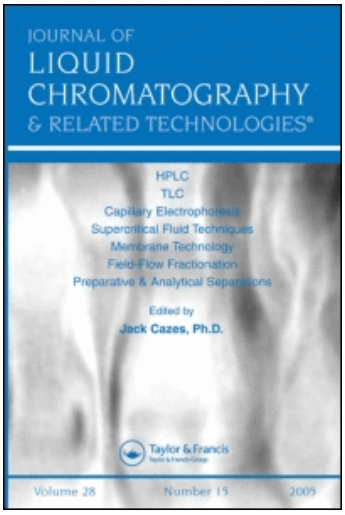

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 13 January 2005

To cite this Article Wang, Yun , Chen, Li-Lan H. and Chien, Yie W.(1999) 'HPLC ANALYSIS OF GONADOTROPIN-RELEASING HORMONE AND ITS ANALOGUES USING BENZOIN AS A FLUOROGENIC REAGENT', Journal of Liquid Chromatography & Related Technologies, 22: 16, 2421 — 2432
To link to this Article: DOI: 10.1081/JLC-100101811
URL: http://dx.doi.org/10.1081/JLC-100101811

J. LIQ. CHROM. & REL. TECHNOL., 22(16), 2421–2432 (1999)

HPLC ANALYSIS OF GONADOTROPIN-RELEASING HORMONE AND ITS ANALOGUES USING BENZOIN AS A FLUOROGENIC REAGENT

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ABSTRACT

Benzoin, which reacts specifically with arginine-containing peptides, was used as a pre-column fluorogenic reagent in the HPLC analysis of gonadotropin-releasing hormone (GnRH) and its synthetic analogues in an attempt to improve their detection sensitivity. The reaction kinetics of benzoin with GnRH and its structurally-related analogues, which all contain an arginine residue in the peptide chain, were investigated in this study. A fluorescence detector (ex 325 nm/em 435 nm) was used to detect benzoin-arginine derivatives. Another set of excitation and emission wavelengths (289 nm/344 nm), where fluorescent activity results from the aromatic amino acid residues, such as Tyrosine and Tryptophan, was used for comparison. The derivatization reaction and the fluorescent derivative product were highly affected by peptide concentration and heating time. The reactivity of arginine at position 8 was found to depend on the primary structure of GnRH and its analogues. However, it was

observed that GnRH and its analogues were extremely unstable, as indicated by the labile nature of GnRH and its analogues under the derivatization conditions used.

INTRODUCTION

Peptide analysis has long attracted considerable interest in biomedical research and pharmaceutical sciences. As a result of its sensitivity, selectivity, and high speed, when compared to conventional chromatographic procedures, high performance liquid chromatography (HPLC) has contributed substantially to the efficiency of peptide analysis in recent years. Reverse-phase HPLC, in particular, has emerged as an important technique for peptide analysis, with analyses being routinely performed using ultraviolet (UV) detection. Many physiologically-important peptides, however, exist at trace levels in mammalian tissues and biological fluids, often below the limit of UV detection.

Fluorescence detection is able to increase detection sensitivity; nevertheless, it is only suitable for peptides or proteins which contain aromatic amino acids, such as Tryptophan, Tyrosine and Phenylalanine. Pre- or post-column derivatization with fluorogenic reagents is commonly used to improve the detection limit of proteins/peptides. The fluorogenic reagents frequently used, such as ninhydrin,[1] 9,10-phenanthraquinone,[2,3] fluorescamine,[4] and o-phthalaldehyde,[5] are known to react mainly with primary or secondary amino groups.[6] For the peptides containing no primary or secondary amino groups, such as gonadotropin-releasing hormone (GnRH), these reagents are not useful. Benzoin, on the other hand, has been investigated for the pre- and post-column fluorogenic derivatization of arginine-containing peptides, such as GnRH[7] and substance P.[8] Benzoin is known to have no fluorescence activity on its own; however, it is able to form a highly fluorescent derivative upon reaction with arginine-containing peptides in an alkaline solution.[7,9,10]

The objectives of this study are to investigate the feasibility of using benzoin as a pre-column fluorogenic reagent for the HPLC analysis of GnRH and its analogues, to optimize the fluorescent yield, and to characterize the structure-reactivity relationship of the derivatization of GnRH and its three synthetic analogues with benzoin.

EXPERIMENTAL

Materials

Unless otherwise stated, all chemicals were ACS grade or better and were used as received. All solutions were prepared using 18 MA water from a Mini Quad type I reagent-grade water system (Photronix, Medway, MA). GnRH and

its three synthetic analogues were purchased from Bachem (King of Prussia, PA). All GnRH peptides were at least 99% pure. Methylcellosolve (HPLC grade) was supplied by Sigma (St. Louis, MO). Benzoin and mercaptoethanol were ordered from Aldrich Chemical Co. (Milwaukee, WI). Tris (hydroxymethyl)aminomethane was provided by Mallinckrodt (Paris, KY). Potassium hydroxide pellets and concentrated hydrochloric acid (37.3%) were obtained from Baker Analytical (Phillipsburg, NJ). Acetonitrile (HPLC grade), and trifluoroacetic acid (TFA, 99.8%) were purchased from Fisher Scientific (Fair Lawn, NJ).

Preparation of Reagents and Solutions

All solutions for derivatization were freshly prepared in 10 mL scale for each experiment. Benzoin (5 mM) was prepared by dissolving 10.60 mg of benzoin in 10 mL of 2-methoxyethanol (methylcellosolve). β-Mercaptoethanol (70 μL) and sodium sulfite (0.2521 g) were dissolved and diluted to a volume of 10 mL with distilled water to form a solution containing 0.1 M β-mercaptoethanol and 0.2 M sodium sulfite. Potassium hydroxide (0.4489 g) was dissolved in sufficient water to obtain a concentration of 0.8M. Tris (0.6057 g) was dissolved in 8 mL of 1 N HCl and adjusted to 10 mL with distilled water to form 0.5 M Tris buffer in 0.8 M HCl.

TFA in water (0.1% (v/v)) and TFA in ACN (0.1% (v/v)) were prepared by adding 1 mL of TFA to 1 L of each solvent. It was filtered before use.

Derivatization Procedure

The derivatization procedure was adopted from Kai, et al.[10] GnRH solutions were prepared in an isotonic phosphate (0.01 M) buffered saline solution with a pH of 7.4. The concentration of GnRH and analogues varied from 0.1 μg/mL to 100 μg/mL. A 100 μL portion of peptide solution was used for the derivatization. Benzoin (50 μL of 5 mM) in methylcellosolve was added to 100 μL of GnRH solution in a glass microvial, followed by 50 μL of 0.1 M β-mercaptoethanol solution in 0.2 M sodium sulfite. Then, 100 μL of 0.8 M potassium hydroxide was added into each vial under cooling conditions (ice-bath). Microvials, which were crimp-sealed to prevent evaporation, were placed in a boiling water-bath to accelerate the derivatization reaction. The heating time ranged from 0 to 60 sec to study derivatization kinetics. Immediately after heating, each microvial was cooled in an ice-bath to condense any vapor that was formed within the microvial during heating and to slow down the reaction. Acidified Tris buffer (100 μL, pH 8.5) was injected into each vial to stop the

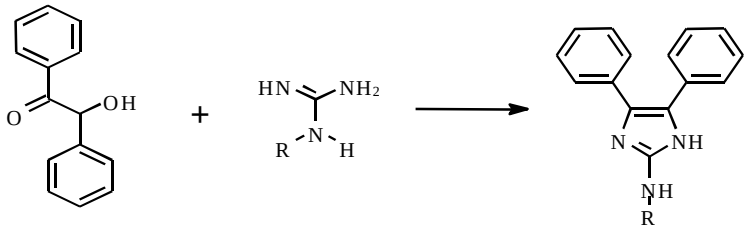
reaction at the final step of derivatization. The samples were kept at refrigerator temperature during storage and HPLC analysis.

HPLC Instrument/Conditions

A Hewlett Packard 1090 system with a multi-wavelength UV detector and a fluorescence detector (HP 1046) in series was used. A Vydac reverse-phase column (5 μm, RP-4, 250 x 4.6 mm) was used with 0.1%(v/v) TFA in water (mobile phase A)/0.1% (v/v)TFA in ACN (mobile phase B) as the mobile phase. A linear gradient system was employed in which mobile phase A decreased from 80% to 50% during the first 12 minutes and remained at 50% afterwards at a constant flow rate of 0.7 mL/minute. The column temperature was maintained at 30°C to minimize any effect due to fluctuation of room temperature. A five-minute post time period allowed the system and column to re-equilibrate after each run. The samples were monitored simultaneously for UV and fluorescence absorbance. The UV Diode Array Detector (DAD) was set to detect at 280/220 nm and the fluorescence detector (HP1046A) was used at 325 nm for excitation and 435nm for emission. The samples were reanalyzed under the same conditions (I) with a different fluorescence detection wavelength (289 nm ex /344 nm em). All samples were analyzed twice at different fluorescence detection wavelengths.

RESULTS AND DISCUSSION

Arginine-containing peptides can be derivatized to produce fluorescence activity through condensation of the guanidine moiety of the arginyl residue with benzoin. The highly fluorescent derivative, 2-substituted amino-4,5-diphenylimidazole, is produced by the following reaction:[9,11]



Benzoin Guanidine Moiety Fluorescent Product

Fluorometric detection of the derivatives from pre- and post-column derivatization was reportedly more sensitive than conventional UV detection.[7]

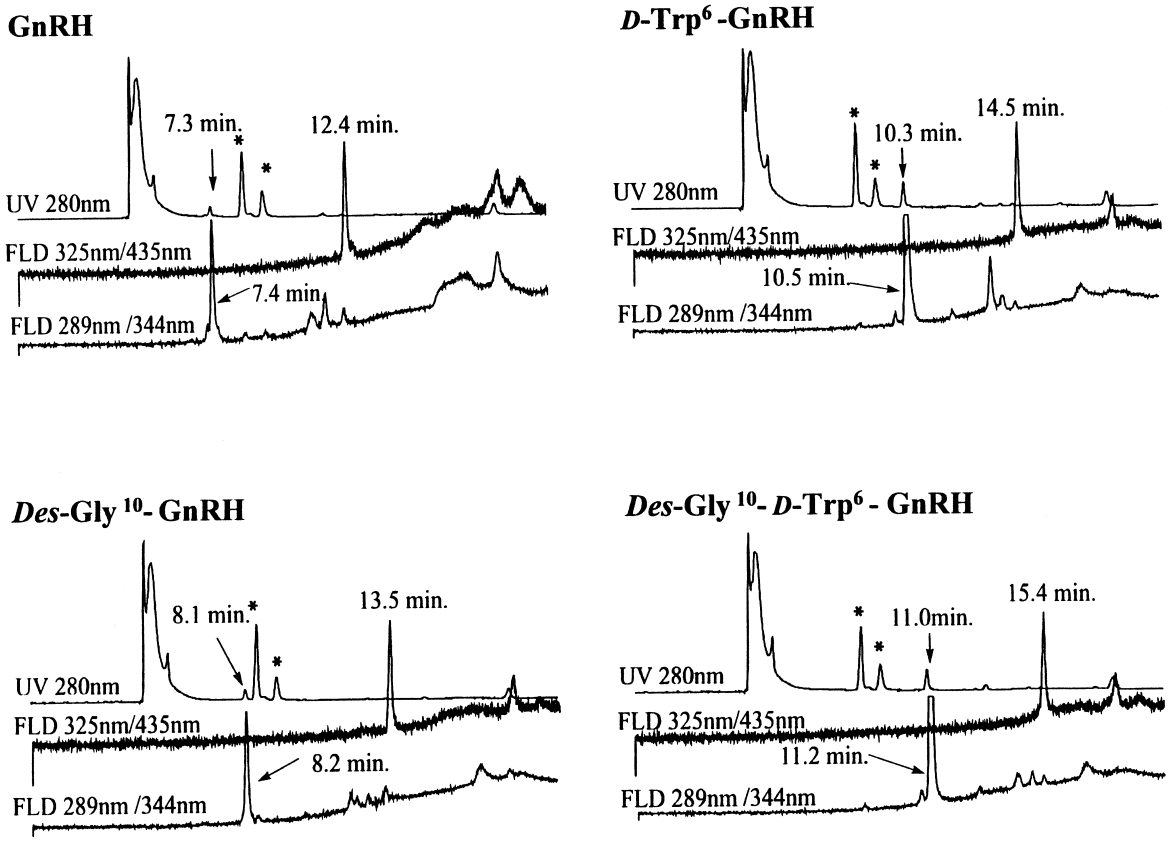


Figure 1. Chromatograms of GnRH and its analogues after derivatization (without heating), as determined by HPLC. The chromatograms obtained from UV 280 nm and FLD 289 nm/344 nm signals indicate the unreacted GnRH and its analogues, while FLD 325 nm/435 nm signals show the benzoin-derivatized products of GnRH and its analogues. "*" are the derivatization reagents.

GnRH is a physiologically-important decapeptide which controls human reproductive functions. It stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn, trigger the release of sex steroids from ovaries or testes. GnRH and all the analogues used in this study contain an arginine (Arg) residue at position 8. This Arg is expected to react with benzoin to yield a highly fluorescent derivative; and thus, increase the detection limit. Reactivity of benzoin with arginine and other arginine-containing peptides has been documented;[7,10] however, the feasibility and suitability of using benzoin as a fluorogenic agent for the detection of GnRH has not been addressed. Furthermore, the conditions for benzoin/arginine derivatization reaction is yet to be optimized.

The primary structure of GnRH and three structurally related analogues used in the present study are shown below:

Natural GnRH (N): pyr-Glu-His-Trp-Ser-Tyr-gly-Leu-Arg-Pro-Gly-NH_2

desGlyGly^{10}-Pro^{9}-GnRH (G) pyr-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-**NHEt**

D-Trp^{6}-GnRH (T): pyr-Glu-His-Ser-Tyr-**Trp**-Leu-Arg-Pro-Gly-NH_2

D-Trp^{6}-desGly^{10}-Pro^{9}-GnRH(GT): pyr-Glu-His-Trp-Ser-Tyr-**Trp**-Leu-Arg-Pro-**NHEt**

In order to increase the fluorescence sensitivity of benzoin-arginine derivatives, a slightly alkaline mobile phase (pH 8.5) was suggested by Kai et al.[7,12] However, the packing material of the LiChrosorb RP-18 column is only stable at pH 2~8. The packing material dissolved and degraded in the mobile phase at pH 8.5 and was lost rapidly under the conditions used by Kai et al. Therefore, an acidic condition, 0.1% TFA in water and ACN, which is commonly used for HPLC analysis of peptides, was used. Figure 1 illustrates the UV and FLD chromatograms for 100 μg/mL of GnRH and its analogues after derivatization with benzoin in the absence of heating.

Significant amounts of parent peptides eluted at 7.3, 8.1, 10.3, and 11.0 minutes for GnRH (N), *des*Gly^{10}-Pro^{9}-GnRH (G), D-Trp^{6}-GnRH (T), and D-Trp^{6}-*des*Gly^{10}-Pro^{9}-GnRH (GT), respectively. GnRH and its analogues eluted in the order of their corresponding lipophilicities. The benzoin-derivatized products of each peptide eluted at 12.4, 13.5, 14.5, and 15.4 minutes, respectively, without change in the order of the lipophilicities of the parent peptides.

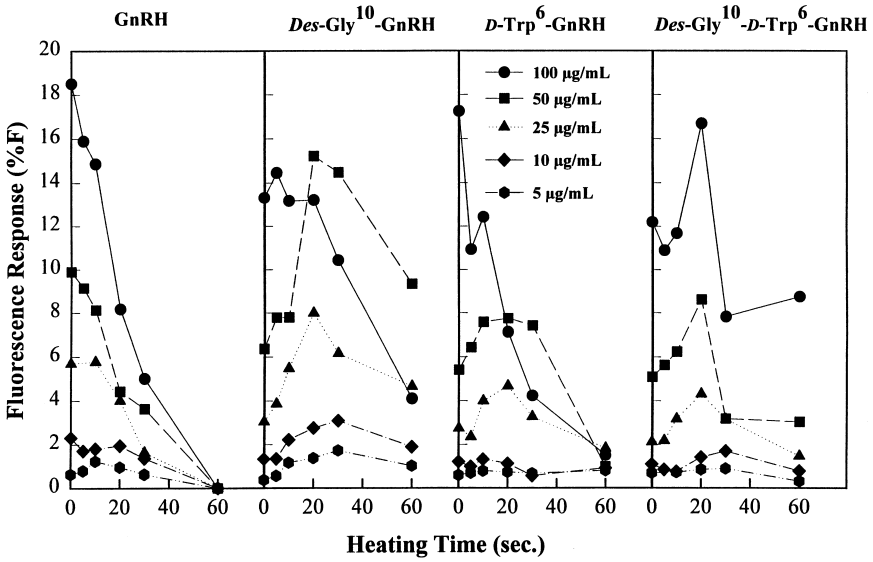


Figure 2. Fluorescence responses (benzoin-derivatized GnRH and analogues) as a function of heating time for various concentrations of GnRH and analogues.

Chromatograms (FLD 325 nm/435 nm) in Figure 1 and the profiles of benzoin-arginine fluorescence as a function of heating time in Figure 2 illustrate that significant fluorescence activity of the derivatized peptide product was obtained at zero heating time. The observations suggested the derivatization started immediately upon mixing of all solutions even at room temperature.

It is clear from Figure 2 that fluorescence activity of benzoin-derivatized GnRH and its analogues depended on both the heating time and the concentration of parent peptides. For GnRH and D-Trp^{6}-GnRH (decapeptides), at high concentration (>50 µg/mL), the benzoin derivatization reaction was observed to be spontaneous and heating was not needed. For low concentration samples (< 50 µg/mL), heating time of 10 sec was required to achieve maximum fluorescence activity. Longer heating times (>10 sec) reduced the fluorescence activity, possibly as a result of the degradation of parent peptides or derivatized peptides. For nonapeptide GnRH analogues, such as *des*Gly^{10}-Pro^{9}-GnRH and D-Trp^{6}- *des*Gly^{10}-Pro^{9}-GnRH, a longer heating time (20-30 sec) was required to achieve maximum fluorescence at all concentrations.

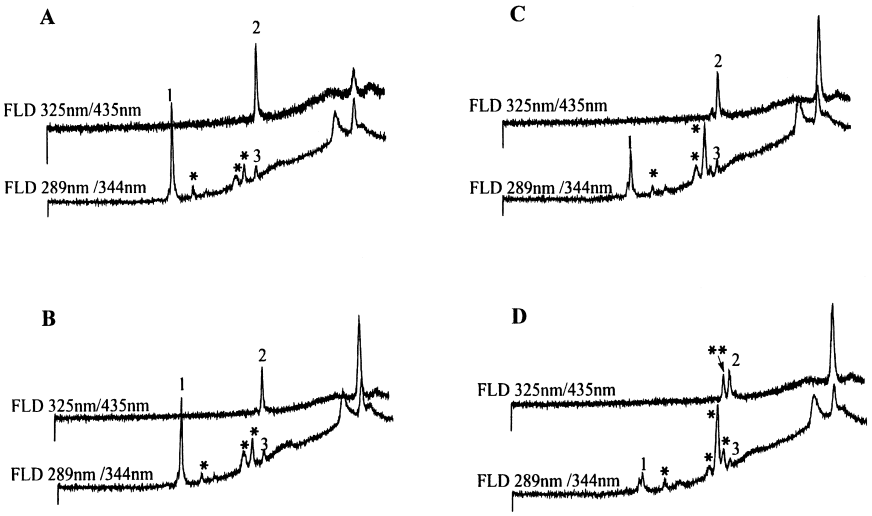


Figure 3. Chromatograms of GnRH and its benzoin derivatives following derivatization of GnRH (50μg/mL) for various duration of heating time. (A): 0 sec, (B): 5 sec, (C): 10 sec, (D): 20 sec. Peak 1: GnRH; Peak 2: FLD 325 nm/435 nm signal of benzoin-derivative; Peak 3: FLD 289 nm/344 nm signal of benzoin-derivative; Peak *: fragments GnRH; Peak**: secondary deriv. of the break-down of derivatized GnRH.

These observations demonstrate the structure-reactivity dependence of GnRH and its analogues with benzoin. The absence of Gly at position 10 resulted in greater energy requirement for maximum fluorescence activity. The observation illustrates that the Gibb's free energy required for initiating the derivatization of arginine at position 8 was higher for GnRH nonapeptides than that for GnRH decapeptides. The replacement of D-Trp at position 6 did not have any significant effect on the benzoin-arginine derivatization reaction.

The stability of GnRH and its analogues under the derivatization conditions was the authors' major concern. A U-shaped pH-stability profile, with greatest stability at pH 4, has been reported for GnRH.[13] GnRH is very labile when the solution pH deviates from 4. Figures 3 ~ 6 are the chromatograms of GnRH and its analogues, the benzoin-derivatives and the degraded fragments at two fluorescence detection wavelengths, with various heating times. For FLD detection, 325nm ex/435 nm em was reported to be the maximum excitation and emission wavelengths for benzoin-derivatized peptides;[7,12] and thus, these wavelengths were used for the detection of benzoin-derivatized peptides.

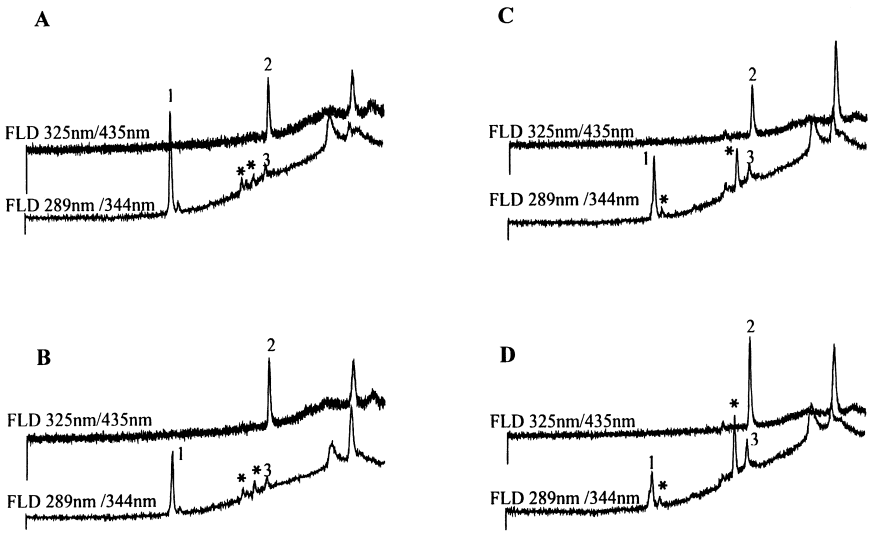


Figure 4. Chromatograms of *des*Gly[10]-Pro[9]-GnRH and its benzoin derivatives following derivatization reaction of *des*Gly[10]-Pro[9]-GnRH(50 μg/mL) for various duration of heating time. (A): 0 sec, (B): 5 sec, (C): 10 sec, (D): 20 sec. Peak 1: *des*Gly[10]-Pro[9]-GnRH; Peak 2: FLD 325 nm/435 nm signal of benzoin-derivative; Peak 3: FLD 289 nm/344 nm signal of benzoin-derivative; Peak*: fragments of *des*Gly[10]-Pro[9]-GnRH; Peak**: secondary derivative or the break-down product of derivatized *des*Gly[10]-Pro[9]-GnRH.

FLD 289 nm/344 nm was chosen for the detection of aromatic amino acids, such as tyrosine, tryptophan, and phenylalanine.[14] Significant amounts of degradation products from parent peptides (FLD 289nm/344nm) and benzoin-derivatized peptides (FLD 325nm/435nm) were observed in Figures 3-6.

Peaks "1" are the unreacted parent GnRH peptide and its analogues. Peaks "2" are their benzoin derivatives. Peaks "*" are the degraded fragments from parent GnRH peptides. Peaks labeled "**" are either the secondary derivatives or the degraded benzoin-derivatized products.

The results strongly suggest that significant degradation took place for both parent peptide and benzoin-derivatized products of GnRH and its analogues under the derivatization conditions. The results further indicate that the deletion of glycine from GnRH prevented the formation of "**" peaks and also diminished the breakdown of parent peptides under boiling temperatures for up

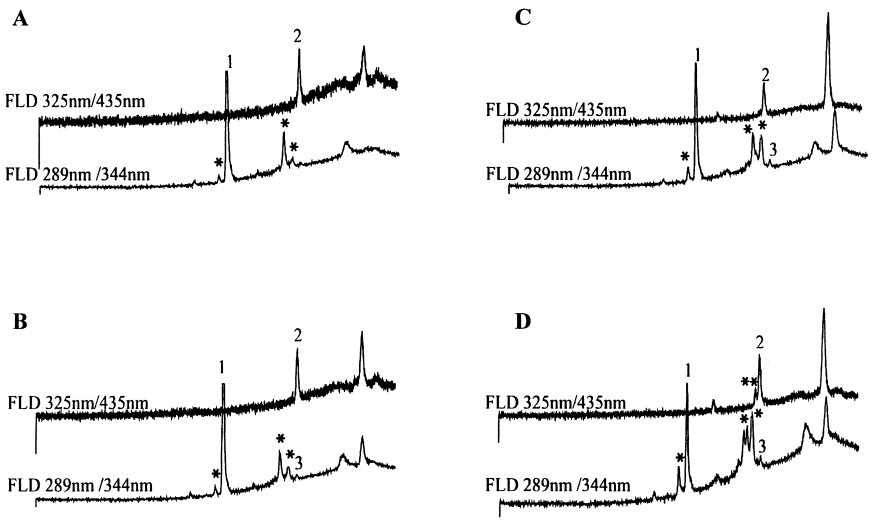


Figure 5. Chromatograms of D-Trp^6-GnRH and its benzoin derivatives following derivatization reaction of D-Trp^6-GnRH (50 µg/mL) for various duration of heating time. (A): 0 sec, (B): 5 sec, (C): 10 sec, (D): 20 sec. Peak 1: D-Trp^6-GnRH; Peak 2: FLD 325 nm/435 nm signal of benzoin-derivative; Peak 3: FLD 289 nm/344 nm signal of benzoin-derivative; Peak*: fragments of D-Trp^6-GnRH; and Peak**: secondary derivative or the break-down product of derivatized D-Trp^6-GnRH.

to 20 sec (delayed appearance of "**" peaks and the lower heights of "*" peaks in Figure 4 & 6). The replacement of Gly at position 6 with D-Trp did not improve the stability of GnRH analogues or the derivatized peptides under the derivatization conditions.

CONCLUSION

Important requirements for using benzoin as a pre-column derivatizing agent to improve the analytical sensitivity of GnRH or any other delicate arginine-containing peptides are: 1) the derivatization condition cannot be harmful to parent peptides, and 2) a single fluorescent derivative with higher fluorescent activity than parent peptides should be formed. Neither requirements was achieved by using benzoin as a pre-column fluorogenic reagent for GnRH and its analogues. For GnRH decapeptides, more than one fluorescent derivative was formed as soon as the reaction mixture was heated for up to 20 sec. The derivatization reaction was both concentration- and time-dependent. The percentage yield varied with the concentration of parent

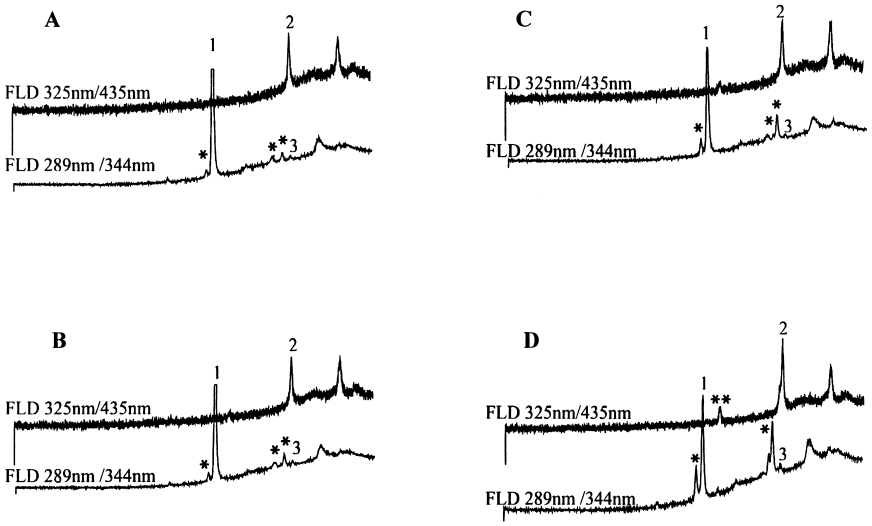


Figure 6. Chromatograms of D-Trp^{6}-*des*Gly^{10}-Pro^{9}-GnRH and its benzoin derivatives following derivatization reaction of D-Trp^{6}-*des*Gly^{10}-Pro^{9}-GnRH (50μg/mL) for various duration of heating time. (A): 0 sec, (B): 5 sec, (C): 10 sec, (D): 20 sec. Peak 1: D-Trp^{6}-*des*Gly^{10}-Pro^{9}-GnRH; Peak 2: FLD 325 nm/435 nm signal of benzoin-derivative; Peak 3: FLD 289 nm/344 nm signal of benzoin-derivative; Peak*: fragments of D-Trp^{6}-*des*Gly^{10}-Pro^{9}-GnRH; Peak**: secondary derivative or the break-down product of derivatized D-Trp^{6}-*des*Gly^{10}-Pro^{9}-GnRH.

peptides and the reactivity depended on the relative position of arginine in the peptide chain. The reaction efficiency was found to be lower for longer heating times due to the formation of degradation products from both parent and derivatized peptides. Moreover, the derivatization reaction did not improve the detection sensitivity for GnRH or its analogues. The detection limit was found to be 5 μg/mL, which is the same as the detection sensitivity achieved by FLD 289 nm/344 nm without derivatization. Therefore, using benzoin as a pre-column fluorogenic agent to improve the detection limit GnRH and its analogues was not suitable or feasible due to the labile nature of these peptides under the derivatization conditions.

ACKNOWLEDGMENT

The authors thank the Parke-Davis Chair Endowment for providing financial support to this undergraduate honors research project.

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Received January 7, 1999
Accepted January 27, 1999
Manuscript 4976

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