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Sensitive analytical method for the novel H_1-receptor antagonist HSR-609 in human plasma and urine by high-performance liquid chromatography with pre-column fluorescent derivatization

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Abstract

A simple and sensitive method for quantitation of HSR-609 (I) in human plasma and urine was developed using HPLC with the fluorescence labelling reagent 4-(N,N-dimethylaminosulfonyl)-7-N-piperazino-2,1,3-benzoxadiazole (DBD-PZ). Compound I was extracted from human plasma and urine, and derivatized by reaction with DBD-PZ in the presence of Mukaiyama reagent A, an equimolar solution of 2,2′-dipyridyl disulfide (DPDS) and triphenylphosphine (TPP) in acetonitrile. The reaction mixture was cleaned up by liquid–liquid extraction following the derivatization. The conjugate was analyzed by ion-pair HPLC with fluorometric detection. The quantitation limits for I were 0.5 ng/ml in plasma and 5 ng/ml in urine. Using this method, plasma concentration and urinary excretion of I were studied after oral administration of I to human volunteers.

Keywords: Derivatization, LC; H_1-Receptor antagonist; HSR-609

1. Introduction

HSR-609 (3-[4-(8-fluoro-5,11-dihydro[1]benzoxepino[4,3-b]pyridin-11-ylidene)piperidino]propanoic acid, I) is a novel non-sedating H_1-receptor antagonist [1]. As the detection limit of I was 10 ng/ml using HPLC with UV detection in an animal study, and the clinical dose of I is lower than the dose in the animal study, it was necessary to establish a more sensitive assay method for the determination of I in plasma and urine. We therefore attempted to develop an HPLC method employing pre-column derivatization with a fluorescence labelling reagent.

Many kinds of fluorescence labelling reagents, such as 9-anthryldiazomethane (ADAM) [2–5], 3-bromomethyl-6, 7-dimethoxy-1-methyl-2-(1H)-quinoxalinone (Br-DMEQ) [6,7], 1-bromomethylpyrene [8] and 4-bromomethyl-6,7-dimethoxycoumarin (Br-MDC) [9], have been reported for the determination of compounds having a carboxylic group. Most of them are utilized for the detection of endogenous compounds such as prostaglandins [2,5], fatty acids [3,6] and bile acids [8], although a few applications to xenobiotics have been reported [4,7,9]. Simple derivatization procedures with fluorescent amines, such as dansyl-semipiperazide (DSP), monodansylcadaverine (MDC) and DBD-PZ have also been reported. However, DSP is not commercially available and MDC requires recrystallization prior to use [10–13]. In order to achieve sensitive detection, the

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excess reagent as well as interfering substances must be removed prior to HPLC. DBD-PZ has the advantage that the fluorescence excitation and emission spectra of its adducts are red-shifted compared with those of the reagent itself, by 19 nm for excitation and 7 nm for emission, and the reaction can be carried out at room temperature [12,13]. Even if the separation of the adducts from unreacted excess reagent is incomplete on the chromatogram, interference by the free reagent can be minimized by detection at the red-shifted excitation and emission wavelengths. We therefore chose DBD-PZ as the labelling reagent and employed the ion-pair HPLC method for the sensitive quantitation of I in human plasma and urine.

2. Experimental

2.1. Materials

The structures of I and PY-670 (internal standard; I.S.) are shown in Fig. 1. These compounds were synthesized in our laboratory [1,14]. DBD-PZ, DPDS and TPP were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Acetonitrile and sodium dodecylsulfate (SDS), purchased from Wako Chemicals (Tokyo, Japan), were of liquid chromatographic and of biochemical grade, respectively. Other reagents were of analytical grade.

2.2. Extraction from plasma

To 0.5 ml of plasma sample, 1 ml of acetonitrile and 0.05 ml of the I.S. aqueous solution (100 ng/ml) were added. The mixture was centrifuged at 1300 *g*

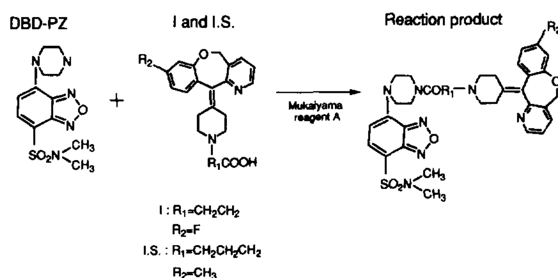

Fig. 1. Derivatization of I and I.S. with DBD-PZ.

for 5 min at room temperature. The supernatant was taken and acidified with 0.05 ml of 1 *M* HCl and then 6 ml of diethyl ether were added. This mixture was shaken for 5 min, centrifuged at 1300 *g* for 5 min at room temperature and the organic layer was discarded. To the aqueous layer, 0.01 ml of 5 *M* NaOH, 0.1 ml of 0.5 *M* sodium phosphate buffer (pH 7.0) and 6 ml of chloroform were added. The whole mixture was shaken for 5 min, centrifuged at 1300 *g* for 5 min at room temperature and the organic layer was taken and evaporated to dryness.

2.3. Extraction from urine

To 0.5 ml of urine sample, 0.05 ml of the I.S. aqueous solution (1000 ng/ml), 0.05 ml of 1 *M* HCl and 6 ml of diethyl ether were added. Subsequent procedures were the same as mentioned above for plasma samples.

2.4. Derivatization and subsequent clean-up procedure

Optimization of the derivatization reaction of I with DBD-PZ was examined with respect to the concentration of the derivatization reagent (0.5–10 m*M*), the concentration of Mukaiyama reagent A (5–100 m*M*) and the reaction time (2–60 min). The identification of the derivatized I and the I.S. was carried out by Frit-FAB LC–MS on a DX-303 mass spectrometer (JEOL, Tokyo, Japan). Glycerol was used as a matrix. HPLC conditions were as follows: column, TSK gel ODS-80TM (100×4.6 mm I.D.); mobile phase, acetonitrile–water–acetic acid (700:300:1, v/v); flow-rate, 1.2 ml/min.

The extracted residue from plasma or urine was reacted with 0.05 ml of DBD-PZ in acetonitrile (plasma, 2 m*M*; urine, 20 m*M*) and 0.05 ml of Mukaiyama reagent A (plasma, 50 m*M*; urine, 20 m*M*). DBD-PZ solution and Mukaiyama reagent A were prepared immediately before use and the reaction was carried out in the dark according to Toyo'oka et al. [12]. The mixture was allowed to stand at room temperature for 30 min, then 0.1 ml of 0.1 *M* HCl and 1 ml of diethyl ether were added. The whole mixture was mixed for 30 s and centrifuged at 1300 *g* for 5 min at room temperature and the

organic layer was discarded. A 25-μl aliquot of the aqueous layer was injected into the HPLC apparatus.

2.5. HPLC apparatus and conditions

An LC-10A pump (Shimadzu, Kyoto, Japan) was used and the detector was an RF-550A fluorescence spectrophotometer (Shimadzu), which was set at an excitation wavelength of 440 nm and an emission wavelength of 550 nm. All samples were injected using a WISP 712 automatic sample processor (Waters, Milford, MA, USA). The analytical column was a 5 μm TSK gel-ODS 80 TM (100×4.6 mm I.D., Tosoh, Japan). The peak areas were obtained from a C-R4AX Chromatopak integrator (Shimadzu).

The mobile phase was 10 m*M* citrate buffer (pH 2.0)–acetonitrile (51:49, v/v) containing 10 m*M* SDS. The flow-rate of the eluent was 1.5 ml/min.

2.6. Pharmacokinetics in human volunteers

Four groups of six healthy male volunteers participated in this study after having given their informed consent. They were given oral doses of 5, 10, 20 and 40 mg of I as tablets after overnight fasting. Samples of blood (10 ml each) were collected with heparinized syringes at 0, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12 and 24 h after dosing. Plasma samples were obtained by centrifugation. Urine samples were collected 0–6, 6–12, 12–24 and 24–48 h after dosing. All samples were stored at −20°C until analysis. Compound I was stable in plasma and urine on storage at −20°C for 22 months, or at room temperature for 1 day.

3. Results and discussion

3.1. Effect of clean-up after derivatization

To establish a highly sensitive assay method, it was necessary to clean up the reaction solution after derivatization. To select the most effective clean-up solvent, 0.1 *M* HCl, 0.1 *M* sodium phosphate buffer (pH 7.0) or 0.1 *M* NaOH was added to the reaction mixture and the whole mixture was washed with *n*-hexane, chloroform, diethyl ether or ethyl acetate. The diethyl ether washing under the acidic condition was the most effective in removing interfering substances. The effect of this clean-up procedure is illustrated in Fig. 2. Various mobile phases were tested. It was established that the separation of derivatized I from peaks of the reagent blank without SDS was insufficient (Fig. 3A), but derivatized I was

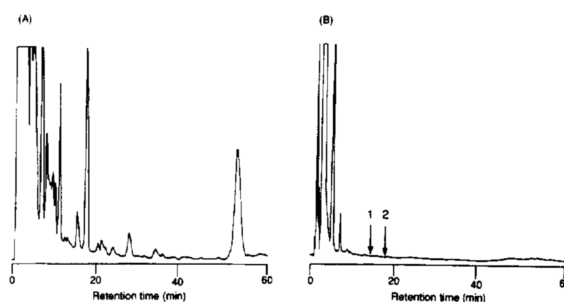

Fig. 2. Effect of clean-up after derivatization. Drug-free plasma samples were extracted and derivatized. Reaction conditions: DBD-PZ, 2 m*M*; Mukaiyama reagent A, 20 m*M*; reaction time, 30 min; reaction temperature, room temperature. (A) No treatment, (B) treatment. Positions at which compounds would be eluted, if they were present, are indicated by arrows: 1=I; 2=I.S.

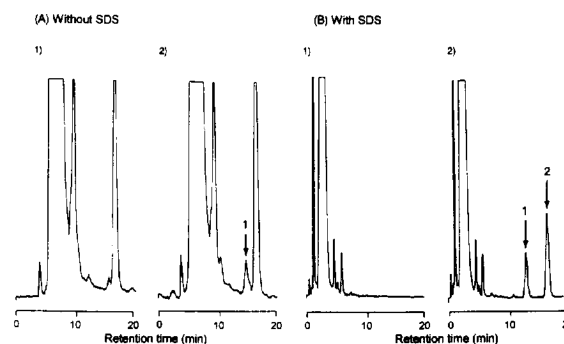

Fig. 3. Effect of SDS in the eluent. (A) 10 m*M* citrate buffer (pH 2.0)–acetonitrile (51:49, v/v), flow-rate 0.3 ml/min; (B) 10 m*M* citrate buffer (pH 2.0)–acetonitrile (51:49, v/v) containing 10 m*M* SDS, flow-rate 1.5 ml/min. (1) Reaction reagent blank, (2) sample containing I (500 ng/ml) and I.S. (1000 ng/ml). Peaks: 1=I; 2=I.S. Samples were derivatized with 20 m*M* DBD-PZ in the presence of 20 m*M* Mukaiyama reagent A. After 30 min reaction at room temperature, the reaction solution was subjected to clean-up as described in the text.

successfully separated from the blank peaks by the ion-pair HPLC method using SDS (Fig. 3B).

3.2. Optimization of derivatization reaction

The optimum concentrations of DBD-PZ and Mukaiyama reagent A and the optimum time for the derivatization in plasma were examined (Fig. 4). Constant peak areas of derivatized I and I.S. were obtained when the DBD-PZ concentration was more than 2 m*M*, the concentration of Mukaiyama reagent A was more than 20 m*M* and the reaction time was more than 20 min. Therefore, the derivatization reaction was carried out under the following conditions: DBD-PZ, 2 m*M*; Mukaiyama reagent A, 50 m*M*; reaction time, 30 min. In the same way, the optimum conditions for the derivatization in urine were decided to be DBD-PZ, 20 m*M*; Mukaiyama reagent A, 20 m*M*; reaction time, 30 min. The peak at *m/z* 662, derived from the protonated molecular ion ($[M+H]^+$) of derivatized I, was detected at 4.8 min after injection by Frit-FAB LC–MS. The peak at *m/z* 672, derived from $[M+H]^+$ of derivatized I.S., appeared at 5.2 min after the injection. Furthermore, unreacted I was not detected (below 1%) when the derivatized I at the concentration of 200 ng/ml was analyzed under the following conditions, which could separate I from the derivatized I and reagent blank: column, TSK gel ODS-80TM (150×4.6 mm I.D.); mobile phase, 0.05 *M* potassium biphosphate solution–acetonitrile (75:25, v/v); flow-rate, 1.0 ml/min; detection, UV at 254 nm. These results indicated that the derivatization of I was complete.

3.3. Chromatography

Typical chromatograms of extracts from human plasma and urine are shown in Fig. 5. Compound I was not completely separated from a small blank peak in plasma, but the top of the small peak was distinct even at high concentrations of I, such as 200 ng/ml. Therefore, interference by this peak in the determination of I could be nullified by use of the vertical resolution method.

3.4. Linearity and quantitation limit

Calibration curves were constructed by plotting the peak-area ratio of the drug against the internal standard versus drug concentration in spiked plasma and urine samples. The correlation coefficients calculated by linear least-squares regression analysis were 0.99987 for plasma and 0.99979 for urine over the concentration ranges 0.5–200 ng/ml and 5–2000 ng/ml, respectively. The minimum quantifiable concentration was 0.5 ng/ml in plasma and 5 ng/ml in urine and the values of the coefficient of variation

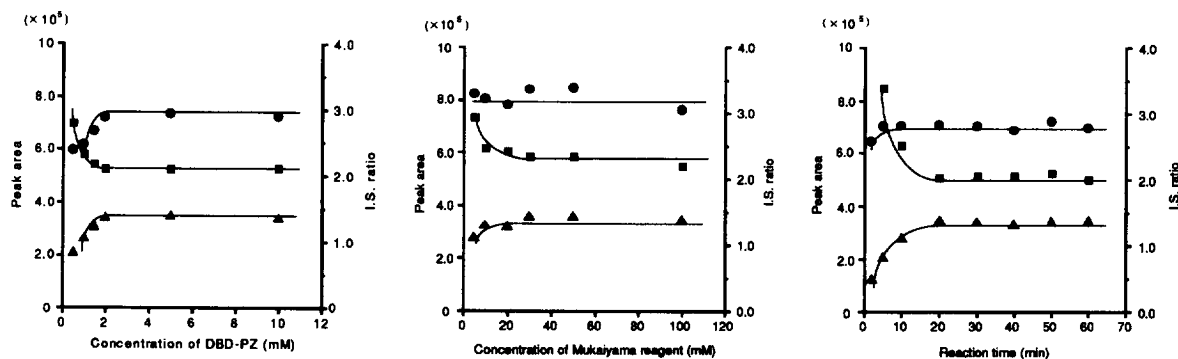

Fig. 4. Optimization of derivatization reaction. (Left) Effect of the concentration of DBD-PZ on the peak areas of I and I.S., and on the I.S. ratio; (middle) effect of the concentration of Mukaiyama reagent A on the peak area of I and I.S., and on the I.S. ratio; (right) effect of the reaction time on the peak area of I and I.S., and on its I.S. ratio. (●) I, (▲) I.S., (■) I.S. ratio.

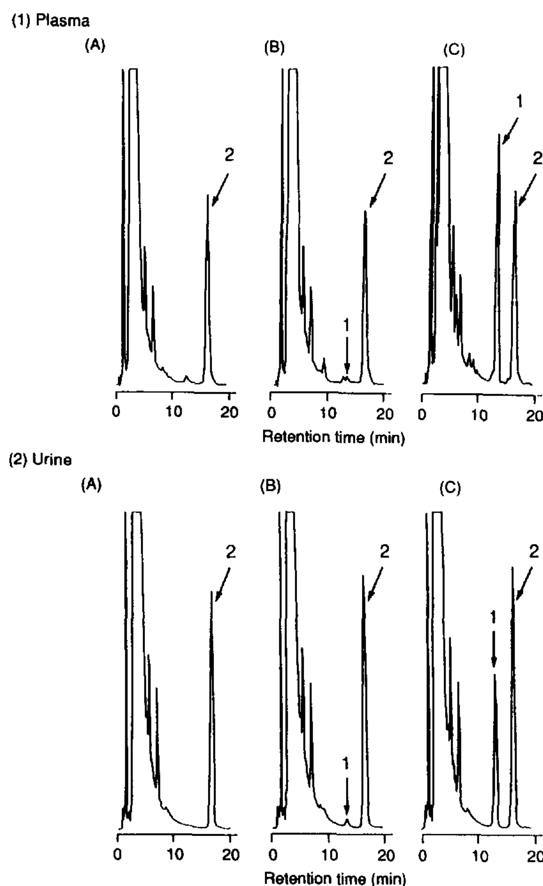

Fig. 5. Typical chromatograms of extracts from human plasma and urine (1). Plasma: (A) drug-free plasma containing only I.S. (100 ng/ml); (B) sample containing I (2 ng/ml) and I.S. (100 ng/ml); (C) sample containing I (100 ng/ml) and I.S. (100 ng/ml). (2) Urine: (A) drug-free urine containing only I.S. (1000 ng/ml); (B) sample containing I (20 ng/ml) and I.S. (1000 ng/ml); (C) sample containing I (500 ng/ml) and I.S. (1000 ng/ml). Detector sensitivity for urine samples was one-tenth of that for plasma samples.

(C.V., $n=9$) of the slope were 2.9% for plasma and 2.9% for urine.

3.5. Reproducibility, accuracy and stability

In the assay method, the total extraction yields were 79.3±1.8% (mean±S.D., $n=3$) in plasma and 81.0±3.7% ($n=3$) in urine at the concentration of 200 ng/ml and 2000 ng/ml, respectively. The accuracy, precision and reproducibility of the method in the intra-day assay were examined with spiked plasma and urine samples (Table 1). The values of the C.V. were 0.6–11.3% for plasma and 1.3–5.7% for urine. The repeatability for plasma and urine was also examined using three different matrices (Table 2). The values of the C.V. were 0.3–1.1% for plasma and 0.5–2.2% for urine. The derivatized I in the mobile phase was stable in the autosampler at room temperature for 1 day.

3.6. Application to biological samples

The pharmacokinetics of I in healthy humans was investigated using the present method. The average plasma concentrations after oral administration of I are shown in Fig. 6. The method was capable of measuring plasma levels of I up to 24 h after dosing, except for the point 24 h after administration of 5 mg, with a quantitation limit of 0.5 ng/ml. The average plasma concentration peaked at 1.0–1.4 h after dosing (C_{max}; 63.6–483.7 ng/ml) and the C_{max} and AUC values increased proportionally to the administered dose (5–40 mg).

The cumulative urinary excretion after oral administration of I is shown in Fig. 7. The average urinary excretion of I within 48 h amounted to more than 70% of the dose and was independent of the administered dose (5–40 mg).

4. Conclusion

Sensitive quantitative detection of I in human plasma and urine was achieved by ion-pair HPLC with pre-column fluorescent derivatization using a newly developed fluorescent reagent, DBD-PZ. Detection of I using the pre-column derivatization with DBD-PZ was about twenty times more sensitive than the previous method using a UV detector. The new assay method is not only suitable for routine assay of I in biological samples, but also should be applicable for the sensitive determination of other xenobiotics having a carboxylic group.

Table 1
Intra-day assay validation of I in human plasma and urine

Sample	Added (ng/ml)	Found (ng/ml) (mean±S.D., n=6)	Coefficient of variation (%)	Accuracy (%)
Plasma	1.00	0.99±0.11	11.3	−1.08
	5.00	5.08±0.17	3.4	1.57
	20.0	20.3±0.6	3.0	1.64
	100	99.1±0.6	0.6	−0.89
Urine	10.0	10.1±0.6	5.7	0.53
	50.0	50.6±2.1	4.1	1.13
	200	203±3.1	1.5	1.66
	1000	998±13	1.3	−0.91

Table 2
Repeatability among different matrices

Sample	n	Added (ng/ml)	Found (ng/ml) (mean±S.D.)	Coefficient of variation (%)
Plasma				
No. 1	3	200	198±2.2	1.1
No. 2	3	200	205±0.7	0.3
No. 3	3	200	203±2.0	1.0
Mean±S.D. (n=9)			202±3.5	1.7
Urine				
No. 1	3	2000	1991±10	0.5
No. 2	3	2000	1933±43	2.2
No. 3	3	2000	1975±37	1.8
Mean±S.D. (n=9)			1966±39	1.9

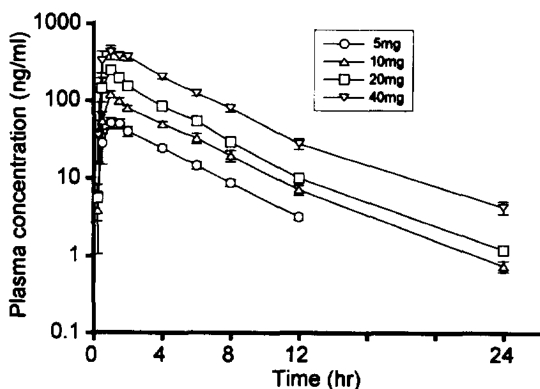

Fig. 6. Plasma concentrations after single oral administration of I. Each point represents the mean±S.E. of six volunteers. When the bar showing the standard error of data is omitted, the standard error is within the size of the symbol.

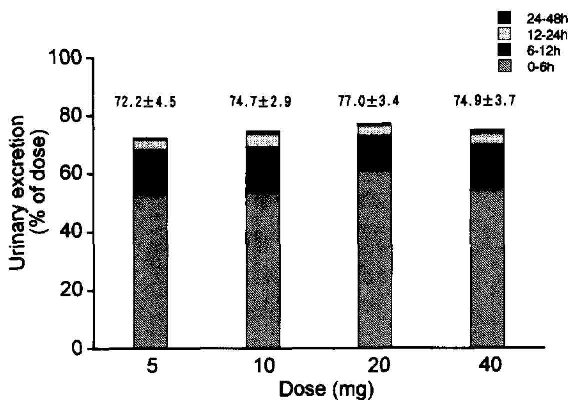

Fig. 7. Urinary excretion after single oral administration of I. Each value represents the mean±S.E. of six volunteers.

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