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High-performance liquid chromatographic determination of a cysteine protease inhibitor and its ethyl ester in mouse serum and muscle by pre-column fluorescence derivatization

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SUMMARY

A simple and sensitive high-performance liquid chromatographic method has been developed for the determination of a cysteine protease inhibitor (E-64-c) and its ethyl ester in mouse serum and muscle samples. After deproteinization with acetone, E-64-c is converted into a fluorescent derivative by reaction with 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone. The derivative is separated on a reversed-phase column by isocratic elution of aqueous acetonitrile and monitored fluorimetrically. The ethyl ester is hydrolysed to E-64-c by carboxyl esterase and then derivatized in the same way as E-64-c. The limits of detection, at a signal-to-noise ratio of 3, of E-64-c and the ester are 500 pmol/ml in serum (10 μl) and 300 pmol/g in muscle (20 mg), corresponding to ca. 0.5 pmol each in a 50-μl injection volume. The method allows the determination of E-64-c and the ethyl ester in mouse serum and muscle after oral administration of these compounds.

INTRODUCTION

(+)-(2*S*,3*S*)-3-[(*S*)-3-Methyl-1-(4-guanidinobutylcarbamoyl)butylcarbamoyl]-2-oxiranecarboxylic acid was isolated from a culture of *Aspergillus japonicus* [1]. It was found to be a potent inhibitor of cysteine protease [1,2]. Many chemical analogues of this compound were synthesized and evaluated to obtain superior protease inhibitors suitable for medical application [2–5].

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Among them, (+)-(2*S*,3*S*)-3-[(*S*)-3-methyl-1-(3-methylbutylcarbamoyl)-butylcarbamoyl]-2-oxiranecarboxylic acid (E-64-c, I) (Fig. 1) was selected as a strong candidate because of its maximal inhibitory activity against lysosomal cathepsin and calcium-activated neutral protease [3,5], and it was expected to be a possible therapeutic drug for Duchenne muscular dystrophy [6]. However, a positive effect was not always observed when I was administered orally to experimental animals [6]. The ethyl ester of I (EST, II) (Fig. 1) was found to be more effective as an oral drug for the treatment of muscular dystrophy in experimental animals [6].

Methods for the determination of both I and II have been required not only for pharmacokinetic studies but also for the therapeutic evaluation of which drugs can enter the target organs. No method for monitoring I and II in biological samples appears to be available.

We have recently found that II can be enzymically hydrolysed to I with carboxyl esterase, and also that I can be labelled with 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (Br-DMEQ), a fluorescent reagent for carboxylic acids [7] (Fig. 1).

This paper describes the conditions for the hydrolysis of II and the fluores-

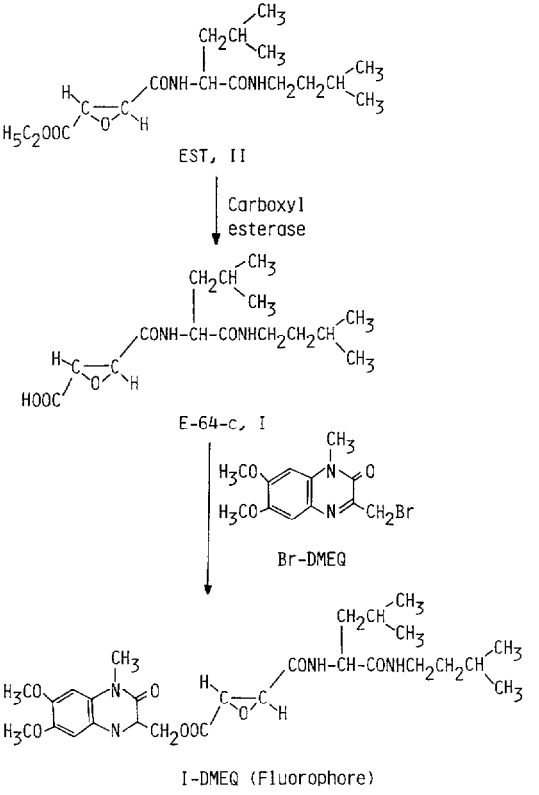

Fig. 1. Fluorescence derivatization of I and II with Br-DMEQ.

cence derivatization of I with Br-DMEQ. The aim was to establish a simple and sensitive method for the quantification of I and II in mouse serum and muscle by means of high-performance liquid chromatography (HPLC) with fluorescence detection. The distribution of I and II in the serum and muscle of mice after oral administration of either I or II was also examined.

EXPERIMENTAL

Chemicals

Deionized, distilled water was used. I and II were products of Taisho Pharmaceutical (Tokyo, Japan). Br-DMEQ was obtained from Dojindo Labs. (Kumamoto, Japan). Carboxyl esterase (EC 3.1.1.1) from porcine liver was purchased from Sigma (St. Louis, MO, U.S.A.). Other chemicals were of reagent grade.

Administration and sample preparation

Inbred mice (C57BL, five weeks old, weight 18 ± 3 g) were used. The mice received a 50 mg/kg oral dose of I or II suspended in aqueous 5% (w/v) arabic gum through a conductor needle. After the administration, the mice were fed only with water until being killed by exsanguination from a carotid artery. The blood was collected in a centrifuge tube and serum was separated. Trapezius muscle was also quickly collected, washed with saline and stored at $-80\,^{\circ}C$ after removing the saline with filter-paper.

For the determination of I, a 10-μl portion of serum was mixed with 50 μl of water (or standard aqueous solution of I for calibration graph). The sliced muscle (20 ± 2.5 mg) was homogenized with 50 μl of water (or standard aqueous solution of I). To the serum and muscle samples, 200 μl of acetone were added for deproteinization. After centrifugation at 1000 *g* for 5 min, the supernatants of the serum and muscle samples were used for fluorescence derivatization.

For the determination of II, a 10-μl portion of serum was mixed with 50 μl of water (or standard aqueous solution of II). The sliced muscle (20 ± 2.5 mg) was homogenized with 50 μl of water (or standard aqueous solution of II). Portions (5 μl) of 300 U/ml carboxyl esterase in 0.1 *M* phosphate buffer (pH 8.0) were added to the serum and muscle samples, respectively. The mixtures were then incubated at room temperature ($25 \pm 4\,^{\circ}C$) for 30 min. Acetone (200 μl) was added to each incubation mixture. The deproteinized serum and muscle samples were used for fluorescence derivatization.

Fluorescence derivatization

A 20-μl portion of the sample solution was mixed with 10 μl of 100 m*M* potassium hydrogencarbonate in aqueous 20% (v/v) N,N′-dimethylformamide (DMF) and 70 μl of 4 m*M* Br-DMEQ in DMF. The mixture was heated at

100°C for 20 min in the dark. A 50-μl portion of the final reaction mixture was used for HPLC assay.

HPLC conditions and apparatus

The HPLC system consisted of a Tosoh 803D high-performance liquid chromatograph equipped with a Rheodyne 7125 syringe-loading sample injector valve (50-μl loop) and a Hitachi F-1000 fluorescence spectrophotometer fitted with a 12-μl flow-cell operating at an emission wavelength of 460 nm and an excitation wavelength of 380 nm. A column (15 cm×0.4 cm I.D.) of TSK gel ODS-120T (particle size, 5 μm; Tosoh, Tokyo, Japan) was used. Two mixtures of acetonitrile and water (40:60 and 95:5, v/v) were used for a stepwise gradient elution. The column was first eluted with the 40% acetonitrile for 25 min, followed by the 95% acetonitrile for the next 30 min; the column was then equilibrated with the 40% acetonitrile for 10 min before analysis of the next sample. The flow-rate was 0.8 ml/min. The column temperature was ambient (25±4°C).

Uncorrected fluorescence excitation and emission spectra of the HPLC fractions were measured with a Hitachi MPF-4 spectrofluorometer in 10 mm×10 mm quartz cells; spectral band widths of 10 nm were employed for both excitation and emission monochromators.

The concentration of I was evaluated by the peak height in chromatograms. The concentration of II was calculated by subtracting the concentration of I in the same sample before the hydrolysis of II.

RESULTS AND DISCUSSION

HPLC separation

The fluorescent derivative of I was completely separated from the large peaks due to the reagent blank on a reserved-phase column, TSK gel ODS-120T, by stepwise gradient elution with acetonitrile (Fig. 2). The retention time of the I peak was 20.0 min. The HPLC fraction of the I peak showed excitation and emission maxima at wavelengths of 380 and 460 nm, respectively. The components of the reagent blank were retained on the column for at least 60 min when 40% (v/v) acetonitrile eluent was run. However, 95% (v/v) acetonitrile eluted the blank components from the column within 45 min. Therefore, the 95% (v/v) acetonitrile eluent was used for ca. 15 min after the elution of the I peak. A buffer of either 10 m*M* phosphate buffer or 10 m*M* acetate buffer (both pH 4.0) in the mobile phase had no effect on the separation and peak height for I.

Fig. 3 shows typical chromatograms of I in serum and muscle obtained from a mouse dosed orally with I. The fluorescent peak for I was separated from other substances in both the samples. In the chromatograms obtained with the

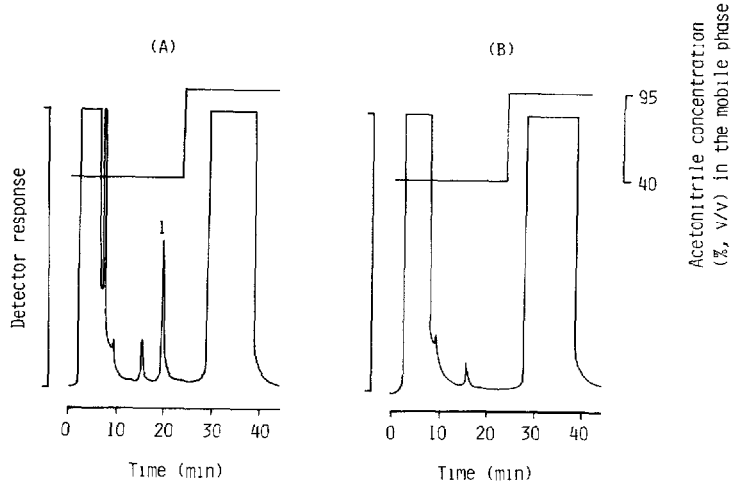

Fig. 2. Chromatograms of the DMEQ derivative of (A) I and (B) the reagent blank. Peaks: 1 = I; others = the reagent blank.

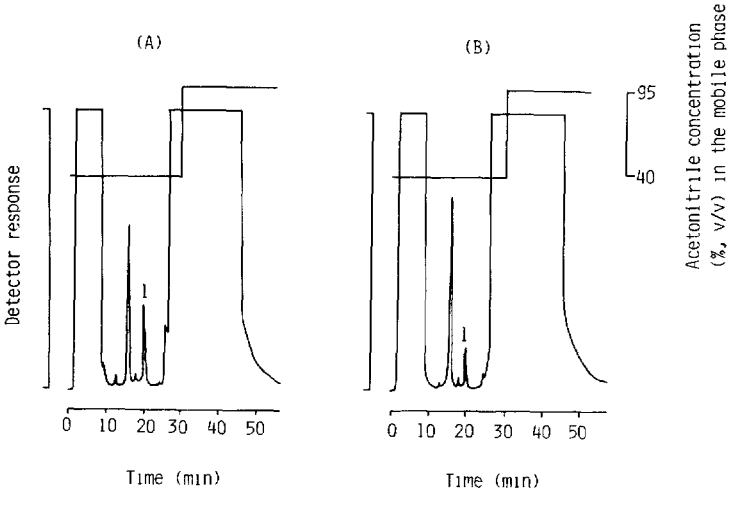

Fig. 3. Chromatograms of the DMEQ derivative of I in (A) serum and (B) muscle. Samples were taken 4 h after oral administration of I (50 mg/kg) to a mouse. Peaks: 1 = I; others = the endogenous substances in serum or muscle and the reagent blanks. Concentrations of I: (A) 8.5 nmol/ml in serum; (B) 3.8 nmol/g in muscle.

drug-free serum and muscle samples, no peak at the retention time of the I peak arose and the other peaks were observed as in Fig. 2.

Peaks of fluorescent derivatives from synthetic carboxylic acids (palmitic acid, oleic acid, lactic acid and maleic acid) were not observed within 1 h by the same chromatography as that for Fig. 2. In addition, α-keto acids (*p*-hy-

droxyphenylpyruvic acid, phenylpyruvic acid), amino acids (arginine, tyrosine), saccharides (glucose, fructose), amines (histamine, tyramine), aldehydes (formaldehyde, benzaldehyde), ketones (acetone, acetophenone) and phenols (3.5-xylenol, epinephrine) did not give fluorescent products by the present derivatization procedure.

Fluorescence derivatization

Br-DMEQ has been reported to react in acetonitrile with carboxylic acids in the presence of both potassium carbonate powder and 18-crown-6 ether, which serves to accelerate the reaction by including potassium ion in its molecule [7]. Under these conditions, however, the undissolved potassium carbonate in the reaction mixture often stopped the flow of eluent at the sample loop or the inlet of the HPLC column. Therefore, the production of the fluorescent derivative of I was investigated in the presence of potassium salts (potassium carbonate, potassium hydrogencarbonate and potassium chloride) or organic bases (pyridine, triethylamine and tetra-*n*-butylammonium hydroxide), which were miscible in the reaction mixture containing 2% (v/v) water.

Of these, potassium hydrogencarbonate gave a good yield of the I derivative. The optimum concentration of potassium hydrogencarbonate was 5 m*M* for the derivatization of I in water with Br-DMEQ (Fig. 4, curve b). However, the production of the derivative of I in drug-free serum was fairly low (Fig. 4, curve a). A concentration of potassium hydrogencarbonate higher than 100 m*M* was required for the derivatization of I in the serum samples. The same was also true for the muscle samples. Under these conditions, 18-crown-6 ether (5 m*M* in the reaction mixture) for the fluorescence derivatization did not have any effect on the yield of the I derivative.

Water was necessary for dissolving the potassium salt, though the derivati-

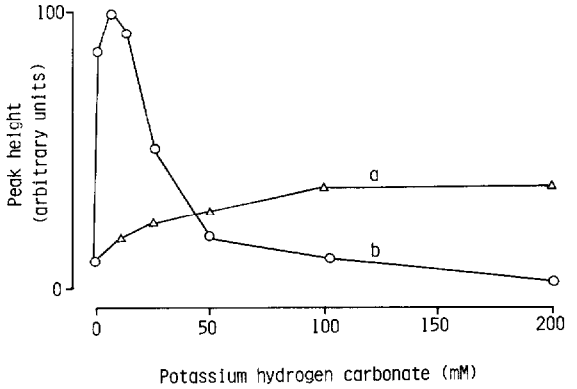

Fig. 4. Effect of the concentration of potassium hydrogencarbonate on the derivatization of I. A 10-μl portion of 10 nmol/ml I was added to (a) drug-free serum or (b) water and treated as in the recommended procedure with various concentrations of potassium hydrogencarbonate.

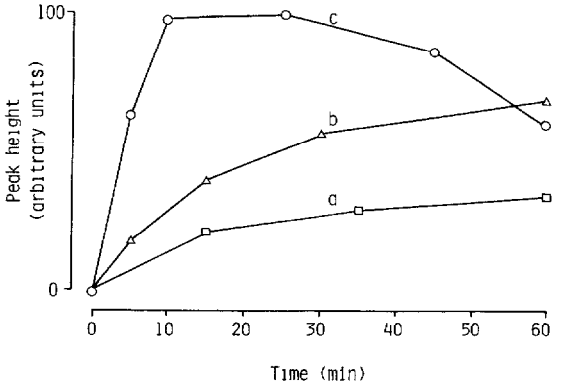

Fig. 5. Effects of reaction time and temperature on the derivatization of I. A 10-μl portion of 10 nmol/ml I was added to drug-free serum and treated as in the recommended procedure at (a) 37°C, (b) 60°C and (c) 100°C for various periods.

zation was inhibited by the presence of water at a concentration higher than 4% (v/v) in the reaction mixture. Thus, a 10-μl portion of 100 m*M* potassium hydrogencarbonate in DMF–water (4:1, v/v) was used for the recommended procedure; the concentration of water in the reaction mixture was 2% (v/v).

DMF, acetonitrile, dimethyl sulphoxide (DMSO), methanol, dioxane and acetone were examined as the reaction solvent. The product peaks were not observed when methanol, dioxane or acetone was used. The highest peak of the product was achieved by the use of DMF, and it was two to three times higher than that obtained with acetonitrile or DMSO.

Maximum yield of the fluorescent derivative was obtained at a Br-DMEQ concentration higher than 3 m*M*; 4 m*M* Br-DMEQ was therefore employed. Higher temperatures allowed I to form the derivative more rapidly (Fig. 5). At 100°C, a maximum rate of the formation was achieved for a reaction time of 20 min; heating at 100°C for 20 min was adopted in the procedure. The fluorescent derivative of I was stable for at least 24 h when the final reaction mixture was kept at room temperature (25±4°C) in the dark.

Determination of I and II

The deproteinization of serum and muscle samples was examined by use of organic solvents such as acetonitrile, DMF and acetone. Of these, acetone gave the greatest recovery of I. The recoveries of I added to the drug-free serum and muscle at concentrations of 20 nmol/ml in serum and 10 nmol/g in muscle were 98.7±1.2 and 95.3±2.2% ($n=5$, in each instance), respectively. The derivatization was not affected by the presence of acetone at a concentration lower than 20% (v/v) in the reaction mixture. Therefore, a 20-μl portion of the supernatant of the deproteinized sample was used for the derivatization.

Compound II was hydrolyzed by incubating with carboxyl esterase (100–500

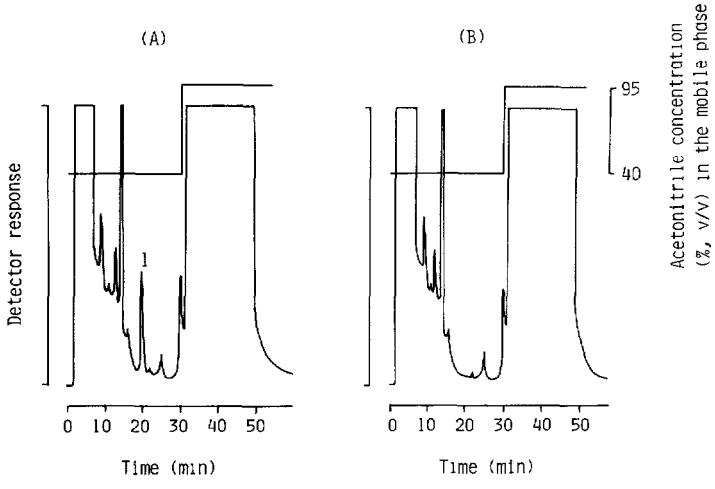

Fig. 6. Chromatograms of the DMEQ derivative of I obtained by enzymic hydrolysis of a drug-free serum (A) containing II and (B) without II. A 10-μl portion of 10 nmol/ml II or water was added to the drug-free serum and treated as in the recommended procedure Peaks: 1 = I; others = the reagent blank and endogenous substances in serum.

U in the incubation mixture) at room temperature (25 ± 4°C) for 30 min. The yield of I from II was 98 ± 2% under the conditions. Fig. 6 shows chromatograms obtained after the enzymic hydrolysis of a drug-free serum with and without II. The fluorescent derivative of I was certainly produced in the sample by the hydrolysis of II followed by the reaction with Br-DMEQ.

The calibration graphs of peak height versus amount of I or II added to drug-free serum and muscle samples were linear through the origin in the concentration ranges 0–0.5 μmol/ml and 0–0.8 μmol/g, respectively. The correlation coefficients (r) for the straight lines of these graphs were 0.998–0.999. Therefore, the quantification of I or II in the biological samples after the drug administration was performed by the standard addition method. The lower detection limits (signal-to-noise ratio = 3) of I and II were 500 pmol/ml in serum and 300 pmol/g in muscle. These values corresponded to ca. 0.5 pmol per an injection volume of 50 μl.

The precision of the present method was established with six replicated intra-assays. The relative standard deviations were 4.7 and 5.2% for the mean concentration of 25 nmol I or II per ml of serum and 5.5 and 6.1% for the mean concentration of 20 nmol I or II per g of muscle, respectively.

Distribution of I and II

The concentration–time graphs for I in serum and muscle of mice dosed orally with I (50 mg/kg) are shown in Fig. 7A. The concentrations of I in both serum and muscle reached maximum values at 1 h after administration and

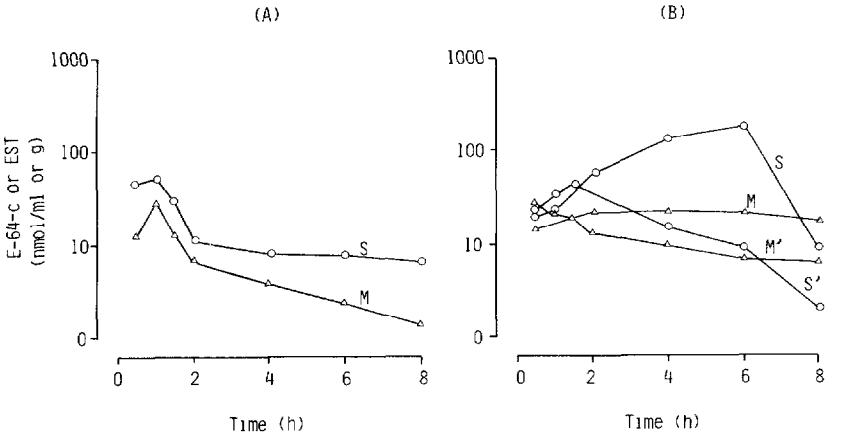

Fig. 7. Concentrations of I and II in serum and muscle of mice ($n=3$ in each case of the sampling) after oral administration of (A) I and (B) II. Dose: 50 mg/kg each. Curves: S, I in serum; M, I in muscle; S', II in serum; M', II in muscle.

then decreased slowly until 8 h; the half-life was ca. 8.7 h in serum and ca. 5.2 h in muscle.

With the administration of II (Fig. 7B), the half-life of II was ca. 4.0 h in serum and ca. 5.3 h in muscle. Compound I in the samples of serum and muscle dosed with II was detected before hydrolysis. Compound II was metabolized to I in the mouse body. The I concentration became greater than the II concentration in serum and muscle at 0.8 and 1.5 h, respectively, and reached maximum values at 4 and 6 h in serum and muscle, respectively. The I concentration (126 nmol/ml) in serum 6 h after the administration of II was approximately fourteen times higher than that following the same dose of I. These results demonstrate that I can be more readily distributed into muscle tissue by the administration of II.

The proposed HPLC method permits the sensitive determination of I and II in 10 μl of serum and 20 mg of muscle from mice dosed with either I or II. Thus, this method may be useful in the chemotherapeutic and pharmacokinetic studies of these drugs.

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