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Bioanalytical method development and validation using incurred samples—Simultaneous quantitation of ramipril and ramiprilat in human EDTA plasma by LC–MS/MS

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ABSTRACT

A new method development and validation approach is proposed in order to develop a reliable method for the simultaneous quantitation of ramipril and ramiprilat in the presence of numerous labile metabolites. This new approach involves the usage of a synthesized labile acyl glucuronide of ramipril as well as individual and pooled incurred (study) samples in the development and validation process. Following the method validation and prior to its application to a large clinical study, a mini pilot study was performed to evaluate the performance of the method. When the samples from the mini pilot study were analyzed by two different scientists, 100% of the results from incurred sample reanalysis (ISR) matched within 8% of difference and the mean differences were 0.21% and 1.40% for ramipril and ramiprilat, respectively. The validated concentration range reported in this article is 0.2–80 ng/mL for both analytes. Various stabilities, such as bench-top, autosampler, freeze/thaw, and long-term, were also successfully evaluated. The key to the success were low sample processing temperature (4 °C), proper choice of sample extraction procedure, and adequate chromatographic conditions to obtain good peak shape without the need of derivatization and baseline separation between the analytes and their glucuronide metabolites.

1. Introduction

Bioanalytical methods are usually developed and validated by using spiked biological matrix samples, i.e. fortifying control (blank) with different amounts of analyte. While this approach is generally effective, it is not uncommon that bioanalytical issues arise upon applying a validated method to the analysis of incurred (study) samples, which are usually quite different from the spiked samples [1]. Such differences include the absence of various phase I or phase II metabolites and formulation-related components in the spiked samples. Therefore, it is unlikely that any interference or back-conversion reaction associated with any of the metabolites or formulation-related components can be observed during the conventional method development and validation procedures. Once the validated method is applied to the analysis of incurred samples, unreliable or irreproducible results would be obtained. To avoid the occurrence of such situations, a different approach is desirable, particularly when there are labile metabolites and potential back-conversions of the metabolites into their parent compounds [2].

The method development of ramipril and ramiprilat is such a bioanalytical case. Ramipril is a potent and specific angiotensin-converting enzyme (ACE) inhibitor that lowers peripheral vascular resistance without affecting heart rate. After its adsorption, ramipril is rapidly hydrolysed to the active metabolite ramiprilat (refer to Fig. 1 for chemical structures). The metabolism also yields ramipril and ramiprilat diketopiperazine and various glucuronide metabolites [3–4]. Some of these metabolites, such as acyl and N-glucuronides, are especially labile and are subject to potential back-conversions during sample collection, storage and/or extraction, and after sample processing as well when they are co-extracted. Though a few methods have been published either for ramipril only or for both ramipril and ramiprilat [5–8], the evaluations of metabolite back-conversion and demonstration of method reproducibility for incurred sample analysis are lacking despite the fact that the potential stability issues were noticed [4,9]. For example, Persson et al. observed and identified an interfering N-glucuronide of ramipril [4]. In addition, as glucuronides

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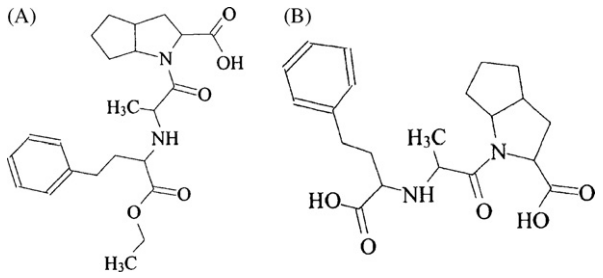

Fig. 1. Chemical structures of ramipril (A) and ramiprilat (B).

could easily be fragmented in-source to the respective parent compounds, chromatographic separation of the analytes from their respective glucuronides was necessary [10]. However, it is not clear that this had been achieved in the published methods due to their short retention time [5–8].

In light of the aforementioned potential metabolite back-conversions and lack of adequate addressing of this issue in the published methods, an extensive investigation is desirable to evaluate potential metabolite back-conversions during sample collection, storage, extraction, and mass spectrometric detection as well as how to avoid and reduce such conversions supported by the demonstration of the reproducibility of incurred sample analysis for ramipril and ramiprilat. In this article, a new method development and validation procedure is proposed by using ramipril acyl glucuronide and incurred samples in the process in order to develop a reliable method for the simultaneous quantitation of ramipril and ramiprilat in the presence of many labile metabolites. Furthermore, it is proposed to perform a mini pilot study to evaluate the performance of validated methods prior to their application to large clinical studies in cases where metabolite back-conversion is suspected, so as to ascertain their robustness and minimize potential bioanalytical issues that could jeopardize studies.

2. Experimental

2.1. Chemicals and reagents

Ramipril was purchased from the United States Pharmacopeia (USP, Rockville, Maryland, USA). Ramprilat, rampril-d_3, ramiprilat-d_3, and ramipril acyl glucuronide were obtained from SynFine Research (Richmond Hill, Ontario, Canada). Methanol (Omnisolv), formic acid, and acetic acid (glacial, AnalaR) were obtained from EMD (Toronto, Ontario, Canada). Ammonium formate (AnalaR) and ammonium hydroxide (ACS) were obtained from Sigma (Oakville, Ontario, Canada). Human EDTA K_2 plasma was obtained from Valley Biomedical (Winchester, Virginia, USA). Water was produced in-house by a Milli-Q water system (Milford, Massachusetts, USA). High purity liquid nitrogen was supplied by Prodair (Mississauga, Ontario, Canada).

2.2. Stock solutions, calibration standards and quality control samples

The stock solutions were prepared in methanol at the concentrations of 100 μg/mL for the two analytes and ramipril acyl glucuronide and 40 μg/mL for the internal standards. All intermediate and working solutions were prepared by the successive dilutions of the stock solutions in methanol. Calibration standards were prepared in control human EDTA K_2 plasma at the concentrations of 0.2, 0.4, 2, 8, 16, 32, 64, and 80 ng/mL. Quality control samples were prepared at the concentrations of 0.2, 0.6, 28, 60, and 80 ng/mL.

2.3. Sample processing

Three hundred microliters (300 μL) of human EDTA K_2 plasma sample was aliquoted (after being thawed at 4 °C or in an ice/water bath for frozen samples) and mixed with 150 μL of internal standard and 1.5 mL of 1% (v/v) acetic acid solution at 4 °C. The mixture was loaded on a Bond Elut C18 cartridge (200 mg, 3 cc, Varian, Palo Alto, California, USA). After two successive washings with 1% acetic acid solution and methanol (2 mL each), 2 mL of elution solution (basified methanol with ammonium hydroxide) was used to eluate the analytes. The eluate was evaporated at 40 °C for 25 min and the residuals were reconstituted in 150 μL of reconstitution solution, 50% (v/v) methanol in water. The reconstituted samples were kept at 4 °C prior to and during the injection.

2.4. LC–MS/MS conditions

The LC system consisted of a solvent delivery module (Hewlett Packard series 1100 from Agilent, Palo Alto, California, USA), an autosampler (PE series 200 of Perkin Elmer, Shelton, Connecticut, USA), and Platinum C18 column (100 mm × 4.6 mm, 3 μm, Alltech, Deerfield, Illinois, USA) operated at 55 °C. The mobile phase was a mixture of methanol/water (70:30, v/v) with 15 mM ammonium formate and the flow rate was 1 mL/min. The injection volume was 20 μL.

Mass spectrometric detection was carried out with a Sciex API 4000 equipped with a TurboIonSpray interface (MDS Sciex, Toronto, Ontario, Canada). The ion source was operated in the negative mode. The MRM transitions were m/z 415.3 → 154.1 amu and 387.3 → 154.1 for ramipril and ramiprilat, respectively. The TurboIonSpray voltage and temperature were set at −2000 V and 650 °C, respectively. The declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) voltages were set at −82, −35, and −4 V for ramipril and its internal standard (IS). The DP, CE, and CXP for ramiprilat and its internal standard were set at −53, −29, and −2 V, respectively. The Analyst software (version 1.4.1, MDS Sciex) was used for data acquisition and processing. Calibration curves were constructed using the respective analyte/IS peak area ratios with a weighted ($1/C^2$) least-squares linear regression.

2.5. Measurement of ramipril impurity in ramipril acyl glucuronide reference standard

Freshly prepared ramipril acyl glucuronide stock solution was diluted to 2 μg/mL in the reconstitution solution and analyzed against a calibration curve of ramipril prepared also in the same reconstitution solution. The same amounts of internal standards were added to both the diluted ramipril acyl glucuronide and calibration standard samples. The amount of ramipril impurity was estimated by the amount of ramipril detected in the ramipril acyl glucuronide sample divided by the amount of ramipril acyl glucuronide spiked.

2.6. Recovery evaluation

The recovery of the analytes was each evaluated at three different concentration levels (low, medium, and high quality controls). For each concentration level, the mean analyte responses of six quality control replicates were compared with those of six extracted control plasma samples post-extraction spiked with appropriate amounts of the analytes. The recovery of the internal standards was determined in a similar way except that it was evaluated at only one concentration level for each internal standard and the mean internal standard response was from 18 samples, instead

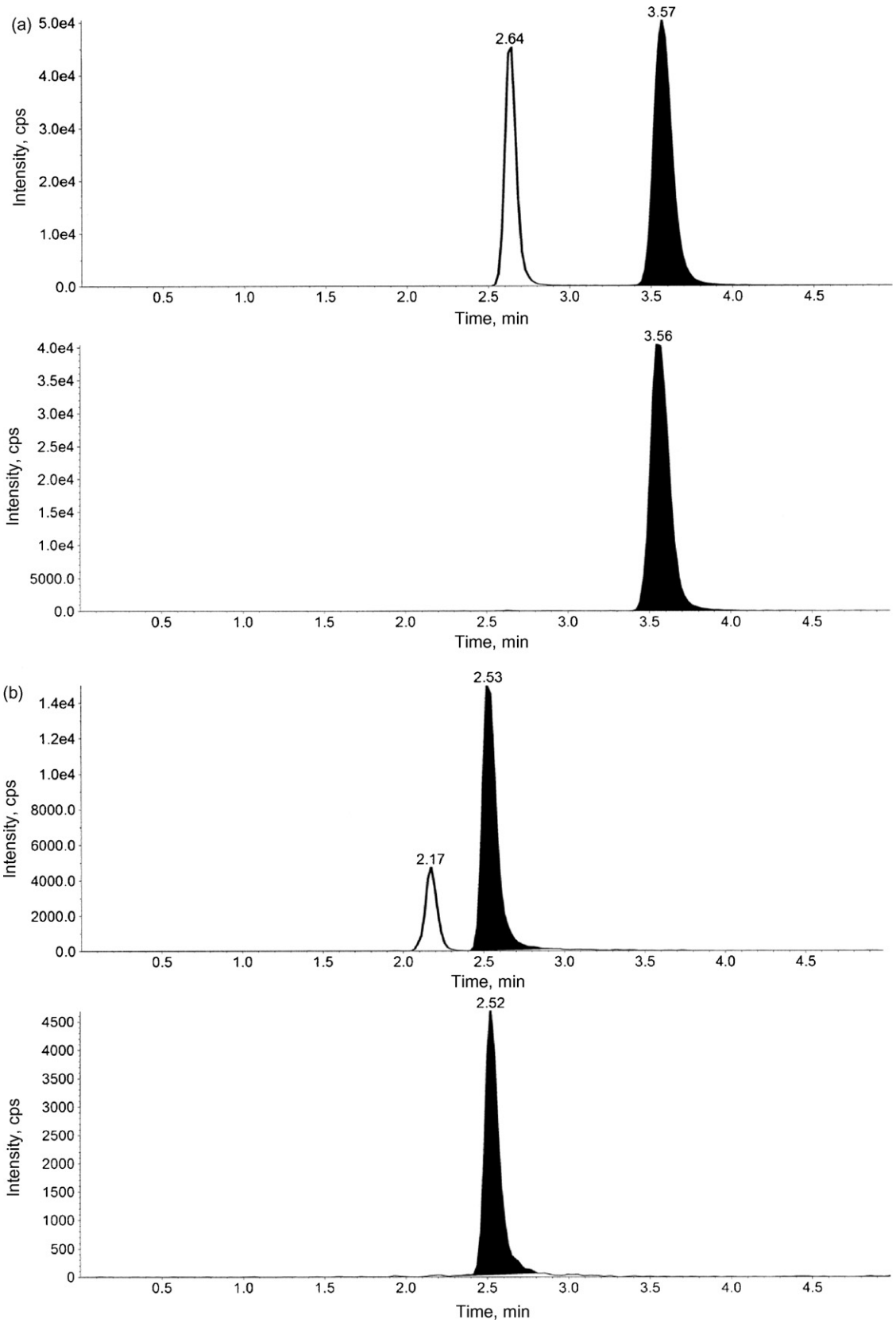

Fig. 2. Representative chromatograms for an incurred sample (1 h after the administration of a single 10 mg oral dose of ramipril). (a) Ramipril; (b) ramiprilat. The un-shaded peaks were due to in-source fragmentation of glucuronides, which were not observed in spiked calibration standard and quality control samples. The peaks of the internal standards are shown in the lower panels.

Table 1
Evaluation of matrix effect on the quantitation of ramipril and ramiprilat.

Matrix lot	Ramipril			Ramiprilat		
	Mean[a] conc. (pg/mL)	Bias[b] of mean (%)	CV (%)	Mean conc. (pg/mL)	Bias of mean (%)	CV (%)
1	143.30	−4.47	5.07	150.18	0.12	9.69
2	150.93	0.62	2.54	158.87	5.91	2.84
3	147.66	−1.56	1.17	158.67	5.78	2.55
4	148.75	−0.83	6.52	151.53	1.02	5.23
5	149.05	−0.63	6.07	143.76	−4.16	9.53
6	155.07	3.38	1.78	153.47	2.31	6.08
7	152.77	1.85	5.38	148.53	−0.98	4.04
8	146.25	−2.50	2.07	147.09	−1.94	3.43
9	157.37	4.91	1.92	155.87	3.92	6.81
10	153.16	2.10	3.22	153.73	2.49	6.23
Average	150.43	0.29	3.57	152.17	1.45	5.64

[a] Mean concentration of 3 measurements.
[b] Both analytes were prepared at the concentration of 150 pg/mL.

of 6, because the same internal standard concentrations were used for all quality control samples.

2.7. Matrix effect

Ten randomly selected analyte and IS-free control human EDTA K_2 plasma lots were individually spiked at low quality control level. Then, three replicates from each lot were analyzed by following the sample processing procedure described above.

2.8. Pooling and analysis of incurred samples

The samples from two rejected studies (each with a single dose of 10 mg ramipril) were first thawed at 4 °C and then three representative pools of incurred samples (Pool 1, Pool 2, and Pool 3) were prepared as the relative amounts of the glucuronide metabolites vary over time. The sampling times chosen for Pool 1 were 0.5, 0.667, 0.833, and 1 h. For Pool 2, the sampling times were 1.25, 1.5, 2, and 2.5 h. For Pool 3, the sampling times were 4, 5, 6, and 8 h. These pooled samples were then aliquoted and analyzed on different occasions with 6 replicates each according to the sample processing procedure.

2.9. Evaluation of extraction ruggedness

To evaluate the ruggedness of the sample extraction procedure, the following stressed conditions were compared with the normal sample processing (comparison); Test 1: sample loading mixture, i.e. the mixture of aliquoted sample, IS, and buffer, was kept at 4 °C for 1 h prior to being loaded on solid-phase extraction (SPE) cartridges; Test 2: sample loading mixture was kept at room temperature for 1 h; Test 3: the SPE eluate was kept at room temperature for 1 h prior to evaporation; Test 4: extension of evaporation time for 20 more minutes, i.e. from 25 to 45 min; Test 5: combination of all the stressed conditions from tests 1 to 4, i.e. the sample loading mixture was kept at 4 °C and room temperature for 1 h each; the SPE eluate was kept at room temperature for 1 h prior to evaporation and the evaporation was extended for 20 more minutes.

2.10. Stability evaluation

For sample collection and handling stability, fresh human EDTA K_2 whole blood was spiked with the analytes at the low QC level (600 pg/mL) and with ramipril acyl glucuronide at 60 ng/mL. This spiked whole blood sample was split into two aliquots (A and B). Aliquot A was incubated for 10 min in an ice/water bath, centrifuged at 4 °C and the resulting plasma was used as comparison sample. Aliquot B was incubated in the ice/water bath for 120 min, centrifuged at 4 °C and kept in the ice/water bath for another 182 min prior to harvesting plasma. The resulting plasma (stability sample) was analyzed with the comparison sample in the same batch to access the percentage of change during the sample collection process. To account for the amount of ramipril associated with the impurity of ramipril acyl glucuronide reference standard, the same amount of ramipril acyl glucuronide (60 ng/mL) was spiked in the fresh blood and processed in the same way as aliquot A. The amount

Table 2
Accuracy and/or precision of spiked quality controls (QC) and pooled incurred samples.

Sample	Ramipril						Ramiprilat					
	Inter-day (between-run)			Intra-day (within-run)			Inter-day			Intra-day		
	Mean[a] (pg/mL)	% CV	% Bias	Mean[b] (pg/mL)	% CV	% Bias	Mean (pg/mL)	% CV	% Bias	Mean (pg/mL)	% CV	% Bias
LLQC[c]	–[d]	–	–	197.80	4.03	−1.98	–	–	–	197.81	5.54	0.41
Low QC	605.52	3.38	0.02	608.29	3.30	0.48	578.48	2.46	−2.12	576.89	0.90	−2.39
Medium QC	27622.03	2.82	−2.23	26836.23	2.01	−5.01	26893.30	2.23	−2.49	26387.74	1.15	−4.32
High QC	59124.68	2.58	−2.34	57496.87	1.03	−5.03	58093.46	2.50	−1.70	56618.64	1.72	−4.20
ULQC[e]	–	–	–	78116.41	2.24	−3.23	–	–	–	78291.76	0.92	−0.64
Pool 1	10109.58	3.44	–	10227.83	1.55	–	5700.21	2.02	–	5712.34	1.84	–
Pool 2	1914.43	3.20	–	1925.31	2.18	–	16753.70	2.11	–	16960.22	1.68	–
Pool 3	<LLOQ[f]	–	–	<LLOQ	–	–	9558.21	2.17	–	9650.49	0.82	–

[a] Measurements of 30 for QC samples and 18 for pooled incurred samples.
[b] Measurements of 6.
[c] Lower limit quality control.
[d] Not performed or not applicable.
[e] Upper limit quality control.
[f] Lower limit of quantitation.

Table 3
Evaluation of method ruggedness using pooled incurred samples collected at time points near the Cmax of ramipril.

		Comparison	Test 1	Test 2	Test 3	Test 4	Test 5
Ramipril	Mean[a] conc. (pg/mL)	11458.31	11133.42	10724.87	10744.35	11002.74	10699.10
	CV (%)	2.99	2.25	4.44	1.14	1.67	3.19
	% Change	N/AP[b]	−2.84	−6.40	−6.23	−3.98	−6.63
Ramiprilat	Mean conc. (pg/mL)	2760.43	2740.97	2712.67	2724.62	2756.25	2755.15
	CV (%)	3.43	2.26	2.79	0.51	1.14	4.79
	% Change	N/AP	−0.70	−1.73	−1.30	−0.15	−0.19

[a] Mean of three replicates.
[b] Not applicable.

of ramipril detected in this sample was later deducted from both the comparison and stability samples prior to the calculation of the percentage of change.

For autosampler (post-preparative) stability, 12 replicates each of the low and high quality control (QC1 and QC3) samples were extracted with a calibration curve but only 6 replicates (comparison samples) were injected with the calibration curve. The remaining 6 replicates (stability samples) were kept at room temperature and at 4 °C under ambient laboratory conditions for specific periods of time. Then, these stability samples were injected with another freshly extracted calibration curve. The mean concentration of stability samples was compared with that of the comparison samples for both the low and high QC levels.

As to the other stabilities, aliquots of pooled or individual incurred samples were first analyzed against a freshly prepared calibration curve to obtain time 0 comparison values. After going through the respective stressed conditions, such as freeze and thaw cycles, left at room temperature, 4 °C, or −20 °C for different durations, those stressed samples (stability samples) were analyzed against a new freshly prepared calibration curve. The results of stability samples were compared with the time 0 comparison values to determine the % of change. Unless otherwise specified, both stability and comparison samples were analyzed in six replicates for the pooled incurred samples and once for the individual incurred samples.

3. Results and discussion

3.1. Method development

There were two main challenges associated with the method development for ramipril and ramiprilat, i.e. chromatographic separation and potential metabolite back-conversions.

The first chromatographic challenge was to achieve adequate chromatographic separation between the two analytes and their respective metabolites, particularly glucuronide metabolites, because glucuronides can be fragmented in the ionization source to yield their parent analytes. This phenomenon could potentially bias the quantitation of ramipril and ramiprilat as the molecular fractions arising from the fragmented glucuronides would also be measured through the same MRM transitions as those used to quantitate the analytes of interest. Another chromatographic challenge was to obtain good peak shape for both analytes to insure peak homogeneity and reproducibility throughout a run. While many of the common C18 columns were tested, such as Zorbax SB-C18, SymmetryShield RP18, and ACE C18, no satisfactory chromatography was obtained. Peak splitting and broadening were usually observed, probably due to potential structural rotations [5]. Eventually, satisfactory chromatography was obtained with a combination of proper column choice, mobile phase composition, and column temperature. As shown in Fig. 2, not only well-defined and symmetrical peaks were obtained, but also, base-line separation was obtained between ramipril, ramiprilat, and their glucuronide metabolites.

Generally speaking, derivatization could be an alternative solution to improve chromatography, such as retention and peak shape, given the chemical features of both analytes (Fig. 1) and a bioanalytical method based on SPE with acidified methanol elution and post-extraction derivatization was initially validated "successfully". However, the multiple potential labile metabolites present in incurred samples would not withstand the harsh conditions usually associated with the derivatization process. For example, when ramipril acyl glucuronide, one of the most labile and most abundant metabolites, was added into a prepared QC, it was observed that up to 0.688% of ramipril acyl glucuronide could convert back to ramipril during a sample processing procedure with derivatization. By removing the derivatization step and using basic elution, the conversion rate of ramipril acyl glucuronide to ramipril could be significantly reduced to 0.089%. It should be noted that the measured ramipril impurity (0.195%) in the reference standard of ramipril acyl glucuronide has been deducted during the estimation of the conversion rates.

However, even with this improved condition, it would be extremely difficult to prevent the back-conversion from occuring completely considering the fragile nature of ramipril acyl glucuronide and its relatively high concentration in incurred samples (estimated as high as 60 ng/mL for a single dosage of 10 mg and 8 ng/mL for a single dose of 1.25 mg based on internal data and the reference [3]). In other words, it is unlikely that the amount of ramipril generated by the back-conversion of 60 ng/mL of ramipril

Table 4
Validated stabilities of ramipril and ramiprilat using pooled incurred samples.

	Ramipril			Ramiprilat		
	Pool 1	Pool 2	Pool 3	Pool 1	Pool 2	Pool 3
Post-preparative (autosampler) stability: % Change after 3 h at room temperature and 76 h at 4 °C	7.59	4.56	N/AP[a]	5.23	5.65	4.53
Reinjection reproducibility: % Change after 63 h at 4 °C	2.25	−0.60	N/AP	−0.09	−0.02	−1.49
Short-term stability of analyte in matrix: % Change after 25 h at 4 °C	−4.70	−4.95	N/AP	−3.21	−1.09	−2.99
Freeze and thaw cycle stability						
% Change after 4 cycles (−20 °C/4 °C)	−2.82	−3.92	N/AP	−1.94	−2.33	−3.38
% Change after 4 cycles (−80 °C/4 °C)	−3.79	−4.48	N/AP	−1.16	−0.72	−2.76

[a] Not applicable because both comparison and stability samples showing concentrations less than the lower limit of quantitation.

Table 5
Comparison of ramiprilat stability in individual incurred samples at room temperature and 4 °C.

Sampling time (h)	Time 0 concentration (pg/mL)	After 24 h at 4 °C (pg/mL)	After 24 h at RT[a] (pg/mL)	% Change at 4 °C	% Change at RT
0.5	117.82	97.79	352.35	−17.00	199.06
0.667	272.97	300.05	510.72	9.92	87.10
0.833	512.18	498.54	668.15	−2.66	30.45
1	747.30	794.33	853.18	6.29	14.17
1.25	992.02	1105.95	1068.06	11.48	7.67
1.5	1353.35	1572.46	1428.29	16.19	5.54
2	2324.66	2230.30	2248.25	−4.06	−3.29
2.5	3510.55	3556.01	3155.44	1.29	−10.12
3	3885.11	3794.18	3107.30	−2.34	−20.02
3.5	3872.34	3644.57	3903.83	−5.88	0.81
4	4041.65	4006.12	4121.74	−0.88	1.98
5	3908.92	4152.21	3751.68	6.22	−4.02
6	3778.16	3681.81	3508.64	−2.55	−7.13
8	3345.35	3200.58	3221.58	−4.33	−3.70
10	2381.33	2302.59	2242.41	−3.31	−5.83
Mean % of change				0.56	19.51

[a] Room temperature.

acyl glucuronide could be reduced to such an extent that it would be less than 20% of the lower limit of quantitation (LLOQ), i.e. 40 pg/mL. This is one of the reasons why the use of individual incurred samples is important to investigate such phenomenon and evaluate method performance (shown later in this article).

As to mass spectrometric detection, both positive and negative MRM modes were tested and in-source fragmentation of glucuronides to parent analytes was observed in both modes. Negative MRM mode was chosen over positive MRM mode because of its improved signal to noise ratio (S/N) for extracted samples, particularly for ramiprilat. The key mass spectrometer parameters, such as DP, CE, CXP, gas pressures, and turbo ion spray temperature, were optimized to obtain the highest sensitivity. Apparently, some of these parameters like DP and turbo ion spray temperature would also have an impact on the in-source fragmentation of the glucuronides. However, further experiments were not performed on how these parameters would influence the degree of in-source fragmentation of the glucuronides because base-line separation of the glucuronides from the parent analytes has already been achieved and there might be conflicts in optimal values of DP and turbo ion spray temperature in terms of achieving the highest sensitivity for the analytes or the lowest in-source fragmentation of the glucuronides.

3.2. Recovery

High absolute recoveries were obtained for both analytes. The recoveries of ramipril at low, medium, and high QC concentration levels were 86.84, 87.39, and 84.22%, respectively. The recovery of ramipril internal standard was 89.98%. For ramiprilat, the recoveries at low, medium, and high QC concentration levels were 73.29, 80.59%, and 76.00%, respectively. The recovery of its internal standard was 81.75%. The slightly lower recovery of ramiprilat might be due to its higher hydrophilicity than that of ramipril (refer to their retention time during separation in Fig. 2). Since 100% methanol washing was employed during the solid-phase extraction, yet high and reproducible recoveries were still obtained, there must be more than one retention mechanisms on the Bond Elut C18 cartridges used. However, no additional efforts were made to ascertain what the exact mechanism was.

3.3. Matrix effect

As shown in Table 1, accurate and precise quantitative results at low QC concentration level (150 pg/mL) were obtained independent of matrix sources, which demonstrates the absence of matrix effect on the quantitation of ramipril and ramiprilat. It should be noted that these results were from the matrix effect test associated with the lower range method (50–10,000 pg/mL intended for studies of 1.25 mg dosage), which has the same sample extraction and separation procedures as the high range method reported in this article. Matrix effect with the high range method was not evaluated because it was assumed internally that no further matrix effect test was necessary once the matrix effect with the low range method met the acceptance criteria.

3.4. Accuracy and precision using spiked samples and pooled incurred samples

At first, the accuracy and precision of the method were evaluated by using spiked samples. Both the accuracy and precision for ramipril and ramiprilat are satisfactory (Table 2). The range

Table 6
Long-term stability of ramipril and ramiprilat in matrix using individual incurred samples.

Sampling time (h)	Ramipril		Ramiprilat	
	Time 0 concentration (pg/mL)	% Change after 32 days at −20 °C	Time 0 concentration (pg/mL)	% Change after 32 days at −20 °C
0.167	<99.80	N/AP[a]	<101.40	N/AP
0.5	16256.89	−2.24	1242.66	−3.07
0.833	11797.98	−2.80	5506.13	7.67
1.25	6342.33	2.20	10398.51	0.51
2	1169.55	−7.61	14743.18	−8.43
3	472.64	−11.62	12811.26	−7.53
4	403.29	3.21	10828.19	1.48
6	251.41	−5.05	7551.89	−2.91
10	158.18	4.79	4284.36	−1.28
Mean % of change		−2.39		−1.70

[a] Not applicable.

validated in this article is 0.2 to 80 ng/mL for both analytes. However, the same method has also been validated for a range of 50 to 10,000 pg/mL for clinical studies of lower dosage (data not shown in this article due to less extensive tests using incurred samples).

As mentioned in the introduction, the successful validation of spiked samples may not guarantee the accuracy and precision of incurred samples in situations where there are several labile metabolites, such as ramipril acyl glucuronide. Therefore, it is very much desirable and necessary to evaluate the method with incurred samples. Ideally, individual incurred samples should be analyzed repeatedly to obtain the true performance of the method during in-study applications. However, due to the limited sample volume collected at each time point and the potential large number of incurred samples to be tested, an alternative approach was taken. Specifically, three representative pools of incurred samples (Pool 1, Pool 2, and Pool 3) were prepared from two rejected studies. These pools are representative of the sample matrix composition near the Tmax of ramipril, Tmax of ramiprilat, and their elimination phase, respectively. Each pool was analyzed by two different persons on three different days with 6 replicates per pool per run. As shown in Table 2, the precision and reproducibility of incurred sample pools are also satisfactory.

3.5. Evaluation of extraction ruggedness

To evaluate the ruggedness of the sample extraction procedure, various samples went through the stressed conditions mentioned in the experimental section, including (a) ramipril only QC sample (80 ng/mL); (b) ramiprilat only QC sample (80 ng/mL); (c) ramipril and ramiprilat mixture QC sample; (d) control blank fortified with 60 ng/mL of ramipril acyl glucuronide; (e) pooled incurred samples corresponding to Cmax and elimination phase. No significant conversions between ramipril and ramiprilat or from metabolites to parent compounds, such as ramipril acyl glucuronide to ramipril, were observed in all the test samples. As representative data, the results of pooled incurred samples collected near the Cmax of ramipril are shown in Table 3. These results demonstrate that the extraction method is very rugged.

3.6. Sample collection stability in the presence of ramipril acyl glucuronide

Even at the presence of 100-fold of ramipril acyl glucuronide, the % change in ramipril concentration was as low as 0.09% after 110 min in whole blood and an additional 182 min in plasma over erythrocytes at 4 °C, which indicates that there was no significant back-conversion of ramipril acyl glucuronide to ramipril during the sample collection process. For ramiprilat, the measured percentage of change (-1.19%) during this sample collection process was also insignificant.

3.7. Evaluation of other stabilities using pooled or individual incurred samples

Other important stabilities, such as autosampler, bench-top, and freeze–thaw stabilities, were also successfully validated using pooled incurred samples as shown in Table 4. One of the critical factors in the successful validation of these stabilities is to keep incurred samples or processed samples at 4 °C, instead of room temperature. To prove this, further bench-top stability comparison tests were performed between room temperature and 4 °C using samples spiked with ramipril acyl glucuronide or individual incurred samples. The results from these tests clearly demonstrate the back-conversions from metabolites to the analytes at room temperature and their noticeable impacts on the quantitation of both ramipril and ramiprilat. As representative data, the results of ramiprilat are shown in Table 5. For a low concentration ramiprilat sample, the error caused by metabolite back-conversion at room temperature could be as high as 200%. However, it should be noted that there would be no difficulty in validating those stabilities at room temperature should only spiked plasma QC samples were used.

In addition, long-term stability in matrix was evaluated using individual incurred samples (Table 6). The mean percentages of change after a storage period of 32 days in a −20 °C freezer were −2.39% and −1.70% for ramipril and ramiprilat, respectively. These results demonstrate that ramipril and ramiprilat are stable during long-term storage in the presence of various metabolites including acyl glucuronides. It should be noted these data were obtained with calibration curves with an LLOQ of 100 pg/mL instead of 200 pg/mL, due to the fact that time 0 comparisons for this long-term stability assessment were established early in the method development stage and the final concentration range had not been finalized at that time.

3.8. Mini clinical pilot study

Despite the aforementioned encouraging results, it would be still preferable to perform a mini clinical pilot study to analyze freshly collected individual incurred samples prior to using the validated method for large pivotal studies for the following reasons. First of all, it is unrealistic or even impossible to evaluate all potential labile metabolites during the method development and validation. Secondly, even though various efforts, such as sample processing at 4 °C, mild extraction conditions, baseline separation of the glucuronides from the respective analytes, have been made to reduce the potential conversion of the fragile glucuronides to parent analytes to the minimum, the conversion of fragile glucuronides has not been avoided completely. Thirdly, the metabolite compositions in the aged samples from the rejected studies might be different from those of the samples collected freshly.

To this end, 51 fresh incurred samples were collected from 3 subjects (17 samples per subject) and analyzed by two research scientists within an interval of 6 days using the same aliquots. The percent differences, i.e. 100 × (difference of the two values)/the average of the two, range from −5.82 to 5.13% with the average of −0.21% for ramipril. For ramiprilat, the percent differences range from −5.27 to 8.00% with the average of 1.40%. These results satisfactorily demonstrate the high level of reproducibility and robustness of the method that can be expected during the analysis of individual incurred samples.

As shown in Fig. 2A and B, it is possible to monitor the responses of the in-source fragmentation peaks of the glucuronides. Accordingly, the area ratios of these peaks to the corresponding IS peaks could be plotted against sampling time to obtain a rough idea as how the concentrations of these glucuronides would change over the time. Shown in Fig. 3A and B are the concentration-related profiles from the subject that showed most extensive glucuronidation. These profiles could explain why reproducible and precise results were obtained despite the fact that the potential conversion of ramipril acyl glucuronide to ramipril may not be avoided completely. Specifically, when the concentration of ramipril acyl glucuronide is high, the concentration of ramipril is also high. Even though there might be small amount of back-conversion from ramipril glucuronide to ramipril, it would not cause significant relative error in the measured concentration of ramipril because the concentration of ramipril is high and the percentage of the back-conversion of the acyl glucuronide is 0.089% or lower. On the other hand, when the concentration of ramipril is low, the concentration of ramipril acyl glucuronide is also low so that the potential impact of the back-conversion is insignificant on data accuracy and reproducibility. For ramiprilat, the back-conversion of ramiprilat

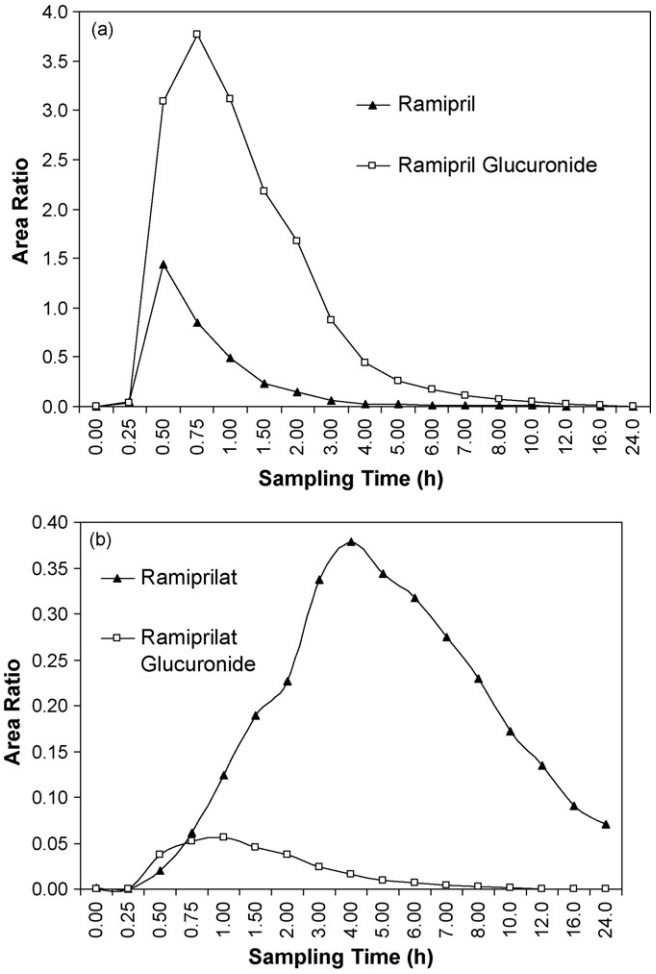

Fig. 3. Time course concentration-related profiles of ramipril, ramiprilat, and their glucuronides. (a) ramipril and ramipril glucuronide; (b) ramiprilat and ramiprilat glucuronide.

glucuronide to ramiprilat is of much less concern because much lower concentration of ramiprilat glucuronide was presented in the incurred samples for most of the sampling points (Fig. 3B). However, at some early sampling hours, such as 0.5 h, relatively higher glucuronide concentration was observed, which could explain why high % of change was obtained for the 0.5 h sample after being left at room temperature for 24 h (Table 5).

3.9. Application to a bioequivalence study

The aforementioned method has also been applied to the analysis of incurred samples from a large bioequivalence study. As expected, its application was successful and no bioanalytical issue related to metabolite back-conversion was observed during sample analysis. This is the result of the comprehensive investigation that had been performed in the course of the method development and pre-study validation phases. A total of 1101 incurred samples collected from 30 subjects were analyzed with a reassay rate of only 1.1%. All the bioanalytical batches for incurred samples were accepted and the study met bioequivalence acceptance criteria.

4. Conclusions

A robust, precise, and accurate method has been developed and validated as per current regulatory standards to reliably measure ramipril and ramiprilat in human EDTA K_2 plasma. Also, it has been demonstrated that the development and validation of bioanalytical methods using samples spiked with a parent drug may not be reliable in cases where the drug undergoes extensive phase II metabolism and where phase II metabolites can back-convert to the parent drug at any step of the bioanalytical process, i.e. from sample collection to LC–MS/MS analysis. It has been shown that the use of ramipril acyl glucuronide metabolite and pooled and individual incurred samples was critical to the method development and validation of a highly reproducible and rugged method for the simultaneous quantitation of ramipril and ramiprilat in human plasma. In addition, robustness and reproducibility of the validated method was further assessed by performing a mini pilot study in which potential problems relating to metabolite back-conversion or other bioanalytical issues were comprehensively addressed under conditions mimicking those prevailing during the conduct of bioequivalence clinical studies.

Although this approach may be somewhat more time consuming and cost more than the conventional one using spiked samples, it is deemed that its relative costs are significantly offset by the potential benefits one may achieve. Indeed, the methods developed and validated under such stringent conditions are more robust and should ensure a higher confidence level in the overall reliability of the bioanalytical data.

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