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DETERMINATION OF *p*-HYDROXYBESTATIN IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING FLUORESCENCE DETECTION

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SUMMARY

A high-performance liquid chromatographic method is described for the fluorimetric determination of *p*-hydroxybestatin (an active metabolite of bestatin) in human serum. *p*-Hydroxybestatin is formylated in an alkaline medium in the presence of chloroform, and converted to a fluorescent derivative with 1,2-diamino-4,5-dimethoxybenzene. This derivative is then separated on a reversed-phase column (TSK gel ODS-120T) with isocratic elution. The detection limit of *p*-hydroxybestatin in serum is 15 ng (46 pmol) per ml serum (115 pg in a 100-μl injection volume). This method is simple and sensitive enough to determine *p*-hydroxybestatin in serum (200 μl) from muscular dystrophic patients and from healthy subjects dosed with bestatin.

INTRODUCTION

p-Hydroxybestatin (Fig. 1) is a major metabolite of bestatin [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-*S*-leucine] in man [1], and has been studied as a possible therapeutic drug for cancer, resistant infections and muscular dystrophy [2—5]. *p*-Hydroxybestatin and bestatin inhibit aminopeptidase B and enhance delayed-type hypersensitivity to the same degree [6]. For the determination of *p*-hydroxybestatin in serum, together with bestatin, a gas chromatographic—mass spectrometric (GC—MS) method has been reported [1]. A more readily available method of determination is needed for the therapeutic and pharmacokinetic studies of bestatin.

We previously reported a simple method for the determination of bestatin and *p*-hydroxybestatin by high-performance liquid chromatography (HPLC) with fluorescence detection [7]. Although the method allows the determination of bestatin in serum from subjects dosed with bestatin, the sensitivity of the method is not high enough to determine *p*-hydroxybestatin, which occurs at low concentrations in serum.

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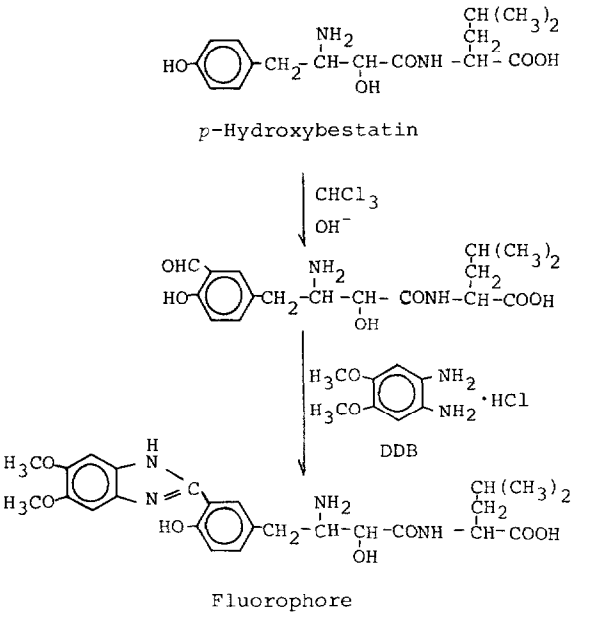

Fig. 1. Fluorescence derivatization of *p*-hydroxybestatin with DDB.

We have found that *p*-hydroxybestatin can be converted to a highly fluorescent derivative with 1,2-diamino-4,5-dimethoxybenzene (DDB) (a fluorescence derivatization reagent for aromatic aldehydes [8, 9]) in a weakly acidic solution after formylation by the Reimer-Tiemann reaction [10,11] (Fig. 1). This derivative is then separated from endogenous substances in the serum and from components of the reagent blank by reversed-phase HPLC with isocratic elution, thus giving a sensitive and simple method for the quantification of *p*-hydroxybestatin in serum.

Leucine enkephalin (LEK, Tyr-Gly-Gly-Phe-Leu) and methionine enkephalin (MEK, Tyr-Gly-Gly-Phe-Met), both of which are tyrosine-containing peptides that can be converted to fluorescent derivatives by formylation followed by the DDB reaction, were also examined with regard to the conditions of chromatographic separation.

EXPERIMENTAL

Reagents and solutions

All chemicals were of analytical reagent grade, unless indicated otherwise. Deionized and distilled water was used. *p*-Hydroxybestatin and bestatin are the products of Nihon Kayaku (Tokyo, Japan). LEK and MEK were purchased from Sigma (St. Louis, MO, U.S.A.). DDB monohydrochloride was obtained from Dojindo Labs. (Kumamoto, Japan).

DDB solution (1.3 m*M*) was prepared by dissolving 5.3 mg of DDB monohydrochloride in 20 ml of water. This solution should be used within 2 h. Acetate–hydrochloric acid buffer (50 m*M*, pH 2.2) was prepared by dissolving 6.8 g of sodium acetate trihydrate in 900 ml of water, adjusting the pH to 2.2 with concentrated hydrochloric acid and diluting the solution with water

to 1000 ml. Hydrogen peroxide solution (1.0 m*M*) was freshly prepared from 10 m*M* hydrogen peroxide stock solution that had been previously standardized by an acid permanganate titration. Sera from patients with Duchenne's muscular dystrophy (male, 5—9 years of age) were supplied from National Nishibeppu Hospital (Beppu, Oita, Japan).

Apparatus

The HPLC system consisted of a Toyo Soda 803D high-performance liquid chromatograph equipped with a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) and a Shimadzu RF 530 fluorescence spectrometer fitted with a 12-μl flow-cell operating at an emission wavelength of 425 nm and at an excitation wavelength of 325 nm. The column was TSK gel ODS-120T (particle size 5 μm; 150 × 4 mm I.D.; Toyo Soda, Tokyo, Japan). This column can be used for more than 1000 injections with only a small decrease in the theoretical plate number. Uncorrected fluorescence excitation and emission spectra were measured with a Hitachi MPF-4 spectrofluorimeter in quartz cells (optical pathlength, 10 × 10 mm).

Procedure

A 200-μl portion of serum was mixed with 100 μl of water [or *p*-hydroxybestatin standard solution (0.1—1.0 μg/ml) for the calibration curve] and 200 μl of 2.5 *M* perchloric acid to deproteinize the serum. The mixture was centrifuged at 800 *g* for 10 min. To 200 μl of the supernatant, 200 μl of 2.6 *M* potassium hydroxide were added. The mixture was centrifuged at 800 *g* for 5 min to precipitate potassium perchlorate. The supernatant (250 μl) was placed in a screw-capped vial (45 × 12 mm I.D.), to which were added 100 μl of chloroform. The mixture was heated in a heating module (Pierce, Rockford, IL, U.S.A.) at 70°C for 8 min to formylate *p*-hydroxybestatin, which was then cooled in ice-water for ca. 1 min. To the mixture, 50 μl of 14.0 *M* acetic acid, 300 μl of DDB solution and 50 μl of 1.0 m*M* hydrogen peroxide solution were successively added, with cooling in ice-water. The mixture was heated in the heating module at 70°C for 10 min to develop the fluorescence and was then cooled. A 100-μl portion of the aqueous layer was injected into the chromatograph.

The mobile phase was acetonitrile—50 m*M* acetate—hydrochloric acid buffer, pH 2.2 (9:41). The flow-rate was 0.8 ml/min (pressure 98 bar). The column temperature was ambient (20—27°C).

RESULTS AND DISCUSSION

HPLC conditions

Fig. 2 shows the chromatograms obtained with a standard mixture of *p*-hydroxybestatin, MEK and LEK, and with water for the reagent blank. The fluorescent derivative from *p*-hydroxybestatin was well separated from those of MEK and LEK, and from the components of the reagent blank. The eluates from peaks 1, 2 and 3 in Fig. 2a have fluorescence excitation spectra with maxima around 350 nm and emission spectra with maxima around 425 nm.

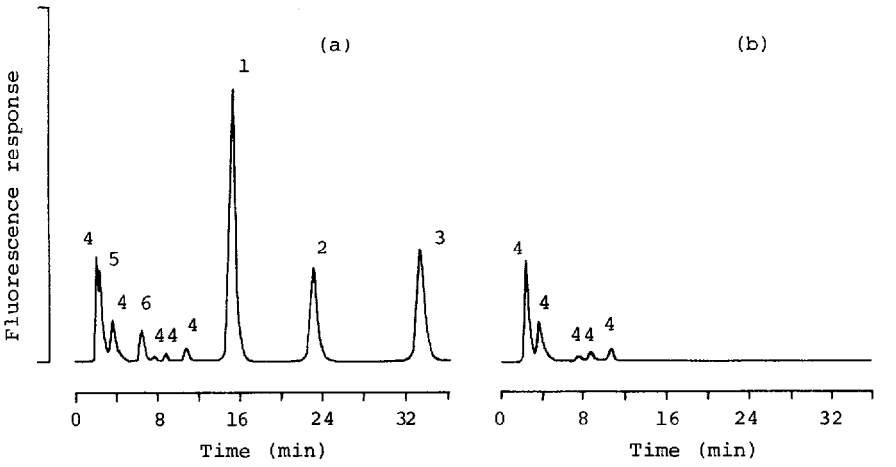

Fig. 2. Chromatograms of (a) a mixture of *p*-hydroxybestatin, MEK and LEK and (b) the reagent blank. Portions (200 μl) of a standard solution (5 nmol/ml, each) and water for the blank were treated according to the procedure. Peaks: 1 = *p*-hydroxybestatin; 2 = MEK; 3 = LEK; 4 = reagent blank; 5 = by-products from MEK and LEK; 6 = by-product from MEK.

The concentration of acetonitrile in the mobile phase affects the separation of the peaks. At a concentration higher than 35%, the peak for *p*-hydroxybestatin partially overlaps the nearest peak of the blank, while a concentration of 13% or lower causes a delay in the elution with a broadening of all the peaks. An acetonitrile concentration between 13 and 20% provides a satisfactory separation of the peaks for *p*-hydroxybestatin and the blank. An acetonitrile concentration in the range 15—25% gives higher peaks for *p*-hydroxybestatin, LEK and MEK. Therefore, 18% acetonitrile was used for the mobile phase in the recommended procedure. When methanol was used instead of acetonitrile in the mobile phase, the peaks for the compounds were badly broadened. Acetate—hydrochloric acid buffer in the mobile phase does not affect the retention times of any of the peaks at concentrations of 10—100 m*M*; 50 m*M* was selected for convenience. The pH of 50 m*M* acetate—hydrochloric acid buffer has an effect on the retention times and the heights of the peaks for the compounds. The retention times increase with decreasing pH in the range 1.3—6.0. The DDB derivatives fluoresce most intensely at pH 1.3—3.5. Thus, a 50 m*M* acetate—hydrochloric acid buffer of pH 2.2 was employed in the recommended procedure. Phosphate buffer (50 m*M*, pH 2.2) or potassium chloride—hydrochloric acid buffer (50 m*M*, pH 2.2) can be used as alternatives to the acetate—hydrochloric acid buffer.

Formylation and derivatization

The formylation of *p*-hydroxybestatin proceeds rapidly at temperatures higher than the boiling point of chloroform (Fig. 3). The height of the peak for *p*-hydroxybestatin reaches a maximum and is constant after heating at 70 or 80°C for 8 min. No by-product peak from *p*-hydroxybestatin was observed in the chromatogram; 8-min heating at 70°C was therefore employed as optimum.

Potassium hydroxide concentration has a considerable effect on the rate of

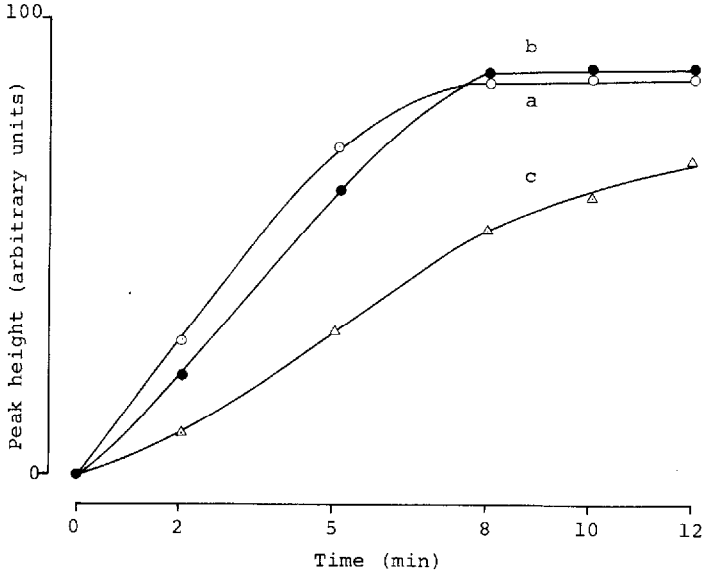

Fig. 3. Effects of reaction time and temperature on the formylation of *p*-hydroxybestatin. A 200-μl portion of 5 nmol/ml *p*-hydroxybestatin solution was treated by the recommended procedure for various reaction times at different reaction temperatures. (a) 80°C; (b) 70°C; (c) 60°C.

formylation. At potassium hydroxide concentrations higher than 0.8 *M* in the reaction mixture, the height of the peak for *p*-hydroxybestatin decreases; a concentration of 0.8 *M* was therefore used to obtain maximum peak height.

Chloroform in quantities of 50—200 μl in the reaction mixture gives maximum and constant peak height for *p*-hydroxybestatin; 100 μl was selected for the recommended procedure. Shaking the reaction mixture during the formylation does not affect the peak height.

The derivatization reaction with DDB proceeds most effectively at pH 3.3 in aqueous acetic acid. At a pH higher than 5.0 or lower than 1.8, the reaction does not occur.

The reaction proceeds rapidly at 70°C in the presence of hydrogen peroxide. The height of the peak for the fluorescent derivative reaches a maximum after heating for 10 min and then decreases slightly with time. Hydrogen peroxide in a concentration range of 60—100 μ*M* in the reaction mixture gives a maximum peak height; a concentration of 77 μ*M* was selected as optimum. Sodium periodate (4—10 μ*M* in the reaction mixture) can be used as an alternative to hydrogen peroxide. In the absence of such oxidizing agents, the reaction was not initiated for the first 20 min or more. This lag time was probably due to some reducing substances produced during the formylation.

Higher temperatures allow the derivatization reaction to proceed more rapidly and a maximum peak height for *p*-hydroxybestatin can be attained by heating at 70—100°C for 7 min.

Determination of p-hydroxybestatin in serum

Deproteinization of serum can be achieved by adding 2.0—3.0 *M* perchloric

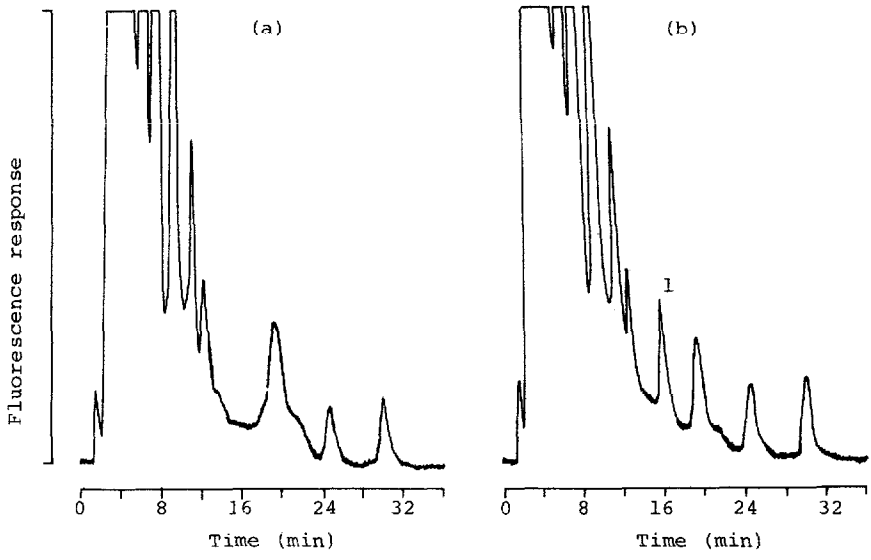

Fig. 4. Chromatograms of (a) drug-free serum and (b) serum at 4 h after oral administration of bestatin to a healthy man. Dose: 4.17 mg/kg. Peaks: 1 = *p*-hydroxybestatin; others = endogenous substances in the serum and reagent blank. Serum concentrations of *p*-hydroxybestatin are 0.0 ng/ml (a) and 165 ng/ml (b).

acid (final concentration 0.8—1.2 *M*) to the serum; 2.5 *M* perchloric acid was employed in the procedure. The use of trichloroacetic acid (final concentration 0.5 *M*) caused low recovery of *p*-hydroxybestatin (approximately 65%).

Fig. 4 shows the chromatograms obtained with drug-free serum from a healthy man and with serum from a man dosed with bestatin. No interfering peaks arose in the drug-free serum around the retention time of *p*-hydroxybestatin. The eluate from the peak for *p*-hydroxybestatin in the chromatogram (Fig. 4b) showed identical fluorescence spectra to those from *p*-hydroxybestatin standard.

The calibration curve for *p*-hydroxybestatin is linear in the relationship between peak height and concentration (0--25 nmol/ml) of *p*-hydroxybestatin in the serum, with a correlation coefficient of 0.999. The limit of detection for *p*-hydroxybestatin was 15 ng (46 pmol) per ml serum (115 pg in a 100-μl injection volume) at a signal-to-noise ratio of 2.

The recovery of *p*-hydroxybestatin (0.8 μg/ml) added to drug-free serum is 98 ± 1.8% (mean ± S.D., n=5). The precision was established by repeated determinations (n=7) using a serum obtained at 4 h after oral administration of bestatin (250 mg) to a healthy man (body weight 60 kg). The coefficient of variation was 3.9% for a mean concentration of 165 ng/ml of serum.

Fig. 5 shows the serum concentrations of *p*-hydroxybestatin and bestatin after oral administration of bestatin (single dose, 4.17 mg/kg) to a healthy man. The concentrations of bestatin in serum were determined by the previous method [7]. The concentration of *p*-hydroxybestatin reached a maximum value at 4 h after the administration, while that of bestatin reached a maximum at 3 h.

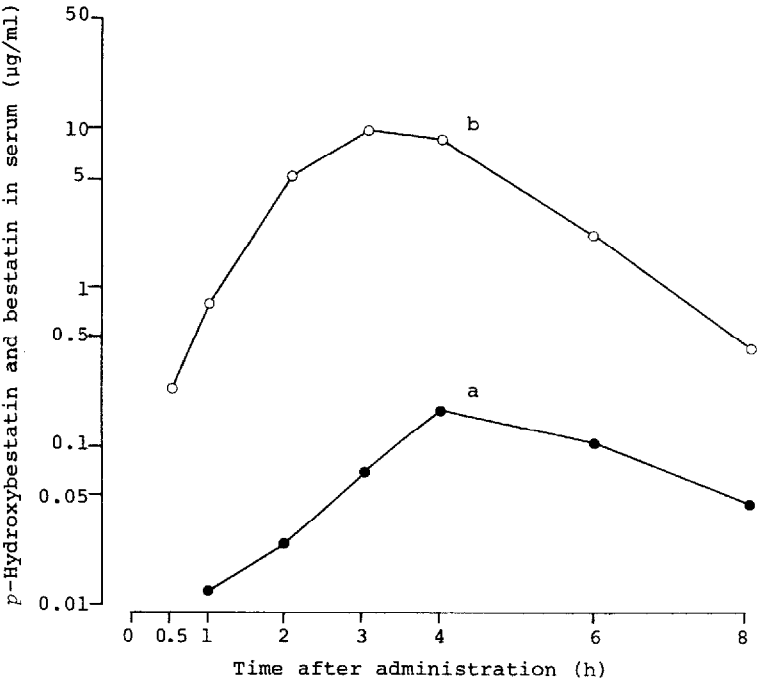

Fig. 5. Serum concentrations of (a) *p*-hydroxybestatin and (b) bestatin after oral administration of bestatin in a single dose to a healthy man at 1 h after feeding. Dose: 250 mg. Body weight and age: 60 kg, 26 years.

TABLE I

CONCENTRATIONS OF *p*-HYDROXYBESTATIN AND BESTATIN IN SERA AFTER ORAL ADMINISTRATION OF BESTATIN TO PATIENTS WITH DUCHENNE'S MUSCULAR DYSTROPHY

Bestatin (6.67 mg/kg) was orally administered three times a day (morning, noon and evening) for six weeks. Concentrations of *p*-hydroxybestatin and bestatin were determined in sera obtained at 1 h after the last administration in the morning.

Patient	Age (years)	Weight (kg)	Concentration (μg/ml)		Ratio*
			p-Hydroxybestatin	Bestatin	
A	9	20.0	0.190	4.18	0.045
B	7	22.5	0.330	5.98	0.055
C	7	19.0	0.200	6.26	0.032
D	9	23.4	0.365	5.06	0.072
E	8	20.0	0.275	5.20	0.053
F	5	16.6	0.350	4.18	0.084

*The concentration of *p*-hydroxybestatin was divided by that of bestatin in the same sample.

Table I shows the concentrations of *p*-hydroxybestatin and bestatin in sera from patients with Duchenne's muscular dystrophy who had been orally dosed with bestatin for several weeks. The concentration ratios of *p*-hydroxybestatin to bestatin in sera from the patients were larger than that (0.014)

for a healthy volunteer at 1 h after the dosage. The data may reflect that *p*-hydroxybestatin accumulates in the bodies of the patients dosed with bestatin.

The proposed method is simple and approximately 27 times more sensitive than the previous HPLC method [7]. The study provides the first practical method for the quantification of *p*-hydroxybestatin in serum. This HPLC method should be useful for routine analyses of *p*-hydroxybestatin in therapeutic and biomedical studies of bestatin.

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