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Short Communication

Simultaneous determination of a new inhibitor of acyl CoA:cholesterol acyltransferase, YM17E, and five metabolites using high-performance liquid chromatography with electrochemical detection

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ABSTRACT

We describe a reversed-phase high-performance liquid chromatographic method for the determination in plasma of YM17E (I), an inhibitor of acyl CoA:cholesterol acyltransferase, and its five metabolites using electrochemical detection. This method enables simultaneous quantification of I and five active metabolites. The plasma sample is extracted by a one-step solid-phase extraction using a SepPak C_{18} cartridge, with high recovery and reproducibility of the analytes. The method is sensitive and the limits of determination are 0.5 ng/ml for I and 1 ng/ml for metabolites M1, M2-a, M2-b, M3 and M4. This method is applicable to rat, dog and human plasma, and is useful for pharmacokinetic studies.

INTRODUCTION

YM17E,1,3-bis[[1-cycloheptyl-3-(*p*-dimethylaminophenyl)ureido]methyl]benzene dihydrochloride (I), is a newly developed compound being evaluated as a regulator of serum cholesterol level and as an anti-atherosclerotic agent [1]. It has been shown to be a potent of ACAT, acyl CoA:cholesterol acyltransferase, inhibiting rabbit microsomal ACAT with a median inhibitory concentration (IC_{50}) of $4.4 \cdot 10^{-8}$ *M* and lowering serum cholesterol levels in cholesterol-fed animal models *in vivo* [2]. Therapeutic use of ACAT inhibitors is expected in hyperlipidaemia and atherosclerosis, but no ACAT inhibitor is yet in clinical use.

We have previously identified and characterized five N-demethylated metabolites of I (Fig. 1) using rat liver microsomal fractions and LC–MS analysis [3]. Further study revealed that each of these five metabolites also has considerable pharmacological activity against ACAT *in vitro*, and can lower serum cholesterol levels *in vivo* [4]. These findings make it important to investigate the pharmacokinetic profile of this agent and to determine what concentration is required to produce a pharmacological effect.

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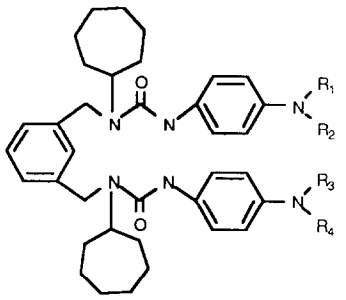

	R1	R2	R3	R4
I	CH_3	CH_3	CH_3	CH_3
M1	CH_3	CH_3	CH_3	H
M2-a	CH_3	H	CH_3	H
M2-b	CH_3	CH_3	H	H
M3	CH_3	H	H	H
M4	H	H	H	H
IS	C_3H_7	H	C_3H_7	H

Fig. 1. Molecular structures of I, the five metabolites and internal standard (I.S.).

In this study we developed an HPLC method for the determination of I and five metabolites in rat, dog and human plasma. This method allows the simultaneous determination of I and five active metabolites with appropriate accuracy and precision.

EXPERIMENTAL

Chemicals

I, five metabolites (M1, M2-a, M2-b, M3 and M4) and the internal standard were obtained from Yamanouchi Central Research Laboratory. Other chemicals were of reagent grade. Membrane filters were purchased from Nippon Millipore (Yonezawa, Japan).

Standard solution

Stock solution containing I, M1, M2-a, M2-b, M3 and M4 was prepared at a concentration of 10 μg/ml each in acetonitrile. This solution is stable for at least six months at −80°C. The solution was diluted with dimethyl sulfoxide to appropriate concentrations and a 50-μl aliquot was spiked with blank plasma. Internal standard was prepared at a concentration of 1 μg/ml in methanol just before use.

HPLC instrumentation

The HPLC system consisted of a L-6000 pump (Hitachi, Tokyo, Japan), a 712 WISP autosample processor (Waters, Milford, MA, USA), a CTO-6 column oven and a C-R4A recorder (Shimadzu, Kyoto, Japan). Reversed-phase HPLC was carried out with a phenylethyl-bonded silica column (Cosmosil 5PE; particle size 5 μm, 250 mm × 4.6 mm I.D., Nacalai Tesque, Kyoto, Japan). The mobile phase consisted of 20 m*M* disodium hydrogenphosphate adjusted to pH 5 with 10 m*M* citric acid–acetonitrile (2:8). The column temperature was 50°C and the flow-rate of the mobile phase was 1.0 ml/min. The mobile phase buffer was filtered through a 0.22-μm membrane filter (GS type) before mixing with acetonitrile. Analytes were detected with a Coulochem 5100A electrochemical detector (ESA, Bedford, MA, USA), which consisted of a 5020 guard cell and a 5011 analytical cell. The guard cell was set at +0.3 V and placed in line before the autosample processor to remove the interfering components in the mobile phase by electrolysis. The analytical cell, consisting of dual electrodes, detectors 1 and 2 (D1 and D2), was operated in the oxidative screen mode. The potentials of D1 and D2 were +0.03 V and +0.25 V, respectively. The signal from D2 was recorded.

Sample preparation

A 1-ml aliquot of plasma was diluted with 9 ml of 100 m*M* Tris–HCl (pH 7.4) and applied to a SepPak C_{18} cartridge (Classic type, Waters), which was conditioned by washing with 5 ml of methanol and 10 ml of water before use. The cartridge was washed sequentially with 10 ml of water, 5 ml of 50% methanol and 0.5 ml of methanol, and the analytes were then eluted with 1.5 ml of methanol. As internal standard, 50 μl of methanol solution (1 μg/ml) were added to the eluted fraction. After mixing, the samples were stored at −30°C until analysis. A 100-μl volume of the

eluted fraction was injected onto the HPLC system.

Human studies

Compound I (150 mg) was given by oral administration to six healthy male volunteers after a meal. Blood samples were collected in heparinized syringes and the plasma was stored at −30°C until use. I in plasma is stable for at least five months under these conditions.

RESULTS AND DISCUSSION

Selection of a suitable cell potential

The potential for simultaneous detection of I and five metabolites was selected from the voltammogram shown in Fig. 2. Using a mobile phase of buffer–acetonitrile (45:55, v/v) of pH 3, maximum responses for all analytes were obtained at +0.23 V and maintained up to +0.35 V. All metabolites showed closely similar voltammograms, and stable responses could be obtained from all analytes when the electrode potential was set at +0.23 V at pH 3. However, these responses decreased slightly when pH was greater than 4.

High-performance liquid chromatography

Representative chromatograms of this method are shown in Fig. 3. I, M1, M2-a, M2-b, M3 and M4 were separated from each other and from biological substances, with retention times of approximately 21, 17, 14.5, 13.8, 12 and 10 min, respectively. The internal standard was eluted at 30 min. The metabolites M2-a and M2-b, both of which are desdemethylated I, could not be separated using an octadecyl-bonded silica column. We sought a column that could separate these metabolites properly, and found a phenyl-bonded silica column to be successful but unsuitable for sensitive determination because it resulted in broader peaks than other column types. We chose Cosmosil 5PE, a phenylethyl silica-packed column, which could separate all analytes and the internal standard with appropriate peak shape and favourable resolution performance.

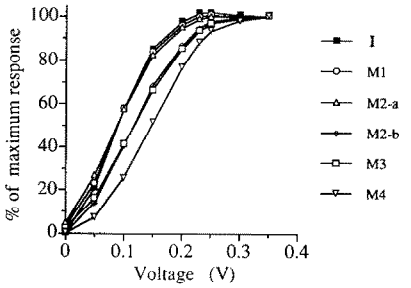

Fig. 2. Electrochemical detection response curves of I and its five metabolites. The detector was operated in oxidative mode. The response represents the percentage of the maximum response at 0.35 V.

Sample extraction

The recovery of analytes using this method was more than 90% in rat and dog plasma and more than 80% in human plasma. The internal standard was not eluted from a SepPak cartridge under the above conditions, although it could be eluted from the cartridge conditioned with a low-pH buffer. However, when we used a cartridge conditioned in this way, the chromatogram was not suitable for quantification because of unfavourable peaks or an unstable baseline. We therefore decided to add internal standard after solid-phase extraction.

We tried to concentrate the eluate fraction (1.5 ml) to enhance sensitivity, but the evaporation process or sequential liquid extraction unexpectedly reduced the recovery of these analytes, especially M3 and M4, probably because of degradation by treatment. Hence, this eluate fraction was injected directly onto the HPLC system without concentration. The recovery of analytes from human plasma was somewhat less than that from rat or dog plasma, but they were eluted quantitatively and validated as described blow.

Accuracy and precision

In this method, the calibration curve for I was linear over the range 0.5–200 ng/ml, and those for M1–M4 were linear from 1 to 200 ng/ml. Correlation coefficients were not less than 0.999. Accuracy and precision were determined by measuring the concentration of three to five different plasma samples, each spiked with I or its metabo-

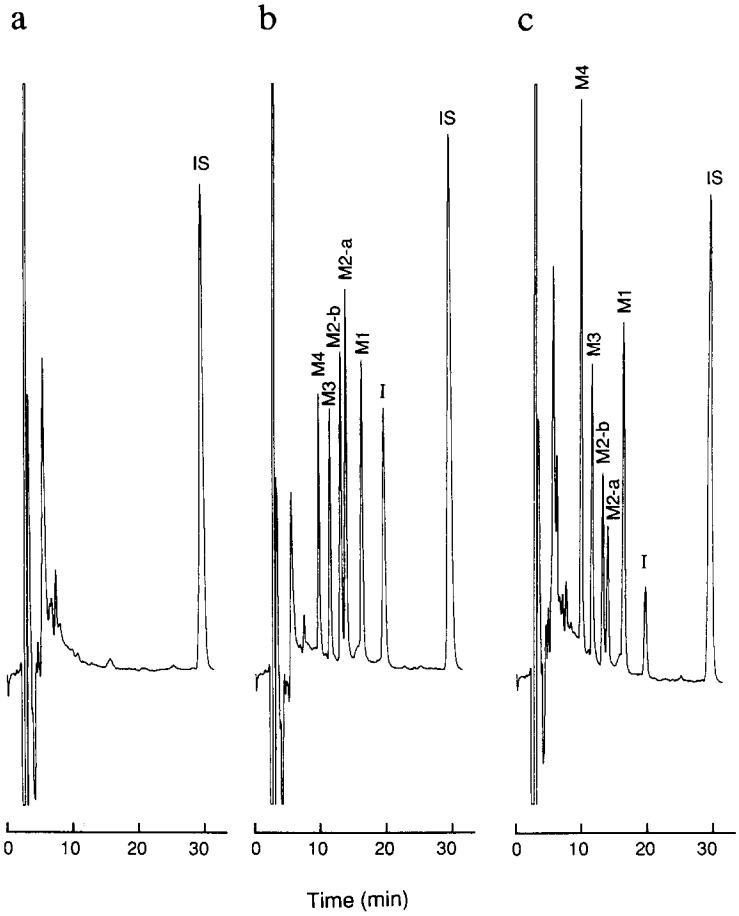

Fig. 3. Typical chromatograms obtained using this method. (a) Control human plasma; (b) control human plasma spiked with 20 ng/ml of each standard analyte; (c) plasma sample from a male volunteer given 150 mg of I orally.

lites at specified concentrations. The accuracy and precision of this method are shown in Table I. Both accuracy and precision were within 20%, but were slightly greater at 0.5 and 1 ng/ml, these being the quantification limits of I and the five metabolites, respectively.

Application of this method

Plasma concentrations of I and five metabolites after oral administration of I at 150 mg to six healthy male volunteers were measured using this method. The results are shown in Fig. 4. The concentrations of I reached maximum at 4.2 h after dosing and could be detected at up to 24 h. Interestingly, the time of peak concentration of I, M1, M2-a, M2-b, M3 and M4 was prolonged relative to the degree of demethylation, suggesting the sequential formation of these metabolites. This method was considered to be applicable to pharmacokinetic studies in rats, dogs and humans.

TABLE I

ACCURACY AND PRECISION OF ASSAY FOR YM17E AND FIVE METABOLITES IN PLASMA (n = 5)

Compound	Concentration prepared (ng/ml)	Concentration measured (mean ± S.D.) (ng/ml)	Precision (C.V.) (%)	Accuracy (△) (%)
I	0.5	0.50 ± 0.08	16.3	− 0.1
	1.0	1.05 ± 0.08	7.3	+ 5.0
	5.0	5.15 ± 0.44	8.6	+ 3.0
	50.0	52.6 ± 4.80	9.1	+ 5.2
	200.0	205.0 ± 9.15	4.5	+ 2.6
M1	1.0	1.14 ± 0.09	7.9	+14.0
	5.0	5.34 ± 0.49	9.2	+ 6.8
	50.0	53.6 ± 5.00	9.3	+ 7.2
	200.0	203.5 ± 10.7	5.4	+ 1.7
M2-a	1.0	1.03 ± 0.17	16.0	+ 3.0
	5.0	5.37 ± 0.68	12.7	+ 7.4
	50.0	54.1 ± 5.40	10.0	+ 8.2
	200.0	199.3 ± 11.5	5.7	− 0.4
M2-b	1.0	1.14 ± 0.11	9.6	+14.0
	5.0	5.26 ± 0.55	10.5	+ 5.2
	50.0	53.4 ± 5.20	9.7	+ 6.8
	200.0	200.4 ± 12.0	6.0	+ 0.2
M3	1.0	0.96 ± 0.07	7.3	− 4.0
	5.0	5.21 ± 0.56	10.7	+ 4.2
	50.0	53.9 ± 5.20	9.6	+ 7.8
	200.0	197.4 ± 13.6	6.8	− 1.3
M4	1.0	1.15 ± 0.12	10.4	+15.0
	5.0	4.99 ± 0.56	11.2	− 0.2
	50.0	53.2 ± 4.80	9.0	+ 6.4
	200.0	194.5 ± 21.8	10.9	− 2.5

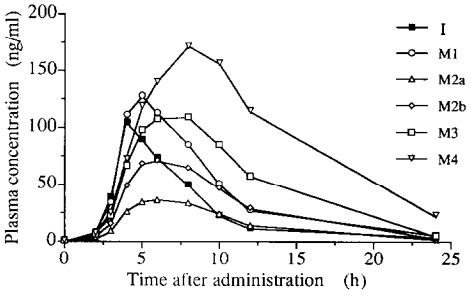

Fig. 4. Plasma concentrations of I and its five metabolites in healthy male volunteers given an oral dose of 150 mg of I. Each value represents the mean concentration from six subjects.

REFERENCES

1 N. Ito, K. Matsuda, T. Yasunaga, T. Araki, K. Iwaoka, Y. Iizumi and I. Ohhata, *4th Chemical Congress of North America and 202nd ACS National Meeting, New York, Aug. 25–30, 1991*, Abstract No. 109.

2 Y. Iizumi, Y. Yanagita, Y. Masuyama, H. Ohtsuka, H. Kakuta, N. Fujimori, N. Itoh, K. Matsuda and I. Ohata, *9th International Symposium on Atherosclerosis, Rosemont, IL, Oct: 6–11, 1991*, Abstract No. 96.

3 T. Uchida, T. Usui, T. Teramura, T. Watanabe and S. Higuchi, *Drug Metab. Dispos.*, submitted for publication.

4 Y. Izumi, personal communication.