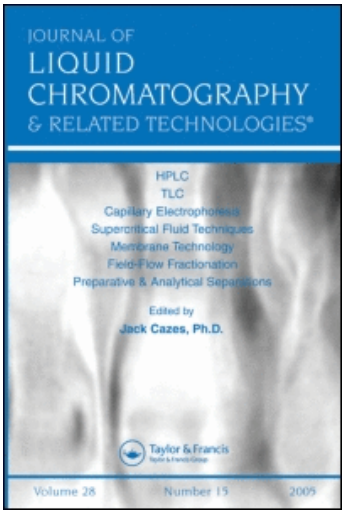

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

HPLC in the Analysis and Separation of Pharmaceutically Important Peptides

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To cite this Article Krummen, K.(1980) 'HPLC in the Analysis and Separation of Pharmaceutically Important Peptides', Journal of Liquid Chromatography & Related Technologies, 3: 9, 1243 — 1254
To link to this Article: DOI: 10.1080/01483918008062775
URL: http://dx.doi.org/10.1080/01483918008062775

JOURNAL OF LIQUID CHROMATOGRAPHY, 3(9), 1243-1254 (1980)

HPLC IN THE ANALYSIS AND SEPARATION OF PHARMACEUTICALLY IMPORTANT PEPTIDES

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INTRODUCTION

Since the introduction of alkylsilanized silicas of small particle diameter as stationary phases for peptide separations several years ago (1-4), the importance of reversed phase high performance liquid chromatography (RP-HPLC) has been growing continuously and is now a well established method for the assay of biological or synthetic peptides.

Several reviews dealt with various aspects of the applications of HPLC to peptides (5-9). The present review is dedicated to the use of HPLC for the assay of pharmaceutically important peptides.

GENERAL FEATURES OF PEPTIDE SEPARATIONS BY HPLC

Most of the work published was performed by using chemically modified silicas as stationary phases i.e. alkylsilanized (C8 or C18)-, alkylphenyl- or cyanoalkyl-silicas, and only few separations were reported on other stationary phases, e.g. polystyrene - divinylbenzene copolymers (10) or ion exchangers (11). Bonded phases have the advantage of high efficiency and good reproducibility, but they can only be used within a pH-range of about 2 to 7.5.

Mobile phases consisting of mixtures of an aqueous buffer with an organic modifier, e.g. acetonitrile, methanol etc. are usually employed. The parameters influencing the chromatography, e.g., the type of stationary phase, the type and the amount of the organic modifier, the salt concentration in the mobile phase, the pH, the temperature and the flow rate were investigated for several groups of peptides, among these oxytocin and vasopressins (12,13), somatostatin, insulin and others (14). A thorough treatment covering 32 hormonal peptides and some proteins was published by O'Hare and Nice(15,16). A recent study including vasopressins, oxytocin, ACTH, β-endorphin, glucagon and insulin stems from Biemond et al (58).

The nature of the organic modifier affords a limited degree of selectivity, some peak reversals may occur (12,13,15). Of great importance is the salt concentration in the mobile phase and to some extent, also the pH. A minimal salt concentration is mandatory for efficient chromatography (58). Deletion of the salts results usually in broad peaks and marked loss of resolution (15). Some peptides, e.g. Lys^8-vasopressin, could not be eluted at all without salts (12). Phosphate buffers are extensively used because of their low UV absorbance. Volatile buffers such as ammonium formiate are preferred if the eluted substances must be collected for further investigation. The kind of salt used has in most cases a minor influence upon the separation (17). It was found that chloride could be substituted for phosphate (15) without negative effect.

A special buffer (triethylammonium phosphate) was recommended for high resolution and high recovery especially for large peptides which could not be eluted otherwise (14). Changing the pH of the mobile phase is a suitable means to solve separation problems, especially for peptides with basic amino acids. Lys^8-vasopressin and Orn^8-vasopressin could be separated in alkaline media only (12). However, most of the separations were performed in the pH-range of 2 to 4.

Usually higher efficiency and shorter elution time ensue from an elevation in temperature (15). This effect is, however, sometimes accompanied by a decrease of selectivity (14). Most seperations were therefore performed at room temperature.

Cationic and anionic ion pairing and column modifying reagents such as hexanesulfonate, sodium dodecylsulfate or tetraalkylammonium salts have been used to control the elution of the peptides. Extensive studies were carried out by the group of Hancock, Bishop and Hearn (8, 18). Recently a study was published by Kinkel et al. (19).

The elution time of peptides is very sensitive to the content of the organic modifier in the mobile phase. Even slight changes may dramatically alter the elution time (14, 15, 20). This effect can be utilized advantageously for the preconcentration of aqeous peptide solutions on top of the column (21), for example the determination of oxytocin in low dosage ampoules (31) and the assay of somatostatin in plasma extracts (45).

The separation of different peptides from each other was explained in terms of the hydrophobicity of the side chains of the amino acids, but in some cases conformational and hydrophobic properties of the whole molecule and its size had to be taken into account (15,20,22). It was found that the retention time of smaller peptides ($\leq$ 15 amino acids) correlated with the lipophilicity of the peptides, expressed in terms of the fragmental hydrophobic constants of the amino acids according to Rekker (23).

Detection is usually performed at wavelengths between 200 and 220 nm, yielding very low detection limits for most peptides. Even lower limits and enhanced selectivity can be achieved with pre- or post column derivatisation. Post column derivatisation of free NH_2-groups with fluorescamine or o-phthalaldehyde has been extensively used (24-26, 28, 29). Precolumn derivatization was employed for oxytocin, vasotocin and Arg^8-vasopressin (30).

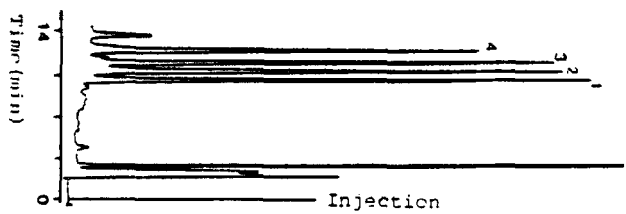
INDIVIDUAL PEPTIDES

Oxytocin, Lys^8- Vasopressin and similar Nonapeptides

Krummen and Frei separated Oxytocin from Lys^8-vasopressin and 3 other closely related peptides on alkylsilanized silicas (12). The method was used for the quantitation of these hormones in pharmaceutical dosage forms by taking advantage of preconcentration effects (31, 32). Dosage forms containing 2 I.U./ml could be examined using injection volumes up to 750 µl. The results were in satisfactory accordance with those of the bioassays (31, 33).

The high selectivity of the chromatographic system is demonstrated in Fig. 1, which shows the separation of oxytocin from 3 of its diastereoisomers.

Larsen et al (13, 34) separated oxytocin from 7 of its diastereoisomers. The elution order depended on the kind of organic modifier and to a lesser extent on pH.



1 = oxytocin 3 = [D-Tyr^2,D-Gln^4]oxytocin
2 = [D-Gln^4]oxytocin 4 = [D-Tyr^2]oxytocin

Figure 1 : Chromatogram of 100 µl of a mixture of oxytocin and diastereoisomers (= 20 µg/ml each). Gradient elution beginning with 30% of phase B with a linear increase of 1%/min up to 50% of phase B. Conditions were as follows: column RP 18, 5 µ, 125 x 4.6 mm; flow rate 1.0 ml/min; pressure at column inlet 160 bars; UV monitor at 215 nm. In (33).

Oxytocin, vasotoxin and Arg^8- vasopressin were determined in individual rat posterior pituitaries (30,35). The gland extract was deproteinized and cleaned up by passage through copper Sephadex. The resulting peptide fraction was allowed to react with fluorescamine, and the fluorophors were separated by RP-HPLC. The same chromatographic procedure was applied for the purity testing of synthetic oxytocin, which could be separated from 16 similar peptides (38).

The separation of oxytocin from vasopressin on a cation exchange column was also reported (28). RP-HPLC was used for the content uniformity testing of oxytocin tablets (36) and for the in process control of the oxytocin synthesis (37).

Adrenocorticotropic hormone (ACTH), Enkephalins and related peptides

Many groups are working in this field, which is of major interest from the pharmaceutical and biological standpoint. Accordingly, a great number of papers appeared and only the most recent ones will be reviewed here. For earlier papers reference is made to the previous reviews. Excellent separations of a large number of hormonal peptides have been achieved by O'Hare and Nice (15) with phosphate buffer 0.2 M - acetonitrile gradients. The optimum chromatographic conditions were thoroughly explored. 21 analogues of ACTH were separated by Terabe et al (20) with tartrate buffer-acetonitrile systems containing butanesulfonate. The ion pair RP-HPLC technique was chosen because ACTH contains many basic amino acid residues. The effect of the nature of the residues upon the elution order of the peptides was discussed.

Ligand exchange chromatography on a copper-ammonia-silica-column was applied to enkephalin degradation studies in mouse brain (50). Trifluoroacetic acid was recommended for ion-pair RP-HPLC of opioid peptides (51). Radioimmunoassay was used to quantify peptides isolated by RP-HPLC from biological samples (52, 29).

An acetonitrile-ammoniumacetate gradient with triethylamine gave sharp peaks for β_h-endorphin and related peptides and was useful for checking their purity (54). Chromatographic conditions for the assay of tetracosactrin were investigated in an attempt to eventually replace the bioassay in the Pharmacopeia (36).

Insulin

Dinner and Lorenz (53) separated bovine insulin (51 amino acids) by RP-HPLC from the by-products most commonly encountered during its purification. Isocratic elution with a 0.2 M ammonium sulfate/acetonitrile mixture was used. Bovine insulin was separated from similar proteins such as porcine insulin. Seemingly minor structural differences among the investigated proteins, e.g. the substitution of one asparagine by aspartic acid, resulted in significantly different retention times. A very good reproducibility was found. Insulin was also chromatographed on controlled porosity glass beads bonded with glyceryl propylsilane as stationary phase (40). RP-HPLC separations of insulin and desaminoinsulin (36, 58), of human, porcine and bovine insulin (20,58) and of insulin from other peptides (14,15, 39, 47) were published.

RP-HPLC was used for the analysis and purification of insulin related peptides prepared by solid phase synthetic procedures. Mobile phases containing β-mercaptoethanol allowed the direct analysis of insulin reduction products (41).

Somatostatin

Somatostatin and its analogs were separated and purified on μBondapak C18 columns with triethylammonium phosphate-acenitrile mixtures (42, 43). Another study reports the separation of six analogs of somatostatin which were prepared by solid phase synthesis of their corresponding diastereomeric mixtures followed by their complete resolution by preparative partition chromatography (44). RP-HPLC with gradient elution afforded resolution, whereas thin layer chromatography was inadequate to resolve the diastereoisomeric mixtures.

RP-HPLC was used in a study to elucidate the in vivo degradation of somatostatin in the rat after intravenous injection (45). Preparative RP-HPLC was employed for the purification of somatostatin and its chemical precursor (60).

Calcitonin

The purity of solid-phase synthesized calcitonin was tested by RP-HPLC (46). The method was also applied to isolation and separation of the components of a mixture of calcitonin like proteins secreted by human tumor-cells in vitro (56). However, no complete resolution of the components could be achieved. RP-HPLC may not be inherently capable of completely resolving all components of a natural protein mixture.

Antibiotics

Commercial gramicidin claimed to have 100 % biological activity was separated into 5 peaks, which were suggested to be various conformers (22). A thorough study of bacitracin was performed by Tsui (3). Structurally similar polypeptide components of bacitracin powder were separated on µBondapak C18 with gradient elution. The polymyxins, a group of closely related cyclic decapeptides, were also examined. Recently Fong et al. (59) separated the polymyxins B_1 and B_2 isocratically with 22,5 % acetonitrile in aqueous tetramethyl-ammonium chloride.

VARIOUS PEPTIDES

The luteinizing hormone-releasing hormone (LH-RH), a decapeptide, and two analogs were separated by Terabe (20). Separations were also reported by other workers (14, 46).

Aprotinin (bovine pancreatic trypsin inhibitor of Kunitz), a polypeptide with 58 amino acids, was assayed on a PP 8 column by isocratic elution with a triethyl ammonium phosphate-acetonitrile mixture. An excellent reproducibility was observed (55).

The insulin antagonist glucagon, a peptide with 29 amino acids was chromatographed by RP-HPLC (1, 58) and on "glycophase G" (40). A trifluoracetic acid-diethylamine-buffer was also recommended for RP-HPLC of this compound (61).

Synthetic peptides of the angiotensin type were separated both isocratically (57) and with a 0,1 M phosphate buffer-acetonitrile gradient (22).

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