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Sensitivity enhancement and matrix effect evaluation during summation of multiple transition pairs—case studies of clopidogrel and ramiprilat

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ABSTRACT: Sensitivity enhancement via summation of multiple MRM transition pairs is gaining popularity in tandem mass spectrometric assays. Numerous validation experiments describing the assays for two model substrates, clopidogrel and ramiprilat, were performed. The quantitation of clopidogrel was achieved by the summation of transition pairs *m/z* 322.2 to *m/z* 212.0 and *m/z* 322.2 to *m/z* 184.0, while that of ramiprilat was achieved by the summation of transition pairs *m/z* 389.2 to *m/z* 206.1 and *m/z* 389.2 to *m/z*156.1. The use of summation approach achieved sensitivities of >2 fold for both compounds as compared with the reported single MRM transition pair assays. The validation experiments addressed some important assay development issues, such as: (a) lack of impact of matrix effect; (b) unequivocal verification of the percentage contribution of each MRM transition pair towards sensitivity; (c) sensitivity enhancement factor achieved by summation approach of MRM transition pairs; and (d) accurate prediction of quality control samples using summation approach vs a single MRM transition pair. In summary, the appropriateness of using two MRM transition pairs for quantitation was demonstrated for both clopidogrel and ramiprilat. Additionally, pharmacokinetic application of the MRM transition pair assays using a summation approach was established for the two compounds. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: clopidogrel; ramiprilat; LC/MS/MS; MRM; assay; sensitivity enhancement

Introduction

The innovative use and versatility of tandem mass spectrometric assays has been confirmed by some recent publications that have adopted the summation of transition pairs to increase the sensitivity of the assay for a number of compounds (Srinivas, 2009a). Therefore, a higher sensitivity has been achieved for several important compounds and/or metabolites such as capsaicin (Beaudry and Vachon, 2009), torcetrapib (Trivedi *et al.*, 2008), bulaquine and primaquine (Nitin *et al.*, 2003), midazolam and 4-hydroxymidazolam (Link *et al.*, 2007), and rivastigmine (Bhatt *et al.*, 2007) by the application of the summation of response of multiple transition pairs. In an elegant overview on this topic, several important considerations during the validation of assays involving summation of transition pairs have been discussed. Such considerations include: (a) appropriate selection of stable transition pairs; (b) thorough evaluation of matrix effect for the individual transition pair; (c) correlation and/or contribution of the transition pairs towards the quantitation of the compound(s) in question.

Several methods are available for the determination of clopidogrel (Shin and Yoo, 2007; Nirogi *et al.*, 2006; Lainesse *et al.*, 2004) and ramiprilat (Zhu *et al.*, 2002) using tandem mass spectrometry. Both compounds produce stable and intense multiple MRM transition pairs as evidenced by the positive ion mass spectral data and therefore are amenable for the proposed summation approach of multiple MRM transition pairs to improve sensitivity. The selection of clopidogrel and ramiprilat (Fig. 1) as substrates for this case study provided a contrast in that clopidogrel was the parent entity converting into an active metabolite, while ramiprilat was an active metabolite formed from the parent ramipril.

While establishing quantitative LC/MS/MS assays for clopidogrel and ramiprilat, the application of multiple transition pairs for the enhancement of sensitivity of the chosen compounds was considered. In this paper, we report the various validation experiments employed (a) to tease out the matrix effects at each transition pair; and (b) to confirm sensitivity enhancement with

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Abbreviations used: API, atmospheric pressure ionisation; AUC_{inf}, area under the plasma concentration vs time curve from time t = zero to t = infinity; C_{max}, peak plasma concentration; HPLC, high-performance liquid chromatography; MRM, multiple reaction monitoring, LCMS/MS, triple quad liquid chromatography mass spectrometry; IS, internal standard; T_{max}, time taken to reach peak plasma concentration; $t_{½}$, elimination half-life.

percentage contribution established from each of the two transition pairs towards the sensitivity. To the best of our knowledge such validation experiments have not been reported earlier and therefore our work may provide some future directions on this important validation aspect of LC/MS/MS assays that use multiple transition pairs. The two model compounds, clopidogrel and ramiprilat, chosen in our bioanalytical investigation provided the opportunity to utilize two stable MRM transition pairs for a summation approach and the existence of literature LC/MS/MS data for a single transition pair for each of the two compounds enabled us to compare the sensitivity data obtained from our work.

Methods and Materials

Chemicals and Reagents

Clopidogrel and deuterated clopidogrel (internal standard) were purchased from Vardha Biotech (Mumbai, India). Ramiprilat and enalaprilat (internal standard) were obtained from Actavis Pharma (Hafnarfirdi, Iceland). Solvents such as methanol and formic acid were purchased from Spectrochem (Mumbai, India), acetonitrile was obtained from J.T. Baker (Phillipsburg, USA) and ethyl acetate and *n*-hexane were purchased from Merck (Mumbai, India). Drug-free plasma samples were obtained from Lotus Laboratories (Bangalore, India).

Extraction, Chromatographic and Mass Spectrometric Conditions

Two independent tandem mass spectrometric assays were employed for the determination of both clopidogrel and ramiprilat from plasma samples. The conditions for the extraction, separation, detection and quantification for the two compounds are discussed below.

Instrumentation

- Chlopidogrel—chromatographic separation was carried out using Agilent1200 HPLC. Mass spectra were obtained using a Sciex API 4000 mass spectrometer. Analyst 1.4.2 software was used for data acquisition.
- Ramiprilat—chromatographic separation was carried out using Agilent1100 HPLC. Sciex API 3000 mass spectrometer (ramiprilat) equipped with a turbo-ion spray. Analyst 1.4.1 software was used for data acquisition.

Sample preparation. Clopidogrel was extracted by liquid–liquid extraction (LLE) using an ethyl acetate–*n*-hexane mixture. Ramiprilat was extracted using solid-phase extraction (SPE) with Oasis 1 cm^3 cartridges. The organic phase after either LLE or SPE was subjected to dryness under a gentle stream of nitrogen and dissolved in mobile phase. A 25 µL aliquot of clopidogrel or 5 µL aliquot of ramiprilat was injected on to the column.

Chromatography. The separation of clopidogrel and its deuterated internal standard was achieved using a mobile phase comprising a mixture of 2 mM ammonium acetate–acetonitrile (30:70, v/v) using a Ascentis C_{18} analytical column (4.6 × 50 mm, 5 µm, Waters Corporation). In order to achieve an optimal separation the mobile phase flow rate was kept at 0.5 mL/min. Under these conditions, the elution of clopidogrel and internal standard was achieved within 5.5 min.

The separation of ramiprilat and its internal standard, enalaprilat, was carried out using a mobile phase comprising of a mixture of 0.1% formic acid–methanol (20:80, v/v) using a Discovery C_8 analytical column (150 × 50 mm, 5 µm; Supelco). The separation was found to be optimal at a mobile phase flow rate of 0.8 mL/min. The retention times of both ramiprilat and enalaprilat were within 4 min.

Mass spectrometer related transition pairs for quantitation. A turboionspray platform, employing a positive mode, was used for ionization and detection of clopidogrel and ramiprilat, and their respective internal standards. Using a dwell time of approximately 200 msecs, multiple transition pairs for each analyte were followed for quantification. The transition pairs followed for clopidogrel included: *m/z* 322.2 to *m/z* 212.0 and *m/z* 322.2 to *m/z* 184.0 and for ramiprilat were *m/z* 389.2 to *m/z* 206.1 and *m/z* 389.2 to *m/z*156.1. d_3-clopidogrel and enalaprilat were used as internal standard for the quantification of clopidogrel and ramiprilat, respectively. The transition pair used for d_3-clopidogrel was *m/z* 326.1 to *m/z* 215.1and for enalaprilat was *m/z* 349.3 to *m/z* 206.1.

Validation Parameters

Sensitivity enhancement. The relative sensitive enhancement was calculated using the response factor obtained for the standard representing lower limit of quantitation for clopidogrel (1.007 pg/mL) and ramiprilat (0.150 ng/mL) using the following general equation:

$$\% \text{ enhancement} = \text{two MRM pair summation factor} / \text{single MRM response factor}$$

Relative contribution of each transition pair. The contribution of each transition pair towards the concentration(s) of quality control samples for both clopidogrel (5.099, 14.243. 989.102, and 1978.204 pg/mL) and ramiprilat (0.152, 0.421, 7.020 and 17.550 ng/mL) was evaluated separately. Additionally, calibration standard data were inspected for the relative contribution of each MRM transition pair over the studied calibration curve ranges for both clopidogrel and ramiprilat.

Matrix effect evaluation. The matrix effect was evaluated by using six independent lots of matrix of same source. Analyte detector responses in matrix samples spiked post extraction were compared with detector responses observed in unextracted standard prepared at similar concentrations (Hubert *et al.*, 1999).

Correlation between each MRM transition pair versus summed MRM transition pairs. Separate regression lines were computed for both clopidogrel and ramiprilat using the individual response factor for each MRM transition pair and compared with the regression line obtained from the summation factors for the two transition pairs. The concentration of the QC samples for both clopidogrel and ramiprilat was additionally predicted using the regression lines for each MRM transition pair for comparison with predictions made by the summation approach.

Results

The positive Q1 mass spectrums of clopidogrel/ramiprilat and the respective associated product ion mass spectrum of both clopidogrel and ramiprilat are shown in Figs 2 and 3. The regres-

sion lines computed using the response factors of the summed MRM transition pairs and response factors from the two individual MRM transition pairs for clopidogrel (summation: $y = 0.00063x + -0.000145$, $r^2 = 0.9988$; MRM1 = $y = 0.000368x + -0.000158$, $r^2 = 0.9989$; MRM2 = $0.00026x + -0.000119$, $r^2 = 0.9987$) and ramiprilat (summation: $y = 0.0822x + 0.00179$, $r^2 = 0.9982$; MRM1 = $y = 0.0532x + 0.00131$, $r^2 = 0.9968$; MRM2 = $0.029x + 0.000719$, $r^2 = 0.9983$) are provided in Figs 4 and 5, respectively.

Regardless of clopidogrel or ramiprilat, the summation approach of two transition pairs resulted in better sensitivity as compared with the reported assays that utilized a single transition pair for quantitation (Table 1). The increased sensitivity observed for clopidogrel and ramiprilat was due to an enhancement factor of at least 1.70 obtained for each transition pair (Table 2). As depicted in Tables 3 and 4, validation of quality control samples for both clopidogrel and ramiprilat suggested that both transition pairs contributed almost in similar proportions across the concentration ranges (i.e. approximately 58–65% from MRM1 pair and approximately 35–40% from MRM2 pair). The relative contribution of the MRM pairs in the calibration standards for either clopidogrel or ramiprilat appeared to be consistent over the applied calibration curve ranges (data not shown). The validation for possible matrix effects at each of the two MRM transition pairs showed negligible impact for either clopidogrel or ramiprilat (Tables 5 and 6). Further validation of the summation approach vs the use of single MRM transition pair suggested consistent prediction of the QC concentrations regardless of the use of transition MRM pairs either alone and/or in combination for clopidogrel and ramiprilat (Tables 7 and 8).

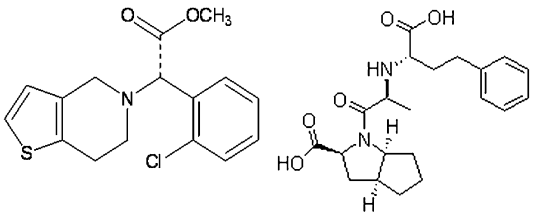

Figure 1. Structures of clopidogrel and ramiprilat.

The pharmacokinetic application of summation approach of MRM transition pairs is depicted for clopidogrel and ramiprilat in Figs 6 and 7, respectively. The pharmacokinetic profiles obtained for either clopidogrel or ramiprilat was consistent with reported literature values. The C_{max}, T_{max}, AUC_{inf} and $t_{½}$ were 705.01 pg/mL, 1.67 h, 2226.71 pg h/mL and 3.41 h, respectively for clopidogrel and the corresponding values for ramiprilat were 5.65 ng/mL, 5.00 h, 255.51 ng h/mL and 74.64 h, respectively.

Discussion

The use of both clopidogrel and ramiprilat as model substrates provided us an opportunity to evaluate several aspects of the

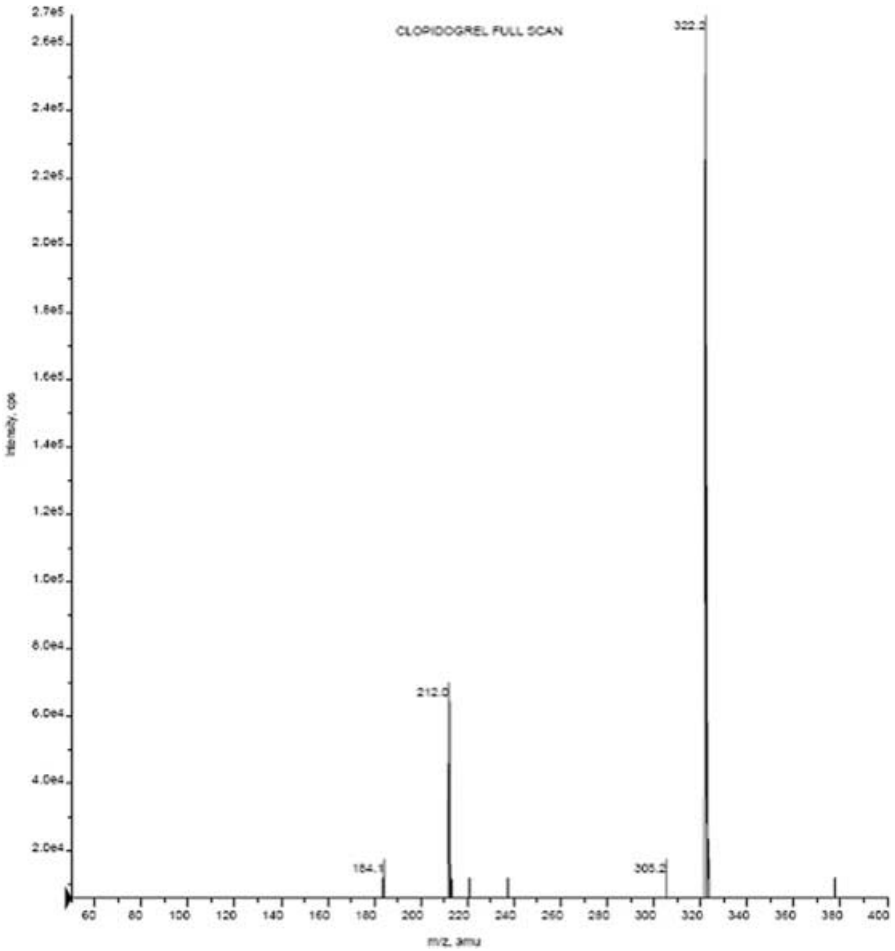

Figure 2. Mass fragmentation patterns for the display of transition pairs for clopidogrel (positive mode) with the molecular ion.

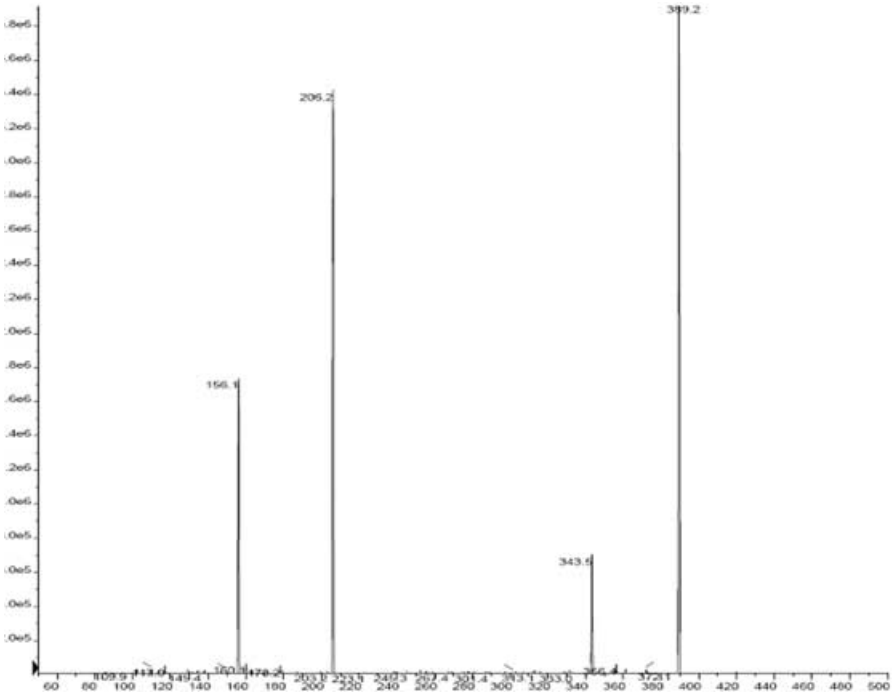

Figure 3. Mass fragmentation patterns for the display of transition pairs for ramiprilat (positive mode) with the molecular ion.

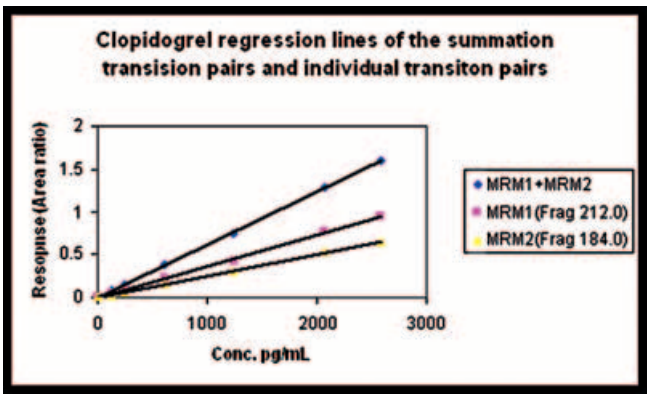

Figure 4. Regression lines of the summation transition pairs and individual MRM transition pairs for clopidogrel.

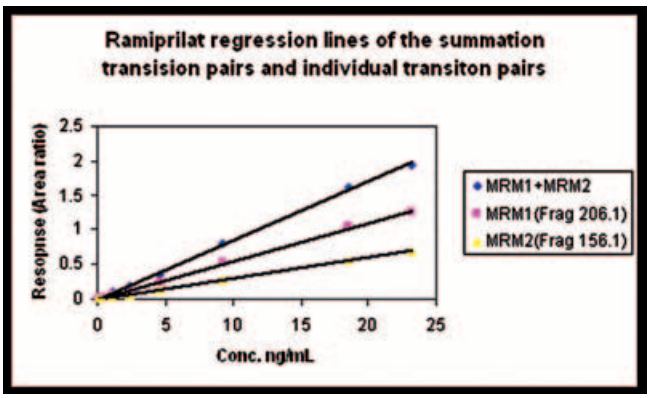

Figure 5. Regression lines of the summation transition pairs and individual MRM transition pairs for ramiprilat.

Table 1. Comparison of lower limit of quantitation—Summation of transition pairs vs individual MRM transition pair

Compound	Literature Reference Authors	MRM transition pair	LOQ	Summation approach (current method)
Clopidogrel	Shin and Yoo (2007)	322.2 → 211.9	10 pg/mL	1 pg/mL[a]
	Nirogi *et al.* (2006)	322 → 211	5 pg/mL	
Ramiprilat	Zhu *et al.* (2002)	389.2 → 206.1	0.5 ng/mL	0.15 ng/mL
	Lu *et al.* (2006)	389.2 → 206.1	0.25 ng/mL	

[a] One precision and accuracy batch was performed.

Table 2. Sensitivity enhancement factor at LOQ—comparison of response factors of the summation of MRM transition pairs versus single MRM transition pair

Compound	Response of MRM transition pair (summation)	MRM 1 Response	MRM 1 Enhancement factor	MRM 2 Response	MRM 2 Enhancement factor
Clopidogrel	2336.5	1365	1.71	851.5	2.74
Ramiprilat	1197	768.2	1.71	441.8	2.71

Enhancement factor = response of summation transition pairs/response of individual pair.

Table 3. Percentage contribution by the individual MRM transition pairs for the various quality control samples of clopidogrel

Concentration (pg/mL)	N	Summation of MRM transition pairs 322.2 to (212.0 + 184.0) (average area 100%)	MRM 1 322.2 to 212.0 (%)	MRM 2 322 to 184.0 (%)
5.099	6	2336.5	1365 (58.4%)	851.5 (36.5%)
14.243	6	6462.3	3762.2 (58.2%)	2569.5 (39.8%)
989.102	6	450,632.5	264,792.3 (58.7%)	185,569.3 (41.2%)
1978.204	6	888,054.6	521,094.2 (58.7)	366,069 (41.2)

Table 4. Percentage contribution by the individual MRM transition pairs for the various quality control samples of ramiprilat

Concentration (ng/mL)	N	Summation of MRM transition pairs 389.2 to (206.1 + 156.1) (average area 100%)	MRM 1 389.2 to 206.1 (%)	MRM 2 389.2 to 156.1 (%)
0.152	6	1197.0	768.2 (64.2%)	441.8 (36.9%)
0.421	6	3213.5	2073.8 (64.5%)	1171.2 (36.4%)
7.020	6	56710.7	36,180.5 (63.8%)	20185.5 (35.6%)
17.550	6	145158.2	93,842.0 (64.6)	51,339.0 (35.4)

Table 5. Contribution of MRM transition pairs on matrix effects—clopidogrel

Matrix lots	Matrix factor Summation 322.2 to (212.0 + 184.0)	Matrix factor MRM 1 322.2 to 212.0	Matrix factor MRM 2 322.2 to 184.0
Lot 1	1.001	1.027	0.989
Lot 2	1.028	1.045	0.990
Lot 3	1.004	1.005	1.006
Lot 4	1.018	1.024	1.027
Lot 5	0.968	0.983	0.957
Lot 6	1.025	1.069	0.970
Average	1.0038	1.0168	0.9938

Table 6. Contribution of MRM transition pairs on matrix effects—ramiprilat

Matrix lots	Matrix factor Summation 389.2 to (206.1 + 156.1)	Matrix factor MRM 1 389.2 to 206.1	Matrix factor MRM 2 389.2 to 156.1
Lot 7	0.983	0.975	0.937
Lot 8	0.909	0.868	0.923
Lot 9	0.858	0.875	0.856
Lot 10	0.919	0.904	0.881
Lot 11	0.915	0.868	0.890
Lot 12	0.864	0.869	0.844
Average	0.9168	0.898	0.897

summation approach of multiple MRM transition pairs now being increasingly considered in the LC/MS/MS analysis to increase the sensitivity of many compounds (Beaudry and Vachon, 2009; Trivedi *et al.*, 2008; Link *et al.*, 2007; Bhatt *et al.*, 2007; Nitin *et al.*, 2003).

Interestingly, for both compounds, one of the MRM transition pair (i.e. *m/z* 322.2 to *m/z* 212.0 for clopidogrel and *m/z* 389.2 to *m/z* 206.1 for ramiprilat) contributed more significantly for the summed response factor. It was not surprising that these single transition pairs have been considered for the quantification of the two compounds (Shin and Yoo, 2007; Nirogi *et al.*, 2006; Lu *et al.*, 2006; Zhu *et al.*, 2002). However, the other lesser contributing MRM transition pair (i.e. *m/z* 332.2 to *m/z* 184.0 for clopidogrel and *m/z* 389.2 to *m/z* 156.1 for ramiprilat) appeared to be equally accurate and precise for the prediction of the QC samples across the chosen range for both compounds.

Although the two IS (d_3-clopidogrel and enaliprilat) for the assays provided us with the opportunity to use a summation approach for quantification of the levels of the internal standard, we used a single transition pair for determination of the IS to avoid the additional complexities for the assay.

The evaluation of matrix effects specifically performed for each transition pair suggested consistent effects between the transition pairs for either clopidogrel or ramaprilat. The matrix effect

Table 7. Relationship of clopidogrel QC samples assayed by individual MRM transition pair versus the summation approach

Nominal concentration (pg/mL)	MRM 1 322.2 to 212.0		MRM 2 322.2 to 184.0		Summation of MRM transition pairs 322.2 to (212.0 + 184.0)	
	Found (pg/mL)	%CV	Found (pg/mL)	%CV	Found (pg/mL)	%CV
5.099	5.61	5.23	5.03	7.05	5.41	2.80
14.243	14.64	3.63	14.19	4.13	14.49	2.45
989.102	977.73	0.44	970.39	0.64	972.61	0.49
1978.204	1947.51	0.46	1937.77	0.34	1940.27	0.30

Average of six determinations.

Table 8. Relationship of ramiprilat QC samples assayed by individual MRM transition pair versus the summation approach

Nominal concentration (ng/mL)	MRM 1 389.2 to 206.1		MRM 2 389.2 to 156.1		Summation of MRM transition pairs 389.2 to (206.1 + 156.1)	
	Found (ng/mL)	%CV	Found (ng/mL)	%CV	Found (ng/mL)	%CV
0.152	0.14	14.30	0.15	8.31	0.14	10.25
0.421	0.41	7.29	0.42	11.48	0.41	4.45
7.020	7.18	2.77	7.25	2.42	7.29	2.72
17.550	18.85	0.99	18.64	0.82	18.86	0.79

Average of six determinations.

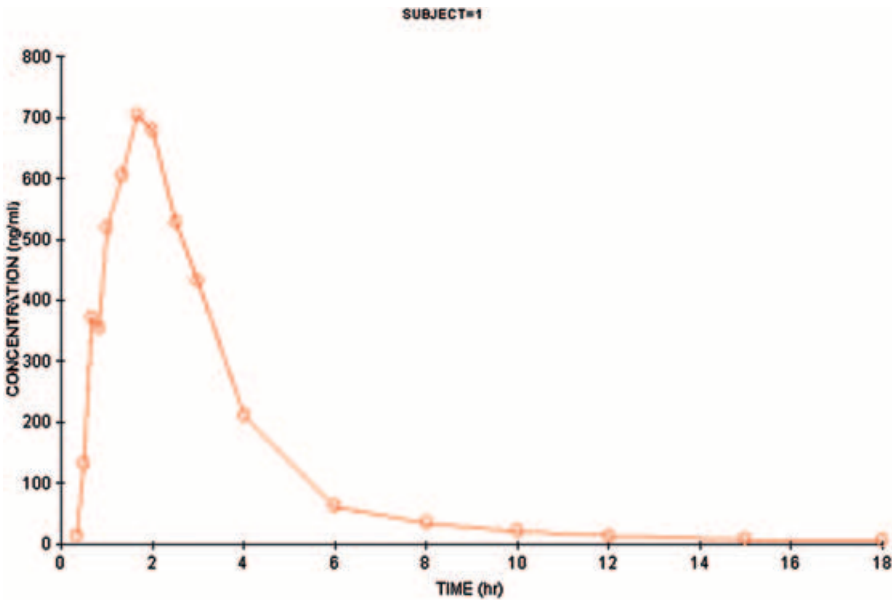

Figure 6. Plasma curve profile for clopiclogrel (single subject only).

for clopidogrel appeared to be negligible with a slight positive bias of one MRM pair cancelling the slight negative bias for the other MRM transition pair. Interestingly, the matrix effect observed for ramiprilat suggested a slight suppression (approximately 10%) and appeared to be similar for both MRM transition pairs. Nevertheless, the performance of the assay for ramiprilat is accurate in predicting the concentrations of QC samples, suggesting negligible impact of the matrix effects. As suggested by Srinivas (2009b), the existence of matrix effects should not pose an issue as long as it is fully understood and comprehensively validated not to impact the bias of the quantitative determination of the analyte in question.

The prediction of QC samples using the individual MRM transition pairs which exactly overlapped with those predicted by the summation approach provided strong evidence for interchangeable use of either of the two transition pairs, if it became necessary, for the determination of clopidogrel and ramiprilat concentrations from pharmacokinetic samples.

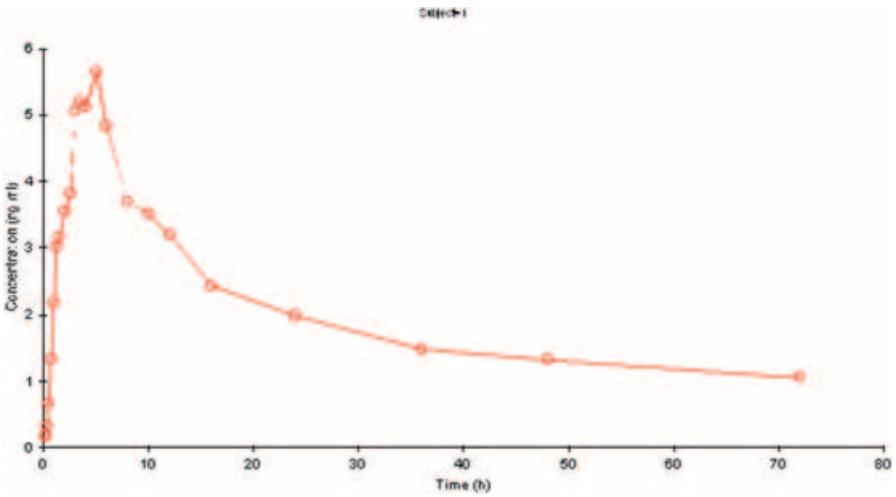

Figure 7. Plasma curve profile for ramiprilat (single subject only).

Conclusions

The present work confirmed the enhanced sensitivity achieved by the summation of multiple MRM transition pairs of both clopidogrel and ramiprilat in comparison to assays that employed single MRM transition pair. Our approach, of conducting a series of validation experiments to support the summation approach of multiple MRM transition pairs, should provide an initial framework for the development of assays based on summation approach of MRM transition pairs for other compounds.

References

Beaudry F and Vachon. Quantitative determination of capsaicin, a transient receptor potential channel vanilloid 1 agonist, by liquid chromatography quadrupole ion trap mass spectrometry: evaluation of in vitro metabolic stability. *Biomedical Chromatography* 2009; **23**: 204–211.

Bhatt J, Subbaiah G, Kambli S, Shah B, Nigam S, Patel M, Saxena A, Baliga A, Parekh H and Yadav G. A rapid and sensitive liquid chromatographic–tandem mass spectrometry (LC-MS/MS) method for the estimation of rivastigmine in human plasma. *Journal of Chromatography B* 2007; **852**: 115–121.

Hubert P, Chiap P, Crommen J, Boulanger B, Chapuzet E, Mercier N, Bervoas-Martin S, Chevalier P, Grandjean D, Lagorce P, Lallier M, Laparra MC, Laurentie M and Nivet JC. The SFSTP guide on the validation of chromatographic methods for drug bioanalysis Washington conference to the laboratory. *Analytica Chimica Acta* 1999; **391**: 135–148.

Lainesse A, Ozalp Y, Wong H and Alpan RS. Bioequivalence study of clopidogrel bisulfate film-coated tablets. *Arzneimittel-Forschung/Drug Research* 2004; **54**: 600–604.

Link B, Haschke M, Wenk M and Krahenbuhl S. Determination of midazolam and its hydroxy metabolites in human plasma and oral fluid by liquid chromatography/electrospray ionization ion trap tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* 2007; **21**: 1531–1540.

Lu XY, Shen-Tu JZ and Liu J. High performance-mass spectrometric analysis of ramipril and its active metabolite ramiprilat in human serum: application to a pharmacokinetic study in the Chinese volunteers. *Journal of Pharmaceutical and Biomedical Analysis* 2006; **40**: 478–483.

Nirogi RV, Kandikere VN, Shukla M, Mudigonda K, Maurya S and Boosi R. Quantitation of clopidogrel in human plasma by sensitive liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* 2006; **20**: 1695–1700.

Nitin M, Rajanikanth M, Lal J, Madhusudanan KP and Gupta RC. Liquid chromatography-tandem mass spectrometric assay for the simultaneous determination of bulaquine and its metabolite, primaquine, in monkey plasma. *Journal of Chromatography B* 2003; **793**: 253–263.

Shin BS and Yoo SD. Determination of clopidogrel in human plasma by liquid chromatography/tandem mass spectrometr: application to a clinical pharmacokinetic study. *Biomedical Chromatography* 2007; **21**: 883–889.

Srinivas NR. Sensitivity enhancement in tandem liquid chromatographic mass spectrometric assays by summation of two transition ion pairs—perspectives. *Journal of Separation Science* 2009a; **32**: 483–486.

Srinivas NR. Dodging matrix effects in liquid chromatography tandem mass spectrometric assays—compilation of key learnings and perspectives. *Biomedical Chromatography* 2009b; **23**: 451–454.

Trivedi RK, Dubey PK, Mullangi R and Srinivas NR. Development and validation of a sensitive LC-MS/MS method with electrospray ionization using multiple ions for quantitation of torcetrapib in hamster and dog plasma. *Biomedical Chromatography* 2008; **22**: 316–326.

Zhu Z, Vachareau A and Neirinck L. Liquid chromatography–mass spectrometry method for determination of ramipril and its active metabolite ramiprilat in human plasma. *Journal of Chromatography B* 2002; **778**: 297–306.

