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Original Article

HPLC-MS/MS METHOD FOR SIMULTANEOUS QUANTIFICATION OF VILDAGLIPTIN, METFORMIN, AND METFORMIN-RELATED COMPOUNDS IN TABLETS

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ABSTRACT

Objective: The present study developed and validated a high-performance liquid chromatography-tandem mass spectrometry method for simultaneous determination of vildagliptin, metformin, and metformin-related compounds (A, B, and C) in tablets.

Methods: Chromatographic separations were achieved on a C8 column (150 × 4.6 mm, 5 µm particle size) maintained at 25°C. The isocratic mobile phase consisted of acetonitrile/water/formic acid (20:80:0.1, *v/v/v*), eluted at 800 µL/min. The volume of injection was 20 µL and the electrospray source was operated in positive ion mode.

Results: Data from validation studies demonstrated that the new method was highly selective, sensitive (limits of detection ≤ 0.6 ng/mL), precise (RSD < 5%), and accurate (RE < 5%). All of the calibration curves showed excellent coefficients of correlation ($r \geq 0.99$) over the ranges of 5-150 ng/mL for vildagliptin, 250-2000 ng/mL for metformin, and 2.5-25 ng/mL for metformin-related compounds A, B, and C. Quantification of these compounds in tablets showed that they contained from 95.2-101.2% (vildagliptin) and 97-104.4% (metformin) of the stated level. No metformin-related compounds were detected using this highly sensitive method.

Conclusion: This new method is presented as an alternative approach to the quality control of tablets containing vildagliptin, metformin, and metformin-related compounds.

Keywords: Diabetes, HPLC-MS/MS, Metformin, Metformin-related compound, Tablets, Vildagliptin.

INTRODUCTION

Failures of the secretion and/or activity of insulin causes a group of metabolic diseases named *diabetes mellitus* (DM). By 2030, more than 300 million people worldwide are expected to be diabetic and 90-95% of these individuals will have type 2 DM (T2DM) [1, 2].

Treatment of T2DM begins with lifestyle modification and pharmaceutical monotherapy. Metformin (MET) is the first-line drug used in oral therapy for T2DM in diabetics who do not respond to dietary and lifestyle changes. The mechanism of action of MET is not completely understood, but it primarily appears to act by inhibiting gluconeogenesis and thereby suppressing hepatic glucose release [3]. If the goals of glycemic control are not achieved using MET monotherapy, combination therapy involving agents with complementary mechanisms of action is the next logical step in the management of T2DM [4]. Recently, newer agents such as vildagliptin (VIL) have been used in association with MET for the treatment of T2DM. VIL is a potent and selective dipeptidyl peptidase-4 inhibitor that prevents the rapid degradation of endogenous glucagon-like peptide-1 and glucose-dependent insulinotropic peptide. VIL reduces hyperglycemia by increasing insulin secretion and decreasing the levels of glucagon in diabetic patients [5]. The advantages of combined MET and VIL therapy include superior efficacy to that of monotherapy, with a reduced risk for hypoglycemia and weight gain, as compared with other classes of oral antihyperglycemic agents [6, 7].

Some analytical methods have been described in the literature for the determination of VIL and MET in tablets; these include infrared spectroscopy [8, 9] and ultraviolet spectrophotometry [10]. However, none of these could monitor VIL and MET simultaneously, because of their low selectivity.

High-performance liquid chromatography (HPLC) has proved to be the most suitable technique for routine analyses due to its high sensitivity, reproducibility, and ability to separate compounds from a range of different matrices, allowing excellent separation and reducing analysis times. Despite these advantages, some of the available HPLC methods can only monitor VIL and MET separately [11-15] and in other cases, simultaneous determination does not include MET-related compounds A (MTA), B (MTB), and C (MTC) [16-18], as recommended by The European and United States Pharmacopoeias [19, 20]. To improve selectivity, some HPLC methods used long wavelengths (263 and 293 nm) but this resulted in a loss of sensitivity, because VIL and MET are poor chromophore molecules [16, 18]. In other cases, sensitivity was improved by using shorter wavelengths (210-220 nm), but the method had poor selectivity [17]. None of the currently-available HPLC methods for the determination of MET-related compounds has sufficient selectivity to monitor MTA, MTB, and MTC in the same run, and only two of the methods cited above were fully validated in accordance with international regulations [11, 12]. This validation is a key step in the process of proving that a method is suitable for its intended purpose [21].

HPLC-tandem mass spectrometry (MS/MS) has been demonstrated to have several benefits over other techniques, such as sensitivity and the ability to analyze pharmaceutical drugs in complex matrices with high selectivity. HPLC-MS/MS is a promising technique with the potential to allow the simultaneous quantification of VIL, MET, MTA, MTB, and MTC. The present study was devoted to the development and validation of an HPLC-MS/MS method for simultaneous quantification of these compounds in tablets. The method developed was highly selective and sensitive, and was capable of detecting low concentrations of the target compounds in tablet matrices.

MATERIALS AND METHODS

Chemicals and reagents

The VIL standard (98.0% purity) was obtained from Ontario Chemicals (Guelph, ON, Canada). Standards of MET hydrochloride

(99.7% purity), MTA (100% purity), MTB (100% purity), and MTC (100% purity) were obtained from United States Pharmacopoeia (Rockville, MD, USA). Acetonitrile and methanol (HPLC grade) were purchased from Tedia (Fairfield, CA, USA) and ultrapure water was obtained from a Milli-Q system (Milford, MA, USA). Formic acid (98-100% purity) was purchased from Merck (Darmstadt, Germany).

Fixed dose tablets of MET hydrochloride (850 mg; from different manufacturers), VIL (50 mg; Galvus®, Novartis Corporation), and VIL/MET hydrochloride (50/500, 50/850, and 50/1000 mg; Galvus Met®, Novartis Corporation) were obtained from local markets in Curitiba, Brazil.

Standard solutions

Stock solutions (1 mg/mL) of VIL, MET, MTA, MTB, and MTC were prepared in methanol and stored at 4°C. Working standard solutions were freshly prepared by direct dilution of these stock solutions in acetonitrile/water/formic acid (20:80:0.1 *v/v/v*).

Sample preparation

For VIL and MET quantification, twenty tablets of each fixed dose combination (Chemicals and reagents section) were weighed separately and powdered. A mass equivalent to 1 mg of VIL and 10, 17, or 20 mg of MET (according to the tablet dosage) was accurately weighed and transferred to a 10 mL volumetric flask. Methanol (5 mL) was added to the flask and the samples were sonicated for 10 min. The volume was completed with the same solvent and the samples were centrifuged for 10 min at 4000 rpm at room temperature (Eppendorf 5810-R, Hamburg, Germany). The supernatant was diluted (1:2000 *v/v*) in acetonitrile/water/formic acid (20:80:0.1 *v/v*).

For MTA, MTB, and MTC quantification, the samples were prepared essentially as described above; however the final dilution was 1:200 *v/v*. All samples were filtered through a polyvinylidene fluoride (PVDF) syringe filter (13 mm, 0.22 µm, Millipore Millex, Billerica, MA, USA) before HPLC-MS/MS analysis.

HPLC-MS/MS conditions

These analyses were performed on an Agilent 1200 HPLC system (Santa Clara, CA, USA) equipped with a G1312B binary pump, G1379B degasser, and G1316B column oven, coupled to an Applied Biosystems MDS Sciex API 3200 Triple Quadrupole Mass Spectrometer (Foster City, CA, USA) equipped with a syringe pump (Havard 22 Dual Model, Harvard Apparatus, Holliston, MA, USA) and an electrospray ionization (ESI) source. The HPLC-MS/MS system was connected to a CTC Sample Manager (Model 2777, Waters Corporation, Milford, MA, USA). Chromatographic separation was achieved using an XBridge C8 (150 × 4.6 mm, 5 µm) column maintained at 25ºC. The isocratic mobile phase consisted of a mixture of acetonitrile/water/formic acid (20:80:0.1 *v/v*). The flow rate was 800 µL/min and the injection volume were 20 µL. The MS ESI source operated in positive ion mode and analyte quantification was achieved using multiple reaction monitoring (MRM). Data acquisition was performed with the MS workstation using Analyst 1.4 software (MDS Sciex, Concord, Canada). High-purity nitrogen was produced using a PEAK Scientific Instruments generator (Chicago, IL, USA). The ion source parameters were: curtain gas (CUR) 10 psi; collision-activated dissociation (CAD) 6 psi; nebulizer gas (GS1) 45 psi; turbo gas (GS2) 40 psi; ion spray voltage (IS) 5500V, and temperature 600°C. The ion transitions and the individual compound parameters, including declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE), and cell exit potential (CXP) are summarized in Table 1.

Table 1: Compound-dependent parameters and ion transitions

Analyte	$[M+H]^+$ (*m/z*)	Transition (*m/z*)	DP[a] (eV)	EP[b] (V)	CEP[c] (V)	CE[d] (V)	CXP[e] (V)
VIL	303.90	154.1	21	8	16	25	6
		97.1				35	4
MET	130.05	60.1	21	6.5	10	17	4
		71.1				27	4
MTA	84.90	68.2	20	5	7.5	20	3
MTB	116.00	60.1	26	8	10	17	4
		57.1				29	4
MTC	155.20	71.1	36	6.5	10	35	4
		85.1				27	4

VIL: vildagliptin; MET: metformin; MTA: MET-related compound A; MTB: MET-related compound B; MTC: MET-related compound C; [a]declustering potential; [b]entrance potential; [c]collision cell entrance potential; [d]collision energy; [e]cell exit potential

Validation of the analytical method

The HPLC-MS/MS method was validated according to the International Conference on Harmonization guidelines [21]. The parameters evaluated were selectivity, limits of detection and quantification, linearity, precision, and accuracy.

Selectivity

The selectivity was evaluated for each fixed dose matrix by comparing the slopes of two analytical curves. The first analytical curve was produced by analysis of a serial dilution of the working standard solutions to achieve concentrations of 10, 25, 50, 75, and 100 ng/mL for VIL; 170, 425, 850, 1275, and 1700 ng/mL for MET; and 5, 7.5, 10, 12.5, and 15 ng/mL for MTA, MTB, and MTC.

The second curve was constructed as follows: Initially, the samples were prepared as described in the sample preparation section, however, the supernatant was diluted 1:20000 (*v/v*) in the mobile phase. The diluted samples were then spiked with the analytes to achieve the same concentrations used to prepare the first curve. Both of these curves were prepared in triplicate and Student's t-test was used to compare their slopes.

Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were estimated from the signal-to-noise ratio. To perform this assay, stock solutions were diluted in mobile phase until the smallest detectable peaks were observed. The LOD was estimated at a signal-to-noise of 3:1 and the LOQ was estimated as the peak at a signal-to-noise ratio of at least 10:1.

Linearity

Seven concentrations of each analyte were analyzed in triplicate and calibration curves were produced to confirm that there was a linear relationship between concentration and analyte peak area.

To conduct this study, stock solutions were diluted in mobile phase to obtain the following concentrations: 5, 10, 25, 50, 75, 100, and 150 ng/mL VIL; 250, 500, 750, 1000, 1250, 1500, and 2000 ng/mL MET; 2.5, 5, 7.5, 10, 12.5, 15, and 25 ng/mL MTA, MTB, and MTC. The slope, intercept and regression coefficient (r) was calculated by weighted 1/x linear regression for VIL, MTA, MTB and MTC and $1/x^2$ for MET.

Precision and accuracy

Precision was evaluated using the intra-day and inter-day reproducibility of measurements of three replicate analyses of working standard solutions at 25, 50, and 75 ng/mL for VIL; 500, 750, and 1000 ng/mL for MET; and 5, 12.5, and 25 ng/mL for MTA, MTB, and MTC. These measurements were conducted by the same analyst over a short period of time. The inter-day precision was determined by a second analyst after two consecutive days. The precision was expressed as the percentage relative standard deviation (RSD%) of these measurements.

Accuracy was measured in triplicate using the method of addition of the standard. To perform this assay, twenty tablets of Galvus Met® (50 mg of VIL and 850 mg of MET) were weighed and powdered. A mass equivalent to 1 mg of VIL and 17 mg of MET was accurately weighed and transferred to a 10 mL volumetric flask. Methanol was added to the flask and the samples were sonicated and centrifuged. The supernatant was diluted (1:20000 *v/v)* in acetonitrile/water/formic acid (20:80:0.1 *v/v*) and analyzed. Then, the diluted sample was spiked with standard solutions at the same concentrations used for the precision assay. The amounts of the analytes recovered were calculated by subtracting the levels of the analytes detected in the spiked samples from those obtained in the original (unspiked) samples. Accuracy was expressed as the percentage relative error (RE%) of the amount recovered, compared with the standard concentrations.

RESULTS AND DISCUSSION

Method development

The MS and MS/MS conditions were automatically optimized by direct infusion of individual working standard solutions of VIL, MET, MTA, MTB, and MTC. In the MRM mode, the DP, EP, CEP, CE, and CXP parameters were optimized for each analyte and the two most intense fragments were selected. For MTA, only one fragment ion was monitored (*m/z* 68.2) because the fragments showed low signal intensity after MS/MS optimization. At the end of the MS/MS optimization, the positive ion mode was found to be the most efficient ionization mechanism for each analyte. The structures of all the compounds analyzed are shown in Fig. 1.

The optimization of the source parameters (CUR, CAD, ISV, GS1, GS2, and temperature) was accomplished through flow-injection analysis (FIA), which coupled the HPLC with the MS. The pump was operated at 200 µL/min using an isocratic system with a mobile phase consisting of acetonitrile/water/formic acid (50:50:0.1 *v/v/v*).

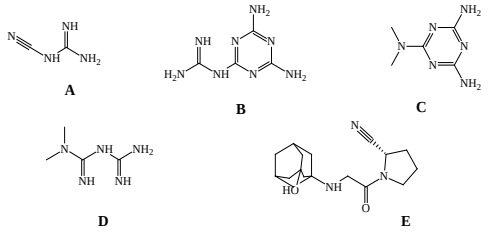

Fig. 1: Chemical structures of (A) metformin (MET)-related compound A, (B) MET-related compound B, (C) MET-related compound C, (D) MET, and (E) vildagliptin

To identify the optimum chromatographic conditions, mobile phases with several combinations of acetonitrile, methanol, and water were tested. Different types of column (C18 and C8), and variations in flow rate (400-800 µL/min) and column oven temperature (25-40°C) were also compared to achieve the best sensitivity, peak shape, and the shortest run times.

The initial experiments were carried out on a C18 column using isocratic mobile phases consisting of acetonitrile/water/formic acid (10:90:0.1 *v/v/v*) or methanol/water/formic acid (10:90:0.1 *v/v/v*), both at flow rates of 400 µL/min. This analysis identified that the methanol-containing mobile phase did not result in adequate peak shapes for all the compounds and the C18 column did not promote efficient retention of MET.

Thus, acetonitrile was selected as the organic solvent in the mobile phase and a C8 column was evaluated as the stationary phase. The use of a C8 column improved the MET retention time and an excellent peak shape was observed for all the compounds. However, extensive run time (10.8 min) was observed for VIL and consequently, the proportion of acetonitrile in the mobile phase was modified from 10 to 15, 20, and 25%. These experiments showed that chromatography was optimized using an isocratic mobile phase composed of acetonitrile/water/formic acid (20:80:0.1 *v/v/v*), eluted at 800 µL/min, at a column oven temperature of 25°C. Representative chromatograms for analytes are shown in Fig. 2.

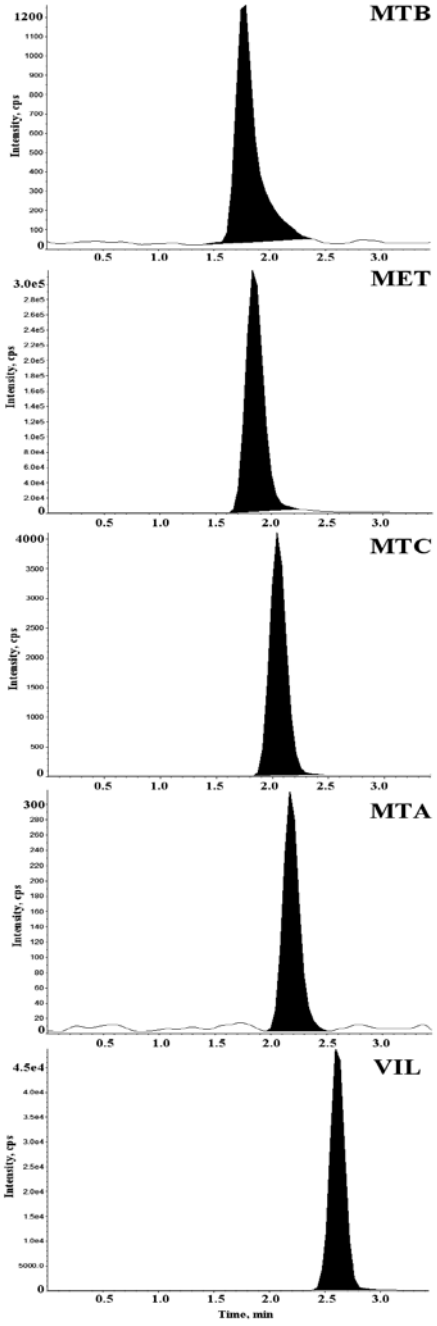

Fig. 2: High-performance liquid chromatography-tandem mass spectrometry chromatograms of metformin (MET), MET-related compound A (MTA), MET-related compound B (MTB), MET-related compound C (MTC), and vildagliptin (VIL)Method validation

This novel HPLC-MS/MS method offered several advantages over other methods described in the literature, especially in terms of specificity, sensitivity, speed (3.5 min), and the linear range of detection achieved with acceptable accuracy and precision. This method was uniquely developed for simultaneous determination of VIL, MET, MTA, MTB, and MTC. The new HPLC-MS/MS method was also fully validated in accordance with current international regulations. Thus ensuring reliable results.

Selectivity

As demonstrated in Table 2, the comparison of the regression equations of the analytical curves (standard solutions and spiked matrix) did not identify any significant differences (Student's t-test, $p > 0.05$). The developed method was therefore considered to be selective.

LOD and LOQ

The high sensitivity of the method developed in the present study was demonstrated its low LOD, estimated at 0.5 ng/mL for VIL and MET and 0.6 ng/mL for MTA, MTB, and MTC. The LOQ was estimated at 1.5 ng/mL for VIL, 2.5 ng/mL for MTA, and 2 ng/mL for MET, MTB, and MTC.

Linearity

The calibration curves for VIL, MET, MTA, MTB, and MTC showed an excellent linearity with a correlation coefficient (r) > 0.99. The precision (RSD) for each compound was < 10% and the accuracy was < 11.6%. The regression equations and correlation coefficients were y = 8790x + 2330 (r = 0.9991) for VIL, y = 2000x + 1200000 (r = 0.9962) for MET, y = 246x + 255 (r = 0.9942) for MTA, y = 1300x + -457 (r = 0.9936) for MTB, and y = 3190x + 2130 (r = 0.9978) for MTC.

Precision and accuracy

The precision and accuracy data are shown in Table 3. The intra-day precision ranged from 0.59-4.98%, whereas the inter-day precision ranged from 0.47-4.91%. The level of accuracy was satisfactory, with RE ranging from 0.13-4.88%.

Table 2: Statistical comparison of the slopes of the analytical curves for the determination of selectivity

Matrices	VIL *(p-value)*	MET *(p-value)*	MTA *(p-value)*	MTB *(p-value)*	MTC *(p-value)*
Galvus®	0.591	-	-	-	-
Galvus Met® 50/500	0.483	0.442	0.791	0.512	0.060
Galvus Met® 50/850	0.323	0.546	0.346	0.192	0.282
Galvus Met® 50/1000	0.834	0.140	0.754	0.670	0.580
MET manufacturer A	-	0.318	0.475	0.276	0.883
MET manufacturer B	-	0.772	0.347	0.238	0.943
MET manufacturer C	-	0.675	0.258	0.732	0.606
MET manufacturer D	-	0.542	0.234	0.768	0.774
MET manufacturer E	-	0.884	0.187	0.639	0.365

VIL: vildagliptin; MET: metformin; MTA: MET-related compound A; MTB: MET-related compound B; MTC: MET-related compound C. Data shown represent the *p* values (Student's t test 95% confidence) for comparison of analytical curves prepared in triplicate.

Table 3: Precision and accuracy of high-performance liquid chromatography-tandem mass spectrometry analyses

Compound	Concentration (ng/mL)	Precision		Accuracy (RE %)
		Intra-day (RSD %)	Inter-day (RSD %)	
VIL	25	1.59	4.23	4.88
	50	3.71	4.82	3.36
	75	1.13	4.35	2.21
MET	500	4.63	4.39	0.26
	750	0.59	3.91	1.57
	1000	2.86	3.89	0.13
MTA	5	3.90	3.80	3.00
	12.5	4.32	4.67	2.13
	25	2.73	3.73	1.93
MTB	5	4.98	3.79	4.17
	12.5	2.75	4.25	4.73
	25	2.37	2.85	1.87
MTC	5	3.78	3.68	4.07
	12.5	2.07	4.91	3.00
	25	2.84	0.47	4.53

VIL: vildagliptin; MET: metformin; MTA: MET-related compound A; MTB: MET-related compound B; MTC: MET-related compound C. RSD: relative standard deviation, RE: relative error.

Sample analysis

The new method was successfully applied to real samples containing VIL (Galvus® 50 mg), 850 mg MET hydrochloride (from five different manufacturers), and VIL associated with MET at different doses (Galvus Met® containing VIL/MET at 50/500, 50/850, and 50/1000 mg). The results are shown in Table 4.

All of the MET hydrochloride tablets analyzed were compliant with the United States Pharmacopoeia (95-105%) [20]. The VIL content ranged from 95.2-101.2%. No impurities of MET were detected using this highly sensitive method. Based on the results obtained. The new method was considered a reliable alternative for quality control analyses of tablets containing VIL and MET.

Table 4: Drug content determined in commercial formulations using high-performance liquid chromatography-tandem mass spectrometry

Sample	Content (%)				
	VIL	MET	MTA	MTB	MTC
Galvus®	95.2	-	-	-	-
Galvus Met® 50/500	101.2	102.0	ND	ND	ND
Galvus Met® 50/850	96.8	101.7	ND	ND	ND
Galvus Met® 50/1000	96.5	98.6	ND	ND	ND
MET manufacturer A	-	97.0	ND	ND	ND
MET manufacturer B	-	104.1	ND	ND	ND
MET manufacturer C	-	98.6	ND	ND	ND
MET manufacturer D	-	104.4	ND	ND	ND
MET manufacturer E	-	102.8	ND	ND	ND

VIL: vildagliptin; MET: metformin; MTA: MET-related compound A; MTB: MET-related compound B; MTC: MET-related compound C. ND: not detected; -: not present in the formulation.

CONCLUSION

A fast and sensitive HPLC-MS/MS method was developed for quantitative determination of VIL, MET hydrochloride, and MET-related compounds A, B, and C in tablets. Data from the validation study demonstrated that the new method was selective, linear, precise, and accurate. This novel HPLC-MS/MS method was also highly sensitive, allowing the detection of MET-related compounds at low concentrations. Analysis of real samples demonstrated the applicability of the new method. Based on these results, the new HPLC-MS/MS method could significantly improve the quality control of pharmaceutical preparations containing these drugs and can be easily applied to routine analysis.

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CONFLICT OF INTERESTS

The authors confirm that they have no conflicts of interest in relation to the contents of this article.

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