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DETERMINATION OF BESTATIN IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A simple method for the determination of bestatin and its major metabolite in man, *p*-hydroxybestatin, in human serum was investigated; the method employs high-performance liquid chromatography with fluorescence detection. Bestatin and *p*-hydroxybestatin are oxidized to phenylacetaldehyde and *p*-hydroxyphenylacetaldehyde, respectively, with periodate, which are then converted into fluorescent compounds with 4,5-dimethoxy-1,2-diaminobenzene. The compounds are separated by reversed-phase chromatography on LiChrosorb RP-18. The detection limits of bestatin and *p*-hydroxybestatin are 0.2 and 0.4 μg/ml serum, respectively. This method permits the precise determination of bestatin in serum (20 μl) from patients administered bestatin. *p*-Hydroxybestatin in serum can not be measured by this method because of its low concentration (less than the detection limit).

INTRODUCTION

Bestatin, (2*S*, 3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-S-leucine, inhibits aminopeptidase B and leucine aminopeptidase, and enhances delayed-type

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hypersensitivity [1, 2]. Bestatin has been studied as a possible therapeutic drug for cancer, resistant infection and muscular dystrophy.

For the determination of bestatin in serum, only a gas chromatographic—mass spectrometric (GC—MS) method has been reported [3]. This method also permits the determination of *p*-hydroxybestatin, which is a major metabolite in man but occurs at low concentration in serum. For the biomedical investigattions of bestatin and for drug monitoring during experimental therapy, a readily available method of determination was required.

We have developed a simple method for the determination of bestatin and *p*-hydroxybestatin in minute amounts of human serum by high-performance liquid chromatography (HPLC) with fluoresence detection. This method is based on the determination of phenylacetaldehyde and *p*-hydroxyphenylacetaldehyde formed from bestatin and *p*-hydroxybestatin, respectively, by oxidation with periodate. The aldehydes are converted into fluorescent compounds by reaction with 4,5-dimethoxy-1,2-diaminobenzene (DDB), a fluorescent derivatization reagent for aromatic and arylaliphatic aldehydes [4, 5]. The fluorescent products are separated by reversed-phase HPLC on LiChrosorb RP-18. Drug-free serum spiked with bestatin and *p*-hydroxybestatin was used as a model sample to establish suitable conditions for a general analytical method. Sera from patients administered bestatin were subjected to analysis by the method.

EXPERIMENTAL

Materials and reagents

All chemicals were of reagent grade, unless otherwise noted. Deionized and distilled water was used. Bestatin and *p*-hydroxybestatin are the products of Nihon Kayaku (Tokyo, Japan). DDB monohydrochloride was prepared as described previously [4].

The DDB solution (1.3 m*M*) was prepared by dissolving 26.5 mg of DDB monohydrochloride in 100 ml of 0.3 *M* hydrochloric acid and should be used within 3 h. Normal sera were obtained from healthy volunteers (male, 22—52 years of age) in this laboratory in the usual manner. Sera from patients with muscular dystrophy (male, 4—10 years of age) were supplied from National Nishibeppu Hospital (Beppu, Oita, Japan).

Apparatus

A Toyo Soda 803 D high-performance liquid chromatograph equipped with a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) and a Shimadzu RF 530 fluorescence spectromonitor fitted with a 12-μl flow-cell operating at an emission wavelength of 390 nm and an excitation wavelength of 320 nm was used. The column was LiChrosorb RP-18 (particle size, 5 μm; 150 × 4 mm I.D.; Japan Merck, Tokyo, Japan). This column can be used for more than 1000 injections (with only a small decrease in the theoretical plate number) when washed with aqueous acetonitrile (1:1, v/v) at a flow-rate of 0.8 ml/min for about 30 min after every day of analyses. Uncorrected fluorescence excitation and emission spectra were measured with an Hitachi MPF-4 spectrofluorimeter in quartz cells (optical path-length, 10 × 10 mm);

spectral band-widths of 5 nm were used in both the excitation and emission monochromators.

Procedure

Sample serum (20 μl) was mixed with 20 μl of water and 200 μl of 4.2 m*M* acetic acid. The mixture was heated for about 5 min in a boiling water-bath and centrifuged at approximately 1000 *g* for 5 min. To 100 μl of the supernatant were added 50 μl of 1.5 *M* ammonium hydroxide and 25 μl of 3 m*M* sodium periodate, and the mixture was allowed to stand at room temperature (approximately 25°C) for 20 min. The excess periodate was decomposed by the addition of 25 μl of 12 m*M* sodium sulphite. To the mixture, 200 μl of DDB solution were added and the mixture was warmed at 37°C for 50 min; then, 50 μl of 1.0 *M* sodium hydroxide were added. Drug-free serum (20 μl) was treated in the same way as for the sample serum. A 100-μl volume of the final mixture was injected into the chromatograph.

The mobile phase was a mixture of 0.1 *M* Tris · hydrochloric acid buffer (pH 8.7) and acetonitrile (3:1, v/v). The flow-rate was 0.8 ml/min (approximately 75 kg/cm^2). The column temperature was ambient (approximately 25°C). The net peak heights due to bestatin and *p*-hydroxybestatin in the sample serum were calculated. The amounts of bestatin and *p*-hydroxybestatin were calibrated by means of the standard addition method: 20 μl of water added to the serum in the procedure were replaced by 20 μl of a standard mixture of bestatin and *p*-hydroxybestatin (0.7 or 3 μg/ml each).

RESULTS AND DISCUSSION

HPLC conditions

Fig. 1 shows the chromatograms obtained with a standard mixture of bestatin and *p*-hydroxybestatin and with water for reagent blank. The chromatogram (Fig. 1a) was identical to that obtained when a mixture of phenylacetaldehyde and *p*-hydroxyphenylacetaldehyde was treated directly with DDB as in the procedure. The retention times for the DDB derivatives of bestatin and *p*-hydroxybestatin are 4.5 and 10.5 min, respectively. The eluates from peaks 1 and 2 in Fig. 1a have fluorescence excitation spectra with maxima at 325 and 327 nm, respectively, and emission spectra with maxima at 391 and 382 nm, respectively.

The concentration of acetonitrile in the mobile phase affects the separation of the peaks. At a concentration greater than 45%, the peaks for bestatin and *p*-hydroxybestatin partially overlap that of the blank, while a concentration of 20% or less causes delay in the elution with broadening of the peaks for bestatin and *p*-hydroxybestatin. A concentration of acetonitrile less than 30% provides a satisfactory separation of the peaks for DDB and *p*-hydroxybestatin. Greater concentration of acetonitrile gives higher peaks for bestatin and *p*-hydroxybestatin in a range 20—40%. Therefore, 25% acetonitrile in the mobile phase was used for the procedure recommended. When methanol was used in place of acetonitrile in the mobile phase, the half-widths of the peaks doubled. Tris · hydrochloric acid buffer in the mobile phase does not affect the retention times of any of the peaks at concentrations of 0.05—1.0 *M*;

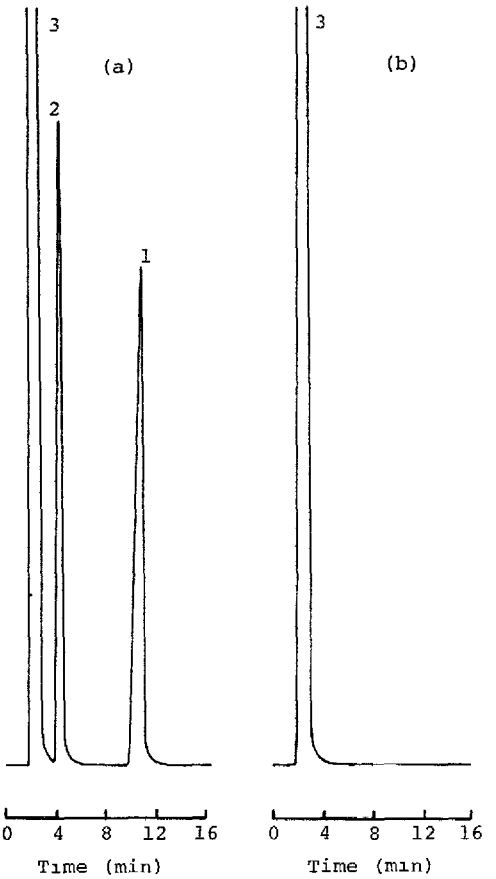

Fig. 1. Chromatograms of (a) DDB derivatives of bestatin and *p*-hydroxybestatin, and (b) reagent blank. Aliquots (20 μl) of a standard mixture of bestatin and *p*-hydroxybestatin (2 μg/ml each), and of water for blank, were treated according to the procedure. Peaks: 1, bestatin; 2, *p*-hydroxybestatin; 3, DDB.

0.1 *M* was selected for convenience. The pH of 0.1 *M* Tris · hydrochloric acid buffer only slightly influences the retention times of the peaks for bestatin and *p*-hydroxybestatin in the range 7.0—9.0, but has a considerable effect on the peak heights. Both peaks increase in height with increasing pH in the range 7.0—9.0. Since the column packing LiChrosorb RP-18 can be used in the limited range of pH less than about 9.0, a 0.1 *M* Tris · hydrochloric acid buffer of pH 8.7 was employed in the recommended procedure.

Oxidation and derivatization

Bestatin and *p*-hydroxybestatin are oxidized effectively to phenylacetaldehyde and *p*-hydroxyphenylacetaldehyde, respectively, in ammonium hydroxide solution with sodium periodate solution at a concentration of 1.5 m*M* or greater for bestatin and at 2 m*M* or greater for *p*-hydroxybestatin; 3 m*M* was used for the simultaneous oxidation of the compounds. Ammonium hydroxide solution in a concentration range 0.8—3.0 *M* gives maximum and constant heights of peaks for both compounds; 1.5 *M* was selected as optimum.

The formation of the aldehydes is dependent on the temperature and time of the oxidation procedure. At room temperature (22—27°C) the peak heights reach their maxima after standing for 18 min and remain constant for 25 min or longer, while at 0°C the heights do not reach their maxima even after standing for 40 min. Thus, the oxidation was carried out at room temperature for 20 min. Excessive periodate interferes with the derivatization of the aldehydes with DDB, and should therefore be decomposed with sodium sulphite. Sodium sulphite gives maximum and constant peak heights in a concentration range of 10—14 m*M*; a 12 m*M* solution was employed in the procedure. The recoveries of phenylacetaldehyde and *p*-hydroxyphenylacetaldehyde from bestatin and *p*-hydroxybestatin respectively (2 μg/ml each) under the conditions of the oxidation procedure were 99.3 ± 0.8% (mean ± S.D., $n = 6$).

The resulting aldehydes should be derivatized in dilute hydrochloric acid with DDB. Thus, DDB was dissolved in the acid for convenience. The acid concentration in the range 0.2—0.4 *M* gives maximum and constant peak heights; a 0.3 *M* solution was employed as optimum. The DDB solution gives the most intense peaks at a concentration greater than about 1.0 m*M* in the case of both bestatin and *p*-hydroxybestatin; 1.3 m*M* was used as a sufficient concentration. The peak heights reach maximum and constant values after warming at 37—50°C for more than 30 min in the case of bestatin and for more than 40 min in the case of *p*-hydroxybestatin, and a higher temperature allows the peaks to develop more rapidly, but heating at 100°C for more than 10 min causes a decrease in peak heights. Therefore, warming at 37°C for 50 min was selected as optimum. The resulting mixture is made slightly alkaline by the addition of 1.0 *M* sodium hydroxide to stabilize the DDB derivatives. The final mixture is stable for more than 2 h at room temperature. The limits of detection for bestatin and *p*-hydroxybestatin in a standard mixture were 90 and 68 pg per injection volume of 100 μl, respectively, at a signal-to-noise ratio of 2.

Determination of bestatin and p-hydroxybestatin in serum

Serum should be deproteinized, otherwise the column packing for HPLC is considerably damaged. The deproteinization can be done by adding 4.0—4.5 m*M* acetic acid (final concentration 3.3—3.8 m*M*, final pH approximately 5) to water-diluted serum and heating at 100°C for more than 3 min; 4.2 m*M* acetic acid and 5 min heating were employed in the procedure. When serum was deproteinized with perchloric acid (final concentration 1.0 *M*) in the usual manner, a large peak arose very close to the peak for bestatin and interfered with the determination of bestatin. The use of trichloroacetic acid (final concentration 0.5 *M*) caused low recoveries of bestatin and *p*-hydroxybestatin (approximately 50 and 40%, respectively).

Fig. 2 shows typical chromatograms obtained with drug-free serum from a healthy man and the serum spiked with bestatin and *p*-hydroxybestatin. Peaks for bestatin and *p*-hydroxybestatin (Fig. 2a, peaks 1 and 2) are successfully separated under the conditions of HPLC from other peaks due originally to the sample serum. The eluates from peaks 1 and 2 in Fig. 2a have the same fluorescence excitation and emission spectra as those of the eluates from peaks 1 and 2 in Fig. 1a, respectively.

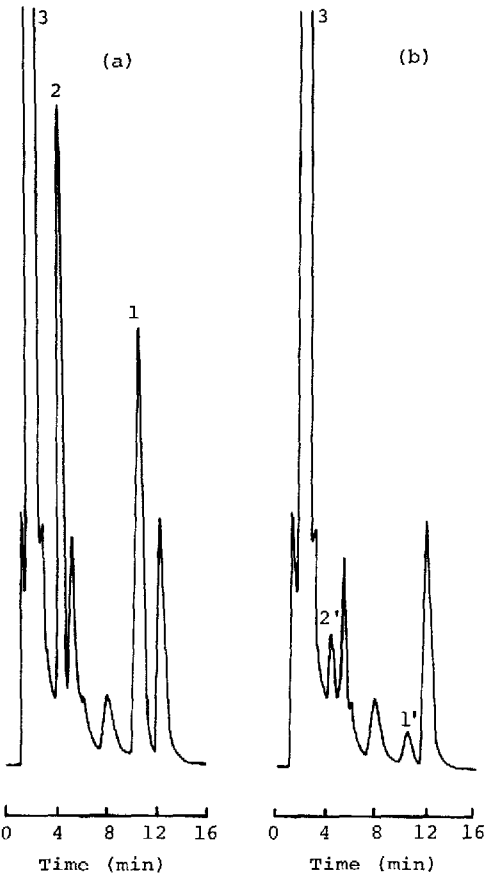

Fig. 2. Chromatograms obtained with (a) drug-free serum spiked with bestatin and *p*-hydroxybestatin (2 μg/ml each) and (b) the drug-free serum, according to the procedure. Peaks: 1, bestatin; 2, *p*-hydroxybestatin; 3, DDB. For peaks 1′ and 2′, see text

Small peaks in the chromatogram of the drug-free serum (peaks 1′ and 2′ in Fig. 2b) have exactly the same retention times as those of peaks 1 and 2 in Fig. 2a, respectively, and increased in height in proportion to increasing amounts of serum (10—100 μl). These peaks were also observed even when serum was treated without the oxidation procedure, and could not be separated from the peaks for bestatin and *p*-hydroxybestatin by any changes in HPLC conditions. Moreover, peaks 2′ and 1′ were observed even when another reversed-phase column, μBondapak phenyl, was used in place of the LiChrosorb RP-18 column. In this case, the peak height ratios of peak 1′ to peak 1 and peak 2′ to peak 2 were almost same as those ratios in Fig. 2, respectively, although the resolution of the derivatives from bestatin and *p*-hydroxybestatin on μBondapak phenyl was slightly worse than that on LiChrosorb RP-18. The fluorescence excitation and emission spectra of the eluate from peak 1′ are identical in shape and maxima with those of the eluate from peak 1, while the eluate from peak 2′ has rather complex spectra with excitation maxima at 300, 327 and 340 nm, one of which (the maximum at 327 nm) is identical with that for peak 2, and emission maxima at 366 and 382 nm, the latter being identical with the maximum for peak 2.

The above observations indicate that the component of peak 1′ in Fig. 2b should be the DDB derivative of phenylacetaldehyde which may be present in serum as an intermediate metabolite of phenylalanine [6]. Peak 2′ in Fig. 2b is ascribable to some substances occurring in serum, which may include *p*-hydroxyphenylacetaldehyde as a metabolite of tyrosine [6—8]. In addition, the identification of peak 2′, for which the HPLC fraction was collected from ten analyses and successively lyophilized, was carried out by electron-impact mass spectrometry. However, the mass spectrum obtained did not show a peak of the molecular ion (*m/z* 284) corresponding to the fluorescent product from *p*-hydroxyphenylacetaldehyde because of the small amount of the product and decomposition of the product during the collection of peak 2′ during HPLC.

In any case, the heights of peaks 1′ and 2′ should be subtracted from those in the serum sample in calculating peak heights in the procedure. If drug-free serum corresponding to the serum sample is not available, a pooled serum can be used, because almost constant heights of peaks 1′ and 2′, corresponding to approximately 0.13 μg/ml bestatin and approximately 0.28 μg/ml *p*-hydroxybestatin, respectively, were observed with individual drug-free sera from healthy subjects and patients with muscular dystrophy (coefficients of variation for the heights of peaks 1′ and 2′ were 4.8 and 5.0%, respectively; n = 30 each). Other unidentified peaks in the chromatograms in Fig. 2 were not studied.

Linear relationships were observed between the peak heights and the amounts of bestatin and *p*-hydroxybestatin added in the range 0.2—30 μg/ml to drug-free serum. The recoveries of bestatin and *p*-hydroxybestatin added to drug-free serum at concentrations of 3.0 μg/ml each, were 78.2 ± 1.2% and 78.0 ± 1.4% (mean ± S.D., n = 5 each), respectively.

The limits of detection for bestatin and *p*-hydroxybestatin were 0.2 and 0.4 μg/ml serum, respectively. The limit was defined as the amount giving 1.5 times the height of the peak in drug-free serum for the reason that such amounts of these compounds can be measured fairly precisely, as described below.

The precision was established with respect to repeatability using drug-free sera with added bestatin and *p*-hydroxybestatin. The coefficients of variation were 4.3, 3.0 and 1.8% for 0.2, 0.5 and 3.0 μg/ml bestatin, and 2.7 and 2.0% for 0.4 and 3.0 μg/ml *p*-hydroxybestatin, respectively (n = 10 in each case).

Fig. 3 shows examples of the time—concentration curves of the sera of patients administered bestatin orally in single doses. The chromatograms of sera from the subjects were the same in their pattern as those of drug-free sera with added bestatin and *p*-hydroxybestatin. However, the metabolite, *p*-hydroxybestatin, could not be measured precisely because its concentrations in sera were estimated to be less than 100 ng/ml over 24 h after the administration of bestatin. Miyazaki [9] and Koyama et al. [3] reported that most (about 70%) of the bestatin administered orally was excreted as the unchanged form in urine within 24 h, and that a maximum value of *p*-hydroxybestatin concentration in the serum, reached at 2—3 h after the administration of bestatin, was about 1% of that of the maximum serum concentration of bestatin. The serum concentration of bestatin was at a maximum 30 min after oral administration and then decreased nearly at a first-order rate as shown in

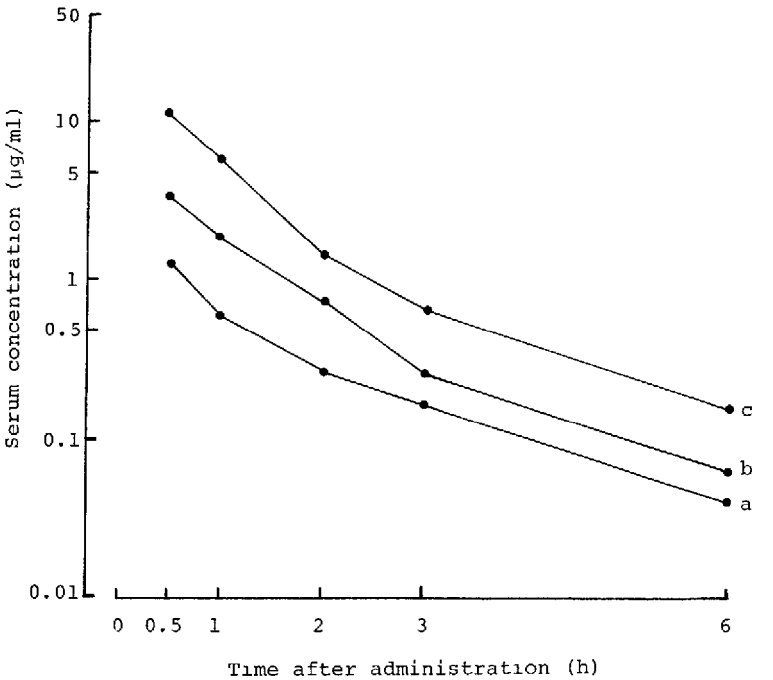

Fig. 3. Serum concentration of bestatin after oral administration of bestatin to patients (male) with muscular dystrophy. Doses of bestatin, body weights and ages: (a) 12 mg, 17 kg, 5 years; (b) 25 mg, 17 kg, 5 years; (c) 60 mg, 16 kg, 9 years.

TABLE I

COMPARISON OF SERUM CONCENTRATIONS OF BESTATIN OBTAINED BY THE PRESENT HPLC METHOD AND THE GC—MS METHOD [3]

	Serum concentration (μg/ml)	
	HPLC	GC—MS
	7.13	7.97
	4.83	4.41
	4.80	4.40
	0.70	0.87
	0.32	0.37
	0.31	0.30
Mean	3.02	3.05
± S.D.	2.95	3.09

Fig. 3. The biological half-life was 0.7—1.0 h. The pattern of the curves was almost identical with that obtained by the GC—MS method [3].

Comparison with the GC—MS method was made using sera of patients administered bestatin (Table I). The values of bestatin obtained by the present HPLC method are in close agreement with those obtained by the GC—MS method.

This study provides the first HPLC method for the determination of

bestatin. The method is precise and has adequate sensitivity to measure bestatin in 20 μl of serum, though the sensitivity is not enough to measure low concentrations of *p*-hydroxybestatin in serum. The method is readily performed and may therefore be applied for routine use in biomedical studies of bestatin.

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