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Short Communication

Simultaneous determination of a new gastrointestinal prokinetic agent (HSR-803) and its metabolites in human serum and urine by high-performance liquid chromatography using automated column-switching

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ABSTRACT

A method based on high-performance liquid chromatography using column-switching is described for the simultaneous determination of HSR-803 and its metabolites in human serum and urine. The system uses a six-port valve with a Nucleosil CN pre-column for on-line sample clean-up, and direct injection of samples. The limits of quantitation in serum and urine were 5 and 20 ng/ml for HSR-803 and 50 and 200 ng/ml for the metabolites, respectively. The coefficients of variation for the intra- and inter-day accuracies were between 0.8 and 7.1% for each compound. This method was applied to the pharmacokinetic studies in humans after oral administration of HSR-803.

INTRODUCTION

HSR-803 (N-[4-[2-(dimethylamino)ethoxy]-benzyl]-3,4-dimethoxybenzamide hydrochloride, I, Fig. 1) is a newly synthesized gastrointestinal prokinetic agent, which has anti-acetylcholinesterase and dopamine D_2 antagonistic activity [1]. M1 (II, Fig. 1) and M2 (III, Fig. 1) were identified as metabolites of I in rats and dogs [2]. Compound III, a main metabolite of I, has an N-oxide group. In general, N-oxides are thermolabile [3–5], and the inherent polarity of the N-oxide group results in low extraction efficiencies with organic solvents [4–8]. Further, it has been reported that an amine oxide could be reduced to a corresponding amine during the extraction of serum [5].

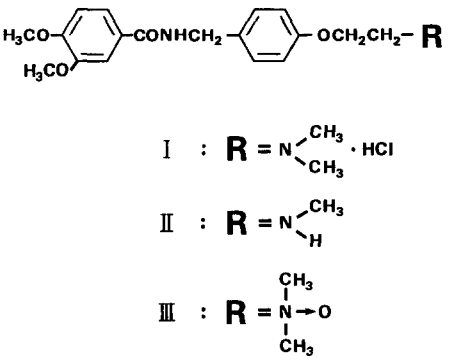

Fig. 1. Structures of I, II and III.

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Recently, the column-switching technique has been increasingly used instead of extraction procedures in sample clean-up of biological fluids for high-performance liquid chromatographic (HPLC) analysis [9–12]. This paper describes a method for the simultaneous determination of I and its metabolites in human serum and urine. Automated column switching is used, which allows for direct injection of the sample.

EXPERIMENTAL

Materials and reagents

Compounds I, II and III were synthesized in our laboratory. Acetonitrile (Kantoukagaku, Tokyo, Japan) was HPLC grade, and other reagents were commercially available and of analytical-reagent grade. The buffer solutions for mobile phase were filtered with the membrane filter Millipore HV 0.5 μm (Millipore, Yonezawa, Japan).

Instrumentation

The chromatographic system consisted of two pumps (LC-3A, Shimadzu, Kyoto, Japan and 600E, Waters, Milford, MA, USA), an automatic sample injector (710, Waters) and a six-port automated switching valve (Waters). A Nucleosil CN column (25–40 μm, 10 mm × 4.0 mm I.D., Macherey & Nagel, Duren, Germany) and a TSK gel ODS-80T_M column (5 μm, 150 mm × 4.6 mm I.D., Tosoh, Tokyo, Japan) were used as the pre-column and analytical column, respectively. A guard column packed with TSK gel ODS-80T_M (5 μm, 10 mm × 4.0 mm I.D., Tosoh) was connected before the analytical column. The fluorescence detector (820-FP, Jasco, Tokyo, Japan) was set at an excitation wavelength of 308 nm and an emisison wavelength of 344 nm. The chromatograms were recorded on a chromatographic data analyser (C-R4AX, Shimadzu).

Column-switching operation

After injection of 200 μl of serum or 50 μl of urine sample onto the pre-column, which had been previously equilibrated with mobile phase A (0.1 *M* phosphate buffer, pH 7.0), the column was washed for 4 min with mobile phase A at a flow-rate of 1.0 ml/min. The substances adsorbed on the pre-column were then introduced into the analytical column with mobile phase B, acetonitrile–0.05 *M* phosphate buffer (pH 5.5) (1:4, v/v), by switching the six-port valve to back-flush mode for 1 min. The six-port valve was then returned to its initial position. The analytical column was disengaged from the pre-column, and the latter was equilibrated with mobile phase A ready for the next injection. The separation was carried out with mobile phase B at a flow-rate of 1.0 ml/min.

Accuracy and stability

Standard solutions were prepared by adding an aqueous solution of I, II and III to pooled human serum or urine to give concentrations of 0.05–2 or 0.2–10 μg/ml, respectively. Calibration curves were obtained by least-squares regression of the peak area *versus* the sample concentration. Intra-day variation and accuracy were assessed by analysis of spiked control samples ($n = 6$) at each concentration. Inter-day variation and accuracy were assessed by six replicate analyses of spiked control samples over two months. Stability studies of I, II and III were performed at 25 and −20°C in serum and urine samples (0.2 μg/ml in serum, 2.0 μg/ml in urine).

Drug administration

Serum and urine samples were obtained from six healthy male volunteers. Volunteers were given a single oral dose of I (100 mg) in tablets. Blood was collected at 0 (predose), 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h after administration. The serum was prepared in the usual manner. Spontaneous urine was collected in polypropylene tubes at intervals of 0–4, 4–8, 8–12, 12–24, 24–48 and 48–72 h post-dose. Serum and urine samples were stored at −20°C until analysis. Pharmacokinetic parameters were estimated by the non-linear least-squares regression computer program, NONLIN84 [13].

RESULTS AND DISCUSSION

Sample clean-up by liquid–liquid extraction

We initially attempted to clean up samples containing I and its metabolites by liquid–liquid extraction under basic conditions, because of the amino group in the structures of these compounds. Although the procedure was done as rapidly as possible, III was converted into I. Furthermore, when III in human serum was allowed to stand under extraction conditions at room temperature, III was partially converted into I as a function of time. We thus concluded that sample clean-up by liquid–liquid extraction was not good for the determination of I and its metabolites.

Studies of column-switching techniques

Establishing the conditions for the pre-column is very important for obtaining good recoveries and sufficient sample clean-up, especially in this case to avoid the conversion of III into I.

By using Nucleosil CN as the pre-column packing and 0.1 *M* phosphate buffer (pH 7.0) as the washing solvent, serum and urine samples could be directly injected and more than 95% of I and its metabolites were recovered without any conversion of III into I. To avoid broadening of the peaks, the back-flush mode was adopted as the connection method for pre-column.

Chromatography

Typical chromatograms are shown in Fig. 2. No interfering peaks from endogenous compounds were observed in either blank serum or urine. The retention times of I, II and III were 13, 15 and 20 min, respectively. The peaks of I, II and III were well separated.

Stability, variation and accuracy

The calibration curve of I was divided into two concentration ranges because the concentrations of I in serum and urine have wide ranges. The low concentration range was 0.005–0.1 μg/ml for serum and 0.02–0.5 μg/ml for urine. The high concentration range was 0.1–2 μg/ml for serum and 0.5–10 μg/ml for urine. On the other hand, a single calibration curve was used for II and III. The concentration ranges were 0.05–2 μg/ml for serum and 0.2–10 μg/ml for urine. The least-squares regression fit of each calibration curve showed good linearity over the corresponding range ($r > 0.999$). The variation and accuracy are shown in Table I. Both their intra- and inter-day coefficients of variation (C.V.) were below 10%. The reproducibility and accuracy were satisfactory. The limits of quantitation for I in serum and urine were 5 and 20 ng/ml, respectively. The limits of quantitation for the metabolites in serum and urine were 50 and 200 ng/ml, respectively. Each compound in serum and urine was sufficiently stable for up to seven days at 25°C and up to three months at −20°C.

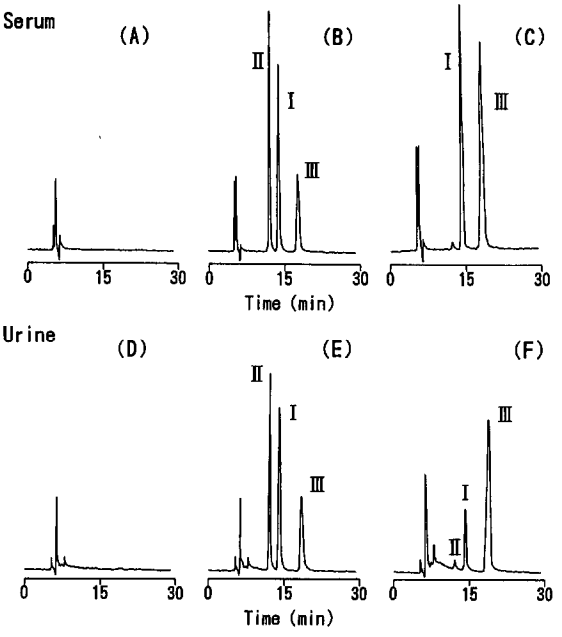

Fig. 2. Typical chromatograms of serum and urine samples. (A) Human blank serum; (B) human serum spiked with 0.5 μg/ml each of I, II and III; (C) 30-min post-dose serum sample (I: 0.69 μg/ml; III: 1.46 μg/ml) from a human subject; (D) human blank urine; (E) human urine spiked with 2.0 μg/ml each of I, II and III; (F) 4 h post-dose urine sample (I: 0.75 μg/ml; II: 0.08 μg/ml; III: 82.29 μg/ml) from a human subject.

Pharmacokinetic data

Fig. 3 shows the serum concentration–time curves of I and III in six healthy volunteers after

TABLE I

VARIATION AND ACCURACY OF THE ASSAY OF I, II AND III FROM SPIKED SERUM AND URINE SAMPLES[a]

Compound	Concentration (μg/ml)	Intra-day				Inter-day			
		Serum		Urine		Serum		Urine	
		Accuracy (%)	C.V. (%)	Accuracy (%)	C.V. (%)	Accuracy (%)	C.V. (%)	Accuracy (%)	C.V. (%)
I	0.01	100.5	2.2	–[b]		100.3	4.9	–[b]	
	0.05	98.9	2.2	99.6	3.8	98.6	2.6	100.2	6.6
	0.2	101.2	1.7	98.7	3.0	102.8	1.6	99.0	4.8
	1	100.4	1.1	98.3	2.2	100.2	1.2	100.2	3.4
	5	–[b]		100.2	2.0	–[b]		98.8	1.7
II	0.05	100.2	3.4	–[b]		98.9	6.4	–[b]	
	0.2	101.5	3.7	92.2	2.0	103.2	1.4	93.3	5.2
	1	100.2	1.1	100.9	3.2	100.8	1.9	102.2	1.3
	5	–[b]		100.9	1.8	–[b]		101.2	0.9
III	0.05	100.2	1.2	–[b]		96.1	5.4	–[b]	
	0.2	97.9	2.6	92.3	5.5	102.2	3.5	94.9	7.1
	1	98.2	1.3	101.3	3.2	100.4	2.1	101.9	3.5
	5	–[b]		103.0	1.6	–[b]		100.7	0.8

[a] $n = 6$.
[b] Not tested.

oral administration of I (100 mg). Compound I was rapidly absorbed, and the serum concentration of I reached the maximum level at 0.67 ± 0.11 h. The maximum concentration (C_{max}) of I was 0.65 ± 0.08 μg/ml. The elimination half-life ($t_{1/2}$) and the area under serum concentration–time curve ($AUC_{0-\infty}$) were 6.15 ± 0.24 h and 2.09 ± 0.37 μg h/ml, respectively. The C_{max} of III was 4.62 ± 0.40 μg/ml, obtained at 1.83 ± 0.17 h. The $t_{1/2}$ and $AUC_{0-\infty}$ were 6.59 ± 0.19 h and 42.43 ± 2.79 μg h/ml, respectively. Compound II was detected at 0.25–4 h, but the concentration was less than 50 ng/ml at each point.

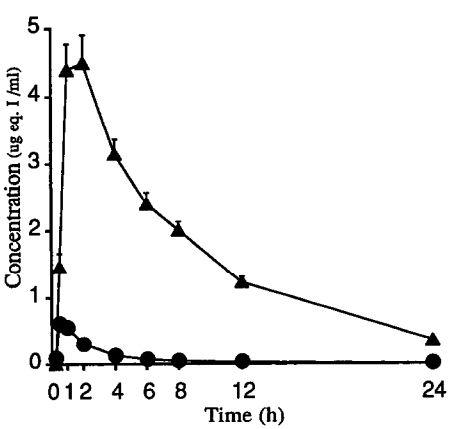

Fig. 3. Serum levels of I (●) and III (▲) in human subjects after oral administration of I at a dose of 100 mg.

Urinary excretion of I, II and III in six healthy volunteers after oral administration of I (100 mg) was 4.37 ± 0.98; 0.58 ± 0.10 and 76.60 ± 4.49% of dose, respectively, within 72 h after the administration. The total urinary recovery of I, II and III was 78.54 ± 4.86% of dose.

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