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Short communication

Optimization and validation of a high-performance liquid chromatographic method with UV detection for the determination of pyrrole–imidazole polyamides in rat plasma

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Abstract

A simple and sensitive high-performance liquid chromatography (HPLC) method utilizing UV detection was developed for the determination of plasma pyrrole (Py)–imidazole (Im) polyamides in rats and applied to the pharmacokinetic study of compounds. After deproteinization of plasma with methanol, Py–Im polyamides were analyzed with a reversed-phase TSK-GEL ODS-80T$_M$ (4.6 mm × 15.0 cm TOSOH Co., Japan) column maintained at 40 °C. The mobile phase solvent A was 0.1% acetic acid and the solvent B was HPLC-grade acetonitrile (0–10 min, A: 100–20%, B: 0–80% linear gradient; 10–15 min, A: 40%, B: 60%). The flow rate was 1.0 ml/min. The detection wavelength was set at 310 nm. The method was used to determine the plasma concentration time profiles of Py–Im polyamides after intravenous injection.

Keywords: Pyrrole–imidazole polyamide; HPLC; Rat plasma; Pharmacokinetics

1. Introduction

Pyrrole (Py)–imidazole (Im) polyamides are small synthetic molecules composed of the aromatic rings of *N*-methylpyrrole and *N*-methylimidazole amino acid [1]. Py–Im polyamides were purified by HPLC using Chemcobond 5-ODS-H column, 0.1% acetic acid and acetonitrile 0–50% linear gradient, 40 min. The detection wavelength was set at 254 nm [2,3]. Synthetic polyamides can bind to specific nucleotide sequences in the minor groove of double-helical DNA with high affinity and specificity. Sequence-specific DNA recognition by Py–Im polyamide depends on the side-by-side paring of Py and Im; the Py/Im pair targets the CG base pair, Im/Py recognizes the GC base pair, and Py/Py binds to both AT and TA base pairs [4]. Various types of sequence-specific DNA-binding Py–Im polyamides have been developed to regulate gene expression by targeting the promoter regions of enhancer and transcription factor binding elements in vitro [5]. Recently, Matsuda et al. [6] reported that the Py–Im polyamide targeting the rat transforming growth factor (TGF)-β_1 is a novel gene-silencing agent for the treatment of progressive renal diseases in Dahl-S rats. These observations suggested that the Py–Im polyamide could be a useful bioprobe for molecular biology and a potential medicine.

The aims of the present study are to develop a sensitive and specific HPLC assay for the quantitation of Py–Im polyamides in rat plasma and to propose its application to pharmacokinetic studies.

2. Experiment

2.1. Chemicals and reagents

Py–Im polyamides (A) and (B) were provided by Gentier Biosystems Co., Ltd. (Japan). Their chemical structures are

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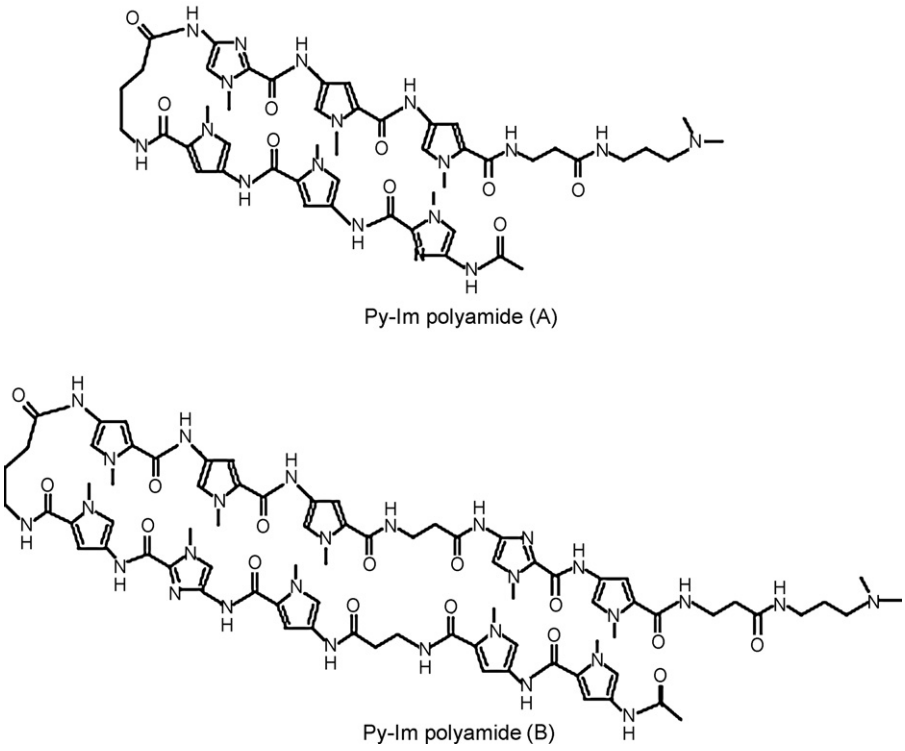

Fig. 1. Chemical structures of Py–Im polyamides (A) and (B). The molecular weight of Py–Im polyamide (A) is 1035.12 and that of Py–Im polyamide (B) is 1665.78.

shown in Fig. 1. Py–Im polyamide (A) was composed of Ac-ImPyPy-ImPyPy-β-Dp (Dp: *N*,*N*-dimethylaminopropylamide). Py–Im polyamide (B) was composed of Ac-PyPy-β-PyIm-Py-PyPyPyβ-ImPy-β-Dp. The molecular weights of Py–Im polyamides (A) and (B) were calculated from the sum of the standard atomic weights of all the atoms [7]. Py–Im polyamide (A) was 1035.12 and Py–Im polyamide (B) was 1665.78. Acetonitrile and methanol were HPLC-grade from Wako Pure Chemical Industries, Ltd. Acetic acid was of the highest quality from Kanto Chemical Co., Inc. The water was purified by distillation.

2.2. Chromatographic system

The analyses of Py–Im polyamides were carried out using a Shimadzu LC-20A HPLC system (Tokyo, Japan) with a reversed-phase TSK-GEL ODS-80T_M (4.6 mm × 15.0 cm, TOSOH Co., Japan) column maintained at 40 °C. The mobile phase solvent A was 0.1% acetic acid and the solvent B was acetonitrile (0–10 min, A: 100–20%, B: 0–80% linear gradient; 10–15 min, A: 40%, B: 60%). The flow rate was set at 1.0 ml/min. The detection wavelength was set at 310 nm. Injection volume was 30 μl.

2.3. Animals and blood sampling

Male Wistar rats (14 weeks old) weighing 280–300 g were obtained from Sankyo Lab Service Corporation. The animals were maintained in a temperature-controlled room on a 12-h light:12-h dark cycle and allowed free access to food and tap water. A polyethylene tube (0.58 mm, I.D. 0.96 mm, O.D.) was inserted into the left femoral artery and right jugular vein of the rat while under anesthesia with pentobarbital sodium (Dainippon Sumitomo Pharma Co.). The cannulation treatment was performed at least 1 day prior to the experiment. A Py–Im polyamide aqueous solution was injected into the jugular vein at a single dose of 2.0 mg/kg. Blood samples (0.5 ml) were collected at 0, 10, 30, 60, 90 and 120 min after the injection. After each sampling, the blood drawn was replaced with an equal volume of saline.

2.4. Preparation of plasma samples

Blood samples were centrifuged in a 1.5 ml microcentrifuge at 10,000 × *g* at 4 °C. Each sample was immediately transferred to a heparinized microcentrifuge tube and centrifuged for 10 min. Collected plasma samples (50 μl) were then vortex-mixed with 100 μl of methanol for 10 s. The mixture was centrifuged for 5 min to precipitate protein. Supernatants were centrifuged for 5 min again and 30 μl of each supernatant was directly injected into the chromatograph.

2.5. Stock solution and standards

Primary stock solutions of Py–Im polyamide (A) or (B) (2.0 mg/ml) were prepared in water and stored at 4 °C until analysis. Working solutions with concentrations of 20 and 200 μg/ml were prepared by adding water. Py–Im polyamide (A) or (B) calibration standards were prepared fresh daily at concentrations of 0, 1, 2, 5, 10, 20, 50, 100 and 200 μg/ml by spiking 45 μl of blank rat plasma with 5 μl of water (for "zero" standard sample) or Py–Im polyamides (A) and (B) working solution. In the same manner, quality control (QC) samples at concentrations of

1, 5, 20, and 100 μg/ml were freshly prepared to evaluate the accuracy and precision of the HPLC method.

2.6. Linearity of calibration curve

Calibration curves were constructed by plotting the peak area of Py–Im polyamide. The linearity of the calibration curves was evaluated by linear regression analysis.

2.7. Recovery

To assess the extraction recovery, two series of Py–Im polyamide QC samples were prepared as described above one with rat plasma and another without plasma. The set without plasma was prepared by adding water instead of plasma. The extraction recovery was determined from the ratio of peak area of water standard to that of the corresponding plasma standard.

2.8. Sensitivity

The lower limit of quantification (LLOQ) was determined during the evaluation of the linear range of the calibration curve. LLOQ was defined as the lowest concentration yielding a precision with coefficients of variation (CV) of less than 20% and accuracy within 15% of the theoretical value (i.e., accuracy between 85 and 115%) for both intra- and inter-day analysis.

2.9. Accuracy and precision

For the intra- and inter-day precision and accuracy of the assay, Py–Im polyamide (A) and (B) QC samples were prepared as described above. The intra-day precision was calculated from the CV for QC samples in three replicate analyzed on the same day, and the inter-day precision was determined by the analysis of QC samples in three consecutive days. The accuracy of each set of measurements was calculated by comparing the means with nominal values, and was expressed in percent. The criterion for the acceptability of precision was that the relative standard deviation for each concentration level did not exceed ±15% except for the LLOQ, which did not exceed ±20%. Similarly, for accuracy, the mean value did not exceed ±15% of the nominal concentration except for the LLOQ, where the limit was the LLOQ.

Table 1
Recovery of Py–Im polyamides (A) and (B) from plasma ($n = 3$)

Theoretical concentration (μg/ml)	Recovery (%)			
	Py–Im polyamide (A)		Py–Im polyamide (B)	
	Mean ± S.D.	CV (%)	Mean ± S.D.	CV (%)
1	83.7 ± 7.5	8.9	109.0 ± 1.2	1.1
5	103.5 ± 4.5	4.4	87.3 ± 4.4	5.1
20	97.4 ± 1.7	1.7	93.4 ± 11.1	11.9
100	97.7 ± 2.7	2.8	92.7 ± 2.8	3.0

2.10. Assay application

The present method was used to determine the concentrations of Py–Im polyamides (A) and (B) in rat plasma after intravenous injection of Py–Im polyamide (A) and (B) aqueous solutions.

3. Results and discussion

3.1. Separation

The UV absorbances of Py–Im polyamides (A) and (B) were determined from wavelengths of 200–400 nm. Py–Im polyamides (A) and (B) had a maximum UV absorption at 310 nm (data not shown). Therefore, the UV detection wavelength at 310 nm was measured. Chromatograms of blank plasma and plasma spiked with Py–Im polyamides (A) and (B) (5 μg/ml) are shown in Fig. 2. Py–Im polyamides (A) and (B) were well separated from co-extracted material under the described chromatographic conditions at approximate retention times of 9.7 and 10.5 min, respectively. The peak shapes were satisfactory and completely resolved from one another. No interference with constituents from the plasma matrix was observed.

3.2. Linearity of calibration curves

Calibration curves for Py–Im polyamides (A) and (B) were linear over the concentration range of 1–200 μg/ml in rat plasma. The mean (±S.D.) regression equation from three replicate calibration curves on different days for Py–Im polyamide (A) was $Y = 24{,}206\ (\pm 1413)X - 7715(\pm 7112)$ and for Py–Im polyamide (B) was $Y = 32{,}876(\pm 924)X - 8753(\pm 12{,}146)$, where Y is

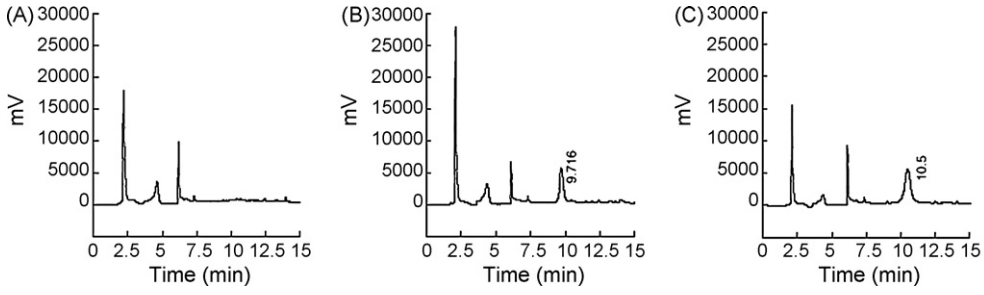

Fig. 2. Representative chromatograms of (A) blank rat plasma, (B) blank rat plasma spiked with Py–Im polyamide (A) (5 μg/ml), and (C) blank rat plasma spiked with Py–Im polyamide (B) (5 μg/ml). Approximate retention times: Py–Im polyamide (A) (5 μg/ml) = 9.7 min; Py–Im polyamide (B) (5 μg/ml) = 10.5 min.

Table 2
Accuracy and precision of the HPLC method for determining

Theoretical concentration (μg/ml)	Intra-day			Inter-day		
	Observed concentration (mean ± S.D.; μg/ml)	Accuracy (%)	CV (%)	Observed concentration (mean ± S.D.; μg/ml)	Accuracy (%)	CV (%)
Py–Im polyamide (A) concentration in plasma samples ($n = 3$)						
1	1.0 ± 0.1	102.2	5.3	1.1 ± 0.2	106.7	14.4
5	5.6 ± 0.1	111.8	1.6	5.3 ± 0.3	106.3	6.4
20	20.3 ± 0.3	101.4	1.4	18.3 ± 1.8	91.3	9.7
100	107.7 ± 4.0	107.7	3.7	103.6 ± 3.7	103.6	3.5
Py–Im polyamide (B) concentration in plasma samples ($n = 3$)						
1	1.0 ± 0.1	102.8	10.0	1.0 ± 0.2	102.2	15.0
5	5.0 ± 0.2	100.8	3.5	5.2 ± 0.2	104.0	3.1
20	19.5 ± 0.1	97.5	0.6	18.2 ± 1.4	90.8	7.9
100	103.7 ± 2.7	103.7	2.6	103.2 ± 3.3	103.2	3.2

peak area and X is the concentration of the analyte. R^2 of Py–Im polyamide (A) was 0.9997(±0.0003) and R^2 of Py–Im polyamide (B) was 0.9983(±0.0018).

3.3. Recovery

The efficiencies of the extraction procedure for the two analytes at QC levels (1, 5, 20, and 100 μg/ml) are presented in Table 1. The recovery ranged between 83.7 and 103.5% for Py–Im polyamide (A) and 87.3 and 109.0% for Py–Im polyamide (B).

3.4. Precision, accuracy and sensitivity

Table 2 shows the intra- and inter-day precision and accuracy of Py–Im polyamides (A) and (B). The intra- and inter-day accuracies (% deviation) were within ±20% for the LLOQ and ±15% for the other QC samples. The intra- and inter-day assay precisions (CV) were also within the acceptable range of 20% for LLOQ and 15% for the other QC samples. The LLOQ was determined as 1 μg/ml for both Py–Im polyamides (A) and (B).

3.5. Application

The plasma concentrations of Py–Im polyamides (A) and (B) were determined at 0, 10, 30, 60, 90 and 120 min after injection. The plasma concentration time profile after intravenous administration (2.0 mg/kg) of Py–Im polyamides (A) and (B) in the rat are shown in Fig. 3. The retention time of Py–Im polyamide (A) extracted from plasma (4.5 μg/ml) and water (5.5 μg/ml) were the same time at 9.8 min. The retention time of Py–Im polyamide (B) extracted from plasma (2.5 μg/ml) and water (5.6 μg/ml) were the same at 10.5 min. The respective retention time of Py–Im polyamides extracted from plasma and water were consistent.

In conclusion, the newly developed HPLC method for the determination of plasma Py–Im polyamides in rats is simple, sensitive and specific, and the method can be used for the analysis of large numbers of plasma samples in other species. The assay was validated to apply the requirements of pharmacokinetic studies.

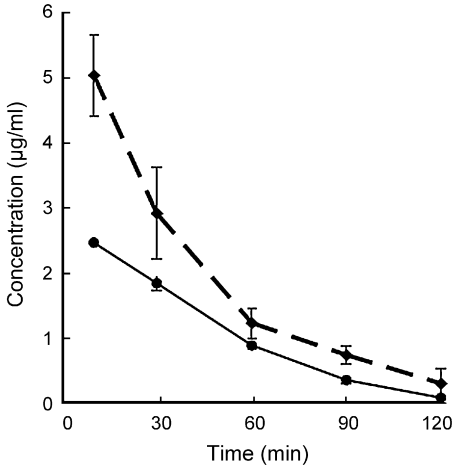

Fig. 3. Plasma concentration time profile after intravenous administration (2.0 mg/kg) of Py–Im polyamides (A) (♦) and (B) (●). The data was shown as the mean ± S.D. ($n = 3$).

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