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Determination of pyrrole–imidazole polyamide in rat plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Pyrrole (Py)–imidazole (Im) polyamides synthesized by combining *N*-methylpyrrole and *N*-methylimidazole amino acids have been identified as novel candidates for gene therapy. In this study, a sensitive method using liquid chromatography–tandem mass spectrometry (LC–MS/MS) with an electrospray ionization (ESI) source was developed and validated for the determination and quantification of Py–Im polyamide in rat plasma. Py–Im polyamide was extracted from rat plasma by solid-phase extraction (SPE) using a Waters Oasis® HLB cartridge. Separation was achieved on an ACQUITY UPLC HSS T3 (1.8 μm, 2.1 × 50 mm) column by gradient elution using acetonitrile:distilled water:acetic acid (5:95:0.1, v/v/v) and acetonitrile:distilled water:acetic acid (95:5:0.1, v/v/v). The method was validated over the range of 10–1000 ng/mL and the lower limit of quantification (LLOQ) was 10 ng/mL. This method was successfully applied to the investigation of the pharmacokinetics of Py–Im polyamide after intravenous administration.

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1. Introduction

Pyrrole (Py)–imidazole (Im) polyamides are synthesized by combining *N*-methylpyrrole and *N*-methylimidazole amino acids [1–3]. Various combinations have enabled the recognition and binding to specific nucleotide sequences of double-helical DNA with high affinity [1]. Sequence-specific DNA-binding Py–Im polyamides inhibit gene expression by targeting the promoter regions of enhancer and transcription factor binding sites [4]. Moreover, Py–Im polyamide designed to bind to the activator protein-1 binding site of the rat transforming growth factor-β1 (TGF-β1) promoter was reported to significantly inhibit TGF-β1 promoter activity and the expression of TGF-β1 mRNA and protein in rat mesangial cells [5]. Therefore, Py–Im polyamides have been identified as novel candidates for gene therapy.

The molecular sizes of various types of Py–Im polyamides with the hairpin structure are much larger than those of normal candidate compounds used in medicine. For specific binding to the base sequence, Py–Im polyamides should be much longer and larger. For these large compounds, a determination method is required. The measurement of Py–Im polyamides of large molecular weight was developed and validated by high-performance liquid chromatography (HPLC) [6]. This method was applicable to pharmacokinetic study. However, the lower limit of quantification (LLOQ) for the measurement of Py–Im polyamides by HPLC was 1 μg/mL. In pharmacokinetic study, to investigate the pharmacokinetic characteristic for many hours, more sensitive methods like liquid chromatography–tandem mass spectrometry (LC–MS/MS) are required. Short and small polyamides of structure similar to that of Py–Im polyamide with *N*-methylpyrrole and *N*-methylimidazole were analyzed by LC–MS/MS [7–9]. However, in these analyses, the fragmentation of polyamide was mainly reported. To the best of our knowledge, no sensitive, quantitative or validated methods for the determination of Py–Im polyamide in biological samples using LC–MS/MS have been reported. Therefore, the aim of this study is to develop and validate a sensitive LC–MS/MS method for the

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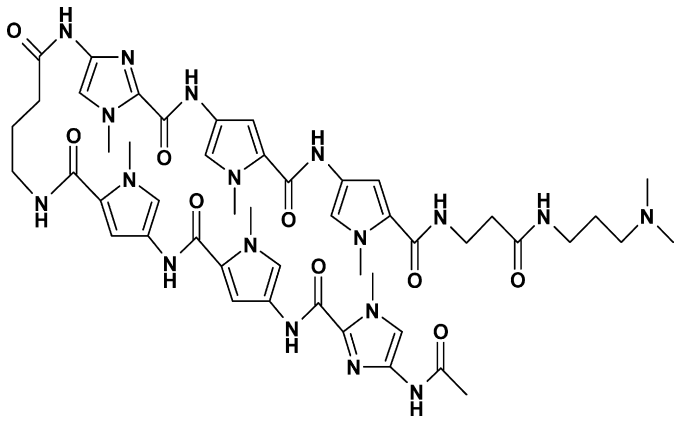

Fig. 1. Chemical structure of Py–Im polyamide.

determination and quantification of Py–Im polyamide in rat plasma, as well as for the determination of the pharmacokinetics of Py–Im polyamide after intravenous administration in rats.

2. Experimental

2.1. Chemicals and reagents

Py–Im polyamide was purchased from Gentier Biosystems Co., Ltd. (Kyoto, Japan). Py–Im polyamide composed of Ac–ImPyPy–ImPyPy–β–Dp (β: β-alanine, Dp: *N*,*N*-dimethylaminopropylamide) was designed (Fig. 1). The molecular weight of Py–Im polyamide calculated on the basis of standard atomic weights [10] was 1035.12, and the mono-isotopic mass was 1034.49. HPLC-grade methanol, HPLC-grade acetonitrile, LC–MS-grade acetic acid, and LC–MS-grade distilled water were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). All the other reagents used were of the highest quality and purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

2.2. Animals and drug administration

Three male Wistar rats (280–300 g) were obtained from Sankyo Labo Service Corporation (Tokyo, Japan). They were housed in a temperature-controlled room in a 12-h light–dark cycle and were given food and water *ad libitum*. They were acclimatized for at least one week before the experiments. A polyethylene tube (SP45; 0.58 mm I.D., 0.96 mm O.D., Natsume Seisakusho Co., Ltd.) was inserted into the right jugular vein and left femoral artery of the rats under anesthesia with pentobarbital sodium (Kyoritsu Seiyaku Corporation). After cannulation, the rats were left alone for at least one night before the experiments. Py–Im polyamide dissolved in water was administered intravenously into the jugular vein of the rats at a dose of 2.0 mg/kg. Blood samples (0.5 mL) were collected into heparinized 1.5 mL microtubes 30, 60, 90, 120, 150, 180, and 240 min after administration. After sampling, the collected blood was replaced with an equal volume of saline. Plasma was separated by centrifugation at 10,000 × *g* and 4 °C for 10 min and stored −20 °C until use. The animal study was approved by the Animal Ethics Committee of the College of Pharmacy, Nihon University.

2.3. Chromatography and mass spectrometry conditions

Chromatographic separation was performed with an ACQUITY Ultra Performance LC (UPLC) system (Nihon Waters K.K., Tokyo, Japan) with a pump, PDA detector, and autosampler using an ACQUITY UPLC HSS T3 (1.8 μm, 2.1 × 50 mm, Nihon Waters K.K., Tokyo, Japan) column with in-line filter, maintained at 40 °C. The autosampler temperature was maintained at 15 °C. The liquid flow rate was set at 0.30 mL/min. The mobile phase of solvent A was acetonitrile:water:acetic acid (5:95:0.1, v/v/v) and solvent B was acetonitrile:water:acetic acid (95:5:0.1, v/v/v). Samples were eluted by gradient with solvents A and B. The gradient started at the mobile phase A–B (95:5%), changed linearly to A–B (45:55%) until 2 min, washed with A–B (0:100%) until 3.5 min, and equilibrated at the initial condition until 5.5 min. The injection volume was 10 μL. The UPLC system was coupled with a Quattro Premier XE (Nihon Waters K.K., Tokyo, Japan) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source and operating in the positive mode. MassLynx version 4.1 software (Nihon Waters K.K., Tokyo, Japan) was used to control all the parameters of UPLC and MS and to collect the data. An MS scan to obtain the precursor ion of the analyte was performed (refer to Fig. 2). In the MS scan, Py–Im polyamide represents a cluster of ions near *m*/*z* 1036, 519 and 346, which corresponds to $[M+H]^+$, $[M+2H]^{2+}$, and $[M+3H]^{3+}$, respectively, owing to the presence of many isotopes. Quantitation was performed using the multiple reaction monitoring (MRM) to study precursor → product ion transitions for Py–Im polyamide (*m*/*z* 518.60 → 288.16). Figs. 2 and 3 show the mass spectra's precursor and product ions for the analyte, respectively. Source- and compound-dependent parameters were optimized as follows: capillary voltage, 0.50 kV; cone voltage, 27.0 V; source temperature, 120 °C; desolvation temperature, 400 °C; cone gas flow, 50 L/h; RF lens at 0.2 V (RF lens voltage focuses ions toward the center of the quadrupole); multiplier, 700 V; desolvation gas flow, 800 L/h (nitrogen); collision gas flow, 0.30 mL/min (argon); collision energy, 20.0 eV; ion energy 1, 0.5; ion energy 2, 2.0; LM and HM 1 resolution, 10; LM and HM 2 resolution, 10; dwell time, 0.25 s; interscan delay, 0.02 s. Precursor and product ion were evaluated at different cone voltages and collision energies. The capillary voltage giving the highest peak area of MRM transitions (*m*/*z* 518.60 → 288.16) was investigated by changing capillary voltages.

2.4. Data evaluation

QuanLynx, a component of MassLynx, was used for the generation of each calibration curve. The output was based on a least-squares linear regression analysis, with weighting factor $(1/x^2)$, of the peak area of Py–Im polyamide against the nominal analyte concentration. The least-squares regression line was not forced through the origin (0, 0), and no blank (zero concentration) samples were included in the calibration curve. The concentrations of quality control (QC) and unknown samples were determined by back calculation (interpolation) using the calibration curve.

2.5. Preparation of stock solutions and working solution

The master stock solution of Py–Im polyamide was prepared by dissolving the compound in water at 2.0 mg/mL. In addition, a working stock solution was prepared at 200 μg/mL by diluting 2.0 mg/mL master stock solution with water. The working stock solution was stored at −20 °C until use. Working solutions were serially diluted with water:acetonitrile (1:1, v/v) to obtain the desired concentrations.

2.6. Standard and quality control preparation

The calibration curve samples and QC samples were prepared by spiking 54 μL of blank rat plasma with 6 μL of appropriate working solution. Calibration curve samples were made at concentrations of 10, 20, 50, 100, 200, 500, 750, and 1000 ng/mL and QC samples were prepared at concentrations of 10, 20, 200, and 1000 ng/mL by diluting the highest concentration of the working solutions in the same manner as the calibration curve samples. Rat samples were

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