Pre-Column Fluorescence Derivatization of Peptides Containing Arginine Aldehyde Moiety in High-Performance Liquid Chromatography

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Key Words
Column liquid chromatography
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Summary
A sensitive reversed-phase high-performance liquid chromatographic method with pre-column fluorescence derivatization has been developed for the determination of DLL-MePhe-Pro-Arg-H (LY-DLL, LY-294468). In the search for a derivatization method for the determination of the tripeptide aldehyde, which is present in equilibrium structures in aqueous solution, it was found that the guanidino group of the argininal residue was not converted into a fluorescent derivative by reaction with benzoine. However, if the LY-DLL was first converted into a LY-DLL-TRIS adduct, a fluorescent product could be obtained by the reaction of LY-DLL with TRIS in TRIS(HCl) buffer (pH 8.5) followed by benzoine in the presence of beta-mercaptoethanol and sodium sulphite in an alkaline medium.

Introduction
D-MePhe-Pro-Arg-H (Figure 1) (LY-DLL, LY-294468) is an anticoagulant with specific thrombin inhibitor

![Diagram of derivatization process]

Figure 1
Flowsheet of TRIS-benzoine derivatization of LY-DLL
A:aldehyde hydrate form of LY-DLL
B,B': two amino cyclol forms of LY-DLL
C:free aldehyde form of LY-DLL
R₁=D-MePhe-Pro
X=OH or O-CH₂-C(CH₂OH)₂-NH₂
R₁-NH₂-CH₂-C(CHOH)₂-NH₂

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action [1–3]. In our search for a sensitive method for the determination in biological fluids of the tripeptide aldehyde containing an arginine-aldehyde moiety, the use of benzoin, a fluorogenic reagent for the guanidino group has been studied. Benzoin reacts with N-substituted guanidines to yield 2-substituted amino-4,5-diphenyl-imidazole [4–6]. Our first attempts revealed that the fluorescence intensity was only a few percent of that shown by the corresponding tripeptide acid. This is in agreement with a study on leupeptin, containing an arginine aldehyde group [4] which found that the fluorescence intensity obtained from leupeptin was only 10 % of that corresponding to an equimolar concentration of arginine. This phenomenon may be due to the equilibrium structures of the peptidyl or acyl arginine aldehyde in aqueous solution [7, 8] (Figure 1) the guanidino group of which – at least in part – is blocked by the aldehyde function. Therefore, a reaction of tripeptide aldehyde should be performed in which the guanidino group is free and able to react with the fluorogenic reagent benzoin.

Experimental

Instrumentation

The apparatus consisted of an LKB liquid chromatograph (LKB pumps, Model 2150, a controller, Model 2152, an auto-injector, Model 2157, a multitemp, Model 2219, a multiwavelength UV-visible detector, Model 2151 set at 215 nm. The fluorescence of the eluate was monitored by a Shimadzu RF-530 detector (325 nm ex., 435 nm em.). The reversed-phase columns were as follows: UltraPac TSK-ODS-120T 5 μm, 250 × 4.6 mm (LKB, Bromma, Sweden), Polygosil-60 C-18 10 μm, 250 × 4.6 mm (Macherey Nagel, Düren, Germany) and Neisorb BST-8 phenyl 10 μm, 250 × 4.6 mm (Bioseparation Technologies Co., Budapest, Hungary). Bond Elut C-18 tubes (1 ml) (Varian, Analytichem Internationa), LC-CN SPE tubes (1 ml) (Supelco, Inc. Supelco Park, Bellefonte PA, USA) and Amberlite-XAD, Type 2, particle size 0.3–1.0 mm tubes (1 ml) (Serva, Heidelberg, Germany) were used for the extraction of LY-DLL from plasma. A PENELSON 900 Series Intelligent Interface was used for analogue-digital signal transformation. For the integration of the data Nelson System’s Model 2600 Chromatography Software Rev. 5.00 M3 1988 was used.

Elution Systems

System A: Eluent A: 0.5 mol/l TRIS(HCl)

pH 8.5 : water : methanol : TEA
(15 : 35 : 50 : 0.1)

Eluent B: 0.5 mol/l TRIS(HCl)

pH 8.5 : water : methanol : TEA
(15 : 5 : 80 : 0.1)

The methanol concentration was increased linearly from 50 % to 80 % in 30 min (from 0 % B to 100 % B in 30 min) followed by isocratic elution (100 % B for 5 min). The reequilibrium time between gradient runs was 5 min (with eluent A).

System B: Methanol: 0.1 mol/l phosphate buffer
pH 6.85 (30 : 70)

System C: 0.1 mol/l phosphate buffer
pH 2.2 : acetonitrile (90 : 7)

Reagents and Solutions

Acetonitrile, methanol and triethylamine (TEA) were from Merck (Darmstadt, Germany), all other substances were of special grades. The DLL-MePhe-Pro-Arg-H-sulphate (LY-DLL), DLD-MePhe-Pro-Arg-H-sulphate (LY-DLD) and the internal standard, benzoyloxy-carbonyl arginine hydrochloride (Z-Arg.OH(HCl)) were synthesized in house. 0.5 mol/l TRIS(HCl) buffer (pH 8.5) solution was prepared as follows: – TRIS(HCl) (30.3 g) was dissolved in 400 ml water, the pH was adjusted to 8.5 with concentrated hydrochloric acid; the solution was then diluted with water to 500 ml and stored at 4 °C.

Benzoin solution (4 mmol/l) was prepared by dissolving 85 mg benzoin in 100 ml of methycellosolve. The solution was stable at room temperature for a week. Beta-mercaptoethanol (0.1 mol/l-sodium sulphite/0.2 mol/l/solution was prepared as follows: – beta-mercaptoethanol (0.78 g) and sodium sulphite (Na2SO3) (2.52 g) were dissolved in 80 ml water and diluted with water to 100 ml. The solution was stable for a week at room temperature. Sodium hydroxide solution (2 mol/l) was prepared by dissolving 8.0 g sodium hydroxide in 100 ml of water.

Procedures

Preparation of standard solutions. The stock solutions were prepared by weighing 10 mg of LY-DLL and internal standard into a 10 ml volumetric flask and dissolving it in 0.1 mol/l phosphate buffer (pH 2.2) (0.1 mol/l NaH2PO4 to pH 2.2 was adjusted with 47 % phosphoric acid). Working solutions containing 1 ng/μl, 10 ng/μl and 100 ng/μl concentrations of LY-DLL and internal standard (10 ng/μl) were prepared freshly each working day by diluting the stock solutions with 0.1 mol/l phosphate buffer (pH 2.2). All standard solutions were stored at 4 °C.

Extraction. To a 1 ml plasma sample 1 ml of 0.5 mol/l TRIS(HCl) buffer (pH 8.5), 5 μl TEA and 150 ng of internal standard were added. The sample was passed through the Bond Elut C-18 cartridge conditioned with 5 ml of methanol and 5 ml of distilled water. The cartridges were rinsed with 5 ml of distilled water and dried in vacuo. LY-DLL and the internal standard were eluted with 0.8 ml of 0.2 % HCl-methanol. The eluate was collected in a 0.7 ml vial to which 100 μl of 0.5 mol/l TRIS(HCl) buffer (pH 8.5) had been added. The solution was concentrated to 10 μl with nitrogen at room temperature.