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High-throughput quantification of perindopril in human plasma by liquid chromatography/tandem mass spectrometry: application to a bioequivalence study

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A simple, sensitive and rapid high-performance liquid chromatography/positive ion electrospray tandem mass spectrometry method was developed and validated for the quantification of perindopril in human plasma. Following liquid-liquid extraction, the analytes were separated using an isocratic mobile phase on a reversed-phase column and analyzed by mass spectrometry in the multiple reaction monitoring mode using the respective $[M+H]^+$ ions, *m/z* 369/172 for perindopril and *m/z* 417/234 for the internal standard. The method exhibited a linear dynamic range of 0.1–100 ng/mL for perindopril in human plasma. The lower limit of quantification was 0.1 ng/mL with a relative standard deviation of less than 6.1%. Acceptable precision and accuracy were obtained for concentrations over the standard curve range. A run time of 2.0 min for each sample made it possible to analyze more than 450 human plasma samples per day. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic, bioavailability and bioequivalence studies. Copyright © 2006 John Wiley & Sons, Ltd.

Perindopril (Fig. 1) is the first member of a new chemical class of non-peptide angiotensin II receptor antagonists. The first approved indication for perindopril is for hypertension.[1] Recent evidence suggests that perindopril may also offer a novel therapeutic strategy for Parkinson's disease.[2]

Perindopril is a long-acting angiotensin-converting enzyme inhibitor (ACEI), which displays similar pharmcodynamic properties to other agents in this class. Among ACEIs, perindopril is a mono-acid mono-ester prodrug which is hydrolyzed *in vivo* into an active diacid metabolite, perindoprilat.[1] Perindopril is rapidly and extensively absorbed and reaches a maximum plasma concentration about 1 h after oral administration.[3] The bioavailability for perindopril has been reported to be 95% and not influenced by food.[4] However, food does reduce the conversion of perindopril into perindoprilat.[5]

The bioanalytical component of a pharmacokinetic study requires an analytical method with simplicity, selectivity, sensitivity, small volume requirements and rapid turnaround time. A few methods for the quantification of perindopril in biological fluids have been reported. These methods are based on radioimmunoassay[6,7] or gas chromatography/mass spectrometry (GC/MS).[8] The radioimmunoassay has been described by Doucet *et al.*[6] and the assay sensitivity was 0.5 ng/mL. Radioimmunoassays often lack specificity, especially for drugs that are extensively metabolized. In the GC/MS method, a sensitivity of 100 pg/mL was achieved using 2 mL of plasma sample.

Quantification of drugs in biological matrices by liquid chromatography/tandem mass spectrometry (LC/MS/MS) is becoming more common due to the improved sensitivity and selectivity of this technique. In recent years, we have reported a number of bioanalytical procedures using LC/MS/MS with high sample throughput capacity.[9–12] We now report for the first time an LC/MS/MS method developed and validated for the quantification of perindopril in a low volume (0.2 mL) of human plasma. It was essential to establish a method capable of quantifying perindopril at concentrations down to 100 pg/mL. At the same time, it was expected that this method would be efficient in analyzing a large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of perindopril.

EXPERIMENTAL

Chemicals

Perindopril erbumine drug substance was obtained from Glenmark Pharmaceutical Ltd. (Ankleshwer, India) and ramipril (internal standard, IS) was obtained from Torrent Research Centre (Ahmedabad, India). Chemical structures are presented in Fig. 1. HPLC-grade LiChrosolv acetonitrile was purchased from Merck (Darmstadt, Germany).

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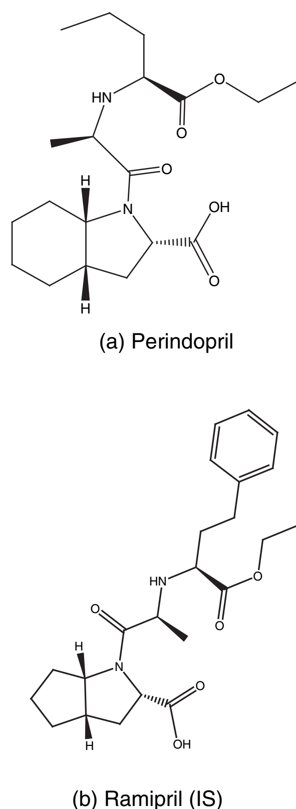

Figure 1. Chemical structures for perindopril and the IS (ramipril).

Table 1. Main working parameters of the tandem mass spectrometer

Parameter	Value
Source temperature, °C	250
Dwell time per transition, ms	200
Ion source gas 1, psi	30
Ion source gas 2, psi	30
Curtain gas, psi	20
Collision gas, psi	5
Ion spray voltage, V	5500
Entrance potential, V	10
Declustering potential, V	70 (Analyte) and 56 (IS)
Collision energy, V	27 (Analyte) and 30 (IS)
Collision cell exit potential, V	10 (Analyte) and 6 (IS)
Resolution	Unit
Mode of analysis	Positive
Ion transition for perindopril, *m/z*	$369.4 \pm 0.5/172.4 \pm 0.5$
Ion transition for ramipril, *m/z*	$417.4 \pm 0.5/234.3 \pm 0.5$

Ammonium acetate, orthophosphoric acid, diethyl ether and dichloromethane were purchased from Merck (Worli, Mumbai, India). HPLC-grade water from a Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

LC/MS/MS instrument and conditions

The HPLC SIL HTC system (Shimadzu Corporation, Kyoto, Japan) is equipped with LC-AD VP binary pump, a DGU20A5 degasser, and a SIL-HTC autosampler equipped with a CTO-10AS VP thermostatted column. The chromatography was on Inertsil ODS-3V (5 μm, 4.6 × 150 mm) at a temperature of 30°C. The isocratic mobile phase composition was a mixture of 10 mM ammonium acetate/acetonitrile (30:70, v/v), which was pumped at a flow rate of 1.5 mL/min with split ratio of 10:90.

Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using multiple reaction monitoring (MRM). A turbo electrospray ionization (ESI) interface in positive ionization mode was used. The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed on Analyst 1.4.1 software package (SCIEX).

Sample preparation

Standard stock solutions of perindopril (1 mg/mL) and the IS (1 mg/mL) were separately prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in water/methanol (50:50, v/v; diluent). The IS working solution (250 ng/mL) was prepared by diluting its stock solution with diluent. Working solutions (0.2 mL) were added to drug-free human plasma (9.8 mL) as a bulk, to obtain perindopril concentration levels of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 ng/mL, as a single batch at each concentration. Quality control (QC) samples were also prepared as a bulk on an independent weighing of standard drug, at concentrations of 0.1 (lower limit of quantification (LLOQ)), 0.3 (low), 40 (medium) and 80 ng/mL (high), as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in microcentrifuge tubes (Tarson, 1.5 mL) and stored in the freezer at below −20°C until analyses.

A plasma sample (0.2 mL) was pipetted into a 15-mL glass tube and then 20 μL of IS working solution (250 ng/mL) and 50 μL of orthophosphoric acid (2.5%) were added and vortex-mixed for 30 s. Then 3 mL of the extraction mixture, diethyl ether/dichloromethane (7:3, v/v), were added and the sample was vortex-mixed for 3 min. The organic layer (2 mL) was transferred to a glass tube and evaporated to dryness using an evaporator at 40°C under a stream of nitrogen. Then the dried extract was reconstituted in 250 μL of mobile phase and a 10-μL aliquot was injected into the chromatographic system.

Bioanalytical method validation

A calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma processed with the IS) and ten non-zero samples covering the total range 0.1–100 ng/mL, including the LLOQ. The calibration curves were generated using the analyte to IS peak area ratios by weighted ($1/x^2$) least-squares linear regression on consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the above criteria, including acceptable LLOQ and upper limit of quantification.

The within-batch precision and accuracy were determined by analyzing six sets of QC samples in a batch. The between-

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batch precision and accuracy were determined by analyzing six sets of QC samples on three different batches. The QC samples were randomized daily, processed and analyzed in a position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of the batch. The acceptance criteria for within- and between-batch precision were 20% or better for LLOQ and 15% or better for the other concentrations, and the accuracy were $100 \pm 20\%$ or better for LLOQ and $100 \pm 15\%$ or better for the other concentrations.

Recovery of perindopril from the extraction procedure was determined by a comparison of the peak area of perindopril in spiked plasma samples (six each of low, medium and high QCs) with the peak area of perindopril in samples prepared by spiking extracted drug-free plasma samples with the same amounts of perindopril at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted QC samples ($n = 6$) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography.

The stability of the analyte and IS in human plasma under different temperature and timing conditions, as well as their stability in the stock solutions, was evaluated. QC samples

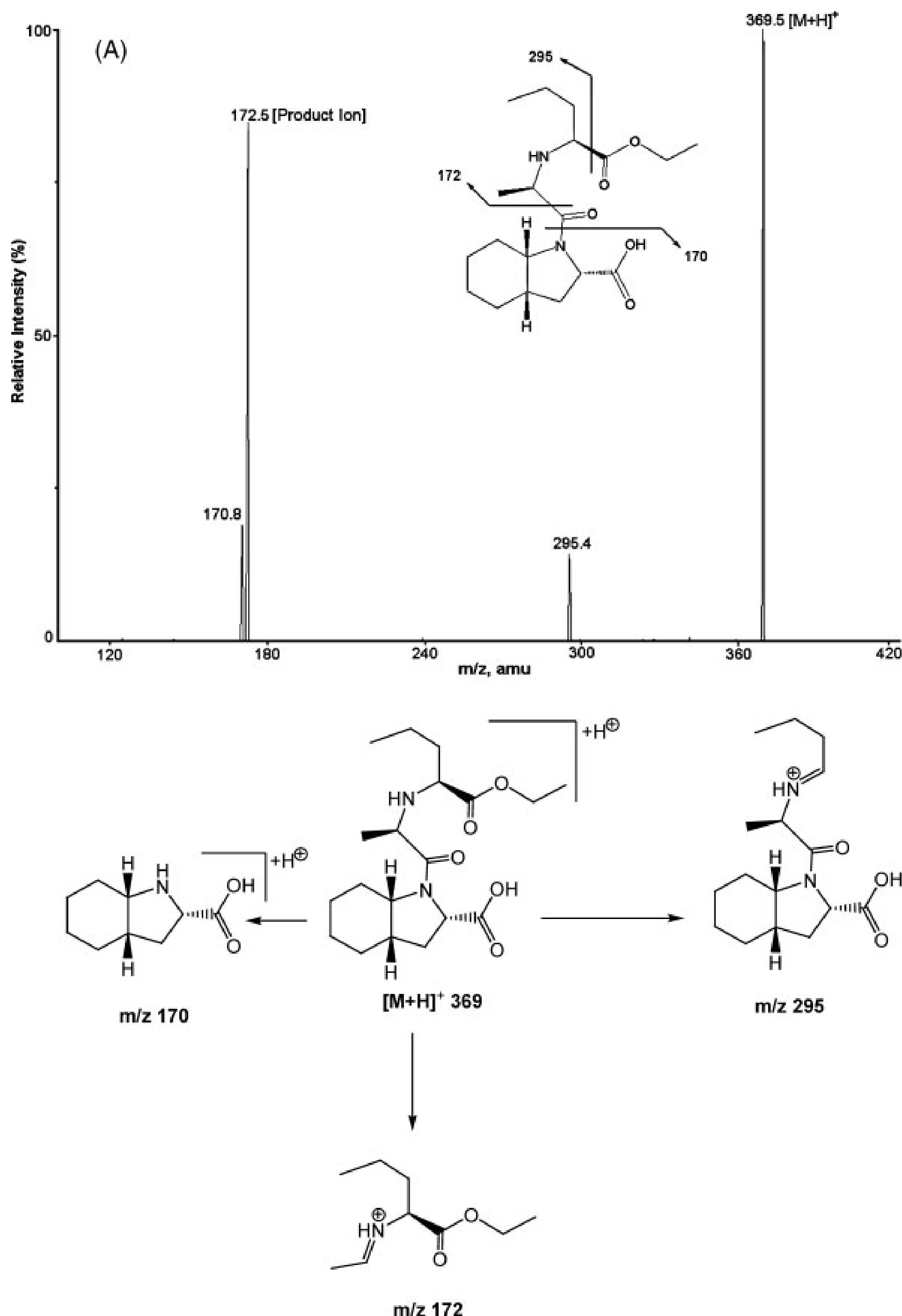

Figure 2. Full scan positive ion turboIonspray product ion mass spectra and the proposed patterns of fragmentation of (A) perindopril and (B) ramipril (IS).

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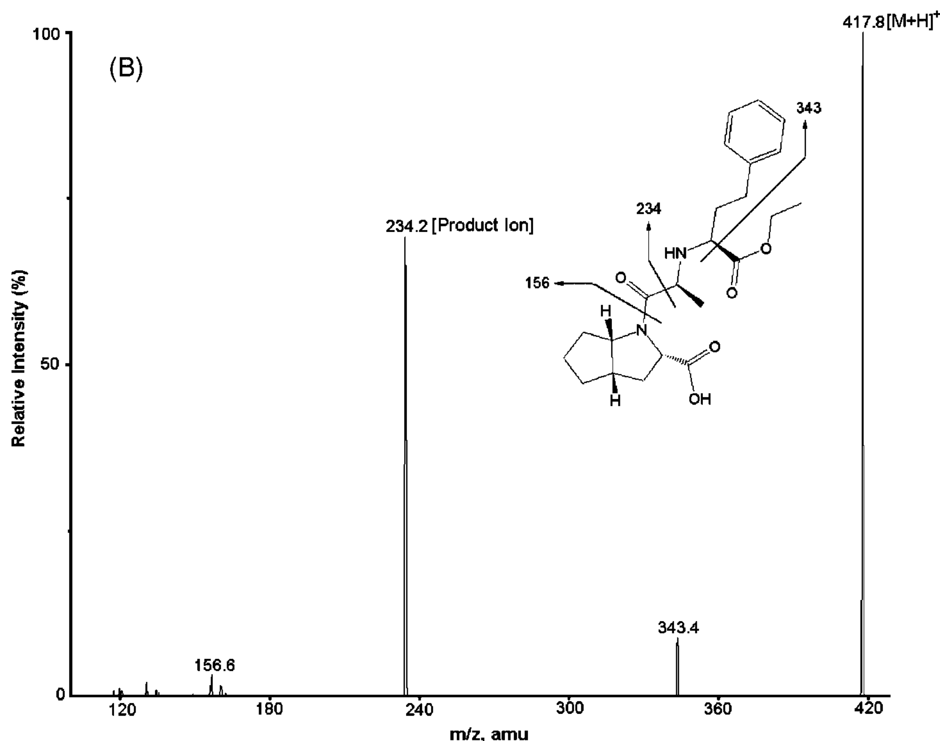

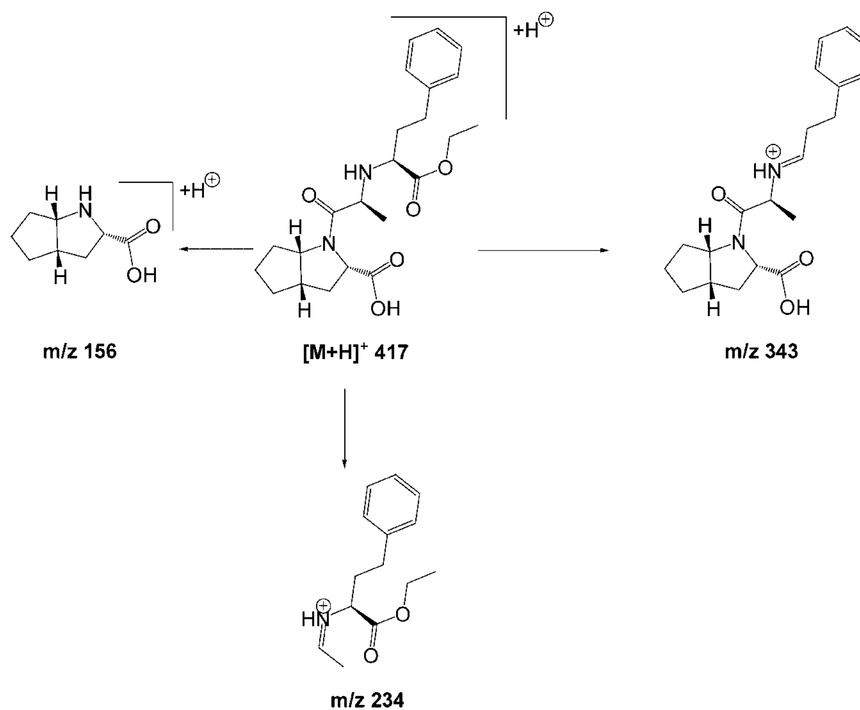

Figure 2. (Continued).

were subjected to short-term room temperature conditions, to long-term storage conditions (−20°C), and to freeze/thaw stability studies. All the stability studies were conducted at two concentration levels (0.3 and 80 ng/mL as low and high values) with six determinations for each.

RESULTS AND DISCUSSION

Mass spectrometry

In order to develop a method with the desired LLOQ (100 pg/mL), it was necessary to use MS/MS detection, as MS/MS methods provide improved limit of detection for trace-mixture analysis. The inherent selectivity of MS/MS detection was also expected to be beneficial in developing a selective and sensitive method. The product ion mass spectra, and their proposed rationalizations in terms of fragmentation patterns, of perindopril and the IS are illustrated in Fig. 2. $[M+H]^+$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The low intensity peak at *m/z* 295 is presumably due to an ion formed by the loss of the ethoxycarbonyl moiety, whereas cleavage (to the carbonyl

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group after protonation on the ring N atom[13] can give rise to the major fragment ion at *m/z* 172 (Fig. 2). A similar fragmentation pattern was observed for the IS ramipril. The most sensitive mass transitions were from *m/z* 369 to 172 for perindopril and *m/z* 417 to 234 for the IS.

LC-MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus, the MRM technique was chosen for the method development. The MRM state file parameters were optimized to maximize the response for the analyte. The parameters presented in Table 1 are the result of this optimization.

Method development

The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analyte and IS, as well as a short run time. It was found that a mixture of 10 mM ammonium acetate/acetonitrile (30:70, v/v) could achieve this purpose and was finally adopted as the mobile phase. The high proportion of organic solvent eluted the analyte and the IS at retention times of 1.22 and 1.32 min, respectively. A flow rate of 1.5 mL/min produced good peak shapes and permitted a run time of 2.0 min.

Liquid-liquid extraction (LLE) was used for the sample preparation in this work. LLE can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effect in LC/MS/MS analyses. A mixture of diethyl ether and dichloromethane (7:3, v/v) was found to be optimal, which can produce a clean chromatogram for a blank plasma sample. The average absolute recoveries of perindopril from spiked plasma samples was $82.0 \pm 1.4\%$ and the recovery of the IS was $84.1 \pm 1.0\%$ at the concentration used in the method (250 ng/mL). Recoveries of the analytes and IS were good and it was consistent, precise and reproducible. Therefore, the method has proved to be robust in high-throughput bioanalysis.

Choosing the appropriate IS is an important aspect to achieving acceptable method performance, especially with LC/MS/MS, where matrix effects can lead to poor analytical results. Ideally, isotopically labeled internal standards for all analytes should be used, but these are not commercially available. Therefore, we opted for ramipril, structurally relevant to perindopril and commercially available. In addition, its retention behavior is similar to that of the target analyte. Clean chromatograms were obtained and no significant direct interferences in the MRM channels at the relevant retention times were observed. However, in ESI, signal suppression or enhancement may occur due to co-eluting endogenous components of the sample matrix. The importance of including the evaluation of matrix effect in any LC/MS/MS method is outlined in an excellent paper by Matuszewski and co-workers.[14] Their data strongly emphasize the need to use a blank matrix from (at least five) different sources/individuals instead of using one blank matrix pool to determine method precision and accuracy. Therefore, all validation experiments in this method were performed with matrices obtained from different individuals. In addition, validation experiments were performed using haemolytic and strongly lipemic matrices. As all data falls within the guidelines, we conclude that the degree of matrix effect was sufficiently low to produce acceptable data and the method can be considered as valid.

Table 2. Precision and accuracy data of back-calculated concentrations of calibration samples for perindopril in human plasma

Concentration added (ng/mL)	Concentration found (mean ± SD n = 5) (ng/mL)	Precision (%)	Accuracy (%)
0.1	0.09 ± 0.00	0.5	95.3
0.2	0.22 ± 0.00	2.0	108.0
0.5	0.51 ± 0.03	5.7	101.9
1	0.99 ± 0.03	3.1	99.0
2	1.98 ± 0.02	1.1	98.9
5	4.97 ± 0.08	1.7	99.5
10	10.00 ± 0.30	3.0	99.9
20	20.78 ± 0.24	1.2	103.4
50	50.29 ± 0.97	1.9	100.6
100	98.29 ± 1.68	1.7	98.3

Method performance and validation

The ten-point calibration curve was linear over the concentration range 0.1–100 ng/mL. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$ and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$ weighing factor, giving a mean linear regression equation for the calibration curve of: $y = 0.0176\ (\pm 0.0008)x + 0.0021\ (\pm 0.0007)$, where y is the peak area ratio of the analyte to the IS and x is the concentration of the analyte. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.9998 ± 0.0001; Table 2 summarizes the calibration curve results.

The selectivity of the method was examined by analyzing (n = 6) blank human plasma extract (Fig. 3(A)) and an extract spiked only with the IS (Fig. 3(B)). As shown in Fig. 3(A), no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte. Similarly, Fig. 3(B) shows the absence of direct interference from the IS to the MRM channel of the analyte. Figure 3(C) depicts a representative ion chromatogram for the LLOQ (0.1 ng/mL). Excellent sensitivity was observed for a 10-μL injection volume; the LLOQ corresponds to ca. 4 pg on-column.

The MRM chromatograms obtained from an extracted plasma sample of a healthy subject who participated in a bioequivalence study conducted on 12 subjects are depicted in Fig. 4. Perindopril was identified and was quantified as 61.6 ng/mL.

The LLOQ is defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision, and was found to be 0.1 ng/mL in human plasma. The mean response for the analyte peak at the method sensitivity limit (0.1 ng/mL) was ≈10-fold greater than the mean response for the peak in eight blank human plasma samples at the retention time of the analyte.

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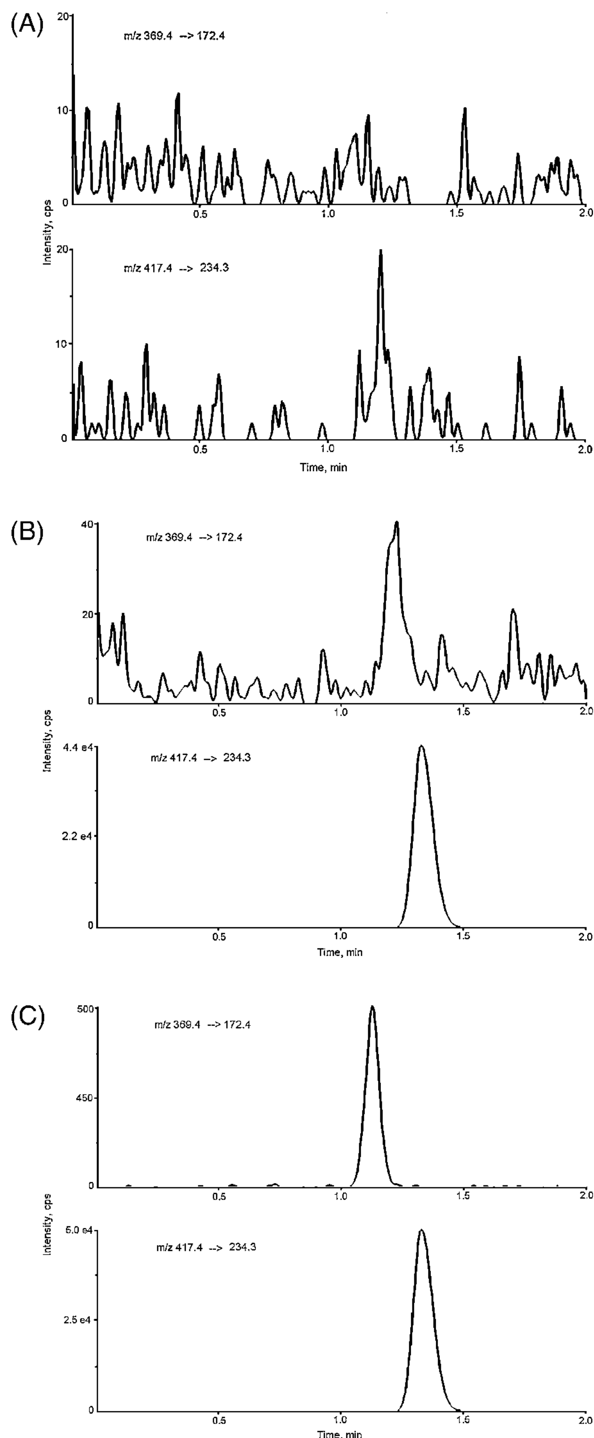

Figure 3. MRM chromatograms for perindopril and the IS resulting from analysis of: (A) blank (drug and IS free) human plasma; (B) zero sample (drug-free spiked with IS) human plasma; and (C) 0.1 ng/mL (LLOQ) of perindopril spiked with the IS.

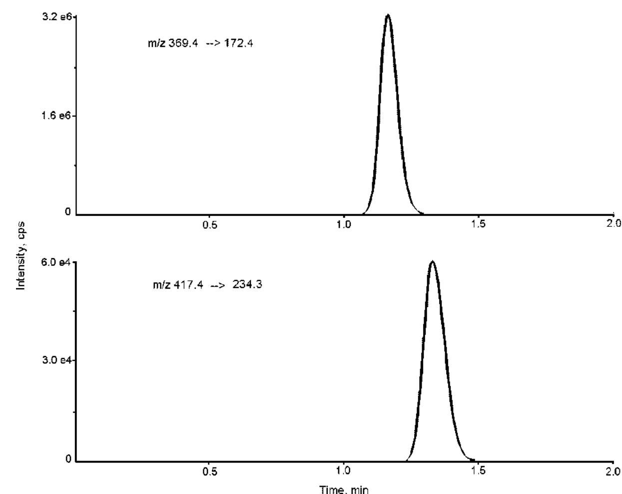

Figure 4. MRM chromatograms resulting from the analysis of a subject's plasma sample after the administration of a 4-mg oral single dose of perindopril. The sample concentration was determined to be 61.6 ng/mL.

The between-batch precision at the LLOQ was 6.1%, and the between-batch accuracy was 96.6% (Table 3). The within-batch precision was 3.2% and the accuracy was 96.6% for perindopril.

The middle and upper quantification levels of perindopril ranged from 0.3 to 80 ng/mL in human plasma. For the between-batch experiments the precision ranged from 2.4 to 6.0% and the accuracy from 96.2 to 102.0% (Table 3). For the within-batch experiments the precision and accuracy for the analyte met the acceptance criteria (<±15%).

Injector carryover (memory effect) was assessed by injecting a series of upper limit of quantification samples interspersed with blank reconstituted solvent samples. Carryover for perindopril (80 ng/mL) was found to be 0.056% and carryover for the IS (250 ng/mL) was found to be 0.086%.

Stability studies

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (around 24 h). Samples were extracted and analyzed as described above and the results are given in Table 4. These results indicate reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of QC samples kept in the autosampler for 24 h was also assessed. The results indicate that solutions of perindopril and the IS can remain in the autosampler for at least 24 h without showing significant loss

Table 3. Precision and accuracy of the method for determining perindopril concentrations in plasma samples

	Within-batch precision (n = 5)			Between-batch precision (n = 5)		
Concentration added (ng/mL)	Concentration found (mean ± SD) (ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± SD) (ng/mL)	Precision (%)	Accuracy (%)
0.1	0.1 ± 0.0	3.2	96.6	0.1 ± 0.0	6.1	96.6
0.3	0.3 ± 0.0	2.1	102.0	0.3 ± 0.0	6.0	102.0
40	40.0 ± 0.9	0.2	100.1	40.0 ± 1.0	2.4	100.1
80	76.9 ± 0.2	0.3	96.2	77.0 ± 2.5	3.2	96.2

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Table 4. Stability of perindopril in human plasma

Sample concentration (ng/mL; n = 6)	Concentration found (ng/mL)	Precision (%)	Accuracy (%)
Short-term stability for 24 h in plasma			
0.3	0.3	3.9	101.2
80	77.0	2.6	96.0
Three freeze/thaw cycles			
0.3	0.3	2.3	101.4
80	81.4	1.1	101.7
Autosampler stability for 24 h			
0.3	0.3	3.5	109.0
80	78.6	2.2	98.2
Stability for 55 days at below −20°C			
0.3	0.3	3.6	96.9
80	78.8	2.4	98.5

in the quantified values, indicating that samples should be processed within this period of time (Table 4).

The data representing the stability of perindopril in plasma at two QC levels over three freeze/thaw cycles are given in Table 4. These tests indicate that the analyte is stable in human plasma for three freeze/thaw cycles, when stored at below −20°C and thawed to room temperature.

Table 4 also summarizes the long-term stability data for perindopril in plasma samples stored for a period of 55 days at below −20°C. The stability study of perindopril in human plasma showed reliable stability behavior, as the means of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicate that storage of perindopril in plasma samples at below −20°C is adequate, and no stability-related problems would be expected during routine analyses for pharmacokinetic, bioavailability or bioequivalence studies.

The stability of the stock solutions was tested and established at room temperature for 4 h, 8 h, 21 h and under refrigeration (~4°C) for 55 days (data not shown). The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

Application

The method was applied to determine the plasma concentration of perindopril following a single 4-mg oral administration to 12 healthy subjects. Figure 5 shows the mean plasma concentration–time profile of six healthy subjects each receiving a 4-mg oral dose of perindopril under fasting conditions.

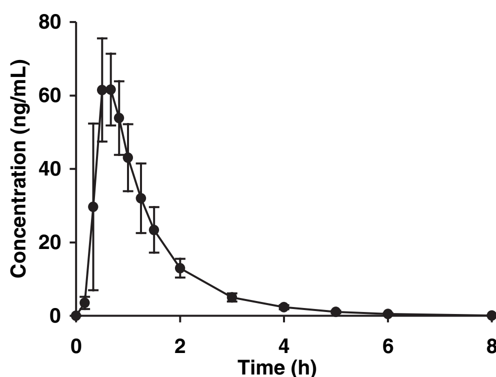

Figure 5. Representative data showing mean plasma concentration–time profile of six healthy subjects after the administration of an oral single dose of 4 mg of perindopril under fasting conditions.

CONCLUSIONS

In summary, the method is described for the quantification of perindopril in human plasma by LC/MS/MS in positive ESI mode using multiple reaction monitoring and fully validated according to commonly accepted criteria.[15] The current method has shown acceptable precision and adequate sensitivity for the quantification of perindopril in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies. The desired sensitivity of perindopril was achieved with an LLOQ of 0.1 ng/mL, which has a within- and between- batch coefficient of variance (CV) of 3.2 and 6.1%, respectively. Many variables related to the electrospray reproducibility were optimized for both precision and sensitivity to obtain these results. The simplicity of the method, and using rapid liquid-liquid extraction and sample turnover rate of 2.0 min per sample, make it an attractive procedure in high-throughput bioanalysis of perindopril. The validated method allows quantification of perindopril in the 0.1–100 ng/mL range.

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