Journal of Chromatography, 345 (1985) 259—265
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2801

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR MONITORING LEUPEPTIN IN MOUSE SERUM AND MUSCLE BY PRE-COLUMN FLUORESCENCE DERIVATIZATION WITH BENZOIN

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(First received April 23rd, 1985; revised manuscript received July 15th, 1985)

SUMMARY

A high-performance liquid chromatographic method is described for the determination of leupeptin, a possible therapeutic drug for muscular dystrophy, in mouse serum and muscle. Leupeptin is reduced with sodium borohydride to leupeptinol, and then converted to a fluorescent derivative with benzoin. The derivative is separated on a reversed-phase column (LiChrosorb RP-18) with isocratic elution and determined with fluorescence detection. The detection limits of leupeptin in serum and muscle are 250 pmol/ml (107 ng/ml) and 500 pmol/g (214 ng/g), respectively, corresponding to approximately 150 fmol each in a 100-μl injection volume. This method is simple and sensitive enough to permit the quantification of leupeptin in biological samples from mice dosed with leupeptin.

INTRODUCTION

Leupeptin is a peptide having a guanidino moiety in its molecule, which inhibits the in vitro activity of various proteases such as plasmin, trypsin, papain and cathepsin B [1, 2]. Therefore, leupeptin has been studied as a therapeutic drug for muscular dystrophy, owing to its non-immunogenic and low-toxic nature, with the suggestion that leupeptin might inhibit the proteolytic degradation of muscle cells in vivo [3—6]. However, the therapeutic benefit of leupeptin administered to dystrophic animals was not always recognized [7, 8].

For an investigation of the therapeutic efficacy of leupeptin on the disease, it is important to examine whether this inhibitor can enter into the muscle cells with a concentration high enough to inhibit protease activity. But, a

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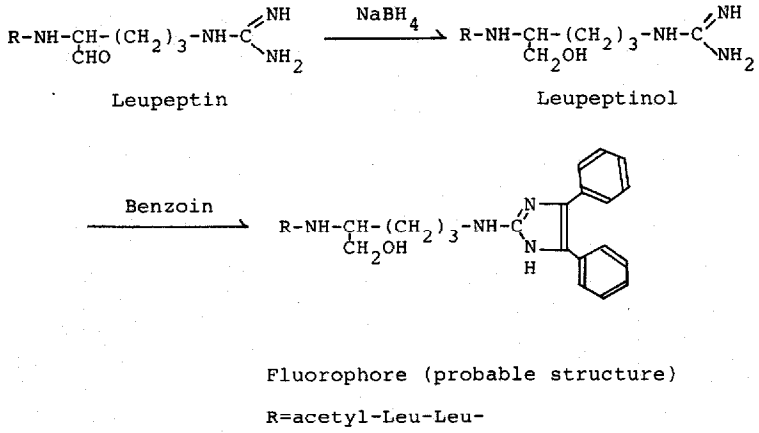


Fig. 1. Fluorescence derivatization of leupeptin with benzoin.

practical method for monitoring leupeptin in biological samples is not available.

We previously developed a pre-column fluorescence derivatization method of high-performance liquid chromatography (HPLC) for arginine-containing peptides (angiotensins I, II and III) by using a fluorogenic reagent, benzoin [9]. Benzoin gives highly fluorescent derivatives of compounds with guanidino moieties in alkaline medium in the presence of β-mercaptoethanol (stabilizer for the fluorescent products) and sodium sulphite (suppresser of blank fluorescence) [10—12].

We have recently found that leupeptin can form a fluorescent derivative provided that leupeptin is converted to leupeptinol by reduction with sodium borohydride prior to the benzoin reaction (Fig. 1); leupeptin itself does not react directly with benzoin, probably because of Schiff-base formation by reaction of the guanidino moiety with the formyl group in the molecule. The present study aims to develop a simple and sensitive HPLC method by means of the pre-column fluorescence derivatization with benzoin for the quantification of leupeptin in serum and muscle from mice dosed with leupeptin. Arginine and leupeptin-related peptides such as Leu-Arg and leupeptinic acid (acetyl-Leu-Leu-Arg) were also used for examining the fluorescence derivatization and HPLC separation, since these compounds are conceivable metabolites of leupeptin.

EXPERIMENTAL

Chemicals and solutions

Deionized and distilled water was used. Leupeptin, leupeptinol, leupeptinic acid and Leu-Arg are the products of Nihon Kayaku (Tokyo, Japan). Tris(hydroxymethyl)aminomethane (Tris; Wako, Osaka, Japan) was purified as described previously [9]. Other chemicals were of reagent grade. The reagent solutions used for the fluorescence derivatization were prepared as described previously [11].

Apparatus

The HPLC system consisted of a Hitachi 635A high-pressure pump, a

Rheodyne 7120 syringe-loading injector (100-μl loop) and a Hitachi F1000 HPLC fluorescence spectrometer fitted with a 12-μl flow-cell. The column was LiChrosorb RP-18 (particle size 5 μm; 150 × 4 mm I.D.; Japan Merck, Tokyo, Japan). Uncorrected fluorescence excitation and emission spectra were measured with a Hitachi MPF-4 spectrofluorimeter in 10 × 10 cm quartz cells.

Administration of leupeptin and sample preparation

Inbred mice (C57BL, five weeks old, weight ca. 10 g) were used. The mouse received a 50 mg/kg intravenous dose of leupeptin dissolved in saline via its tail vein, or a 100 mg/kg oral dose of leupeptin dissolved in saline by using a conductor needle. The administration was performed 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. The blood was collected in a centrifuge tube and serum was separated. A 40-μl portion of serum was mixed with 160 μl of water (or standard solutions of leupeptin for the calibration graph) and 200 μl of 1.5 *M* perchloric acid. The mixture was then centrifuged at 800 *g* for 10 min for deproteinization.

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 1.0 ml of ice-water (or standard solutions of leupeptin for the calibration graph) and then centrifuged at 1000 *g* for 20 min at 0—2°C. Protein in 200 μl of the supernatant was precipitated with 200 μl of 1.5 *M* perchloric acid by the same procedure as that for serum.

Portions (200 μl) of the supernatants prepared from serum and muscle were neutralized with 100 μl of 1.0 *M* potassium carbonate. The resulting mixtures were used as sample solutions.

Procedure

Reduction of leupeptin. A 100-μl portion of the sample solution (or a standard mixture of leupeptin and its related compounds) was mixed with 50 μl of 40 m*M* sodium borohydride and then left to stand at room temperature (20—27°C) for 5 min. A 50-μl portion of 1.5 *M* hydrochloric acid was then added to the reaction mixture to decompose excess sodium borohydride.

Fluorescence derivatization. To the above reaction mixture (200 μl), 100 μl each of 5 m*M* benzoin (in methylcellosolve) and an aqueous solution containing 0.1 *M* β-mercaptoethanol and 0.2 *M* sodium sulphite, and 200 μl of 1.5 *M* potassium hydroxide were added, with cooling in ice-water. The mixture was heated in a boiling water-bath for 90 s. Then, a 200-μl portion of an acidic solution containing 1.0 *M* hydrochloric acid and 0.5 *M* Tris—hydrochloric acid buffer (pH 8.5) was added to adjust the pH to ca. 8.5. A 100-μl portion of the final reaction mixture was used for HPLC.

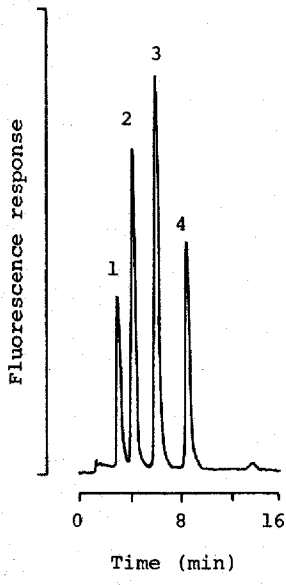
HPLC conditions. The mobile phase was a mixture of methanol, 0.5 *M* Tris—hydrochloric acid buffer (pH 8.5) and 40 m*M* tetra-*n*-butylammonium chloride (7:1:2). The flow-rate was 0.8 ml/min. The fluorescence of the eluate was monitored at 425 nm emission against 325 nm excitation. The LiChrosorb RP-18 column can be used for more than 600 injections with only a small decrease in the theoretical plate number. The column temperature was ambient (20—27°C).

RESULTS AND DISCUSSION

HPLC separation and fluorescence derivatization

The benzoin derivatives obtained from a standard solution of leupeptin, leupeptinic acid, Leu-Arg and arginine can be separated mutually by reversed-phase HPLC with isocratic elution (Fig. 2). Tetra-*n*-butylammonium chloride in the mobile phase serves to separate the derivative of leupeptinic acid from those of Leu-Arg and arginine (Fig. 3). The other conditions for HPLC separation are similar to those reported previously for the benzoin derivatives of angiotensins [9].

The leupeptin peak corresponds to the fluorescent derivative of leupeptinol. Leupeptin can be converted to leupeptinol with a maximum yield (90%) when it is reacted with sodium borohydride in a concentration range 27—270 m*M* at room temperature (20—27°C) for 3 min or longer. The excess borohydride should be decomposed by acidification with dilute hydrochloric acid before the benzoin reaction; without this decomposition, the yields of the fluorescent derivatives are lower, approximately 10% of those obtained according to the recommended procedure. The fluorescence derivatization of leupeptin, leupeptinic acid and Leu-Arg with benzoin, affording single fluorescent derivatives with each maximum yield, occurs in 0.37 *M* potassium hydroxide solution with heating at 100°C for 90 s.



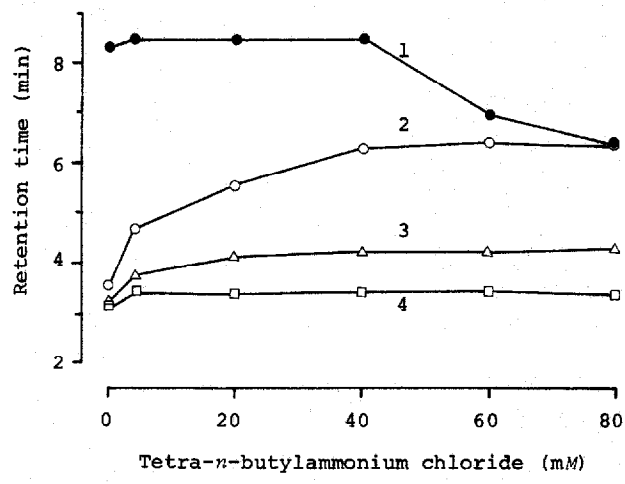


Fig. 2. Chromatogram of the benzoin derivatives obtained with a standard mixture of leupeptin, leupeptinic acid, Leu-Arg and arginine. Portions (100 μl) of the standard solutions (10 nmol/ml) were treated by the recommended procedure. Peaks: 1 = arginine; 2 = Leu-Arg; 3 = leupeptinic acid; 4 = leupeptin.

Fig. 3. Effect of tetra-*n*-butylammonium chloride concentration, added to the mobile phase, on the HPLC separation of the benzoin derivatives of leupeptin and its related compounds. Curves: 1, leupeptin; 2, leupeptinic acid; 3, Leu-Arg; 4, arginine. HPLC conditions: mobile phase, methanol—0.5 *M* Tris—hydrochloric acid buffer (pH 8.5)—0—80 m*M* tetra-*n*-butylammonium chloride (7:1:2); other conditions, see text.

A linear relationship is obtained between the peak heights and the amounts (0—1.0 nmol) of leupeptin, leupeptinic acid or Leu-Arg used for the derivatization. The lower limits of detection for leupeptin, leupeptinic acid and Leu-Arg are 150, 100 and 130 fmol per 100-μl injection volume, respectively, at a signal-to-noise ratio of 2. For repeated derivatizations of the three compounds (0.5 nmol each), the coefficients of variation of their peak heights (n = 8) are less than 3%.

Determination of leupeptin in serum and muscle

Fig. 4 shows typical chromatograms obtained with serum and muscle from a mouse dosed with leupeptin. The peak owing to leupeptin is detected apparently in both of the samples. However, the leupeptin-related peptides, leupeptinic acid and Leu-Arg, cannot be detected because various endogenous compounds elute at the same retention times.

Identification of the leupeptin peak is carried out on the basis of the retention time of the leupeptin standard and also by co-chromatography of the standard and the sample with a different eluent, i.e. using a lower methanol concentration (65%) than that used for the procedure (the retention time for leupeptin is 17.0 min). The eluate from the peak also shows fluorescence excitation (maximum, 325 nm) and emission (maximum, 435 nm) spectra identical to those from the leupeptin standard. No peak is observed at the

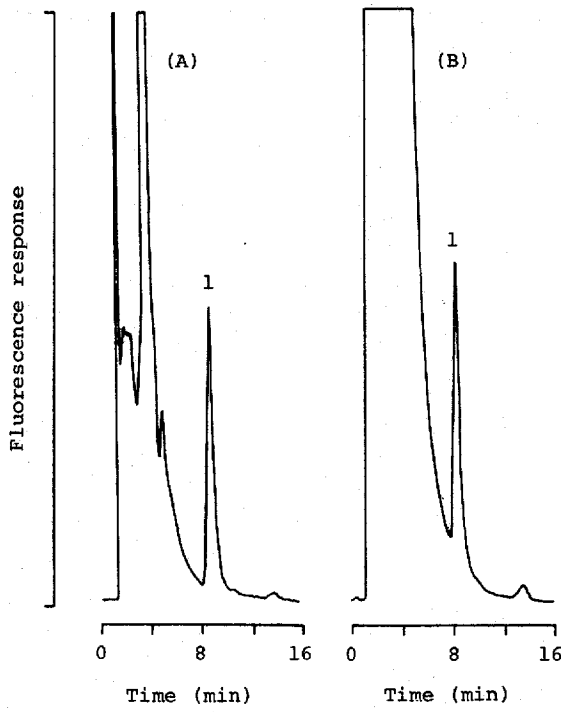


Fig. 4. Chromatograms obtained with (A) serum and (B) muscle at 1 h after intravenous administration of leupeptin to a mouse. Dose: 50 mg/kg. Peaks: 1 = leupeptin; others = endogenous substances in serum or muscle. Concentrations of leupeptin: A, 17.9 nmol/ml in serum; B, 13.2 nmol/g in muscle.

retention time for leupeptin when the drug-free sample of serum or muscle is treated in the same way.

Calibration graphs for leupeptin in serum and muscle are linear up to concentrations of 125 nmol/ml and 50 nmol/g, respectively. The minimum determinable concentration of leupeptin is 250 pmol/ml in serum and 500 pmol/g in muscle. The recoveries of leupeptin (5.0 nmol/ml or 5 nmol/g in the drug-free serum and muscle are 83.0 ± 5.6 and 68.0 ± 2.4% (mean ± S.D., n = 5), respectively.

Fig. 5 shows time—concentration curves obtained by the determination of leupeptin in serum and muscle of mouse after oral (100 mg/kg) and intravenous (50 mg/kg) administration of leupeptin. The leupeptin concentration in serum after oral administration decreases biexponentially, with half-lives of 0.9 h for the former phase and 9.6 h for the latter phase (Fig. 5A, a). In contrast, after intravenous administration, the leupeptin concentration in serum decreases almost at a first-order rate; the half-life is approximately 0.7 h (Fig. 5A, b). The cause of this difference in the disappearance rate of leupeptin remains unknown. However, such a change of elimination rate of drugs from serum is occasionally observed in dose-dependent pharmacokinetics [13]. On the other hand, the leupeptin concentration in muscle after intravenous administration is approximately five-fold higher in a 5-h period than that in muscle after oral administration, and its disappearance rate from muscle is not significantly different between oral and intravenous administrations (Fig. 5B). The data suggest that the distribution of leupeptin in the target organ may be affected by the method of dosing.

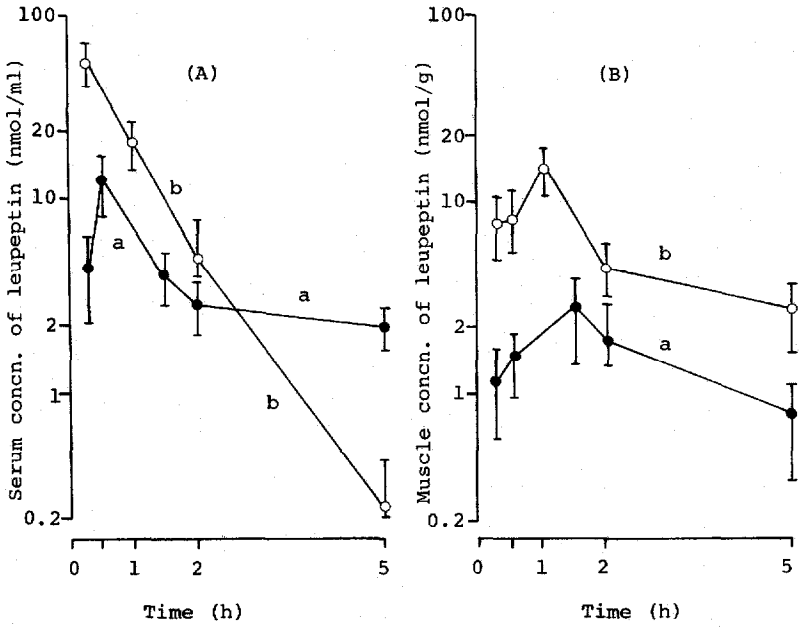


Fig. 5. Concentrations of leupeptin in (A) serum and (B) muscle of mouse after oral and intravenous administration of leupeptin. Administrations: a, oral (100 mg/kg in single doses); b, intravenous (50 mg/kg in single doses). Mean values of the concentrations of leupeptin obtained with five mice are plotted. Bar of each point represents the maximum and minimum values.

This study provides the first practical method for the quantification of leupeptin in biological samples. The proposed HPLC method is simple and rapid and, moreover, it offers the necessary specificity and sensitivity to permit the quantitative determination of leupeptin in 40 μl of serum and in 0.1 g of muscle from a mouse dosed with leupeptin. The method can thus be applied routinely in the therapeutic and biomedical study of leupeptin for muscular dystrophy.

ACKNOWLEDGEMENTS

We are grateful to Drs. H. Umezawa and T. Aoyagi (Institute of Microbial Chemistry) for encouragement throughout this work, and to Dr. H. Miyazaki (Nihon Kayaku Co.) for the generous gift of leupeptin-related peptides. This work was partly supported by a Grant-in-Aid for New Drug Development Research from the Ministry of Health and Welfare, Japan.

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