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Development and validation of a highly sensitive LC-MS/MS method for quantitation of dexlansoprazole in human plasma: application to a human pharmacokinetic study

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ABSTRACT: A highly sensitive, specific and simple LC-MS/MS method was developed for the simultaneous estimation of dexlansoprazole (DEX) with 50 µL of human plasma using omeprazole as an internal standard (IS). The API-4000 LC-MS/MS was operated under multiple reaction-monitoring mode using electrospray ionization. A simple liquid–liquid extraction process was used to extract DEX and IS from human plasma. The total run time was 2.00 min and the elution of DEX and IS occurred at 1.20 min. This was achieved with a mobile phase consisting of 0.2% ammonia–acetonitrile (20:80, v/v) at a flow rate of 0.50 mL/min on an X-terra RP 18 (50 × 4.6 mm, 5 µm) column. The developed method was validated in human plasma with a lower limit of quantitation of 2 ng/mL for DEX. A linear response function was established for the range of concentrations 2.00–2500.0 ng/mL ($r > 0.998$) for DEX. The intra- and inter-day precision values for DEX met the acceptance criteria as per FDA guidelines. DEX was stable in the battery of stability studies, viz. bench-top, auto-sampler and freeze–thaw cycles. The developed assay method was applied to an oral bioequivalence study in humans. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: DEX; LC-MS/MS; method validation; human plasma; pharmacokinetics

Introduction

Dexlansoprazole (DEX; CAS no. 138530-94-6; Fig. 1), chemically (*R*)-(+) 2-{[3-methyl-4-(2, 2, 2-trifluoroethoxy) pyridin-2-yl] methylsulfinyl}-1H-benzo[d]imidazole, is a proton pump inhibitor that is marketed under the brand name Dexilant (Kapidex was renamed Dexilant in the USA to avoid name confusion). Chemically, it is an enantiomer of lansoprazole. It is available commercially as delayed release capsules (30 and 60 mg; www.druglib.com/druginfo/kapidex/). DEX is the newest addition to the proton pump inhibitor (PPI) class and is approved for heartburn associated with symptomatic nonerosive gastroesophageal reflux disease (GERD), the healing of erosive esophagitis (EE) and the maintenance of healed EE. DEX is a PPI that suppresses gastric acid secretion by specific inhibition of (H^+, K^+)-ATPase in the gastric parietal cell. DEX MR is a Dual Delayed Release (DDR) formulation of DEX, with two distinct drug release periods to prolong the plasma DEX concentration–time profile and extend the duration of acid suppression. Clinical studies show that DEX MR produces a dual-peak pharmacokinetic profile that maintains therapeutic plasma drug concentrations longer than lansoprazole, with a single-peak pharmacokinetic profile, and increases the percentage of time that intragastric pH is >4 (Metz *et al.*, 2009a; Sharma *et al.*, 2009).

DEX is extensively metabolized in the liver by oxidation, reduction and subsequent formation of sulfate, glucuronide and glutathione conjugates to inactive metabolites. Oxidative metabolites are formed by the cytochrome P450 (CYP) enzyme system, including hydroxylation mainly by CYP2C19, and oxidation to the sulfone by CYP3A4.

CYP2C19 is a polymorphic liver enzyme that exhibits three phenotypes in the metabolism of CYP2C19 substrates; extensive metabolizers (*1/*1), intermediate metabolizers (*1/mutant) and poor metabolizers (mutant/mutant). DEX is the major circulating component in plasma regardless of CYP2C19 metabolizer status. In CYP2C19 intermediate and extensive metabolizers, the major plasma metabolites are 5-hydroxy DEX and its glucuronide conjugate, while in CYP2C19 poor metabolizers DEX sulfone is the major plasma metabolite. In food-effect studies in healthy subjects receiving DEX under various fed conditions compared with fasting, increases in C_{max} ranged from 12 to 55%, increases in AUC ranged from 9 to 37%, and t_{max} varied (ranging from a

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Abbreviations used: CYP, cytochrome P450; DEX, dexlansoprazole; ER, erosive esophagitis; GERD, gastroesophageal reflux disease; PPI, proton pump inhibitor.

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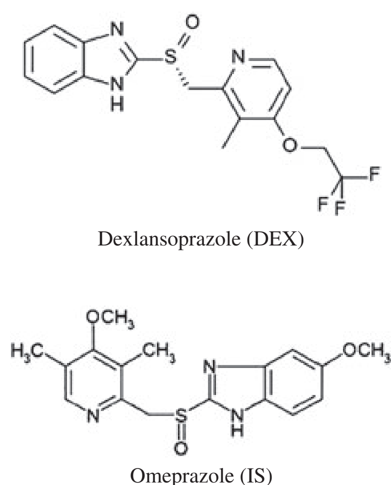

Figure 1. Structural representation of dexlansoprazole and omeprazole.

decrease of 0.7 h to an increase of 3 h). No significant differences in mean intragastric pH were observed between fasted and various fed conditions. However, the percentage time that intragastric pH exceeded 4 over the 24 h dosing interval decreased slightly when DEX was administered after a meal (57%) relative to fasting (64%), primarily owing to a decreased response in intragastric pH during the first 4 h after dosing. Because of this, while DEX can be taken without regard to food, some patients may benefit from administering the dose prior to a meal if post-meal symptoms do not resolve under post-fed conditions (Lee *et al.*, 2009; Sharma *et al.*, 2009; Zhang *et al.*, 2007; Vakily *et al.*, 2009)

DEX has a unique dual delayed-release formulation, which releases the drug at two time points: the first peak occurs 1–2 h after administration and the second occurs within 4–5 h after administration (Metz *et al.*, 2009a; Aslam and Wright, 2009).

A significant number of analytical HPLC methods have been described for the determination of lansoprazole enantiomers in biological fluids such as plasma, serum and liver microsomes (Katsuki *et al.*, 1996, 2001; Borner *et al.*, 1998; Masa *et al.*, 2001; Kim *et al.*, 2002; Miura *et al.*, 2004). A two-dimensional HPLC method has also been used for the enantiomeric content of DEX in biological matrices by direct injection (Gomes *et al.*, 2010). Following its improved pharmacokinetic behavior compared with the *R* isomer, i.e. lansoprazole (Aslam and Wright, 2009), no established quantification methods in human plasma of DEX have been published. Herein we present a highly sensitive LC-MS/MS method with several advantages over earlier reported methods, viz. increased specificity, simple liquid–liquid extraction, use of a commercial drug (omeprazole) as the IS, low quantity of human plasma (50μL) with a 5 μL injection volume and a shorter run time of 2.0 min. The newly validated LC-MS/MS method was successfully used for human pharmacokinetic study.

Experimental

Chemicals and reagents

DEX (purity 99.6%) was procured from CTO Unit VI, Dr Reddy's Laboratories Ltd, India. Omeprazole (purity 98.9%) was from FTO III, Dr Reddy's Laboratories Ltd, India. All the compounds were found to be >98.5% purity as determined by chromatographic (HPLC) analysis. HPLC-grade acetonitrile and analytical-grade ammonia were purchased from Merck Specialties Pvt. Ltd, Mumbai, India. All aqueous solutions including the buffer for the mobile phase were prepared with Milli Q (Millipore, Milford, MA, USA) grade water. K_2EDTA was purchased from a registered blood bank, Secunderabad, India, and stored at −20°C prior to use.

HPLC operating conditions

A Shimadzu (Shimadzu, Japan) LC system equipped with a degasser (DGU-20A5) and a Binary pump (LC-20AD) along with an auto-sampler (SIL-HTc) was used to inject 5 μL aliquots of the processed samples onto an X-terra RP_{18} (50 × 4.6 mm, 5 μm, Waters, UK) column, which was kept at ambient temperature (24 ± 2°C). The isocratic mobile phase, a mixture of 0.2% ammonia–acetonitrile (20:80, v/v), was filtered through a 0.45 μm membrane filter (XI5522050; Millipore, USA or equivalent), then degassed ultrasonically for 5 min and delivered at a flow rate of 0.5 mL/min into the mass spectrometer electrospray ionization chamber.

Mass spectrometry operating conditions

Quantitation was achieved by MS/MS detection in positive ion mode for analyte and IS using an MDS Sciex (Foster City, CA, USA) API-4000 mass spectrometer, equipped with a Turboionspray™ interface at 400°C. The common parameters, viz. curtain gas, nebulizer gas, auxillary gas and collision gas were set at 20, 30, 40 and 5 psi, respectively. The compounds parameters, viz. declustering potential, collision energy, collision exit potential and entrance potential for DEX and IS were 42, 5, 5 and 10 V and 40, 5, 10 and 10 V, respectively. Detection of the ions was performed in the multiple reaction monitoring mode, monitoring the transition of the *m/z* 370.3 precursor ion to the *m/z* 252.2 product ion for DEX, and *m/z* 346.1 precursor ion to the *m/z* 198.3 product ion for IS. Quadrupoles Q1 and Q3 were set on unit resolution. The dwell time was 200 ms. The analytical data were processed by Analyst software (version 1.5.1).

Preparation of stock and standard solutions

Primary stock solutions of DEX for preparation of standard and quality control (QC) samples were prepared from separate weighings. The primary stock solutions were prepared in methanol (1000 μg/mL). The IS stock solution of 1000 μg/mL was prepared in methanol. The stock solutions of DEX and IS were stored at 4°C, and were found to be stable for one month (data not shown). They were successively diluted with methanol–water (50:50, v/v) to prepare working solutions for preparation of a calibration curve. Another set of working stock solutions of DEX was made in methanol (from primary stock) for preparation of QC samples. Working stock solutions were stored at approximately 4°C for a week (data not shown). Appropriate dilutions of DEX stock solution was made in methanol–water (50:50, v/v) to produce working stock solutions. Working stocks were used to prepare plasma calibration standards. A working IS solution (125 ng/mL) was prepared in methanol. Calibration samples were prepared by spiking 50 μL of control human plasma with the appropriate working solution of the analyte (10 μL) and IS (10 μL) on the day of analysis. Samples for the determination of precision and accuracy were prepared by spiking control human plasma in bulk with DEX at appropriate concentrations [for DEX, 2.00 (lower limit of quantitation, LLOQ), 6.00 (low quality control, LQC), 1127.48 (medium quality control, MQC) and 2127.32 (high quality control, HQC) ng/mL] and 50 μL plasma aliquots were distributed into different tubes. All the samples were stored at −65 ± 15°C.

Recovery

The efficiency of DEX and IS extraction from human plasma was determined by comparing the responses of the analytes extracted

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from replicate QC samples ($n=6$) with the response of analytes from post extracted plasma standard sample at equivalent concentrations (Dams *et al.*, 2003) by liquid/liquid extraction process. Recoveries of DEX were determined at LQC, MQC and HQC concentrations [for DEX, 6.00 (LQC), 1127.48 (MQC) and 2127.32 (HQC) ng/mL], whereas the recovery of the IS was determined at a single concentration of 125 ng/mL.

Sample preparation

A simple liquid–liquid extraction method was followed for extraction of DEX from human plasma. To an aliquot of 50 µL plasma, IS solution (20 µL of 125 ng/mL) was added and mixed for 15 s on a cyclomixer (Remi Instruments, Mumbai, India). After the addition of 1 mL of ethyl acetate, the mixture was vortexed for 2 min, followed by centrifugation for 5 min at 3000 rpm on Multifuge 3^{SR} (Heraus, Germany). The organic layer (1.8 mL) was separated and evaporated to dryness at 40°C using a gentle stream of nitrogen (Turbovap®, Zymark®, Kopkinton, MA, USA). The residue was reconstituted in 500 µL of the mobile phase and 5 µL was injected onto LC-MS/MS system.

Validation procedures

A full validation according to the FDA guidelines (US DHHS *et al.*, 2001) was performed for the assay in rat plasma/brain.

Specificity and selectivity. The specificity of the method was evaluated by analyzing human plasma samples from at least six different lots to investigate the potential interferences at the LC peak region for analytes and IS.

Matrix effect. The effect of human plasma constituents over the ionization of DEX and IS was determined by comparing the responses of the post extracted plasma QC samples ($n=6$) with the response of analytes from neat standard samples (10 µL of required working stock sample spiked into 240 µL of methanol instead of blank plasma) at equivalent concentrations (Hubert *et al.*, 1999; Dams *et al.*, 2003). The matrix effect was determined at low and high concentrations, viz. 6.00 and 2127.32 ng/mL, whereas the matrix effect over the IS was determined at a single concentration of 125 ng/mL.

Calibration curve. The eight point calibration curve (2.00, 4.00, 10.00, 125.0, 375.0, 1250, 2000 and 2500 ng/mL) was constructed by plotting the peak area ratio of each analyte–IS against the nominal concentration of calibration standards in human plasma. Following the evaluation of different weighing factors, the results were fitted to linear regression analysis with the use of $1/x^2$ (x = concentration) weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.99 or better. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LLOQ, which was set at ±20% (US DHHS *et al.*, 2001).

Precision and accuracy. The intra-assay precision and accuracy were estimated by analyzing six replicates containing DEX at four different QC levels [for DEX, 2.00 (LLOQ), 6.00 (LQC), 1127.48 (MQC) and 2127.32 (HQC) ng/mL] in plasma. The inter-assay precision was determined by analyzing the four levels QC samples on four different runs. The criteria for acceptability of the data included accuracy within ±15% deviation (SD) from the nominal values and a precision of within ±15% relative standard deviation (RSD) except for LLOQ, where it should not exceed ±20% of SD (US DHHS *et al.*, 2001).

Stability experiments. The stability of DEX and IS in the injection solvent was determined periodically by injecting replicate preparations of processed plasma samples for up to 22 h (in the auto-sampler at 4°C) after the initial injection. The peak areas of the analyte and IS obtained at the initial cycle were used as the reference to determine the stability at subsequent points. Stability of DEX in plasma during 8 h (bench-top) was determined at ambient temperature (24 ± 2°C) at two concentrations (for DEX, 6.00 and 2127.32 ng/mL; in six replicates). Freezer stability of DEX in human plasma was assessed by analyzing the LQC and HQC samples stored at −65 ± 15°C for at least 100 days. The stability of DEX in human plasma following three freeze–thaw cycles was assessed using QC samples spiked with DEX. The samples were stored at −65 ± 15°C between freeze–thaw cycles. The samples were thawed by allowing them to stand (unassisted) at room temperature for approximately 2 h. The samples were then returned to the freezer. The samples were processed using the same procedure as described in the 'Sample Preparation' section. Samples were considered stable if assay values were within the acceptable limits of accuracy (i.e. ±15% SD) and precision (i.e. ±15% RSD).

Pharmacokinetic study

A pharmacokinetic study was performed in healthy male subjects. The ethics committee approved the protocol and the volunteers provided informed written consent. Following oral administration of 60 mg of DEX tablet, blood samples were obtained into polypropylene tubes containing K_2EDTA solution as anti-coagulant at pre-dose, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, 20, 24 and 36 h. Plasma was harvested by centrifuging the blood using a Biofuge (Hereaus, Germany) at 1760***g*** for 5 min and stored frozen at −30 ± 10°C until analysis.

An aliquot of 50 µL of thawed plasma samples were spiked with IS and processed as mentioned in the 'Sample Preparation' section. Along with study samples, QC samples at low, medium and high concentrations were assayed in duplicate and were distributed among unknown samples in the analytical run. The criteria for acceptance of the analytical runs encompassed the following: (i) not more than 33% of the QC samples were greater than ±15% of the nominal concentration; and (ii) not less than 50% at each QC concentration level must meet the acceptance criteria. Plasma concentration–time data for DEX were analyzed by a noncompartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, CA, USA).

Results

Liquid chromatography

The feasibility of various mixture(s) of solvents, such as acetonitrile and methanol, using different buffers, such as ammonium acetate, ammonium formate and formic acid, along with altered flow rates (in the range of 0.1–0.5 mL/min) was tested for complete chromatographic resolution of DEX and IS (data not shown). The resolution of peaks was achieved with 0.2% ammonia–acetonitrile (20:80, v/v) with a flow rate of 0.5 mL/min, on an X-terra RP $_{18}$ (50 × 4.6 mm, 5 µm), and was found to be suitable for the determination of electrospray response for DEX and IS.

Mass spectroscopy

In order to optimize ESI conditions for DEX and IS, quadrupole full scans were carried out in positive-ion detection mode. During a direct infusion experiment, the mass spectra for DEX and IS revealed peaks at *m/z* 370.3 and 346.1, respectively, as protonated molecular ions, $[M+H]^+$. The product ion mass spectrum for DEX shows the formation of characteristic product ions at *m/z* 119.3, 136.1, 205.00, 234.3 and 252.2 (Fig. 2), and that for the IS shows the formation of characteristic product ions at *m/z* 149.4, 168.4 and 198.3. Following detailed optimization of mass spectrometry conditions (provided in the 'Instrumentation and Chromatographic Conditions' section) *m/z* 370.3 precursor

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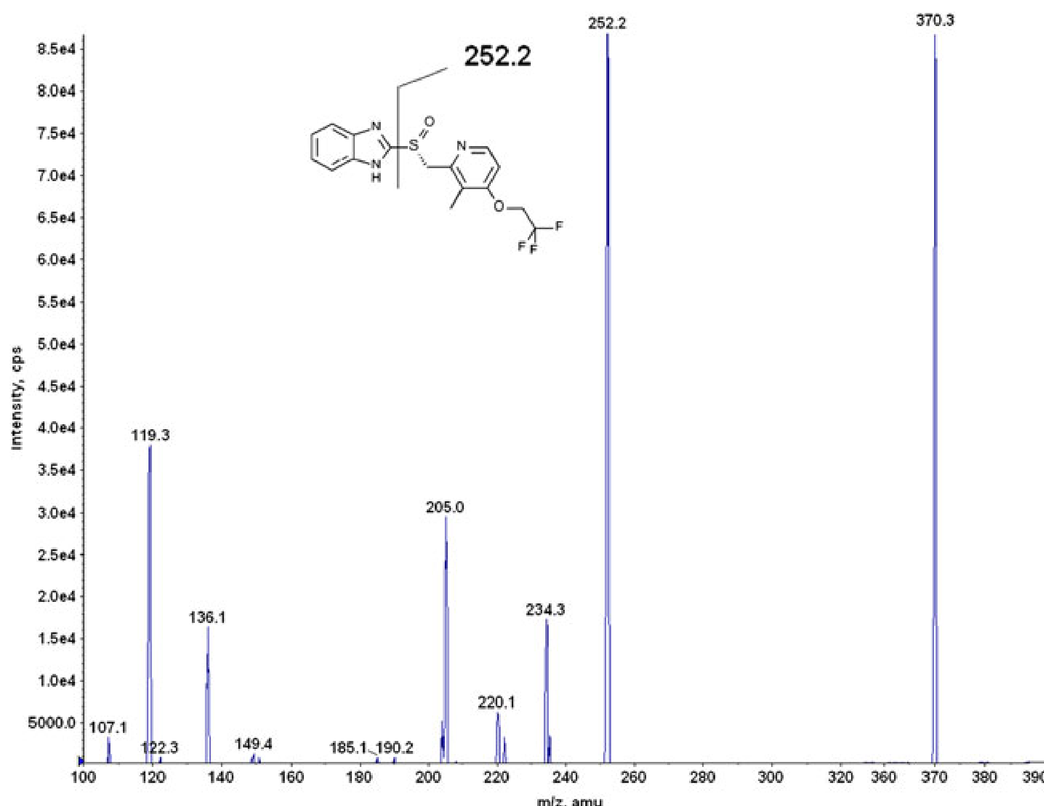

Figure 2. MS/MS spectra of dexlansoprazole showing prominent precursor to product ion transitions.

ion to the *m/z* 252.2 was used for quantification for DEX. Similarly, for IS *m/z* 346.1 precursor ion to the *m/z* 198.3 was used for quantification purpose.

Recovery

A simple liquid–liquid extraction with ethyl acetate proved to be robust and provided the cleanest samples. The results of the comparison of neat standards vs plasma-extracted standards were estimated for DEX [for DEX, 6.00 (LQC), 1127.48 (MQC) and 2127.32 (HQC) ng/mL]. The mean recoveries for DEX were found to be 88.75 ± 3.14, 86.15 ± 2.00 and 87.51 ± 3.66%. The recovery of IS at 125 ng/mL was 96.60 ± 4.42%.

Matrix effect, specificity and selectivity

Average matrix factor values (matrix factor = response of post-spiked concentrations/response of neat concentrations) obtained were −0.03 (coefficient of vatiation, CV, 3.03%, $n = 6$) and +0.04 (CV, 4.16%, $n = 6$) for DEX in human plasma at LQC (6.00 ng/mL) and HQC (1127.48 ng/mL) concentrations, respectively. No significant peak area differences were observed. The matrix effect on IS was found to be +0.02 (CV, 6.55%, $n = 12$) at the tested concentration of 125 ng/mL. Overall it was found that the plasma extract has a small impact on the ionization of analyte and IS.

Figure 2 shows a typical overlaid chromatogram for the control human plasma (free of analytes and IS), human plasma spiked with DEX at LLOQ and IS and an *in vivo* plasma sample obtained at 5 h after oral administration of DEX. No interfering peaks from endogenous compounds were observed at the retention times of analytes and IS in the matrix. The retention time of DEX and IS was 1.20 and 1.21 min, respectively, figure 3 shows the representative chromatograms of DEX and IS. The total chromatographic run time was 2.0 min.

Calibration curve

The plasma homogenate calibration curve was constructed using eight calibration standards (viz. 2.00–2500 ng/mL). The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) vs concentration, and fitted to $y = mx + c$ using a weighing factor ($1/x^2$). The average regression ($n = 4$) was found to be ≥0.995. The lowest concentration with RSD < 20% was taken as the LLOQ and was found to be 2.00 ng/mL. The percentage accuracy observed for the mean of back-calculated concentrations for four calibration curves for DEX was within 96.3–100.8%m while the precision (% CV) values were 1.47–6.08 for DEX.

Accuracy and precision

Accuracy and precision data for intra- and inter-day plasma samples for DEX are presented in Table 1. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variability limits.

Stability

The predicted concentrations for DEX at 6.00 and 2127.32 ng/mL samples deviated within ±15% of the nominal concentrations

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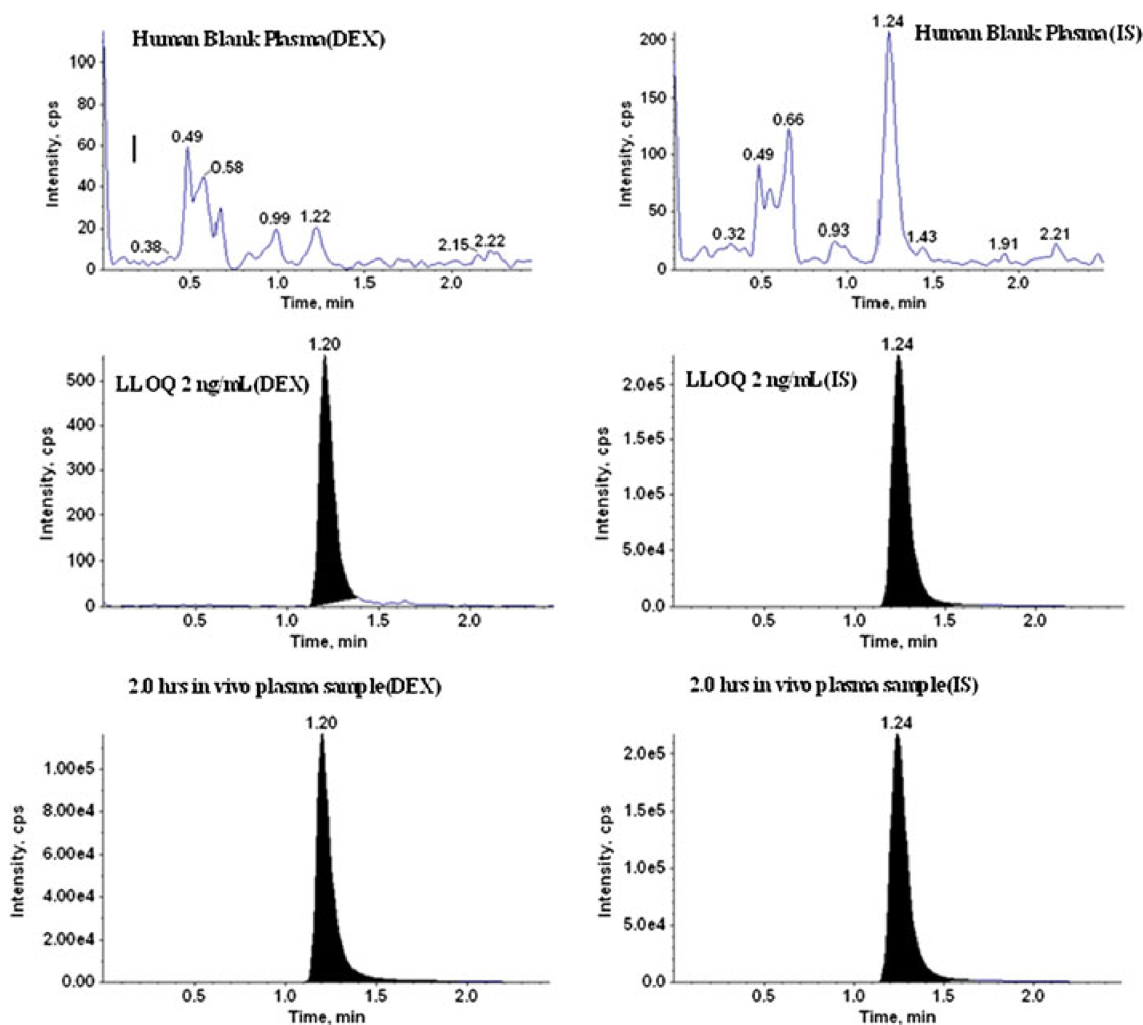

Figure 3. Typical multiple reaction-monitoring mode chromatograms of dexlansoprazole (left panel) and IS (right panel) in (a) human blank plasma and (b) human plasma spiked with dexlansoprazole at LLOQ (2 ng/mL) and IS; (c) a 2.0 h in vivo plasma sample showing dexlansoprazole peak obtained following an oral dose of Delixent tablets to healthy humans.

in a battery of stability tests, viz. in-injector (22 h), bench-top (8 h), three repeated freeze–thaw cycles and freezer stability at −65 ± 15°C for at least 100 days (Table 2). The results were found to be within the assay variability limits during the entire process.

Pharmacokinetic study

The present method was applied to the analysis of plasma samples obtained from 20 healthy human volunteers following oral administration of 60 mg of DEX tablet as a part of a bioequivalence study. The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of DEX and in humans. Figure 4 depicts the mean plasma concentration vs time profile of DEX in these volunteers.

Discussion

Even though there are some significant analytical methods for the identification of the enantioselectivity of lansoprazole enantiomers in biological fluids (Katsuki *et al.*, 1996, 2001; Borner *et al.*, 1998; Masa *et al.*, 2001; Kim *et al.*, 2002, Miura *et al.*, 2004), to the best of our knowledge, no published LC-MS/MS methods are available for the determination of DEX in human plasma following identification of advantages in its pharmacokinetic behavior over its enantiomer lansoprazole (Aslam and Wright, 2009) in humans. The main advantage of DEX is not with the enantiopure substance but the pharmaceutical formulation, hence achiral assay of DEX is sufficient to assess the bioequivalence, monitor adverse events and ensure the safety of subjects (http://wpedia.goo.ne.jp/enwiki/Discovery and Development_of_Proton_Pump_Inhibitors; Metz *et al.*, 2009b; Aslam and Wright, 2009).

Validated methods are essential for the determination of DEX concentrations in pre-clinical species plasma (for pre-clinical pharmacokinetics and toxic kinetic studies) and for clinical studies. It also finds applicability in *in vitro* plasma protein binding studies across the species and efficacy studies. The current validated method developed for DEX is very simple and sensitive and it utilizes a low quantity of aliquot volume (50 µL) and s short run time of 2.0 min for each sample analysis. No interferences from endogenous plasma components or other sources were found in plasma samples. The maximum on-column loading was much smaller, i.e. 2.5 ng/mL for ULOQ (2500 ng/mL). It provided a maximum cycle time for both the instrument and

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Table 1. Intra and inter-day precision determination of DEX quality controls in human plasma

Theoretical concentration (ng/mL)	Run	Measured concentration (ng/mL)			
		Mean	SD	RSD	Accuracy (%)
Intraday variation (six replicates at each concentration)					
2.00	1	1.91	0.04	2.12	95.5
	2	1.93	0.03	1.73	96.3
	3	1.97	0.05	2.57	98.3
6.00	1	5.95	0.12	1.96	99.2
	2	5.92	0.13	2.14	98.6
	3	5.89	0.25	4.21	98.1
1127.48	1	1157.15	12.69	1.10	102
	2	1139.67	13.18	1.16	101
	3	1158.31	9.91	0.86	102
2127.32	1	2213.42	59.28	2.68	104
	2	2183.59	32.05	1.47	102
	3	2310.91	56.15	2.43	108
Inter-day variation (18 replicates at each concentration)					
2.00		1.93	0.05	2.39	96.7
6.00		5.96	0.12	2.00	99.4
1127.48		1151.71	14.30	1.24	102
2127.32		2235.97	73.44	3.28	105

RSD, Relative standard deviation (SD × 100/mean).

Table 2. Stability data of DEX quality controls in human plasma

Nominal concentration (ng/mL)	Stability	Mean ± SD,[a] $n = 6$ (ng/mL)	Accuracy (%)[b]	Precision (% CV)
6.00	0 h (for all)	6.12 ± 0.09	102	1.45
	Third freeze–thaw	6.26 ± 0.17	104	2.72
	8 h (bench-top)	6.37 ± 0.25	106	3.94
	22 h (in-injector)	6.14 ± 0.26	102	4.17
	100 days at −80°C	6.26 ± 0.20	104	3.25
2127.32	0 h (for all)	2182.31 ± 39.75	102	1.82
	Third freeze–thaw	2104.88 ± 10.20	98.9	0.48
	8 h (bench-top)	2153.54 ± 26.98	101	1.25
	22 h (in-injector)	2159.65 ± 35.87	101	1.66
	100 days at −80°C	2211.59 ± 48.08	103	2.17

[a]Back-calculated plasma concentrations; [b](mean assayed concentration/mean assayed concentration at 0 h) × 100.

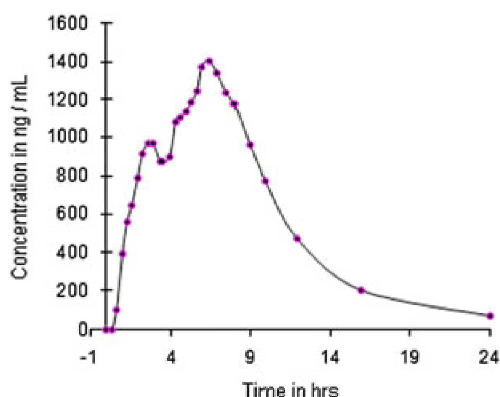

Figure 4. Mean ± SD plasma concentration–time profile of dexlansoprazole in human plasma following oral dosing of dexlansoprazole.

the column used for analysis. Owing to the low sensitivity (LLOQ 2 ng/mL) of the assay and based on the signal-to-noise values for 2 ng/mL, its LLOQ can be lowered further if required, and offers a suitable platform for the determination of DEX in clinical studies. Sample preparation is very simple and involves liquid–liquid extraction of plasma with ethyl acetate. The applicability of the method in clinical pharmacokinetic studies has been demonstrated in more than 120 healthy humans.

Conclusion

In summary, we have developed and validated a simple, sensitive, specific and reproducible LC-MS/MS assay to quantify DEX in human plasma. From the results of all the validation parameters, we can conclude that the present method is useful for pharmacokinetic studies with desired precision and accuracy.

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