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

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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.

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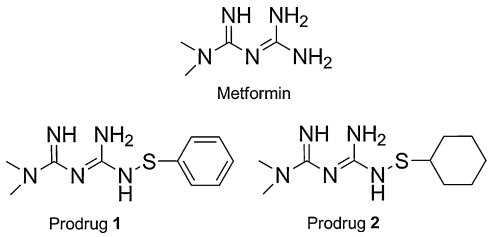

Fig. 1. Structures of metformin and two novel prodrugs of metformin.

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