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Liquid chromatography–tandem mass spectrometry method for simultaneous quantification of bisoprolol, ramiprilat, propranolol and midazolam in rat dried blood spots

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ABSTRACT

Dried blood spot (DBS) sampling represents a suitable method for pharmacokinetic studies in rats, particularly if serial sampling is needed. To study the pharmacokinetics of drugs in a rat heart failure (HF) model, we developed and validated a method for the simultaneous determination of bisoprolol, ramiprilat, propranolol and midazolam in DBS samples. Bisoprolol and ramipril are widely used in the treatment of HF, and midazolam and propranolol are markers of hepatic metabolism, which can be altered in HF. A 20 μL sample of rat blood was pipetted onto Whatman 903 Protein Saver Card and allowed to dry. The whole spot was excised and 300 μL of solvent (methanol with 10% ultrapure water and 0.1% formic acid) was added. After mixing and incubating the sample in an ultrasonic bath, a mixture of isotopically labeled internal standards was added. After centrifugation, the extracts were cleaned on an Ostro™ plate and analyzed using liquid chromatography–tandem mass spectroscopy. The method was successfully validated. No significant interference was observed in the retention times of analytes or internal standards. The intraday and interday accuracy and precision were within a ±15% interval. The method was linear in the range 5–250 μg/L and the lower limit of quantification was 5 μg/L for all four analytes. The absolute matrix effect ranged from 98.7% for midazolam to 121% for ramiprilat. The recovery was lowest for ramiprilat and highest for propranolol. Samples were stable at all tested temperatures. The method has been used successfully in a real-time pharmacokinetic study in rats.

1. Introduction

In any pharmacokinetic study, the pharmacokinetic profile of a drug is assessed by sampling at multiple time points. In such studies in humans, several milliliters of blood can be withdrawn on any single occasion without affecting the patient's health [1], but in rats the blood volume that can be withdrawn is considerably smaller [2]. An average rat weighing 200 g has approximately 13 mL of blood in circulation [3], thus is not possible to obtain 1 mL of blood at each time point for determination of the drug concentration in plasma. In the past, one animal or even a group of animals frequently had to be killed at each time point; DBS sampling, on the other hand, requires only 10–20 μL of blood at each time point, thus pharmacokinetic profiles can be determined using multiple blood samples from an individual rat. The use of rat DBS samples, instead

Abbreviations: DBS, dried blood spot; EMA, European Medicines Agency; FDA, Food and Drug Administration; LLOQ, lower limit of quantification; ME, matrix effect; RSD, relative standard deviation; QC, quality control sample; QC_l, quality control sample of 15 μg/L (low concentration); QC_m, quality control sample of 75 μg/L (medium concentration); QC_h, quality control sample of 200 μg/L (high concentration).

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of regular plasma samples, significantly reduces the number of animals needed for experiments and allows repeated determination of pharmacokinetics in the same animals (e.g. in a disease model) [4].

The main objective of this work was to develop and validate an analytical method that would enable simultaneous determination of four different drugs (bisoprolol, ramiprilat, midazolam and propranolol) in rat DBS samples. The method was developed to study the pharmacokinetics of these drugs in a rat heart failure model, using a multiple-sampling-times strategy in a single rat. The study protocol was designed to minimize the number of animals needed to achieve the study goals. Bisoprolol, a beta blocker, and ramipril, an angiotensin-converting-enzyme inhibitor, are widely used in the treatment of heart failure [5]. They have an important role in improving survival and reducing the number of hospitalizations in patients with chronic heart failure [6]. In this condition, blood flow to the liver is frequently reduced [7] or the liver is congested [8], thus hepatic metabolism is affected in a way that modifies drug pharmacokinetics. Propranolol is a marker of a hepatic blood flow and midazolam is a marker of intrinsic hepatic metabolism and activity of the cytochrome enzyme CYP3A4 [9,10]. Together, their pharmacokinetics provide information on the hepatic metabolism of drugs with high and low extraction ratios and thus help to explain possible changes in the metabolism of other drugs.

The technique of DBS sampling has gained popularity in recent years, and methods for measurement of bisoprolol, ramipril, propranolol or midazolam concentrations in human DBS have been developed [11–16]. Some of the methods measure combinations of drugs, but no method has yet been published for the specific combination of midazolam, propranolol, bisoprolol and ramiprilat. Moreover, with the exception of propranolol, no method has been established for measurement of the concentrations of these drugs in rat DBS samples [17]. We present the development and validation of an analytical method for simultaneous quantification of four marker drugs in rat DBS: midazolam, propranolol, bisoprolol and ramiprilat.

2. Experimental

2.1. Chemicals and materials

Bisoprolol fumarate and ramiprilat were purchased from Sequoia Research Products Ltd., Pangbourne, UK. Propranolol hydrochloride was obtained from Fluka–Sigma–Aldrich, Buchs, Switzerland, and midazolam from Cerilliant, Round Rock, TX, USA. For *in vivo* experiments, the same substances were used, except for midazolam (Midazolam Torrex parenteral solution 1 mg/mL, Chiesi Pharmaceuticals GmbH, Austria). The internal standards bisoprolol-D5, ramiprilat-D5 and racemic propranolol-D7 were purchased from Santa Cruz Biotechnology, CA, USA. [$^{13}C_6$]-midazolam was provided by Alsachim, Illkirch Graffenstaden, France.

Ultrapure water was obtained using the Milli-Q Advantage A10 Ultrapure Water Purification System (Millipore Corporation, Bedford, MA, USA). Methanol Chromasol V® was purchased from Sigma–Aldrich, Steinheim, Germany. Formic acid 98–100% Suprapur® was obtained from Merck KGAa, Darmstadt, Germany. Whatman 903 Protein Saver Cards (Whatman, UK) were used for spotting the blood samples. Spots were manually excised with scissors to ensure that the whole spot was included in the analysis. Ostro™ (Waters, USA) 96-well plates were used for the final removal of phospholipids from samples. The samples of whole blood of Wistar Han rats with K2-EDTA anticoagulant were obtained in accordance with the law on the protection of animals in the Republic of Slovenia and the Veterinary Administration of the Republic of Slovenia.

2.2. Working solutions

Stock solutions of bisoprolol, ramiprilat and propranolol were prepared by diluting an accurately weighed amount of each drug in methanol to yield drug concentrations of 1 mg/mL. Midazolam stock solution was purchased in the form of a 1 mg/mL methanol solution. The primary solution was prepared by mixing 25 μL of each stock solution and further diluting it to a final volume of 5 mL to obtain a mixture of all four drugs at a concentration of 5 mg/L for each one. Working solutions were prepared by diluting appropriate volumes of the primary solutions with a mixture of 25% methanol and 75% 0.1% formic acid.

Stock solutions of internal standards (bisoprolol-D5, ramiprilat-D5, racemic propranolol-D7 and [$^{13}C_6$]-midazolam) were prepared by dissolving weighed amounts in methanol to yield concentrations of 1 mg/mL. An appropriate volume of each solution was diluted with methanol to yield a mixture of internal standards: bisoprolol-D5 500 μg/L, ramiprilat-D5 1000 μg/L, propranolol-D7 1000 μg/L and [$^{13}C_6$]-midazolam 400 μg/L. All solutions were stored at −20 °C and brought to room temperature before use.

2.3. Preparation of calibration standards and quality control samples

Calibration standards and quality control (QC) samples were prepared by adding 20 μL of an appropriate working solution to 380 μL of blank rat blood, which was a mixture of blood obtained from three untreated rats. Samples were stirred for 15 min at 1300 rpm and 37 °C, and then 20 μL of each blood sample was spotted directly onto Whatman protein saver cards using a volumetric pipette (Eppendorf Multipette Plus). The cards were allowed to dry for at least 2 h at room temperature before processing or storage. For each drug, the concentrations of the calibration standards were 0, 5, 10, 25, 50, 100, 175 and 250 μg/L; the QC concentrations were 15 (QC_l), 75 (QC_m), and 200 μg/L (QC_h).

2.4. Extraction of dried blood samples

Whole DBS were cut out of the Whatman cards with scissors and transferred to 1.5 mL Eppendorf microtubes. A 300 μL volume of solvent (methanol with 10% ultrapure water and 0.1% formic acid) was added and vortex mixed for 1 min before the microtubes were put in an ultrasonic bath for 10 min. The tubes were then shaken on an orbital shaker for 45 min at 100 rpm (room temperature) before the addition of 35 μL of the internal standards mixture to each sample. Following centrifugation (10 min, 15,000 × *g*, 5 °C), portions of supernatant (280 μL) were transferred to an Ostro plate and filtered to a 96-well collection plate for liquid chromatography–tandem mass spectrometry analysis.

2.5. Chromatographic and mass spectrometry conditions

After extraction, centrifugation and purification, the DBS samples were analyzed using an Agilent 1290 Infinity liquid chromatography system coupled to a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, USA). The injection volume was 1 μL and the sample needle was washed with 80% methanol for 10 s after each injection. Chromatographic separation was achieved on a Kinetex C18 50 mm × 2.1 mm column with 2.6 μm particles, guarded by a C18 guard column (Phenomenex, Torrance, USA) at 50 °C. The mobile phase consisted of 0.1% formic acid in Milli-Q water (A) and 100% acetonitrile (B); the flow rate was set at 0.5 mL/min with the following gradient (time, % of mobile