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High-performance liquid chromatographic determination of peptides in biological fluids by automated pre-column fluorescence derivatization with fluorescamine

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ABSTRACT

Peptides containing a free α- or ε-amino group react with fluorescamine under mild alkaline conditions to generate a highly fluorescent but unstable reaction product and, consequently, practical high-performance liquid chromatographic (HPLC) approaches to analysis have typically involved the use of postcolumn derivatization. An automated precolumn approach is reported in which peptides are reacted with fluorescamine just prior to HPLC analysis by a commercially available autoinjector with derivatization capabilities. The autoinjector added base and fluorescamine reagent solutions to a sample vial containing peptide analytes, and the derivatization reaction was allowed to proceed for 5 min at room temperature prior to injection into the HPLC system. The derivatized peptides were analyzed by reversed-phase HPLC with fluorescence detection (excitation at 390 nm; emission 470-nm cut-off filter) on an octylsilica column. Optimization of the precolumn reaction conditions and the use of narrower HPLC columns (2 mm I.D.) resulted in a typical on-column detection limit of 30–50 fmol of peptide, which was substantially lower than that in previously reported post-column methods. This approach was applied to the HPLC of several naturally occurring and synthetic peptides containing α- and ε-amino groups. In combination with solid-phase extraction, prior to automated precolumn fluorescence derivatization and chromatographic analysis, the methodology was used for the determination of a synthetic growth hormone-releasing peptide in plasma samples.

INTRODUCTION

The development of novel peptide analogues as therapeutic agents has recently been of increasing interest in drug development. As many synthetic peptide analogues are extremely potent, and often administered at a low dosage, the development of highly sensitive and specific analytical methodology to support pharmacokinetic and disposition studies is a challenging problem for this class of molecules. This is especially true for high-performance liquid chromatographic (HPLC) assays, as many synthetic peptide analogues lack an appropriate chromophoric or fluorescent group which would allow detection of these peptides at the sub-picomole level. Enhancing the detectability of such peptides by chemical modification is one possible solution, and for peptides containing a free α- or ε-amino group, selective derivatization (pre-

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or postcolumn) can be achieved with several fluorescent reagents to allow HPLC measurement at high sensitivity.

Although fluorescamine was discovered two decades ago [1–5] as a derivatization reagent for the fluorescence detection of primary amines, peptides and proteins, its use for postcolumn reaction detection [6–8] in HPLC has been limited owing to the high cost of reagent. The use of fluorescamine in precolumn derivatization methods for HPLC has also been restricted owing to the limited stability of the fluorescent product formed [9]. Owing to these, and other, practical limitations, previously reported methods for the HPLC of peptides with fluorescence detection following pre- and postcolumn derivatization with fluorescamine have suffered from a lack of routine applicability and/or sensitivity.

This paper describes the development of HPLC methodology for the determination, in plasma samples, of synthetic peptides containing a free α- or ε-amino group by automated reaction with fluorescamine using a commercially available autoinjector with sophisticated precolumn derivatization capabilities. The approach involved preliminary isolation of the peptide from plasma prior to automated reaction with fluorescamine and subsequent HPLC. The derivatized peptides were separated by reversed-phase HPLC and detected with a spectrofluorimeter. The use of narrower HPLC columns and optimization of the precolumn derivatization conditions resulted in an on-column detection limit in the range 30–50 fmol (signal-to-noise ratio = 3). Although we developed this methodology primarily to study a series of synthetic lysine-containing peptides which release growth hormone in several species, the method, owing to its high sensitivity, has general applicability in the detection of several naturally occurring peptides such as bradykinin and the angiotensins.

EXPERIMENTAL

Chemicals and materials

The synthetic lysine-containing peptides (Fig. 1), known to release growth hormone in several species, were supplied by Drug Substances and Products, SmithKline Beecham Pharmaceuticals (Swedeland, PA, USA). Naturally occurring peptides, such as bradykinin and angiotensins, were purchased from Sigma (St. Louis, MO, USA). HPLC-grade water (Millipore, Bedford, MA, USA) was used in the mobile phases and in the preparation of buffers and standard solutions. Fluorescamine and trifluoroacetic acid (TFA, 99%) were obtained from Pierce (Rockford, IL, USA). HPLC-grade methanol and acetonitrile were obtained from J. T. Baker (Phillipsburg, NJ, USA). All other chemicals were of analytical-reagent grade and obtained from local sources. Weak cation-exchange (CBA) solid-phase extraction cartridges (1 ml) and a Vac-Elut manifold were purchased from Analytichem International (Harbor City, CA, USA).

PEPTIDE	SK&F	STRUCTURE
I	110679	NH_2-L-HIS-D-TRP-L-ALA-L-TRP-D-PHE-L-LYS-NH_2
II	110910	NH_2-HIS-D-PHE-ALA-PHE-D-PHE-LYS-NH_2

Fig. 1. Structures of synthetic lysine-containing peptides.

Tris buffer, 10 m*M* (pH 7.0), was prepared by dissolving tris(hydroxymethyl) aminomethane (1.21 g) and triethylamine (2.8 ml) in 1 l of HPLC-grade water adjusting the pH to 7.0 with phosphoric acid.

Standard solutions

Stock standard solutions of peptide analytes were prepared by dissolving the appropriate amount of the compounds in methanol to obtain a 1 mg/ml solution. Working standard solutions were prepared by diluting a portion of the stock standards solutions with 0.1 *M* citrate buffer (pH 4.0) to give a final solution with concentrations ranging from 0.1 to 10 μg/ml. The working standard solutions were used in the generation of chromatographic data and calibration graphs. The stock and working standard solutions were stored at 4°C for not more than 2 weeks.

Extraction of synthetic lysine-containing peptides from plasma

An aliquot of plasma (0.5 ml) containing the synthetic peptide as a standard or as an unknown was mixed with 50 μl of an internal standard solution (1 μg/ml) in a 75 × 12 mm I.D. borosilicate tube. A CBA solid-phase extraction column was conditioned by successive washings with 1 ml of methanol and 1 ml of water. Following application of the plasma sample to this cartridge, the sample tube was rinsed with 1 ml of water, which was applied to the column also. The CBA columns was then washed successively with 1 ml of 1% TFA in water, 3 ml of water and 1 ml of 50% methanol. The peptide was then eluted from the column with 2 ml of 2% (w/v) ammonium acetate in methanol and collected in a 75 × 12 mm I.D. borosilicate tube. The methanol was evaporated under a gentle stream of nitrogen and the residue was reconstituted in 100 μl of 0.1 *M* citrate buffer (pH 4.0)–acetonitrile (75:25, v/v). The sample was then subjected to automated precolumn derivatization and HPLC.

Automated precolumn derivatization

A portion (20 μl) of the reconstituted plasma extract or standard solution was transferred to a limited-volume vial and loaded on the autoinjector. A 20-μl volume of potassium phosphate buffer was added to the sample vial by the autosampler followed by 30 μl of fluorescamine solution. The resulting solution was mixed by purging air into the sample vial and the reaction was allowed to proceed at room temperature. A portion of the reaction mixture (10–50 μl) was then injected for HPLC analysis.

High-performance liquid chromatography

The HPLC system consisted of a Hitachi 665A-12 high-pressure gradient semimicro solvent-delivery system (EM Science, Cherry Hill, NJ, USA), a Hitachi F-1000 fluorescence detector (EM Science) and an Varian (Sunnyvale, CA, USA) autoinjector with precolumn reagent addition and mixing capabilities (Model 9090). Chromatographic separations were carried out on a 25 cm × 2.0 mm I.D. Ultrasphere 5-μm octylsilica column (Beckman Instruments, Palo Alto, CA, USA), maintained at 50°C, at a flow-rate of 300 μl/min. The gradient mobile phase eluents utilized were Tris buffer (pH 7.0) and methanol. The specific gradient conditions that were employed for the chromatographic separation of various peptide analytes are described in detail under Results and Discussion. Mobile phase eluents were filtered through a 0.2-μm

nylon 66 filter and degassed before use. Detection was accomplished by excitation at 390 nm while monitoring the fluorescence emission using a 470-nm cut-off filter. An automated laboratory system (Access Chrom, PE/Nelson, Cupertino, CA, USA) was used for data acquisition and processing. Chromatographic peak-height data were collected and used for the generation of calibration graphs.

RESULTS AND DISCUSSION

The need to measure endogenous bioactive peptides and their synthetic analogues in biological fluids requires highly sensitive and specific analytical methods. The application of HPLC to such a problem typically requires derivatization in order to improve the native detectability of the peptide analyte. For many peptide analytes containing a free α- or ε-amino group, derivatization with one of the many fluorescence reagents available for primary amines is an attractive approach to high-sensitivity detection by HPLC. Of the reagents available, *o*-phthaldialdehyde and fluorescamine are most commonly used owing to their commercial availability in pure form, ease of handling and high fluorescence quantum yields. Fluorescamine has several advantages in routine use, including a higher rate of reactivity with primary amines, including amino acids, peptides and proteins [3]. Moreover, fluorescamine itself is non-fluorescent, as are the hydrolysis products formed during aqueous reaction, and this provides an extremely low background interference level for both pre- and postcolumn derivatization applications in HPLC.

Previous HPLC approaches using fluorescamine for the analysis of peptides have typically employed either postcolumn or manual precolumn derivatization. In general, precolumn derivatization is often preferred over postcolumn reaction methods in HPLC because a higher sensitivity can be achieved owing to elimination of baseline flow noise, band broadening and dilution effects resulting from the postcolumn addition of reagent solutions. Moreover, the cost of fluorescamine can make its use for postcolumn reaction detection in HPLC prohibitive. However, aside from the need to control the reaction conditions precisely to ensure reproducibility, precolumn derivatization methods using fluorescamine also suffer from the limited stability of the peptide derivatives in the alkaline reaction medium following formation. The use of the Varian 9090 autoinjector for automation and precise controle of derivatization, followed by chromatographic injection immediately after reaction, resolves the issues associated with the use of fluorescamine in precolumn derivatization for the HPLC of peptides with fluorescence detection.

Optimization of precolumn reaction conditions

Optimization of the precolumn derivatization conditions was achieved using gradient elution HPLC of the model synthetic peptides I and II. The HPLC conditions used an initial mobile phase composition of Tris Buffer (pH 7.0)–methanol (50:50, v/v) and, following injection, the methanol concentration was increased to 65% over 10 min, held for 7 min and then cycled to the initial conditions in 2 min. Using these HPLC conditions, the precolumn reaction parameters were systematically varied, as described below, during repetitive injections of a standard solution of peptide, and the resulting chromatographic peak height observed was used as a measure of fluorescence response for optimization purposes.

The effect of fluorescamine concentration on precolumn derivatization was examined by varying the concentration from 1 to 5 mg/ml. The results indicated that a fluorescamine concentration $\geqslant$ 2 mg/ml gave the maximum fluorescence intensity for these model peptides. In order to minimize chromatographic difficulties associated with excess of reagent, a fluorescamine concentration of 2 mg/ml was adopted. The effect of pH on the reaction was examined by varying the pH of the phosphate buffer from 7.0 to 10.0. The results indicated that maximum fluorescence intensity was observed at pH 9.0 using phosphate buffer. The effect of ionic strength of the buffer was also studied by varying its molarity from 0.1 to 2, while maintaining the fluorescamine concentration at 2 mg/ml and the pH of the buffer at 9.0. The results demonstrated that a buffer molarity of 0.5 was necessary to maintain the reaction medium at the optimum pH of 9.0. The optimum conditions reported here may vary slightly with different peptides and buffers [3].

Using the reagent conditions established above, the effect of reaction time was examined by allowing the derivatization to proceed for times ranging from 1 to 30 min. The results showed that maximum fluorescence intensity was observed at a reaction time of between 1 and 5 min. Longer reaction times provided no increase in fluorescence signal owing to apparent instability of the peptide derivatives, which resulted in the appearance of additional chromatographic signals. A reaction time of 5 min was therefore adopted. Hence the optimum phosphate buffer and fluorescamine concentrations and reaction time were 0.5 *M*, 2 mg/ml and 5 min, respectively, and these conditions were used in all subsequent work.

The precision of the automated fluorescamine derivatization method described here was examined by repetitive derivatization of 23 pmol of peptide I and 25 pmol of peptide II and injecting 10 μl of reaction mixture into the HPLC system. The method displayed suitable chromatographic peak-height reproducibility for the routine determination of peptides, yielding a relative standard deviation (R.S.D.) of 7.8% for I and 5.3% for II (Table I).

Detection of synthetic lysine-containing peptides

Fig. 2 shows the results obtained from HPLC of synthetic lysine-containing peptides using the automated precolumn fluorescamine derivatization approach described here. These peptides have been demonstrated *in vivo* to cause the release of growth hormone in several species following intravenous administration. Chromatographic analysis was accomplished using gradient elution on a 2 mm I.D. octylsilica column followed by fluorescence detection. The initial mobile phase composition used was Tris buffer (pH 7.0)–methanol (50:50, v/v). Following injection, the methanol concentration was increased to 65% over a period of 10 min. The peptides were well separated despite only minor structural differences. Although peptides I and II contain both an α- and an ε-amino group, which provide two potential sites for derivatization, no evidence for multiple derivative formation was observed. The synthetic peptides examined provided a single reproducible chromatographic peak for analysis. Under these conditions, the limit of detection for these synthetic peptides ranged from 30 to 50 fmol injected on-column.

Application of this methodology to the determination a synthetic lysine-containing peptide was exemplified by an HPLC assay developed to determine the concentration of SK&F 110679 (I) in plasma samples. SK&F 110910 (II) was used as an

TABLE I

CHROMATOGRAPHIC REPRODUCIBILITY OF AUTOMATED PRECOLUMN DERIVATIZATION

Injection[a]	Fluorescence intensity[b]	
	I	II
1	33 352.0	31 119.0
2	29 549.0	30 281.0
3	30 977.0	32 089.0
4	28 060.0	28 457.0
5	24 439.0	28 011.0
6	28 241.0	29 868.0
7	29 582.0	30 103.0
8	29 385.0	33 439.0
9	28 636.0	29 947.0
10	28 286.0	29 698.0
Mean	29 050.7	30 301.2
S.D.	2273.4	1601.2
R.S.D. (%)	7.8	5.3

[a] An aliquot (20 μl) of standard solution containing 23 pmol of peptide I and 25 pmol of peptide II was repetitively analysed using the methods described in the text.

[b] Fluorescence intensity as measured by chromatographic peak height (μV).

internal standard. The assay involved solid-phase extraction of the peptide from plasma, as a preliminary isolation step, followed by quantitative gradient HPLC with the methodology described here. Typical chromatograms of a drug-free plasma sam-

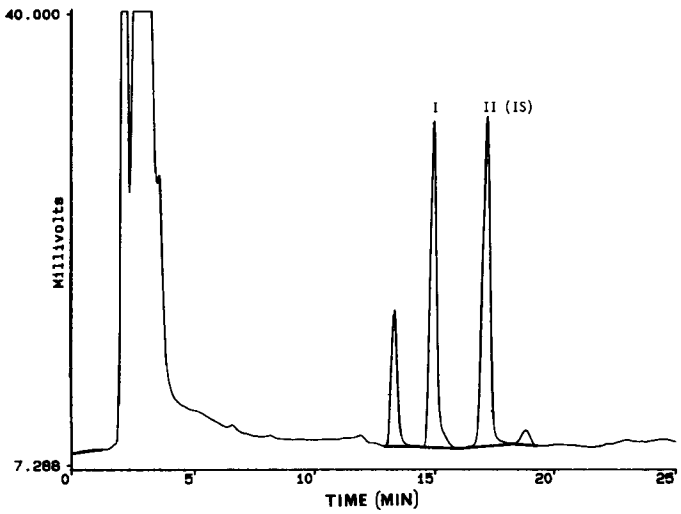

Fig. 2. Chromatogram of an aqueous standard solution of lysine-containing synthetic peptides. A solution containing 20 ng of each peptide was derivatized as described under Experimental and 3 ng were injected onto the column. See text for chromatographic conditions and peak identification.

ple and a plasma sample with 57 pmol of SK&F 110679 added (*ca.* 8 pmol injected on-column) are shown in Fig. 3. The initial mobile phase composition was Tris buffer (pH 7.0)–methanol (55:45, v/v). After injection, the methanol concentration was increased to 65% in 10 min and held for 7 min. The retention times of I and the internal standard (II) were 16.4 and 18.2 min, respectively. No interferences from endogenous compounds were observed and the drug and internal standard were well separated. The mean recovery of I from plasma samples was >90%. Plasma concentrations as low as 5 pmol/ml of I, which corresponded to an injected amount of *ca.* 0.7 pmol, have been determined using this approach. The assay was linear over the plasma

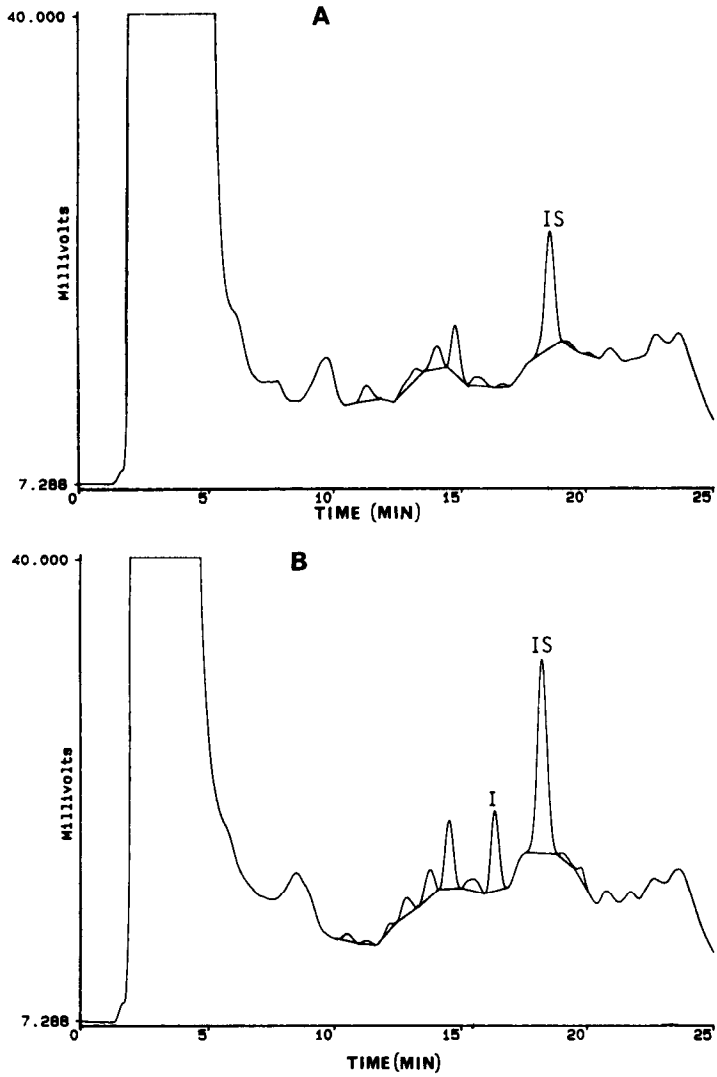

Fig. 3. Chromatograms of extracts of (A) drug-free plasma and (B) a plasma sample containing 57 pmol/ml of peptide I. See text for chromatographic conditions.

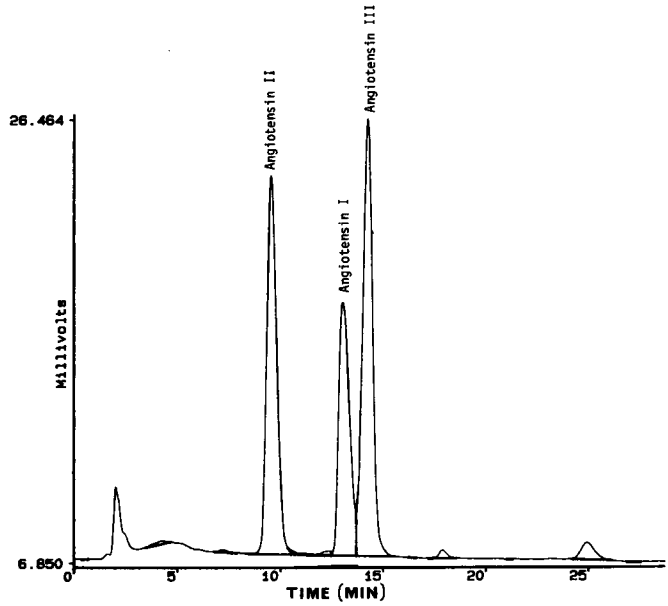

Fig. 4. Chromatogram of an aqueous standard solution of angiotensin I, II and III. The concentration of each peptide injected onto the column was 3 ng. See text for chromatographic conditions.

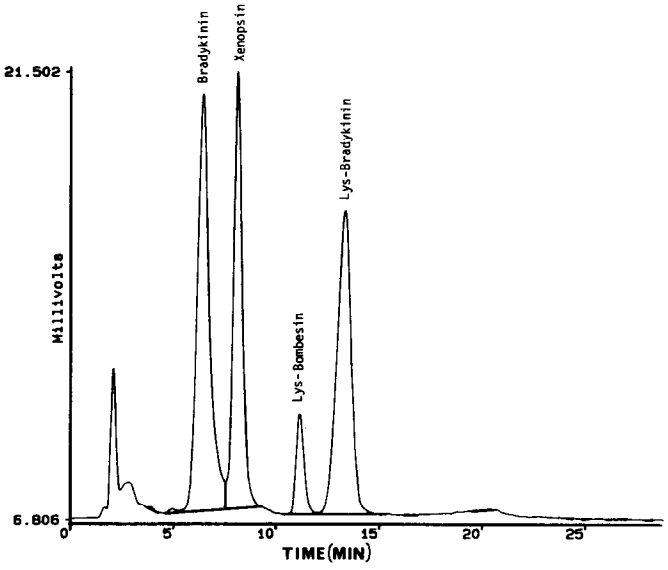

Fig. 5. Chromatogram of an aqueous standard solution of bradykinin, Lys-bradykinin, xenopsin and Lys-bombesin. The concentration of each peptide injected onto the column was 3 ng. See text for chromatographic conditions.

concentration range 5–500 pmol/ml. Correlation coefficients for plasma calibration graphs were typically >0.99.

Application to naturally occurring peptides

The methodology reported here has also been applied to the HPLC detection of naturally occuring peptides containing free α- and ε-amino groups. Fig. 4 shows a gradient HPLC separation of angiotensins I, II and III on a 2 mm I.D. octylsilica column. The initial mobile phase was Tris buffer (pH 7.0)–methanol (60:40, v/v). Following injection, the methanol concentration was increased to 65% over a period of 10 min and held for 10 min. The detection limit for these peptides was in the range 30–50 fmol injected on-column. Fig. 5 shows the gradient elution of bradykinin, Lys-bradykinin, xenopsin and Lys-bombesin on a 2 mm I.D. octylsilica column. The initial mobile phase was Tris buffer (pH 7.0)–methanol (50:50, v/v), then the methanol concentration was raised to 65% over 10 min and held for 10 min. Xenopsin and Lys-bombesin have a blocked N-terminal amino group but contain a lysine residue in the peptide chain. Bradykinin and the angiotensins, on the other hand, do not contain a lysine residue, but have a free N-terminal amino group.

In conclusion, the HPLC methodology described here permitted the specific and highly sensitive detection of the peptides examined. Injection of a variety of other peptides that did not contain either a free α- or ε-amino group resulted in a lack of fluorescence response. The molecules examined demonstrated that the method is capable of detecting peptides with either or both a free α- or ε-amino group. The detection limits reported here are substantially lower than those decribed in previous reports for fluorescamine postcolumn detection of similar peptides. The sensitivity of the method allowed its successful application to the determination of synthetic peptides in plasma samples.

REFERENCES

1 M. Weigele, J. F. Blount, J. P. Tengi, R. C. Czaijkowski and W. Leimgruber, *J. Am. Chem. Soc.*, 94 (1972) 4052.
2 M. Weigele, S. L. DeBernardo, J. P. Tengi and W. Leimgruber, *J. Am. Chem. Soc.*, 94 (1972) 5927.
3 S. Udenfriend, S. Stein, P. Bohlen and W. Dairman, *Science*, 178 (1972) 871.
4 S. Udenfriend, S. Stein, P. Bohlen and W. Dairman, in M. Johanes (Editor) *Proceedings of Third American Peptide Symposium*, Ann Arbor Sci. Publ., Ann Arbor, MI, 1972, p. 655.
5 N. Nakai, C. Y. Lai and B. L. Horecker, *Anal. Biochem.*, 58 (1974) 563.
6 S. Stein, P. Bohlen, J. Stone, W. Dairman and S. Udenfriend, *Arch. Biochem. Biophys.*, 155 (1973) 202.
7 P. Kucera and H. Umagat, *J. Chromatogr.*, 255 (1983) 563.
8 T. D. Schlabach, *J. Chromatogr.*, 266 (1983) 427.
9 T. K. Narayanan and L. M. Greenbaum, *J. Chromatogr.*, 306 (1984) 109.