Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 469–474

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Determination of metformin and its prodrugs in human and rat blood by hydrophilic interaction liquid chromatography

Kristiina M. Huttunen[a,b,*], Jarkko Rautio[a,b], Jukka Leppänen[a,b], Jouko Vepsäläinen[b,c], Pekka Keski-Rahkonen[a,b]

[a] Department of Pharmaceutical Chemistry, University of Kuopio, P.O. Box 1627, FI-70211 Kuopio, Finland
[b] Biocenter Kuopio, University of Kuopio, P.O. Box 1627, FI-70211 Kuopio, Finland
[c] Department of Biosciences, University of Kuopio, P.O. Box 1627, FI-70211 Kuopio, Finland

ARTICLE INFO

Article history:
Received 4 March 2009
Received in revised form 29 April 2009
Accepted 29 April 2009
Available online 7 May 2009

Keywords:
Hydrophilic interaction liquid chromatography (HILIC)
Metformin
Prodrug

ABSTRACT

Simple and specific hydrophilic interaction liquid chromatography (HILIC) method with ultraviolet (UV) detection was developed for the simultaneous determination of highly water-soluble metformin and its more lipophilic prodrugs in human and rat blood samples. The sample preparation was accomplished by precipitating proteins with acetonitrile, which enabled the direct injection of supernatants to the HPLC. Chromatographic separation was performed on an analytical normal phase silica column using a mixture of 0.01 M ammonium acetate pH 5.0 and acetonitrile (40:60, v/v) as a mobile phase at flow rate of 1 ml/min and at the wavelength of 235 nm. The method was validated in terms of specificity, linearity, accuracy, precision, recovery, and analyte stability. The UV-HILIC method was suitable for detecting both metformin and one of its more lipophilic prodrugs simultaneously in human and rat blood samples.

1. Introduction

Metformin, *N*,*N*-dimethyl imidodicarbonimidic diamide (Fig. 1), is an oral antihyperglycemic agent that has been widely used in the management of type 2 diabetes mellitus for decades [1,2]. Unfortunately, this effective but highly basic anti-diabetic agent is fully protonated under physiological conditions and therefore slowly and incompletely absorbed from gastro-intestinal tract. Metformin has absolute bioavailability about 40–60% and at effective doses (0.5–2 g per day) it causes frequently uncomfortable gastrointestinal adverse effects [3–7]. Prodrugs are pharmacologically inactive or less active bioreversible derivatives of drug molecules utilised to improve the unfavourable physicochemical or pharmaceutical properties of parent drug molecules [8–10]. Accomplishing good membrane permeability for high passive transcellular absorption after oral administration by masking hydrogen bonding groups of an active compound is probably one of the most commonly introduced prodrug strategy. We have recently applied a novel sulfenamide prodrug strategy [11,12] to metformin to achieve improved permeability and oral absorption of metformin [13].

Several high-performance liquid chromatography (HPLC) methods, such as reversed-phase [14–16], normal phase [17–19], ion-pair [20,21], and cation-exchange [22–26] with different detection methods, like UV [14–25] and tandem mass spectrometry [27–31] have been developed and used for the determination of metformin in biological samples. However, most of these methods cannot be applied to simultaneous detection of highly polar metformin and less polar prodrugs of metformin due to their distinct physicochemical characteristics (based on our own studies that are discussed in Section 3.1). Furthermore, many of these methods have long running times, lack sensitivity, or have complex extraction procedures. Hydrophilic interaction liquid chromatography (HILIC) technique has proved to be a powerful way to separate polar analytes with reversed retention compared to the traditional reversed-phase chromatography, and thus, the HILIC method has gained the ground extensively in recent years [32–34]. The HILIC stationary phases are typically bare silica or silica derivatized with different polar functional groups, like amine, amide, cyano, and diol groups, while the mobile phase is an aqueous–organic mixture, often buffered water:acetonitrile. Generally, polar analytes are retained strongly on the stationary phase as the organic solvent proportion is increased in the mobile phase.

In the present study, simple and specific HILIC method was developed and validated for simultaneous determination of metformin and its two novel metformin prodrugs **1** and **2** (Fig. 1) in human or rat serum samples and the method was applied in the preliminary stage of study for determination of metformin and the prodrugs **1** and **2** from rat plasma samples. In the future, the method will be applied also to human studies.

* Corresponding author. Tel.: +358 40 3553 684; fax: +358 17 162 252.
E-mail address: Kristiina.Huttunen@uku.fi (K.M. Huttunen).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.04.033

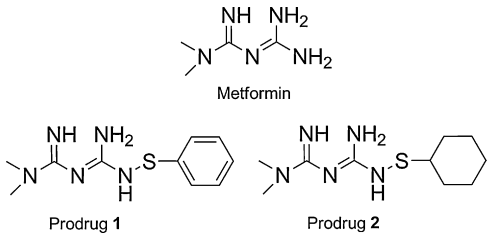

Fig. 1. Structures of metformin and two novel prodrugs of metformin.

2. Experimental

2.1. Chemicals and reagents

Metformin hydrochloride (1,1-dimethylbiguanide HCl), 1,1,3,3-tetramethylguanidine (TMG) and ethylenediaminetetraacetic acid disodium (EDTA) was purchased from Sigma–Aldrich (St. Louis, MO, USA), ultra-gradient HPLC-grade acetonitrile (ACN), HPLC gradient grade methanol and sodium chloride from J.T. Baker (Denventer, The Netherlands), ammonium acetate, sodium phosphate monobasic dihydrate and sodium phosphate dibasic dihydrate from Riedel-de Haën (Seelze, Germany). All reagents were of commercial high purity quality and they were used without further purification. The prodrugs **1** and **2** were synthesized and characterized as previously described [13]. Water was purified using a Milli-Q Gradient system (Millipore, Milford, MA, USA).

2.2. Biological material and animals

The pooled human and rat sera were obtained from normal human donors or control rats, respectively, and collected aseptically from whole blood. The pooled human and rat sera were stored at −80 °C until used. Adult male Wistar rats weighing 250 ± 5 g were supplied by the National Laboratory Animal Centre (Kuopio, Finland). Rats were housed in stainless steel cages on a 12 h light (07:00–19:00) and 12 h dark (19:00–07:00) cycle at an ambient temperature of 22 ± 1 °C with a relative humidity of 50–60%. All experiments were carried out during the light phase. Tap water and food pellets (Lactamin R36; Lactamin AB, Södertälje, Sweden) were available *ad libitum*. All procedures were reviewed and approved by the Animal Ethics Committee at the University of Kuopio.

2.3. Instrumentation and chromatographic conditions

The analyses were performed on the HPLC system, which consisted of a Agilent 1100 binary pump, a 1100 micro vacuum degasser, a HP 1050 Autosampler and a HP 1050 variable wavelength detector (operated at 235 nm) (Agilent Technologies, Waldbronn, Germany). The chromatographic separations were achieved on a Supelco Supelcosil LC-Si analytical column (4.6 mm × 250 mm, 5 μm) (Supelco Inc., Bellefonte, PA, USA) by using isocratic elution of acetonitrile and 10 mM ammonium acetate buffer (pH 5.0) with a ratio of 60:40 (v/v) at the flow rate 1.0 ml/min at room temperature.

2.4. Calibrations and quality control standards

Stock solutions (1 mg/ml) of metformin in 80% ACN, the prodrugs **1** and **2** in 90% ACN and stock solution (5.2 mg/ml) of 1,1,3,3-tetramethylguanidine (TMG, I.S.) in methanol were prepared and stored at +4 °C. Calibration standards (1, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μg/ml) were prepared daily from the stock solutions by mixing 100 μl of pooled human serum, 200 μl of ice-cold ACN, 200 μl of known amounts of metformin or the prodrug **1** or **2**, and 40 μl of I.S. stock solution. The mixtures were centrifuged at 14 000 rpm for 10 min and the supernatants were placed in HPLC-vials.

Quality control (QC) samples (10, 25, 50, 75, 100 μg/ml) were prepared daily from the stock solutions by mixing 100 μl of pooled human serum, 200 μl of ice-cold ACN, 200 μl of known amounts of metformin or the prodrug **1** or **2**, and 40 μl of I.S. stock solution. The mixtures were centrifuged at 14 000 rpm for 10 min and the supernatants were placed in HPLC-vials.

Reference control standards (10, 25, 50, 75, 100 μg/ml) were prepared daily from the stock solutions by mixing 100 μl of 50 mM phosphate buffer pH 7.4, 200 μl of ice-cold ACN, 200 μl of known amounts of metformin or the prodrug **1** or **2**, and 40 μl of I.S. stock solution. The mixtures were centrifuged at 14 000 rpm for 10 min and the supernatants were placed in HPLC-vials.

2.5. Assay validation

Assay performance was evaluated through determination of specificity, linearity, accuracy, precision, recovery, and stability. Specificity was evaluated by comparing the chromatograms obtained from the reference control standards of metformin, the prodrugs **1** and **2**, and the internal standard with a blank sample of the drug-free pooled human serum for interference of endogenous compounds. The calibration standards (1, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μg/ml) were analyzed before each analytical batch and the calibration curves were constructed by plotting peak area ratio (y) of metformin or the prodrug **1** or **2** to the internal standard versus nominal concentrations of each analyte (x). Linearity was evaluated using linear regression analysis. Intra-day and inter-day accuracy as well as precision were determined by analyzing six parallel QC samples at each concentration level (10, 25, 50, 75 and 100 μg/ml) on the same day and on three different days. Accuracy was calculated by comparing the mean experimental concentrations of assayed QC samples with their nominal values, and percentage values were used as an index. Relative standards deviation (RSD) of the concentrations was used as an index of intra-day and inter-day precision. Furthermore, a separate system suitability test was determined daily just before analyzing each set of samples by performing six replicate injections of reference control sample (50 μg/ml). RSD of peak areas and retention times were calculated and accepted with criteria of 2% and 0.5%, respectively. Recoveries of metformin and the prodrugs **1** and **2** were determined by comparing the mean experimental concentrations of assayed QC samples (10, 25, 50, 75 and 100 μg/ml) with the mean experimental concentrations of assayed reference control standards (10, 25, 50, 75, 100 μg/ml). Stabilities of metformin and the prodrug **1** and **2** in QC samples (10, 50, 100 μg/ml) were evaluated after 24 h at room temperature and after three freeze and thaw cycles (freezing at −80 °C for 24 h and completely thawing at room temperature) by comparing the mean experimental concentrations of assayed QC samples before and after the stability period, and percentage values were used as an index. Stock solution stabilities were also evaluated after one week, two weeks, and 1 month at +4 °C.

2.6. Application

The method was used to determine the concentration of metformin and the prodrugs **1** and **2** in plasma samples from rats after intravenous administration of metformin and the prodrugs **1** and **2** to evaluate the bioconversion of the prodrugs to the parent drug *in vivo*. Metformin and the prodrug **2** were also administered orally to rats to evaluate the absorption and bioavailability of these compounds. The procedures for the pretreatment of rats including the cannulation of the right jugular and/or left femoral veins (for intravenous drug administration and blood sampling) are reported in earlier studies [13]. Metformin hydrochloride (35 mg/kg, 53 μmol) and the prodrug **1** (50 mg/kg, 53 μmol) were dissolved in 0.9% NaCl solution and the prodrug **2** (67 mg/kg, 69 μmol) was dissolved in

10% HP-β-CD in 0.6% NaCl solution. The solutions were administered *via* the jugular or femoral vein over 0.5 min (total injection volume of 0.3 ml) to the rats. An approximately 200 μl aliquot of blood was collected *via* the jugular vein at given time points after intravenous administration of metformin or the prodrugs. The drawn blood volume was substituted by 0.9% NaCl immediately after each blood sampling and the samples were analyzed by HPLC immediately after sample preparation.

Metformin hydrochloride (140 mg/kg, 0.2 mmol) was dissolved in 0.9% NaCl solution and the prodrug **2** (100 mg/kg, 0.1 mmol) was dissolved in 10% HP-β-CD in 0.6% NaCl solution. The solutions were administered orally (total injection volume of 1.0 ml) to the rats using a feeding tube and the blood samples were collected as described above.

2.7. Sample preparation

An approximately 200 μl aliquot of blood from rats after intravenous or oral administration of metformin or the prodrugs **1** or **2** in a 2.0 ml polypropylene microcentrifuge tube was centrifuged with 20 μl of 3% ethylenediaminetetraacetic acid disodium (EDTA) in 0.7% NaCl solution for 5 min at 14 000 rpm immediately after each blood sampling. 100 μl aliquot of each plasma sample and 40 μl of 5.2 mg/ml I.S. stock solution were vortexed (about 10 s) with 400 μl of ice-cold ACN in a 2.0 ml polypropylene microcentrifuge tube to precipitate proteins and quench the degradation reaction. The samples were centrifuged 10 min at 14 000 rpm and kept on ice until the supernatants were injected into the HPLC system.

3. Results and discussion

3.1. Method development

In the present study, a bare silica column was used for the chromatographic separation for highly polar metformin and its prodrugs **1** and **2** (Fig. 1), since the very hydrophilic metformin was easily retained on the stationary phase under any elution condition [31–33,35]. In the development of the method, C18 and C8 analytical columns with different buffer systems (with and without ion pair reagents) were also tested, but the peak symmetries and retention times were unsatisfactory. It was difficult to separate metformin from the biological material without an ion pair reagent, while the prodrugs eluted very early from the column when using the one. Furthermore, these conditions required complex gradient elution methods in order to elute the prodrugs (in case of non-ion pair method) or metformin (in case of ion-pair method) out of the column. A good separation between metformin, less polar prodrugs, and I.S. was accomplished with silica column by using ammonium acetate buffer pH 5.0–ACN (40:60) as a mobile phase. In the HILIC approach higher ACN content in the mobile phase was used to retain polar compounds (metformin and I.S.) to the stationary phase while less polar compounds (the prodrugs **1** and **2**) eluted earlier from the column.

Since metformin and 1,1,3,3-tetramethylguanidine (TMG, I.S.) are strongly basic and rather polar molecules and the more lipophilic prodrugs of metformin are still quite hydrophilic (log *D* values −0.76–0.49) [13], we used a simple and rapid protein precipitation and extraction as a sample preparation method for serum and plasma samples. Although it has been commented that the use of protein precipitation sacrifices sensitivity and is not effective in removing endogenous substances [19,21,23], we found that a plasma to ACN ratio of 1:4 is the most effective method for complete protein precipitation and compound extraction from the proteins. Furthermore, the supernatants were injected directly into the HPLC after protein precipitation, which also makes the method very simple.

TMG was used as an internal standard in the present study, even though it is more polar than metformin and the prodrugs **1** and **2**, and prolongs the total running time of the assay. It was challenging to find a suitable structurally related internal standard, since more lipophilic biguanidines, like phenformin and buformin, have retention times close to the prodrugs **1** and **2**. Due to the high polarity, TMG does not bind to plasma proteins, is easy to extract, and exhibits a similar recovery as metformin and the prodrugs **1** and **2** (data not shown). The selected amount of the I.S. was rather high, since the range of the method (1–100 μg/ml) was very wide, and therefore presented a peak broadening and tailing of the I.S. caused by silanol interactions of this highly basic compound (Fig. 2). However, this can be overcome by using a smaller amount of I.S. or stronger mobile phase (25 or 50 mM). In the present method, the peak tailing did not cause any problems with accuracy or precision.

3.2. Assay performance

The proposed method is suitable for simultaneous quantification of metformin and the prodrugs **1** or **2** in human serum samples. Since the present method was applied to preliminary bioactivation and pharmacokinetic studies of metformin and its prodrugs in rats, the specificity, accuracy, precision, and recovery were analyzed also from rat serum samples ($n = 3$, data not shown). The method was validated for human serum, since the method will be used later on for the studies with other animals and humans.

The method is specific, since metformin, the prodrugs **1** and **2**, and I.S. were totally separated and no interfering peaks from endogenous components of pooled human or rat serum were observed (Fig. 2). Retention times were approximately 9.5 min for metformin, 6.2 min for prodrug **1**, 6.5 min for prodrug **2**, and 11.8 min for I.S. at the flow rate 1 ml/min. Since we did not have a column oven and the room temperature was unstable during the time of study (summer), the retention times varied slightly between the days of analysis. However, this was not a problem, because the method was specific (no interfering peaks were observed) and the identification of the compounds was always based on the standards. We also found that the flow rate can be accelerated up to 2 ml/min without excessively increasing the pressure in the HPLC. With the higher flow rate, the retention times of metformin, I.S., and the prodrugs **1** and **2** can be halved and the total running time of the assay can be reduced to less than 10 min. However, we used 1 ml/min flow rate during the method validation.

The calibration curves were linear over the concentration range 1–100 μg/ml. For this validation, the lower limit of quantification (LLOQ) was set at 1 and 10 μg/ml was used as a lowest QC sample in the validation. The mean equation and the mean correlation coefficients of the calibration curves ($n = 3$) with standard deviation shown in parentheses are presented in Table 1. Deviation of the calibration standards from their nominal concentrations was always less than 20% for LLOQ and less than 15% for standards other than LLOQ. LLOQ was also reproducible in terms of accuracy (80–120%) (data not shown). The values obtained for intra-day and inter-day precision and accuracy as well as for recoveries are shown in Table 2. For all QC levels of metformin and the prodrugs **1** and **2**, the intra-day precision was less than 6% (RSD) and the mean accuracy was within 100 ± 14% whereas the inter-day precision was less than

Table 1
Linearity of the method ($n = 3$).

Compound	Equation of calibration curve	Correlation coefficient
Metformin	0.029 (0.005) × −0.032 (0.050)	0.994 (0.005)
Prodrug **1**	0.019 (0.003) × −0.070 (0.033)	0.996 (0.003)
Prodrug **2**	0.010 (0.001) × −0.017 (0.010)	0.997 (0.002)

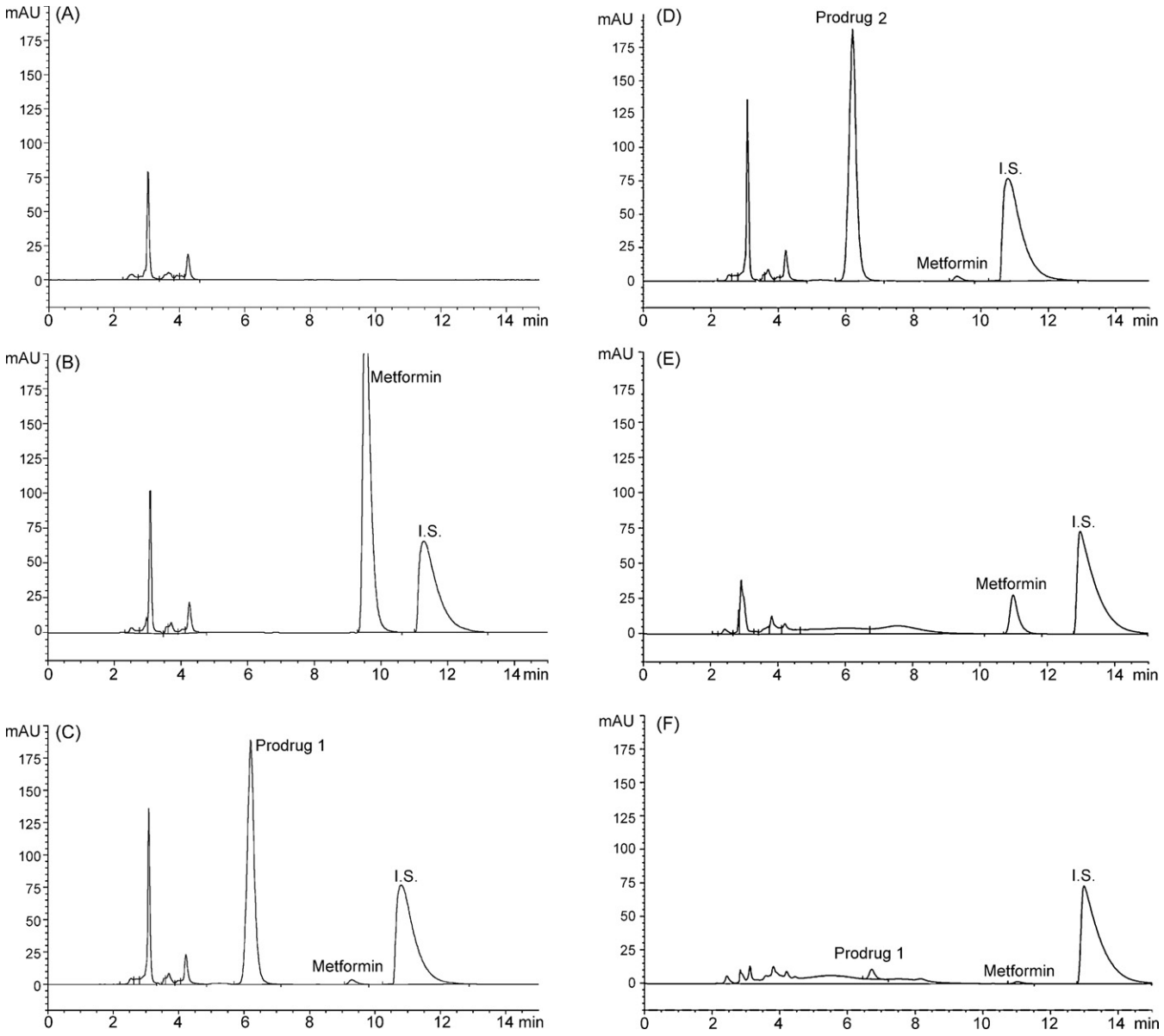

Fig. 2. Representative chromatogram of (A) a blank serum; (B) serum spiked with 50 μg/ml of metformin + I.S.; (C) serum spiked with 50 μg/ml of the prodrug **1** + I.S.; (D) serum spiked with 50 μg/ml of the prodrug **2** + I.S.; (E) serum from a rat containing 6.8 μg/ml of metformin 6 min after i.v. administration of 8.78 mg of metformin in 0.9% NaCl-solution + I.S.; (F) serum from a rat containing 1.2 μg/ml of the produg **1** and less than 1 μg/ml of metformin 20 min after p.o. administration of 25 mg of the prodrug **1** in 10% HP-β-CD in 0.6% NaCl-solution + I.S.

8% (RSD) and the mean accuracy was within 100 ± 10%. The mean recoveries at all QC levels of metformin and the prodrugs **1** and **2** were within 100 ± 10%. The mean concentration values obtained on three separate days are presented in Table 3 and they did not show significant differences from each other.

The stock solutions of metformin and the prodrug **2** were stable for four weeks at +4 °C whereas the stock solution of the prodrug **1** was stable for only two weeks and the stock solution of I.S. was stable for less than one week at +4 °C. Therefore, the stock solution of I.S. was freshly prepared daily before the analyses. The short-term stability (24 h at room temperature) and the freeze and thaw stability of metformin and the prodrugs **1** and **2** are presented in Table 4. Metformin and the prodrug **2** were not degraded significantly (more than 10%) at the concentrations of 50 and 100 μg/ml at room temperature within 24 h. However, the stabilities of these compounds were not acceptable at the concentration of 10 μg/ml. The prodrug **1** was unstable at these conditions, and therefore, these samples were injected into HPLC and analyzed immediately after sample preparation. Thus, the freeze and thaw stability of the prodrug **1** was not determined and precautions were always taken when handling these samples. In contrast, metformin and the prodrug **2** were not degraded significantly (more than 20%) during three freeze (24 h at −80 °C) and thaw cycles.

The chemical stability of the prodrugs at various pH values and in oxidative conditions has been evaluated previously [13]. As a result the prodrug **1** was unstable in acidic, basic, and oxidative conditions while the prodrug **2** showed higher stability in all tested conditions.

The method was also validated with rat serum samples and it proved to be specific (no interfering peaks were observed), accurate (100 ± 10%), and precise (RSD less than 6%) for metformin and the prodrugs **1** and **2**, and the recoveries of each compound were within 100 ± 11%.

Table 2
Intra-day ($n = 6$) and inter-day ($n = 3$) accuracy and precision of the method, and recoveries ($n = 6$) of metformin and the prodrugs **1** and **2** in human serum samples.

Nominal concentration (μg/ml)	Accuracy (%)[a]		Recovery (%)[b]		Precision (RSD%)
	Intra-day	Inter-day	Intra-day	Inter-day	
Metformin					
10	111.08 ± 3.49	109.08 ± 4.81	6.20	8.00	110.17 (7.79)
25	99.93 ± 3.81	101.85 ± 2.19	3.81	6.62	96.71 (2.01)
50	97.95 ± 4.71	100.23 ± 4.69	4.82	5.50	103.29 (5.57)
75	102.45 ± 5.27	98.52 ± 3.39	1.62	5.59	92.10 (5.96)
100	102.60 ± 5.16	103.26 ± 0.84	2.53	7.13	103.45 (6.96)
Prodrug **1**					
10	109.37 ± 2.09	108.60 ± 10.32	1.91	5.77	105.81 (5.69)
25	100.71 ± 6.12	100.95 ± 2.39	6.07	6.40	97.85 (7.60)
50	99.86 ± 3.85	98.35 ± 1.47	3.85	6.78	100.10 (7.13)
75	102.56 ± 2.45	101.11 ± 1.82	2.50	7.89	98.38 (3.93)
100	105.90 ± 7.22	102.82 ± 2.92	6.00	5.51	104.81 (9.83)
Prodrug **2**					
10	113.65 ± 2.37	110.47 ± 16.14	4.08	6.73	104.04 (3.53)
25	98.33 ± 3.06	100.71 ± 5.33	2.23	4.27	95.88 (4.33)
50	99.16 ± 7.31	99.19 ± 8.75	2.15	4.47	101.34 (11.05)
75	97.41 ± 5.72	96.40 ± 5.26	1.18	4.57	102.64 (6.37)
100	102.23 ± 6.13	102.62 ± 5.46	5.32	5.59	99.78 (7.24)

[a] Mean values ± SD.
[b] RSD% values presented in parentheses.

Table 3
Inter-day accuracy and precision of the method on three different days ($n = 3$).

Nominal concentration (μg/ml)	Day 1	Day 2	Day 3
	Metformin (mean ± 95% confidence intervals, μg/ml)		
10	10.261 ± 0.708	10.408 ± 0.856	11.366 ± 0.739
25	26.100 ± 2.060	25.874 ± 2.153	24.982 ± 0.999
50	52.593 ± 2.436	51.872 ± 2.853	48.981 ± 2.479
75	78.763 ± 4.058	75.107 ± 2.148	75.202 ± 1.515
100	104.938 ± 11.693	100.791 ± 3.497	100.705 ± 3.164
	Prodrug **1** (mean ± 95% confidence intervals, μg/ml)		
10	10.193 ± 0.508	10.987 ± 0.688	10.937 ± 0.220
25	24.972 ± 1.799	25.153 ± 0.905	25.178 ± 1.605
50	50.279 ± 2.831	50.219 ± 4.715	49.932 ± 2.018
75	75.380 ± 1.821	72.998 ± 3.092	77.127 ± 2.023
100	101.710 ± 7.854	105.167 ± 5.237	104.030 ± 7.745
	Prodrug **2** (mean ± 95% confidence intervals, μg/ml)		
10	11.043 ± 1.244	10.757 ± .0461	11.365 ± 0.249
25	24.464 ± 1.692	25.124 ± 0.588	24.584 ± 0.804
50	49.650 ± 1.458	50.316 ± 1.136	49.581 ± 3.841
75	72.654 ± 1.458	76.327 ± 0.945	73.058 ± 4.511
100	101.071 ± 5.766	104.849 ± 5.856	102.232 ± 6.443

3.3. Application

The present method was applied for the determination of metformin and the prodrugs **1** and **2** in plasma samples from rats after intravenous and oral administration instead of serum samples to ease rapid blood sampling during the studies. Therefore, the calibration standards were also prepared in rat plasma samples in these studies. This was acceptable, since the used anticoagulant, EDTA, did not interfere with the peaks of the analytes. The mean plasma concentration–time curves of metformin after intravenous administration of metformin and the prodrug **2** to rats are illustrated in Fig. 3. The approximate area under the plasma concentration–time

Table 4
Stability of metformin and the prodrugs **1** and **2** at room temperature (short-term stability) and after repeated freezing and thawing (mean values ± SD, $n = 3$).

Nominal concentration (μg/ml)	Metformin (%)	Prodrug **1** (%)	Prodrug **2** (%)
Short-term stability			
10	120.60 ± 6.06	50.82 ± 1.58	70.90 ± 2.46
50	106.41 ± 3.56	36.91 ± 1.19	97.58 ± 0.56
100	105.89 ± 3.39	45.61 ± 1.55	111.75 ± 3.22
Freeze and thaw stability			
10	101.76 ± 0.93	–[a]	81.98 ± 7.40
50	82.04 ± 3.95	–[a]	113.71 ± 5.71
100	80.29 ± 2.38	–[a]	113.05 ± 8.11

[a] Not determined.

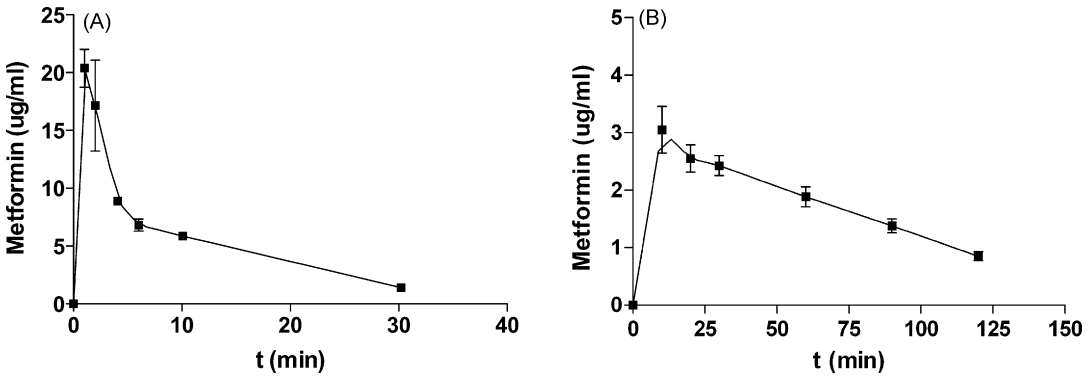

Fig. 3. Mean plasma concentration–time profile of metformin (■) after intravenous administration of metformin (A) and the prodrug **2** (B) at the dose of 35 mg/kg and 67.0 mg/kg, respectively, to rats (mean ± SD, $n = 3$).

curve ($AUC_{0-\infty}$) of the prodrug **2**, measured in longer time period, was similar to the $AUC_{0-\infty}$ of metformin, which indicates that the prodrug **2** was quantitatively bioconverted to metformin *in vivo* after intravenous administration. Furthermore, the bioavailability of metformin after oral administration was increased from 43% to 63% with this prodrug strategy. Therefore, it can be concluded from these preliminary results that the method is suitable for the simultaneous determination of metformin and its more lipophilic prodrugs in rat plasma/serum samples and can be used for further studies with humans. For these studies, the method can be easily adapted to the mass spectrometry to improve the sensitivity of the method. Furthermore, these novel prodrugs of metformin could be used to enhance the absorption of metformin, which may improve the clinical usefulness of this widely used anti-diabetic agent.

4. Conclusions

A HILIC method for the simultaneous determination of metformin and its more lipophilic prodrugs was successfully developed and validated. The method proved to be simple, specific, accurate, and precise over the range 1–100 μg/ml and it will be suitable for pharmacokinetic and bioavailability studies of metformin and its prodrugs in human or rat blood samples.

Acknowledgements

We thank Professor Tomi Järvinen and Marko Lehtonen, M.Sc., for the great scientific contribution, as well as Mrs. Tiina Koivunen, Ms. Miia Niskanen and Pedro Diamantino, M.Sc., for their skilful technical assistance. The work was financially supported by the National Graduate School of Organic Chemistry and Chemical Biology, the Finnish Funding Agency for Technology and Innovation (Tekes), the Research and Funding Foundation of Farmos, the Diabetes Research Funding Foundation and the Emil Aaltonen Foundation, the Alfred Kordelin Foundation (the Gust. Komppa fund), the Finnish Konkordia Foundation, and Jenny and Antti Wihuri Foundation.

References

[1] M.B. Davidsen, A.L. Peters, Am. J. Med. 102 (1997) 99–110.
[2] D. Kirpichnikov, S.I. McFarlane, J.R. Sowers, Ann. Intern. Med. 137 (2002) 25–33.
[3] G.T. Tucker, C. Casey, P.J. Phillips, H. Connor, J.D. Ward, H.F. Woods, Br. J. Clin. Pharmacol. 12 (1981) 235–246.
[4] P. Dandona, V. Fonseca, A. Mier, A.G. Beckett, Diabetes Care 6 (1983) 472–474.
[5] A.J. Krentz, R.E. Ferner, C.J. Bailey, Drug Saf. 11 (1994) 223–241.
[6] A.J. Garber, T.G. Duncan, A.M. Goodman, D.J. Mills, J.L. Rohlf, Am. J. Med. 103 (1997) 491–497.
[7] G. Belcher, C. Lambert, G. Edwards, R. Urquhart, D.R. Matthews, Diabetes Res. Clin. Pract. 70 (2005) 53–62.
[8] P. Ettmayer, G.L. Amidon, B. Clement, B. Testa, J. Med. Chem. 47 (2004) 2393–2404.
[9] V.J. Stella, Expert Opin. Ther. Patents 14 (2004) 277–280.
[10] J. Rautio, H. Kumpulainen, T. Heimbach, R. Oliyai, D. Oh, T. Järvinen, J. Savolainen, Nat. Rev. Drug Discov. 7 (2008) 255–270.
[11] V.R. Guarino, V. Karunaratne, V.J. Stella, Bioorg. Med. Chem. Lett. 17 (2007) 4910–4913.
[12] J.N. Hemenway, K. Nti-Addae, V.R. Guarino, V.J. Stella, Bioorg. Med. Chem. Lett. 17 (2007) 6629–6632.
[13] K.M. Huttunen, A. Mannila, K. Laine, E. Kemppainen, J. Leppänen, J. Vepsäläinen, T. Järvinen, J. Rautio, submitted for publication.
[14] R. Huupponen, P. Ojala-Karlsson, J. Rouru, M.J. Koulu, J. Chromatogr. 583 (1992) 270–273.
[15] M.S. Arayne, N. Sultana, M.H. Zuberi, Pak. J. Pharm. Sci. 19 (2006) 231–235.
[16] V. Porta, S.G. Schramm, E.K. Kano, E.E. Koono, Y.P. Armando, K. Fukuda, C.H. Serra, J. Pharm. Biomed. Anal. 46 (2008) 143–147.
[17] K.H. Yuen, K.K. Peh, J. Chromatogr. B Biomed. Sci. Appl. 710 (1998) 243–246.
[18] C.-L. Cheng, C.-H. Chou, J. Chromatogr. B Biomed. Sci. Appl. 762 (2001) 51–58.
[19] H. Amini, A. Ahmadiani, P. Gazerani, J. Chromatogr. B Biomed. Sci. Appl. 824 (2005) 319–322.
[20] A. Zarghi, S.M. Foroutan, A. Shafaati, A. Khoddam, J. Pharm. Biomed. Anal. 31 (2003) 197–200.
[21] S. AbuRuz, J. Millership, J. McElnay, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 798 (2003) 203–209.
[22] B.G. Charles, N.W. Jacobsen, P.J. Ravenscroft, Clin. Chem. 27 (1981) 434–436.
[23] O. Vesterqvist, F. Nabbie, B. Swanson, J. Chromatogr. B Biomed. Sci. Appl. 716 (1998) 299–304.
[24] A.R. Bonfigli, S. Manfrini, F. Gregorio, R. Testa, I. Testa, G. De Sio, G. Coppa, Ther. Drug Monit. 21 (1999) 330–334.
[25] M. Zhang, G.A. Moore, M. Lever, S.J. Gardiner, C.M. Kirkpatrick, E.J. Begg, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 766 (2002) 175–179.
[26] N. Koseki, H. Kawashita, M. Niina, Y. Nagae, N. Masuda, J. Pharm. Biomed. Anal. 36 (2005) 1063–1072.
[27] K. Heinig, F. Bucheli, J. Pharm. Biomed. Anal. 34 (2004) 1005–1011.
[28] Y. Wang, Y. Tang, J. Gu, J.P. Fawcett, X. Bai, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 808 (2004) 215–219.
[29] X. Chen, Q. Gu, F. Qiu, D. Zhong, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 802 (2004) 377–381.
[30] M.A. Marques, A. de, S. Soares, O.W. Pinto, P.T. Barroso, D.P. Pinto, M. Ferreira-Filho, E. Werneck-Barroso, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 852 (2007) 308–316.
[31] H.N. Mistri, A.G. Jangid, P.S. Shrivastav, J. Pharm. Biomed. Anal. 45 (2007) 97–106.
[32] A.J. Alpert, J. Chromatogr. 499 (1990) 177–196.
[33] T. Ikegami, H. Fujita, K. Horie, K. Hosoya, N. Tanaka, Anal. Bioanal. Chem. 386 (2006) 578–585.
[34] Z. Hao, B. Xiao, N. Weng, J. Sep. Sci. 31 (2008) 1449–1464.
[35] N.C. van de Merbel, G. Wilkens, S. Fowles, B. Oosterhuis, J.H.G. Jonkman, Chromatographia 47 (1998) 542–546.