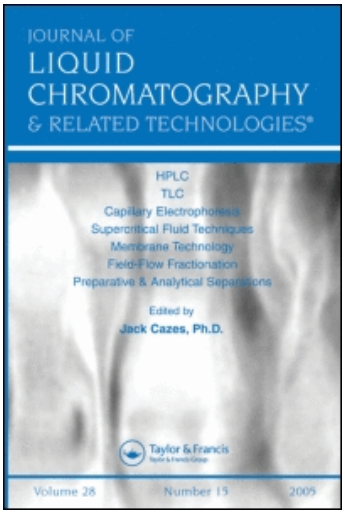

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Pre-Column Derivatization in HPLC of Amino Acids and Peptides

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PRE-COLUMN DERIVATIZATION IN HPLC OF AMINO ACIDS AND PEPTIDES

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The development of high preformance liquid chromatography with permanently bonded non-polar, so called reversed phase packings has been useful in the separation of a variety of peptides and amino acid derivatives. Limits to the detectability of underivatized substances are on the order of several nanomolar. Sensitivity enhancement became very important for the analysis and determination of peptide hormones, their metabolites and fragments, trace constituents and amino acids existing in very little quantity in biological and clinical samples. Therefore chemical derivatization techniques have been introduced into HPLC, too. By derivatization not only sensitivity enhancement , but selectivity can be achieved.

Pre- and post column derivatization have been applied in liquid chromatography. Post-column techniques are very well known by different types of amino acid analyzers and other instruments based on them.

Pre-column techniques offer some further advantages: simple procedure, economy, no restrictions by the solvent system and change of retention behaviour. The derivatization reaction must be rapid and quantitative, or at least reproducible. Formation of artifacts or of several derivatives of one compound can occasionally occur.

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For pre-column derivatization we have worked with dansyl-chloride, fluorescamine and o-phthaldialdehyde as reagents.

Chromatographic separations were performed on a laboratory assembled intrument of which the principal components were a reciprocating piston pump (Type 1515; Orlita, Giessen, G.F.R.) and a variable-wavelength photometer fitted to a 10 µl flow-cell (Model 212; Cecil, Cambridge, Great Britain). Column effluents were monitored at 215, 254 or 280 nm depending on the materials. For detection of fluorophores Waters M 420 fluorimeter was coupled to the instrument . Injection was made by Rheodyne injector. The efficiency of separations was increased by gradient elution. Peptides were synthetized by the Peptide Research Group of Hungarian Academy of Sciences.

Dansylchloride is well established as a reagent for the detection of amino acids and as an N-terminal reagent for peptides and proteins allowing detection at very high sensitivity. The conditions under which it reacts with amino and other functional groups in proteins and the stability of the resulting derivatives to acid hydrolysis have been extensively studied. Dansyl-amino acids are both fluorescent and ultraviolet absorbing. Some HPLC methods have also been developed for separation of dansyl-amino acids.

Wilkinson (1) applied reversed phase columns (µ Bondapak C_{18}, Spherisorb ODS) with linear gradient formed from acetonitrile and sodium phosphate buffers of approximately neutral pH. In order to avoid the problem of reproducibility, that arises from gradient the best possible resolution of the Dns-amino acids under isocratic conditions was sought. It was found, that the addition of glacial acetic acid to the solvent system used, resulted in faster elution of the Dns-amino--acids, but more importantly reduced tailing of the peaks (2). Using these experiences we have elaborated a method for separation of the three dansylated lysines: α, ε and bis-Dns-lysine. Resolution was achieved on PARTISIL-PAC 10 column isocratically by acetonitril-water eluent containing 1% acetic acid. Sharp peaks were obtained (see Fig.1.). The method was used in the analysis of branched polypeptides, for example Poly(Lys(Ala)m). The different bond types of lysines were determined.

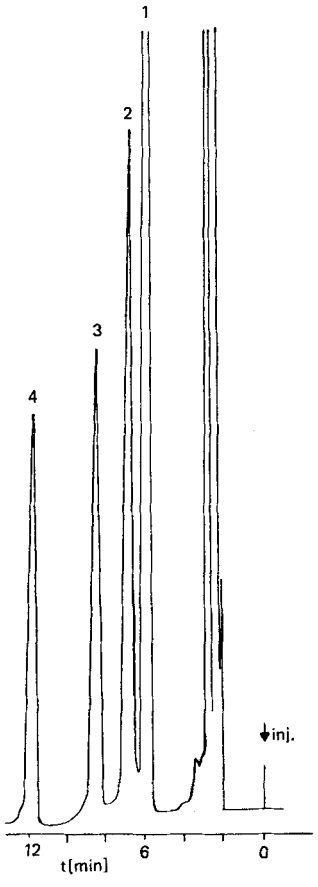

FIGURE 1. Chromatogram of dansylated lysines.
Column: Partisil PAC-10µm, 250 X 4.6mm
Flow: 1.0 ml/min, Pressure: 60 bar
Solvent: AcN-H2O-AcOH, 35:65:1
Detection: UV, 254 nm
Peaks: 1. ε-Dns-Lys k' 2.0
2. bis-Dns-Lys k' 2.4
3. α-Dns-Lys k'3.25
4. Dns-Ala k' 4.9

Recently fluorescamine (FLUORAM) and o-phthaldialdehyde (OPA) ha been used as very sensitive reagents to make fluorescent labelled amino acids and peptides.

Fluorescamine (4-phenylspiro-furan-2(3H)-1'phtalan)--3,3'-dione) reacts with primary amino groups to produce highly fluorescent derivatives. The half time of the reaction is 200-500 msec for most amino acids at pH 9. In post column techniques it is very well known. By pre-column derivatization unfortunately in the case of amino acids two fluorescent derivatives are formed because of second ring closure . Therefore it can be used well in the case of peptides with free amino groups. Live (4) reported results on the HPLC analysis of FLURAM derivatives of oxytocin, [Arg^8]-vasopressin and 16 analogs on Whatman-Partisil-10 ODS column. Wu and coworkers (5) demonstrated, that FLURAM-enkephalins are separated from each other using Tris-methanol solvent and RP-18 column. The nanogram level of sensitivity of this method compares favourably with other HPLC methods using UV, electrochemical or post-column fluorimetry detection.

o-Phthaldialdehyde reacts with amino groups in alkaline media in the presence of a reducing agent such as 2-mercaptoethanol to form a substituted isoindole. It is a highly fluorescent derivative which may be excited at 340 nm and emits at 455 nm. Lindroth (6) and Hodgin (7) elaborated HPLC-method for separation of OPA-amino acids using RP columns.

The potential of pre-column derivatization by FLUORAM and OPA has been demonstrated in our laboratory for monitoring fragments of α-MSH (α-melanocyta stimulating hormone).

The primary structure of α-MSH is

CH_3CO-Ser^{1}-Tyr-Ser-Met-Glu^{5}-His-Phe-Arg^{8}-Trp-Gly^{10}-Lys-Pro-Val^{13}-NH_2

The derivatization reagent was prepared by dissolving 27 mg of OPA in 0.5 ml of ethanol. Boric acid solution (0.4M) was adjusted to pH 9.5 with 1M NaOH. 20 microliters of 2-mercapto-ethanol and the OPA solution was added to 5 ml of borate buffer. The reagent mixture was allowed to stand for 24 hrs prior to use. 100 microliters of reagent was used for labelling of 20-30 μg peptide fragments. The structure of the derivatives were investigated, too.

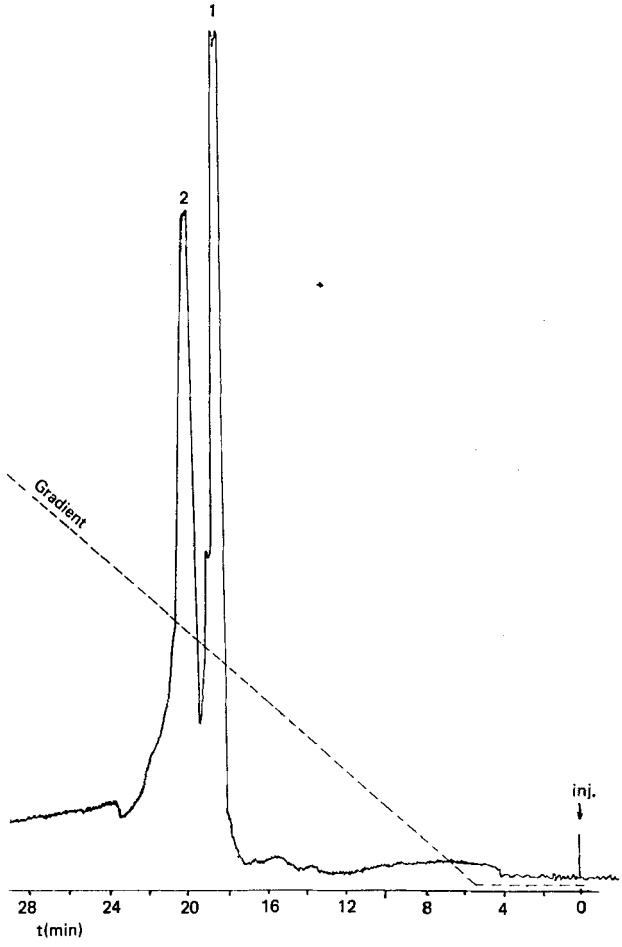

FIGURE 2. Chromatogram of o-phthalaldehyde derivatives of alpha-MSH fragments.
Column: ODS-Hypersil-5µm, 125 X 4 mm.
Flow: 1.4 ml/min, Pressure: 70 Bar
Solvent: AcN-H2O-TFA
A: 15:85:0.1 B: 90:10:0.1
Gradient: 2.2%B/min (-------UV profile)
Detection: Waters M-20 Fluorimeter
Peaks: 1. 0.3 microgram H-8-10-OH fragment, k' 8.5
2. 0.2 microgram H-11-13-NH2, k' 9.2

The efficiency of the separation was increased by gradient elution using acetonitrile-water eluent containing 0.1% trifluoroacetic acid (see Fig.2).

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