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First LC–MS/MS electrospray ionization validated method for the quantification of perindopril and its metabolite perindoprilat in human plasma and its application to bioequivalence study

Deepak S. Jain [a,b], Gunta Subbaiah [b], Mallika Sanyal [c], Umesh C. Pande [a], Pranav Shrivastav [a,*]

[a] *Department of Chemistry, School of Sciences, Gujarat University, Navrangpura, Ahmedabad 380 009, India*

[b] *Analytical Development Laboratory, Research Center, Torrent Pharmaceutical Limited, At Village Bhat, Gandhinagar 382 428, India*

[c] *Chemistry Department, St. Xaviers' College, Navrangpura, Ahmedabad 380009, India*

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Abstract

A high throughput bioanalytical method based on solid phase extraction and liquid chromatography–tandem mass spectrometry (LC–MS/MS), has been developed for the estimation of perindopril and its metabolite perindoprilat, an angiotensin-converting enzyme inhibitor in human plasma. Ramipril was used as internal standard (IS). The extraction of perindopril, perindoprilat and ramipril from the plasma involved treatment with phosphoric acid followed by solid phase extraction (SPE) using hydrophilic lipophilic balance HLB cartridge. The SPE eluate without drying were analyzed by LC–MS/MS, equipped with turbo ion spray (TIS) source, operating in the negative ion and selective reaction monitoring (SRM) acquisition mode to quantify perindopril and perindoprilat in human plasma. The total chromatographic run time was 1.5 min with retention time for perindopril, perindoprilat and ramipril at 0.33, 0.35 and 0.30 min. The developed method was validated in human plasma matrix, with a sensitivity of 0.5 ng/ml (CV, 7.67%) for perindopril and 0.3 ng/ml (CV, 4.94%) for perindoprilat. This method was extensively validated for its accuracy, precision, recovery, stability studies and matrix effect especially because the pattern of elution of all the analytes appears as flow injection elution. Sample preparation by this method yielded extremely clean extracts with very good and consistent mean recoveries; 78.29% for perindopril, 76.32% for perindoprilat and 77.72% for IS. The response of the LC–MS/MS method for perindopril and perindoprilat was linear over the range 0.5–350.0 ng/ml for perindopril and 0.3–40 ng/ml for perindoprilat with correlation coefficient, $r \geq 0.9998$ and 0.9996, respectively. The method was successfully applied for bioequivalence studies in human subjects samples with 4 mg immediate release (IR) formulations.

Keywords: LC–MS/MS; SRM; Perindopril; Perindoprilat; Human plasma

1. Introduction

Perindopril erbumine is the *tert*-butylamine salt of perindopril, the ethyl ester of a nonsulfhydryl angiotensin-converting enzyme (ACE) inhibitor [1]. Perindopril (2*S*,3a*S*,7a*S*)-1-[(*S*)-1-carboxybutylalanyl] hexahydro-2-indolinearboxylic acid 1-ethyl ester compound with *tert*-butylamine (1:1). Perindopril is the free acid form of perindopril erbumine, a pro-drug and metabolized in vivo by hydrolysis of the ester group to form perindoprilat, the biologically active metabolite. Perindopril is a pro-drug for perindoprilat, which inhibits ACE in human subjects and animals. The mechanism through which perindoprilat lowers blood pressure is believed to be primarily inhibition of ACE activity. ACE is a peptidyl dipeptidase that catalyzes conversion of the inactive decapeptide, angiotensin I, to the vasoconstrictor, angiotensin II. Angiotensin II is a potent peripheral vasoconstrictor, which stimulates aldosterone secretion by the adrenal cortex, and provides negative feedback on rennin secretion. Inhibition of ACE results in decreased plasma angiotensin II, leading to decreased vasoconstriction, increased

* Corresponding author. Tel.: +91 79 26300969.
E-mail address: pranav_shrivastav@yahoo.com (P. Shrivastav).

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plasma rennin activity and decreased aldosterone secretion. The latter results in diuresis and may be associated with a small increase of serum potassium.

Oral administration of perindopril tablets results in its rapid absorption with peak plasma concentration occurring at approximately 1 h. The absolute oral bioavailability of perindopril is about 75%. Following absorption, approximately 30–50% of systematically available perindopril is hydrolyzed to its active metabolite, perindoprilat, which has a mean bioavailability of about 25%. Peak plasma concentration of perindoprilat is attained in 3–7 h after perindopril administration. The presence of food in the gastrointestinal tract does not affect the rate or extent of absorption of perindopril but reduces bioavailability of perindoprilat by about 35%. Perindopril is extensively metabolized following oral administration with only 4–12% of the dose recovered unchanged in the urine. Six metabolites resulting from hydrolysis, glucuronidation and cyclization via dehydration have been identified. These include the active ACE inhibitor, perindoprilat (hydrolyzed perindopril), perindopril and perindoprilat glucoronides, dehydrated perindoprilat and the diastereoisomers of dehydrated perindoprilat. In humans hepatic esterase appears to be responsible for the hydrolysis of perindopril. Like perindopril, the active metabolite perindoprilat, also exhibits multiexponential pharmacokinetics following the oral administration of perindopril tablets.

To meet the demands for clinical pharmacokinetic studies, a rapid, selective, sensitive and robust analytical method is highly desirable. Very few analytical procedures, applied mainly to pharmaceutical formulations have been reported for the determination of perindopril which include HPLC [2,3], gas chromatography [4], radioimmunoassay [5], derivatization-gas chromatography [6], spectrophotometry [7,8] and capillary electrophoresis [9]. Recently, Rahman et al. have optimized and validated an initial rate method for perindopril erbumine in commercial dosage forms [10]. Tsaconas et al. have analyzed perindopril and perindoprilat in plasma by derivatization-gas chromatography–mass spectrometry [11] with 2 ng/ml as lower limit of quantification. These methods however do not meet modern drug development needs with respect to an efficient extraction procedure, shorter analytical run time and sensitivity, as they require prior derivatization and laborious extraction procedure.

The objective of this study was to develop and validate a high throughput LC–MS/MS method for routine measurement of perindopril and its active metabolite perindoprilat in human plasma in support of clinical findings. LC–MS/MS being a dominant tool for clinical bioanalysis because of its speed and selectivity, we have developed for the first time a simple, high throughput and sensitive LC–MS/MS detection for the analysis of as low as 0.5 and 0.3 ng/ml for perindopril and perindoprilat, respectively, using 0.75 ml of human plasma. This sensitive method was validated as per FDA guidelines [12] ensures the estimation of perindopril and its metabolite with desired accuracy and precision for elimination phase concentration in 24 healthy human volunteers for bioequivalence or bioavailability studies.

2. Experimental

2.1. Materials and instrumental conditions

2.1.1. Reagents

Perindopril was procured from Glenmark (Mumbai, India) while perindoprilat as well as ramipril (IS) were procured from Torrent Research Centre (Ahmedabad, India). Water used for the LC–MS/MS was prepared using Milli Q water purification system from Millipore (Bangalore, India). Methanol of HPLC grade was purchased from JT Baker (Phillipsburg, USA). Ammonia used for mobile phase buffer preparation was of molecular biology grade and Suprapure phosphoric acid was obtained from Merck (Darmstadt, Germany).

Solutions: 0.1% (v/v) aqueous ammonia solution was prepared. Mobile phase ratio of buffer to methanol was 20:80 (v/v). Control buffered (citrate phosphate dextrose) human plasma was procured from Green Cross blood bank (Ahmedabad, India) and was stored at −20 °C.

2.1.2. Instrumentation

Chromatographic instrument interfaced with MS was from PerkinElmer (Shelton CT, USA), PE 200 series pump, PE 200 series autosampler and PE 200 series column oven (Concord, Ontario, Canada), while triple quadruple mass spectrometer used was API-4000, manufactured by MDS SCIEX (Toronto, Canada). All the parameters of LC and MS were controlled by Analyst software version 1.4.

2.1.3. Liquid chromatographic conditions

The LC part of LC–MS/MS consisted of PerkinElmer, PE 200 series modules of pump, column oven and autosampler were used for the reversed-phase liquid chromatographic condition settings. Pump was operated at 300 μl/min flow rate; autosampler temperature was set at 10 °C. Chromatographic column used was from Thermo Electron Corporation, type X Terra MS C8, 30 mm × 2.1 mm (length × inner diameter), with 3.5 μm particle size and was maintained at 35 °C in column oven. The mobile phase consisted of 0.1% (v/v) aqueous ammonia solution:methanol in ratio of 20:80 (v/v). The LC run time was 1.5 min.

2.1.4. Mass spectrometric conditions and data acquisition

Ionization of analytes was carried out using electro spray ionization technique (TIS interface of the API 4000) with negative polarity and selective reaction monitoring (SRM) mode to monitor the ions with *m/z* of 368.10 (parent ion) and 168.1 (product ion) for perindopril; 339.3 (parent) and 168.1 (product) for perindoprilat and 415.3 (parent ion) and 166.1 (product ion) for IS (Fig. 1). For perindopril, perindoprilat and IS the source parameters maintained were Gas 1 (GS1): 30.0 psig, Gas 2 (GS2): 30.0 psig, ion spray voltage (IS): −4500.0 V, turbo heater temperature (TEM): 450.0 °C, interface heater (Ihe): ON, entrance potential (EP): −10.0 V, collision activation dissociation (CAD): 5 psig, curtain gas (CUR): 20 psig, while the declustring potential (DP), collision energy (CE) and cell exit potential (CXP) applied were −80, −62.5 and −35 V for perindopril,

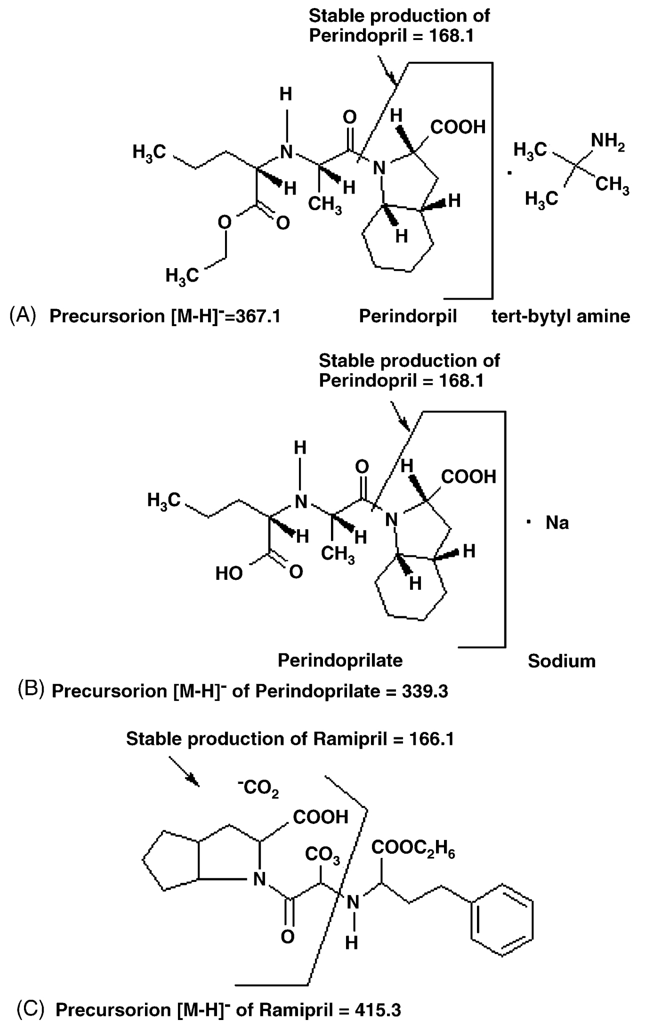

Fig. 1. Precursor ion $[M-\mathrm{H}]^-$ and probable product ion of (A) perindopril, (B) perindoprilat and (C) ramipril.

−34, −20 and −10 V for perindoprilat and −93, −34 and −21 V for IS, respectively. The perindopril and perindoprilat analysis data were acquired and quantified using Analyst software version 1.4.

2.1.5. *Preparation of standard stocks and plasma samples (calibration standard and quality control samples)*

The standard stock solutions of 100 μg/ml for perindopril, perindoprilat and ramipril (IS) were prepared by dissolving their requisite amount in methanol. These stock solutions were further diluted appropriately to get an intermediate concentration of 10 μg/ml for perindopril, perindoprilat and IS, respectively. Combined working solution of perindopril and perindoprilat of different concentrations required for spiking plasma calibration and quality control samples were subsequently prepared using the standard and intermediate stock solutions. IS working solution of 250 ng/ml was prepared using the intermediate stock of 10 μg/ml and was used as internal standard in plasma samples preparation. All the standard stock, intermediate stock and working stock solutions were prepared and stored at 2–8 °C until use. Drug free plasma, i.e. control (blank) plasma was withdrawn from the deep freezer and allowed to get completely thawed before use. Five percent spiking with combined working stock solution of perindopril and perindoprilat was done in blank plasma to achieve their desired concentration for calibration and quality control (QC) samples. The spiked QC samples at all the levels were stored at −70 °C for stability studies. The stabilities of spiked QC samples were compared with freshly prepared quality control samples.

2.1.6. *Protocol for sample extraction*

The control samples of human plasma were taken out from −70 °C freezer and kept at room temperature for 30–45 min for thawing. The samples were vortexed adequately using a vortex mixer before pipetting. Using a micropipette, 0.75 ml of plasma was transferred into an eppendorff micro-tube. To these tubes 50 μl of working solution of ramipril (250 ng/ml) was added and vortexed to mix. To the same tube 20 μl of concentrated *o*-phosphoric acid was added followed by 0.5 ml of water and vortexed again to mix. These samples were loaded on HLB cartridge previously conditioned with 1 ml methanol followed by 1 ml of 2% (v/v) aqueous acetic acid. Washing of the cartridge loaded with sample was done with 2 ml water followed by 1 ml, 5% methanol prepared in water and then again with 1 ml water. Vacuum was then applied for 5 min to remove the aqueous part. Perindopril, perindoprilat and IS were eluted with 0.5 ml of methanol and 5 μl of the eluate was directly injected in the LC–MS in partial loop mode.

3. Results

3.1. *Selectivity*

Selectivity is the ability of the method to distinguish and quantify the analyte in the presence of endogenous and/or exogenous interferences. Figs. 2 and 3 demonstrates the selectivity results with the chromatograms of blank plasma, zero standard (plasma sample with only internal standard) and the peak response of perindopril and perindoprilat at lower limit of quantification (LLOQ) (0.5 and 0.3 ng/ml, respectively). The solid phase extraction method employed gave very good selectivity for the analysis of perindopril, perindoprilat and IS in the blank plasma. The chromatograms show excellent peak shape for perindopril, perindoprilat and IS. No endogenous interferences were found at the retention times for perindopril, perindoprilat or IS. The retention time (RT) was short for both which makes it suitable for routine analysis. Test for selectivity was carried out in different lots of blank plasma; four different lots of buffered blank plasma, four different lots of heparinised blank plasma, one lot of lipemic blank plasma and one lot of haemolysed blank plasma, processed by the same solid phase extraction procedure and analyzed to determine the extent to which endogenous plasma components may contribute to the interference at the retention time of analytes and the internal standard. The area observed at the RT of perindopril and perindoprilat was less than 20% at the LLOQ (0.5 ng/ml) area, where as the area

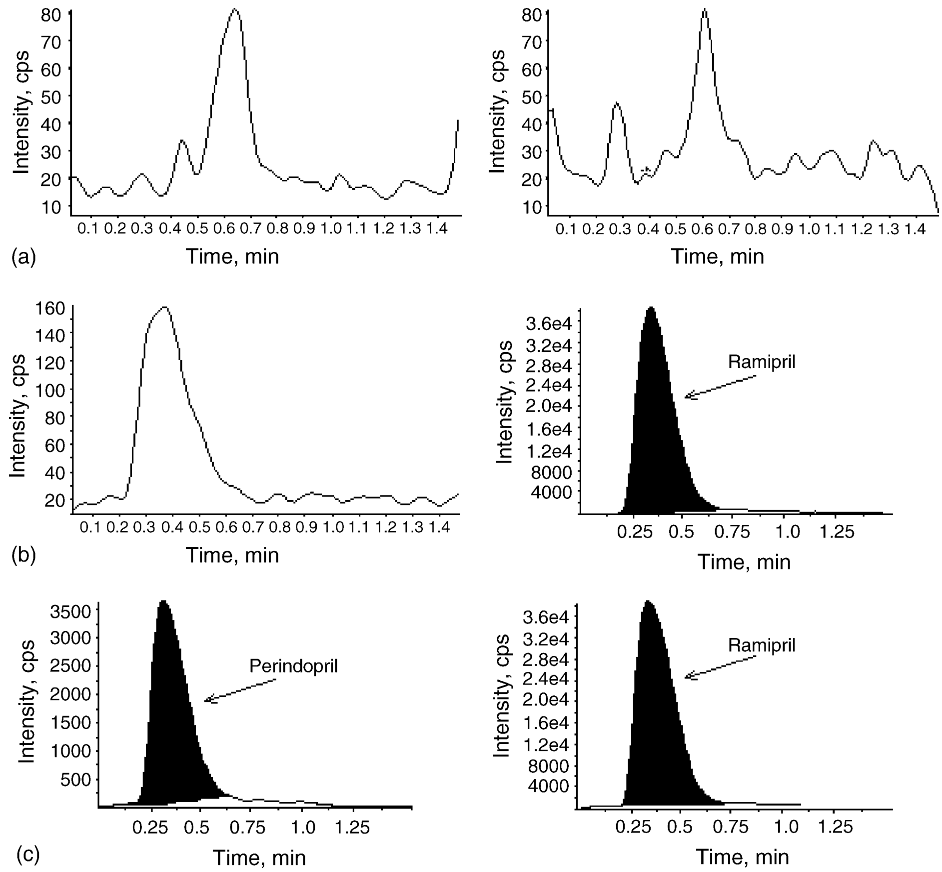

Fig. 2. Representative chromatograms of (a) blank plasma; (b) zero standard; (c) lower limit of quantification (0.5 ng/ml) for perindopril.

Table 1
Selectivity check for perindopril, perindoprilat and ramipril (IS) in 10 different lots of plasma

Sample name	Perindopril		Perindoprilat		Ramipril (IS)	
	Area	% Area	Area	% Area	Area	% Area
LLOQ-1	19521	NA	13032	NA	425027	NA
LLOQ-2	18235	NA	12429	NA	429621	NA
LLOQ-3	19145	NA	13560	NA	441560	NA
LLOQ-4	17953	NA	12931	NA	444335	NA
LLOQ-5	16708	NA	12185	NA	437906	NA
Mean	18878	NA	12731	NA	427324	NA
Blank-1[a]	0	0.00	0	0.00	0	0.00
Blank-2[a]	0	0.00	0	0.00	0	0.00
Blank-3[a]	0	0.00	0	0.00	0	0.00
Blank-4[a]	0	0.00	0	0.00	0	0.00
Blank-5[b]	0	0.00	0	0.00	0	0.00
Blank-6[b]	0	0.00	0	0.00	0	0.00
Blank-7[b]	0	0.00	0	0.00	0	0.00
Blank-8[b]	0	0.00	0	0.00	0	0.00
Blank-9[c]	0	0.00	0	0.00	0	0.00
Blank-10[d]	0	0.00	0	0.00	0	0.00

NA: not applicable. The area observed at the RT of perindopril and perindoprilat are <20% of the mean area of LLOQ. The mean area observed at the RT of IS are <5% of the mean area of IS.

[a] Buffered blank plasma.

[b] Heparinised blank plasma.

[c] Lipemic blank plasma.

[d] Haemolysed blank plasma.

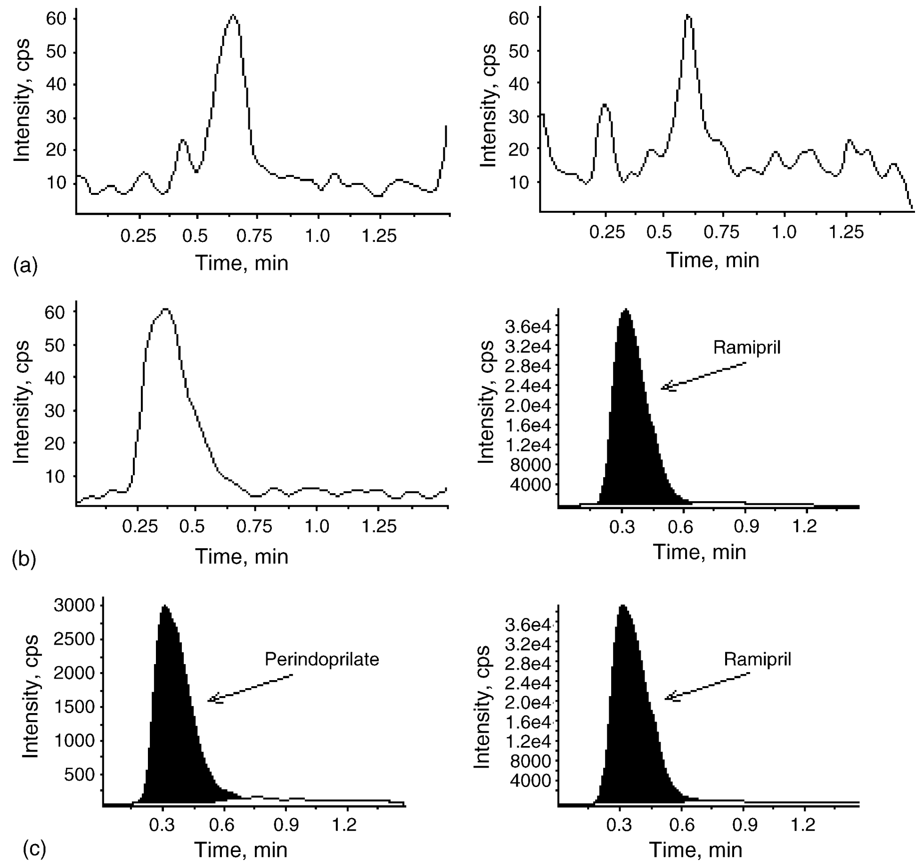

Fig. 3. Representative chromatograms of (a) blank plasma; (b) zero standard; (c) lower limit of quantification (0.3 ng/ml) for perindoprilat.

observed at the RT of IS was less than 5% the area of IS concentration used in sample preparation (Table 1). The aim of performing selectivity check with these different types of plasma samples is to ensure the quality of the results of study sample analysis.

3.2. Linearity

The linearity of the method was determined by analysis of standard plots associated with a 10-point standard calibration curve. Best-fit calibration curves of peak area ratio versus concentration were drawn. The concentration of perindopril and perindoprilat was calculated from the simple linear equation using regression analysis of spiked plasma calibration standard with the reciprocate of the drug concentration as a weighting factor (1/concentration, i.e. $1/x$); $y = mx + c$.

The perindopril calibration curves were linear from 0.5 to 350.0 ng/ml with correlation coefficient of $r \geq 0.9998$ while for perindoprilat calibration curves were linear from 0.3 to 40.0 ng/ml with correlation coefficient of $r \geq 0.9996$ within five calibration curves. The precision values obtained for slopes, and correlation coefficient 'r' from five linearities of perindopril was 2.32 and 0.01%, while for perindoprilat they were found to be 1.07 and 0.01%, respectively. The observed mean back calculated concentration with accuracy (%) and precision (% CV) of five linearities are given in Table 2.

3.3. Recovery

The percentage recovery of perindopril and perindoprilat was determined by comparing the mean area of five replicates each of extracted quality control samples; low quality control (LQC), middle quality control (MQC) and high quality control (HQC) samples with mean area of freshly prepared un-extracted LQC, MQC and HQC samples. The overall mean recovery for perindopril at LQC, MQC and HQC was 75.51, 80.65 and 78.72% and for perindoprilat was 76.04, 77.04 and 75.87%, respectively, with variability (% CV) between them of 3.32% for perindopril and 0.83% for perindoprilat. The recovery of IS was found to be 77.72%. Thus, the consistency in quantitative recoveries of perindopril, perindoprilat and IS upholds the extraction procedure for its application to routine sample analysis.

3.4. Precision and accuracy

Precision of the method is the degree of agreement among the individual test results when the procedure is applied to multiple samples. The intra-assay precision and accuracy were evaluated by five replicate analysis for perindopril and perindoprilat at four concentration levels viz. LLOQ, LQC, MQC and HQC each on the same analytical run. Inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs. The intra-batch coefficient of variation for perindopril was between 1.54 and 7.67% while for perindoprilat it was between

Table 2
Back calculated concentration of calibration standards (CS) from respective calibration curves of perindopril and perindoprilat

	CS-1	CS-2	CS-3	CS-4	CS-5	CS-6	CS-7	CS-8	CS-9	CS-10
Perindopril concentration (ng/ml)										
Added concentration (ng/ml)	0.5	1.0	5.0	14.0	35.0	70.0	140.0	210.0	280.0	350.0
Linearity	0.486	1.043	4.99	13.673	34.099	71.386	140.183	219.615	277.861	342.17
	0.49	0.986	5.028	14.344	35.604	69.497	138.453	207.01	289.681	344.41
	0.458	1.007	5.018	14.347	37.356	70.576	135.498	209.731	282.659	348.85
	0.498	1.012	4.935	14.399	33.228	71.497	140.959	210.127	281.291	347.55
	0.484	0.939	5.07	14.547	35.837	71.51	142.016	209.22	274.355	351.52
n	5	5	5	5	5	5	5	5	5	5
Mean (ng/ml)	0.483	0.997	5.008	14.262	35.225	70.893	139.422	211.141	281.169	346.900
Accuracy (%)	96.640	99.740	100.164	101.871	100.642	101.276	99.587	100.543	100.418	99.114
S.D.	0.015	0.038	0.050	0.339	1.606	0.871	2.550	4.888	5.746	3.683
CV (%)	3.12	3.86	1.00	2.38	4.56	1.23	1.83	2.32	2.04	1.06
Perindoprilat concentration (ng/ml)										
Added concentration (ng/ml)	0.3	0.6	1.0	2.0	4.0	8.0	16.0	24.0	32.0	40.0
Linearity	0.307	0.605	0.975	2.022	3.993	8.115	15.363	23.432	33.125	39.963
	0.274	0.536	1.036	2.13	4.486	8.127	15.266	23.977	32.273	39.797
	0.279	0.632	0.998	1.977	4.126	8.358	14.876	24.494	32.031	40.13
	0.283	0.58	1.019	2.089	4.031	8.375	15.199	24.823	31.856	39.646
	0.259	0.611	0.966	2.2	4.16	8.209	15.837	23.727	32.06	39.868
n	5	5	5	5	5	5	5	5	5	5
Mean (ng/ml)	0.280	0.593	0.999	2.084	4.159	8.237	15.308	24.091	32.269	39.881
Accuracy (%)	93.467	98.800	99.880	104.180	103.980	102.960	95.676	100.378	100.841	99.702
S.D.	0.017	0.037	0.029	0.088	0.195	0.124	0.348	0.565	0.501	0.181
CV (%)	6.22	6.20	2.93	4.22	4.69	1.50	2.27	2.35	1.55	0.45

1.63 and 4.94%. The intra-batch accuracy values for perindopril were found to be between 90.00 and 110.20% while for perindoprilat it was between 99.11 and 113.00%. The inter-batch coefficient of variation for perindopril was between 2.83 and 9.63% while for perindoprilat it was between 3.17 and 8.77%. The inter-batch accuracy values for perindopril were found to be between 81.80 and 114.40% while for perindoprilat it was between 82.67 and 113.00%. The comprehensive results for intra-assay and inter-assay precision are given in Table 3.

3.5. Assessment of the matrix effect in bioanalysis

Matrix effect was checked with total six different lots of plasma which included four lots of normal control heparinised plasma, one lot of lipemic control heparinised plasma and one lot of haemolysed control heparinised plasma. Three samples each of LQC and HQC were prepared from different lots of plasma (in total 36 QC samples) and checked for the inaccuracy in all the QC samples. This was performed with the aim

Table 3
Perindopril and perindoprilat intra-assay and inter-assay precision and accuracy

Quality control samples	Conc. added (ng/ml)	Perindopril intra-assay					Perindopril inter-assay				
		n	Mean conc. found (ng/ml) [a]	RE (%)	S.D.	CV (%)	*n*	Mean conc. found (ng/ml) [b]	RE (%)	S.D.	CV (%)
LLOQ	0.5	5	0.501	0.16	0.038	7.67	14	0.498	−0.37	0.048	9.63
LQC	1.5	5	1.498	−0.15	0.047	3.12	15	1.525	1.67	0.059	3.88
MQC	105	5	109.664	4.41	1.684	1.54	15	109.834	1.70	3.111	2.83
HQC	240	5	251.458	4.77	8.032	3.19	15	244.523	1.88	9.283	3.8
Quality control samples	Conc. added (ng/ml)	Perindoprilat intra-assay					Perindoprilat inter-assay				
		n	Mean conc. found (ng/ml) [a]	RE (%)	S.D.	CV (%)	*n*	Mean conc. found (ng/ml) [b]	RE (%)	S.D.	CV (%)
LLOQ	0.3	5	0.323	7.67	0.016	4.94	15	0.298	0.81	0.026	8.77
LQC	0.9	5	0.946	5.13	0.043	4.514	15	0.925	2.83	0.044	4.76
MQC	12	5	12.510	4.25	0.274	2.19	15	12.699	5.83	0.402	3.17
HQC	28	5	29.494	5.34	0.482	1.63	15	29.169	4.18	1.071	3.67

RE: relative error, S.D.: standard deviation, CV: coefficient of variance, *n*: total number of observations for each concentration.
[a] Mean of five replicate observations at each concentration.
[b] Mean of 15 observations recorded over 3 different analytical runs (5 replicates/run).

Table 4
Matrix effect in six different lots of normal lots of plasma for perindopril and perindoprilat

	Perindopril LQC (1.5 ng/ml)						Perindopril HQC (240 ng/ml)					
	Lot-1	Lot-2	Lot-3	Lot-4	Lot-5	Lot-6	Lot-1	Lot-2	Lot-3	Lot-4	Lot-5	Lot-6
Cal. conc. (ng/ml)	1.439	1.570	1.525	1.476	1.426	1.462	245.135	247.506	256.524	244.821	238.978	252.513
RE (%)	−4.07	4.67	1.64	−1.59	−4.90	−2.51	2.14	3.13	6.89	2.01	−0.43	5.21
	Perindoprilat LQC (0.9 ng/ml)						Perindoprilat HQC (28 ng/ml)					
	Lot-1	Lot-2	Lot-3	Lot-4	Lot-5	Lot-6	Lot-1	Lot-2	Lot-3	Lot-4	Lot-5	Lot-6
Cal. conc. (ng/ml)	0.873	0.964	0.929	0.965	0.886	0.884	29.597	29.370	30.310	28.920	27.978	29.411
RE (%)	−3.01	7.11	3.26	7.19	−1.50	−1.83	5.70	4.89	8.25	3.29	−0.08	5.04

RE: relative error.

to see the matrix effect of these different lots of plasma on the back calculated value of QC's nominal concentration especially because the pattern of elution of all the analytes appears as flow injection elution. The results found were well within the acceptable limit of ±15% with no adverse matrix effects as shown in Table 4. This clearly proves that the elution of endogenous matrix peaks in the dead volume time did not affect the elution pattern of perindopril, perindoprilat and IS peak in the assay. Therefore, the method of extraction for both the analytes and IS from plasma was rugged enough and gave accurate and consistent results when applied to real patient samples.

3.6. Stability study

Plasma being the matrix for extraction of perindopril and its metabolite perindoprilat, with mean bioavailability of 65–70% for perindopril and only 20% for perindoprilat, encouraged us to give prime consideration for the development of a method which precisely and quantitatively extracts perindopril and perindoprilat from the stored plasma (stability) samples. Hence stability experiments were performed very exhaustively with this method to evaluate their stability in stocks solutions and in plasma samples under different conditions. The conditions which occurred during actual study sample analysis were simulated in method validation stability studies, such as: stock solution stability of perindopril, perindoprilat and IS, stability in plasma at room temperature, extracted sample stability (process stability at 10 °C), freeze thaw stability and long term stability at −70 °C. The results obtained were well within the acceptable limit. IS stock solution was also found to be stable.

Stock solution of perindopril, perindoprilat and IS were stable at room temperature for 24 h and at 2–8 °C for 10 days with mean percent change within ±5%. Perindopril and perindoprilat in control human plasma at room temperature was stable at least for 6 h. Perindopril and perindoprilat in the autosampler at 10 °C was found to be stable up to 48 h (process stability). Perindopril and perindoprilat was found to be stable for at least three freeze and thaw cycles. The perindopril and perindoprilat spiked plasma samples stored at −70 °C for long term stability experiment were found stable for at least 222 days. The values for the percent change for the above stability experiment are compiled in Table 5.

3.7. Application of the method on human subjects

The proposed validated method was successfully applied for the assay of perindopril and perindoprilat in healthy adult male human subject samples who received 4 mg immediate release reference and test formulations under fasting conditions. The design of the study comprised of "a randomized, open label, single dose, two treatments, two periods, two sequence crossover bioequivalence study of perindopril and perindoprilat for 4 mg immediate release formulation in 24 healthy human subjects". The subjects were briefed regarding the protocol and risk involved in the study. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA [13]. The samples were processed based on the proposed extraction protocol for quantification of perindopril. The method was sensitive enough to monitor the perindopril plasma concentration up to 12 h while perindoprilat up to 72 h. In all approximately 1200 samples including the calibration, QC and volunteer samples were run and analyzed in only 5 days and the precision and accuracy for calibration and QC samples were well within the acceptable limits. The mean pharmacokinetic profile for the treatment, under fasting condition is presented in Figs. 4 and 5. These

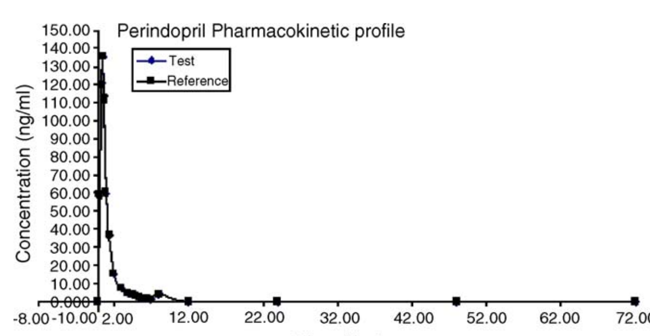

Fig. 4. Mean pharmacokinetic profile of 23 healthy male subjects for test and reference 4 mg IR formulation.

Table 5
Stability results of (a) perindopril and (b) perindoprilat

Stability experiments	Storage condition	Mean comparison, sample conc. found (ng/ml)	S.D.	Mean stability, sample conc. found (ng/ml)	S.D.	% Mean change at quality control level
(a)						
Bench top	Room temperature (6 h)	1.538, 235.678	0.026, 3.760	1.546, 237.658	0.036, 3.116	LQC: 0.53, HQC: 0.84
Process (extracted sample)	Autosampler (5 °C, for 48 h)	1.539, 246.433	0.090, 7.779	1.497, 243.945	0.015, 2.679	LQC: −2.76, HQC: −1.01
Freeze and thaw stability in plasma	After third FT cycle at −70 °C	1.553, 241.626	0.047, 6.623	1.543, 226.233	0.047, 11.364	LQC: −0.67, HQC: −6.37
(b)						
Bench top	Room temperature (6 h)	0.887, 29.526	0.021, 1.406	0.908, 28.747	0.022, 0.583	LQC: 2.41, HQC: −2.64
Process (extracted sample)	Autosampler (5 °C, for 48 h)	0.943, 28.488	0.042, 0.967	0.936, 28.317	0.011, 0.963	LQC: −0.74, HQC: −0.60
freeze and Thaw stability in plasma	After third FT cycle at −70 °C	0.934, 29.222	0.046, 0.823	0.986, 29.309	0.047, 2.453	LQC: 5.60, HQC: 0.30
Long term stability in human plasma	For 222 days at −70 °C	0.936, 12.390, 28.507	0.026, 0.210, 1.213	0.956, 11.903, 27.532	0.037, 0.264,1.189	LQC: 2.12, MQC: −3.94, HQC: −3.42

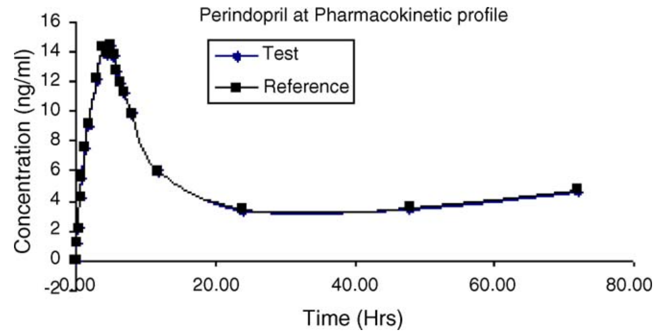

Fig. 5. Mean pharmacokinetic profile of 23 healthy subjects for test and reference 4 mg IR formulation.

observations confirm the bioequivalence of the test sample 4 mg (immediate release tablet) with the reference product in terms of rate and extent of absorption. Further, there was no adverse event during the course of the study. Thus the assay procedure for perindopril in plasma samples demonstrated the linearity, precision and sensitivity needed for the pharmacokinetic studies of this drug.

4. Results and discussion

It was a challenge for us to develop a method for the quantitative extraction of perindopril and perindoprilat, from human plasma. This analytical method was developed and validated for assaying perindopril and perindoprilat in therapeutic concentration range for the analysis of routine samples. During development, tuning of MS parameters in both positive and negative ionization modes was carried out for perindopril, perindoprilat and IS. However, the response found was much higher in negative ionization mode for all, while it was higher only for perindopril and ramipril in positive ionization mode. Use of ammonia in the mobile phase further enhanced the response of perindopril, perindoprilat and ramipril with low background noise, resulting in higher selectivity. Therefore, the negative ionization mode was selected for the entire study. Particular effort was directed towards method development to improve the method ruggedness during the SPE, sample analysis and transferability. The extraction of the analytes from plasma was tried with liquid–liquid extraction using different solvents viz. diethyl ether, tetra butyl methyl ether (TBME), TBME and hexane etc., but all efforts resulted in inconsistent recoveries, longer extraction time, higher background and hence poor sensitivity. In an attempt to get cleaner extracts, solid phase extraction was carried out. The pre-treatment of HLB cartridge with 2% acetic acid was done to reduce the basic matrix retention and increase the retention of acidic analytes. Addition of *o*-phosphoric acid helped in breaking the drug protein binding and maintaining the acidic analytes to remain in nonionic lipophilic form, hence maximizing their retention on hydrophilic lipophilic balance (HLB) stationary phase. Subsequent thorough washings resulted in minimized polar matrix interference and increased specificity.

It has been well established in the industry that use of a compound as internal standard with same analytical behaviour to that of analyte and having same ionization and extraction efficiency can significantly improve the method ruggedness. A good internal standard should track the analyte during the extraction and compensate for any analyte on the column and any inconsistent response due to matrix effects. This is also established with almost same recovery of IS compared to all other analytes. Ramipril was therefore used as IS. All the significant efforts directed towards the extraction procedure, resulted in achieving the required LLOQ with best specificity. The method described is sensitive and highly reproducible for the analysis of perindopril and perindoprilat in human plasma using LC–MS/MS. The basic underlying advantage of this optimized method is that, it utilizes only 0.75 ml of plasma. Also the method involves minimum usage of organic solvent, with no drying or reconstitution step. Five microliters of the final eluate is directly injected into LC–MS to give 3.75 pg/injection for perindopril and 2.25 pg/injection for perindoprilat as quantification limit. No extra labour of derivatization as required in GC or GC–MS to increase the sensitivity. The limit of quantification for perindopril (0.5 ng/ml) and perindoprilat (0.3 ng/ml) achieved is the lowest concentration required for a typical perindopril pharmacokinetic study (4 mg), and still lower quantification can be achieved by drying and reconstituting the eluate in 50 μl (applicable for lower dose samples). With the retention time for perindopril, perindoprilat and ramipril (IS) at 0.33, 0.35 and 0.30 min, respectively, in dead volume time and the precision of LLOQ's for all analytes demonstrates the consistency of extraction procedure which was confirmed by the recovery and matrix effect experiment. The method was more selective and specific because the data acquisition was performed with SRM mode and thus the need to separate perindopril, perindoprilat and IS chromatographically is not mandatory. Overall, this short extraction procedure and analytical run time, with method validated for all the stability studies ensures high throughput and renders it suitable for routine volunteers sample analysis.

5. Conclusion

The objective of this work was to develop a high throughput and a sensitive method to estimate perindopril and its active metabolite perindoprilat in human plasma, especially in the absorption and elimination phase after oral administration of 4 mg formulation. The advantage of using SPE is that it gives cleaner and consistent extracts with minimum matrix effect. The SPE eluate (5 μl) was directly injected onto LC–MS/MS without any prior concentration and reconstitution steps, which increases the sensitivity and selectivity of the analysis. The run time per sample analysis of 1.5 min suggests the high throughput of the proposed method. Moreover, the limit of quantification is low enough to monitor at least five half life of perindopril and perindoprilat concentration with good intra and inter-assay reproducibility (%CV) for the quality controls. From the results of all the validation parameters, we conclude that the method can be highly useful for the therapeutic drug monitoring both for analysis of routine samples of single dose or multiple dose pharmacokinetics and also for the clinical trial samples with precision, accuracy and high throughput.

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