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Determination of Ramipril and its Active Metabolite Ramiprilat from Plasma by Liquid Chromatography-Mass Spectrometry for Bioequivalence Studies

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Ramipril and ramiprilat were isolated by solid phase extraction using PROSPEKT-2, which is an online solid phase extractor. This method is suitable for bioequivalence studies following single dose in healthy volunteers. This assay achieved higher sensitivity and better specificity for the analysis of ramipril and ramiprilat in human plasma. The limit of quantitation of 0.25 ng/mL for ramipril and ramiprilat was thus attainable by high performance liquid chromatography mass spectrometry (HPLC-MS/MS). The internal standard proved to be a good internal standard for this assay. No significant interference caused by endogenous compounds was observed. This simple and rapid assay can be successfully used in pharmacokinetic studies of ramipril and ramiprilat.

Key Words: Ramipril, Ramiprilat, Captopril, Solid Phase Extraction, HPLC-MS/MS, Human plasma.

INTRODUCTION

Ramipril is a second generation angiotensin converting enzyme (ACE) inhibitor. It is a prodrug and is hydrolyzed *in vivo* to release the active metabolite ramiprilat which has long elimination half-life[1]. It is a 2-aza-bicyclo[3.3.0]-octane-3-carboxylic acid derivative. It is a white, crystalline substance soluble in polar organic solvents and buffered aqueous solutions. Ramipril melts between 105 and 112°C. Its empirical formula is $C_{23}H_{32}N_2O_5$ (m.w. 416.5). Ramiprilat, the diacid metabolite of ramipril, is a non-sulfhydryl angiotensin converting enzyme inhibitor. Ramipril is converted to ramiprilat by hepatic cleavage of the ester group. The chemical name for Ramipril is 2-[N-[(S)-1-ethoxycarbonyl-3-phenylpropyl]-L-alanyl]-(1S,3S,5S)-2-azabicyclo[3.3.0]octane-3-carboxylic acid and ramiprilat is (2S,3αS,6αS)-1[(S)-N-[(S)-1-carboxy-3-phenylpropyl]alanyl]-octahydrocyclopenta[b]pyrrole-2-carboxylic acid, 1-ethyl ester[1].

A high performance liquid chromatography mass spectrometry (HPLC-MS/MS) method was used for the determination of ramipril and ramiprilat in heparinized human plasma[2]. Captopril was used as internal standard because its chemical properties and mass spectral fragments were similar to those of ramipril

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and ramiprilat. Selection of mobile phase components was a critical factor in achieving good chromatographic peak shape and resolution. Good separation of the target compounds and short run time were obtained using an elution system of methanol : water (90 : 10%) (v/v).

Following oral administration of ramipril, peak plasma concentrations of ramipril are reached within 1 h. The extent of absorption is at least 50–60% and is not significantly influenced by the presence of food in the GI tract, although the rate of absorption is reduced. Cleavage of the ester group (primarily in the liver) converts ramipril to its active diacid metabolite, ramiprilat. Peak plasma concentrations of ramiprilat reached within 2–4 h after drug intake. The serum protein binding of ramipril is about 73% and that of ramiprilat about 56% *in vitro*; these percentages are independent of the blood, concentrations of ramipril and ramiprilat increase with increased dose, but are not strictly dose-proportional. The 24 h AUC for ramiprilat, however, is dose-proportional over the 2.5–20 mg dose range concentration over the range of 0.01–10 mcg/mL[3].

EXPERIMENTAL

Instrument: HPLC-MS/MS system using turbo ion spray ionization (ESI).

Biological matrix: Pooled human plasma, commercially procured and chromatographically analyzed to ensure non-interference.

CHROMATOGRAPHIC CONDITIONS

Mobile phase	Water : Methanol (10 : 90)	Column	Ace C18 (2.1 × 50 mm) 10 μ
Detector	Perkin-Elmer Sciex API 3000 LC-MS system using turbo ion spray ionization (ESI)	Scan	MRM
Internal standard	Captopril	Polarity	Positive
Flow rate	0.25 mL/min	Pause time	5 min
Injection volume	50 μL		

Preparation of aqueous and plasma standards

Stock solutions of ramipril, ramiprilat and internal standard of 1 mg/mL were prepared in methanol, respectively. Standard solutions containing a mixture of ramipril and ramiprilat of concentration 0.1, 1 and 10 μg/mL were also prepared using methanol.

Calibration standards of mixture of ramipril and ramiprilat (0.25, 0.50, 1.0, 4.0, 5.0, 15.0, 25.0, 40.0 ng/mL) and an LOQ sample at 0.25 ng/mL were prepared by spiking appropriate amounts of the standard solutions in control plasma obtained from healthy human non-smoking volunteers. Quality samples were prepared in the Hank control plasma at the concentrations of 0.25, 0.40, 10.0 and 30.0 ng/mL.

Sample preparation

Following extraction procedure was used for preparation of biological matrix samples *i.e.*, all calibration levels, QC samples and volunteer's plasma samples before injecting into HPLC-MS/MS system.

Step	Name	Comments
1	New catridge	Left clamp.
2	Solvation	With 1 mL methanol. Dispensing flow 5000 μL/min.
3	Equilibration	With 1 mL water. Dispensing flow 5000 μL/min.
4	Start autosampler	—
5	Sample application	Injection volume 500 μL. Dispensing flow 2000 μL/min.
6	Wash catridge	With 1 mL water. Dispensing flow 5000 μL/min.
7	Input	—
8	Output	Start MS and start LC.
9	Elution	With methanol.
10	Solvation	With 1 mL methanol. Dispensing flow 5000 μL/min.
11	Equilibration	With 1 mL water. Dispensing flow 5000 μL/min.
12	Move catridge	From left clamp to the tray.

Assay validation

Specificity: 10 bags of fresh frozen plasma obtained from different sources were analyzed to ensure non-interference.

Linearity and sensitivity: A calibration curve in the range of 0.25–40.0 ng/mL was constructed by plotting the area ratios of ramipril and ramiprilat to internal standard against ramipril and ramiprilat concentrations in plasma. LOQ was established based on an S/N ratio of 5.

Precision and accuracy: The precision of the assay was determined by replicate analyses of four different concentrations LOQ (0.25 ng/mL), LQC (0.4 ng/mL), MQC (10 ng/mL) and HQC (30 ng/mL). Intra-day precision was determined by repeated analysis of each of QC sample on 1 d (n = 5) and the inter-day precision and accuracy were determined by repeated analyses on four consecutive days (n = 1 series/day). The concentration of each sample was determined using calibration standards prepared on the same day.

Stability: Analytes at low and high concentrations were tested for freeze-thaw (5 cycles), bench top stability (up to 24 h), auto sampler stability (up to 24 h), long-term stability (12 weeks) and stock solution stability (up to 24 h).

Extraction recovery: The absolute recovery of ramipril and ramiprilat through extraction procedures were determined at low, medium and high concentrations by external standard method. Known amounts of ramipril, ramiptilat and internal standard were added to human plasma prior to extraction. The concentration of ramipril and ramiprilat was calculated using the calibration curves prepared on the same day and was compared to nominal concentration to estimate extraction recovery.

Pharmacokinetics and study: Each of 24 healthy male volunteers received 1 × 5 mg capsule of ramipril after overnight fasting. Blood samples were drawn at appropriate intervals centrifuged to obtain plasma samples.

Conditions for ESI-MS: The ESI mass spectrum at a fragment voltage of 30 and 32 V showed that the protonated molecular ion $[M + H]^+$ of ramipril, ramiprilat and internal standard was at 417.50, 389.30 and 218.10, respectively. By increasing the fragmentor voltage, the fragmentation pattern of these protonated molecular

ions were observed. The product ion mass spectrum of this protonated molecule with the most intensive product ions was observed at m/z 234.1 and 206.1 for ramipril and ramiprilat, respectively. By monitoring this product ion, a highly sensitive assay for ramipril and ramiprilat was developed. The intensity of product ion of ramipril and ramiprilat at m/z was compared at fragmentor voltages of 15, 20 and 50 V in order to determine the optimal collision energy. The result showed that the highest sensitivity was obtained using a fragmentor voltage of 30 and 32 V for ramipril and ramiprilat, respectively. Therefore, a fragmentor voltage of 30 and 32 V was used to carry out LC-ESI-MS in the MRM mode. At this collision energy the most intensive product ion of I.S. protonated molecular ion was at m/z 116.2.

RESULTS AND DISCUSSION

TABLE-1
RESULTS OF METHOD VALIDATION

Test	Acceptance criteria	Results for ramipril	Results for ramiprilat	Conclusion
Specificity	Non interference at the retention time of Ramipril, Ramiprilat and Internal standard	No interfering peak at the retention time of ramipril	No interfering peak at the retention time of ramiprilat and internal standard was found	Method was found to be specific
Sensitivity	S/N more than 5 : 1	7.31 : 1	5.40 : 1	Method was found to be sensitive
Linearity	Regression to be more than 0.95	0.9995	0.9974	Method was found to be linear
Precision and accuracy	Intra day	Intra day	Intra day	
	1. % Accuracy for			
	LOQ: 80–120%	88.54–90.12%	89.56–97.28%	Method was found to be precise and accurate
	LQC: 85–115%	95.22–105.64%	95.24–101.85%	
	MQC: 85–115%	99.77–111.56%	94.28–99.65%	
	HQC: 85–115%	89.44–99.78%	94.33–109.55%	
	2. % RSD for			
	LOQ: nmt 20%	3.01–6.48%	6.86–9.80%	
	LQC: nmt 15%	1.78–8.62%	4.24–8.90%	
	MQC: nmt 15%	1.41–5.54%	1.61–7.22%	
	HQC: nmt 15%	1.66–11.91%	1.50–3.77%	
	Inter day	Inter day	Inter day	
	1. % Accuracy for			
	LOQ: 80–120%	89.36%	93.65%	
	LQC: 85–115%	100.38%	98.93%	
	MQC: 85–115%	106.59%	96.90%	
	HQC: 85–115%	94.42%	101.27%	
	2. % RSD for			
	LOQ: nmt 20%	5.90%	8.92%	
	LQC: nmt 15%	10.83%	7.48%	
	MQC: nmt 15%	9.00%	6.10%	
	HQC: nmt 15%	8.73%	5.33%	
Recovery	Precise, consistent and reproducible	32.54–35.83%	31.85–35.83%	Recovery was found to be precise, consistent and reproducible

Application

The method described above was successfully applied to the pharmacokinetic study in which plasma concentrations of ramipril and ramiprilat in 24 healthy volunteers were determined up 120 h after the administration of 5 mg capsule. The pharmacokinetic parameter values are calculated. The maximum plasma concentration of 18.28–20.25 ng/mL for Ramipril and 12.63–14.22 ng/mL for ramiprilat after the administration.

Efficacy results

The 90% confidence intervals for ln-transformed parameters for ramipril are summarized below:

Parameters	Geometric mean		T/R	90% confidence intervals for ln-transformed data
	Test (T)	Reference (R)		Lower-Upper %
C_{max} (ng/mL)	16.92	15.39	109.98	92.25–131.11%
AUC_{0-t} (h.ng/mL)	12.65	12.36	102.29	90.34–115.82%
$AUC_{0-\infty}$ (h.ng/mL)	13.11	12.66	103.51	91.53–117.05%

The 90% confidence intervals for ln-transformed parameters for ramiprilat are summarized below:

Parameters	Geometric mean		T/R	90% confidence intervals for ln-transformed data
	Test (T)	Reference (R)		Lower-Upper %
C_{max} (ng/mL)	12.18	11.38	107.05	97.00–118.14%
AUC_{0-t} (h.ng/mL)	126.63	114.08	111.00	102.07–120.73%
$AUC_{0-\infty}$ (h.ng/mL)	134.35	121.03	111.01	101.83–121.02%

Conclusion

This assay achieved higher sensitivity and better specificity for the analysis of ramipril and ramiprilat in human plasma. The limit of quantitation of ng/mL for ramipril and ramiprilat was thus attainable by HPLC-MS/MS. The internal standard proved to be good internal standard for this assay. No significant interference caused by endogenous compounds was observed. This simple and rapid assay can be successfully used in pharmacokinetic studies of ramipril and ramiprilat.

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