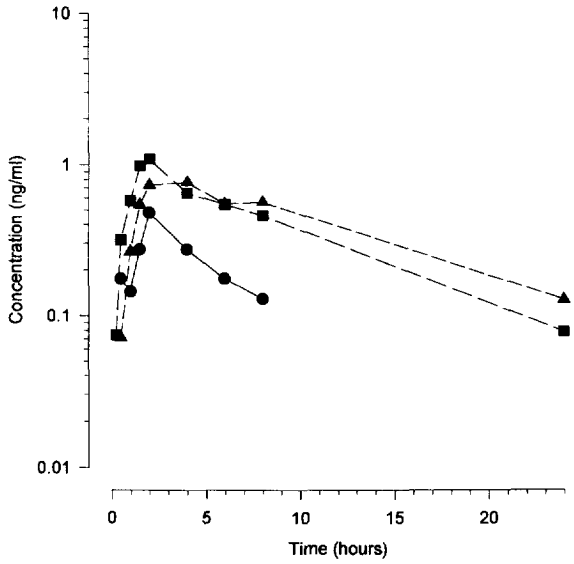

Fig. 5. Profile of DEX plasma concentration versus post-dose sampling time for subjects dosed perorally with 30 mg DEX hydrobromide from a commercial cough formulation.

accuracy across the entire range of spiked concentrations was, in general, within 4% of the target value. The precision, as assessed by the R.S.D. for the replicate analysis, was less than 10% at all levels, and was typically less than 5%. Attempts to inject larger sample volumes or to further concentrate extracts were unsuccessful in further lowering the LOQ. Matrix components isolated during the sample preparation procedure caused degradation of the HPLC peak shape, as well as ion suppression effects during the ESI process, when larger volumes of sample were injected. It is likely that an improved sample preparation scheme may result in an improvement in the LOQ.

3.6. Human DEX pharmacokinetic profile

Plots of the plasma DEX levels versus post-dose sampling time for three subjects, following a p.o. dose of 30 mg DEX hydrobromide from a commercial cough formulation, are shown in Fig. 5. Pharmacokinetic parameters calculated from these data are shown in Table 2. Clearly, all three subjects were classed as fast metabolizers based on the low C_{max} values obtained for DEX.

4. Conclusion

The combination of SPE sample preparation with HPLC–MS–MS analysis on a short, high-resolution column resulted in a rapid, selective and sensitive method for the determination of DEX in human plasma. Sample analysis times of less than 2 min and

Table 2
DEX human pharmacokinetic parameters

Subject	K_e (h^{-1})	$T_{1/2}$ (h)	MRT (h)	C_{max} (ng/ml)	T_{max} (h)	AUC_∞ (ng-h/ml)	Clr. (l/h/f)	$V_{d(ss)}$ (l/f)
1	0.22	3.177	5.2	0.476	2	2.921	10 271	52 926
2	0.11	6.400	8.7	1.082	2	10.051	2985	25 828
3	0.09	7.970	11.5	0.758	4	11.427	2625	30 155
Mean	0.14	5.85	8.4	0.772	2.7	8.1	5294	36 303
S.D.	0.07	2.44	3.2	0.303	1.2	4.6	4314	14 557
%R.S.D.	51.09	41.78	37.6	39.280	43.3	56.1	81	40

K_e=elimination rate constant; $T_{1/2}$=half-life; MRT=mean residence time; C_{max}=maximum observed plasma concentration; T_{max}=observation time of maximum concentration; AUC=area under plasma concentration curve; Clr.=clearance; $V_{d(ss)}$=volume of distribution.

a LOQ of 50 pg/ml, greater than 10-fold lower than previous methods, were achieved. The improved LOQ enabled DEX plasma levels to be monitored for at least 24 h in plasma samples obtained from human subjects following p.o. dosing with a commercially available cough formulation.

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