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Simultaneous Quantification of Ramipril and Ramiprilat in Drug Formulations: A Clinical LC-MS/MS Study

Khaled W. Omari[1]*, Ahmed Abu-Awwad[2], Basel Arafat[3], Gianina Macovei[4], TLucretia Boltez[4], Zaher Abdel Baki[1], Eyad Mallah[6]* and Tawfiq Arafat[7]

1. College of Engineering and Technology, American University of the Middle East, Kuwait
2. Faculty of Pharmacy, Jerash University, Amman –Jordan
3. Faculty of Medical Sciences and Public Health, Anglia Ruskin University, Chelmsford, UK
4. Antibiotice S.A. 1, Valea Lupului, Iaşi, 707410, Romania
5. College of Engineering and Technology, American University of the Middle East, Kuwait
6. Faculty of Pharmacy and Medical Sciences, University of Petra, Amman –Jordan
7. The Jordan Center for Pharmaceutical Research, Amman –Jordan

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*Corresponding author
Khaled W. Omari
Email: khaled.omari@aum.edu.kw

*Corresponding author
Eyad Mallah
Email: emallah@uop.edu.jo

ABSTRACT

Ramipril is a drug controlling hypertension. Human health suffers greatly as a result of uncontrolled hypertension, which can exist without symptoms. Ramiprilat is an active metabolite component. Two drugs were studied in 36 healthy adults. The drugs are the test (Atb®) and the reference (Tritace®). This concept is crucial in the pharmaceutical industry during the development process. The maximum concentration (C_{max}) of Ramipril and its metabolite (Ramiprilat) were determined throughout drug administration in healthy subjects. All analysis was performed using LC-MS/MS. The method was validated over a range of 0.200 – 25.000 ng/mL in which linearity (R^2) was 0.9983, accuracy was 99.9%, precision (CV) was less than 20%, and the Lower Limit of Quantitation was 0.200 ng/mL. Stability and other parameters were discussed. The time needed to reach this concentration (T_{max}) and the time-drug concentration area under the curve (AUC) were most agreed upon. The two drugs were proven to be interchangeable. Both have similar pharmacokinetics and bioequivalence profiles. Consequently, the drugs are bioequivalent and considered alternatives to one another pharmaceutically. For example, 90% confidence intervals (C.I.) for the intra-individual ratios (test/reference) for pharmacokinetic parameters were C_{max}, and AUC_{0-t} for total exposure C.I. Log – transformed Values were 80-125%.

Keywords: chromatographic/spectrometric techniques, hypertension, metabolite, organic medicinal, pharmaceutical chemistry, pharmacokinetics.

INTRODUCTION

The incidence of high blood pressure (hypertension) worldwide is expected to increase by 30% in 2025 (Muñoz-Durango *et al*. 2005). Uncontrolled hypertension severely affects humans' health. Hypertension can exist without symptoms, causing body damage and complications such as heart attack, heart failure, and sudden cardiac death. Monitoring and maintaining healthy blood pressure can reduce damage to arteries, heart, brain, kidneys, eyes, and sexual dysfunction (Momoniat *et al*. 2019). Guidelines for the prevention, diagnosis, risk assessment, and treatment of hypertension have already been established (Rabi *et al*. 2020; Whelton *et al*. 2017; Williams *et al*. 2018; WHO 2021; Antza *et al*. 2021).

Hypertension is greatly affected by the renin-angiotensin-aldosterone system, which maintains the tonicity of vascular cells and regulates both volumes of the extracellular fluid and arterial pressure. A failure in this system can disrupt blood pressure (Patel 2017). Hypertension and cardiovascular diseases should be controlled using angiotensin-converting enzyme (ACE) inhibitors (Messerli *et al*. 2018) by preventing vasoconstriction that targets hypertension and congestive heart failure (Lu *et al*. 2020). Ramipril is an ACE inhibitor ((2S,3aS,6aS)-1[(S)-N [(S)-1-Carboxy-3-phenylpropyl] alanyl] octa

hydrocyclopenta [b]pyrrole-2-carboxylic acid, 1 ethyl ester; figure 1. (A) Ramipril structure) that expands the blood vessels and lowers blood pressure (Kosacka *et al*. 2022) and enhances blood and oxygen delivery to the heart (Yusuf *et al*. 2008). Ramipril minimizes cardiovascular death, peripheral arterial disease, stroke, and myocardial infarction (MI and heart attack) (Ruggenenti *et al*. 2021).

After the oral administration of ramipril, it would be hydrolyzed in the liver, where it becomes an end-product known as the metabolite. This metabolite is called ramiprilat. It is an active inhibitor that can bind to the ACE system. It also inhibits the formation of angiotensin II from angiotensin I (Levitt and Schoemaker 2006). Consequently, this inhibition diminishes the action of angiotensin II as an effective vasoconstrictor. As a result, ramipril and ramiprilat play critical roles in hypertension treatment. Ramipril and ramiprilat can be analyzed using Ramipril and ramiprilat can be analyzed using methods including ramipril-membrane-based ion-selective electrode (Arida *et al*. 2009), LC-MS/MS (Iao-yang *et al*. 2006), and spectrophotometric approach (Afieroho *et al*. 2012).

This study primarily aimed to conduct a randomized comparison of a single-dose, two-period, two-treatment, two-sequence crossover open-label study to determine the bioequivalence of orally administered ramipril (5 mg ramipril per tablet) between the Atb® (test) and Tritace® (reference) drugs in healthy adults under fasting conditions. The pharmaceutical chemistry for both treatments was also evaluated. Ramiprilat (figure 1. (B) Ramiprilat structure) is the metabolite that was also examined.

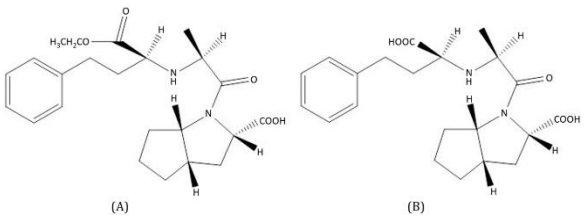

Figure 1. (A) Ramipril and (B) Ramiprilat structures.

The novelty of our study is to add to the literature with a fully validated investigation of ramipril and ramiprilat, as reported in the text and supplemental data, comparing two different medicines. This helped to establish the clinical equivalency of the two formulations, which included varied medication strengths. This assures that the new medicine has the same therapeutic effect as the original created one, protecting patient safety.

MATERIALS AND METHODS

This study was performed following the ICH HARMONISED GUIDELINE: INTEGRATED ADDENDUM TO ICH E6(R1): GUIDELINE FOR GOOD CLINICAL PRACTICE E6(R2) dated November 9, 2016, and the local laws and regulations (ICH 2016). Consent was obtained from every participant before commencing the study. The Institutional Review Board of the Jordan Center for Pharmaceutical Research (JCPR) approved the clinical investigation, ensuring that the rights and welfare of human participants are carefully considered (Appendix 16.1.3.1). Moreover, the Jordan Food and Drug Administration (JFDA) (JFDA 2011) reviewed the study protocol based on the JFDA law, and approval was obtained (Appendix 16.1.8.6).

All 36 randomized participants who were enrolled and completed the study were aged 18–48 years, had an average weight based on the body mass index, were smokers/non-smokers, and were non-alcoholic males (Supplementary Data S1). A serum creatinine test was performed during screening, at 8 h post-drug administration during each period, at 24 h if the serum creatinine level increased during the two periods at 8 h post-drug administration (≥50% above the baseline value), before the second period and at the follow-up examination.

A potassium level test was also performed during screening, at 8 h post-drug administration during each period, before the second period, and at the follow-up examination. Kidney and liver function tests were within the laboratory's average values during screening. Blood hematology and chemistry were performed during screening and follow-up examination. Serology and urinalysis were performed during screening, see Supplementary Data S2-S4. An electrocardiograph (ECG) was performed during screening, before the second period, and at the follow-up examination. Physical assessment and clinical evaluation were performed during screening and at the follow-up examination. Polymerase chain reaction test for coronavirus disease 2019 was performed on day 0 in both periods. Participants had to remain seated on beds upright for the first 6 h after drug administration; then, they were allowed to lie, sit, or walk.

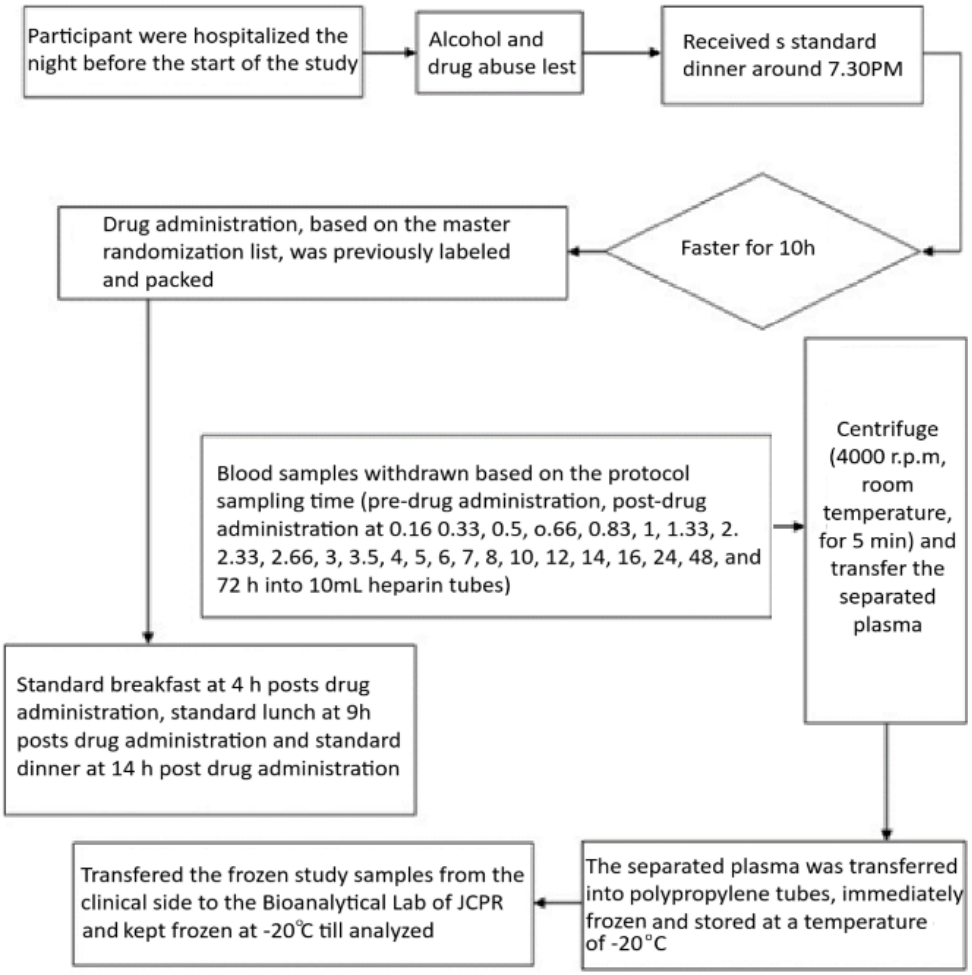

Figure 2. Overall study design flow chart.

Before drug administration, the blood pressure was not <110/70 mmHg. The participants were clinically tested before starting the study, including hematology, differential leukocyte count, biochemistry, immunology, urinalysis, serum creatinine, and potassium levels (Supplementary Data S2). Clinical testing during and after the study (follow-up) was established in Supplementary data S3 and S4, respectively.

No significant changes were observed from screening regarding vital signs. ECG was performed during screening, before the second period, and at the follow-up examination; all findings were within the normal ranges. No clinically relevant abnormalities were observed during the physical examination. Vital signs (blood pressure and heart rate) were unremarkable at pre-drug administration, during the study, and post-study. The total amount of blood withdrawn during the study was 443 mL, including the blood drawn for laboratory examinations and stock plasma.

Noteworthy, for drug-drug interactions, administration of any prescribed systemic or topical medication within 2 weeks before the start of the study and treatment with drugs known to alter the effective metabolic systems, such as barbiturates, phenothiazines, cimetidine, omeprazole, and warfarin, were not allowed within the last 30 days from the study. Participants taking one of the following medications were excluded from the study as they interact with ramipril: non-steroidal anti-inflammatory drugs such as ibuprofen or indometacin and aspirin, ephedrine, noradrenaline or adrenaline, sacubitril/valsartan, chemotherapy, ciclosporin, furosemide, spironolactone, triamterene, amiloride, potassium salts, trimethoprim alone or in combination with sulfamethoxazole and heparin, prednisolone,

allopurinol, procainamide, temsirolimus, sirolimus, everolimus, vildagliptin, racecadotril, angiotensin II receptor blocker or aliskiren, diabetes medications such as oral glucose-lowering medicines and insulin, and lithium.

Study Design and Procedures

An open-label, randomized, single oral dose, two-period, two-sequence, two-treatment, laboratory-blinded, crossover study, was conducted for both the test and reference drugs under fasting conditions. A single oral dose of one tablet containing 5 mg of ramipril was administered under fasting conditions during each study period. A 14-day wash-out period between administrations was respected.

Plasma concentrations were analyzed using the mass spectrometry detection method (LC-MS/MS) with selective quantification of ramipril and ramiprilat (ramipril's active metabolite) in the plasma. Plasma sample analysis continued up to 16 h post-drug administration for ramipril quantification and 72 h post-drug administration for ramiprilat quantification. During each study period, separated by a wash-out phase of 14 days, each participant was given, in a crossover design and under fasting conditions, a single dose of one tablet of either the test or reference drug, with approximately 240±5 mL of water after 10 h at least fasting overnight. In each study period, a (2×8 mL) blood sample was collected pre-drug administration and a series of 24×7 mL blood samples were collected at the following times: 0.16, 0.33, 0.5, 0.66, 0.83, 1, 1.33, 1.66, 2, 2.33, 2.66, 3, 3.5, 4, 5, 6, 8, 10, 12, 14, 16, 24, 48, and 72 h post-drug administration (Figure 2).

Period II procedure was the same as the period I in the above flow chart, switching the two treatments based on the master randomization list. Serum creatinine and potassium level tests were performed before the second period. No significant changes were observed from screening regarding vital signs. ECG was performed during screening, before the second period, and follow-up examination for all participants; findings were all within normal limits. No clinically relevant abnormalities were observed at physical examination. The determination of ramipril and ramiprilat (ramipril's active metabolite) plasma concentrations was performed using a validated specific high-performance liquid chromatography-mass spectrometry detection method (LC-MS/MS) at JCPR. For detailed method validation, see Supplementary data S5. The lower quantification limits of ramipril and ramiprilat are 0.200 ng/ml and 0.100 ng/ml, respectively. These LLOQ values for the analytes were sufficient to achieve the aim of the study. The validation study was performed as per the FDA Guidance for Industry, Bioanalytical Method Validation (FDA *et al*. 2018), and European Medicines Agency, Guideline on bioanalytical method validation (CHMP 1922).

Statistical Analysis

The primary concern in bioequivalence assessment was to limit the risk of erroneously accepting bioequivalence. This risk, also known as "consumer's risk," must be limited to <5%. Bioequivalence can be concluded if 90% confidence intervals for intra-individual ratios (test/reference) for pharmacokinetic parameters were maximum concentration (C_{max}) and area under the curve for total exposure (AUC_{0-t}). The CI log-transformed values were 80–125%.

Confidence intervals were determined using log-transformed data using the parametric method. Ratios of means were calculated using the least square mean (LSM) for both untransformed and log-transformed C_{max} and AUC_{0-t} for ramipril. The geometric mean values were reported for log-transformed parameters. Ratios of means were expressed as the percentage of the LSM for the reference formulation. The sample size was determined based on a bioequivalence range of 80.00–125.00% for C_{max} and AUC_{0-t} for ramipril. In addition to a consumer's risk of <5%, an analysis of variance (ANOVA) coefficient of variation of approximately 30.0% and a mean difference of approximately 20% between preparations are considered. A total number of 36 participants completed the study. In the case of bioequivalence trials, the power is only of limited importance since it is a value that represents the probability of proving the existing bioavailability and, as such, is usually regarded as "producer risk." The statistical analysis of C_{max} and AUC_{0-t} comprises the ANOVA with sequence, the participant (sequence), product, and period effects for all untransformed pharmacokinetic parameters and after a logarithmic data transformation. Point estimates and 90% confidence intervals for the mean ratios of pharmacokinetic parameters were calculated after a logarithmic data transformation. The following products were compared: test vs. reference product. The bioequivalence is based on ramipril. However, ramiprilat (ramipril's active metabolite) data are considered supportive data.

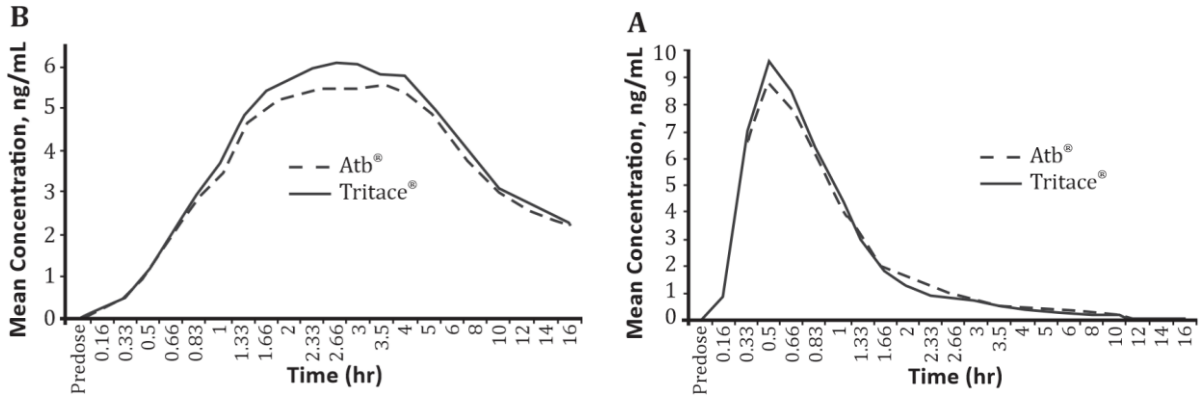

Figure 3. (A) Ramipril and (B) Ramiprilat concentrations after the administered drugs vs. time.

Table I. Pharmacokinetic parameters of ramipril for the test and reference formulations in 36 participants

Parameter		Test	Reference	The ratio of geometric mean	90% confidence interval (C.I., %)
C_{max} (ng/mL)	Geometric mean	9.652	10.163	95.0	83.83–107.59
	Arithmetic mean	11.175	11.792		
	(CV %)	54.30	50.53		
	SD	6.0683	5.9584		
AUC_{0-t} (hr.ng/mL)	Geometric mean	8.605	8.973	95.9	91.04–101.00
	Arithmetic mean	9.525	9.826		
	(CV %)	47.81	41.60		
	SD	4.5542	4.0879		
AUC_{0-inf} (hr.ng/mL)	Geometric mean	9.330	9.098	98.1	92.12–104.37
	Arithmetic mean	10.399	10.039		
	(CV %)	48.56	44.29		
	SD	5.0502	4.4469		
T_{max} (hr)	Median	0.50	0.58		
	Arithmetic mean	0.68	0.69		
	(CV %)	58.24	61.31		
	SD	0.395	0.421		
	N	36	36		
$T_{½}$ (hr)	Geometric mean	1.02	0.99		
	Arithmetic mean	1.56	1.32		
	(CV %)	147.26	95.63		
	SD	2.296	1.260		
	N	32	31		

RESULTS AND DISCUSSION

Clinical investigation

The Supplementary Data (S5) included a detailed method validation that was extensively presented. The two formulations were studied according to the established approach employed in the clinical investigation.

The bioequivalence of the ramipril product Atb® (test) (Batch No.: T491013, expiry date: 09/2022), where each tablet that contains 5 mg of ramipril was assessed versus the product Tritace® (reference) (Batch No. OU007, Expiry date: 03/2023), where each tablet that contains 5 mg of ramipril, in a two-way single-dose crossover study.

Intake or administration of any prescribed systemic or topical medication within 2 weeks before starting the study and treatment with drugs known to alter the effective metabolic systems such

as barbiturates, phenothiazines, cimetidine, omeprazole, and warfarin within the last 30 days from the study were not recommended.

The ramipril and ramiprilat concentrations were monitored over time after administering the drug to each participant. Ramipril concentrations following the administration of the test and reference drugs vs. time, at the peak time of 0.5 h, the ramipril concentration was 9.6 ng/mL in the reference and 8.9 ng/mL in the test (Figure 3 A). However, the overall behaviour is generally almost the same for both drugs. At 10 h, the ramipril concentration in the reference was at the lower limit of quantitation (LLOQ) 0.2 ng/mL and was < LLOQ for the test. For results and details associated with the test and reference, see Supplementary data S6 and S7, respectively. Ramiprilat concentrations following the administration of the test and reference drugs vs. time, the ramiprilat concentration over time follows almost the same pattern (Figure 3 B). At the peak time of 2 to 5 h, the maximum ramiprilat concentration was 6.1 ng/mL in the reference compared with 5.5 ng/mL in the test. This relatively long peak time makes the metabolite a more effective ACE inhibitor. For results and details related to the test and reference drugs, see Supplementary Data S8 and S9, respectively.

The duration of sampling (72 h) was chosen considering the most extended terminal elimination half-life of ramiprilat, i.e., approximately 9–18 h. The wash-out period of a minimum of 14 days was selected based on the terminal elimination half-life of ramipril and ramiprilat. After more than five half-lives, a pharmacokinetic carry-over effect can be excluded. Bioequivalence was assessed by determining the 90% CIs for the log-transformed ratio (test/reference) for bioequivalence parameters: C_{max} and AUC_{0-t} for ramipril.

The first pharmacokinetic-targeted parameters considered where the area under the plasma concentration-time curve from time zero to the last plasma sampling time post-drug administration and the area under the plasma concentration-time curve from time zero to infinity hours post-drug administration (AUC_{0-t} and $AUC_{0-\infty}$ for ramipril), which were calculated using the trapezoidal method. The second pharmacokinetic parameter was the maximum concentration of the drug in plasma samples (C_{max}); these data can be found from the plasma profile of each participant.

The primary concern in bioequivalence assessment was to limit the risk of erroneously accepting bioequivalence. This so-called consumer's risk must be limited to <5%. Bioequivalence can be concluded if 90% CIs for intra-individual ratios (test/ reference) for both pharmacokinetic parameters of ramipril, $C_{max,}$ and AUC_{0-t}, were within 80–125% of the CI log-transformed values. The CIs were determined using log-transformed data with the parametric method. Ratios of means were calculated using the LSM for both untransformed and log-transformed C_{max} and AUC_{0-t} for ramipril.

Pharmacokinetic parameters (C_{max} and AUC_{0-t}) for ramipril used for bioequivalence assessment were determined from the concentration data using non-compartmental analysis. The result showed that all 90% C.I. (obtained by ANOVA) were within the predefined ranges (Table I), in which the geometric mean values were reported for log-transformed parameters. Ratios of means were expressed as the percentage of the LSM for the reference formulation. For more information related to ramiprilat, see Supplementary data S10. The non-zero baseline for ramiprilat concentration data after administering the test and reference ramipril in two periods was ≤ 5% of the C_{max} values, although it has the biggest difference values. Moreover, ramiprilat data showed no significant differences between both drugs (Supplementary data S11 and S12).

The absorption rate of ramipril, as expressed by C_{max} following the administration of both products, was comparable. Both products were slightly related to the maximum plasma concentrations. The extent of ramipril absorption, as expressed by AUC following the administration of both products, was comparable. Because the 90% CI of the geometric mean is fully contained within 80 to 125%, no significant difference was found between the areas under the plasma concentration-time curve for both products.

Pharmacokinetic parameters and elimination rate constant (K_{el}) of ramipril and ramiprilat were obtained from the plasma concentration-time data following the administration of ramipril for the test and reference using a non-compartmental approach after a single oral administration were similar (Figure 4). The elimination rate constant (K_{el}) for ramipril and ramiprilat. For more detailed results, see Supplementary data S13 and S14, respectively. For ramiprilat pharmacokinetics after the administration of ramipril for the test and reference, see Supplementary data S13 and S14,

respectively. Time intervals used in calculating the elimination rate constant (K_{el}) for ramipril and ramiprilat are shown in Tables S15 and S16, respectively.

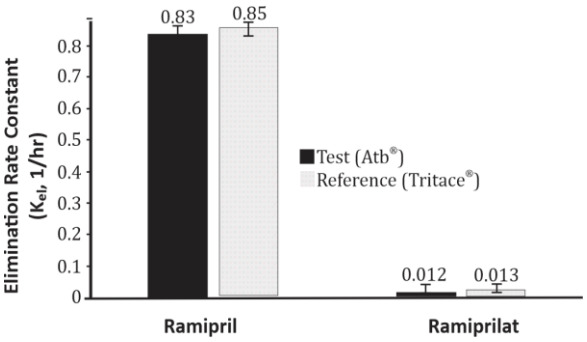

Figure 4. The elimination rate constant (K_{el}) for ramipril and ramiprilat post administering the drugs.

In contrast, all available biochemistry data for both drugs were thoroughly measured and published in both the text and the supplementary data. There were no significant variations in mean values between the test and reference medication parameters (<5% risk at 90% confidence intervals).

CONCLUSION

A simple, developed, and validated method to analyse ramipril and ramiprilat using LC-MS/MS was evaluated. This single-dose study found that the test and reference products met the regulatory criteria for bioequivalence in these fasting healthy volunteers who were treated with ramipril as the main ingredient. The ramipril formulation of the test is on par compared with the reference. Both are bioequivalent in terms of the rate and extent of absorption. Ramiprilat is an active metabolite that can last at a peak of 2–5 h and act as an ACE system inhibitor. The efficient compatibility between the two drugs was observed, including C_{max}, AUC, T_{max}, $T_{1/2}$, and K_{el}. Both drugs were safe, without adverse effects for any participant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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