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Sensitive high-performance liquid chromatographic determination of 2,2′-[(2-aminoethyl)imino]diethanol bis-(butylcarbamate) and its metabolites in human serum following pre-column derivatization with *o*-phthalaldehyde and stabilization of the derivatives

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ABSTRACT

A high-performance liquid chromatographic method for the sensitive determination of 2,2′-[(2-aminoethyl)imino]diethanol bis(butylcarbamate) (I) and its metabolites in human serum has been developed. The method was based on a pre-column derivatization with *o*-phthalaldehyde. The derivatives were stabilized at least for 24 h at 4°C by using N-acetyl-L-cysteine as a thiol and by eliminating the excess *o*-phthalaldehyde in the reaction mixture by solvent extraction and the addition of an ammonium salt after the reaction. The recoveries and reproducibilities in human serum spiked with I and its two metabolites were satisfactory, and the responses were linear over a wide range of analyte concentrations. The detection limits of I and its metabolites, II and III, in serum were 0.5, 4 and 2 ng/ml, respectively, at a signal-to-noise ratio of 5. The method was satisfactorily applied to the clinical study of I.

INTRODUCTION

The dihydrochloride of 2,2′-[(2-aminoethyl)-imino]diethanol bis(butylcarbamate) (I, Fig. 1) is a novel drug with a potent antiarrhythmic activity [1]. In preliminary studies using animals, low serum concentrations of I and its oxidized metabolites (II and III) were observed after intravenous administration of I [2]. Therefore, a sensitive method for quantification of I, II and III in biological materials was required for clinical studies. As there is no chromophore in the structure (Fig. 1), I exhibits poor UV absorption. Also, aliphatic amines such as I usually have no electrochemical activity. Thus, application of chemical derivatization was attempted for the sensitive determination of I, II and III.

High-performance liquid chromatography (HPLC) using chemical derivatization has been effectively applied for the sensitive determination of various amino compounds with dansyl chloride [3], 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole [4], naphthalene-2,3-dicarboxyaldehyde [5], phenyl isothiocyanate [6] and *o*-phthalaldehyde (OPA) [7]. Of these reagents, OPA is one of the most sensitive and gives 1,2-substituted isoindole derivatives reacting with primary amines and thiols. Although the isoindoles, *i.e.* OPA derivatives, exhibit an intense fluorescence response, they are known to be too labile for samples pre-

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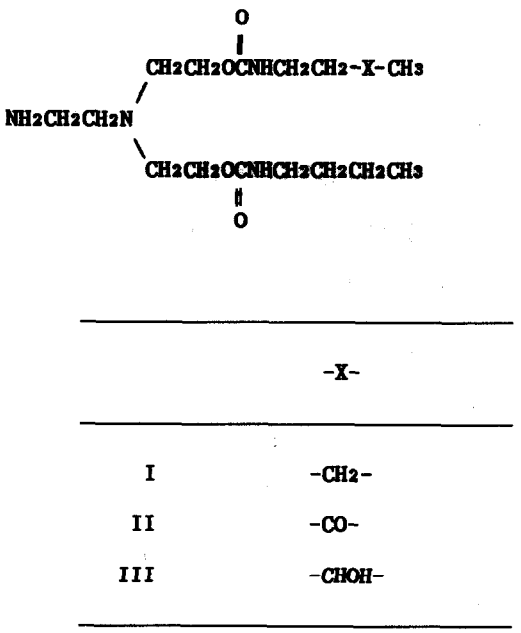

Fig. 1. Molecular structures of I, II and III.

pared by pre-column derivatization to be analysed overnight. Therefore, post-column derivatization has usually been performed for routine analysis [7,8]. However, post-column derivatization shows a high noise response induced by the reagents, which disturbs the sensitive determination of drugs. In our preliminary study on I, the sensitivity obtained with post-column derivatization (detection limit 5 ng/ml) was inadequate.

Thus, we decided to apply pre-column derivatization by stabilizing the OPA derivatives of I, II and III in order to establish a sensitive HPLC determination method. We then applied the method to the clinical study of I.

EXPERIMENTAL

Chemicals

Compounds I, II and III (Fig. 1) were synthesized as the dihydrochloride, dioxalate and dioxalate, respectively, in the Research and Development Division, Takeda Chemical Industries (Osaka, Japan). Methanol, acetonitrile, ethyl acetate, dichloromethane and *n*-hexane were of HPLC grade. OPA and 2-mercaptoethanol (2-ME) were of biochemical grade. All the reagents, including sodium hydroxide (amino acid autoanalysis grade), sodium tetraborate (pH measurement grade) and N-acetyl-L-cysteine (reagent grade), were purchased from Wako (Osaka, Japan). β-Mercaptopropionic acid (β-MPA, masking grade) and non-fluorescence water (fluorometric analysis grade) were purchased from Dojin (Kumamoto, Japan). Deionized water was further purified using a Milli-Q water purification system (Nihon Millipore, Yonezawa, Japan). All other chemicals were of reagent grade.

A 50 m*M* acetate buffer (pH 6) was prepared by adding a 50 m*M* acetic acid to a 50 m*M* sodium acetate solution and adjusting the pH to 6.

N-Acetyl-L-cysteine and β-MPA were purified, N-acetyl-L-cysteine by double recrystallization from non-fluorescence water and β-MPA (35 μl) by dissolving in 3 ml of 0.01 *M* sodium tetraborate buffer (pH 10) and washing with 6 ml of dichloromethane [12]. After centrifugation at 1400 *g* for 5 min, 2 ml of the upper layer were added to 1 ml of methanol (β-MPA solution). This solution was freshly prepared before derivatization.

OPA reagent 1 consisted of OPA (4 mg), 2-ME (50 μl) and methanol (3 ml). OPA reagent 2 consisted of OPA (4 mg), N-acetyl-L-cysteine (50 mg) and methanol (3 ml). These reagents were prepared freshly every day.

Instruments and HPLC conditions

The HPLC system consisted of two LC-6A pumps and an SIL-6B autosampler, all controlled by an SCL-6B controller (all from Shimadzu, Kyoto, Japan), and an F-1050 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). A Waters 741 data module (Waters, Osaka, Japan) was used to measure the peak heights of the analytes. Samples for HPLC were cooled at 4°C by a WIG 7000A cooling system (Ishido, Kyoto, Japan) during analysis. A YMC ODS column (A type, 5 μm particle size, 150 mm × 4.6 mm I.D.; Yamamura Chemicals, Kyoto, Japan) was used at ambient temperature. The flow-rate was 1 ml/min. The column eluate was monitored by the emission at 450 nm (excitation at 340 nm).

TABLE I

GRADIENT ELUTION PROGRAMME OF I, II AND III IN SERUM

Time (min)	Concentration (%)	
	A	B
0	75	25
10	75	25
20	67	33
28	53.5	46.5
39	53.5	46.5
39.1	0	100
45	0	100
45.1	75	25
60	(Stop)	

To investigate the stability of the OPA derivatives, an isocratic elution mode was adopted. The mobile phase was 50 m*M* acetate buffer (pH 6)–acetonitrile (66:34, v/v) in the case of 2-ME and N-acetyl-L-cysteine, and 50 m*M* sodium acetate (pH 7.8)–acetonitrile (67:33, v/v) in the case of β-MPA. For the analyses of I, II and III in serum samples, a gradient elution mode was adopted using two mobile phases: A, 50 m*M* acetate buffer (pH 6)–acetonitrile (90:10, v/v); B, 50 m*M* acetate buffer (pH 6)–acetonitrile (30:70, v/v). The gradient elution programme is indicated in Table I.

Stability investigation of OPA derivatives

When 2-ME and N-acetyl-L-cysteine were used as thiols, the working solutions were prepared with 150 μl of a standard solution of I (1 μg/ml), 850 μl of 0.1 *M* sodium tetraborate buffer (pH 10) and 500 μl of OPA reagent 1 or reagent 2, respectively. In the case of β-MPA, the working solution was prepared with 30 μl of a standard solution of I (1 μg/ml), 270 μl of 0.1 *M* sodium tetraborate buffer (pH 10), 150 μl of methanolic OPA solution (2.7 mg/ml) and 450 μl of β-MPA solution. These mixtures were allowed to stand for 2 min at room temperature. The final concentrations of reagents were as follows: OPA, 3.3 m*M*; N-acetyl-L-cysteine and β-MPA, 35 m*M*; 2-ME, 70 m*M*.

To eliminate excess OPA, an ammonium phosphate buffer (170 μl, pH 9) was added after the derivatization, and for the solvent extraction, 6 ml of ethyl acetate–*n*-hexane (8:2, v/v) were used.

Aliquots of 50 μl of the resulting mixtures were injected into HPLC apparatus.

Serum sample preparation

A serum sample (300 μl) was deproteinized with 3 ml of acetonitrile containing 0.17% propylene glycol. After centrifugation (1400 *g*, 3 min), the supernatant was evaporated under a stream of nitrogen gas at 40°C. The residue was dissolved with 200 μl of 0.1 *M* sodium tetraborate buffer (pH 10) and 100 μl of OPA reagent 2 were added to the solution. The reaction mixture was allowed to stand for 2 min at room temperature, washed with 6 ml of ethyl acetate–*n*-hexane (8:2, v/v) and 50 μl of 0.1 *M* ammonium phosphate buffer (pH 9) were added to the mixture. A 50-μl aliquot of the resulting mixture was injected into the HPLC apparatus.

RESULTS AND DISCUSSION

Stabilization of the OPA derivatives of I, II and III

For the stabilization of OPA derivatives of amines, the selection of β-MPA was reported [10,11]. Tawa *et al.* [12] reported the good stability of the OPA–β-MPA adduct of sisomicin, in which no degradation was observed for at least 6 h after the reaction. We first applied this reagent system to the pre-column derivatization of I, but the fluorescence intensity fell to 80% in 4 h at 4°C.

It has been suggested that degradation of isoindole derivatives is initiated by the attack of water

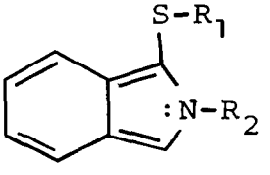

Fig. 2. Molecular structure of the OPA derivatives. R_1 and R_2 are substituents of thiol and primary amine used for derivatization with OPA, respectively.

at the C-1 position of the isoindole derivatives, that excess OPA in the reaction mixture should catalyse the degradation, and that increasing the steric bulk of the thiol side-chain should enhance the stability of the isoindole derivatives (Fig. 2) [9]. Therefore, the stability of the OPA derivative of I would be enhanced by increasing the steric bulk of the thiol side-chain and by eliminating the excess OPA.

The effect of the steric bulk of thiol side-chain was investigated using 2-ME, β-MPA and N-acetyl-L-cysteine as thiols. N-Acetyl-L-cysteine is known to give isoindole derivatives with primary amines and OPA [13]. As shown in Fig. 3, the stability of the derivatives was enhanced as the steric bulk increased (N-acetyl-L-cysteine > β-MPA > 2-ME). With regard to the stability of the isoindole derivatives, the importance of steric bulk of the N-substituents (analytes) in the isoindole derivatives was reported previously [9]. The large steric bulk of the N-substituent of sisomicin compared with that of I would contribute to the stability of the OPA derivative of sisomicin [12]. By using N-acetyl-L-cysteine, the large steric bulk would compensate for the small steric bulk of I and make the OPA–N-acetyl-L-cysteine adduct of I more stable than the OPA–β-MPA adduct.

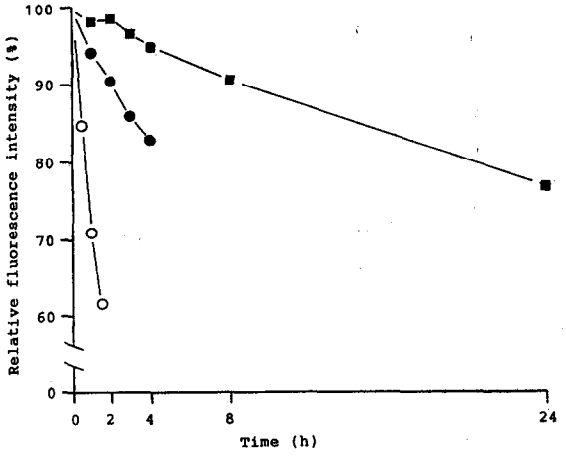

Fig. 3. Effect of the steric bulk of the thiol side-chain on the stability of OPA derivatives of I at 4°C. The fluorescence intensity obtained immediately after the reaction was taken as 100%. Thiols: (○) 2-ME; (●) β-MPA; (■) N-acetyl-L-cysteine.

However, the stability was found to be still inadequate for our purposes.

The effect of the elimination of excess OPA was also investigated. It has been reported that isoindole derivatives become stable when the OPA concentration in the reaction mixture decreased [9,12]. As an estimation of the exact amounts of OPA necessary for the derivatization in clinical samples was difficult, an ammonium salt was added to the reaction mixture to consume the excess OPA by forming the corresponding isoindole. As shown in Fig. 4, more than 90% of the initial fluorescence intensity remained for up to 24 h at 4°C by the addition of ammonium phosphate buffer (pH 9) when N-acetyl-L-cysteine was used, whereas little or no effect on the stability was observed when 2-ME or β-MPA was used. Furthermore, when some of the excess OPA was extracted by organic solvents in advance for the addition of the ammonium phosphate buffer, the OPA derivative of I was almost completely stabilized, and the residual fluorescence intensity was more than 99% up to 24 h at 4°C after the reaction (Fig. 4). The OPA derivatives of II and III were also found to be stable for up to 24 h at 4°C when similarly treated.

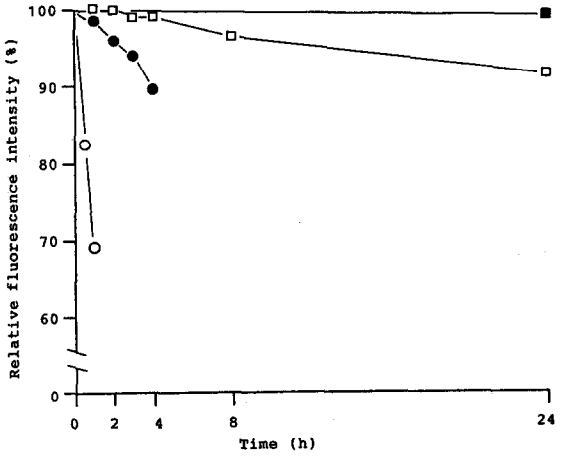

Fig. 4. Effect of the elimination of excess OPA on the stability of OPA derivatives of I at 4°C. The fluorescence intensity obtained immediately after the reaction was taken as 100%. Thiols: (○) 2-ME; (●) β-MPA; (□) N-acetyl-L-cysteine with ammonium phosphate buffer (pH 9); (■) N-acetyl-L-cysteine with solvent extraction and ammonium phosphate buffer (pH 9).

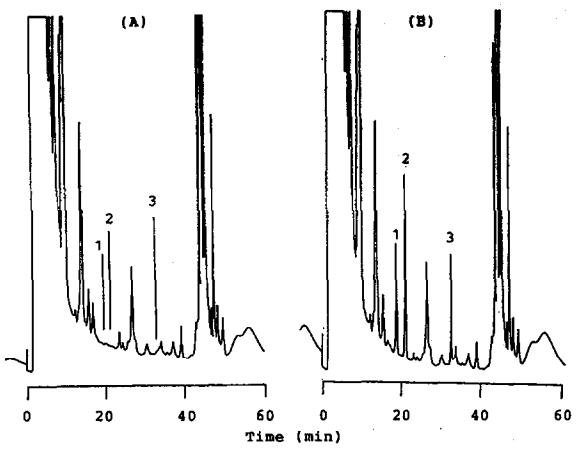

Fig. 5. Typical chromatograms of (A) drug-free serum and (B) serum spiked with I and its metabolites. Peaks: 1 = III (13 ng/ml); 2 = II (67 ng/ml); 3 = I (8.5 ng/ml).

Thus, by using OPA and the bulky N-acetyl-L-cysteine and by eliminating the excess OPA by solvent extraction followed by reaction with an ammonium salt, the resulting isoindole derivatives of I and its metabolites were stabilized sufficiently. This enhanced stability enabled us to perform the sensitive determination of I, II and III by pre-column derivatization with OPA overnight.

Clinical study

Typical chromatograms of human serum spiked with I, II and III and of drug-free serum are shown in Fig. 5. The extract of blank serum showed no peaks at the retention times of I, II and III. The recovery, reproducibility and linearity data were obtained using the serum spiked with I, II and III. The mean recovery and the coefficient of variation (both within-day and between-day assay) were satisfactory (Table II). A linear relationship was obtained between the analyte concentration and the fluorescence response in the range shown in Table III (correlation coefficient > 0.9999). The detection limits of I, II and III in serum were 0.5, 4 and 2 ng/ml, respectively, at a signal-to-noise ratio of 5.

The established determination method was applied to a clinical study of I. Typical serum con-

TABLE II

RECOVERY AND REPRODUCIBILITY IN THE DETERMINATION OF I, II AND III ADDED TO HUMAN SERUM

Compound	Within-day assay (n = 5)			Between-day assay (n = 3)		
	Concentration (ng/ml)	Recovery (%)	C.V. (%)	Concentration (ng/ml)	Recovery (%)	C.V. (%)
I	17	93.4	1.6	25	94.6	1.8
II	670	86.8	2.7	670	87.4	1.8
III	130	88.4	2.1	130	87.8	1.1

TABLE III

REGRESSION DATA IN THE DETERMINATION OF I, II AND III ADDED TO HUMAN SERUM

Compound	Range (ng/ml)	Number of points	Slope	Intercept	Correlation coefficient
I	0.83–83	6	1113	−193	0.9999
	83–8300	7	989	18 967	0.9999
II	13–2670	6	225	−760	0.9999
III	7–1340	6	705	−1392	0.9999

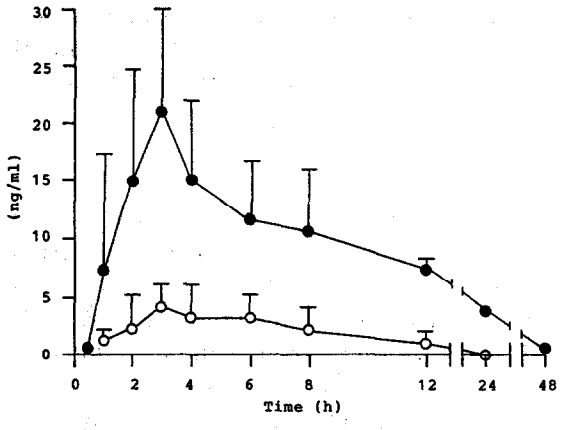

Fig. 6. Serum concentrations of I (●) and III (○) after oral administration of I (25 mg dihydrochloride) to human volunteers (mean + S.D., $n = 3$).

centration–time curves after oral administration of I (25 mg as the dihydrochloride per person) to healthy volunteers are shown in Fig. 6. The concentration of III was *ca.* 20% of that of I, and II was hardly detected in the serum.

CONCLUSION

The stabilization of the OPA derivatives of I and its metabolites was investigated in order to develop a sensitive determination method using pre-column derivatization. When N-acetyl-L-cysteine was used as a thiol and the excess OPA in the reaction mixture was eliminated by both solvent extraction and addition of an ammonium salt, the derivatives were stabilized. This method worked well for the determination of I and its metabolites in human serum. The stabilization technique described would enable the sensitive determination of various primary amines by pre-column derivatization with OPA.

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