Determination of Bestatin and p-Hydroxybestatin in Mouse Muscle and Serum after Oral Administration by High-Performance Liquid Chromatography with Fluorescence Detection

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Summary

Microanalyses of bestatin and p-hydroxybestatin, possible therapeutic drugs for muscular dystrophy, in mouse muscle and serum have been accomplished by high-performance liquid chromatography with fluorescence detection. The drugs are extracted into dilute acetic acid for bestatin and perchloric acid for p-hydroxybestatin. The extracts are converted to fluorescent derivatives by means of the previously reported techniques, and the derivatives are separated on a reversed-phase column (TSK gel ODS-120T) with isocratic elution, respectively. The methods permit the quantification of bestatin and p-hydroxybestatin at concentrations of as low as 900 ng and 130 ng per g in mouse muscle, and 200 ng and 30 ng per ml in mouse serum, respectively. It was found that, in oral administration in the same doses (50 mg/kg each), bestatin is distributed at higher concentrations to the muscle than p-hydroxybestatin, though the concentrations of the drugs are almost equal in the serum.

Introduction

Bestatin, [(2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoyl-S-leucine] is a strong inhibitor of aminopeptidase B and leucine aminopeptidase, and enhances delayed-type hypersensitivity^{1~3)}. Bestatin has been studied as a possible therapeutic drug for muscular dystrophy, cancer and resistant infections⁴⁾. On the other hand, p-hydroxybestatin, [(2S, 3R)-3-amino-2-hydroxy-4-(p-hydroxyphenyl)-butanoyl-S-leucine], is a major active metabolite of bestatin and has about 5-10 times stronger activity than bestatin in inhibiting aminopeptidase B⁵⁾. Therefore, the effect of bestatin in vivo may partly be ascribed to p-hydroxybestatin⁵⁾. For the investigations of the therapeutic efficacy of bestatin and p-hydroxybestatin on muscular dystrophy, it is important to examine whether those drugs can enter into muscle. However, bestatin and p-hydroxybestatin distributed to muscle after administration of the drugs have not been measured.

We previously reported the methods^{6,7)} for the determination of bestatin and p-hydroxybestatin in human serum by reversed-phase high-performance liquid chromatography (HPLC) with pre-column fluorescence derivatization, based on that bestatin is oxidized to phenylacetaldehyde with periodate, which is converted to a fluorescent compound with 1,2-diamino-4,5-dimethoxybenzene^{8,9)} (DDB), a fluorogenic reagent for aromatic aldehydes and that p-hydroxybestatin is derivatized to a fluorescent derivative with DDB after formylation by Reimer-Tiemann reaction¹⁰⁾.

This paper aims to apply the above two HPLC methods to the sensitive determinations of bestatin and p-hydroxybestatin in muscle and serum from mice dosed with bestatin or p-hydroxybestatin, and to investigate muscle and serum levels of the drugs after oral administration.

Experimental

Chemicals and solutions

Deionized and distilled water was used. Bestatin and p-hydroxybestatin were supplied from the Institute of Microbial Chemistry (Tokyo, Japan). DDB monohydrochloride was obtained from Dojindo Laboratories (Kumamoto, Japan). The reagent solutions used for the fluorescence derivatization were prepared as described previously^{6,7)}.

Apparatus and HPLC conditions

The HPLC system consisted of a Toyo Soda 803 D high-performance liquid chromatograph equipped with a Rheodyne 7125 syringe-loading sample injector valve (100- μ l loop) and a Shimadzu RF 530 fluorescence spectrometer fitted with a 12- μ l flow-cell. Emission and excitation wavelengths were 390 and 320 nm for the determination of bestatin, 425 and 345 nm for that of p-hydroxybestatin, respectively. The column was a TSK gel ODS-120 T (particle size, 5 μ m; 150 $\times$ 4 mm I.D.; Toyo Soda, Tokyo, Japan). This column could be used for more than 1000 injections with only a small decrease in the theoretical plate number. The flow-rate was 0.8 ml/min. The column temperature was ambient (approximately 25°C). The mobile phase for bestatin analysis was a mixture of 0.1 M Tris-hydrochloric acid buffer (pH 8.7) and acetonitrile (3 : 1, v/v), and that for p-hydroxybestatin was a mixture of 50 mM acetate hydrochloric acid buffer (pH 2.2) and

acetone:trile (41 : 9, v/v).

Administration of bestatin and p-hydroxybestatin, and sample preparation

Inbred mice (C57BL, five weeks old, weight 17.5 ± 1.6 g) were used. The mouse received 50 mg/kg oral single dose of bestatin or p-hydroxybestatin dissolved in saline by using a conductor needle. The administration was performed ca. 15 min after feeding. Then, only water was given to the mouse until sample preparation. The mouse was killed by exsanguination from a carotid artery. Trapezius muscle of the mouse was quickly taken, sliced, washed with saline and weighed after removing the saline with filter paper. The sliced muscle (0.1 g) was homogenized with a mixture of 150 μ l each of 8.4 mM acetic acid and water, and then centrifuged at 10,000 g for 20 min at 0–2°C. The supernatant was used for the determination. The blood was collected in a centrifuged tube and serum was separated.

Procedure for the determination of bestatin

A 15- μ l portion of the supernatant from muscle homogenate or of serum was mixed with 15 μ l of water and 150 μ l of 4.2 mM acetic acid. The mixture was heated for 5 min in a boiling water-bath and centrifuged at 800 g for 5 min. To 100 μ l of the supernatant were added 50 μ l of 1.5 M ammonium hydroxide and 25 μ l of 3 mM 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 μ l of 12 mM sodium sulphite. To the mixture, 200 μ l of 1.3 mM DDB in 0.3 M hydrochloric acid were added and the mixture was warmed at 37°C for 50 min; then, 50 μ l of 1.0 M sodium hydroxide was added. A 100- μ l volume of the final mixture was injected into the chromatograph. The amounts of bestatin in muscle and serum were calibrated by means of the standard addition method; 150 μ l of water added to the muscle and 15 μ l of water added to the serum in the procedure were replaced by 150 μ l of a standard solution of bestatin for muscle (2 or 5 μ g/ml) and 15 μ l of a standard solution of bestatin for serum (2 or 5 μ g/ml).

Procedure for the determination of p-hydroxybestatin

A 50- μ l portion of the supernatant from muscle homogenate or of serum was mixed with 50 μ l of water and 200 μ l of 1.5 M perchloric acid. The mixture was centrifuged at 800 g for 5 min. To 200 μ l of the supernatant, 200 μ l of 2.6 M potassium hydroxide were added. The mixture was centrifuged at 800 g for 5 min. The supernatant (250 μ l) was placed in a screw-capped vial (45 $\times$ 12 mm I.D.), to which was added a 100- μ l portion of chloroform. The mixture was heated in a heating module (Pierce, Rockford, I.L, U.S.A.) at 70°C for 8 min to formylate p-hydroxybestatin, and then cooled in ice-water for ca. 1 min. To the mixture, 50 μ l of 14.0 M acetic acid, 300 μ l of the DDB solution and 50 μ l of 1.0 mM hydrogen peroxide 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 then cooled. A 100- μ l portion of the aqueous layer was injected into the chromatograph. The amounts of p-hydroxybestatin in muscle and serum were calibrated by means of the standard addition method; 150 μ l of water added to the muscle and 50 μ l of water added to the serum in the procedure were replaced by 150 μ l of a standard solution of p-hydroxybestatin for muscle (0.5 or 2 μ g/ml) and 50 μ l of a standard solution

of p-hydroxybestatin for serum (0.5 or 2 $\mu\text{g}/\text{ml}$).

Results and Discussion

Determination of bestatin and p-hydroxybestatin in mouse muscle and serum

Fig. 1 shows typical chromatograms of bestatin in muscle and serum from a mouse dosed with bestatin. The fluorescent derivative of bestatin was detected at the retention time of 12.5 min, and it was well separated from endogenous fluorescent compounds and the reagent blank. The derivatization and HPLC conditions are actually the same as those reported for bestatin in human serum⁶⁾. The calibration curves for bestatin were linear up to the concentrations of 25 μg per g muscle and 50 μg per ml serum, respectively. The limits of detection were 900 ng (2.9 nmol) per g muscle and 200 ng (650 pmol) per ml serum, respectively, at a signal-to-noise ratio of 2.

Typical chromatograms obtained with muscle and serum from a mouse administered p-hydroxybestatin are shown in Fig. 2. The retention time for the fluorescent derivative of p-hydroxybestatin was 15.0 min and no interfering peaks arose around p-hydroxybestatin peak. The derivatization and HPLC conditions are nearly the same as those for p-hydroxybestatin in human serum⁷⁾. The calibration curves for p-hydroxybestatin were linear up to the concentrations of 25 μg per g muscle and 50 μg per ml serum, respectively. The limits of detection were 130 ng (400 pmol) per g muscle and 30 ng (90 pmol) per ml serum, respectively.

The extraction of bestatin and p-hydroxybestatin from muscle was carried out by homogenizing the sample with 4.2 mM acetic acid. The recoveries of bestatin (5 $\mu\text{g}/\text{g}$)

Fig. 1. Chromatograms of bestatin in (A) muscle and (B) serum at 1 h after oral administration of bestatin to a mouse. Dose: 50 mg/kg. Peaks: 1, bestatin; others, endogenous substances and reagent blank. Concentrations of bestatin: A, 5.70 $\mu\text{g}/\text{g}$ in muscle; B, 7.52 $\mu\text{g}/\text{ml}$ in serum.

Fig. 2. Chromatograms of p-hydroxybestatin in (A) muscle and (B) serum at 1 h after oral administration of p-hydroxybestatin to a mouse. Dose: 50 mg/kg. Peaks: 1, p-hydroxybestatin; others, endogenous substances and reagent blank. Concentrations of p-hydroxybestatin: A, 3.08 $\mu\text{g/g}$ in muscle; B, 6.30 $\mu\text{g/ml}$ in serum.

Fig. 3. Concentrations of bestatin and p-hydroxybestatin in mouse muscle and serum after oral administration (50 mg/kg in single doses) of bestatin. Mean values of the concentrations obtained with three mice were plotted. Curves: a, bestatin in muscle; b, bestatin in serum; c, p-hydroxybestatin in muscle; d, p-hydroxybestatin in serum.

and p-hydroxybestatin ($2 \mu\text{g/g}$) added to a drug-free muscle were 63.0 ± 1.3 and $62.2 \pm 1.0\%$ (mean $\pm$ S.D., $n=5$ in each case), respectively. The use of ice-water, 1 M perchloric acid or 50 mM disodium carbonate in place of the acetic acid resulted in lower recoveries for both bestatin and p-hydroxybestatin; recoveries of 61 and 50% for ice-water, 59 and 55% for 1 M perchloric acid, and 44 and 54% for 50 mM disodium carbonate, respectively, were observed. On the other hand, bestatin and p-hydroxybestatin in serum were completely extracted by heating the sample with 3.5 mM acetic acid and 1.0 M perchloric acid, respectively; recoveries were $96.0 \pm 1.8\%$ for bestatin ($5 \mu\text{g/ml}$) and $100.0 \pm 2.2\%$ for p-hydroxybestatin ($2 \mu\text{g/ml}$) (mean $\pm$ S.D., $n=5$ in each case).

Distribution of bestatin and p-hydroxybestatin in mouse muscle and serum

Fig. 3 shows time-concentration curves obtained by the determination of bestatin and p-hydroxybestatin in mouse muscle and serum after oral administration (50 mg/kg) of bestatin. Muscle concentration of bestatin (Fig. 3, a) is about a half of the serum concentration (Fig. 3, b) at 0.5–1 h, but is at least 1.5 times higher between 1.5 and 6 h after the administration. p-Hydroxybestatin resulted from bestatin was observed in both muscle and serum samples. The concentrations of p-hydroxybestatin in muscle (Fig. 3, c) are higher than those in serum (Fig. 3, d). The bestatin and p-hydroxybestatin concentrations in muscle and serum after oral administration of bestatin seem to decrease biexponentially. Half-lives of bestatin and p-hydroxybestatin in muscle are 0.7 and 1.2 h for the former phase, and 7.6 and 3.9 h for the latter phase, respectively (Fig. 3, a and c). In the case of serum concentration, those of bestatin and p-hydroxybestatin are 0.3

Fig. 4. Concentrations of p-hydroxybestatin in mouse muscle and serum after oral administration (50 mg/kg) of p-hydroxybestatin. Mean values of the concentrations of p-hydroxybestatin obtained with three mice were plotted. Curves: a, p-hydroxybestatin in muscle; b, p-hydroxybestatin in serum.

and 0.5 h for the former phase, and 1.5 and 4.4 h for the latter phase, respectively (Fig. 3, b and d).

Time-concentration curves of p-hydroxybestatin in mouse muscle and serum after the oral administration in the same amount as for bestatin (50 mg/kg) are shown in Fig. 4. The muscle concentrations of p-hydroxybestatin (Fig. 4, a) are between 1/3-1/6 of those in serum (Fig. 4, b), at all the periods after the administration. The p-hydroxybestatin concentrations in muscle and serum after the oral administration decrease biexponentially, with half-lives of 0.5 h for the former phase and 5.3 h for the latter phase in muscle (Fig. 4, a) and those of 0.5 h for the former phase and 3.6 h for the latter phase in serum (Fig. 4, b).

The muscle concentrations of bestatin in the case of bestatin administration (Fig. 3, a) are at least 1.5 times higher than those of p-hydroxybestatin in p-hydroxybestatin administration in the same amount as for bestatin (Fig. 4, a). On the other hand, the serum concentrations of bestatin (Fig. 3, b) are equal or slightly lower than those of p-hydroxybestatin (Fig. 4, b). This suggests that bestatin distributes more readily into muscle cells than p-hydroxybestatin.

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