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Journal of Chromatography B, 668 (1995) 299–307

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Simultaneous determination of citalopram and its metabolites by high-performance liquid chromatography with column switching and fluorescence detection by direct plasma injection

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First received 26 September 1994; revised manuscript received 31 January 1995; accepted 6 February 1995

Abstract

High-performance liquid chromatography with a successive column-switching technique was developed for simultaneous determination of citalopram and its four metabolites in plasma. Plasma samples were injected directly, and the target compounds were purified and concentrated with an inexpensive commercial octadecyl guard column. Then, the six-port valve was switched, and the compounds retained in the column were eluted by the back-flush method using 20 m*M* phosphate buffer (pH 4.6)–acetonitrile (70:30, v/v) containing 0.1% diethylamine and separated with an ODS column. The compounds were assayed with a fluorescence detector at an excitation wavelength of 249 nm and an emission wavelength of 302 nm. At least 30 plasma samples could be treated with an octadecyl guard column. The limits of quantitation of this method were 2.0 ng/ml for citalopram, desmethylcitalopram, didesmethylcitalopram, citalopram propionic acid and citalopram N-oxide. This method was applied to a pharmacokinetic study in dogs and a toxicokinetic study in rats.

1. Introduction

Determination of the plasma concentration of the drug is important for dosage form design, pharmacokinetic studies, and toxicokinetic studies. In developing an assay method for drugs in plasma, not only the sensitivity and reproducibility suitable for the purpose but also labour-saving must be considered because of the need to examine a large number of samples. Liquid–liquid extraction, solid-phase extraction, and the column-switching technique are widely employed for clean-up of drugs in plasma. The column-switching technique allows on-line high-performance liquid chromatography (HPLC) by direct injection of plasma samples, leading to improvements in the efficiency of analysis of a large number of samples [1]. For this reason, it is used frequently for determination of plasma drug levels [2-5]. Pre-columns are used in this method for clean-up of drugs in urine or plasma. Using a guard column described by Kuo et al. [6] as the pre-column in our column-switching HPLC system, we succeeded in simultaneous determination of citalopram and its four metabolites, some of which are basic and others are acidic.

Citalopram (CIT, Fig. 1) is a bicyclic compound known to show little uptake of norad-

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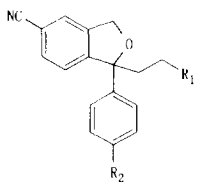
0378-4347/95/$09.50
SSDI 0378-4347(95)00073-9



Compound	R_1	R_2
Citalopram(CIT)	$-CH_2N(CH_3)_2$	F
Desmethylcitalopram(DCIT)	$-CH_2NHCH_3$	F
Didesmethylcitalopram(DDCIT)	$-CH_2NH_2$	F
Citalopram propionic acid(CIT-PA)	$-COOH$	F
Citalopram N-oxide(CIT-NO)	O ↑ $-CH_2N(CH_3)_2$	F
Internal standard(IS)	$-CH_2N(CH_3)_2$	Cl

Fig. 1. Structures of citalopram and its metabolites.

renaline or dopamine and to selectively block the uptake of 5-hydroxytryptamine by serotonergic neurons [7]. Recently, CIT has been reported to be effective for the treatment of emotional disturbances in dementia patients [8] and is presently developed as cerebral circulation and metabolism improvers.

Pharmacokinetic studies of CIT in humans have shown that the drug is rapidly absorbed and slowly eliminated [9,10]. CIT is converted by N-demethylation to desmethylcitalopram (DCIT) and didesmethylcitalopram (DDCIT) and by N-oxidation and deamination to citalopram N-oxide (CIT-NO) and citalopram propionic acid (CIT-PA) [7,11,12]. The metabolites are known to be pharmacologically active, except CIT-PA, but less potent than the parent drug (CIT) [7].

Highly sensitive assay in biological fluids is necessary for pharmacokinetic studies of CIT. There have been reports of the assay of CIT and its metabolites by reversed-phase HPLC [11–14]. CIT and its metabolites DCIT and DDCIT or CIT-PA could be analyzed by this method, but these metabolites could not be extracted simultaneously with highly polarized CIT-NO or acidic CIT-PA. Øyehaug et al. [12] first extracted CIT and its metabolites DCIT and DDCIT, which are basic compounds, from plasma, made the plasma acidic again, and assayed CIT-PA. Reymond et al. [15] reported determination of CIT and its metabolites DCIT, DDCIT, and CIT-PA in plasma by gas chromatography (GC) and GC–mass spectrometry, but these methods require complex pretreatments for extraction and derivatization.

We developed an on-line HPLC method in which the plasma sample is injected directly into HPLC system, the target compound is retained first with a pre-column, eluted from it by the back-flush technique with column switching, and separated and assayed with an analytical column. In this study, we orally administered CIT to dogs and rats and examined changes in the plasma concentrations of CIT and its metabolites by this technique.

2. Experimental

2.1. Materials and reagents

Citalopram hydrobromide (Lu 10-171), desmethylcitalopram hydrochloride (Lu 11-109), didesmethylcitalopram tartrate monohydrate (Lu 11-161), citalopram N-oxide hydrochloride (Lu 11-305), and citalopram propionic acid (Lu 16-073) were used as standard compounds. Lu 10-202 oxalate was used as an internal standard (I.S.). These compounds were provided by H. Lundbeck (Copenhagen, Denmark). Dipotassium hydrogenphosphate, potassium dihydrogenphosphate, and phosphoric acid of the analytical grade quality were purchased from Kokusan Chemical (Tokyo, Japan). Acetonitrile of the HPLC grade was purchased from Wako Pure Chemical Industries (Osaka, Japan). Diethylamine of the analytical grade quality was purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Instrumentation

The HPLC system consisted of a sample pretreatment system and an analytical system. The sample pretreatment system was composed of a Model LC-100P pump with a pre-column and a Model LC-100A autosampler (Yokogawa, Tokyo, Japan). The analytical system was com-

posed of a Model LC-100P pump with an analytical column and a Model RF-540 spectrofluorimeter (Shimadzu, Kyoto, Japan). A LiChrospher 100 RP-18 (4.0 mm × 4 mm I.D., Merck, Darmstadt, Germany) and an Ultron N-C_{18} (150 mm × 4.6 mm I.D., Shinwa Chemical Industries, Kyoto, Japan) were used as the pre-column and the analytical column, respectively. The temperature of the analytical column was maintained at 40°C. The two systems were connected with a Model 7125 six-port valve (Rheodyne, Berkeley, CA, USA) in a Model LC-100T column oven. Switching of the valve, flushing of the mobile phase, acquisition of chromatograms, integration, and processing were controlled with an LC-100 workstation incorporating an NEC PC-9801 microcomputer (NEC, Tokyo, Japan).

2.3. Column-switching procedure

Concentration/clean-up and separation of CIT and its four metabolites in plasma samples were done by the column-switching procedure shown in Fig. 2. The flow-rate was adjusted to 1.0 ml/min with both pumps A and B. Switching of concentration/clean-up, elution, and separation was made by manipulating the 6-port valve. At the beginning of the analysis (time zero), the valve was placed in position 1, and 200 μl of a plasma sample was injected directly from the autosampler. The injected sample dissolved in mobile phase A and first passes the pre-column (C1). Here, CIT and its metabolites are retained firmly, and many contaminants in the sample are drained. The 6-port valve was switched to position 2 for 10 min after injection of the sample. CIT and its metabolites, which were retained near the head of C1, were sent into the analytical column (C2) by back-flushing C1 with mobile phase B using pump B. After perfusion in this position for 2 min, the valve was switched back to position 1. CIT and its metabolites separated by C2 were detected with a fluorescence detector (excitation wavelength 249 nm; emission wavelength 302 nm). During this period, C1 was perfused with mobile phase A and conditioned for analysis of the next sample.

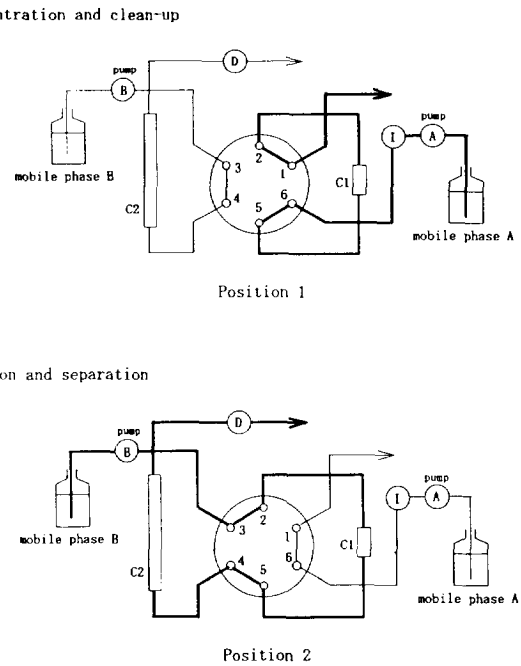


Fig. 2. Schematic diagram of the column-switching HPLC system. Position 1: injection. Position 2: back-flush and loading. Pump A: mobile phase A = 1 m*M* phosphate buffer (pH 3.0). Pump B: mobile phase B = 20 m*M* phosphate buffer (pH 4.6)–acetonitrile (70:30, v/v) containing 0.1% diethylamine. C1: 4.0 mm × 4.0 mm I.D., LiChrospher 100 RP-18 column. C2: 150 mm × 4.6 mm I.D., Ultron N-C_{18} column. I: autosampler. Flow-rate: 1.0 ml/min.

2.4. Mobile phase

Mobile phase A and B were 1 m*M* phosphate buffer (pH 3.0) and 20 m*M* phosphate buffer (pH 4.6)–acetonitrile (70:30, v/v) containing 0.1% diethylamine, respectively.

2.5. Standard solutions

Stock solutions of CIT, DCIT, DDCIT, CIT-NO, and CIT-PA were prepared by dissolving the standard compounds with acetonitrile at a concentration of 1.0 mg/ml (as their respective salts). Standard solutions were prepared by diluting 1 ml of the stock solutions with 100 ml of water, mixing 1 ml of these dilutions with 1 ml of the internal standard solution, and diluting the mixtures with water to 100 ml. These standard

solutions were stored at 4°C. The internal standard solution was adjusted to a concentration of 10 μg/ml with water. The standard solutions were used to examine the recovery rate of the pre-column.

Spiked plasma samples were prepared by adding known amounts of CIT, DCIT, DDCIT, CIT-NO, CIT-PA, and I.S. to drug-free plasma at eight concentration levels (2, 5, 10, 25, 50, 75, 100, and 150 ng/ml) and used for evaluation of the linearity, accuracy, and precision.

2.6. Precision and accuracy

The precision of the method was assessed by the intra- and inter-assay coefficients of variation (C.V.) of the analyses ($n = 5$) of spiked plasma samples. The accuracy of the assay was expressed as: [(determined concentration)/(spiked concentration)] × 100. The inter-assay C.V. was determined every day over a 5-day period. The concentration of each compound in plasma samples was calculated from the aqueous solution with a corresponding concentration.

2.7. Stability

Aliquots of 1 ml of each stock solution were diluted to 10 ml with water. An aliquot of 1 μl of this dilution was spiked to drug-free plasma, placed in a vial for the autosampler, and the residual amounts of CIT and its metabolites at room temperature (25°C) were determined by the method described here. The results were analyzed statistically by *t*-test.

2.8. Data analysis

The observed plasma concentration–time data for each compound were used to determine the maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}). The area under the plasma concentration–time curve (AUC) up to the last sampling point was calculated according to the trapezoidal rule. The mean residence time (M.R.T.) and the elimination half-lives ($T_{1/2}$) were calculated by the moment method. In rats, only the plasma concentration–time curve was examined after repeated oral administration of CIT.

2.9. Studies of plasma samples from dogs and rats

CIT and its metabolites in dog and rat plasma were assayed by this method. CIT was administered to four beagle dogs at 2 mg/kg once orally, and blood was drawn after 0.5, 1, 1.5, 2, 3, 4, 6, 10, and 24 h. In rats, CIT was administered at 100 mg/kg once a day orally for four weeks, and blood was drawn after 1, 4, 8 and 24 h. The blood was centrifuged, and the plasma obtained was stored at −20°C until analysis.

3. Results and discussion

3.1. Evaluation of the pre-column and mobile phase A

Since the effect of clean-up is related to the efficiency of the pre-column and quality of separation, pre-column packing was evaluated. In a preliminary study, commercial pre-column packing such as gel packing and protein-coated packing for biological samples and packing used in guard columns such as octyl and octadecyl were examined, using water as mobile phase A. The retention capabilities of gel and protein-coated pre-columns for CIT and its metabolites were weak, and the compounds were eluted as wastes with the compounds of the biological matrix. However, octyl and octadecyl columns sufficiently retained CIT, DCIT, DDCIT, CIT-NO, and CIT-PA, a high-polarity acidic compound. However, the pre-column used (10 mm × 4.0 mm I.D., particle size 5 μm) was clogged up by protein and other materials in plasma due to direct injection of 200 μl of plasma samples into HPLC, causing an increase in the column pressure, deterioration of the column, and a marked decrease in the resolution. We considered that this was due to the inadequate retention capability and the length of the column, and examined 6 LiChrospher 100 series columns (RP-

18e, RP-18, RP-8e, DIOL, NH2, and CN; all 4 mm × 4.0 mm I.D., particle size 5 μm) of the same separation mode and the same particle size but containing smaller amounts of packing. When water was used as the mobile phase, the recovery rates with octyl and octadecyl columns were 90% or above for various compounds spiked to plasma other than CIT-PA. The recovery rate of CIT-PA was about 50% with the octadecyl column, and no CIT-PA was recovered with the octyl column, probably because CIT-PA was not retained by the columns due to its high polarity and acidity. Then phosphate buffers with various salt concentrations (1, 5, 10, 20 m*M*) and pH (3.0, 5.1, 7.0) levels were used as mobile phase A for the octadecyl column; the recovery rates of various compounds were 90–100% regardless of the salt concentration or pH of the buffer solution. However, as the reproducibility of the retention time of the analytical column deteriorated at pH 5.1 or above, LiChrospher 100 RP-18e guard column and 1 m*M* phosphate buffer (pH 3.0) were chosen as the pre-column and mobile phase A for the pretreatment system. At least 30 plasma samples could be treated with this guard column.

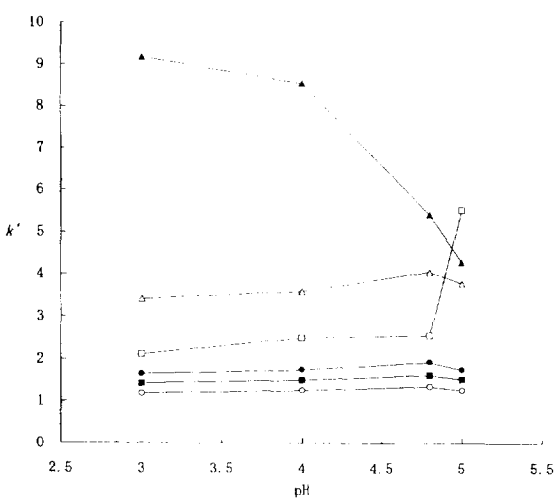


Fig. 3. Effect of buffer pH on capacity factor (k') of (●) CIT, (■) DCIT, (○) DDCIT, (▲) CIT-PA, (□) CIT-NO and (△) I.S.

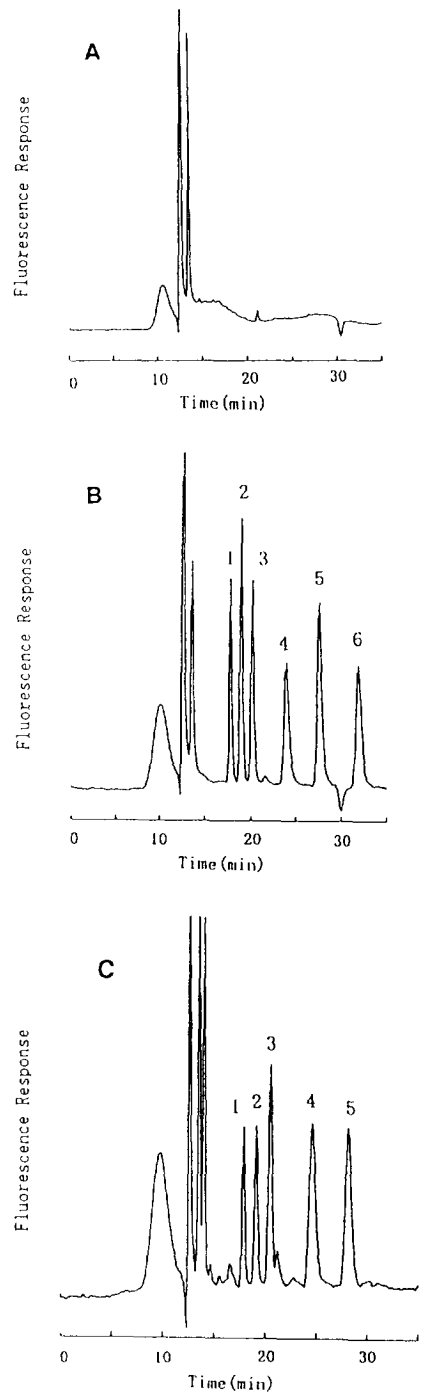


Fig. 4. Typical chromatograms of (A) drug-free plasma, (B) plasma spiked with 100 ng/ml of each compound, and (C) plasma from a dog 2 h after oral administration of citalopram hydrobromide (2 mg/kg). Peaks: 1 = DDCIT; 2 = DCIT; 3 = CIT; 4 = CIT-NO; 5 = I.S.; 6 = CIT-PA.

3.2. Separation with the analytical column

The composition of the mobile phase and the pH and concentration of buffer solutions were evaluated to determine the optimal chromatographic conditions for separation of CIT and its four metabolites with the analytical column. Fig. 3 shows the effects of pH of 20 m*M* phosphate buffer on the capacity factors (k') of CIT and its metabolites. Large changes were observed in the k' values of CIT-NO and CIT-PA, which are high-polarity metabolites, in a range of pH 3.0–5.0. These are considered to be due to changes in the retention force associated with conversion of CIT-NO into non-dissociated molecules and CIT-PA into dissociated molecules. On the basis of these results, the optimal resolution of CIT and its metabolites was considered to be obtained with 20 m*M* phosphate buffer (pH 4.6)–acetonitrile (70:30, v/v). Fig. 4 shows chromatograms obtained under these optimal conditions. All peaks of CIT and its metabolites were separated completely from peaks derived from plasma, and their peak shapes and resolution were also complete.

3.3. Linearity and limit of quantitation

The calibration curves were obtained by analysing spiked plasma samples. Calibration curves for each compound showed good linearity in the concentration range 2–150 ng/ml in plasma, and the correlation coefficients of various compounds were 0.999 or above. The intercept values of the calibration curves were −1.02 to −1.69 in dog plasma and −0.16 to 1.58 in rat plasma (Table 1). The limits of quantitation were 2.0 ng/ml for CIT, DCIT, DDCIT, CIT-NO and CIT-PA.

3.4. Accuracy and precision

The accuracy and precision of the present method were evaluated by analyzing spiked plasma samples with CIT and its metabolites at various concentrations (Table 2). In intra-day assays of dog plasmas, C.V. was 10% or less in all compounds except that it exceeded 10% at low concentrations. In rat plasmas, intra-day assays were not performed, but C.V. values of intra-day assays were similar to those in the dog plasmas (Table 3).

3.5. Stability

Since many plasma samples are expected to be injected directly into HPLC in this method, the stability of spiked plasma sample was examined at room temperature (25°C) over 24 h. Each compound showed no significant difference ($p <$

Table 1
Linear regression parameters for dog and rat plasma

Compound	Slope	Intercept	Correlation coefficient	Concentration range (ng/ml)
Dog				
CIT	0.967	−1.17	0.9994	2–150
DCIT	0.982	−1.60	0.9993	2–150
DDCIT	0.969	−1.69	0.9996	2–150
CIT-NO	1.008	−1.02	0.9991	2–150
CIT-PA	1.051	−1.42	0.9996	2–150
Rat				
CIT	0.900	0.77	0.9997	2–150
DCIT	0.951	−0.16	0.9998	2–150
DDCIT	0.977	1.58	0.9997	2–150
CIT-NO	0.896	0.36	0.9997	2–150
CIT-PA	0.922	0.89	0.9997	2–150

Table 2
Precision and accuracy in the assay of CIT, DCIT, DDCIT, CIT-NO and CIT-PA in dog plasma ($n = 5$)

Added (ng/ml)	Intra-assay			Inter-assay		
	Found (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy (%)	Found (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy (%)
CIT						
2	2.56 ± 0.14	12.43	128			
5	5.18 ± 0.26	11.33	104			
10	8.97 ± 0.28	6.97	90	9.33 ± 0.50	11.86	93
25	21.53 ± 0.34	3.53	86	22.85 ± 0.83	8.07	91
50	44.55 ± 0.40	2.00	89	46.69 ± 0.40	1.90	93
75	70.42 ± 0.71	2.24	94			
100	94.42 ± 0.94	2.23	95	100.23 ± 1.48	3.29	100
150	145.86 ± 1.30	1.99	97			
DCIT						
2	2.34 ± 0.18	17.37	116			
5	4.95 ± 0.28	12.51	98			
10	8.70 ± 0.14	3.64	87	9.09 ± 0.34	8.41	91
25	21.75 ± 0.28	2.91	87	22.31 ± 0.39	3.94	89
50	44.61 ± 0.61	3.08	89	47.12 ± 0.81	3.86	94
75	71.22 ± 0.54	1.70	94			
100	96.77 ± 0.50	1.15	96	93.02 ± 1.15	2.76	93
150	148.42 ± 1.00	1.50	98			
DDCIT						
2	1.80 ± 0.08	10.03	90			
5	4.28 ± 0.14	7.09	85			
10	8.99 ± 0.17	4.28	90	9.56 ± 0.19	4.52	96
25	22.35 ± 0.29	2.85	89	23.08 ± 0.48	4.62	92
50	43.91 ± 0.81	4.12	87	50.78 ± 1.12	4.91	102
75	69.29 ± 0.66	2.11	92			
100	94.98 ± 0.93	2.19	95	92.18 ± 1.18	2.86	92
150	146.68 ± 1.23	1.86	97			
CIT-NO						
2	1.68 ± 0.09	12.55	84			
5	3.89 ± 0.11	6.04	77			
10	8.19 ± 0.40	10.92	81	9.12 ± 0.66	16.28	91
25	23.28 ± 0.27	2.62	93	22.72 ± 0.92	9.03	91
50	48.24 ± 1.14	5.26	96	46.51 ± 0.76	3.66	93
75	76.56 ± 0.89	2.61	101			
100	104.82 ± 1.42	3.03	104	102.02 ± 2.25	4.93	102
150	148.25 ± 2.36	3.55	98			
CIT-PA						
2	1.51 ± 0.08	12.49	76			
5	3.56 ± 0.13	7.91	71			
10	9.50 ± 0.33	7.82	95	7.73 ± 0.37	10.73	77
25	23.28 ± 0.57	5.43	93	21.59 ± 0.81	8.34	86
50	47.15 ± 0.57	2.68	94	46.57 ± 0.98	4.71	93
75	77.84 ± 0.94	2.69	104			
100	103.30 ± 2.16	4.67	104	104.12 ± 1.32	2.83	104
150	155.94 ± 0.84	1.20	104			

Table 3
Precision and accuracy in the assay of CIT, DCIT, DDCIT, CIT-NO and CIT-PA in rat plasma ($n = 3$)

Added (ng/ml)	Found (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy (%)
CIT			
2	2.00 ± 0.06	3.18	100
5	4.80 ± 0.36	7.43	96
10	11.11 ± 0.59	5.31	111
50	46.44 ± 0.66	1.41	93
100	88.62 ± 1.34	1.50	89
150	136.83 ± 0.90	0.65	91
DCIT			
2	2.43 ± 0.32	13.00	122
5	4.63 ± 0.27	5.91	93
10	9.30 ± 0.47	5.01	93
50	47.30 ± 1.05	2.22	95
100	93.16 ± 1.32	1.42	93
150	143.70 ± 0.66	0.46	96
DDCIT			
2	3.72 ± 0.80	21.41	185
5	6.73 ± 0.80	11.85	134
10	11.57 ± 1.11	9.59	115
50	50.95 ± 1.81	3.54	101
100	97.34 ± 0.76	0.77	97
150	150.23 ± 0.91	0.60	100
CIT-NO			
2	2.09 ± 0.20	9.48	105
5	4.62 ± 1.03	22.17	93
10	9.81 ± 0.81	8.29	98
50	45.82 ± 0.64	1.40	92
100	87.53 ± 2.27	2.59	88
150	135.63 ± 2.16	1.59	91
CIT-PA			
2	2.54 ± 0.65	25.53	127
5	4.98 ± 0.07	1.39	100
10	9.14 ± 1.13	12.38	91
50	48.91 ± 0.42	0.85	98
100	94.24 ± 1.30	1.37	94
150	138.09 ± 0.89	0.64	92

0.05) in the concentration as compared with that at the beginning of the study, indicating satisfactory stability (Table 4).

3.6. Application of the method

This method was applied to the assays of CIT and its four metabolites in dog and rat plasmas.

Table 4
Stability of CIT and its four metabolites in dog plasma at 25°C for 24 h (mean ± S.D., $n = 3$)

Compound	Amount remaining (%)	
	8 h	24 h
CIT	101 ± 3.5	99 ± 1.9
DCIT	99 ± 4.0	99 ± 1.1
DDCIT	96 ± 3.0	96 ± 1.1
CIT-NO	101 ± 2.3	103 ± 4.9
CIT-PA	100 ± 1.9	100 ± 1.2

Figs. 5 and 6 show mean plasma concentration–time profiles of CIT, DCIT, DDCIT, CIT-NO, and CIT-PA in dog and rat plasmas. When CIT was administered to dogs once orally, the concentrations of CIT, DCIT, and CIT-NO increased rapidly after the administration to a C_{max} about 1 h after the administration and decreased thereafter. The concentration of DDCIT remained high after C_{max}. CIT-PA was detected in only a trace amount. Table 5 shows mean values of pharmacokinetic parameters in dogs. On repeated oral administration of CIT to rats, the concentration of DCIT was higher than those of CIT, DDCIT, CIT-NO and CIT-PA. In dogs, CIT-NO was detected, but little CIT-PA was observed. The results in rats were opposite to those in dogs, suggesting species differences.

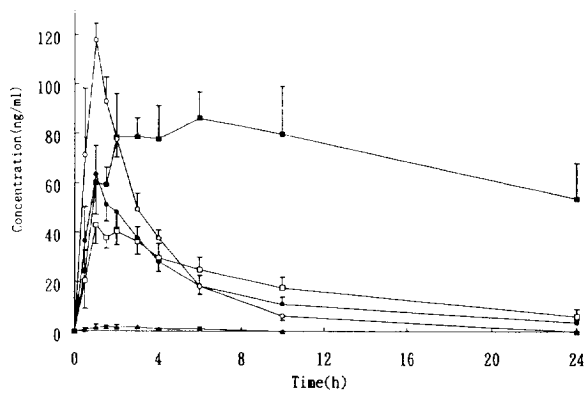


Fig. 5. Mean plasma levels of CIT and its metabolites after oral administration of 2 mg/kg of citalopram hydrobromide to dogs. Each point and bar represent the mean ($n = 4$) and the standard deviation (S.D.), respectively. ● = CIT; □ = DCIT; ■ = DDCIT; ○ = CIT-NO; ▲ = CIT-PA.

Table 5
Pharmacokinetic parameters; each value represents mean ± S.D. ($n = 4$)

Compound	C_{max} (ng/ml)	T_{max} (h)	AUC (ng h ml $^{-1}$)	$T_{1/2}$ (h)	MRT (h)
CIT	63.4 ± 20.2	1.0 ± 0.0	424.6 ± 150.5	9.6 ± 2.7	10.3 ± 2.4
DCIT	43.8 ± 12.6	1.25 ± 0.5	527.4 ± 259.9	8.1 ± 2.7	11.7 ± 3.9
DDCIT	96.0 ± 20.5	5.8 ± 3.7	4072 ± 2346	27.8 ± 9.5	40.4 ± 13.4
CIT-NO	122.7 ± 13.1	0.9 ± 0.3	415.0 ± 80.6	2.2 ± 0.4	3.6 ± 0.3

4. Conclusions

HPLC using the column-switching technique was established for simultaneous determination of CIT and its four metabolites in dog and rat plasmas. By this method, plasma samples are directly injected into HPLC, the target compounds are retained in a pre-column to be purified and concentrated, separated by back-flushing to an analytical column by the column switching method, and detected with a fluorescence detector. Since, in this method, the processes of pretreatment such as extraction, concentration, and purification are connected on-line with the processes of separation and determination, the analysis time was shortened, increasing the usefulness of the method for examination of many samples. It is expected to be applicable to pharmacokinetic studies in humans as well as in dogs and rats.

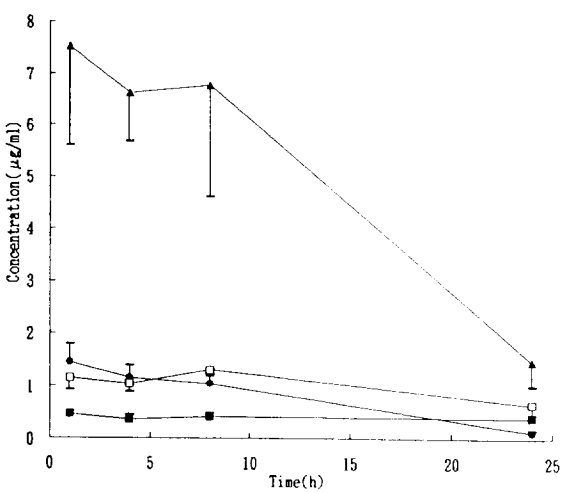


Fig. 6. Mean plasma levels of CIT and its metabolites after administration of citalopram hydrobromide (100 mg/kg) once a day orally for four weeks to rats. Each point and bar represent the mean ($n = 5$) and the standard deviation (S.D.), respectively. ● = CIT; □ = DCIT; ■ = DDCIT; ▲ = CIT-PA.

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