Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
34 (2004) 1005–1011
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

# Fast liquid chromatographic-tandem mass spectrometric (LC–MS–MS) determination of metformin in plasma samples

Katja Heinig*, Franz Bucheli

*F. Hoffmann-La Roche Ltd., Pharmaceuticals Division, Non-Clinical Drug Safety, Bioanalytical Section, Grenzacherstrasse, Building 68/101, CH-4070 Basel, Switzerland*

Received 29 August 2003; received in revised form 8 November 2003; accepted 13 November 2003

**Abstract**

Liquid chromatographic–tandem mass spectrometric (LC–MS–MS) methods for the determination of metformin in plasma from different species are presented. The first method employed a YMC cyano 2 mm × 50 mm, 3 μm analytical column. For minimum sample preparation direct injection of samples after protein precipitation was performed. The polar column used with highly organic mobile phases provided a normal phase retention mechanism. The elution conditions were optimized to obtain reproducible peak areas and good peak shape. A step gradient from 100% acetonitrile to acetonitrile–water 80:20 (v/v) containing 10 mM ammonium acetate and 1% acetic acid was applied, leading to a sample-to-sample cycle time of 2 min. In a second method, a column-switching LC–MS–MS assay for on-line trapping was developed. The analyte and internal standard were trapped on a YMC cyano 2 mm × 10 mm, 5 μm column using acetonitrile–methanol 95:5 (v/v). Elution was performed isocratically in back-flush mode on to the analytical column (YMC cyano 2 mm × 50 mm, 3 μm) using 10 mM ammonium acetate in acetonitrile–water 80:20 (v/v) with 1% formic acid. With this approach, the signal-to-noise ratio was improved and the run time could be shortened to 1 min. Calibration samples were prepared in the matrix to be assayed in the range of 10–10,000 ng/ml. Quality control (QC) samples were prepared at 40, 400 and 4000 ng/ml and interspersed with the unknown study samples in the assays. Deviations for precision and accuracy were less than 20% for the lower limit of quantification (LLOQ) and low QC sample and less than 15% for other calibrators and QCs.


*Keywords:* Metformin; Plasma; LC–MS–MS; Column switching

## 1. Introduction

Metformin is a well-known insulin secretagogue [1]. It has the capability to improve glucose utilization in different in vivo models and is therefore used to evaluate the anti-diabetic potential of other drugs during an acute oral glucose tolerance test (OGTT). This test determines the ability to metabolize the sugar glucose and is normally performed to diagnose type II diabetes. Blood glucose is measured before, and several times after, oral intake of a defined glucose amount and abnormally high levels indicate the occurrence of diabetes. In case of treatment with anti-diabetic drugs, the glucose levels are reduced. The OGTT is

* Corresponding author. Tel.: +41-61-6884105; fax: +41-61-6882908.
*E-mail address:* katja.heinig@roche.com (K. Heinig).

0731-7085/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2003.11.017

employed in drug development. Drug administration is performed prior to glucose and it is determined whether the drug induces a glucose-lowering effect. First the pharmacokinetic profile has to be established to determine the most appropriate time for drug administration. Then the OGTT is performed including measurement of drug exposure in correlation with glucose levels. In the tests of potential new drug candidates, metformin is used for comparison of effects. An analytical method with sufficient sensitivity and selectivity was needed to determine the compound in plasma.

Several HPLC methods with UV detection have been used for the determination of metformin in biological materials. An overview, including the comparison of analytical characteristics, is given by Cheng and Chou [2]. As most of the reported assays are time consuming procedures, which require large sample volumes and lack selectivity and sensitivity, the authors proposed a sensitive and simple analytical method employing protein precipitation, organic wash, and chromatography on a silica column using phosphate/acetonitrile. Other approaches to metformin analysis involve ion pair chromatography on reversal-phase columns (e.g. with the addition of sodium dodecyl sulfate [3]), cation exchange [4,5] and the use of polar columns (cyano [6]) for "normal phase" chromatography. Derivatization procedures to improve retention and detection of the compound were also applied, e.g. with *p*-nitrobenzoyl chloride [7]. Sample preparation consisted of liquid–liquid extraction [7], ion pair extraction [8], protein precipitation [4], solid phase extraction [9] or ultrafiltration [5].

Liquid chromatography–tandem mass spectrometry (LC–MS–MS) is an efficient analysis tool providing low detection limits, reduced influence of interferences and the possibility for short run times. Therefore, it is often the method of choice in pharmaceutical analysis. To our knowledge, no LC–MS–MS assay to determine metformin has been reported up to now. Our goal was to develop a LC–MS–MS method for the analysis of metformin in plasma, preferably providing short run times and employing simple and fast sample preparation. Complete method validation was not required because the exploratory pharmacokinetic and OGTT studies were performed under non-regulatory conditions. The lower limit of quantification (LLOQ) should not exceed 10 ng/ml.

## 2. Experimental

### 2.1. Chemicals and materials

The drug and the structural analogue used as internal standard (ISTD) were obtained from F. Hoffmann-La Roche (Basle, Switzerland). HPLC-grade acetonitrile (ACN) was purchased from Rathburn (Walkerburn, Scotland). Methanol (MeOH), ethanol (EtOH), acetic acid (HOAc), formic acid, and ammonium acetate ($NH_4OAc$) p.a. were obtained from Merck (Darmstadt, Germany). All solutions were prepared using in-house generated doubly distilled water. Human control plasma was purchased from a blood bank (Blutspendezentrum SRK Beider Basel). Rat, minipig and cynomolgus monkey plasma was obtained from animals used for pharmacokinetic and toxicokinetic experiments in our facility.

### 2.2. Solutions and standards

Stock solutions of internal standard and analyte (500 μg/ml) were prepared in ethanol. Internal standard working solution (ISTD) containing 50 ng/ml to use as protein precipitation solvent for plasma samples was prepared by diluting the stock solution with ethanol. The internal standard working solution was also used for diluting samples with high analyte concentrations. The analyte stock solution (different weightings for calibration standards and quality control samples) was diluted with ethanol–water (70:30, v/v) to obtain spiking solutions. Calibration standards in the range 10–10,000 ng/ml were prepared by spiking human control plasma (volume of spiking solution ≤2% of matrix volume). Quality control (QC) samples at low (40 ng/ml), medium (400 ng/ml) and high (4000 ng/ml) levels were prepared by spiking the matrix to be assayed (drug-free rat, minipig, and cynomolgus monkey plasma). Both calibration standards and QC samples were divided into aliquots of 20 μl in 1.5 ml Eppendorf vials and stored at −20 °C until use.

### 2.3. Sample preparation (off-line)

Study samples were thawed at room temperature together with calibration standards and QC samples. To aliquots of 20 μl plasma, 1 ml of internal standard working solution was added. After short mixing, the

samples were stored for 5–10 min in the deep freezer at approximately −20 °C to achieve optimal protein precipitation. The samples were centrifuged for 5 min at 15,000 rpm (ca. 18,000 × *g*) at 10 °C in a Heraeus Sepatech Megafuge 2.0 R. If the expected sample concentration exceeded the calibration range, the supernatant was diluted with ISTD after centrifugation. High QC samples were treated the same way to provide dilution QC samples. A 20 μl aliquot of the supernatant was injected (10 μl for column-switching).

### 2.4. LC–MS–MS with direct sample injection

The LC system consisted of a L-6200 A pump A and a L-6000A pump B (both Merck-Hitachi, Tokyo, Japan) operating in high-pressure gradient mode using a 75 μl dynamic mixer (Labsource, Reinach, Switzerland), a AS 4000 autosampler (Merck) and a solvent degassing unit SDU 2003 (Labsource). The analytical column was a cyano 2.1 mm × 50 mm column, 3.5 μm particle size, from YMC (Milford, MA, USA). The mobile phases consisted of (A) 10 mM ammonium acetate and 1% acetic acid in acetonitrile–water (80:20, v/v) and (B) pure acetonitrile. After injection of 20 μl sample solution, 100% mobile phase B was pumped for 0.5 min at 0.8 ml/min. Within the next 0.1 min, the mobile phase was switched to 100% A. After elution of metformin and internal standard, the column was rinsed with 100% solvent B for 0.6 min at 1.5 ml/min. The overall run time was 2 min.

### 2.5. LC–MS–MS with column switching

An in-house assembled system (Merck-Hitachi, Tokyo, Japan), as shown in Fig. 1, was applied for column-switching LC–MS–MS. The L-6200 Intelligent Pump (P1) was used for trapping and washing, while the pump L-6000 (P3) served for on-line dilution of the injection solution. For separation on the analytical column, the pump L-6000A (P2) was used. The autosampler was a Merck AS 4000. The solvent degassing unit SDU 2003 and the switching valve (High Speed Valve 7000E) were from Labsource. As trapping column (TC), the YMC cyano column (2.1 mm × 10 mm, 5 μm particle size) was used. The analytical column (AC) was a YMC cyano 2.1 mm × 50 mm column with 3.5 μm particles. Mobile phases were acetonitrile–methanol (95:5, v/v)

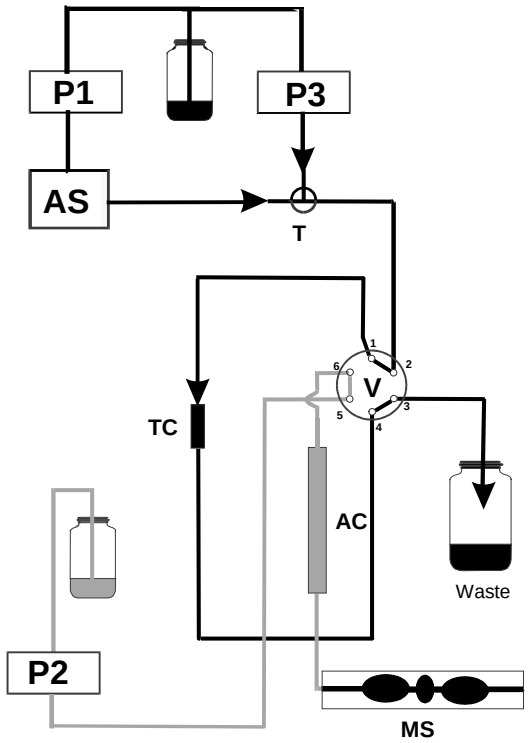



Fig. 1. Scheme of column-switching LC–MS–MS setup.

for on-line dilution, trapping and rinsing and 10 mM ammonium acetate in acetonitrile–water (80:20, v/v) containing 1% formic acid for elution from the trapping column and separation on the analytical column. Aliquots of 10 μl sample solution was transferred to the TC within 0.3 min using pumps P1 and P3 at 1.5 and 1 ml/min, respectively, to reduce the elution strength of the injection solution by on-line dilution. By switching V2, the trapping column was coupled with the analytical column, and the analyte was transferred using pump P2 at 0.6 ml/min within 0.2 min. During this time, the capillaries were rinsed with pump P1. After switching V2 to separate TC and AC, the flow on P2 was reduced to 0.4 ml/min. Metformin and ISTD eluted at 0.8 min. In the meantime, the trapping column was purged at 4 ml/min. The sample-to-sample cycle time was 1 min.

### 2.6. Mass spectrometer

The API III$^+$ triple quadrupole mass spectrometer from PE Sciex (Concord, Ontario, Canada) with

TurboIonSpray™ in positive ion selected reaction monitoring mode (SRM) was used. Nitrogen served as nebulizer, auxiliary and curtain gas, argon was used as collision gas. Gas flow rates, temperatures, ionization voltages, and collision energies were optimized by infusion of 1 ng/μl standard solution of the analyte in methanol–water–acetic acid (50:50:1, v/v) at 20 μl/min and by flow injection analysis at the LC flow rate. The drug was monitored at a transition of $m/z\,130 \rightarrow m/z\,71$ and the internal standard at $m/z\,144 \rightarrow m/z\,88$. Data acquisition was performed with dwell times of 120 ms. The quadrupoles Q1 and Q3 were operated at unit mass resolution (<0.7 Da peak width at half height). Calibration of mass axis and optimization of resolution was performed using a mixture of quaternary ammonium salts.

### 2.7. Quantitation and validation

The calibration curves were established by linear least-squares regression ($1/x^2$ weighting) from peak area ratios (analyte/internal standard) versus nominal concentrations. Seven calibration standards were prepared with concentrations between 10 and 10,000 ng/ml. QC samples, prepared in plasma at three levels (40, 400, and 4000 ng/ml), were analyzed ($n = 5$) and calculated with one set of calibration standards to obtain data on intra-assay precision and accuracy. For analysis of study samples, at least six QC samples (all three concentrations in duplicate) were interspersed with the unknowns. Assay results were valid if at least two-thirds of the QC samples were within ±20% deviation from accuracy.

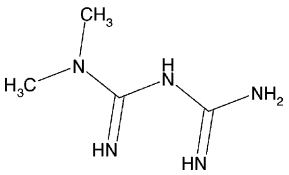
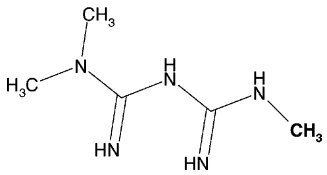
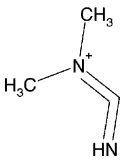
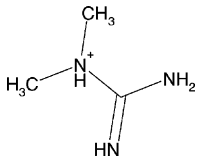
## 3. Results and discussion

Metformin showed a good MS response, the parent ion and product ions possess relatively low masses. A substance with similar structure (one H on $–NH_2$ group substituted by $–CH_3$) was chosen to use as internal standard (ISTD). Table 1 shows the structures and transitions for both substances. For the low molecular mass analyte and its fragments a high noise was expected in MS–MS due to the high background from solvents at lower *m/z* and MS parameters were tuned appropriately to obtain sufficient sensitivity.

Matrix effects were observed with plasma samples when precipitation with the three-fold amount of organic solvent was carried out (small split peaks were observed). An increased ratio of organic solvent–plasma could compensate for this effect and by using only 20 μl of plasma and 1 ml ethanol for precipitation and injecting 20 or 10 μl, good peak shape and sufficient sensitivity was obtained.

Metformin is very polar and therefore difficult to retain on analytical columns. Polar columns used with highly organic mobile phases provide a normal-phase separation mechanism leading to sufficient retention of polar substances. In the literature, a LC–UV

Table 1
Structures and MS–MS transitions with proposed fragments of the compounds

| | Metformin | ISTD |
|---|---|---|
| Transitions | $m/z\,130 \rightarrow m/z\,71$ | $m/z\,144 \rightarrow m/z\,88$ |
| Structures |  |  |
| Fragments |  |  |

method using a cyano column and acetonitrile/buffer as mobile phase in isocratic mode has been described [6]. A content of 40% acetonitrile led to sufficient retention and acceptable peak shape on the 250 mm column in this application. Therefore, we used a YMC cyano analytical column. Various elution conditions were investigated to obtain reproducible peak areas and good peak shape. For minimum sample preparation, direct injection of samples onto a YMC cyano 2 mm × 50 mm, 3 μm analytical column after protein removal was performed. Using acetonitrile contents up to 80% in the eluent, almost no retention of the drug was achieved. With 95% it was completely retained on the column. Isocratic elution with 90% acetonitrile led to a broad analyte peak not suited for quantification but gradient elution improved the peak shape significantly. Starting with 100% acetonitrile and applying a step gradient to acetonitrile-aqueous phase (80:20, v/v) led to narrow peak. The addition of ammonium acetate (10 mM) and acetic acid (1%) to the aqueous phase improved the reproducibility of peak areas and retention times. Extensive reconditioning of the analytical column with pure acetonitrile in between separations was essential for good

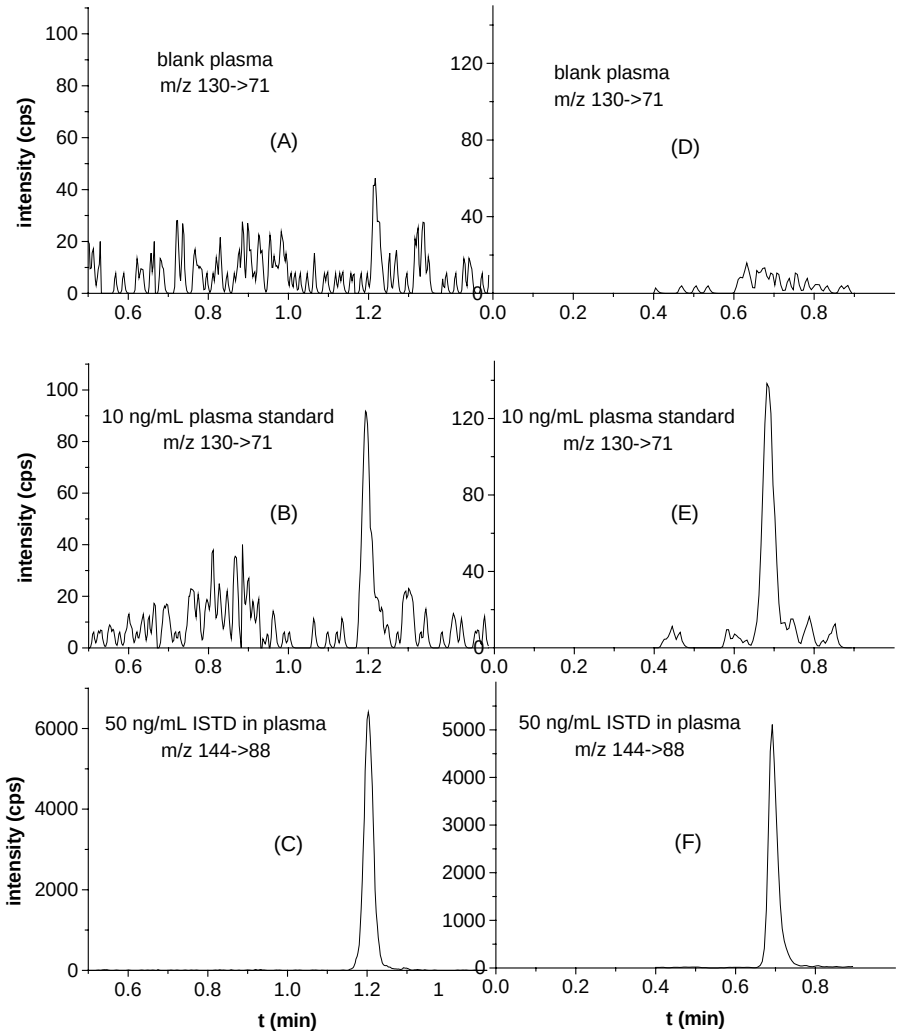



Fig. 2. SRM-LC–MS–MS chromatograms of blank plasma, plasma spiked with metformin at LLOQ of 10 ng/ml and of ISTD in the LLOQ sample using direct injection (A–C) or column switching (D–F).

reproducibility, leading to a sample-to-sample cycle time of 2 min. The internal standard eluted at the same time as the analyte. Fig. 2A–C show the LC–MS–MS chromatograms for blank plasma, for an LLOQ standard in rat plasma and for the internal standard.

The application of an extraction step before LC determination can reduce matrix components and lead to a more robust method. Therefore, a column-switching LC–MS–MS assay for on-line trapping was developed. Trapping and dilution conditions as well as the elution solvent were optimized. Trapping (YMC cyano 2 mm × 10 mm, 3 μm) had to be performed with pure organic solvent to retain the analyte and ISTD. Small amounts of water (e.g. 1%) or acid (e.g. 0.5% acetic acid) in the trapping solvent led to breakthrough of the compounds. A methanol content of 5% in acetonitrile was of advantage to obtain a better analyte peak shape. The ratio of injection flow/dilution flow was optimized to be 1.5/1. The sample-to-sample cycle time was only 1 min. Fig. 2D–F show LC–MS–MS chromatograms for blank plasma, for a standard at LLOQ in plasma and for the internal standard.

Calibration samples were prepared in plasma in the range 10–10,000 ng/ml. The coefficients of variation for five injections of the 10 ng/ml plasma standard were 13.8% for the direct injection method and 11.5% for the column-switching method. Quality control samples were prepared at 40, 400, and 4000 ng/ml. Deviations for precision and accuracy were less than 20% with direct injection and less than 15% with column switching during intra-assay validation (see Tables 2 and 3). Species differences could not be compensated by the internal standard and resulted in deviations from accuracy in the range of 20–30%. Therefore, calibration standards were always prepared in the matrix to be assayed. The overall recovery (including the matrix influence) of metformin from plasma was 58% using the direct injection approach and 77% using the column-switching method (determined by comparing peak areas in plasma with peak areas in ethanolic solution).

Plasma samples from rats, minipigs, and cynomolgus monkeys after oral administration of metformin were successfully analyzed using either the direct injection approach or the column-switching method. The relatively high doses of metformin adminstrated during the studies resulted in high plasma levels and therefore, our LLOQ of 10 ng/ml was sufficient. The column-switching method was preferred for routine analysis because of the shorter run time and better signal-to-noise ratio. Fig. 3 shows the selected ion

Table 2
Intra-assay accuracy and precision (%) of LC–MS–MS with direct injection ($n = 5$)

| | Rat plasma | | Minipig plasma | |
|---|---|---|---|---|
| | Accuracy | Precision | Accuracy | Precision |
| 40 ng/ml | 118.7 | 0.9 | 116.8 | 3.3 |
| 400 ng/ml | 102.7 | 3.2 | 108.8 | 8.7 |
| 4000 ng/ml | 86.8 | 3.0 | 106.3 | 7.9 |

Table 3
Intra-assay accuracy and precision (%) of column-switching LC–MS–MS assay ($n = 5$)

| | Cynomolgus monkey plasma | | Minipig plasma | |
|---|---|---|---|---|
| | Accuracy | Precision | Accuracy | Precision |
| 40 ng/ml | 86.6 | 0.3 | 110.7 | 5.3 |
| 400 ng/ml | 100.4 | 0.9 | 114.3 | 11.9 |
| 4000 ng/ml | 100.1 | 1.4 | 106.6 | 9.0 |

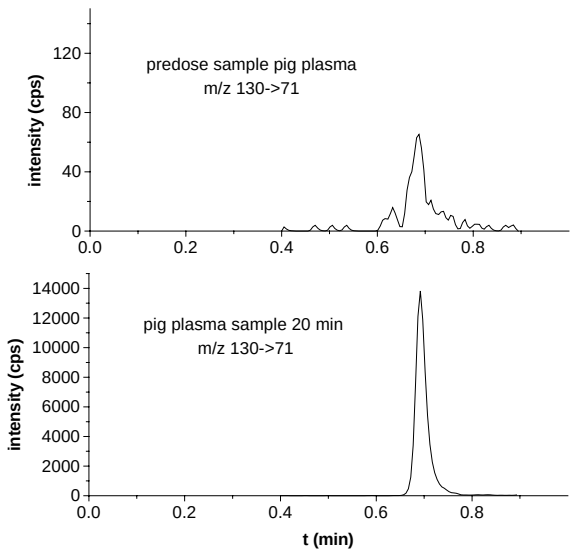



Fig. 3. Column-switching SRM-LC–MS–MS chromatograms of metformin at $m/z$ 130 → 71 from a minipig predose sample and from a sample taken 20 min after oral administration of 250 mg/kg metformin.

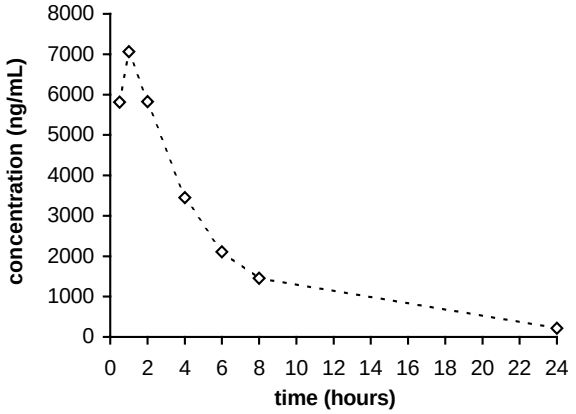



Fig. 4. Concentration–time profile of metformin in cynomolgus monkey plasma after oral administration of 250 mg/kg metformin (column-switching method).

chromatograms for metformin in a predose plasma sample and in a sample obtained 20 min after administration of the compound to a minipig. The small peak in the predose chromatogram corresponds to a concentration five times below the LLOQ and may be due to sample cross-contamination. An example at a pharmacokinetic profile in cynomolgus monkey is shown in Fig. 4. The data have been used for comparison of PK for metformin and potential drug candidates in correspondence to the glucose lowering effect.

## 4. Conclusions

Simple and rapid methods have been developed for the routine analysis of metformin in animal plasma. Liquid chromatographic separation and tandem mass spectrometric detection offered good sensitivity, selectivity, accuracy, and precision. The short run times of only 1–2 min provided high-throughput capabilities. The limit of detection of 10 ng/ml was achieved by using only 20 μl of sample, enabling the measurement of plasma profiles in rat, minipig, and cynomolgus monkey samples from pharmacokinetic and OGTT studies.

## References

[1] C.J. Bailey, General Pharmacology: The Vascular System, vol. 24, 1993, pp. 1299–1309.
[2] C.-L. Cheng, C.-H. Chou, J. Chromatogr. B 762 (2001) 51–58.
[3] A. Zarghi, S.M. Foroutan, A. Shafaati, A. Khoddam, J. Pharm. Biomed. Anal. 31 (2003) 197–200.
[4] M. Zhang, G.A. Moore, M. Lever, S.J. Gardiner, C.M.J. Kirkpatrick, E.J. Begg, J. Chromatogr. B 766 (2001) 175–179.
[5] O. Vesterquist, F. Nabbie, B. Swanson, J. Chromatogr. B 716 (1998) 299–304.
[6] K.H. Yuen, K.K. Peh, J. Chromatogr. B 710 (1998) 243–246.
[7] F. Tache, V. David, A. Farca, A. Medvedovici, Microchem. J. 68 (2001) 13–19.
[8] J. Keal, A. Somogyi, J. Chromatogr. 378 (1986) 503.
[9] R. Huupponen, P. Ojala-Karlsson, J. Rouru, M. Koulu, J. Chromatogr. 583 (1992) 270–273.