RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Hydrophilic interaction chromatography-tandem mass spectrometry based on an amide column for the high-throughput quantification of metformin in rat plasma

Rong Shi, Xining Xu, Jiasheng Wu, Tianming Wang, Yuanyuan Li, Bingliang Ma, Yueming Ma*

Department of Pharmacology, Shanghai University of Traditional Chinese Medicine, Shanghai, China

* Correspondence to: Professor Yueming Ma (Tel: +86-21-51322386, Fax: +86-21-51322386, E-mail: mayueming_117@hotmail.com)

Abstract

Metformin, a biguanide derivative, is the most commonly prescribed medication in the treatment of type 2 diabetes mellitus, among other diseases. Because it is highly polar, determining the concentration of metformin using reversed-phase liquid chromatography is often very challenging. Here, we demonstrate the utility of a novel hydrophilic interaction liquid chromatography method that is based on an amide column with tandem mass spectrometry. Chromatographic separation was achieved using an ACQUITY UPLC bridged ethyl-siloxane/silica hybrid amide column (2.1 × 100 mm, 1.7 μm). The isocratic mobile phase consisted of water and acetonitrile (v/v, 1:9), which both contained 0.06% formic acid and 5 mM ammonium formate at a flow rate of 0.5 mL/min. Data from validation experiments demonstrated that this new method is highly selective, sensitive (2.0 pg on column), and free of matrix and residual effects. The method was also precise (relative standard deviation of <10.1%), accurate (96.9–105.7%), and linear ($r \geq 0.995$) over the ranges of 2–1024 ng/mL for metformin. The developed method was successfully applied to determine the metformin level in the plasma of rats that received a single dose of metformin (100 mg/kg). Thus, this new method can be used as a tool for the clinical monitoring of metformin and for evaluating drug-drug interactions.

Key words: metformin, Amide-HILIC-MS/MS, plasma, pharmacokinetics

Introduction

Reversed-phase liquid chromatography (RPLC) is a powerful and versatile technique that utilizes C_{18}-based silica stationary phases to separate a variety of different compounds. However, using RPLC to separate highly polar analytes and metabolites is often very challenging because of the high matrix effects and the lower sensitivity of the mass spectrometric detection system.[1] Hydrophilic interaction liquid chromatography (HILIC) is a promising alternative to normal-phase liquid chromatography and RPLC for separating polar compounds, as it has a polar stationary phase[1-3]; however, the mobile phase used in HILIC is similar to the phases employed in RPLC.[1, 4, 5] In HILIC, the elution of compounds from the stationary phase is achieved in the order of increasing hydrophilicity. Recently, interest in

using HILIC along with tandem mass spectrometry (HILIC-MS/MS) has increased, particularly for separating peptides,[1, 6] nucleosides,[5] neurotransmitters,[6] polar contaminants in food and environmental samples,[7] pharmaceuticals,[8] and many other compounds that contribute to the development of medicinal chemistry, molecular biochemistry, and metabolomics.[2, 7, 9]

Metformin (Fig. 1A), an oral hypoglycemic agent, is currently the first choice or "gold standard" drug for treating type 2 diabetes and polycystic ovary disease.[10, 11] Moreover, metformin affords protection against diabetes-induced vascular disease and provides therapeutic benefits to patients with several forms of cancer.[11, 12] On the other hand, metformin, a substrate of organic cation transporters and multidrug and toxin extrusion transporters, is used as a transporter probe drug cocktail for assessing transporter-based drug-drug interactions in clinical proposals.[13] Thus, determining the plasma concentration of metformin is important for pharmacokinetic studies, therapeutic drug monitoring, and the optimization of dosing and dosing regimens in antidiabetic therapies.

Several RPLC methods with different detectors, including ultra-violet[14] and tandem mass spectrometry,[15-26] have been developed and used for determining the concentration of metformin in biological samples. Given the high polarity and poor retention of metformin on C_8 and C_{18} reversed-phase columns, highly aqueous mobile phases were used for the analyte retention. Although some studies obtained good sensitivity (0.8–2 ng/mL), complicated evaporation and reconstitution procedures during sample preparation were needed.[17, 19] Other reports exhibited low sensitivity, with the lower limit of quantification (LLOQ) ranging from 4 to 50 ng/mL[14, 15, 21-25]; additionally, these methods required large sample volumes[18, 20] and long run times.[26] Normal-phase,[27] ion-pair,[28-30] and cation-exchange[27] methods have also been used for determining the concentration of metformin, but long run times and poor sensitivity limited the application of these methods.

Recently, a few studies described an HILIC method for determining the metformin concentration in samples.[3, 31-35] However, while some of the published reports exhibited poor sensitivity,[3, 31, 32, 34, 35] others had long run times, troublesome sample preparation methods,[34] or required large sample volumes.[32-35] Additionally, in these previous methods, bare silica and diol silica were used as stationary phases. According to the characteristics of stationary

phases, the strong electrostatic attraction presented by bare silica can cause residual interactions and peak asymmetry in HILIC, thus reducing the separation efficiency.[15, 36] On the other hand, diol columns may slowly release the bonded phase under acidic conditions.[36] Such problems, including column stability, lifetime of the column, and separation reproducibility, can affect the application of these methods.[9]

Bridged ethyl-siloxane/silica hybrid (BEH) particles that are bonded to the high polarity amide groups (BEH Amide) in HILIC can further improve the chemical stability of the stationary phases. Moreover, the BEH Amide column showed a wide pH range and good efficiency and reproducibility,[9, 37] and thus is recommended for efficient and fast separations of highly polar samples.[36] However, few studies have determined the metformin concentration using a HILIC-MS/MS system that is based on a BEH Amide column (Amide-HILIC-MS/MS). Therefore, the aim of the present work was to develop a reliable method for determining the plasma metformin concentration by using Amide-HILIC-MS/MS with an atmospheric chemical pressure ionization (APCI) source. The proposed method has been shown to be potentially useful for the sensitive and quick analysis of metformin from biological matrices.

Experimental

Reagents and materials

Metformin was purchased from the Chinese Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Tetraethylammonium was supplied by Sigma Chemical Co. (St. Louis, MO, USA). The purities of all reference compounds were greater than 98% according to high-performance liquid chromatography (HPLC) analysis. Acetonitrile was of HPLC grade and was purchased from Burdick & Jackson Company (Ulsan, Korea). Ammonium formate and ammonium acetate (HPLC grade) were obtained from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Formic acid and acetic acid (HPLC grade) were purchased from CNW Technologies GmbH Company (Düsseldorf, Germany). Acetic acid (HPLC grade) was purchased from Tedia Company (Fairfield, USA). Ultra-pure water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents were of analytical grade.

Animals

Sprague-Dawley rats of both sexes weighing 230 ± 20 g (Certificate No. SCXK 2012-0002) were provided by the Animal Center of Shanghai University of Traditional Chinese Medicine. They were maintained on a 12-h light-dark cycle in an environmentally controlled breeding room (temperature was 22–24°C, humidity was 50 ± 10%) for 7 days. Animals were fasted for 12 h prior to experimentation, but continued to have free access to water during this time. Animal studies were conducted according to the institute's Guide for the Care and Use of Laboratory Animals and had been approved by the institutional committee of Shanghai University of Traditional Chinese Medicine.

Chromatography and MS conditions

The chromatographic analysis was performed on a Waters ACQUITY^{TM} system (Milford, MA, USA) and a triple-quadrupole mass spectrometer (API 5500, Applied Biosystems, CA, USA) equipped with an APCI source. Chromatographic separation was performed on an ACQUITY UPLC BEH Amide (2.1 × 100 mm, 1.7 μm) column that was maintained at 40°C. The isocratic mobile phase consisted of water and acetonitrile (v/v, 1:9), which both contained 0.06% formic acid and 5 mM ammonium formate (pH 3) at a flow rate of 0.5 mL/min. The ion spray voltage was set to 5 kV in the positive ionization mode. The entrance potential was 10 V and the source temperature was set at 280°C. Nitrogen was used as the nebulizer gas (50 psi), auxiliary gas (50 psi), and curtain gas (40 psi). The selected reaction monitoring mode was employed for quantification. The mass-to-charge transitions that were monitored for the quantification of metformin and tetraethylammonium (internal standard [IS]) were 130.1→71.0 and 130.0→100.3, respectively. The declustering potential, collision energy, and collision cell exit potential were set as follows: 70, 27, and 9 V for metformin; 100, 20, and 15 V for IS, respectively. The total run time for each analytical run was 2 min. Data acquisition and quantitation was carried out using the Analyst software (Applied Biosystems, version 1.5.2).

Standard solution and quality control sample preparation

A stock solution of metformin was prepared in deionized water at a concentration of 0.1 mg/mL. Working calibration standards at concentrations of 2–1024 ng/mL were prepared in blank plasma. Four levels of quality control (QC) working solutions at 4, 32, 256, and 768

ng/mL were prepared in plasma for determining the intra- and inter-day accuracies and precisions.

Sample preparation

To a 15-μL aliquot of plasma sample (i.e., blank plasma, calibration standards, or QC samples), 120 μL of acetonitrile containing 20 ng/mL IS was added and vortex-mixed for 5 min, followed by centrifugation at 16,000 × *g* for 5 min. The supernatant was transferred into a vial, and 2 μL of the aliquot was injected onto the LC–MS/MS system for analysis.

Validation of the method

The linearity of the method was obtained by calculating the regression equation from the peak area ratios of metformin to IS versus the corresponding concentrations of metformin. The limit of detection and LLOQ were defined at the signal-to-noise ratios of 3 and 10, respectively. The intra-day and inter-day accuracies (quantified as relative error) and precisions (% relative standard deviation, RSD%) were assessed by analyzing the QC samples at four different concentrations on one day, and then this experiment was repeated for three consecutive days. In order to evaluate the stability of the method, samples were exposed at room temperature for 2 h or auto-sampler at 4 °C for 12 h, stored at -70°C for 30 days, or treated with three cycles of freezing (-70°C) and thawing (at room temperature; until no ice was observed) to evaluate the pre-preparative stability. The extraction recoveries of the analytes were determined by comparing the peak areas of the analytes that were spiked before extraction to those of the analytes that were spiked after extraction. To determine the matrix effect, the peak areas of the analytes that were spiked after extraction were compared with those of the analytes in neat solution. A dilution integrity experiment was performed using five replicate samples prepared at nominal concentrations (40, 320, 7680 ng/mL), with the samples diluted 10-fold using blank matrix. The diluted samples were analyzed, and the measured mean concentrations were compared with the actual values after the dilution factor was applied.

Rat plasma assays

Sprague-Dawley rats (three males and three females) received a single intragastric gavage (i.g.) administration of metformin (100 mg/kg body weight). A blood sample was drawn into a heparinized tube immediately prior to i.g. administration and at 0.25, 0.5, 1, 2, 4, 6, 8, 12,

24, and 36 h post-i.g. administration. The plasma was isolated and maintained at -70°C until analysis. Plasma concentrations of metformin were measured as described above. Samples that were found to contain concentrations above the upper limit of quantification were diluted with blank plasma and then re-analyzed.

Results and discussion

Development of Amide HILIC–MS/MS methods

HILIC can be defined as a separation mode that combines stationary phases, which are usually used in the normal-phase mode, and mobile phases, which are used in reverse-phase separations. Silica is the most frequently employed chromatographic support for HILIC.[2, 16] Typical applications of HILIC involve highly hydrophilic silica (e.g., bare or chemically modified silica, or polar polymers).[2, 16] It is well known that the chemical characteristics of different stationary phases influence the separation of the targeted compounds, and this also occurs in HILIC. During the development of our new method, we quantitatively analyzed metformin on the following three HILIC columns: Atlantis HILIC Silica column (2.1 × 100 mm, 3 μm particle size), ZIC-c HILIC Silica column (2.1 × 100 mm, 3 μm particle size), and ACQUITY UPLC BEH Amide column (2.1 × 100 mm, 1.7 μm particle size). Though these columns were able to retain metformin, there was a perceptible difference in the column efficiency and column life between the two former columns and the latter. The peak width of the two former columns was more than 1 min; however, the peak width of the latter was only 0.15 min. Under the same chromatographic condition, the amide column can run in half the run time compared to the former columns. Moreover, the two silica columns showed worse column efficiency and broadened peaks (1.5 times the peak width of the initial peak) after only several samples for metformin, while compared to the initial sample analysis, metformin had similar peak widths (still 0.15 min) after 2000 sample injections on the amide column. The amide stationary phase has proven to be particularly useful for the rapid and efficient separation of polar compounds because the retention of these polar compounds is based on their hydrogen bonding interactions with the amide groups of the stationary phase.[2, 16] Amide-HILIC columns packed with particles that are less than 2 μm in size are very promising for highly efficient and fast HILIC separations with good resolution and short

analysis times.[16] Thus, an Amide-HILIC-MS/MS system based on a BEH Amide column (2.1 × 100 mm, 1.7 μm) was used in this study.

Many polar organic solvents can be used in the mobile phase, but in the present study, acetonitrile was used because of its low viscosity and elution strength, its ability to provide efficient separations, and because it does not favor the formation of hydrogen bonds. The amount of water that is used in HILIC separations is very important because HILIC separates compounds by eluting with a strong organic mobile phase against a hydrophilic stationary phase, wherein the elution is driven by increasing the water content in the mobile phase. Therefore, we investigated the effects of using different percentages of water (5–30%). As shown in Fig. 2, increasing the acetonitrile percentage (decreasing the amount of water) significantly increased the retention of metformin.

In addition, the presence of buffers or acids in the mobile phase can greatly influence the separations in HILIC. The phosphate buffer salt used in the method established by the Waters Corporation for the determination of metformin and impurity (http://www.waters.com/webassets/cms/library/docs/720004080en.pdf) is not suitable for the analysis of mass spectrometry. Thus, the effects of the buffer and its pH were subsequently investigated. Several mobile phase additives with different pH, such as 0.06% formic acid and 5 mM ammonium formate (pH 3), 0.01% acetic acid and 5 mM ammonium acetate (pH 5), 5 mM ammonium acetate (pH 6.8), and 0.02% ammonium hydroxide and 5 mM ammonium formate (pH 9), were used to achieve high sensitivity, good peak shape, and sufficient separation. We found that as the pH increased the signal intensity of metformin decreased and another new peak (at the retention time of 1.95 min) was detected in the rat plasma samples (Fig. 3). These two chromatographic peaks were the same in the parent ion and fragment ions. To further verify the peak, following precipitation of the plasma sample, a fraction of the supernatant was decanted and evaporated under a gentle stream of nitrogen at 40°C; then, the residue was reconstituted in the mobile phase and injected. After this treatment, only one peak, which had the same retention time and mass spectra as metformin, was detected. Hence, we speculated that metformin could partly be dissociated in the plasma matrix when the higher-pH mobile phase was used. In order to avoid the dissociation of metformin, an optimum mobile phase was achieved using an aqueous phase and acetonitrile

containing 0.06% formic acid and 5 mM ammonium formate (pH 3).

Though it was reported that ultraviolet (UV) detection was used to detect metformin and impurities (http://www.waters.com/webassets/cms/library/docs/720004080en.pdf), UV detection (218 nm) is not suitable for metformin assays, since it is a small molecule with poor UV absorption in the complicated biological matrix. Therefore, it is necessary to use an MS/MS assay for quantifying metformin in rat plasma. The use of an electrospray ionization source is popular in many studies. Because of the different chromatographic behavior between the C_{18} and hilic columns, the effect of the endogenous matrix on the determination of metformin was not same. In our experiments, we found that the matrix will interfere with the determination of metformin when using an electrospray ionization source (the value of the matrix effect ranged from 60% to 70%), although in other experiments, the electrospray ionization source did not detect the effect of the matrix when using a C18 column. [18, 20, 25] Due to its fundamentally different ionization mechanism, APCI can avoid the risk of matrix effects from endogenous materials. Finally, we performed the quantification of metformin using the ion source of APCI and detected in positive ion mode.

Because of the lack of commercial isotope IS, we used tetraethylammonium as an IS since it has a similar hydrophilicity and similar chromatographic and mass spectrometric behaviors to metformin. Next, we developed a simple one-step protein precipitation procedure. Acetonitrile (120 μL) containing 20 ng/mL IS was added to 15-μL aliquots of the plasma samples, and then, after centrifugation, the high organic extracts were directly injected onto the column without using any additional time-consuming evaporation and reconstitution procedures during sample preparation.

After the above attempts, our newly developed a simple, highly sensitive, specific, reproducible, and high-throughput Amide-HILIC-MS/MS method for quantifying the plasma concentration of metformin not only achieved a higher sensitivity (LLOQ = 2 ng/mL) using a plasma volume of 15 μL (“one drop” plasma) than the sensitivities reported using other HILIC analytical methods,[32-35] but also required a simpler sample preparation procedure and shorter chromatographic run (within 2 min) than previously used methods.[32, 34, 35] Therefore, this method is suitable for performing high-throughput analyses.

Method validation

Specificity was confirmed by extracting the matrixes from six different blank rats and comparing their MS/MS responses at the retention times of metformin and IS. No endogenous interference was observed at the retention times of metformin and IS. Typical chromatograms of blank rat plasma, blank rat plasma spiked with metformin and IS at the LLOQ, and rat plasma acquired 1 h after a single i.g. administration of metfimin are shown in Fig. 4.

The standard calibration curves for the spiked rat plasma showed good linearity from 2 to 1024 ng/mL for metformin with the linear equation of $y = 0.09x + 0.15$ ($r = 0.995$). The limit of detection and LLOQ of metformin in rat plasma were 0.5 ng/mL and 2 ng/mL (2 pg on column), respectively. The accuracy and precision were determined by using the intra- and inter-day measurements of QC samples at different concentration levels. The intra- and inter-day accuracies are given as the difference between the concentrations added and found (Table 1). Thus, the bias and coefficient of variation values were within the recommended Food and Drug Administration guidelines.

Table 1 Intra-day and inter-day precision and accuracy for metformin from quality control (QC) samples. (n = 5 for intra-day, n = 5×3 for inter-day)

	Sample concentration (ng/mL)	Concentration found (ng/mL, mean±SD)			Accuracy (%)	Precision RSD (%)
Intra-day						
	4	3.87	±	0.39	96.9	10.1
	32	32.8	±	2.9	102.6	8.8
	256	238.4	±	17.9	93.1	7.5
	768	812	±	54	105.7	6.7
Inter-day						
	4	4.03	±	0.32	100.7	8.0
	32	32.1	±	2.9	100.3	9.1
	256	249.8	±	19.5	97.7	7.8
	768	803.5	±	48.4	104.6	6.0

Stability was assessed under a variety of conditions, and the results showed that the obtained accuracy value of metformin was 87.7% to 112.2%, with RSD% values below 7.7%

(Table 2). The results for short-term stability suggest that plasma sample can be kept at 4°C for 2 h and sample extracts can be kept at 4°C for up to 12 h. Three 24-h freeze-thaw cycles for these three concentration samples did not appear to affect the quantification of the target analytes. These samples were stored in a freezer at -70°C and remained stable for at least 30 days. These results suggest that matrix samples containing target analytes can be handled under normal laboratory conditions without any significant compound loss.

Table 2 Stability data of metformin in rat plasma exposed to various storage conditions (n = 5)

	Sample concentration (ng/mL)	Concentration found (ng/mL, mean±SD)	Accuracy (%)	Precision RSD (%)
Short-term stability (about 25°C, 2 h)	4	4.06 ± 0.31	101.6	7.7
	32	30.9 ± 1.9	96.5	6.1
	768	750.2 ± 31.9	97.7	4.3
Stability of the ready-to-inject samples (4°C, 12h)	4	4.27 ± 0.28	106.7	6.5
	32	29.7 ± 1.2	92.8	4.1
	768	788.0 ± 42.5	102.6	5.4
three freeze-thaw stability	4	4.49 ± 0.14	112.2	3.2
	32	28.6 ± 1.3	89.4	4.4
	768	770.6 ± 35.8	100.3	4.6
Long-term stability (−70°C, 30 days) and freeze-thaw stability	4	4.37 ± 0.26	109.2	5.9
	32	27.9 ± 0.3	87.1	1.0
	768	735.0 ± 29.5	95.7	4.0

Recovery was analyzed by spiking blank plasma with low, medium, and high concentrations of the analytes prior to and after sample preparation. Metformin showed stable recovery rates. The matrix effect was determined as the difference between the signals of the metformin and IS obtained with and without post-extract blank plasma. Ion suppression or enhancement was therefore not significant with the Amide-HILIC-MS/MS method (Table 3).

Evaluations of the precision and accuracy in the dilution integrity experiment showed RSD% values that were below 3.97%, whereas the accuracy values were within 93.8–108.1%. These results indicate that a 10-fold dilution of the rat plasma samples that contain the analyte is acceptable.

Table 3 Recovery and matrix effect for analyte from nominal concentration samples (n = 5)

Nominal concentration (ng/mL)	Recovery (%) (mean ± SD)	Matrix effect (%) (mean ± SD)
4	91.91 ± 7.07	97.69 ± 6.99
32	103.79 ± 10.66	112.19 ± 2.88
768	99.45 ± 1.20	101.20 ± 2.72

Pharmacokinetic study

Following validation, the method was successfully applied for studying the pharmacokinetics of metformin in rats. The mean plasma concentration-time profile of metformin is shown in Fig. 5. The Cmax was 8.4 ± 1.4 μg/mL and occurred at 1.1 h. The oral half-life of metformin was 8.4 ± 1.3 h and the area under the plasma concentration-time curve (AUC0-∞) was 31.9 μg h/mL.

Conclusions

In summary, we developed and validated a simple, highly sensitive, specific, reproducible, and high-throughput Amide-HILIC-MS/MS assay to quantify metformin in rat plasma. This validated method uses a small sample volume and a simple protein precipitation method and showed good inter-day and intra-day precisions and accuracies over a calibration range of 2–1024 ng/mL. The method was successfully utilized for sample analysis to support pharmacokinetic studies, and can be easily extended to evaluate metformin in other biological matrices. Moreover, it can potentially be applied for evaluating human drug concentrations.

Acknowledgements

The authors are grateful for the financial support from the National Natural Science Foundation of China (81303296, 81273658 and 81202986), Xinglin scholar (2013), Program for Shanghai Innovative Research Team in University (2009), and “085” First-Class Discipline Construction of Science and Technology Innovation (085ZY1205).

The authors have declared no conflict of interest.

References

1. Y. Yang, R. I. Boysen and M. T. Hearn, *Journal of chromatography. A*, 2009, **1216**, 5518-5524.
2. M. R. Gama, R. G. da Costa Silva, C. H. Collins and C. B. G. Bottoli, *TrAC Trends in Analytical Chemistry*, 2012, **37**, 48-60.
3. W. Zhang, F. Han, H. Zhao, Z. J. Lin, Q. M. Huang and N. Weng, *Biomedical chromatography : BMC*, 2012, **26**, 1163-1169.
4. M. S. Lowenthal, E. L. Kilpatrick and K. W. Phinney, *Analytical and bioanalytical chemistry*, 2015, **407**, 5453-5462.
5. H. Wang, Q. Wu, C. Wu and Z. Jiang, *Journal of chromatographic science*, 2015, **53**, 1386-1394.
6. S. Tufi, M. Lamoree, J. de Boer and P. Leonards, *Journal of chromatography. A*, 2015, **1395**, 79-87.
7. A. L. van Nuijs, I. Tarcomnicu and A. Covaci, *Journal of chromatography. A*, 2011, **1218**, 5964-5974.
8. A. L. van Nuijs, I. Tarcomnicu, W. Simons, L. Bervoets, R. Blust, P. G. Jorens, H. Neels and A. Covaci, *Analytical and bioanalytical chemistry*, 2010, **398**, 2211-2222.
9. A. A. Ghfar, S. M. Wabaidur, A. Y. Ahmed, Z. A. Alothman, M. R. Khan and N. H. Al-Shaalan, *Food chemistry*, 2015, **176**, 487-492.
10. M. Kinaan, H. Ding and C. R. Triggle, *Medical principles and practice : international journal of the Kuwait University, Health Science Centre*, 2015, **24**, 401-415.
11. A. Bhat, G. Sebastiani and M. Bhat, *World journal of hepatology*, 2015, **7**, 1652-1659.
12. S. Kordes, M. N. Pollak, A. H. Zwinderman, R. A. Mathôt, M. J. Weterman, A. Beeker, C. J. Punt, D. J. Richel and J. W. Wilmink, *The Lancet Oncology*, 2015, **16**, 839-847.
13. T. Ebner, N. Ishiguro and M. E. Taub, *Journal of pharmaceutical sciences*, 2015, **104**, 3220-3228.
14. A. Rashid, M. Ahmad, M. U. Minhas, I. J. Hassan and M. Z. Malik, *Pakistan journal of pharmaceutical sciences*, 2014, **27**, 153-159.
15. K. Heinig and F. Bucheli, *Journal of pharmaceutical and biomedical analysis*, 2004, **34**, 1005-1011.
16. Y. Wang, Y. Tang, J. Gu, J. P. Fawcett and X. Bai, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 2004, **808**, 215-219.
17. P. P. Kumar, T. E. Murthy and M. V. Basaveswara Rao, *Journal of advanced pharmaceutical technology & research*, 2015, **6**, 118-124.
18. X. R. Liang, X. J. Dai, Y. F. Zhang, J. F. Ding, X. Y. Chen and D. F. Zhong, *Yao xue xue bao = Acta pharmaceutica Sinica*, 2013, **48**, 547-553.
19. X. H. Zhao, B. Song, D. F. Zhong, S. Q. Zhang and X. Y. Chen, *Yao xue xue bao = Acta pharmaceutica Sinica*, 2007, **42**, 1087-1091.
20. K. Sharma, G. Pawar, S. Yadam, S. Giri, S. Rajagopal and R. Mullangi, *Biomedical chromatography : BMC*, 2013, **27**, 356-364.
21. X. Zhang, X. Wang, D. I. Vernikovskaya, V. M. Fokina, T. N. Nanovskaya, G. D. Hankins and M. S. Ahmed, *Biomedical chromatography : BMC*, 2015, **29**, 560-569.
22. B. Jagadeesh, D. V. Bharathi, C. Pankaj, V. S. Narayana and V. Venkateswarulu, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 2013, **930**, 136-145.
23. C. Georgita, F. Albu, V. David and A. Medvedovici, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 2007, **854**, 211-218.
24. C. G. Ding, Z. Zhou, Q. H. Ge, X. J. Zhi and L. L. Ma, *Biomedical chromatography : BMC*, 2007, **21**, 132-138.
25. G. P. Zhong, H. C. Bi, S. Zhou, X. Chen and M. Huang, *Journal of mass spectrometry : JMS*, 2005, **40**, 1462-1471.

26. Y. R. Ma, Z. Rao, A. X. Shi, Y. F. Wang, J. Huang, M. Han, X. D. Wang, Y. W. Jin, G. Q. Zhang, Y. Zhou, F. Zhang, H. Y. Qin and X. A. Wu, *Journal of chromatographic science*, 2015.
27. N. Koseki, H. Kawashita, M. Niina, Y. Nagae and N. Masuda, *Journal of pharmaceutical and biomedical analysis*, 2005, **36**, 1063-1072.
28. S. AbuRuz, J. Millership and J. McElnay, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 2003, **798**, 203-209.
29. A. Zarghi, S. M. Foroutan, A. Shafaati and A. Khoddam, *Journal of pharmaceutical and biomedical analysis*, 2003, **31**, 197-200.
30. J. Keal and A. Somogyi, *Journal of chromatography*, 1986, **378**, 503-508.
31. Y. Hsieh, G. Galviz and J. J. Hwa, *Bioanalysis*, 2009, **1**, 1073-1079.
32. K. M. Huttunen, J. Rautio, J. Leppanen, J. Vepsalainen and P. Keski-Rahkonen, *Journal of pharmaceutical and biomedical analysis*, 2009, **50**, 469-474.
33. A. Liu and S. P. Coleman, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 2009, **877**, 3695-3700.
34. A. M. Mohamed, F. A. Mohamed, S. Ahmed and Y. A. Mohamed, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 2015, **997**, 16-22.
35. R. Pontarolo, A. C. Gimenez, T. M. de Francisco, R. P. Ribeiro, F. L. Pontes and J. C. Gasparetto, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 2014, **965**, 133-141.
36. P. Jandera, *Analytica chimica acta*, 2011, **692**, 1-25.
37. Y. Liu, S. Urgaonkar, J. G. Verkade and D. W. Armstrong, *Journal of chromatography. A*, 2005, **1079**, 146-152.

Figure Legends

Fig. 1 Chemical structures of metformin and tetraethylammonium.

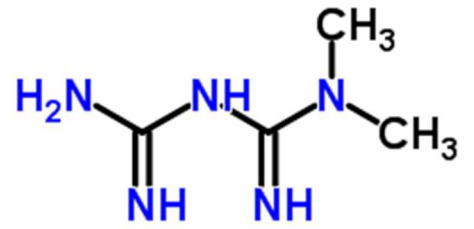
Fig. 2 Retention time of metformin on a BEH amide column as a function of acetonitrile in the mobile phase (%) containing 0.06% formic acid and 5 mM ammonium formate.

Fig. 3 The chromatograms of metformin in rat plasma in different pH mobile phases: (a) the mobile phase containing 0.06% formic acid and 5 mM ammonium formate (pH = 3, solid line) and 5 mM ammonium acetate (pH = 6.8, dashed line); (b) the mobile phase containing 0.01% acetic acid and 5 mM ammonium acetate (pH = 5, solid line) and 0.02% ammonium hydroxide and 5 mM ammonium formate (pH = 9, dashed line).

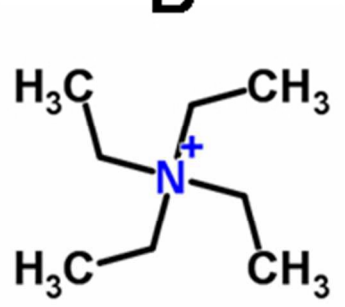
Fig. 4 Typical multiple-reaction monitoring (MRM) chromatograms of metformin (upper panel) and IS (under panel) in (a) rat blank plasma; (b) rat blank plasma spiked with metformin at the lower limit of quantitation (LLOQ; 2.0 ng/mL) and IS; (c) a 24 h in vivo plasma sample showing a metformin peak obtained following oral dosing to rats along with IS.

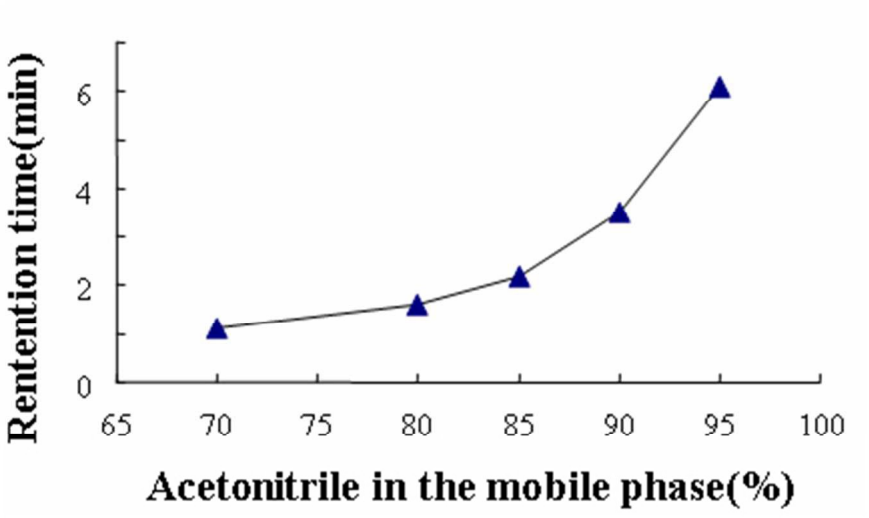
Fig. 5 Mean plasma concentration-time profiles of metformin after oral administration of doses of 100 mg/kg (n = 6, mean ± SD).

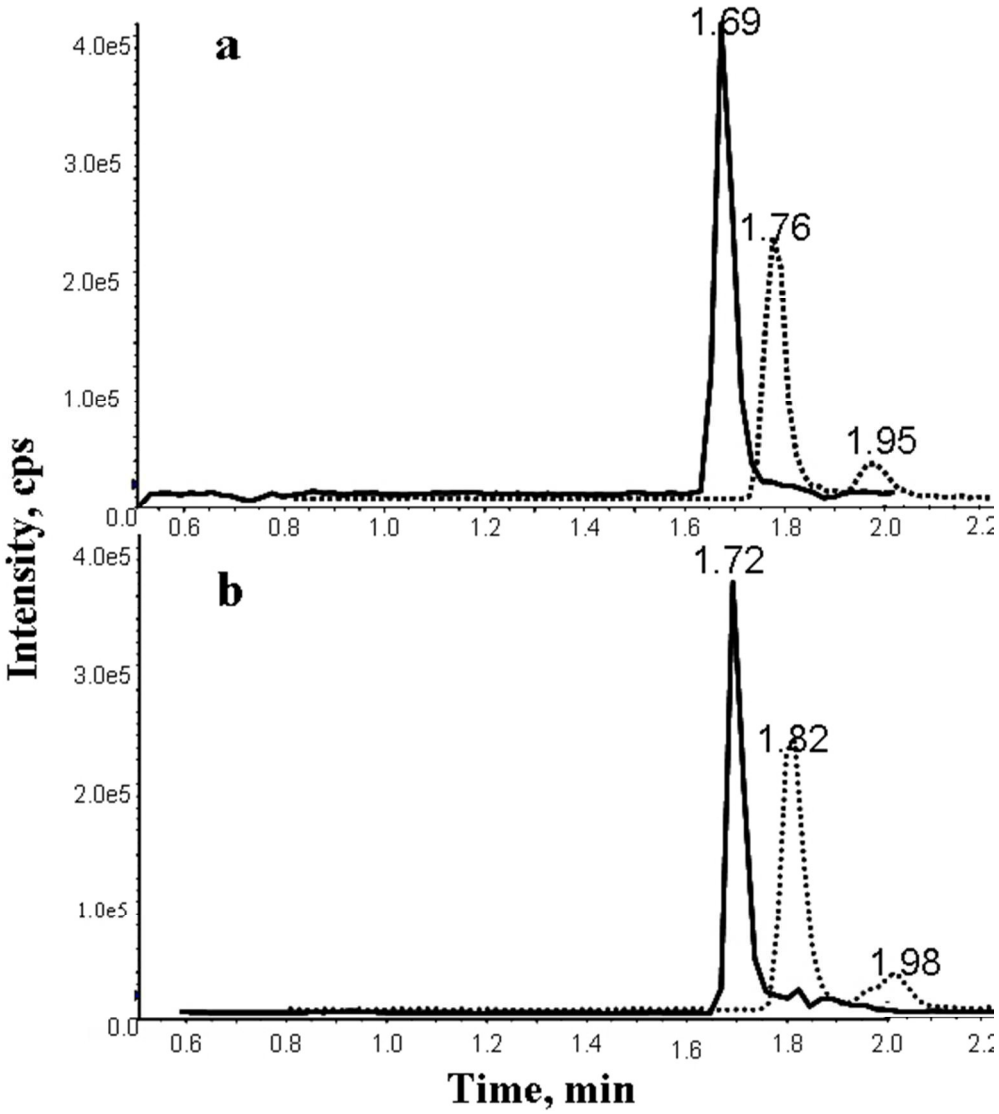
A

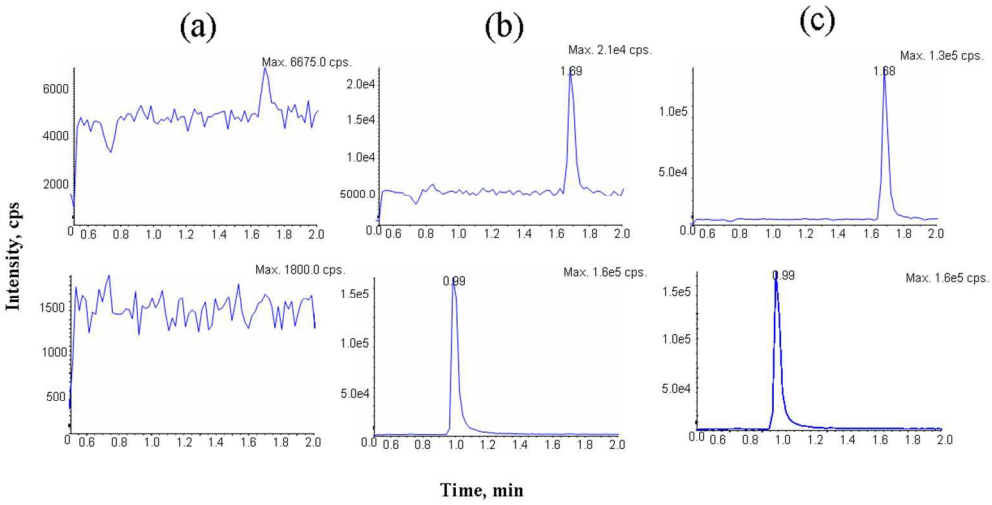


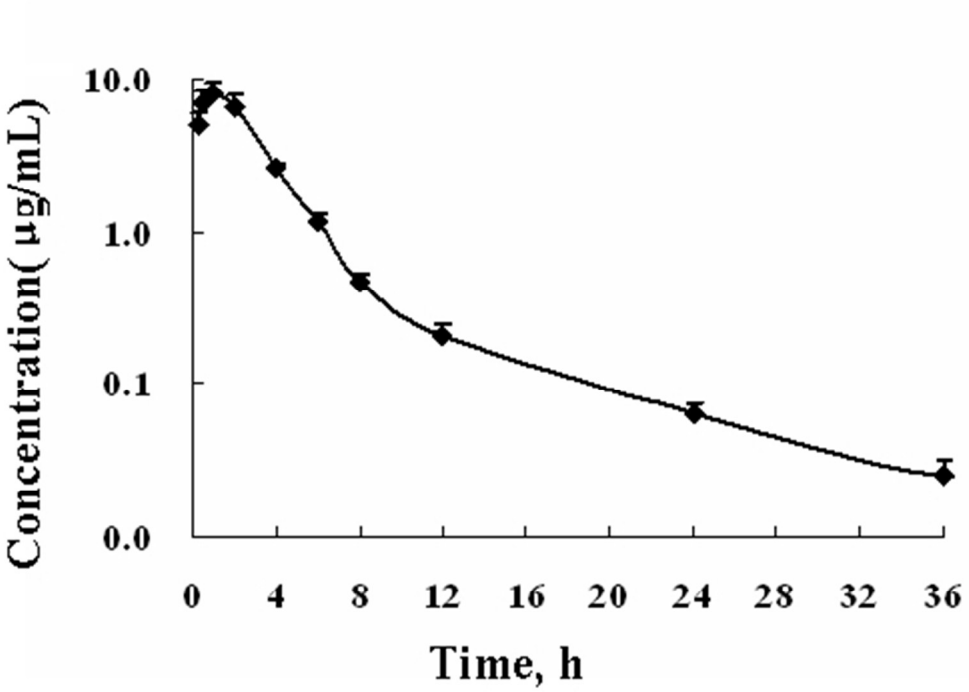
B

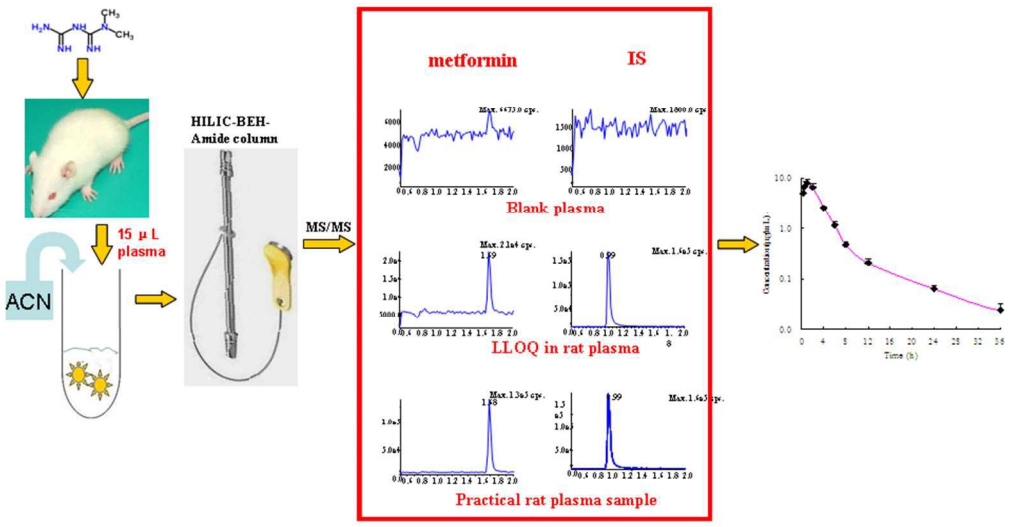












graphic abstract