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Simultaneous Determination of Hydrochlorothiazide, Ramipril and Its Active Metabolite Ramiprilat in Human Plasma by Using Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

This study describes the development and validation of a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous determination of hydrochlorothiazide (HCT), ramipril and its active metabolite ramiprilat in human plasma. Plasma samples were prepared using protein precipitation followed by chromatographic separation on a reversed-phase C18 analytical column with a mobile phase consisting of methanol and 0.2% formic acid in 10 mM ammonium formate solution. Stable isotope-labeled internal standards ramipril D_5, [$^2H_5$] ramiprilat and HCT 15N2 13C D2 were employed to ensure accurate quantification. Detection was performed using electrospray ionization in both positive and negative ion modes under multiple reaction monitoring (MRM) conditions; ramipril and ramiprilat were analyzed in positive ion mode, whereas HCT was monitored in negative ion mode. The method demonstrated linear calibration ranges of 1 - 400 ng/mL for HCT, 0.1 - 70 ng/mL for ramipril, 0.05 - 35 ng/mL for ramiprilat, with correlation coefficients (r^2) exceeding 0.99 for all analytes. Intra- and inter-day precision and accuracy met the accepted bioanalytical validation criteria. No significant matrix effects were observed and the analytes remained stable under various storage and processing conditions. The total chromatographic run time was 5 minutes per sample. The validated method was successfully applied to the analysis of human plasma samples.

Keywords

Hydrochlorothiazide (HCT), Ramipril, Ramiprilat, Human Plasma,

LC-MS/MS

1. Introduction

Hydrochlorothiazide (HCT) is a thiazide diuretic widely used for the treatment of hypertension and fluid retention associated with cardiovascular and renal disorders. It acts by inhibiting sodium-chloride reabsorption in the distal convoluted tubule, promoting diuresis and reducing blood pressure, making it a cornerstone in antihypertensive therapy [1]. Ramipril, an angiotensin-converting enzyme (ACE) inhibitor, is commonly co-administered with HCT to achieve enhanced blood pressure control. Administered as a prodrug, ramipril is rapidly converted in the liver to its pharmacologically active metabolite, ramiprilat, which inhibits ACE activity and prevents the formation of angiotensin II, contributing to vasodilation and cardiovascular protection [2] [3].

The combination of HCT and ramipril offers complementary antihypertensive mechanisms. While HCT reduces plasma volume, it can activate the renin-angiotensin-aldosterone system (RAAS), which may partially counteract its efficacy. Ramipril attenuates this compensatory response by inhibiting ACE, resulting in sustained blood pressure reduction and improved clinical outcomes [4].

To date, several LC-MS/MS methods have been reported for the quantification of ramipril, ramiprilat or HCT individually, as well as for binary combinations. However, these methods typically employ solid-phase extraction (SPE) or liquid-liquid extraction (LLE) and do not include protein precipitation for simultaneous extraction of all three analytes [1]-[9]. Protein precipitation offers several advantages over SPE or LLE: it is simpler, faster, more cost-effective, requires fewer sample manipulation steps and minimizes analyte loss and variability, making it particularly suitable for high-throughput clinical and pharmacokinetic studies. Multi-analyte methods using protein precipitation exist for other antihypertensive drugs, but none have been validated for the simultaneous determination of ramipril, ramiprilat and HCT.

To the best of our knowledge, there are no reported LC-MS/MS methods that simultaneously quantify ramipril, ramiprilat, HCT in human plasma using a protein precipitation-based extraction approach with full validation. Therefore, the present study aims to address this gap by developing a rapid, sensitive and fully validated LC-MS/MS method employing protein precipitation for the simultaneous determination of these three analytes, with stable isotope-labeled internal standards to ensure analytical accuracy and reproducibility.

2. Experimental

2.1. Chemicals and Materials

Ramipril (purity 99.48%), ramiprilat (purity 88.94%), HCT (purity 99.53%), HCT 15N2 13C D2 (purity 92.89%) were generously provided by Clearsynth (India).

Ramipril D5 (purity 98.68%) was obtained from BioOrganics & Applied Materials Pvt. Ltd. (India), while [$^2H_5$]-Ramiprilat (purity 99.5%) was supplied by ALSACHIM (France). HPLC-grade methanol, formic acid and ethanol were purchased from Merck (Darmstadt, Germany), ammonium formate was procured from Supelco (Bellefonte, Pennsylvania, USA). K_2EDTA blank human plasma was sourced from Bioivt (UK). All water used in the study was purified with a Millipore Milli-Q water purification system (USA).

2.2. Stock Solutions, Calibration Standards and QCs

Primary stock solutions of ramipril, ramiprilat and HCT were prepared separately in methanol at concentrations of 1 mg/mL. Stable isotope-labeled internal standards (ramipril D5, [$^2H_5$]-ramiprilat and HCT 15N2 13C D2 were also prepared in methanol at 0.2 mg/mL. The preparation of stock solutions was adjusted according to the certified purity values provided for each reference standard. The weighed amounts of ramipril, ramiprilat and hydrochlorothiazide were corrected for potency based on their reported purity to obtain accurate nominal concentrations. The same approach was applied during the preparation of analyte stock solutions, while internal standard solutions were prepared according to the certified purity values provided by the manufacturers. All stock solutions were stored at -20˚C for long-term use.

Working solutions for calibration standards and quality control (QC) samples were prepared by serial dilution of the primary stocks with methanol to achieve the required concentration ranges. Calibration standards in human plasma were prepared by spiking blank K_2EDTA plasma with appropriate volumes of working solutions to yield final concentrations of 0.1 - 70 ng/mL for ramipril, 0.05 - 35 ng/mL for ramiprilat, 1 - 400 ng/mL for HCT. QC samples were similarly prepared at five concentration levels. The quality control (QC) samples were prepared similarly at concentrations of 0.1 (LLOQ), 0.3 (QC Low), 2 (QC Medium), 21 (QC High), 56 (ULOQ) ng/mL for ramipril, 0.05 (LLOQ), 0.15 (QC Low), 1(QC Medium), 10.5 (QC High), 28 (ULOQ) ng/mL for ramiprilat and 1 (LLOQ), 3 (QC Low), 20 (QC Medium), 120 (QC High), 320 (ULOQ) ng/mL for HCT. All spiked plasma samples for calibration and QC were vortex-mixed thoroughly and stored at −70˚C until analysis. Before LC-MS/MS injection, plasma samples were processed using protein precipitation to remove proteins and obtain clear supernatants suitable for analysis.

2.3. Instrumentation

The LC-MS/MS system (Shimadzu, Japan) consisted of LC-40AD XR solvent pumps, a SIL-40C XR autosampler, a CTO-40S column oven, an FCV-0206 valve unit, a DGU-403 degasser unit, a Shimadzu 8060 tandem mass spectrometer. Chromatographic separation was performed on a GL Sciences InertSustain Swift C18 column (5 μm, 4.6 × 150 mm) maintained at 40˚C. The mobile phase consisted of 0.2% formic acid in 10 mM ammonium formate solution (Mobile Phase

A) and methanol (Mobile Phase B) in a ratio of 30:70 (v/v). The separation was carried out under isocratic conditions at a flow rate of 1 mL/min, with a total run time of 5 minutes. A 15 µL sample was injected using the autosampler, which was maintained at 10˚C.

Mass spectrometric detection was performed using an electrospray ionization (ESI) source in both positive and negative ionization modes, depending on the analyte. High-purity nitrogen, generated by a Peak Scientific NL-60 system, was used as the nebulizing and drying gas. Nebulizing gas flow was set at 3.0 L/min, drying and heating gas flows at 10 L/min, with an ESI voltage of 4500 V. Interface, DL, heat block, desolvation temperatures were maintained at 300, 250, 400, 526˚C, respectively.

Multiple reaction monitoring (MRM) was used for selective and sensitive quantification. Ramipril was monitored at *m/z* 417.100 → 234.100 with ramipril D5 (*m/z* 421.900 → 239.300), ramiprilat at *m/z* 389.100 → 206.100 with [$^2H_5$]-ramiprilat (*m/z* 394.000 → 211.200), both in positive ion mode. Hydrochlorothiazide was analyzed in negative ion mode at *m/z* 296.000 → 205.000 with HCT 15N2 13C D2 (*m/z* 301.000 → 207.000) as the internal standard. Dwell time, Q1 pre-bias, collision energy, Q3 pre-bias were optimized for each analyte to ensure maximum sensitivity and selectivity, with a dwell time of 100 ms applied for all compounds. LabSolutions V5.99 SP2 was used for data acquisition and evaluation of chromatographic data.

2.4. Sample Preparation

Aliquots of 200 µL of plasma were transferred into a 10 mL centrifuge tube, 50 µL of the internal standard solution (10 ng/mL ramipril D5, 25 ng/mL [$^2H_5$]-ramiprilat, 1 µg/mL HCT 15N2 13C D2) was added. The mixture was briefly vortexed for 5 seconds to ensure homogeneity. Protein precipitation was then performed by adding 1 mL of methanol, followed by thorough vortexing for 60 seconds. The samples were subsequently centrifuged at 5500 rpm for 15 minutes to pellet the precipitated proteins. Finally, 15 µL of the clear supernatant was carefully collected and injected into the LC-MS/MS system for analysis. This procedure provided efficient protein removal, minimal matrix effects, reproducible analyte recovery.

2.5. Method Development and Optimization

During method development, several chromatographic conditions were evaluated to achieve optimal separation, peak shape and ionization efficiency for hydrochlorothiazide (HCT), ramipril and ramiprilat. Different reversed-phase columns including C18, C8 and phenyl stationary phases were tested using various combinations of methanol and acetonitrile with aqueous buffers containing formic acid, ammonium acetate and ammonium formate.

The final chromatographic conditions were selected based on peak symmetry,

sensitivity and run time. GL Sciences InertSustain Swift C18 column (5 μm, 4.6 × 150 mm) provided the best chromatographic performance. An isocratic mobile phase consisting of methanol and 0.2% formic acid in 10 mM ammonium formate (70:30, v/v) was chosen as it produced sharp peaks and stable retention times.

MRM transitions were optimized by direct infusion of individual analyte solutions into the mass spectrometer to determine the most abundant precursor-to-product ion transitions.

Under the optimized conditions, the retention times were approximately 2.94 min for ramipril, 2.32 min for ramiprilat and 1.94 min for hydrochlorothiazide, while the internal standards eluted at similar retention times. These retention characteristics ensured adequate selectivity and supported the short total run time of 5 min.

3. Results and Discussion

3.1. Method Validation

A thorough validation of the method was carried out as per the US FDA [10] guidelines and European Medicines Agency Guideline on Bioanalytical Method Validation [11].

The analytical method was fully validated in human plasma to ensure reliability and robustness. Validation parameters included selectivity, assessment of matrix effects, intra- and inter-assay precision and accuracy, extraction recovery, stability under various conditions (freeze-thaw, short-term, long-term, autosampler stability). Additional assessments included whole blood stability, dilution integrity, carryover and stock and working solution stability. The method demonstrated high sensitivity, precision, reproducibility for simultaneous quantification of ramipril, ramiprilat and HCT in human plasma.

3.1.1. Selectivity and Carry-Over

The method's selectivity was confirmed by analyzing blank plasma obtained from eight different sources, including samples exhibiting hemolysis or lipemia, to check for any endogenous interference at the retention times of the analytes and their internal standards. No interfering signals were observed in any of these samples, demonstrating the method's ability to specifically detect the target compounds (**Figure 1**).

Carryover was evaluated to determine the potential for residual analyte signal between consecutive injections within an analytical run. To assess this, an extracted blank plasma sample was injected immediately after the upper limit of quantification (ULOQ) standard in the injection sequence. The blank sample was analyzed using the same injection volume applied to all study samples. No detectable analyte or internal standard response was observed in the blank injection, indicating the absence of significant carryover under the established chromatographic and mass spectrometric conditions.

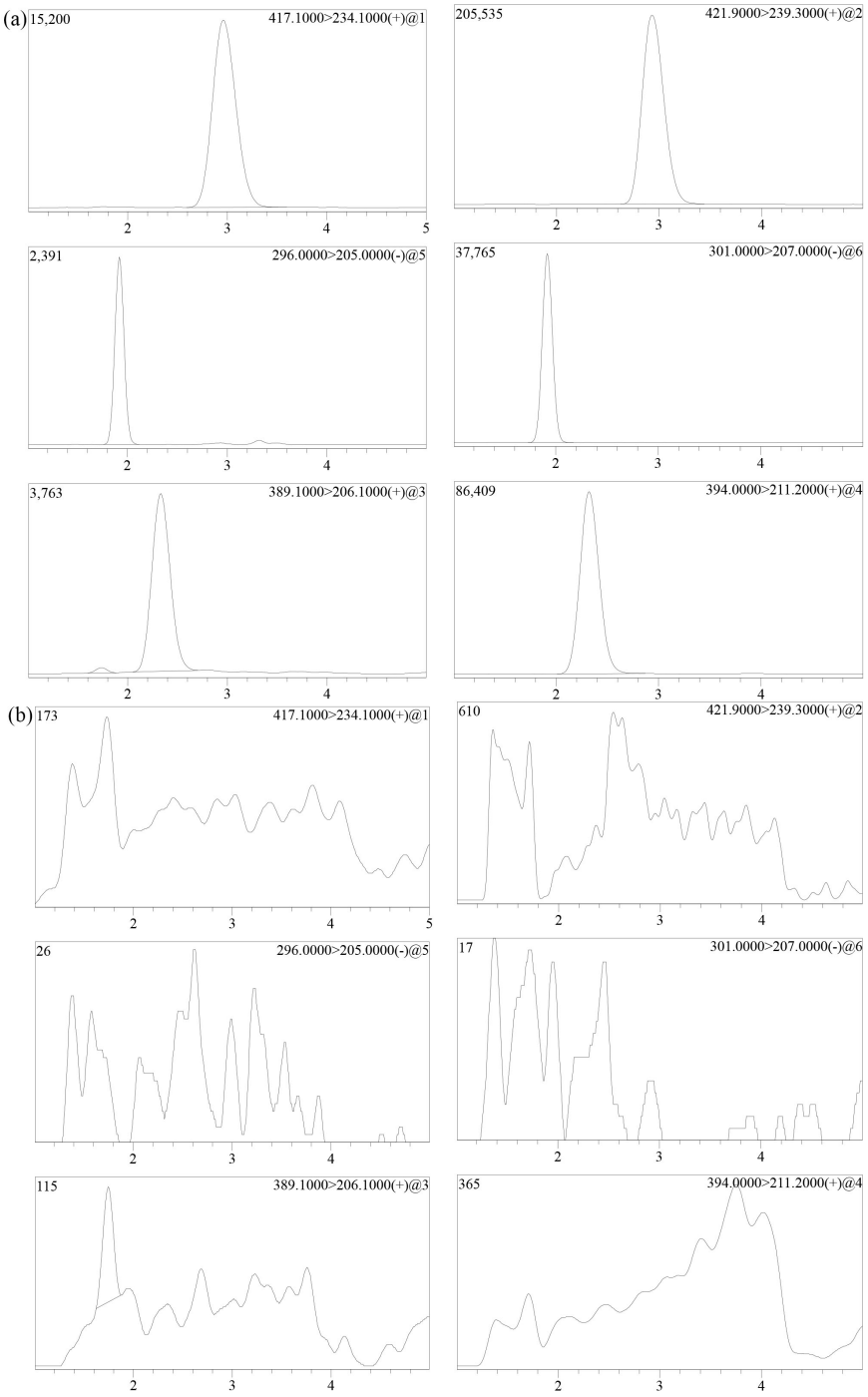

Figure 1. MRM chromatograms of 0.1 ng/mL (LLOQ) for ramipril, 0.05 ng/mL (LLOQ) for ramiprilat and 1ng/mL (LLOQ) HCT spiked with internal standards (a) and blank human plasma (b).

3.1.2. Linearity

For each analyte, calibration curves were prepared using a blank plasma sample (processed without internal standard), a zero sample (processed with internal standard), eight non-zero calibration standards spanning the validated concentration ranges.

The method demonstrated excellent linearity over the validated concentration

ranges of 0.1 - 70 ng/mL for ramipril, 0.05 - 35 ng/mL for ramiprilat, 1 - 400 ng/mL for hydrochlorothiazide (HCT). All calibration curves were regressed using a linear equation with weighting factor of $1/X^2$. Calibration curves constructed during intra- and inter-assay validation runs showed strong correlation, with coefficient of determination (r^2) values consistently greater than 0.9971 for all analytes.

The linearity (correlation coefficient: r^2) of the calibration curves and the accuracy of the back-calculated value at each calibration standard level were evaluated. Curves for intra and inter-assay precision and accuracy were used for calibration curve evaluation. Calibration curves were linear with coefficient of correlation (r^2) values of more than 0.99. The accuracy for calibration standards should be within ±15% of the nominal value at every concentration except for the LLOQ calibration standards, where it should be within ±20% of the nominal value. At least 75% of the eight non-zero standards and a minimum of six calibration standard levels should meet the above criteria in each calibration standard set.

3.1.3. Accuracy and Precision

Quality control (QC) samples were prepared at five concentration levels for ramipril, ramiprilat, (HCT) and evaluated across three independent validation runs, each comprising six replicates per concentration level. The nominal QC concentrations were 0.1, 0.3, 2, 21, 56 ng/mL for ramipril; 0.05, 0.15, 1, 10.5, 28 ng/mL for ramiprilat; and 1, 3, 20, 120 and 320 ng/mL for HCT, thereby encompassing the lower limit of quantification (LLOQ), low, medium, high and upper limit of quantification (ULOQ) levels within the validated analytical range.

Intra-day precision (%CV) ranged from 0.36% to 12.40%, whereas inter-day precision varied between 1.07% and 14.80% for all analytes. Accuracy, expressed as relative error (RE%), ranged from −15.14% to +10.53% across all QC levels (Table 1-6). All precision and accuracy results were within the predefined acceptance criteria (±15% for QC levels and ±20% at the LLOQ), with at least two-thirds of QC samples at each concentration level meeting the regulatory requirements. These findings confirm that the method demonstrates acceptable accuracy and precision across the validated concentration range.

Table 1. Intra-day precision and accuracy of the method for determining ramipril in plasma samples.

	Batch No: 1 (n = 6)			Batch No: 2 (n = 6)			Batch No: 3 (n = 6)		
Nominal Conc. (ng/mL)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)
0.1	0.1029 ± 0.0032	2.90	3.10	0.1010 ± 0.0026	1.00	2.58	0.1003 ± 0.0031	0.28	3.11
0.3	0.2794 ± 0.0062	−6.86	2.23	0.2772 ± 0.0055	−7.59	1.98	0.2754 ± 0.0036	−8.20	1.31
2	1.8576 ± 0.0146	−7.12	0.79	1.8498 ± 0.0296	−7.51	1.60	1.8818 ± 0.0195	−5.91	1.04
21	20.598 ± 0.118	−1.91	0.58	21.840 ± 0.078	4.00	0.36	20.761 ± 0.107	−1.14	0.52
56	60.643 ± 0.409	8.29	0.67	55.570 ± 0.856	−0.77	1.54	57.675 ± 0.866	2.99	1.50

Conc: Concentration, n: Replicates at Each Concentration, RE: Relative Error, CV: Coefficient of Variation, SD: Standard Deviation.

Table 2. Inter-day precision and accuracy of the method for determining rampiril in plasma samples.

	Batch No: 1 - 3 (n = 18)		
Nominal Conc. (ng/mL)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)
0.1	0.1014 ± 0.0030	1.39	2.98
0.3	0.2774 ± 0.0052	−7.55	1.87
2	1.8631 ± 0.0251	−6.85	1.35
21	21.066 ± 0.575	0.32	2.73
56	57.963 ± 2.252	3.51	3.88

Conc: Concentration, n: Replicates at Each Concentration, RE: Relative Error, CV: Coefficient of Variation, SD: Standard Deviation.

Table 3. Intra-day precision and accuracy of the method for determining ramiprilat in plasma samples.

	Batch No: 1 (n = 6)			Batch No: 2 (n = 6)			Batch No: 3 (n = 6)		
Nominal Conc. (ng/mL)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)
0.05	0.05116 ± 0.00161	2.32	3.14	0.04587 ± 0.00668	−8.25	14.56	0.04805 ± 0.00467	−3.90	9.72
0.15	0.14851 ± 0.00505	−1.00	3.40	0.14706 ± 0.00655	−1.96	4.46	0.13729 ± 0.00778	−8.48	5.66
1	0.97183 ± 0.01931	−2.82	1.99	0.97134 ± 0.01217	−2.87	1.25	0.95447 ± 0.02530	−4.55	2.65
10.5	9.984 ± 0.115	−4.91	1.15	10.047 ± 0.113	−4.32	1.12	9.933 ± 0.073	−5.40	0.74
28	28.860 0.213	3.07	0.74	27.901 0.572	−0.35	2.05	26.969 ± 0.560	−3.68	2.08

Conc: Concentration, n: Replicates at Each Concentration, RE: Relative Error, CV: Coefficient of Variation, SD: Standard Deviation.

Table 4. Inter-day precision and accuracy of the method for determining ramiprilat in plasma samples.

	Batch No: 1 - 3 (n = 18)		
Nominal Conc. (ng/mL)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)
0.05	0.04836 ± 0.00503	−3.28	10.40
0.15	0.14429 ± 0.00801	−3.81	5.55
1	0.96588 ± 0.02026	−3.41	2.10
10.5	9.988 ± 0.107	−4.88	1.07
28	27.910 ± 0.913	−0.32	3.27

Conc: Concentration, n: Replicates at Each Concentration, RE: Relative Error, CV: Coefficient of Variation, SD: Standard Deviation.

Table 5. Intra-day precision and accuracy of the method for determining HCT in plasma samples.

	Batch No: 1 (n = 6)			Batch No: 2 (n = 6)			Batch No: 3 (n = 6)		
Nominal Conc. (ng/mL)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)
1	1.0260 ± 0.0719	2.60	7.01	0.8486 ± 0.1052	−15.14	12.40	1.1053 ± 0.1256	10.53	11.36

Continued

3	2.7999 ± 0.2042	−6.67	7.29	2.8774 ± 0.2910	−4.09	10.11	3.1259 ± 0.1742	4.20	5.57
20	20.8831 ± 0.9030	4.42	4.32	20.2127 ± 0.8311	1.06	4.11	20.9396 ± 0.8013	4.70	3.83
120	117.291 ± 2.977	−2.26	2.54	123.966 ± 2.658	3.31	2.14	118.157 ± 1.846	−1.54	1.56
320	334.942 ± 4.914	4.67	1.47	315.382 ± 6.688	−1.44	2.12	324.145 ± 5.724	1.30	1.77

Conc: Concentration, n: Replicates at Each Concentration, RE: Relative Error, CV: Coefficient of Variation, SD: Standard Deviation.

Table 6. Inter-day precision and accuracy of the method for determining HCT in plasma samples.

	Batch No: 1 - 3 (n = 18)		
Nominal Conc. (ng/mL)	Conc. Found mean ± SD; ng/mL	RE (%)	CV (%)
1	0.9933 ± 0.1470	−0.67	14.80
3	2.9344 ± 0.2580	−2.19	8.79
20	20.6785 ± 0.8644	3.39	4.18
120	119.805 ± 3.871	−0.16	3.23
320	324.823 ± 9.882	1.51	3.23

Conc: Concentration, n: Replicates at Each Concentration, RE: Relative Error, CV: Coefficient of Variation, SD: Standard Deviation.

3.1.4. Matrix Effect

The potential impact of plasma matrix components on the quantification of ramipril, ramiprilat and hydrochlorothiazide (HCT) was evaluated using eight distinct plasma sources, including hemolyzed and hyperlipidemic samples. Quality control samples (QC2 and QC4, n = 4 for each level) were prepared in each plasma lot and analyzed against a calibration curve generated from freshly spiked standards. The observed concentrations were compared to the nominal values to assess any matrix-induced deviations. The results indicate that the plasma matrix had minimal influence on analyte detection: relative errors (RE%) for all analytes across different plasma sources were consistently within ±15% and the coefficients of variation (%CV) were below 5% for most measurements, well within the acceptable regulatory limits. These findings demonstrate that the developed LC-MS/MS method is robust and reproducible, with negligible matrix effects. The use of stable isotope-labeled internal standards effectively compensates for minor variations in ionization efficiency, ensuring accurate and precise measurement of ramipril, ramiprilat, HCT in human plasma, regardless of sample origin or condition.

3.1.5. Recovery

The extraction recovery of ramipril, ramiprilat, hydrochlorothiazide (HCT) from human plasma was assessed at three quality control levels. QC samples were prepared at 0.3, 21 and 56 ng/mL for ramipril, 0.15, 10.5 and 28 ng/mL for ramiprilat and 3, 120 and 320 ng/mL for HCT. Recovery was determined by comparing peak responses of analytes in extracted plasma samples to those of corresponding unex-

tracted standards at the same concentrations. Stable isotope-labeled internal standards were used to correct for variability in extraction and ionization efficiency. The mean overall recovery of ramipril, ramiprilat and HCT ranged from 97.26%, 97.42%, 94.32% respectively. Mean recovery of internal standard was 101.44% for ramipril D5, 100.29 % for [$^2H_5$]-ramiprilat and 103.42% for HCT 15N2 13C D2.

3.1.6. Dilution Integrity

Dilution integrity was evaluated using QC samples prepared at 1.7 times the ULOQ for ramipril, ramiprilat and hydrochlorothiazide (HCT). After storage at −70˚C for at least 24 hours and subsequent thawing, samples were diluted with blank human plasma at 1:2 and 1:20 ratios and analyzed against freshly prepared calibration standards. Six replicates (n = 6) were evaluated for each dilution level.

Across both dilution factors (1:2 and 1:20), the overall accuracy (RE%) ranged from −1.21% to 4.42%, while precision (%CV) ranged from 1.75% to 4.32% for all analytes. For all analytes and both dilution levels, precision did not exceed 15%, mean accuracy remained within ±15% of nominal concentrations. Additionally, at least two-thirds of individual QC replicates fulfilled the predefined acceptance criteria.

These results confirm that plasma samples exceeding the validated calibration range can be reliably quantified following 1:2 and 1:20 dilution without compromising analytical performance (Table 7).

Table 7. Summary of dilution integrity results.

Analyte	Dilution	Nominal Conc. (ng/mL)	Mean ± SD (ng/mL)	RE (%)	CV (%)
Ramipril	1:20	119	122.80 ± 2.82	3.20	2.30
Ramipril	1:2	119	124.26 ± 4.81	4.42	3.87
Ramiprilat	1:20	59.5	58.78 ± 2.26	−1.21	3.84
Ramiprilat	1:2	59.5	60.12 ± 2.15	1.04	3.57
HCT	1:20	680	678.88 ± 11.88	−0.17	1.75
HCT	1:2	680	693.78 ± 29.97	2.03	4.32

Conc: Concentration, n: Replicates at Each Concentration, RE: Relative Error, CV: Coefficient of Variation, SD: Standard Deviation.

3.1.7. Stability

The stability of ramipril, ramiprilat and HCT in human plasma was comprehensively evaluated under various storage and handling conditions in accordance with regulatory guidelines. Stability assessments included bench-top (short-term) stability, autosampler stability, freeze-thaw stability, long-term plasma stability at −70˚C and −20˚C, whole blood stability, stock and internal standard stock solution stability and dilution integrity.

1) Bench-top (short-term) stability

Bench-top stability was assessed by keeping plasma QC samples at room tem-

perature for approximately 5 hours prior to sample processing. The determined concentrations at each QC level did not exceed ±15% relative error (RE) from the nominal concentrations. The %CV at each level was ≤15%. These results demonstrate that ramipril, ramiprilat, HCT are stable in human plasma during routine laboratory handling prior to extraction (Table 8).

Table 8. Bench-top stability.

Analyte	Nominal Conc. (ng/mL)	Mean ± SD (ng/mL)	RE (%)	CV (%)
Ramipril	0.3	0.2717 ± 0.0074	−9.45	2.74
Ramipril	56	58.72 ± 0.83	4.86	1.41
Ramiprilat	0.15	0.1463 ± 0.0070	−2.44	4.78
Ramiprilat	28	27.93 ± 0.53	−0.26	1.89
HCT	3	2.9738 ± 0.227	−0.87	7.63
HCT	320	324.61 ± 6.29	1.44	1.94

Conc: Concentration, n: Replicates at Each Concentration, RE: Relative Error, CV: Coefficient of Variation, SD: Standard Deviation.

2) Autosampler stability

The stability of ramipril, ramiprilat, hydrochlorothiazide (HCT) in processed plasma samples was evaluated by storing QC samples in the autosampler at 10˚C for 24 h. Low and high QC samples were analyzed alongside freshly prepared calibration standards. For all analytes, the mean calculated concentrations at both QC levels were within ±15% of their nominal values and the coefficients of variation (CV) did not exceed 6%, demonstrating acceptable precision and accuracy under autosampler conditions. The calibration curves prepared from the autosampler stability batches showed excellent linearity, with correlation coefficients (r^2) greater than 0.993. These results indicate that ramipril, ramiprilat and HCT are stable in processed plasma extracts for at least 24 h at 10˚C, ensuring reliable quantification during routine analysis (Table 9).

Table 9. Autosampler stability.

Analyte	Nominal Conc. (ng/mL)	Mean ± SD (ng/mL)	RE (%)	CV (%)
Ramipril	0.3	0.2858 ± 0.0081	−4.73	2.82
Ramipril	56	61.82 ± 0.59	10.40	0.95
Ramiprilat	0.15	0.1390 ± 0.0037	−7.32	2.65
Ramiprilat	28	29.36 ± 0.30	4.87	1.01
HCT	3	2.72 ± 0.15	−9.22	5.56
HCT	320	338.90 ± 4.61	5.91	1.36

Conc: Concentration, n: Replicates at Each Concentration, RE: Relative Error, CV: Coefficient of Variation, SD: Standard Deviation.

3) Freeze-thaw stability

The freeze-thaw stability of ramipril, ramiprilat, hydrochlorothiazide (HCT) in human plasma was evaluated to simulate repeated sample handling and storage conditions. Low and high QC samples (n = 6) were initially frozen at −70˚C for 24 hours, then thawed unassisted at room temperature (~25˚C). After complete thawing, the samples were refrozen for at least 12 hours, this cycle was repeated four times. Following the fourth cycle, samples were processed with freshly prepared calibration standards and QC samples and analyzed in a single LC-MS/MS run. The results demonstrated that all analytes remained stable after four freeze-thaw cycles. Precision (CV) and accuracy (RE) values were within the regulatory acceptance criteria of ±15%, confirming the robustness of the method (Table 10).

Table 10. The freeze-thaw stability.

Analyte	Nominal Conc. (ng/mL)	Mean ± SD (ng/mL)	RE (%)	CV (%)
Ramipril	0.3	0.2783 ± 0.0127	−7.23	4.58
Ramipril	56	57.98 ± 1.02	3.54	1.76
Ramiprilat	0.15	0.1424 ± 0.0077	−5.07	5.38
Ramiprilat	28	27.51 ± 0.48	−1.77	1.73
HCT	3	2.83 ± 0.11	−5.79	3.74
HCT	320	322.81 ± 6.13	0.88	1.90

Conc: Concentration, n: Replicates at Each Concentration, RE: Relative Error, CV: Coefficient of Variation, SD: Standard Deviation.

4) Whole blood stability

The stability of ramipril, ramiprilat, and HCT in whole blood was evaluated at room temperature (~25˚C) for 2 hours to ensure analytes remain stable during routine sample handling prior to plasma separation. Whole blood QC samples at low and high concentrations (n = 6) were stored at room temperature for 2 hours, then plasma was separated by centrifugation and analyzed against freshly prepared calibration standards.

Results showed that mean concentrations of all analytes remained within ±15% of their nominal values, with precision (%CV) not exceeding 6% at both QC levels. These findings confirm that ramipril, ramiprilat, HCT are stable in whole blood for at least 2 hours at room temperature, supporting reliable sample handling before plasma processing (Table 11).

Table 11. Whole blood stability.

Analyte	Nominal Conc. (ng/mL)	Mean ± SD (ng/mL)	RE (%)	CV (%)
Ramipril	0.3	0.291 ± 0.008	−3.00	2.75
Ramipril	56	55.12 ± 1.21	−1.55	2.19

Continued

Ramiprilat	0.15	0.148 ± 0.006	−1.33	4.05
Ramiprilat	28	27.68 ± 0.52	−1.14	1.88
HCT	3	2.95 ± 0.12	−1.67	4.07
HCT	320	321.40 ± 5.88	0.44	1.83

Conc: Concentration, n: Replicates at Each Concentration, RE: Relative Error, CV: Coefficient of Variation, SD: Standard Deviation.

5) The long-term stability

The long-term stability of ramipril, ramiprilat, HCT in human plasma was evaluated to ensure that analytes remain stable under frozen storage conditions over the duration of the validation. QC samples at low and high concentrations (n = 6) were stored at −70˚C and −20˚C for 7 days (Table 12). After the storage period, samples were thawed and analyzed against freshly prepared calibration standards.

Table 12. Long-term stability.

Analyte	Storage Temperature	Nominal Conc. (ng/mL)	Mean ± SD (ng/mL)	RE (%)	CV (%)
Ramipril	−70˚C	0.3	0.295 ± 0.009	−1.67	3.05
Ramipril	−70˚C	56	55.44 ± 1.12	−1.00	2.02
Ramiprilat	−70˚C	0.15	0.147 ± 0.005	−2.00	3.40
Ramiprilat	−70˚C	28	27.85 ± 0.51	−0.54	1.83
HCT	−70˚C	3	2.97 ± 0.11	−1.00	3.70
HCT	−70˚C	320	322.10 ± 5.70	0.66	1.77
Ramipril	−20˚C	0.3	0.293 ± 0.010	−2.33	3.41
Ramipril	−20˚C	56	55.10 ± 1.18	−1.61	2.14
Ramiprilat	−20˚C	0.15	0.146 ± 0.006	−2.67	4.11
Ramiprilat	−20˚C	28	27.80 ± 0.54	−0.71	1.94
HCT	−20˚C	3	2.96 ± 0.12	−1.33	4.05
HCT	−20˚C	320	321.85 ± 5.88	0.58	1.83

Conc: Concentration, n: Replicates at Each Concentration, RE: Relative Error, CV: Coefficient of Variation, SD: Standard Deviation.

The evaluated storage duration was selected to reflect typical sample storage conditions encountered during routine bioanalytical studies prior to analysis. Stability over 7 days at −70˚C and −20˚C was considered representative of the expected sample handling timeline during validation and early study phases. No significant trends in concentration loss were observed for any analyte and all results remained within the predefined acceptance criteria.

6) Stability in Stock and Working Solutions

The stability of stock and working solutions for ramipril, ramiprilat, hydro-

chlorothiazide and their respective internal standards (ramipril D5, [$^2H_5$]-ramiprilat, HCT 15N2 13C D2) was evaluated under both short-term and long-term storage conditions. Short-term stability was assessed by keeping the solutions at room temperature (~25˚C) for 5 hours, after which they were analyzed alongside freshly prepared solutions. Long-term stability was evaluated by storing the solutions at −20˚C for 8 days, followed by analysis after equilibration to room temperature.

The results demonstrated that all analytes and internal standards remained stable under these conditions. For short-term stability, relative errors (RE) were minimal, well within the acceptance criteria of ±10% for analytes and ±15% for internal standards. Similarly, long-term storage at −20˚C for 8 days showed negligible deviation from nominal values, confirming that the solutions retained their integrity over time.

These findings indicate that both short-term room temperature handling and long-term frozen storage do not compromise the stability of the stock and working solutions, supporting their reliable use in routine LC-MS/MS analysis.

4. Conclusion

A rapid, sensitive and robust LC-MS/MS method was successfully developed and fully validated for the simultaneous quantification of hydrochlorothiazide, ramipril and its active metabolite ramiprilat in human plasma. The method, based on protein precipitation and stable isotope-labeled internal standards, demonstrated excellent selectivity, linearity, precision, accuracy, minimal matrix effects across a wide concentration range. Furthermore, all analytes showed high recovery and stability under various storage and handling conditions. The short chromatographic run time and simple sample preparation make this method highly suitable for high-throughput clinical and pharmacokinetic studies. Overall, the validated assay provides a reliable and efficient tool for simultaneous monitoring of HCT, ramipril, ramiprilat in human plasma, facilitating better pharmacokinetic evaluation and therapeutic monitoring in clinical settings.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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