Research Article

Journal of MASS SPECTROMETRY

Received: 28 June 10 Accepted: 12 August 10 Published online in Wiley Online Library: 1 September 2010

(wileyonlinelibrary.com) DOI 10.1002/jms.1809

Development of a fast LC–MS/MS method for quantification of rilmenidine in human serum: elucidation of fragmentation pathways by HRMS

Lukáš Chytil,[a,b]* Josef Cvačka,[c] Věra Marešová,[a] Branislav Štrauch,[d] Jiří Widimský Jr,[d] Martin Štícha[e] and Ondřej Slanař[b]

Rilmenidine is an alpha 2 adrenoreceptor agonist used in the treatment of mild and moderate hypertension. In this study, a fast and accurate liquid chromatographic method with tandem mass spectrometric detection has been validated in order to assure quantification of rilmenidine in human serum. The fragmentation pathway of protonated rilmenidine was studied using high-resolution mass spectrometry (HRMS). This study compared selectivity, linearity, accuracy, precision, extraction efficiency, matrix effect and sensitivity using common liquid–liquid extraction (LLE) and solid-phase extraction (SPE) procedures. The limit of quantitation for both extraction techniques was 0.1 ng/ml. Several differences between the LLE and SPE have been observed in terms of linearity, accuracy, precision and matrix effect. Additionally, the advantages of SPE included less manual work load and increased recovery of rilmenidine in human serum to approximately 80% (LLE, 57%). The developed method involving SPE was found to be accurate (relative error (RE) <5%), reproducible (relative standard deviation, RSD <7%), robust and suitable for quantitative analysis of rilmenidine in serum samples obtained from patients under antihypertensive treatment.

Keywords: rilmenidine; LC–MS/MS; serum; quantification; HRMS

Introduction

Primary or essential hypertension relates to high blood pressure with no identifiable origin. It represents a major modifiable risk factor for the development of cardiovascular disease. If lifestyle and diet modifications are not satisfactory, medical treatment with antihypertensive drugs is required. Rilmenidine [(*N*-dicyclopropylmethyl)amino-2-oxazoline] is an alpha 2 adrenoreceptor agonist[1] effective after oral administration in the treatment of mild-to-moderate hypertension.[2,3] The hypotensive action of rilmenidine is mediated through a reduction in peripheral sympathetic tone, resulting from a central and possibly also a peripheral action. Rilmenidine also decreases catecholamine release from the adrenal medulla, which might contribute to the antihypertensive effect.

Rilmenidine is a weak base with pK_a of approximately 9.0. At physiological pH (7.4), 97% of the substance is ionized.[4] However, it is a mildly lipid-soluble compound with a true partition coefficient between octanol and water of about 20.[5] Rilmenidine is mainly used as a phosphate salt, which is freely soluble in water. Absorption of rilmenidine (1 mg) in healthy volunteers is rapid, with t_{max} ranging between 1.33 and 1.94 h[6–9] and maximal plasma concentration (c_{max}) between 3.25 and 3.97 ng/ml.[6,7,9]

To meet the requirements for preclinical, biopharmaceutical and clinical pharmacology studies, a rapid, selective, sensitive and robust analytical method is highly desirable. Very few analytical procedures, applied mainly to pharmacokinetic studies, have been reported for the determination of rilmenidine, which include gas chromatography–mass spectrometry (GC–MS)[10–14] and liquid chromatography–tandem mass spectrometry (LC–MS/MS).[9] Other methods such as LC coupled with ultraviolet or fluorescence (LC–UV/FL) detection have not been described to date. GC–MS analysis was performed using a negative chemical ionization and two-step extraction and derivatization[12–14] with the lower limit of quantification (LLOQ) of 0.1[14] and 0.2 ng/ml.[13] These methods, however, do not meet modern bioanalytical needs with respect to a simple extraction procedure or short analytical run time, as they require a prior derivatization procedure. Nowadays, LC–MS/MS is a preferred tool for quantitative bioanalysis because of its speed, selectivity and sensitivity. The LC–MS/MS method[9] was used for

* *Correspondence to: Lukáš Chytil, Institute of Forensic Medicine and Toxicology, 1st Faculty of Medicine, Charles University in Prague, Ke Karlovu 2, 120 00 Praha 2, Czech Republic. E-mail: lukas.chytil@lf1.cuni.cz*

a *Institute of Forensic Medicine and Toxicology, 1st Faculty of Medicine, Charles University in Prague, 121 08 Prague 2, Czech Republic*

b *Institute of Pharmacology, 1st Faculty of Medicine, Charles University in Prague, 121 08 Prague 2, Czech Republic*

c *Institute of Organic Chemistry and Biochemistry Academy of Sciences of the Czech Republic, v.v.i., Flemingovo nám. 2, 166 10 Prague 6, Czech Republic*

d *Third Department of Internal Medicine, 1st Faculty of Medicine, Charles University in Prague, 121 08 Prague 2, Czech Republic*

e *Department of Organic Chemistry, Faculty of Science, Charles University in Prague, 128 43 Prague 2, Czech Republic*

quantification of rilmenidine in plasma samples obtained from volunteers as a background for a bioequivalence study of two rilmenidine formulations. The aim of this published work was to demonstrate pharmacokinetics data obtained after ingestion of rilmenidine, while a detailed description of the analytical method development was not provided. However, it was reported, that the LLOQ was 0.212 ng/ml and other validation parameters were within acceptable criteria.

The objective of this study was to develop and validate a robust, sensitive and high throughput LC–MS/MS method for routine determination of rilmenidine in human serum samples. In the presented work, we have compared two extraction techniques based on a liquid–liquid extraction (LLE) and a solid-phase extraction (SPE). In both extraction procedures, all main validation parameters such a selectivity, extraction efficiency, matrix effect, linearity, sensitivity and an accuracy were investigated. We used high-resolution MS (HRMS) for the identification of product ions of rilmenidine, which are formed from protonated rilmenidine during MS/MS analysis. The LC–MS/MS assay will be useful in a rilmenidine serum concentration measurement in clinical trials as well as for therapeutic drug monitoring.

Experimental

Chemicals and reagents

Standard of pure rilmenidine phosphate (>99.0%) was kindly supplied by Farmak (Olomouc, Czech Republic). Trimipramine-d_3 (99.0%), which was used as internal standard (IS), was obtained from Alltech (Prague, Czech Republic). Formic acid (p.a.), ammonium formate (p.a.) and acetonitrile (HPLC grade) were purchased from Sigma Aldrich (Steinheim, Germany) and *tert*-butyl methyl ether (TBME) (pure) was obtained form Merck (Darmstad, Germany). Deionized water (DI) was produced in-house using a Milli-Q system from Millipore (Bedford, MA, USA). Bond Elut Certify 50 SPE column was purchased from Varian (Palo Alto, CA, USA).

Sample preparation

Liquid–liquid extraction

Serum samples (1 ml) were diluted with 1 ml of borate buffer (pH 9.5). After addition of 10 µl of a methanolic solution of the IS containing 2 µg/ml of trimipramine-d_3 and an appropriate amount of rilmenidine methanolic solution, the samples were mixed for 10 s on a rotary shaker and consequently extracted with 4 ml of TBME. After centrifugation (4000 *g* for 8 min), the organic phase was separated, acidified with 100 µl 1% HCl in methanol (v/v) and evaporated to dryness under a stream of nitrogen at 40 °C. The residue was dissolved in 100 µl of LC mobile phase, and a 5-µl aliquot was injected into the chromatographic system.

Solid-phase extraction

The Bond Elut Certify column was conditioned with 1 ml of methanol, followed by 1 ml of DI water. A mixture of 1 ml of centrifuged serum (4000 *g* for 8 min), 1 ml of 0.1 M phosphate buffer (pH 6.0), and 10 µl of IS working solution (2 µg/ml) was applied to the column. The sample was forced through the bed at a low flow rate by vacuum. The column was subsequently washed with 1 ml of water, 0.5 ml of 0.1 M HCl, 1 ml of methanol/water solution (50 : 50, v/v) and dried for 5 min under vacuum. Analytes were eluted by 2 ml of mixture containing isopropanol, dichloromethane and ammonium hydroxide (80 : 20 : 2, v/v/v). The extract was evaporated to dryness under a stream of nitrogen at 40 °C after addition of 100 µl 1% HCl in methanol (v/v). The residue was redissolved in 100 µl of mobile phase and 5 µL was injected into the chromatographic system.

Liquid chromatography–mass spectrometry

Apparatus

The chromatographic separation was performed on a 1200 rapid resolution LC (RRLC) Agilent (Waldbronn, Germany), consisting of a degasser, binary pump, autosampler and thermostatted column compartment. The MS/MS analysis was performed using a 3200 Q trap triple quadrupole/linear ion trap mass spectrometer with a TurboIonSpray source (MDS Sciex, Ontario Canada). For data analysis was used Analyst software version 1.5.1.

LC conditions

Chromatographic separation was achieved with an Agilent Zorbax Eclipse XBD–C18 column (1.8 µm, 50 × 4.6 mm I.D.), protected by a C18 security guard cartridge (4 × 2 mm I.D.). Isocratic elution occurred with (A) 5 mM ammonium formate with 0.02% formic acid and (B) 0.02% formic acid in acetonitrile (50 : 50, v/v) at a flow rate of 1.15 ml/min. The mobile phase was thermostatted at 30 ± 0.5 °C.

MS/MS conditions

The mass spectrometer was operated in the positive TurboIonSpray mode and selected reaction monitoring (SRM) was used for data acquisition of rilmenidine and IS. The following transitions were monitored: *m/z* 181.1 → 95.1 and 67.1 for rilmenidine and *m/z* 298.2 → 103.1 for IS. The more abundant product ion (*m/z* 95.1) was used for quantification. The MS parameters for the analysis were as follows: ion source temperature 550 °C; ion-spray voltage 2000 V; nebulizer gas 40 psi; auxiliary gas 40 psi; curtain gas 30 psi and medium collision gas. Conditions of mass spectrometric detection were optimized by direct infusion of standard solutions into the MS. The compound-dependent parameter settings are listed in Table 1.

Table 1. Parameters of MS detection for rilmenidine and trimipramine-d_3

	SRM transitions	Dwell time (ms)	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Collision entrance potential (V)	Cell exit potential (V)	Ion ratio, SRM2/SRM1
Rilmenidine	181.1 → 95.1	75	23	6	19	15	3	0.58
	181.1 → 67.1	75	23	6.5	28	15	3	–
Trimipramine-d_3	298.2 → 103.1	75	40	5	25	15	2.8	–

Journal of MASS SPECTROMETRY

High-resolution mass spectrometry

High-resolution exact mass MS/MS spectra were obtained with an LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ion (ESI) source. The spectrometer was operated in the positive ion mode with a mass resolving power of 100 000. The MS/MS experiments were performed in CID and HCD mode with normalized collision energy of 30 and 40%, respectively. Nitrogen was used as sheath/auxiliary gas (35 a.u./5 a.u.) and helium served as the collision gas. The mobile phase was delivered using a Rheos Allegro UHPLC pump (Flux Instruments AG, Switzerland) and consisted of methanol/water (1 : 1), at a flow rate of 50 µl/min. The sample was diluted with the mobile phase and injected using a 2-µl loop. Spray voltage, capillary voltage, tube lens voltage and capillary temperature were 4.3 kV, 40 V, 155 V and 275 °C, respectively. The mass spectra were internally calibrated using sodium adduct of dibutyl pthalate (*m/z* 301.14103) to provide high-accuracy mass measurements within 2.0 ppm.

Assay validation for quantification of rilmenidine in human serum

The following parameters were evaluated for the validation of the LC–MS/MS method for the determination of rilmenidine in human serum: selectivity, sensitivity (limits of detection and quantification), linearity, precision, accuracy, extraction recovery, matrix effect and stability.

Standard working solutions

Standard solutions (200 µg/ml) of rilmenidine or IS were prepared in methanol. Working solutions at rilmenidine concentrations of 2, 0.2 and 0.02 µg/ml were prepared by diluting with mixture of methanol and water (50 : 50, v/v). The IS working solution was prepared by diluting with methanol at the final concentration of 2 µg/ml.

Calibration samples and quality control samples

Blood samples obtained from drug-free volunteers were centrifuged (4000 *g* for 5 min) and the serum was stored at −20 °C prior to analysis. Appropriate amounts of working solutions were added to 1 ml of drug-free human serum to create calibration samples at final concentrations of 0.1, 0.5, 1.5, 3, 6 and 12 ng/ml of rilmenidine (free base). Quality control (QC) samples at concentrations of 0.4 (QC1), 4 (QC2) and 8 (QC3) ng/ml of rilmenidine were prepared daily.

Selectivity

The method selectivity was assessed by analyzing six different lots of pooled blank human serum and by comparing them with spiked serum samples at concentration near to the lowest point of calibration curve. A zero sample (blank sample with IS) was analyzed to check for the absence of ions of the IS in the respective peaks to rilmenidine. Additionally, serum samples from patients receiving other medications frequently used for therapy of cardiovascular diseases (amlodipine, betaxolol, bisoprolol, hydrochlorothiazide, losartan, metoprolol, perindopril, ramipril and telmisartan) were tested to exclude possible interferences.

Linearity

The peak-area ratio of rilmenidine/IS was measured and plotted against the theoretical concentration of the spiked standards. A six-point calibration curve was constructed over the whole concentration range (0.1–12 ng/ml) with a weighting factor of $1/x$. Least square linear regression analysis was performed to determine slopes, intercepts and correlation coefficients (R^2 required to be $\geq$0.99). Replicates ($n = 6$) at each concentration level were prepared by both extraction techniques as described above and the obtained results were compared. The calibration range was defined considering the normal therapeutic concentrations obtained from previously published works.[5,6,9] The working range was extended in comparison to the methods applied in studies with healthy volunteers since overdosed patients are likely to be monitored in real clinical settings.

Accuracy and precision

Accuracy, intra- and interday precision for rilmenidine were evaluated according to the requirements of FDA guidelines on bioanalytical method validation.[15] However, the QC1 level was greater than 3 × LLOQ, which was more suitable for the application of this method in clinical settings with drug levels expected to be higher than in samples from single-dose bioavailability studies. Intraday variation was assessed by six replicate determinations of three concentrations (QC1, QC2, and QC3) over the tested range. Intraday accuracies were expressed as the mean of assays relative to the exact value. The intraday precision of the method was calculated as the relative standard deviation (RSD) of the assay made for intraday accuracy. Interday variation was determined by analyzing replicates of QC samples with the same concentrations for 3 days. Accuracies were calculated as the mean of the assays relative to the nominal value. The interday precision of this method was expresses as the RSD of the assays made for interday accuracy.

Extraction efficiency and matrix effect

Peak areas obtained from QC serum samples and those found by direct injection of mobile phase solutions at the same concentration levels were compared to the calculated extraction efficiency of analyzed compounds. The matrix effect was calculated according to the method by Matuszewski *et al.*[16] as peak areas of the samples spiked after extraction procedure divided by the corresponding areas of the standard solutions dissolved in serum before extraction. Recovery and matrix effect experiments were performed for five different lots of human serum at three concentration levels (QC) of rilmenidine and at one concentration level of IS. Obtained values were converted to a percentage and subtracted from 100 to represent the amount of signal suppressed or enhanced by the presence of matrix.

Limits

The LLOQ refers to the lowest concentration of each compound in human serum, that can be analyzed quantitatively by the LC–MS/MS method with precision less than or equal to 20% and accuracy within 80–120% ($n = 6$). The limit of detection (LOD) is the lowest concentration with a signal to noise ratio higher than 3 : 1. Both parameters were empirically evaluated by analyzing samples with low concentrations of analytes.

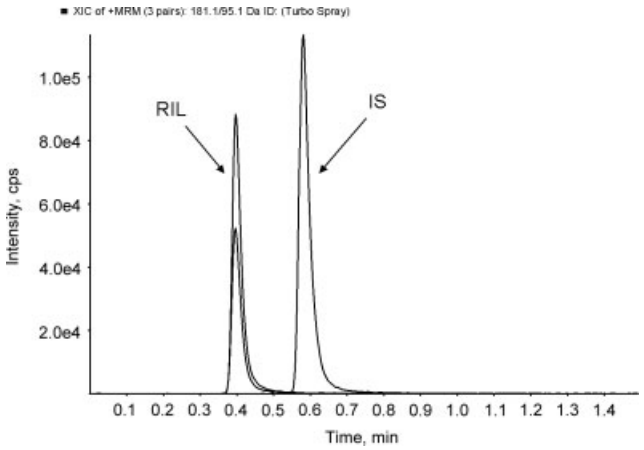

Figure 1. SRM chromatograms obtained after SPE of serum samples collected in a period of 2 h after an oral dose of rilmenidine in dose of 1 mg (3.51 ng/ml).

Stability

Stability was evaluated with human serum fortified with rilmenidine at the three QC concentrations ($n = 6$). Short-term temperature stability was tested during the whole working day at room temperature, at 4 °C for 10 days and at −20 °C for 1 month. QC samples were kept at these storage conditions and calculated concentrations of stability specimens were compared to QC samples prepared freshly on the day of analysis.

Results and Discussion

LC–MS method development

Separation of rilmenidine and IS was performed under acidic conditions. Chromatographic behavior of both analytes on an octadecyl reversed phase packing material was suitable. No extensive tailing was observed during the chromatographic method development. Different ratios of acetonitrile and solution A, temperature and flow rate were tested for obtaining good chromatographic separation. A higher content of acetonitrile (≥90%) resulted in insufficient analytes separation and stability of retention times. Additionally, a high content of acetonitrile gave rise to insufficient response of the analytical system, while a higher content of solution A (≥70%) induced a significantly longer chromatographic run. Finally, using a mobile phase with 50% fraction of acetonitrile provided the best separation and a good response of MS detector was obtained. The optimal flow rate of mobile phase was found to be 1.15 ml/min. The tested column oven temperature changes (20–50 °C) did not substantially affect the time of analysis or response of the MS detector, while the best peak sharpness was observed using 30 °C. Stability of the chromatographic method was evaluated by calculating retention time variability. RSD for retention times was lower than 0.17% for both analytes over 20 consecutive injections. Analyte and IS were eluted within 0.7 min with a total chromatographic run time of 1.5 min (Fig. 1).

The conditions of MS detection were optimized for maximum product ion formation by direct infusion of the single compound of interest. Product spectra of protonated molecule $[M + H]^+$ of rilmenidine and IS are displayed in Fig. 2. For rilmenidine two SRMs were set up, one for quantification and one as a qualifier using one precursor ion and two product ions per compound as shown in Table 1. The ratios of signal intensities of SRM1 and SRM2 and retention time deviations were used for analyte identification in analyzed unknown samples. The RSD of SRM ratios were lower than 2.82% over the whole working range. The transition m/z 298.2 → 103.1 was used for the SRM analysis of trimipramine-d_3. Formation of the most abundant fragment ion m/z 103.1 was analogical to the formation of m/z 101.1 in unlabelled trimipramine.[17,18]

Accurate mass measurement of the product ions of rilmenidine

The fragmentation of protonated rilmenidine was studied using HRMS. The most intense fragment at m/z 95.1 was found to be $C_7H_{11}^+$ (95.08549, −0.4 ppm) and formally explained by elimination of neutral molecule of heterocyclic amine $C_3H_6N_2O$. The ion at m/z 93.1 is $C_7H_9^+$ (93.06985, −0.3 ppm) was formed by loss of $C_3H_8N_2O$. The second most intense signal observed at m/z 67.1 was $C_5H_7^+$ (67.05417, −0.8 ppm) probably originated from $C_7H_{11}^+$ after elimination of ethylene. The loss of C_3H_6 from the same ion gives m/z 55.1 ($C_4H_7^+$; 55.05411, −2.1 ppm). The less abundant ions contain heterocyclic moiety; m/z 99.1 corresponding to $C_4H_7N_2O^+$ (99.05525, −0.4 ppm), m/z 87.1 representing $C_3H_7N_2O^+$ (87.05529, 0.1 ppm). The chemical structure and fragmentation pathway of rilmenidine are displayed in Fig. 3.

Sample preparation

The determination of extraction efficiencies and matrix effects is a major part of bioanalytical LC–MS/MS method validation procedure. For analysis of medications in biological matrices, sample clean up is used to reduce possible ion enhancement or suppression. Serum concentration of rilmenidine is very low, which can lead to trouble during routine clinical analysis. To avoid potential problems with sensitivity it was necessary to find a very effective extraction technique.

Different buffers for alkalization of serum samples and different solvents were tested during optimization of LLE for rilmenidine. The most common buffers used for the extraction of basic drugs are Sörensen's buffer,[19] TRIS buffer[20] and borate buffer[21] and the most frequent organic solvents used for the LLE of drugs are TBME,[22] butyl acetate[23] and dichloromethane.[24] In the initial experiment, rilmenidine (1 ng/ml) was extracted from human serum, using nine different combinations of solvents and buffers. LLE using borate buffer and TBME showed the best results and, therefore, this method was chosen for further comparison of LLE and SPE. Chromatograms obtained by analyzing serum samples extracts from various pHs are displayed in Fig. 4. Similar to the optimization of LLE, different buffers for pH adjustment and various compositions of elution solvents were examined during SPE method development. The best extraction efficiency was observed using the phosphate buffer (pH 6.0), which was also recommended by the column manufacturer. In solvent testing, comparable results were obtained using a mixture of isopropanol, dichloromethane, ammonium hydroxide (80 : 20 : 2, v/v/v) and ethyl acetate, ammonium hydroxide (98 : 2, v/v) for elution of rilmenidine and IS. However, reproducible recoveries were obtained using isopropanolic solution only.

Assay validation

Selectivity

The selectivity of the method was tested by comparing the chromatograms of six different lots of blank human serum. All

Journal of MASS SPECTROMETRY

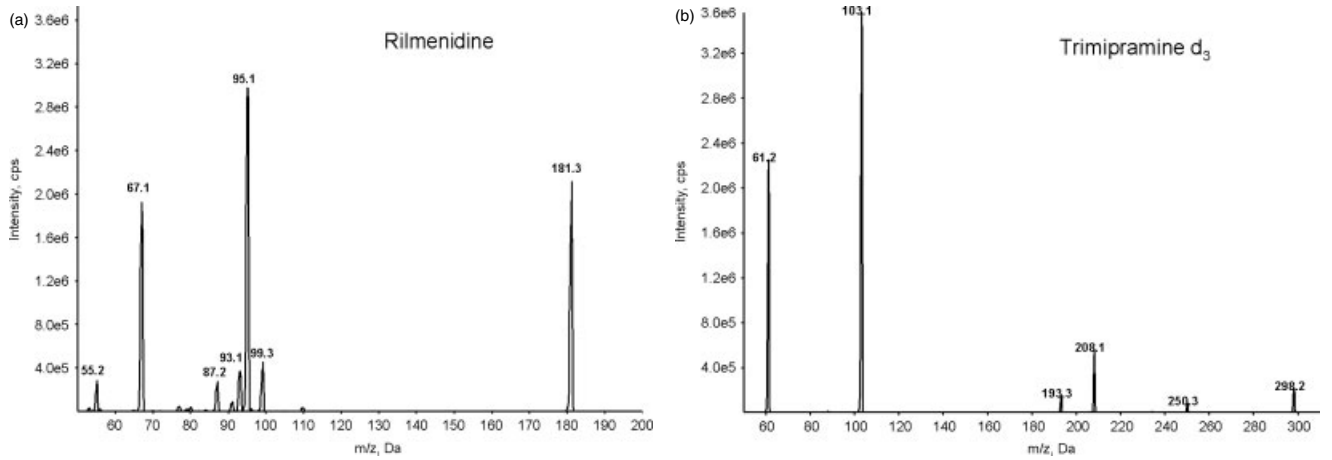

Figure 2. Product ion spectra of $[M + H]^+$ of (a) rilmenidine and (b) trimipramine-d_3, the internal standard.

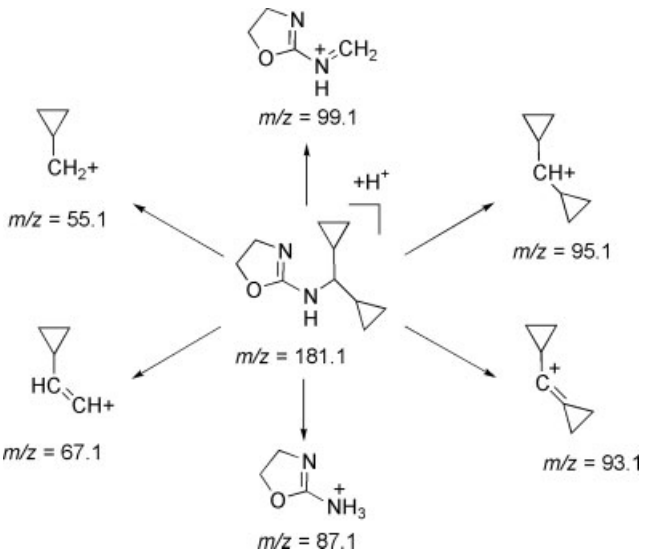

Figure 3. Chemical structure and fragmentation pathways proposed for rilmenidine.

blank serum samples, prepared by both extraction techniques, were found to be free of interferences with respect to both SRM transitions. Analysis of zero samples (blank plus IS) gave no indication of possible interferences from IS. Additionally, no interfering peaks to rilmenidine and IS were found with potentially interfering compounds.

Linearity

Replicates ($n = 6$) of matrix calibrator at six different concentrations from 0.1 to 12 ng/ml were extracted using both described method and consequently analyzed. The back-calculated concentrations of all calibrators were compared with their respective nominal values. If the LLE was used for extraction, it was necessary to exclude some calibrators, whose back-calculated concentrations deviated more than ±15% (±20 LLOQ). Back calculations of the calibrators extracted using SPE were more accurate and the deviation did not exceed 9.8%. The parameters of the calibration curve equations obtained after analysis and computations of calibrators extracted through both methods are listed in Table 2.

Table 2. Comparison of slopes, intercepts and correlations coefficients of calibrations curves obtained after LC–MS/MS analysis of calibrators prepared by two different extraction methods

	a	*b*	R^2	*a*	*b*	R^2
$n = 6$		SPE			LLE	
Mean	1.267	−0.0038	0.9992	0.848	0.0065	0.9972
SD	0.026	0.0011	0.0002	0.077	0.0063	0.002
RSD (%)	2.07	–	–	9.08	–	–

a, slope; *b*, intercept; R^2, correlation coefficient; SD, standard deviation; RSD, relative standard deviation.

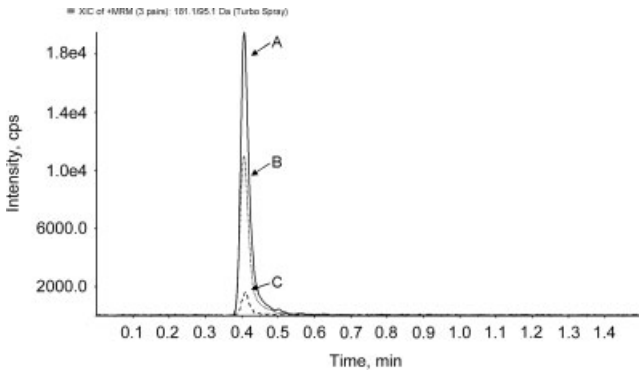

Figure 4. SRM chromatograms of rilmenidine extracted from spiked human serum (1 ng/ml) using liquid–liquid extraction with *tert*-butyl methyl ether. Alkalinization was performed by adding 1 ml of Sörensen's buffer (pH 7.2; A), 1 ml of TRIS buffer (pH 8.1; B) and 1 ml of borate buffer (pH 9.5; C).

Accuracy and precision

QC samples at low (QC1), medium (QC2) and high (QC3) concentration levels were analyzed and calculated on the basis of a freshly prepared calibration curve. QCs and calibrators were prepared using both extraction procedures. The results are summarized in Table 3. The criteria for precision (≤15%) were fulfilled for both tested extraction techniques at each concentration level. However, in the case of LLE, the deviations were significantly higher than for SPE. The data for accuracy were within the

Table 3. Inter- and intraday variation data from the determination of rilmenidine in human serum

	QC1		QC2		QC3	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Intraday variation						
LLE	102.4	7.5	105.4	6.5	106.6	3.9
SPE	104.4	2.5	97.0	5.8	97.7	1.3
Interday variation						
LLE	92.8	9.9	108.7	6.7	117.9	7.0
SPE	96.6	2.9	97.3	6.9	99.3	2.4

QC, quality control; LLE, liquid–liquid extraction; SPE, solid-phase extraction.

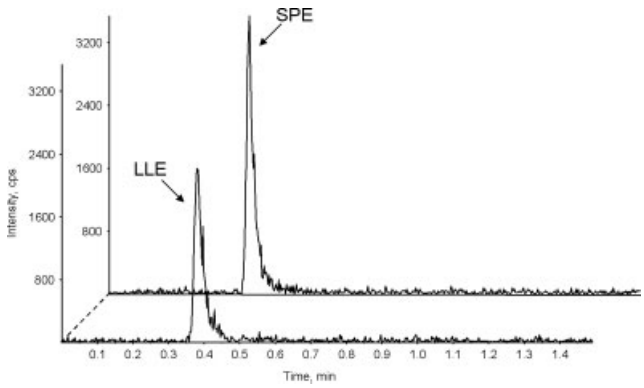

Figure 5. SRM mass chromatograms obtained after analysis of a spiked serum sample containing 0.1 ng/ml (LLOQ) of rilmenidine (*m/z* 181.1 → 95.1).

acceptance limit (±15% of the nominal value), with the exceptions of LLE at the high concentration level. Better results obtained after SPE extraction are probably due to the smaller amount of organic solvent that must be evaporated during preparation.

Extraction efficiency and matrix effect

Generally, solvent selection and evaporation are crucial steps in LLE. LLE requires a large volume of organic solvents that must be evaporated with caution because of suspect volatility of small molecules as rilmenidine. In order to prevent rilmenidine volatility, hydrochloric acid was added to form rilmenidine salt prior to evaporation. The detailed description of LLE optimization is summarized above. SPE simplified specimen preparation and reduced extraction time and solvent consumption compared to LLE. Mean extraction efficiency of rilmenidine at three QC concentrations was higher than 57.1 and 76.0% for LLE and SPE, respectively. The recovery of IS at one concentration level (20 ng/ml) reached 81.0 and 71.1% for LLE and SPE, respectively. The matrix effect was observed regardless of applied extraction technique. The ion enhancement less than 34.8% (<13% RSD) was calculated for LLE, while a level of the ion suppression for SPE of rilmenidine was better than 19.9% (<8% RSD). The ion suppression for IS was better than 17.2% (10% RSD) and 13.4% (5% RSD) for LLE and SPE, respectively. Pretreatment of serum samples by SPE seems to be more functional to minimize the matrix effect and increase extraction efficiency of rilmenidine (Table 4).

Table 4. Comparison of recovery and matrix effect for rilmenidine extracted from human serum using LLE and SPE

	Recovery (%, $n = 5$)			Matrix effect (%, $n = 5$, RSD)		
	QC1	QC2	QC3	QC1 (%)	QC2 (%)	QC3 (%)
LLE	66.1	57.1	71.9	113.2 (13)	134.8 (11)	118.2 (9)
SPE	80.3	76.0	88.4	84.9 (5)	80.1 (5)	94.0 (8)

RSD, relative standard deviation; LLE, liquid–liquid extraction; SPE, solid-phase extraction.

Limits

Computations of LLOQ and LOD were conducted for both extraction techniques. Criteria for LLOQ were fulfilled by the lowest point of the calibration curves, while the empirical determination of LOD with decreasing concentrations of analyte resulted in LOD values of 0.02 and 0.04 ng/ml for SPE and LLE, respectively. Chromatograms of spiked serum sample containing 0.1 ng/ml of rilmenidine obtained after LLE and SPE are presented in Fig. 5.

Stability

Rilmenidine was found to be relatively stable under different storage conditions. The variations in concentrations were within ±9.6% of values obtained from the freshly prepared samples. Thus, storage of serum samples under above-mentioned laboratory conditions was not critical for this method.

Application of the method

The developed assay has been successfully applied to serum samples obtained from five patients under treatment with rilmenidine (1 mg once a day ($n = 3$) and twice daily ($n = 2$)). All the patients were using co-medication of diuretics, statins and/or ACE inhibitors. The blood samples were taken between 2 and 3 h post-dose and at 12 h post-dose in patients using rilmenidine in once a day regime. The samples were transferred into venous blood collection tubes (4 ml, BD Vacutainer, Heidelberg, Germany) containing no additives or gels for serum separations. The concentrations at time near to c_{max} were 3.51 and 3.59 ng/ml in patients using 1 mg per day while ranging between 6.70 and 7.20 ng/ml in patients using 1 mg of rilmenidine twice daily. These values correspond to previously reported c_{max} levels in healthy volunteers[6,7,9] and also demonstrate a linear, dose-dependent increase of drug concentration in the serum. The drug

Journal of MASS SPECTROMETRY

concentrations of the two patients at 12 h post-dose were 0.33 and 0.39 ng/ml, respectively. The representative chromatogram obtained after analysis of serum samples is displayed in Fig. 1.

Conclusion

A novel analytical procedure based on LC–MS/MS is a suitable and valid method for the determination of rilmenidine in serum samples obtained from patients under a treatment with the drug. This LC–MS/MS method, which is described for the first time, is fast, accurate, sensitive and applicable in clinical practice. It was shown to be selective without interferences from the endogenous compounds and co-administered drugs. Matrix effect in terms of ion enhancement or suppression was investigated in this work and seems to be insignificant. Interpretation of the product ion spectra and their correctly structural assignment was performed using HRMS. Application of the method in real samples showed rilmenidine concentrations in line with known pharmacokinetic characteristics of the drug and also demonstrated the ability to determine rilmenidine dose-dependent increase of c_{max} levels.

Acknowledgements

The authors thank Dr Přemysl Indrák (Farmak Olomouc, Czech Republic) for his kind donation of analytical standard of rilmenidine. The authors gratefully acknowledge the technical assistance of Mrs Alena Kopecká. This work was supported by the Academy of Sciences of the Czech Republic (Project No Z4 055 0506) and Czech Ministry of Education (VZ MSMT No 0021620849).

References

[1] P. A. Van Zwieten. Pharmacology of the alpha 2-adrenoceptor agonist rilmenidine. *Am. J. Cardiol.* **1988**, *61*, 6D.
[2] K. Weerasuriya, E. Shaw, P. Turner. Preliminary clinical pharmacological studies of s3341, a new hypotensive agent, and comparison with clonidine in normal males. *Eur. J. Clin. Pharmacol.* **1984**, *27*, 281.
[3] J. P. Fillastre, B. Letac, F. Galinier, G. Le Bihan, J. Schwartz. A multicenter double-blind comparative study of rilmenidine and clonidine in 333 hypertensive patients. *Am. J. Cardiol.* **1988**, *61*, 81D.
[4] M. Remko, M. Swart, F. M. Bickelhaupt. Theoretical study of structure, pka, lipophilicity, solubility, absorption, and polar surface area of some centrally acting antihypertensives. *Bioorg. Med. Chem.* **2006**, *14*, 1715.
[5] P. Genissel, N. Bromet. Pharmacokinetics of rilmenidine. *Am. J. Med.* **1989**, *87*, 18S.
[6] M. Aparicio, M. Dratwa, N. el Esper, J. P. Fillastre, B. Levaltier, R. Lins, A. Meyrier, F. Mignon, J. P. Ryckelynck, J. Sennesael. Pharmacokinetics of rilmenidine in patients with chronic renal insufficiency and in hemodialysis patients. *Am. J. Cardiol.* **1994**, *74*, 43A.
[7] J. L. Reid, V. Panfilov, G. MacPhee, H. L. Elliott. Clinical pharmacology of drugs acting on imidazoline and adrenergic receptors. Studies with clonidine, moxonidine, rilmenidine, and atenolol. *Ann. N. Y. Acad. Sci.* **1995**, *763*, 673.
[8] J. L. Reid. Update on rilmenidine: clinical benefits. *Am. J. Hypertens.* **2001**, *14*, 322S.
[9] G. Groenewoud, L. Potgieter, B. Meyer. Bioequivalence evaluation of rilmenidine in healthy volunteers. *Arzneimittelforschung* **2009**, *59*, 233.
[10] R. L. Lins, R. Daelemans, M. Dratwa, D. Verbeelen, J. Sennesael, B. Brisgand, N. Lameire. Acceptability of rilmenidine and long-term surveillance of plasma concentrations in hypertensive patients with renal insufficiency. *Am. J. Med.* **1989**, *87*, 41S.
[11] C. T. Dollery, D. S. Davies, J. Duchier, B. Pannier, M. E. Safar. Dose and concentration-effect relations for rilmenidine. *Am. J. Cardiol.* **1988**, *61*, 60D.
[12] J. D. Ehrhardt. Gas chromatographic negative ion mass spectrometric assay of 2-dicyclopropylmethylamino-2-oxazoline (s-3341), a new antihypertensive drug. *Biomed. Mass Spectrom.* **1985**, *12*, 593.
[13] H. L. Ung, J. Girault, M. A. Lefebvre, A. Mignot, J. B. Fourtillan. Quantitative analysis of s3341 in human plasma and urine by combined gas chromatography-negative ion chemical ionization mass spectrometry: 15 month inter-day precision and accuracy validation. *Biomed. Environ. Mass Spectrom.* **1987**, *14*, 289.
[14] S. Murray, D. Watson, D. S. Davies. Bistrifluoromethylaryl derivatives for drug analysis by gas chromatography electron capture negative ion chemical ionization mass spectrometry. Application to the measurement of (n-dicyclopropylmethyl)amino-2-oxazoline in plasma. *Biomed. Mass Spectrom.* **1985**, *12*, 230.
[15.] Food and Drug Administration. *FDA Guidance for Industry: Bioanalytical Method Validation. US Department of Health and Human Services*, Food and Drug Administration, Center for Drug Evaluation and Research: **2001**.
[16] B. K. Matuszewski, M. L. Constanzer, C. M. Chavez-Eng. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal. Chem.* **2003**, *75*, 3019.
[17] S. McClean, E. J. O'Kane, W. F. Smyth. Electrospray ionisation-mass spectrometric characterisation of selected anti-psychotic drugs and their detection and determination in human hair samples by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B. Biomed. Sci. Appl.* **2000**, *740*, 141.
[18] B. Doherty, V. Rodriguez, J. C. Leslie, S. McClean, W. F. Smyth. An electrospray ionisation tandem mass spectrometric investigation of selected psychoactive pharmaceuticals and its application in drug and metabolite profiling by liquid chromatography/electrospray ionisation tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2031.
[19] S. St-Hilaire, P. M. Belanger. Simultaneous determinations of tolbutamide and its hydroxy and carboxy metabolites in serum and urine: application to pharmacokinetic studies of tolbutamide in the rat. *J. Pharm. Sci.* **1989**, *78*, 863.
[20] E. Saar, D. Gerostamoulos, O. H. Drummer, J. Beyer. Comparison of extraction efficiencies and LC-MS-MS matrix effects using LLE and SPE methods for 19 antipsychotics in human blood. *Anal. Bioanal. Chem.* **2009**, *393*, 727.
[21] S. Wenzel, R. Aderjan, R. Mattern, I. Pedal, G. Skopp. Tissue distribution of mirtazapine and desmethylmirtazapine in a case of mirtazapine poisoning. *Forensic Sci. Int.* **2006**, *156*, 229.
[22] M. Polinko, K. Riffel, H. Song, M. W. Lo. Simultaneous determination of losartan and exp3174 in human plasma and urine utilizing liquid chromatography/tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2003**, *33*, 73.
[23] S. S. Johansen, J. L. Jensen. Liquid chromatography-tandem mass spectrometry determination of LSD, iso-LSD, and the main metabolite 2-oxo-3-hydroxy-LSD in forensic samples and application in a forensic case. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **2005**, *825*, 21.
[24] S. Mennickent, R. Fierro, M. Vega, M. de Diego, C. G. Godoy. Instrumental planar chromatographic method for determination of carbamazepine in human serum. *J. Sep. Sci.* **2009**, *32*, 1454.

