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Reversed-phase liquid chromatography of the opioid peptides. 3. Development of a microanalytical system for opioid peptides involving microbore liquid chromatography, post-column derivatization and laser-induced fluorescence detection*†‡

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Abstract: A microanalytical system has been developed for the determination of peptides in small samples. Naphthalene-2,3-dicarboxaldehyde–β-mercaptoethanol (NDA–BME) was used as the labelling reagent system as an alternative to NDA–cyanide (NDA–CN) because of the faster labelling when CN was replaced by a thiol. The fluorescence characteristics of the NDA–thiol adducts, N-substituted 1-alkylthiobenz[f]isoindoles (TBIs), were found to be different from the previously described cyanobenz[f]isoindole (CBIs) adducts formed by the reaction of primary amines with NDA–CN. The excitation maximum of the TBI adducts was at 460 nm, which was closer to the 457.9 nm argon-ion laser line, than the 440-nm maximum of the CBI adduct. The limit of detection for leucine enkephalin was 36 fmol (S/N = 3) and linearity was proven for greater than 2 orders of magnitude, from 45 fmol to 9 pmol for an injection volume of 60 nl. The detectability was limited by the high background noise produced by the post-column derivatization system. The utility of the system was demonstrated for the analysis of methionine enkephalin and its potential oxidation products, using leucine enkephalin as a suitable internal standard.

Keywords: *Peptides; opioids, post-column derivatization; microbore liquid chromatography; napthalene-2,3-dicarboxaldehyde; laser-induced fluorescence detection; Ar-ion laser, microanalysis.*

Introduction

The main objective of this research was the development of an ultrasensitive post-column derivatization procedure to be used in combination with microbore liquid chromatography (μLC) and laser-induced fluorescence detection (LIF) for the analysis of opioid peptides. The main advantage of microanalysis is the reduced sample consumption in method development, preformulation studies, stability studies or any other investigation. In addition to its low sample requirements, microbore chromatography has various other advantages over conventional chromatography such as high mass sensitivity and the possibilities of using exotic, expensive mobile phases and highly sensitive laser-based detectors [1]. The main disadvantage of microbore chromatography is the need for specially designed injectors and flow cells with very low internal volumes to minimize extra-column band broadening [1].

The native fluorescence of the opioid peptides (Fig. 1) is generally very weak and some type of derivatization procedure is generally required to access the potentially high sensitivity of LIF detectors. In the present study a miniaturized post-column derivatization was developed based on reaction of the primary-amino function of peptides with naphthlalene-2,3-dicarboxaldehyde in the presence of a

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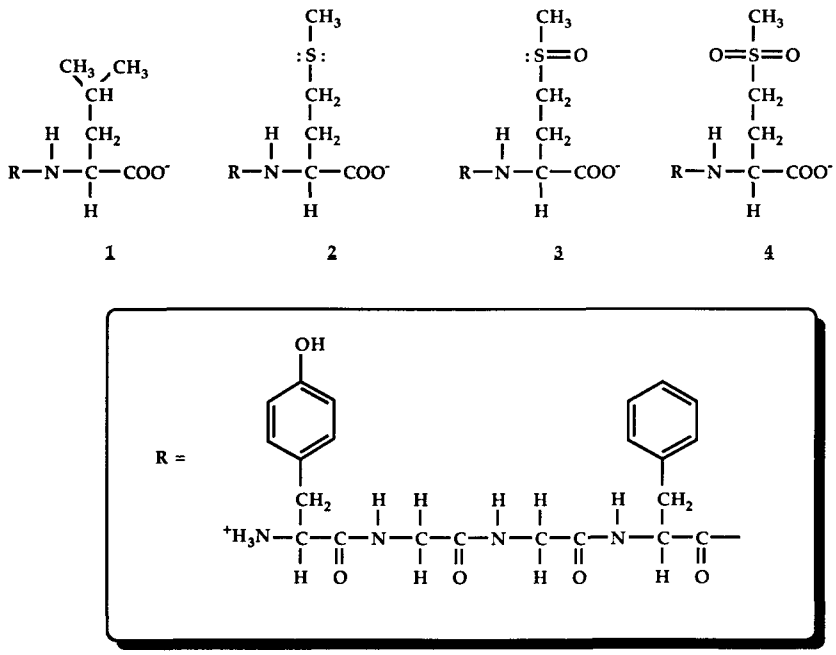

Figure 1
Structures of leucine enkephalin (**1**), methionine enkephalin (**2**), and the sulphoxide (**3**) and sulphone (**4**) of **1**.

nucleophile [2, 3]. The reagent system, naphthalene-2,3-dicarboxaldehyde–cyanide (NDA–CN) has been widely described for the pre-column derivatization of primary amines [2–24]. NDA–CN is related to the *o*-phthaldehyde–thiol (OPA–thiol) reagent system [25] but replacement of the thiol by cyanide increased the stability of the amine-derivatives to auto-oxidation [4]. Unlike OPA–thiol, NDA–CN also produces stable, fluorescent derivatives following reaction with the terminal amino groups of peptides [4, 5, 8, 12, 13, 18, 19, 24, 26]. Furthermore, extension of the aromatic-ring system by the replacement of OPA with NDA shifts the excitation maxima of the derivatives from 325 nm [25] to greater than 420 nm [11]. This shift in the excitation wavelength is important because it permits the available argon-ion (Ar-ion) and helium–cadmium (He–Cd) lasers to be used as excitation sources [19, 20]. Although NDA–CN produces stable fluorescent derivatives of peptides, it is generally unsuitable for post-column work because NDA reacts with cyanide to give fluorescent side products, which, in turn, give rise to an unacceptably high background signal. In addition, whereas the reaction of NDA–CN with unhindered primary amines is rapid, the reaction with the terminal amino group of a peptide may be too slow for post-column derivatizations taking up to 10 min for completion in typical LC solvent systems [12, 13, 26]. Some of the disadvantages of the NDA–CN reagent system for post-column derivatization include decreased reaction rate and increased side products. The main disadvantage of the dialdehyde–thiol combination is poor stability of the derivatives. However, this disadvantage may be less crucial in post-column reactions.

The present work is part of an on-going programme of research concerned with the development of direct chromatographic methods for the sensitive analysis of opioid peptides. Previous reports from this group on the LC analysis of opioid peptides have been concerned with pre-column derivatization using NDA–CN [13, 27], retention prediction [27] and the optimization of one-dimensional and two-dimensional separations [13, 27].

Experimental

Apparatus

Fluorimeter. Fluorescence measurements were made with a Perkin–Elmer Model MPF-66 fluorescence spectrophotometer, a Perkin–Elmer 7500 professional computer and a PR-210 printer.

Liquid chromatograph. The solvent delivery system consisted of Brownlee Laboratories

(Santa Clara, CA, USA) micro-LC gradient syringe pumps. The gradient was generated using the syringe pumps and good mixing achieved with a Brownlee glass bead (100 μm) packed cartridge which had a nominal void volume of 52 μl. The mobile phase was filtered through an Upchurch Scientific, Inc. (Oak Harbor, WA, USA) in-line removable screen filter (2 mm). An injector (Valco, Houston, TX, USA) with a fixed internal loop (0.06, 0.1, 0.2, or 1 μl) was used to introduce the samples. The microbore columns (3 μm, 150 × 1 mm, i.d.) used in the study were ISCO C_{18} (Lincoln, NE, USA). The columns were eluted at ambient temperature (22 ± 1°C).

UV detector. A μLC–10 UV (ISCO, Lincoln, NE, USA) detector and ISCO flow cell (2 mm pathlength and 0.06 μl internal volume) were used for detection at 210 nm.

Laser-induced fluorescence detector. A laser-induced fluorescence (LIF) detector was constructed from commercially available components (Fig. 2). The excitation source was a Coherent Innova 100-20 UV ion-argon laser (Palo Alto, CA, USA) operated in the light regulation mode at 0.2–0.8 W. The laser was tuned to the 457.9 nm line. The output was filtered using a 360 nm high pass cutoff filter. The excitation beam was modulated by an optical chopper (Stanford Research Systems, Palo Alto, CA, USA) positioned in front of the beam which was focused on the quartz-capillary flow cell by a 101.6 nm focal length fused silica lens. Modulation of the excitation source afforded phase sensitive detection of the fluorescence signal with a Stanford Research Systems (Palo Alto, CA, USA) SR510 lock-in amplifier. A modulation frequency of 1.41 kHz was used since the noise spectrum was found to be relatively quiet in that frequency region. Final data acquisition and manipulation were executed by an IBM AT computer and a Nelson Analytical System.

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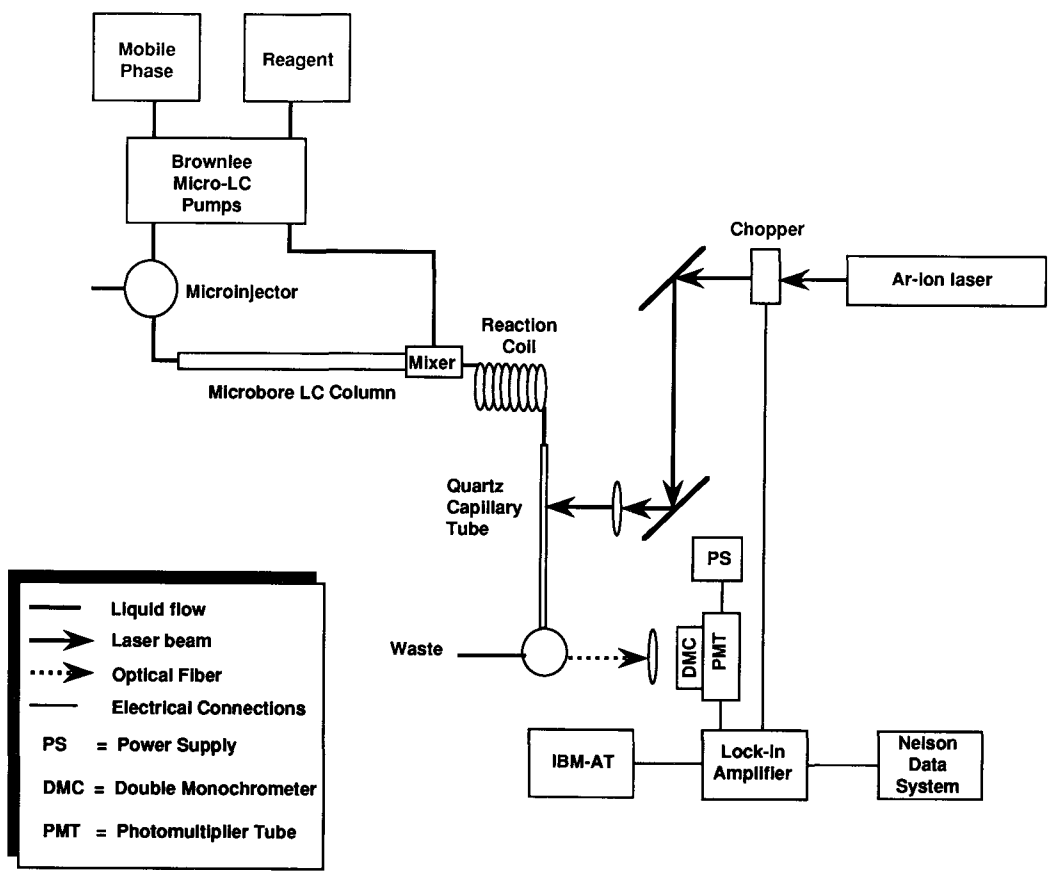

Figure 2
Diagram of the analytical system developed for the micropreformulation of peptides.

double spectrometer (*f*/4) fitted with diffraction gratings (1200 gr mm^{-1}) was used for emission wavelength selection. In general, the optimal signal-to-noise ratio (*S/N*) was obtained when the monochromator slits were fully opened (8 mm) resulting in a bandpass of 15 nm. A photomultiplier tube (PMT) (Hamamatsu R928, Middlesex, NJ, USA) was enclosed in a water-cooled housing and biased at 600 V for the reaction coil volume optimization experiments and at 400, 600, 700 and 800 V for the PMT voltage optimization experiments.

The quartz capillary flow cell was based on that described by Yeung [28], and was constructed of a fused silica quartz capillary and an optical fiber both purchased from Spectran Corporation (Sturbridge, MA, USA). The capillary had an inside diameter of 250 μm and an outside diameter, including the vespel coating, of 400 μm. At the illuminated region of the capillary the vespel coating was burned away and cleaned with methanol. The use of an epoxide adhesive on the nuts at the entry into tee of the silica capillary and the exit of the optical fibre from the tee was found to reduce problems with leakages and the optical fibre slipping downwards. The optical fibre was a step index, UV transmitting fibre with a cladding of 125 mm and a core of 100 mm (Spectran). The capillary was connected to the reaction coil by a reducing union and adjoined with the fibre and exit tubing by a 1/32″ tee connector using Valco (Houston, TX, USA) FS.25 fused silica adapters. A segment of the end of the fibre was stripped of the coating and inserted into the capillary. The fibre was supported by a vespel ferrule which was crimped onto the coating of the fibre. The side port of the tee was fitted with a 1/32″ (o.d.) stainless steel tubing with 0.01″ (i.d.), through which the effluent was channelled to the waste.

Focusing the excitation beam by a 101.6 mm focal length fused silica lens immediately above the fibre resulted in the best *S/N*. The emission collected by the fibre was focused into the monochromator by a 51 mm focal length fused silica lens (*f*/1.3).

Post-column reactor. A post-column reactor was constructed from an ultra-low dead volume (51 nl) mixer and a reaction coil of either polytetrafluorethylene (PTFE, Teflon) or polyetheretherketone (PEEK) tubing. Two different designs of open tubular reactor were compared. In the breadboard design, PEEK tubing (i.d. 0.005″ and o.d. 1/16″) was stitched through electronic circuit board, which held the tubing firmly in a modified serpentine configuration with a small bend radius [29, 30]. The electronic breadboard holes were enlarged using a 5/64″ drill bit. The PEEK tubing was stitched alternatively between two adjacent rows of holes. Thus, the direction of flow was periodically reversed in two dimensions. PTFE tubing (i.e. 0.012″ and o.d. 1/32″) was used to prepare a crocheted reactor coil [31] because PTFE was more flexible than PEEK. This design allowed three-dimensional variation of the flow path.

The mixer was custom built from a Valco 1/8″ to 1/16″ reducing union, with a fine hole drilled perpendicular (0.010″) to the channel to introduce the reagent mixture. A 5 cm length of stainless steel tubing (0.005″ i.d.) was soldered onto the drilled hole. The internal volume of this mixer was 51 nl (calculated from the internal dimensions).

Chemicals and reagents

The chemicals used were of the highest grade available and were obtained from various sources.

Peptides. Leucine enkephalin (**1**), methionine enkephalin (**2**) and methionine enkephalin sulphoxide (**3**) were obtained from Sigma Chemical Company (St Louis, MO, USA) and were used as received. Standard solutions of **1**, **2** and **3** were prepared by accurately weighing 1 mg of each and dissolving in HPLC grade water (1 ml). The standard stock solutions were further diluted with HPLC grade water. The solutions were stored at 4°C in the refrigerator when not in use.

Sodium cyanide. Stock solutions (10 mM) were prepared by dissolving the required amount of the sodium cyanide in HPLC grade water. The solutions were stored at 4°C in the refrigerator, when not in use, and were discarded after 1 week.

Thiols (β-mercaptoethanol, sodium β-mercaptoacetate, 2-mercaptopyridine and 4-mercaptopyridine). Stock solutions (100 mM) were prepared by diluting the required amount of the thiol in HPLC grade acetonitrile. The solutions were stored at 4°C in the refrigerator, when not in use, and discarded after 1 week.

Buffer solutions. Buffer solutions for the derivatization studies and the mobile phase were obtained by preparing separate solutions of monobasic potassium phosphate (20 mM) and dibasic potassium phosphate (20 mM). These solutions were then blended to obtain solutions of appropriate pH (6.5–7.5). The borate buffer (pH 9.5, 100 mM) was prepared by dissolving sodium tetraborate decahydrate in water and adjusting the pH with NaOH.

Procedures

Synthesis. Methionine-enkephalin sulphone (**4**) was synthesized by oxidation of **2** with *m*-chloroperoxybenzoic acid as follows: methionine enkephalin (17.23 mg, 0.03 mmol) was dissolved in methylene chloride (5 ml) and was cooled to −40°C over acetonitrile–dry ice. *m*-Chloroperoxybenzoic acid (11.3 mg, 0.063 mmol) was dissolved in methylene chloride (1 ml) and added dropwise to the previously cooled peptide solution. The reaction was quenched after 6 h, by pouring the reaction mixture into sodium hydrogen carbonate solution (10%, w/v). The resultant solution was extracted with methylene chloride and the extracts were evaporated to dryness. The resultant solid was dissolved in distilled water (10 ml).

Kinetic experiments. The rates of formation of fluorescent adducts produced by the reaction of leucine enkephalin, naphthalene-2,3-dicarboxaldehyde and various nucleophiles (cyanide, mercaptoethanol, and mercaptoacetic acid) were studied by fluorescence spectroscopy. The excitation and emission maxima for the cyanide adducts were 440 and 490 nm, respectively. The excitation and emission wavelengths for the thiol adducts were 460 and 560 nm, respectively. The nucleophile (30 μl, 100 mM/acetonitrile), leucine enkephalin (120 μl, 1.5 mM), and phosphate buffer (1050 μl, 50 mM, pH 6.8) were mixed sequentially in a cuvette. The reaction was initiated by adding NDA (1800 μl, 1 mM) to the cuvette. The solution was mixed by inversion and rate of adduct formation was monitored by detection of the fluorescence at ambient temperature.

Mobile phases. Mobile phase were prepared by filtering phosphate buffer (pH 6.8, 20 mM) through a 0.2 μm cellulose acetate membrane filter and then mixing with the required amount of pre-filtered HPLC grade organic modifier. The resultant mixture was degassed by purging with argon and sonication prior to use. The mobile phase used for isocratic separations was acetonitrile–phosphate buffer (pH 6.8, 20 mM) (17:83, v/v). The A and B mobile phases for gradient work were acetonitrile–phosphate buffer (pH 6.8, 20 mM) (5:95, v/v) and acetonitrile–phosphate buffer (pH 6.8, 20 mM) (65:35, v/v), respectively.

Results and Discussion

Fluorogenic derivatization of opioid peptides

The aim of this part of the study was to develop a reagent system that reached maximum fluorescence emission within 2–3 min when reacted with leucine enkephalin, producing minimal side reactions and resulted in a product with an excitation maximum around 457.9 nm (a convenient emission line for Ar-ion lasers). Generally, the labelling reaction with NDA–CN of peptides takes about 5–10 min [12, 13, 26], which was considered too long for a post-column derivatization. Hence, the derivatization kinetics of NDA and a model peptide, leucine enkephalin were investigated. The effect of changing the nucleophile from cyanide to a thiol, increasing the temperature, organic modifier concentration, mixing ratio, catalyst, nucleophile concentration, and NDA concentration were investigated. All reactions were conducted in a pH 6.8 phosphate buffer [12, 13, 26].

Previously, cyanide has been used as the external nucleophile necessary for the fluorogenic derivatization of primary amines with NDA (Fig. 3). The main disadvantages of cyanide for post-column work are the slow rate of reaction and the fluorescent side products that it produces as a result of its reaction with NDA. Consequently, several thiols, β-mercaptoethanol, β-thiolacetic acid, 2-thiolpyridine, and 4-thiolpyridine, which had been used previously for the fluorogenic reaction of primary amines with OPA, were investigated as possible alternatives to cyanide. Thiopyridines were evaluated; however, they did not give fluorescent products and were abandoned.

β-Mercaptoethanol and β-thiolacetic acid gave fluorescence products which, although less stable than the cyanide derivative, had reaction kinetics more suitable for post-column derivatization (Fig. 4). The reaction times to reach the maximum fluorescence with β-mer-

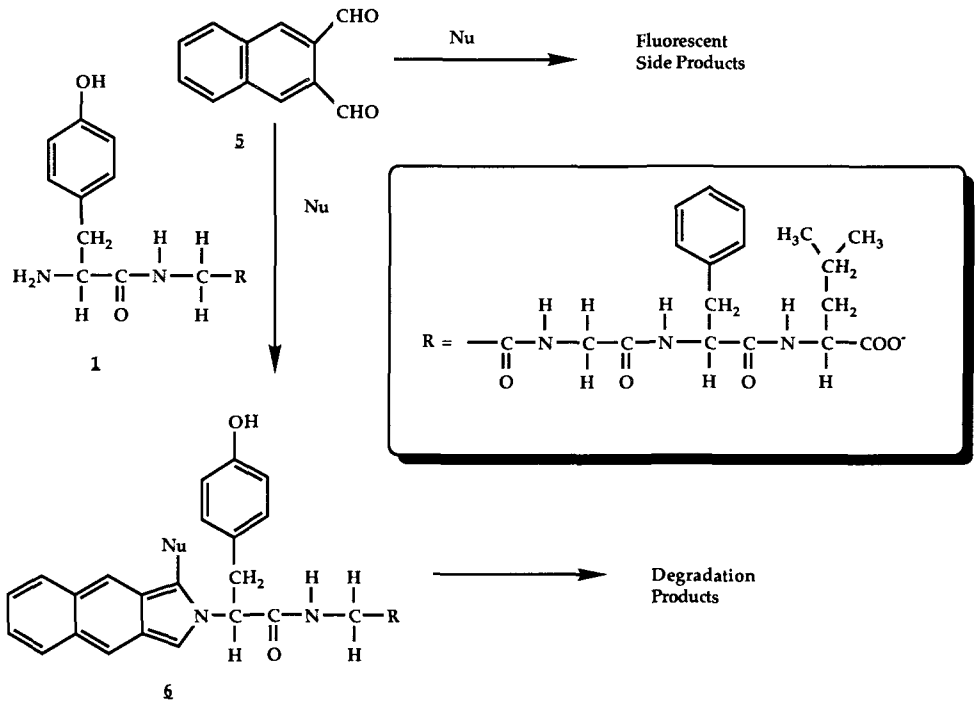

Figure 3
Reactions of naphthalene-2,3-dicarboxaldehyde (**5**) with leucine enkephalin (**1**) in the presence of a nucleophile.

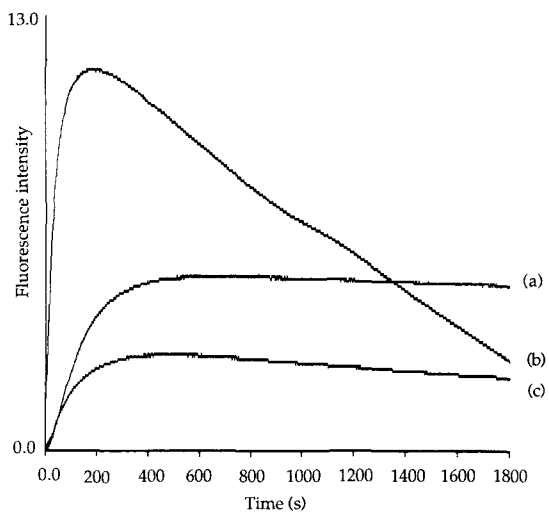

Figure 4
Reaction profiles for the derivatization of leucine enkephalin (6×10^{-5} M) with naphthalene-2,3-dicarboxaldehyde (**5**) (3×10^{-3} M) and (a) cyanide (6×10^{-2} M, λ_{em} 490 nm); (b) β-mercaptoethanol (6×10^{-2} M, λ_{em} 590 nm); and (c) β-mercaptoacetic acid (6×10^{-2} M, λ_{em} 560 nm), as nucleophiles at room temperature (λ_{ex} 460 nm).

captoethanol, β-mercaptoacetic acid and cyanide were approximately 150, 200 and 500 s, respectively. The relative intensities of fluorescence when the excitation was held constant at 460 nm (Fig. 4) provided information on the relative sensitivities that could be achieved for the three derivatives when excited with the Ar-ion laser line at 457.9 nm. The NDA–β-mercaptoethanol system produced the greatest maximum fluorescence intensity under the measurement conditions. Furthermore, the excitation maximum of this TBI derivative was located at 457.9 nm and hence can be excited more efficiently using an argon-ion laser (Fig. 5) compared to CBI derivatives. It should also be noted that both TBI derivatives had excitation maxima at 440 nm which corresponds to one of the two available lines of the He–Cd laser. Therefore, TBI derivatives could potentially be excited by the present Ar-ion laser discussed here or by the He–Cd laser. The background fluorescence for β-mercaptoethanol and β-mercaptoacetic acid was substantially lower than that of cyanide (approximately by a factor of 20 based on the blanks).

The emission spectra of these TBI adducts were also different from that of CBI-leucine enkephalin. The emission maximum was 560 nm compared with 490 nm for the CBI-derivative (Fig. 6). This shift in emission maxima to longer wavelengths was significant because they were substantially removed from the Raman scattering of acetonitrile–water

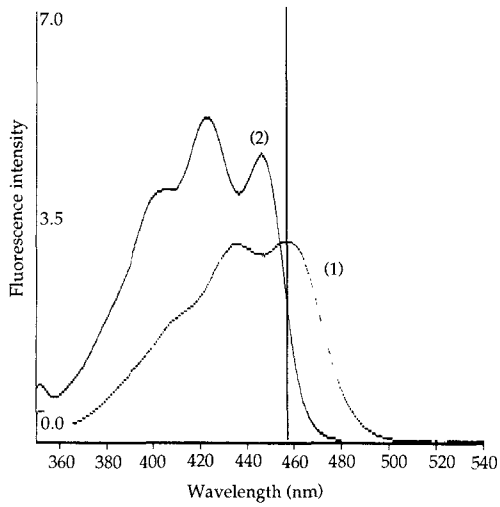

Figure 5
Excitation spectra of the adducts (**6**) of leucine enkephalin (**1**), naphthalene-2,3-dicarboxaldehyde (**5**) and (1) β-mercaptoethanol (λ_{em} = 590 nm) and (2) cyanide (λ_{em} = 490 nm). The vertical line indicates the position of the 457.9 nm argon-ion laser line.

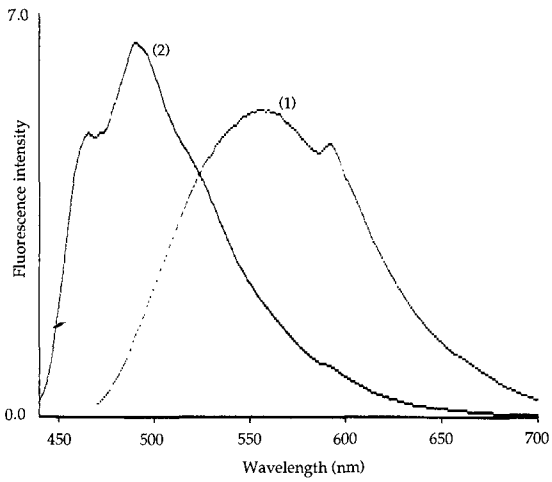

Figure 6
Emission spectra of the adducts (**6**) of leucine enkephalin (**1**), naphthalene-2,3-dicarboxaldehyde (**5**) and (1) β-mercaptoethanol (λ_{ex} = 460 nm) and (2) cyanide (λ_{ex} = 440 nm).

mixtures [32] which is a major noise source in LC–LIF [J. Bostick, Department of Pharmaceutical Chemistry and Center for BioAnalytical Research, University of Kansas, personal communication, 1991]. Based on the results of these fluorescence studies, β-mercaptoethanol was chosen for further investigation as a nucleophile.

The effect of temperature on the time to maximum fluorescence was investigated, using β-mercaptoethanol as the nucleophile and

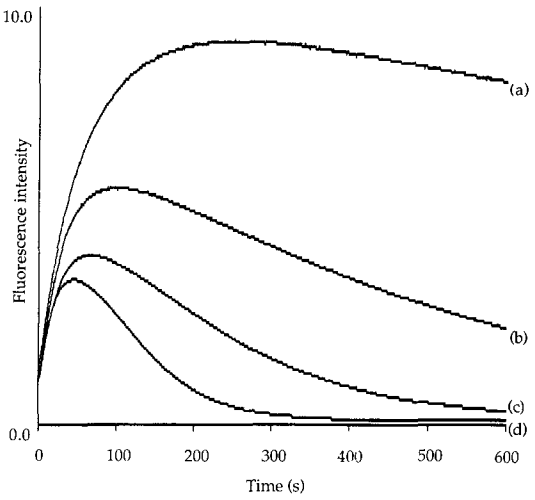

Figure 7
Effect of temperature on the reaction of leucine enkephalin (**1**) (6×10^{-5} M) with NDA (6×10^{-5} M) and β-mercaptoethanol (6×10^{-2} M). λ_{ex} 460 nm; λ_{em} 590 nm. (a) 22°C, (b) 50°C, (c) 70°C and (d) 90°C.

leucine enkephalin as the analyte (Fig. 7). An increase in temperature resulted in a decrease in the time it took to the reach the maximum intensity. For example, increasing the temperature from room temperature to 90°C, the time required to reach maximum was decreased from 200 to 50 s. The maximum intensity also decreased with increasing temperature. For example, the fluorescence intensity decreased by 60% on increasing the temperature from room temperature to 90°C. This decrease in fluorescence intensity with increasing in temperature could be due either to the effect of temperature on the reaction kinetics, a decrease in fluorescence quantum efficiency with increase in temperature or both. Based on the temperature study, increasing the temperature did not seem to be of any practical advantage and subsequent derivatizations with NDA and β-mercaptoethanol were conducted at ambient temperature (22 ± 1°C).

The derivatization reactions described so far were conducted in essentially aqueous solutions containing approximately 1% (v/v) acetonitrile. However, post-column derivatization conditions are necessarily dictated by the composition of the mobile phase, the composition of the derivatization reagent and the volumetric mixing ratio of the two solvent systems. Reversed-phase separations of peptides are typically conducted in buffered, aqueous acetonitrile. Therefore the effects of the acetonitrile on the reaction of NDA, β-

mercaptoethanol and leucine enkephalin were briefly studied. Consistent with previous observations with NDA–CN [13], the rate of the reaction decreased slightly with increasing acetonitrile concentration up to 50% (v/v); but even at the highest concentration of acetonitrile (50%) studied the maximum fluorescence was observed after 59 s. Thus it was concluded that the effect of acetonitrile concentration on the post-column reaction was minimal.

Post-column reactor

The post-column reactor consisted of a mixing tee and reaction coil. The mixing tee was custom built from a microbore-colum end fitting (a Valco 1/8″ to 1/16″ reducing union) such that the column outlet could be mixed directly with the derivatization reagent. Two designs of open tubular reactor, crocheted and breadboard designs, were investigated. PTFE tubing (0.012″, i.d.) and PEEK (0.005″, i.d.) tubing were used for the crocheted [30] and breadboard, modified-serpentine [28, 29] designs, respectively. Table 1 shows the effect the mixer and the reactor coils on the peak variance (σ^2), which was measured from the peak width ($w = 4\sigma$). In these experiments the lengths of the two reaction coils were adjusted so that the internal volumes were the same (23 μl). To eliminate the effects of mixing the eluent with the reagent the reagent flow was stopped and the analyte, leucine enkephalin was detected by UV absorbance at 210 nm. Table 1 shows that the band broadening introduced by the breadboard open tubular reactor design (9%) was much less than that of a crocheted reactor of the same volume (54%). The differences in the contributions of the two types of reactor were attributed to differences in internal diameter of the tubes rather than differences in the configuration.

Figure 8 also demonstrates the advantage of the breadboard reactor design when the reagent was added to the post-column eluant and detection was by LIF. This difference in the performance of the two reactors was also attributed to the difference in the tubing's internal diameter. The breadboard design was chosen for all further work.

The reaction-coil volume was optimized by varying the reactor length and measuring the peak height of CBI-leucine enkephalin following post-column derivatization. Figure 9 shows the effect of the reaction time on detector response. The shape of this plot was similar to that obtained from the spectrofluorometric studies (Fig. 9). The peak height initially increased with increasing the reaction coil volume and went through a maximum; subsequent increases in reaction coil volume resulted in decreased peak height. The initial increase in peak height with increasing in reaction coil volume was due to greater time for the reaction to occur, which resulted in greater extent of the derivatization reaction. Further increase in the reaction coil volume beyond 60 μl resulted in decreased peak height which may be due to degradation of the fluorescent adduct. The optimum reaction coil

Table 1
Effects of the post-column mixer and reactor coil type on the peak variance of leucine enkephalin at 10 μl min^{-1}

Configuration*			
Mixer	Crocheted reactor	Breadboard reactor	Peak variance† (s^2)
−	−	−	14.6
+	−	−	15.8
+	+	−	24.4
+	−	+	17.3

*Column was always in place, + = device was present, − = device was absent.
†Mean of two determinations.

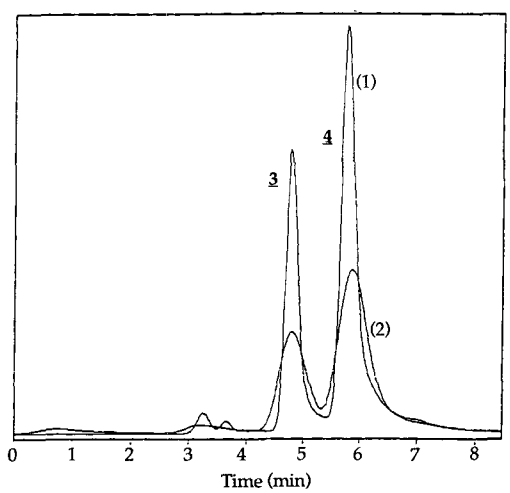

Figure 8
Comparison of the band broadening caused by (1) the breadboard and (2) crocheted post-column reactors. PMT 500 V, laser power 0.9 W, chopper frequency 1.41 kHz, reaction coil volume 220 μl, Column Isco C_{18}, mobile phase acetonitrile–phosphate buffer (10 mM, pH 6.8) (14:86, v/v), mobile phase and reagent flow rates 50 μl min^{-1} each, injection volume 1 μl, sample methionine enkephalin sulphoxide (**3**) (t_r = 5.8 min) and methionine enkephalin sulphone (**4**) (t_r = 4.8 min).

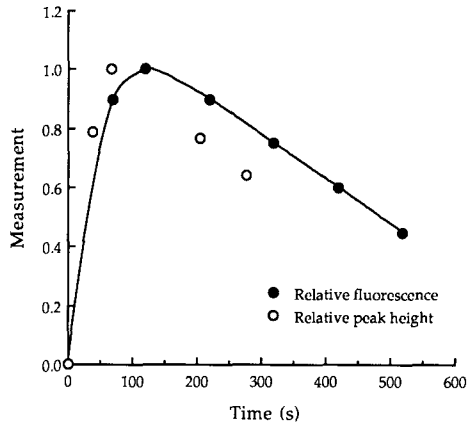

Figure 9
Reaction profile of the derivatization of leucine enkephalin (1) with naphthalene-2,3-dicarboxaldehyde (NDA)–β-mercaptoethanol generated using a fluorometer (●) and post-column derivatization (○). Fluorescence conditions: λ_{ex} 460 nm, λ_{em} 560 nm. Chromatographic conditions as Fig. 8, total flow rate through the reaction coil 20 μl min^{-1}, laser power 0.2 W, 457.9 nm line, chopper frequency 1.41 kHz, monochromator set at 560 nm, slit settings 2 mm (front) and 4 mm (back). Lock-in amplifier sensitivity 5 mV, dynamic range low, time constants: TC1 1 s and TC2 0.1 s. PMT voltage setting 600.

volume under this total flow rate of 20 μl min^{-1} was experimentally determined to be 30 μl. Thus, under these conditions, the derivatization time was 90 s. The general shapes of the reaction profiles generated by the fluorometer and the one-line post-column system were in good agreement (Fig. 9).

The effects of varying the ratio of the flow rate of the post-column reagent to that of the mobile phase on the peak shape, width and height of the leucine enkephalin peak were investigated. The flow rate of the mobile phase was maintained at 10 μl min^{-1} and the flow rate of the reagent was varied from 0 to 40 μl min^{-1}. To eliminate the effects of varying derivatization reaction on the peak height and column efficiency, NDA and the thiol were omitted from the post-column reagent stream and the analyte (leucine enkephalin) was detected by UV absorption at 210 nm. Only marginal decreases in peak height were obtained from dilutions at mobile phase: reagent flow rate ratios of less than 3:1. At higher ratios, the sensitivity was compromised substantially, but this was to be expected because increasing the mixing ratio resulted in greater dilution. Consistent with the previous observations on the effects of the breadboard reactor on column efficiency (Table 1), the peak variances were unaffected by the addition of the reagent.

Laser-induced fluorescence (LIF) detector

The LIF detector (Fig. 2) was constructed according to the specifications described in the experimental section. The illuminated flow cell was estimated to be 10 nl. The sources of noise that were considered likely to have a significant influence on the detection limit of the post-column reactor–LIF system were fluctuation in the detector response and fluctuating background fluorescence arising from the post-column reactor. A maximum value of the *S/N* ratio was obtained at a PMT (photomultiplier tube) potential of 800 V, which was used for subsequent investigation. PMT voltages of greater than 800 V could not be used because the PMT was overloaded.

When the NDA–β-mercaptoethanol reagent system was pumped through the reactor, the rms noise increased from 71 to 327 counts (Fig. 10). The background signal also increased substantially. This proved to be the limiting source of noise in the present detection system. This increase in noise and background signal was attributed to the reaction of the reagent with trace amines in the solvents or side products arising from the reaction of NDA and the thiol. The possibility of the reaction of NDA with β-mercaptoethanol prior to introduction of the reagent stream to the column eluent giving rise to trace amounts of fluorescent side products was investigated by

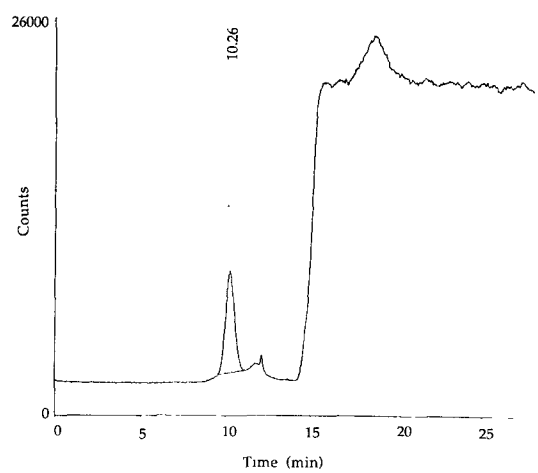

Figure 10
The effect of post-column reagent flow on the noise and background signal of LIF detector. The peak at 10.26 min arose from the injection of solvent. Flow rates of the mobile phase and the reagent mixture were 10 μl min^{-1} each. Other conditions as Fig. 8.

measuring the noise and background when the thiol was added to the mobile phase instead of the reagent mixture containing NDA. This approach did not reduce the noise or the background level and therefore, did not help in reducing the contribution to the fluorescence signal arising from the derivatization chemistry. The increase in noise by a factor of 4.6 suggests that the limit of detection by post-column reaction with NDA may be at least higher by a similar factor compared with pre-column derivatization.

The effect of β-mercaptoethanol concentration on noise was also investigated spectrofluorometrically. The β-mercaptoethanol molar excess ratios to NDA investigated were 2 and 28. The optimum concentration ratio of cyanide to NDA, has been reported previously to be 2 to 1 [2]. The time to reach maximum intensity decreased only slightly when the nucleophile concentration was increased from 0.36 to 5 mM ([NDA] = 0.18 mM). From the limited investigations conducted here, it was not clear whether the increased time to maximum intensity was due to a decrease in the initial rate of reaction or an increase in the rate of degradation of the derivative. All further investigations were conducted with a two-fold excess of β-mercaptoethanol over NDA based on previously known information about the NDA–CN system [2] and the limited investigation carried out on NDA–thiol.

The limit of detection (S/N = 3) for the complete system was calculated from Fig. 11 to be 36 fmol using leucine enkephalin as a test compound and injection volume of 60 nl. This corresponded to an injected concentration of 0.6 μM. Figure 11 shows a chromatogram of 227 fmol of leucine enkephalin for which the RSDs (n = 3) of the peak heights and peak areas were 2.1 and 3.3%, respectively. The linear range of the system was demonstrated with duplicate injections of five solutions of leucine enkephalin whose concentrations ranged from 2 to 150 μM.

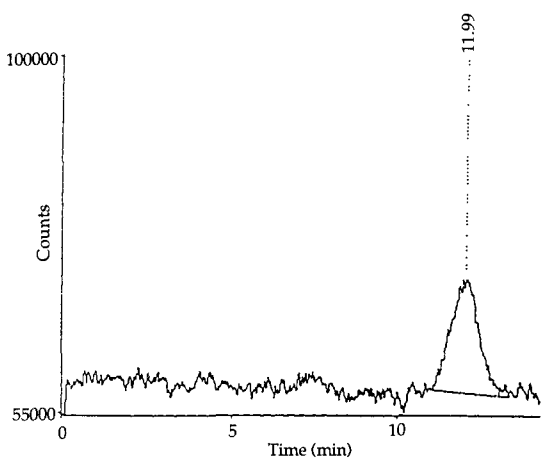

Figure 11
A chromatogram showing 227 fmol of leucine enkephalin injected. PMT 800 V, Laser power 0.9 W, λ_{ex} 457.9 nm, λ_{em} 560 nm, reagent and mobile phase flow rates 10 μl min^{-1} each, injection volume 0.06 μl. Other conditions as Fig. 8.

Chromatographic optimization and characterization of the assembled microanalytical system

Previously, this research group has reported [32] on the applicability of Snyder's theory of gradient elution [33, 34] to the prediction of optimal isocratic conditions for the separation of opioid peptides. In this study, the same technique was applied to the development of an optimized isocratic separation of methionine enkephalin (**1**) from its potential oxidation products, methionine enkephalin sulphoxide (**3**) and methionine enkephalin sulphone (**4**). Leucine enkephalin (**2**) was included as a potentially useful internal standard. In addition to optimizing the separation, a Van Deemter plot (Fig. 12) was constructed to determine the relationship between reduced plate height (h) and reduced linear velocity (v) using **2** as the test compound. In these studies, UV detection (210 nm) was used and the post-column reactor was disconnected because the objective was to determine the efficiency of the chromatographic system. Figure 12 shows that a minimum value of approximately 6 for h was obtained at a value of 1.5 for v. It is not clear why the theoretical optimum value of 2 for h was not obtained, but was probably due to extra-column band broadening in the micro-injector rather than excessive on-column dispersion band broadening in the post column reactor. This corresponds to an optimal flow rate of 10 μl min^{-1}. In practice, somewhat higher flow rates were used (25–50 μl min^{-1}) to decrease analysis times.

A four-peptide mixture (**1–4**) was prepared by mixing methionine enkephalin (7.3×10^{-4} M), leucine enkephalin (1.6×10^{-3} M) and oxidized methionine enkephalin reaction crude mixture in the ratio 2:1:1. Retention times for the four analytes were obtained on the microbore C18 column by elution with a two linear acetonitrile–phosphate buffer (20 mM, pH 6.8) gradients. The initial and

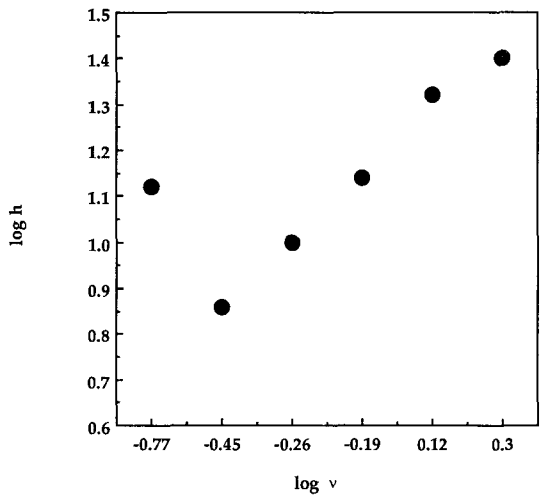

Figure 12
Relationship between the logarithm of the reduced plate height (h) and the reduced velocity (v) (Van Deemter plot) for leucine enkephalin with UV detection. Column Isco C_{18}, mobile phase acetonitrile–phosphate buffer (40 mM, pH 6.7) (20:80, v/v), detection 210 nm, injection volume 1 μl, flow cell volume 0.06 μl.

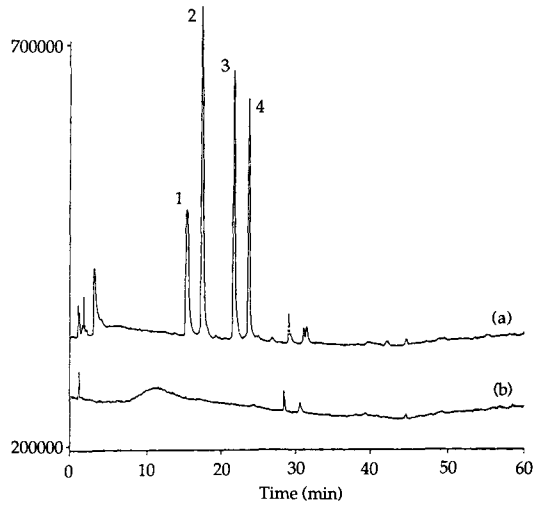

Figure 13
Gradient separation of (1) methionine sulphoxide enkephalin (**3**), (2) methionine sulphone enkephalin (**4**), (3) methionine enkephalin (**2**), and (4) leucine enkephalin (**1**) with UV detection at 210 nm. Gradient from 5 to 65% acetonitrile over 60 min and flow rate 50 μl min^{-1}. (a) Sample, (b) blank.

final concentrations of acetonitrile were 5 and 65% (v/v), respectively. The flow rate was 50 μl min^{-1} and the run times of the two gradients were 20 and 60 min. Figure 13 shows the gradient and separation of the peptides obtained with the 60-min gradient. These gradient data were used to predict the isocratic separation conditions for the four component mixture [32–34]. Figure 14 shows the optimized isocratic separation for the mixture, with UV detection at 210 nm. The sample mixture was diluted by 1:100 and then injected on to the μLC–LIF system (Fig. 14). For LIF detection, the column and reagent flow rates were kept the same (each 50 μl min^{-1}) and the reaction coil volume was increased (220 μl) to allow sufficient delay time for the reaction to occur. The PMT voltage setting was the previously optimized value of 800 V. The reagent mixture solvent was acetonitrile–phosphate buffer (20 mM, pH 6.8) (1:1, v/v) containing NDA (11 mM), β-mercaptoethanol (22 mM).

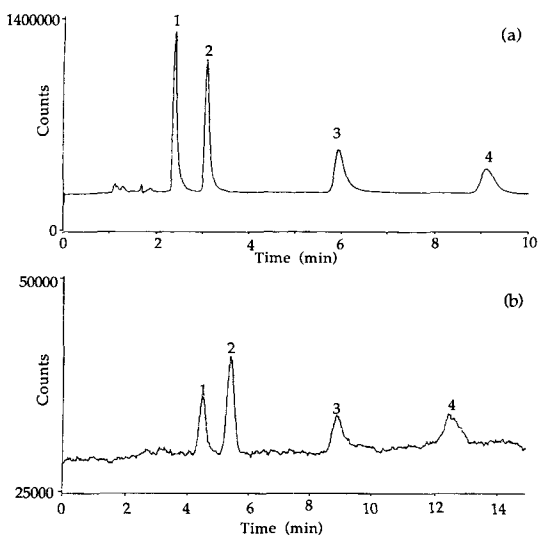

Figure 14
Isocratic separation of the four component peptide mixture, with detection by (a) UV (210 nm) and (b) LIF. Mobile phase acetonitrile–phosphate buffer (20 mM, pH 6.8) (16:84, v/v), column and reagent flow rates were each 50 μl min^{-1}, injection volume 0.06 μl. Peaks as Fig. 12. The amounts of (3) and (4) injected (a) were 26 and 90 pmol, respectively. The mounts on column in (b) were 260 and 900 fmol, respectively. The amounts of (1) and (2) injected were unknown because a crude preparation of these oxidized peptides was used. Other conditions as Fig. 8.

Conclusions

This study has demonstrated the potential of microbore liquid chromatography, post-column fluorogenic derivatization with NDA–thiol and laser-induced fluorescence detection for the microanalysis of peptides. This system may be useful for the analysis of peptides when only small samples are available as is often the

case in the preformulation evaluation of biotechnology products as well as in the analysis of biological samples. To achieve the optimal performance of this ultra-sensitive analytical procedure, each component of the method must be optimized separately and particular attention must be paid to the mutual compatibility of each component. In the present study this involved a careful characterization of the chromatographic system, the post-column reactor and the laser-induced fluorescence detection. The LOD of the method is approximately 36 fmol which translates to a concentration injected (60 nl) of 600 nM. This LOD is determined by contributions to the fluorescent blank arising from the derivatization system. Future efforts at reducing detection limits in post-column systems should be geared toward defining the exact source of the background signal and modifying the reaction conditions to minimize the blank contributions. This LOD is 2–3 orders of magnitude higher than has been demonstrated for pre-column derivatization of peptides using similar primary-amine fluorogenic reagents and LIF detection [1, 3, 5, 8, 10, 12, 15, 16, 19, 20, 24, 26, 35–41]. However, amol-detection limits reported by pre-column methods can only generally be achieved by dilution of standards with mobile phase [1, 3, 5, 8, 10, 12, 15, 16, 19, 20, 24, 26, 35–41]. Furthermore, pre-column derivatization at those low amol levels results in substantial chromatographic interferences, arising from degradation of the reagent, reaction with trace impurities or both, which severely compromise detection limits in real samples. Additionally, pre-column derivatization changes the chromatographic properties of the analyte. Therefore the separation and the reaction conditions have to be optimized simultaneously when using pre-column derivatization. If post-column derivatization of peptides is to be employed then the chromatography can be optimized independently from the derivatization reaction.

A more complete comparison of pre- and post-column derivatization than the one conducted here would include a consideration of mass utilization efficiencies of the two approaches. Although LODs in the amol range can be achieved by the combination of pre-column derivatization and microbore LC, less than 1% of the sample is actually injected onto the column. With post-column derivatization dilution of the sample prior to injection is avoided and, with careful optimization of the reactor, losses in sensitivity and column efficiency due to dilution can be avoided. Overall, post-column derivatization represents a viable alternative to pre-column derivatization for the microanalysis of opioid peptides and the two should be considered complementary rather than competitive approaches.

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