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Short communication

Development and validation of UFLC–MS/MS method for determination of bosentan in rat plasma

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ABSTRACT

A rapid, simple and sensitive UFLC–MS/MS method was developed and validated for the determination of bosentan in rat plasma using etodolac as an internal standard (IS) after liquid–liquid extraction with diethyl ether–chloroform (4:1, v/v). Bosentan and IS were detected using electrospray ionization in positive ion multiple reaction monitoring mode by monitoring the transitions m/z 551.90 → 201.90 and 288.20 → 172.00, respectively. Chromatographic separation was performed on the inertsil ODS-4 column with a gradient mobile phase, which consisted of 0.1% acetic acid with 5 mM ammonium acetate in water for solvent A and 5 mM ammonium acetate in acetonitrile–methanol (50:50, v/v) for solvent B at a flow rate of 0.3 mL/min. The method was sensitive with 0.5 ng/mL as the lower limit of quantitation (LLOQ) and the standard calibration curve for bosentan was linear (r > 0.997) over the studied concentration range (0.5–2000 ng/mL). The proposed method was fully validated by determining specificity, linearity, LLOQ, precision and accuracy, recovery, matrix effect and stability. The validated method was successfully applied to plasma samples obtained from rats.

1. Introduction

Bosentan, [(4-*tert*-butyl-*N*-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl) pyrimidin-4-yl], is a competitive oral dual endothelin receptor antagonist which is non-selective for endothelin A and B receptors (Fig. 1). Bosentan is used as an oral drug for the treatment of pulmonary arterial hypertension [1,2]. It has a high protein binding (98%), especially to albumin and is rapidly absorbed after oral administration. Its bioavailability is 45–50%. The peak plasma concentration occurs within 3–5 h [3,4]. Bosentan is eliminated mainly by hepatic metabolism; renal elimination occurs for only 0.9% of the administered dose [5].

Few studies of high performance liquid chromatography with UV detection (HPLC-UV) [6,7] and liquid chromatography-tandem mass spectrometry (LC–MS/MS) methods have been presented for the determination of bosentan in biological matrices [8–14]. HPLC methods were long chromatographic run time, low sensitivity and, usually had large volumes of biological samples. The analytical time of each run in these reports were 24 min [6] and 16 min [7].

The relatively long chromatographic run time could not satisfy the requirement of high throughput determination of bosentan in plasma. Therefore, mass spectrometric detection coupled to an ultra fast liquid chromatography (UFLC) method has been considered as very important to performing bioanalytical analysis with speed, selectivity and sensitivity.

Therefore, we developed UFLC–MS/MS method for determination of bosentan in rat plasma. Then, the developed method was validated by using linearity, stability, precision, accuracy and sensitivity parameters according to Food and Drug Administration (FDA) guidelines [15].

Finally, plasma samples obtained from rats after oral administration of bosentan were analyzed in order to demonstrate the applicability of the method.

2. Experimental

2.1. Chemicals and reagents

The reference standard of bosentan (purity > 99%) was obtained from Actelion Pharmaceuticals (Allschwil, Switzerland). Etodolac (IS, purity > 99%) was generously supplied by Novagenix Company (Ankara – Turkey). High-purity grade acetonitrile, methanol, acetic

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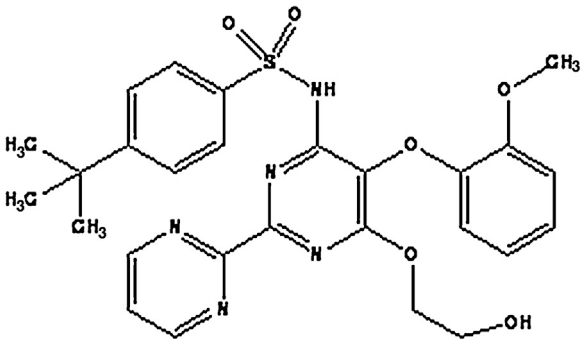

Fig. 1. Chemical structure of bosentan.

acid, ammonium acetate and all other reagents were purchased from Merck (Darmstadt, Germany) and were used without further purification.

2.2. Instrumentation and operation conditions

The Shimadzu UFLC-XR-MS/MS system consisted of an 8030 LC-MS/MS system, a CBM-20ALite system controller, two LC-20ADXR pumps with a micro gradient mixer and DGU-20A3R degasser, one SIL-20ACXR autosampler with a cooling function and, one CTO-20AC column oven (Shimadzu Co., Kyoto/Japan). All of the operations and analysis of data obtained were controlled by lab solutions Main software. Inertsil ODS-4 column (3 μm, 2.1 mm × 50 mm; GL Science, Japan) was employed for the separation at 40 °C. The mobile phase was composed of 0.1% acetic acid with 5 mM ammonium acetate in water for solvent A and 5 mM ammonium acetate in acetonitrile–methanol (50:50, v/v) for solvent B. The gradient was 30% B (0–3.50 min), 95% B (3.51–6.5 min) and 30% B (6.51–10 min). Efficient and symmetrical peaks were obtained at a flow rate of 0.3 mL/min and injection volume of 10 μL. Mass spectrometric detection was performed using ESI ion source in the positive ionization mode; the nebulizing gas, and drying gas flow rates and the ESI voltage were 3 L/min, 15 L/min and 4500 V, respectively. The collision gas was argon and had a pressure of 230 kPa. The gas used for nebulizing and drying was high pure nitrogen. MS data acquisition was conducted with the MRM mode in order to quantify and identify the investigated analytes. Detailed information for MS-parameters is represented in Table 1.

2.3. Preparation of standards and quality control samples

Stock solutions of bosentan and IS were prepared separately in the mobile phase solvent A–solvent B (20:80, v/v) at concentrations of 100 μg/mL. Working solutions of bosentan were prepared by serial dilution of the stock solution in the mobile phase. Calibration standards were prepared by spiking 20 μL of the appropriate standard solutions to 250 μL of blank plasma giving concentrations of 0.5, 5, 25, 100, 500, 1000 and 2000 ng/mL. Quality control (QC) samples were prepared in the same way as the calibration standards, to achieve low, medium and high concentrations of 3, 600 and 1800 ng/mL. IS working solution (1 μg/mL) for routine use was freshly prepared by diluting IS stock solution in the mobile phase. All the solutions were stored at −20 °C until analysis and were brought to room temperature before use. The standard and QC samples were extracted on each analysis run along with the procedure described below.

2.4. Plasma sample preparation

An aliquot of 250 μL plasma sample was transferred to a polypropylene tube; 20 μL of IS solution (100 ng/mL) were added and vortex was mixed for 30 s. 100 μL of methanol and 500 μL of 1 M HCl were added to the mixture. The mixture was vortexed for 30 s and extracted with 5 mL of diethyl ether–chloroform (4:1, v/v) by vortexing for another 60 s. After centrifugation at 3500 rpm for 8 min, the upper organic layer (4.9 mL) was then transferred into another clean polypropylene tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted with mobile phase solvent A–solvent B (20:80, v/v), and transferred into an autosampler vial. An aliquot of 1 μL was injected into the UFLC–MS/MS system for analysis.

2.5. Selection of internal standard

To meet the internal standard, short analysis time, good extraction recovery, chromatographic and mass spectrometric behaviour similar to the analytes is required. Also, analytes should be readily available. We evaluated different analytes such as etodolac, nimesulide, meloxicam, indomethacin, lidocaine, prilocaine and amlodipine as internal standards. Etodolac was selected as the internal standard for its similarity in retention time, mass conditions and extraction efficiency to those of the analytes.

2.6. Method validation

This method was fully validated for selectivity, linearity, precision and accuracy, recovery, matrix effect and stability according to FDA guidance for validation of bioanalytical methods [15].

2.6.1. Selectivity and specificity

The samples were prepared to determine whether endogenous matrix constituents interfere with the mass transitions chosen for bosentan and IS. From six different batches of blank rat plasma, blank (spiked with IS) and the LLOQ (at concentration of 0.5 ng/mL) samples were tested for interferences using the proposed liquid phase extraction procedure and UFLC–MS/MS conditions.

2.6.2. Linearity and sensitivity

The seven-point calibration curve over the concentration range of 0.5–2000 ng/mL was constructed by plotting the peak area ratio of BOS/IS against the nominal concentration of calibration standards in blank rat plasma. Calibration curves of peak area ratio as a function of nominal concentration were linear using weighted (1/×2) linear regression. The LLOQ was defined as the lowest concentration on the calibration curve. It has an acceptable accuracy (relative error, RE) within ±15% and a precision (relative standard deviation, RSD) below 15% can be obtained.

2.6.3. Precision and accuracy

The intra- and inter-day precision and accuracy in rat plasma were evaluated using three QC samples on the same day and on

Table 1
Detailed information of MS parameters for bosentan and IS.

Analyte	Precursor	Product	Dwell time	q1 pre-bias (V)	Collision energy (V)	q3 pre-bias (V)
Bosentan	551.90	201.90	100.00	−26.00	−38.00	−13.00
IS	288.20	172.00	100.00	−15.00	−14.00	−18.00