Precolumn fluorescence derivatization of the antagonist [Arg^6,D-Trp^{7,9},MePhe^8]-Substance P{6–11} with benzoin in high-performance liquid chromatography and selective detection of arginine-containing fragments in its degradation products

Hua Cui[a,b], J. Leon E. Reubsaet[a], Auke Bult[a,*]

[a]Department of Pharmaceutical Analysis, Faculty of Pharmacy, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, Netherlands

[b]Department of Applied Chemistry, University of Science and Technology of China, Anhui, Hefei 230026, China

Abstract

Precolumn fluorescence derivatization for the determination of the antagonist [Arg^6,D-Trp^{7,9},MePhe^8]-Substance P{6–11} (antagonist G) using benzoin in HPLC was studied. Under the conditions chosen (0.067 M NaOH, heating at 100°C for 10 s), a good yield of fluorescent derivatives was obtained and no methodology-related degradation occurred. The detection limit of antagonist G was 0.21 nmol/ml. The method has been applied to the selective and sensitive detection of arginine-containing fragments in degradation products of antagonist G.

Keywords: Derivatization, LC; Pharmaceutical analysis; Antagonist G; Benzoin; Arginine; Amino acids; Peptides

1. Introduction

The antagonist [Arg^6,D-Trp^{7,9},MePhe^8]-Substance P{6–11} (antagonist G) is a synthetic peptide consisting of six amino acids (NH$_2$–Arg–D-Trp–MePhe–D-Trp–Leu–Met–CONH$_2$), which resembles an autocrine growth factor, bombesin, synthesized by small-cell lung cancer (SCLC) cells. It has been found that antagonist G can prevent tumour cells in small cell lung cancer from growing by competing with bombesin [1,2].

It is expected to undergo clinical application in the near future. The stability of antagonist G in aqueous solutions is under investigation in our laboratory [3]. The arginine residue plays an important role in the analysis and degradation of antagonist G. Therefore, there is a need for the selective determination of arginine-containing fragments in degradation products of antagonist G.

The fluorescent derivatization reagents 9,10-phenanthrenequinone [4,5], ninhydrin [6] and benzoin [7–10] have been reported for the selective determination of monosubstituted guanidino

* Corresponding author.

compounds. Of these methods, benzoin as a precolumn and postcolumn derivatization reagent has been used to determine selectively arginine-containing peptides in high-performance liquid chromatography (HPLC) [7–9] and capillary electrophoresis (CE) [10]. This reagent reacts with the guanidino moiety of peptides to form highly fluorescent derivatives, 2-substituted amino-4,5-diphenylimidazoles [11,12]. The method has the advantages of high sensitivity and selectivity. It has been applied to the selective detection of some bioactive peptides containing arginine, such as angiotensins [13] and D-MePhe–Pro–Arg-H [14]. However, we found that the derivatization conditions using benzoin reported in the literature [7–10,13,14] are too drastic to be suitable for antagonist G. Substantial degradation occurred. Therefore, milder derivatization conditions for antagonist G were studied. The developed method was applied to the selective detection of arginine-containing fragments in degradation products of antagonist G.

2. Experimental

2.1. Chemicals

Antagonist G was a gift from the New Drug Development Office of the European Organization for Research and Treatment of Cancer (NDDO–EORTC, Amsterdam, Netherlands). All other chemicals used were of analytical-reagent grade. Deionized water was used.

2.2. Solutions

The reagent solutions used for fluorescence derivatization were prepared as follows [15]: for benzoin solution (4.0 mM), benzoin (0.0213 g) was dissolved in 25 ml of methyl-Cellosolve; for β-mercaptoethanol (0.1 M)–sodium sulphite (0.2 M) solution, β-mercaptoethanol (135.8 μl) and sodium sulphite (0.63 g) were dissolved in water and diluted to 25 ml with water; and for perchloric acid (0.2 M)–phosphate buffer (0.05 M,

pH 8.5) mixture, equal volumes of 0.4 M perchloric acid and 0.1 M phosphate buffer (pH 8.5) were mixed together.

2.3. Derivatization

An 80-μl portion of sample solution was placed in a test vial, to which were added 40 μl each of benzoin and β-mercaptoethanol–sodium sulphite solution and 80 μl of aqueous 0.2 M sodium hydroxide with cooling in ice–water. The vial was sealed tightly. The mixture was heated in a boiling water-bath for 10 s, cooled in ice–water for 5 min and then 80 μl of perchloric acid–phosphate buffer mixture were added. The reaction mixture was kept at 4°C until 10 min before injection. A volume of 20 μl of the final mixture was injected onto the HPLC system.

2.4. Degradation [3]

A 100-μl volume of 0.4 mg/ml antagonist G solution were placed in a test vial, to which were added 100 μl of 0.1 M NaOH or 1.0 M HClO$_4$. The vial was sealed tightly, heated in a water-bath at 85°C for a specified time and then cooled in an ice–water bath for 5 min. A volume of 100 μl of 0.1 M HClO$_4$ or 1.0 M NaOH was added to adjust the pH to about 7. This mixture was used for derivatization.

2.5. HPLC

The HPLC system consisted of a Model 510 high-pressure pump (Waters, Milford, MA, USA), a programmable multi-dimensional injection system (PROMIS, Spark Holland, Netherlands) and a Jasco 821-FP intelligent spectrofluorometric detector (Separations, H.I. Ambacht, Netherlands). The reversed-phase column [LiChroCART RP-18 (125 × 4.0 mm I.D.; particle size 15 μm; Japan Merck, Tokyo, Japan)] was used for isocratic elution with acetonitrile–5 mM phosphate buffer (pH 8.5) (56:44, w/w) at ambient temperature and a flow-rate of 1.0 ml/

min. The fluorescence was detected at 425 nm with excitation at 325 nm.

3. Results and discussion

The specific derivatization of an arginine residue in peptides necessitates fairly drastic reaction conditions (pH 12) to generate an effective nucleophile because of the high pK_a of the guanidine functional group ($pK_a = 12-14$). Hence a good derivatization yield needs a high pH, but most peptides are not stable to extreme alkaline pH. Therefore, it is important to find the optimum conditions under which the peptide does not degrade and the sensitivity of detection is still good. Literature methods were not suitable for antagonist G, as was shown by the appearance of several peaks on the chromatogram and the substantial loss of antagonist G. Therefore, the conditions for fluorescence derivatization for antagonist G were modified. The effect of reaction time and concentration of sodium hydroxide on the intensity of fluorescence was studied. Under the investigated conditions (NaOH 0.05-0.3 M, reaction time 10-120 s and 100°C), the intensity of fluorescence increases with increase in sodium hydroxide concentration and reaction time. With 0.3 M NaOH, a decline in intensity with time is observed. However, degradation of antagonist G also increases with increase in sodium hydroxide concentration and reaction time. Chromatographic information shows that the degradation of antagonist G is negligible at 0.2 M NaOH and 100°C for 10 s (because of dilution, the actual concentration of NaOH in the reaction mixture is 0.067 M), while the intensity of fluorescence is acceptable. At 0.2 M and 60 s, substantial degradation (33%) was observed, as demonstrated by HPLC-UV analysis [3].

The effect of the final pH of the reaction mixture on the fluorescence intensity was studied in the pH range 8.0-13.5. The fluorescence intensity increases slowly from pH 8.0 to 12.0, and decreases sharply beyond pH 12.0. pH 8.5 was chosen because column deterioration will occur at higher pH.
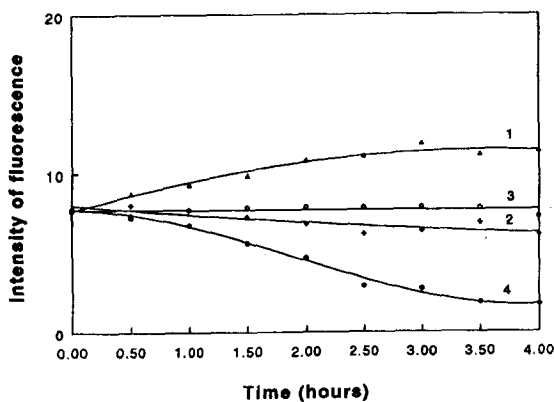

Fig. 1. Stability of the peptide derivative with benzoin. For conditions, see text. (1) The reaction mixture is kept at room temperature and adjusted to pH 8.5 before injection; (2) the reaction mixture is kept at 4°C and adjusted to pH 8.5 before injection; (3) the reaction mixture is adjusted to pH 8.5 after cooling for 5 min and kept at 4°C until 10 min before injection; (4) the reaction mixture is adjusted to pH 8.5 after cooling for 5 min and kept at room temperature until injection.

The stability of the fluorescent derivatives of antagonist G with benzoin was investigated (Fig. 1). The derivatives are stable for at least 4 h when the reaction mixture is cooled in ice-water for 5 min, brought to pH 8.5 and kept at 4°C until 10 min before injection (curve 3). Various variations in this procedure result in less stable fluorescence intensities (curves 1, 2 and 4).

The derivatized peptides can be eluted from the column by isocratic elution with acetonitrile-5 mM phosphate (pH 8.5). The use of Tris-hydrochloric acid (pH 8.5), often mentioned in the literature instead of phosphate buffer, delayed the retention to over 1 h.

The plot of peak height versus the concentration of antagonist G was linear in the range 0.26-210 nmol/ml with correlation coefficients of 0.9913-0.9948. The detection limit at a signal-to-noise ratio of 2 was 0.21 nmol/ml. The sensitivity of the method is higher than that of the UV-HPLC method [3]. The detection limit of the UV-HPLC method was found by us to be 0.84 nmol/ml. In within-day assays, the relative standard deviation ($n = 10$) of the peak heights was
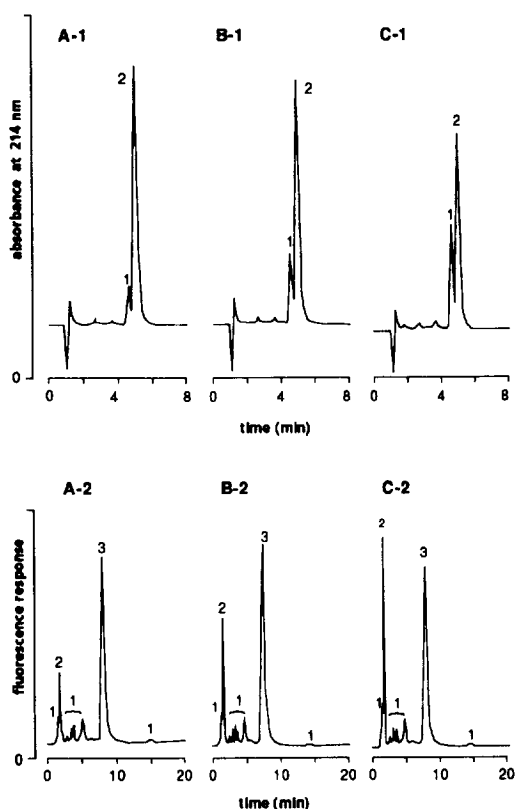
Fig. 2. Chromatograms of the peptide fragments from degradation of antagonist G in acidic solution. A 100-μl volume of antagonist G solution was mixed with 100 μl of 1.0 M perchloric acid, the mixture was heated at 100°C for (A) 5, (B) 15 or (C) 30 min and then 100 μl of 1.0 M NaOH solution were added. A 20-μl volume of the reaction mixture was chromatographed with UV detection (A-1, B-1, C-1). HPLC mobile phase: acetonitrile–0.1 M NaClO$_4$ containing 0.1% TFA (pH 2) (45:55). Peaks: 1 = fragment degraded from antagonist G; 2 = antagonist G. Another 80 μl of mixture were used for fluorescence derivatization and 20 μl of the final reaction mixture were chromatographed with fluorescence detection (A-2, B-2, C-2). HPLC mobile phase: acetonitrile–5 mM phosphate (pH 8.5) (56:44). Peaks: 1 = reagent blank; 2 = fragment degraded from antagonist G; 3 = antagonist G.

0.27% at a concentration of antagonist G of 42 nmol/ml.

The method has been successfully applied to the selective detection of arginine-containing fragments in degradation products of antagonist G. The time courses of degradation of antagonist

G in acidic and alkaline solution are shown in Figs. 2 and 3. Under acidic conditions, one degradation fragment was found with UV detection (Fig. 2 A-1, B-1 and C-1). This fragment was confirmed to contain an arginine residue (Fig. 2 A-2, B-2 and C-2). Under alkaline conditions, at least six degradation fragments were found with UV detection (Fig. 3 A-1, B-1 and C-1). Three of them are arginine-containing fragments according to the present method (Fig. 3 A-2, B-2 and C-2). In all cases, the degree of degradation of antagonist G increases with reaction time. The identification of degradation fragments of antagonist G is under investigation by HPLC–MS [16].

The derivatization method not only offers useful information for the characterization of degradation products of antagonist G, but can also be used for quantitative degradation studies of antagonist G. According to Reubsaet et al. [3], in acidic and alkaline solutions, degradation of antagonist G follows (pseudo)-first-order kinetics. The degradation rate constants (k_{obs}) were calculated at pH/H$_0$ 0.3 and pH/H$_-$ 12.7 with the present method ($k_{obs} = 1.7 \cdot 10^{-4}$ and $5.2 \cdot 10^{-4}$ s^{-1}, respectively) and a stability-indicating UV–HPLC method [3] ($k_{obs} = 1.9 \cdot 10^{-4}$ and $5.6 \cdot 10^{-4}$ s^{-1}, respectively) by measuring the decrease in the antagonist G concentration in the degraded samples at 85°C with time. The results show that the degradation rate constants obtained from both techniques are comparable. Therefore, the present method is also a stability-indicating method in the degradation study of antagonist G.

4. Conclusions

The developed derivatization method is suitable for the selective and sensitive determination of antagonist G and its degradation products containing arginine. The mild derivatization conditions prevent methodology-related degradation of the peptide. The method is also stability-indicating in the degradation study of antagonist G.
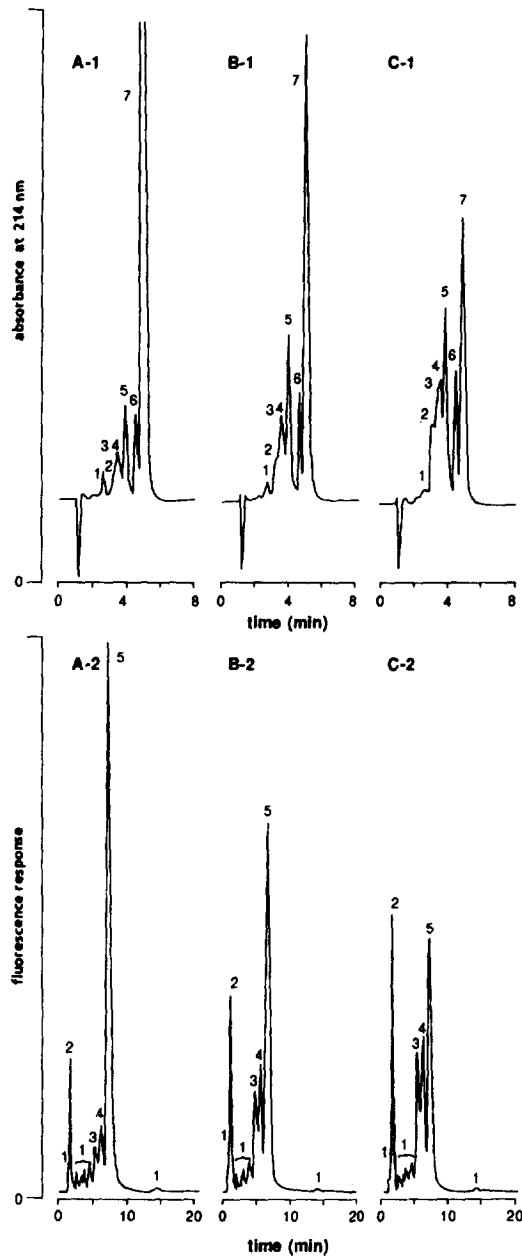
Fig. 3. Chromatograms of the peptide fragments from degradation of antagonist G in alkaline solution. A 100-μl volume of antagonist G solution was mixed with 100 μl of 0.1 M sodium hydroxide, the mixture was heated at 100°C for (A) 5, (B) 15 or (C) 30 min and then 100 μl of 0.1 M HClO$_4$ solution were added. A 20-μl volume of the reaction mixture was chromatographed with UV detection (A-1, B-1, C-1). HPLC mobile phase: acetonitrile–0.1 M NaClO$_4$ containing 0.1% TFA (pH 2) (45:55). Peaks: 1–6 = fragments degraded from antagonist G; 7 = antagonist G. Another 80 μl of mixture were used for fluorescence derivatization and 20 μl of the final reaction mixture were chromatographed with fluorescent detection (A-2, B-2, C-2). HPLC mobile phase: acetonitrile–5 mM phosphate (pH 8.5) (56:44). Peaks: 1 = reagent blank; 2–4 = fragments degraded from antagonist G; 5 = antagonist G.

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