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Determination of peptides by high-performance liquid chromatography with laser-induced fluorescence detection

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Abstract

Peptides were determined by high-performance liquid chromatography (HPLC) with laser-induced fluorescence detection. Detection was based on pre-column fluorescence derivatization of peptides with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) in acetonitrile (MeCN)–0.1 *M* borate buffer (pH 8.0) at 40°C for 10 min. The peptide derivatives were separated on a reversed-phase column with trifluoroacetic acid–MeCN and determined fluorometrically at 530 nm with excitation at 470 nm. The method was applied to the determination of enkephalins in rat brain and to a degradation study of bradykinin in human plasma. Optimization of the reaction conditions and the use of a semi-micro-column (100×2 mm I.D., 2 μm) made the detection limit of the peptides as low as 5–10 fmol. The detection limits of enkephalin and bradykinin were 20 and 5 fmol, respectively, using HPLC with laser-induced fluorescence detection. The method was sensitive enough to permit the quantitative determination of opioid peptides and bradykinin in tissue and plasma samples.

Keywords: Derivatization, LC; Peptides; Bradykinin; Enkephalins

1. Introduction

The development of novel peptide analogues as therapeutic agents has recently been of increasing interest in drug research. As many peptide analogues are extremely potent and often are administered at a low dosage, the development of a highly sensitive and specific analytical method to support pharmacological and pharmacokinetic studies is a challenging problem for this class of molecules.

The application of high-performance liquid chromatography (HPLC) to such a problem typically requires derivatization, in order to improve the native detectability of the peptide analyte, which lacks an appropriate chromophoric or fluorescent group. The use of fluorescence reagents available for primary amines is an attractive approach to high-sensitive detection by HPLC. However, the popular reagents, fluorescamine [1,2] and *o*-phthalaldehyde (OPA) [3], do not react with secondary amino acids such as proline and hydroxyproline. OPA derivatives of amino acids, especially lysine and glycine, are not very stable [4]. 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) has several advantages in routine use, including reactivity with both primary and secondary amines. NBD-F reacts faster than 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) with amino acids [5,6]. Thus, a reaction time of 1 min at pH 8.0 and 60°C is enough for the derivatization of amino acids to give a single peak in HPLC for each amino acid [7]. Previous HPLC approaches using NBD-F for the analysis of proteins have employed post-column derivatization [8,9], because the hydrolyzed product (NBD-OH; 4-hydroxy-7-nitro-2,1,3-benzoxadiazole) of the reagent fluoresces weakly. However, the detection limits for proteins were of

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the picomol level. Pre-column derivatization is often preferred to post-column derivatization in HPLC, because a high sensitivity is achieved due to elimination of baseline flow noise, band broadening and dilution effects resulting from the post-column addition of reagent solutions.

This paper describes the development of a HPLC method for the determination of bioactive peptides by pre-column derivatization with NBD-F. The use of semi-micro columns and optimization of the reaction conditions resulted in a detection limit in the sub-fmol range (signal-to-noise ratio=3). Furthermore, the resulting compounds have relatively long wavelengths of excitation (ca. 470 nm) and emission maxima (530 nm). As the excitation wavelength is close to the wavelength of an argon ion laser (488 nm), the minimum detectable level should be improved with laser-induced fluorescence detection. The method, due to its high sensitivity, has general applicability in the detection of several naturally occurring peptides, such as opioid peptides and bradykinin. Enkephalins have opiate-like activity in the nervous system [10]. Bradykinin is a very potent pro-inflammatory peptide [11]. Measurements of these endogenous bioactive peptides in biological fluid require highly sensitive and specific analytical methods.

2. Experimental

2.1. Chemicals and materials

Bradykinin (BK), des-phe^8-Arg^9-BK (des-8,9-BK), Arg-Pro-Pro-Gly-Phe (1-5-BK), methionine enkephalin and leucine enkephalin were purchased from Sigma (St. Louis, MO, USA). Des-Arg^9-BK (des-9-BK) was purchased from the Peptide Institute (Minoh, Osaka, Japan). Milli-Q (Millipore, Bedford, MA, USA) grade water was used for the mobile phases and for preparation of buffers and standard solutions. NBD-F was obtained from Dojindo Laboratories (Kumamoto, Japan). Trifluoroacetic acid (TFA) was obtained from Wako (Osaka, Japan). Acetonitrile was of HPLC grade (Wako). All other chemicals were of reagent grade and obtained from commercial sources. The synthetic peptides were dissolved in water and stored at −20°C for not more than two weeks.

2.2. Extraction of opioid peptides from rat tissue

The tissues of cortex, striatum and hypothalamus were separated from rat brain. The tissues were stored at −80°C until use. The tissues were quickly washed with saline and weighed after removing the saline with filter paper. A portion (0.2–0.5 g) of the tissue was homogenized with 3 ml of 0.1 *M* HCl [12]. The homogenate was deproteinized with 0.5 ml of 2 *M* $HClO_4$. After centrifugation at 1200 *g* for 10 min, the precipitate was suspended with 2 ml of 0.2 *M* $HClO_4$ and recentrifuged. The supernatants were combined and adjusted to pH 7.0–8.0 by the addition of 2 ml of 1 *M* $NaHCO_3$ and were applied to a Sep-Pak Octyl cartridge (Waters, Milford, MA, USA). The tissue extract was loaded after the cartridge was washed with water and methanol. A series of liquids: 1 ml of water, 2 ml of dichloromethane, 1 ml of water, 3 ml of 0.1 *M* borate buffer (pH 8.5) and 1 ml of water were passed through the cartridge. Finally, the enkephalin-rich fraction was obtained by elution with 1.0 ml of 90% methanol. After evaporation, the residue was dissolved in 80 μl of 0.1 *M* borate buffer (pH 8.5). The solution was allowed to react with 10 μl of 8 m*M* NBD-F and 10 μl of a sample that had been diluted 120-fold was applied to the HPLC system.

2.3. Incubation of bradykinin with plasma

Venous blood was collected from healthy male volunteers in plastic tubes containing 1/10 of its volume of 3.8% sodium citrate. The plasma was stored at −80°C until use. A mixture of 20 nmol of BK and 20 μl of plasma in 560 μl of 50 m*M* Tris–HCl buffer (pH 7.4) was incubated at 37°C for 0–3 h. The reaction was quenched by addition of 100 μl of 30% TCA and the mixture was centrifuged at 1200 *g* for 5 min. A 25-μl volume of supernatant, adjusted to pH 8.0 with 0.1 *M* borate buffer (pH 9.5), was allowed to react with 10 μl of 10 m*M* NBD-F, and 1 μl of the sample solution was applied to the HPLC system.

2.4. High-performance liquid chromatography

The high-performance liquid chromatograph consisted of a Tosoh multi pump CCPM (Tosoh, Tokyo, Japan), a laser-induced fluorescence detector (Tosoh LF-8010) and a thermostatted analytical column. The detector, equipped with a 5-μl flow cell, was employed for detection at 537 nm with excitation at 488 nm (light emission of the argon ion laser). The analytical columns were a TSK-gel Octyl-80Ts (150×4.6 mm I.D., 5 μm; from Tosoh) and a Super-Octyl (100×2.0 mm I.D., 2 μm). The columns were maintained at 40°C with a CO-8000 column oven (Tosoh). The mobile phase eluents used were TFA and acetonitrile. The specific conditions employed for the separation of various peptide analytes are described in detail in Section 3. The peak areas obtained from the LF-8010 monitor were calculated with a D-2500 data processor (Hitachi, Tokyo, Japan). The laser was used at a power of 5 mW, except for measurement of the detection limit, which was at 15 mW.

3. Results and discussion

3.1. Optimization of precolumn reaction conditions

Optimization of the precolumn derivatization conditions for BK and methionine enkephalin with NBD-F was examined using HPLC on an octylsilica column (150×4.6 mm I.D.). The mobile phases used were 0.05% TFA–26% acetonitrile for bradykinin and 0.05% TFA–50% acetonitrile for enkephalin and the flow-rate was 1 ml/min. The effect of the pH of the borate buffer on the reaction of bradykinin with NBD-F was examined by varying the pH from 6.5 to 9.5. The results indicate that maximum fluorescence intensity was obtained at pH 8.0. The effect of temperature on the reaction was also studied by varying the temperature from 4 to 60°C, while maintaining the pH of the buffer at 8.0. A reaction temperature of 40–60°C was optimum for the derivatization.

Using the conditions established above, the effect of reaction time was examined by allowing the derivatization to proceed from 1 to 120 min and overnight at 40°C. The results showed that maximum fluorescence intensity was obtained at a reaction time of between 10 and 15 min. Longer reaction times provided no further increase in fluorescence signal. Hence the reaction conditions of pH 8 (borate buffer), 40°C and 10 min were adopted. The detection limits for BK and enkephalins were 5 and 20 fmol, respectively, (signal-to-noise ratio=3) under the present conditions, with a laser (15 mW)-induced fluorescence detector. These conditions were employed in all subsequent work. The optimum conditions reported here were slightly different from those employed for amino acids reported in the previous paper [7].

3.2. Chromatographic separation and determination of enkephalins in rat tissue

The method reported here has been applied to the HPLC detection of methionine enkephalin (MEK; Tyr-Gly-Gly-Phe-Met) and leucine enkephalin (LEK; Tyr-Gly-Gly-Phe-Leu). These peptides are known to show opiate-like activity in the nervous system. Opioid peptides have been found to act in vivo as neurotransmitters via interaction with opiate receptors. Fig. 1 shows a HPLC separation of MEK and LEK on a 4-mm I.D. octylsilica column. The mobile phase was 0.1% TFA–35% acetonitrile and the flow-rate was 1 ml/min.

Chromatograms of cortex, striatum and hypothalamus tissue extracts of rat brain obtained by the procedure described in Section 2.2 are shown in Fig. 2. The peak of MEK in the tissue sample was identified in each chromatogram, but that of LEK was not observed. A linear relationship was observed between peak area and the amount of enkephalin in the range of 100–1000 pmol. The concentrations of MEK in cortex, striatum and hypothalamus of rat brain, as determined by the present method, were 98, 480 and 400 pmol per g of tissue, respectively. The method was sensitive enough to determine the endogenous enkephalins in brain tissues.

3.3. Application to a degradation study of bradykinin in human plasma

Bradykinin is quickly degraded by peptidases, kininases I and II, in plasma and other biological fluids. The degradation of BK in human plasma has

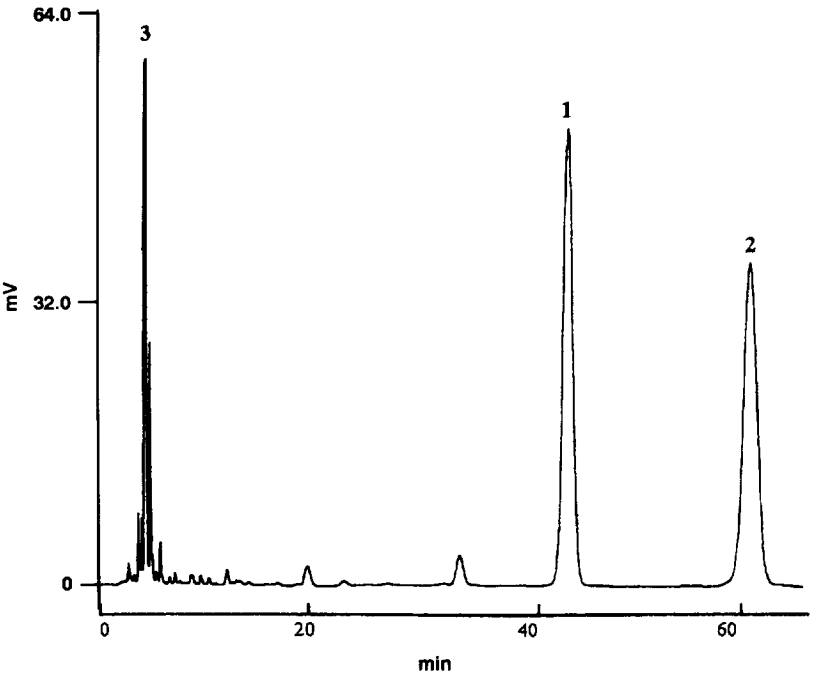

Fig. 1. HPLC separation of a mixture of methionine enkephalin and leucine enkephalin on a 4-mm I.D. octylsilica column. Peak 1, NBD–methionine enkephalin; 2, NBD–leucine enkephalin and 3, NBD-OH.

been reported by several workers [13–15]. The degradation pathways in human plasma are summarized in Fig. 3. As a trace amount of BK exerts remarkable biological activities, a sensitive, selective and specific method for microassaying BK and its metabolites was required for pharmacological studies [15].

Fig. 4 shows a chromatogram of a mixture of BK, des-9-BK, des-8,9-BK and 1-5-BK using precolumn NBD-F derivatization on a 4.6-mm I.D. octylsilica column. The concentration of each peptide injected onto the column was 8 pmol in 10 μl. The chromatographic analysis was accomplished using isocratic elution with 0.1% TFA–25% acetonitrile, with a flow-rate of 1 ml/min.

Linear relations were obtained between the peak areas and the amounts of the peptides in the ranges of 1–5 pmol of BK, des-9-BK, des-8,9-BK and 1-5-BK.

Fig. 5A–B shows chromatograms obtained before (A) and after (B) incubation of BK with plasma at 37°C for 3 h. The retention times of 1-5-BK, des-8,9-BK, BK and des-9-BK were 10, 12, 17 and 38 min, respectively. The results show that BK is cleaved by plasma enzymes to des-9-BK, des-8,9-BK and 1-5-BK.

Fig. 6 shows a chromatogram obtained using a semi-micro (100×2 mm I.D., 2 μm) octylsilica column of a mixture of BK, des-9-BK, des-8,9-BK and 1-5-BK using the precolumn NBD-F derivatization. The mobile phase was 0.1% TFA–26% acetonitrile and the flow-rate was 0.2 ml/min. A 0.1-mm I.D. stianless steel column was used from the injector to the detector in the HPLC system. The limits of detection for BK and its analogues on the semi-micro column were in the 2–25 fmol range (signal-to-noise ratio=3). The sensitivity of the detection of these peptides increased three to ten fold on the semi-micro column.

Bradykinin (BK) is readily hydrolyzed by angiotensin converting enzyme (ACE)/kininase II and the main product is des-8,9-BK. The degradation studies are useful for evaluation of ACE inhibitors, which are potent antihypertensive drugs. These studies are in progress in our laboratory and will be reported elsewhere.

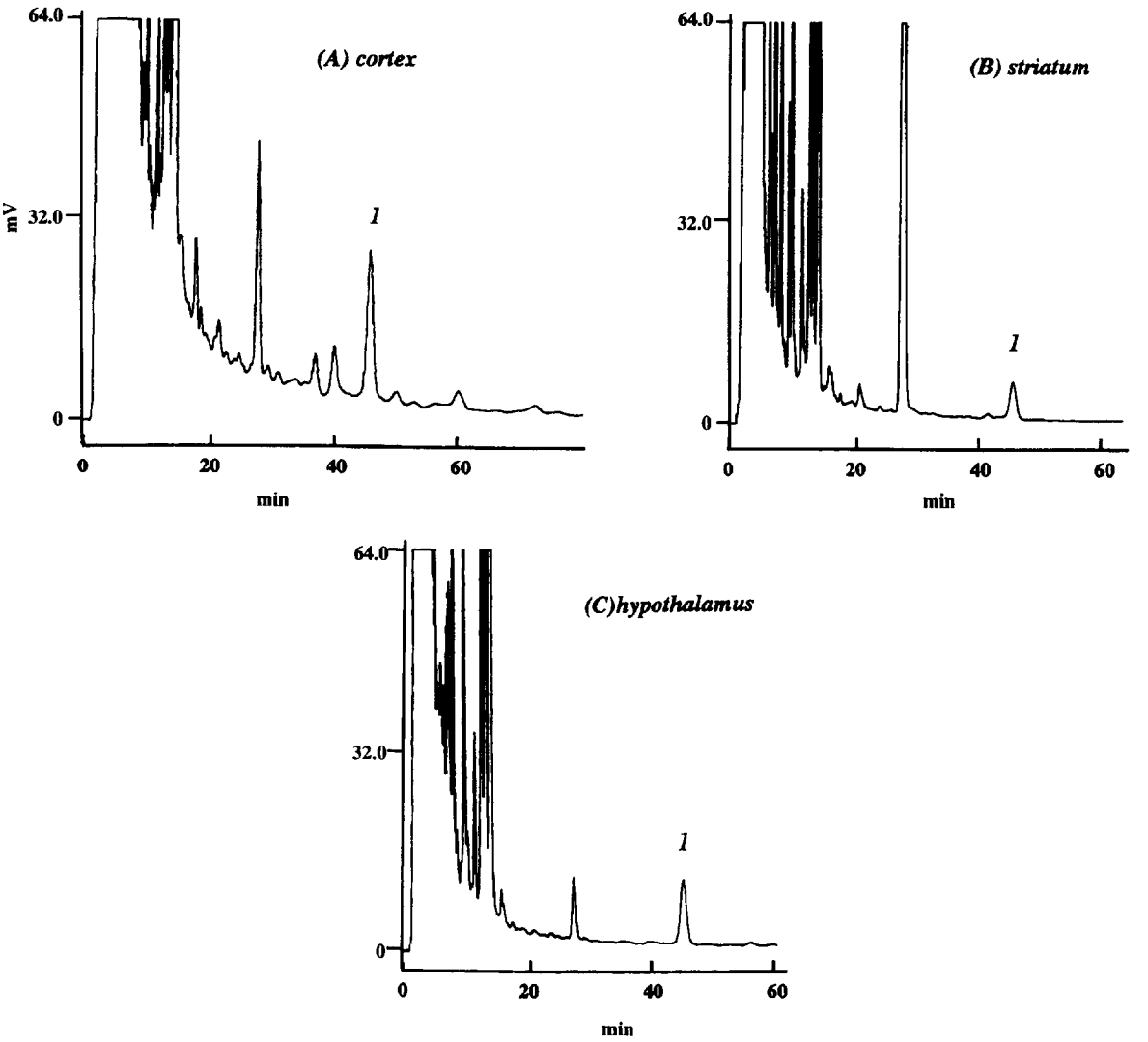

Fig. 2. Chromatograms of cortex, striatum and hypothalamus tissue extracts of rat brain. HPLC conditions were the same as in Fig. 1. Peak 1, NBD–methionine enkephalin.

4. Conclusion

The highly sensitive determination of the peptides was achieved using precolumn derivatization with NBD-F as a fluorigenic reagent by laser-induced fluorescence detection. Optimization of the reaction conditions and the use of a semi-micro-column (100×2 mm I.D., 2 μm) made the detection limit of the peptide as low as 5–10 fmol. The sensitivity of the method allowed it to be applied successfully to the determination of BK and its analogues in plasma and tissue samples.

Acknowledgments

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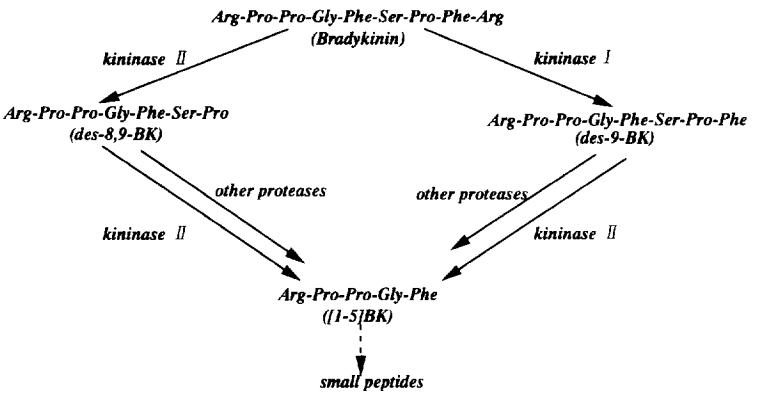

Fig. 3. Summary of the degradation pathway for bradykinin in human plasma.

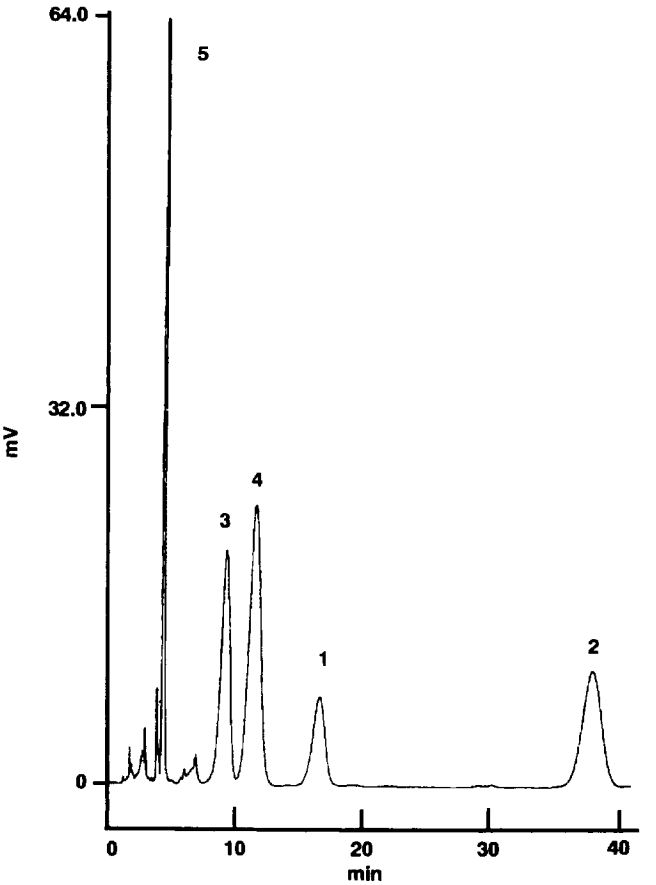

Fig. 4. Chromatogram of a mixture of BK, des-9-BK, des-8,9-BK and 1-5-BK obtained from the precolumn reaction with NBD-F. Peaks: 1, NBD–BK; 2, NBD–des-9-BK; 3, NBD–des-8,9-BK; 4, NBD–1-5-BK and 5, NBD-OH.

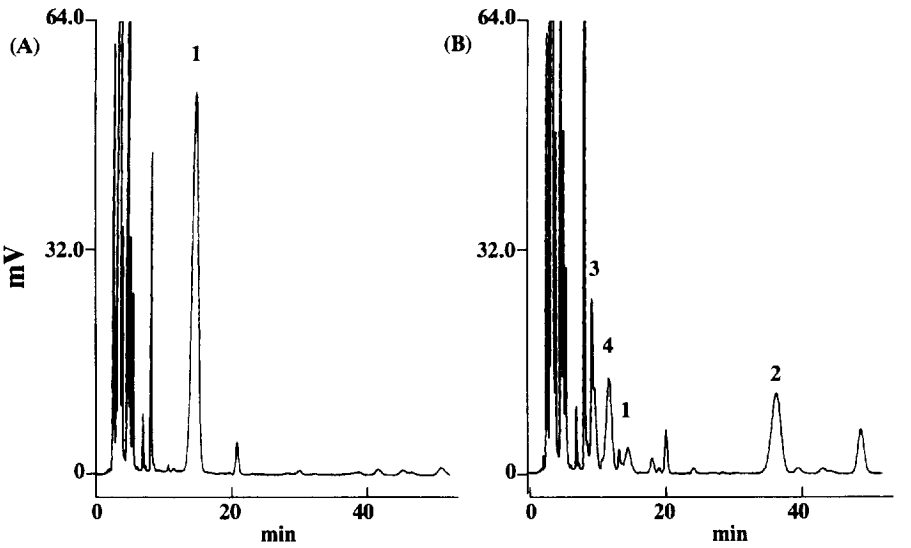

Fig. 5. Chromatograms obtained after incubation of BK with plasma at 37°C for 0 (A) and 3 (B) h. Peak numbers are the same as in Fig. 4.

ments, and Shinobu Oda and Chihiro Aoyama for technical assistance.

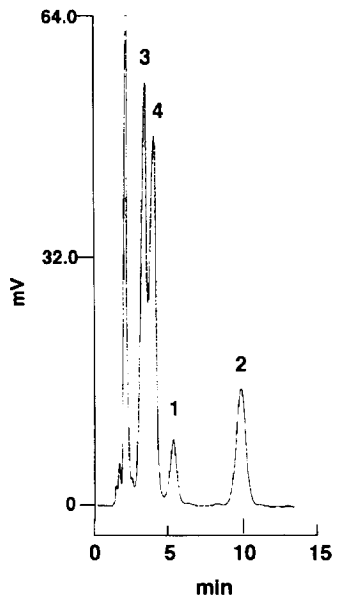

Fig. 6. Chromatogram of a mixture of BK, des-9-BK, des-8,9-BK and 1-5-BK using the precolumn NBD-F derivatization on a semi-micro (100×2 mm I.D.) octylsilica column. Peak numbers are the same as in Fig. 4.

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