Journal of Chromatography, 509 (1990) 245–253
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 200

Ion-exchange high-performance liquid chromatographic separation of peptides with UV photometric and electrochemical detection

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ABSTRACT

The ion-exchange separation of dipeptides and some higher peptides was studied using a Separon HEMA Bio 1000 CM weak cation-exchange column. The effects of the stationary phase properties (particle size, column dimensions) and the mobile phase parameters (flow-rate, composition, pH, organic modifier content) on the separation were studied. Both electrostatic and hydrophobic interactions are involved in the separation; in the series of dipeptides, the retention depends on the hydrophobicity of the amino acid side-chain and its carbon number.

UV photometric (at 210 nm) and electrochemical detection (voltammetric at a copper electrode and tensammetric at a mercury dropping electrode) were tested and their sensitivities compared. UV photometric detection is about ten times more sensitive than electrochemical detection, the sensitivities of amperometric detection with a copper electrode and tensammetric detection being comparable. An advantage of tensammetric detection is that the response is independent of the length of the peptide side-chain; on the other hand, tensammetric detection is very sensitive to the presence of surface-active substances in the mobile phase.

INTRODUCTION

Liquid chromatographic analyses of peptides are important in many fields of science, especially in biochemistry, medicine and biology[1–3].
The most common separation systems are reversed-phase and ion-exchange high-performance liquid chromatography (HPLC) (see, *e.g.*, refs. 3–7); size-exclusion techniques are suitable for group separations of peptides and for analyses of long-chain peptides and proteins[8]. Peptides are usually detected by UV photometry at low wavelengths (*e.g.*, ref. 9), but amperometric detection is also possible, using primarily mercury[10–12] but also carbon[11,13–15] electrodes. Peptides exhibit surface activity and hence it can be assumed that tensammetric detection[16] should also be possible.

Amperometric detection on a passivated copper electrode has been shown[17,18]

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0021-9673/90/$03.50

to be advantageous for many substances that rapidly form stable complexes with copper (II) ions and it has been applied to the detection of short peptides separated in a reversed-phase chromatographic system[19]. However, this method of detection imposes severe limitations on the mobile phase composition; the pH must not be lower than 6 and only certain buffers (*e.g.*, phosphate or carbonate) permit sensitive measurement. The separation of peptides in reversed-phase systems is not optimum at these pH values and substantially lower values are preferable[20–22].

Therefore, in this work we decided to test a newly developed[23] column packing material, HEMA Bio 1000 CM (Tessek, Prague, Czechoslovakia), based on a hydroxyethylmethacrylate–ethylene dimethylacrylate copolymer modified with carboxymethyl groups. It is a weak cation exchanger and hence permits the use of mobile phases with a pH higher than the range permissible with the chemically bonded alkyl phases. We studied the conditions for the separation of short peptides on this stationary phase and the possibilities of combining this separation with UV photometric detection, amperometric detection on a copper electrode and tensammetric detection on a mercury electrode.

EXPERIMENTAL

The measurements were carried out on an HPLC instrument consisting of an LC–UV variable-wavelength photometric detector and an LC–XPD pump (both from Pye Unicam, Cambridge, U.K.), a Rheodyne Model 7125 injector with a 20-μl external loop and and ADLC 1 amperometric detector (Laboratorní Přístroje, Prague, Czechoslovakia) with a voltammetric cell of our own construction[24], containing a tubular copper working, silver/silver chloride (Ag/AgCl) reference and a stainless-steel counter electrode. Tensammetric detection was performed with an EDLC detection cell containing a static mercury drop electrode[25] (Laboratorní Přístroje) and an OH-105 a.c. polarograph (Radelkis, Budapest, Hungary). A TZ 4620 dual-line recorder (Laboratorní Přístroje) was used to record simultaneously the photometric and voltammetric signals.

Prior to measurement, the copper electrode was activated in the mobile phase for 15 min at -0.3 V and then measurement itself was carried out at $+0.15$ V. All the potential values are referred to the Ag/AgCl reference electrode. All the measurements were performed at laboratory temperature (20 ± 2°C).

The characteristics of the column used are given in Table I. The stainless-steel columns were products of Tessek.

The mobile phases involved (a) aqueous phosphate buffers containing various concentrations of NaH_2PO_4 and methanol as the organic modifier, (b) an aqueous acetate buffer and (c) a 0.1 *M* aqueous solution of sodium perchlorate. Before use, the mobile phases were degassed in an ultrasonic bath. The mobile phase pH was adjusted by addition of solutions of phosphoric, acetic or perchloric acid and sodium hydroxide.

The test peptides were obtained from Sigma (St. Louis, MO, U.S.A.). All the other chemicals were of analytical-reagent grade (Lachema, Brno, Czechoslovakia) and were used as received.

TABLE I

CHARACTERISTICS OF THE HEMA COLUMNS AND THEIR PERFORMANCE

Parameter	*Column*			
	I	*II*	*III*	*IV*
Dimensions (length × I.D., mm)	80 × 8	80 × 8	250 × 8	250 × 4
Particle size (μm)	7	10	7	10
Plate number[a]:				
a	940	800	1850	835
b	790	650	1560	700
c	750	580	1480	660
Resolution[a, b]:				
a	1.6	1.5	2.7	1.5
b	1.3	1.1	2.2	1.0
c	1.05	1.0	1.75	0.9

[a] Flow-rate: (a) 0.5, (b) 1.0 and (c) 1.5 ml/min.
[b] Gly–Val and Gly–Leu pair.

RESULTS AND DISCUSSION

Separation

The weakly acidic cation exchanger HEMA Bio 1000 CM exhibits both ion-exchange properties and hydrophobic interactions with the mobile phase and hence is particularly well suited for separations of proteins and their fragments. Four columns with different lengths, inside diameters and particle sizes were tested (see Table I). The narrower column exhibits a hydrodynamic resistance four times greater than that of the wider columns and its efficiency is less than half (columns III and IV). The effect of the particle size is not very pronounced (columns I and II); with a change in particle diameter from 7 to 10 μm, the efficiency decreases by about 18%.

The retention behaviour of the peptides was studied using aqueous phosphate and acetate buffers as mobile phases. The elution orders were similar in the two mobile phases (see Table II), but the phosphate buffer permitted a better resolution of the peptides and a longer lifetime of the column; therefore, the dependences of the peptide retention on the experimental conditions [buffer concentration (Fig. 1), pH (Fig. 2) and the content of the methanol organic modifier (Fig. 3)] were studied with the phosphate mobile phase.

In ion-exchange separations, the retention is primarily determined by electrostatic interactions between the solute and the stationary phase; hence, the effect of the buffer concentration, *i.e.*, the mobile phase ionic strength, should predominate. All the dipeptides studied in this work contain glycine whereas the other amino acids differ in their side-chains. It can be seen from Fig. 1 that the retention of all the dipeptides decrease with increasing ionic strength, in agreement with the theory of ion exchange, except for the two substances containing free carboxylic groups, *i.e.*, Gly–Asp and Gly–Glu, where the dependence is reversed. These two substances are negatively charged under the given experimental conditions and thus cannot be retained

TABLE II

RETENTION DATA FOR DIPEPTIDES

Column I (see Table I); flow-rate, 0.5 ml/min.

Dipeptide	*Side-chain*	*Capacity ratio*	
		5mM phosphate	*2 mM acetate*
Gly–Asp	$-CH_2COO^-$	1.23	0.72
Gly–Glu	$-CH_2CH_2COO^-$	1.51	0.88
Gly–Gly	–H	1.93	1.83
Gly–Ala	$-CH_3$	1.98	–
Gly – Val	$-CH(CH_3)_2$	2.33	2.20
Gly–Met	$-CH_2CH_2SCH_3$	2.35	–
Gly–Leu	$-CH_2CH(CH_3)_2$	2.91	2.71
Gly–Phe	$-CH_2C_6H_5$	4.33	3.93
Gly–Tyr	$-CH_2C_6H_4OH$	5.32	4.87
Gly–Gly–Gly	–H	2.03	–
Gly–Gly–Gly–Gly	–H	2.05	–

through ion exchange on a cation exchanger; it seems that their retention is governed by hydrophobic interactions, possibly combined with a size-exclusion effect.

These dependences demonstrate the dual character of interactions on this stationary phase, ion exchange and hydrophobicity, as pointed out above. The peptides are separated into groups by ion exchange, whereas the separation within a group depends on the hydrophobicity of the amino acid side-chain. For example, the elution

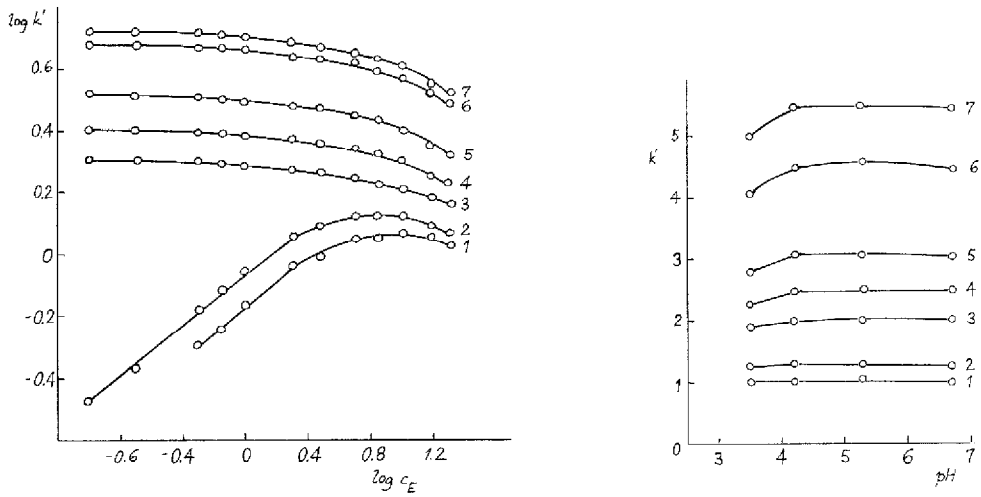

Fig. 1. Dependence of log k' on buffer concentration. Column IV; sodium phosphate buffer; flow-rate, 0.5 ml/min; pH 5.3; UV photometric detection at 210 nm. 1 Gly–Asp; 2 = Gly–Glu; 3 = Gly–Gly; 4 = Gly–Val; 5 = Gly–Leu; 6 = Gly–Phe; 7 = Gly–Tyr.

Fig. 2. Dependence of k' on mobile phase pH. Column I; 10^{-3} M sodium phosphate buffer; flow-rate, 0.5 ml/min; without methanol; UV photometric detection at 210 nm. Substances as in Fig. 1.

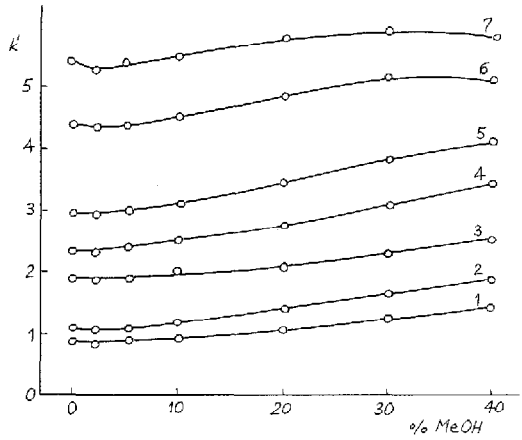

Fig. 3. Dependence of k' on the methanol (MeOH) content in the mobile phase (%, v/v). Conditions as in Fig. 2; pH, 5.3.

order follows the increasing side-chain hydrophobicity in the series Gly–Gly, Gly–Ala, Gly–Val, Gly–Leu and Gly–Phe, with the respective side-chains, –H, $-CH_3$, $-CH(CH_3)_2$, $-CH_2CH(CH_3)_2$ and $-CH_2C_6H_5$.

Vláčil and co-workers[26,27] proposed the following relationship for ion exchange on HEMA columns:

$$\log k' = p + q \log c_E$$

where k' is the capacity ratio, c_E is the concentration and p, and q are empirical constants. As can be seen from Fig. 1, this relationship only holds for low buffer concentrations, whereas a more complex mechanism operates at high concentrations. The side-chain character exerts a decisive effect on the separation, whereas the length of the peptide main chain is unimportant (see Table II, demonstrating poor resolution of Gly–Gly, Gly–Gly–Gly and Gly–Gly–Gly–Gly).

The dependences on pH (Fig. 2) and on methanol content in the mobile phase (Fig. 3) are not pronounced. The retention of Gly–Asp and Gly–Glu does not depend on pH within the region studied, whereas the retention of the other peptides increases slightly with increasing pH. The retention also increases slightly with increasing methanol content from 0 to 40% (v/v).

The plots of the log k' values against carbon number (n) in the peptide side-chain (Fig. 4) are linear for $n > 3$, in agreement with Molnár and Horváth[28].

The separation of dipeptides on the columns tested is shown in Fig. 5.

Detection

UV photometric detection at 210 nm, which is commonly used in the HPLC of peptides[1,3], was also employed. The limits of detection, *ca.* 1 ng in the volume injected, are in agreement with the values given in the literature. The detection sensitivity is highest for peptides with aromatic rings, such as Gly–Tyr and Gly–Phe.

An alternative detection technique is amperometry with a copper working elec-

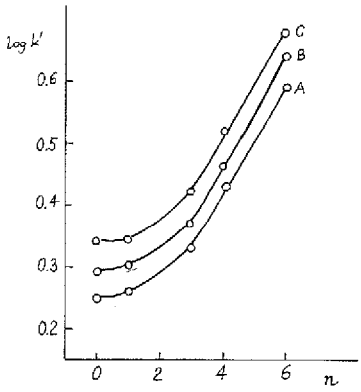

Fig. 4 Dependence of log k' on the number of carbon atoms in the dipeptide side-chain. Column I; flow-rate, 0.5 ml/min; UV photometric detection at 210 nm. Sodium phosphate buffer concentration: (A) $1 \cdot 10^{-2}$, (B) $5 \cdot 10^{-3}$ and (C) $2 \cdot 10^{-3}$ M.

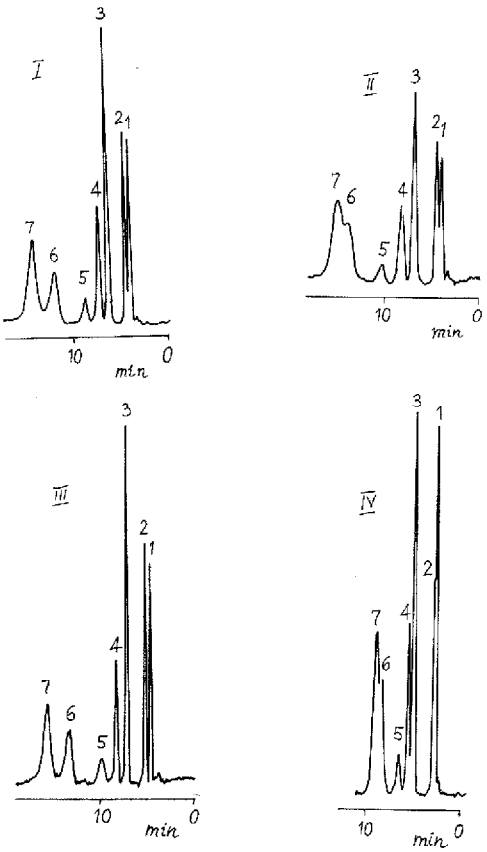

Fig. 5. Separation of dipeptides on the columns tested. 10^{-3} M sodium phosphate buffer (pH 5.3); flow-rate, 0.5 ml/min (columns I, II and IV) and 1.5 ml/min (column III). Substances as in Fig. 1.

trode[17–19]. As can be seen from Table III, the detection parameters for dipeptides are satisfactory, but the limits of detection are about one order of magnitude higher than those obtained by UV photometry. The sensitivity of detection decreases rapidly with increasing length of the peptide chain; among the tripeptides, only Gly–Gly–Gly and Leu–Gly–Gly can be detected, with a limit of detection of about 60 ng; the sensitivity is even poorer for tetrapeptides. The sensitivity of detection can be improved by using low mobile phase flow-rates, as the copper electrode current increases with decreasing flow-rate[18,19]; hence the use of micropacked and possibly also capillary columns would be advantageous. The main advantage of amperometric detection with a copper electrode is its high selectivity, hence the sample pretreatment is substantially simplified in many practical cases (see, *e.g.*, refs. 24 and 29).

TABLE III

CALIBRATION GRAPH PARAMETERS AND DETECTION LIMITS FOR VARIOUS PEPTIDES

(A) Amperometric detection with a copper electrode: mobile phase, aqueous 0.025 *M* NaH_2PO_4 (pH 6.8); flow-rate, 0.5 ml/min; sample size, 0.5 μl; electrode potential, +0.15 V; column I. (B) Tensammetric detection with a dropping mercury electrode: mobile phase, aqueous 0.1 *M* $NaClO_4$ (pH 5.0); flow-rate, 0.4 ml/min; sample size, 20 μl; electrode d.c. potential, −0.38 V; a.c. voltage amplitude, 30 mV; drop time, 3 s; without column.

Peptide	*A*			*B*		
	Slope [nA/(μg/μl)]	*Correlation coefficient*	*Detection limit (ng/μl)*	*Slope [nA/(μg/μl)]*	*Correlation coefficient*	*Detection limit (ng/μl)*
Glycine	1640	1.000	0.09	68.3	0.996	12
Gly–Gly	36.0	0.993	25	65.7	0.999	12
Gly–Gly–Gly	27.3	0.996	25	81.2	1.000	10
Gly–Gly–Gly–Gly	9.4	0.984	100	82.2	0.996	10
Gly–Asp	65.4	1.000	10	–	–	–
Gly–Ala	98.3	0.998	10	–	–	–
Gly–Val	51.7	0.999	10	–	–	–
Gly–Leu	31.7	0.999	25	–	–	–
Gly–Glu	84.8	0.999	10	–	–	–
Gly–Phe	48.8	0.998	25	–	–	–
Gly–Tyr	22.7	0.993	40	–	–	–
Gly–Met	89.9	0.994	10	–	–	–
Leu–Gly–Gly	18.7	0.996	50	487.0	1.000	1.7
Tyr–Ala–Gly–Phe–Cys(Me)	–	–	–	165.0	0.995	5.5
Insulin	–	–	–	13.4	0.994	59.5

Tensammetric detection at a mercury electrode, another selective detection method, was further investigated. It has been found that a hanging mercury drop cannot be used, as it rapidly becomes passivated and the measuring sensitivity and reproducibility are poor; the results are satisfactory with a dropping mercury electrode. The dependence of the response on the applied d.c. potential (Fig. 6) indicates that the optimum potential is in the range −0.36 to −0.40 V. The tensammetric response increases with increasing surface area of the mercury drop, with increasing drop time (up to a value of 3 s and then remains constant) and with increasing

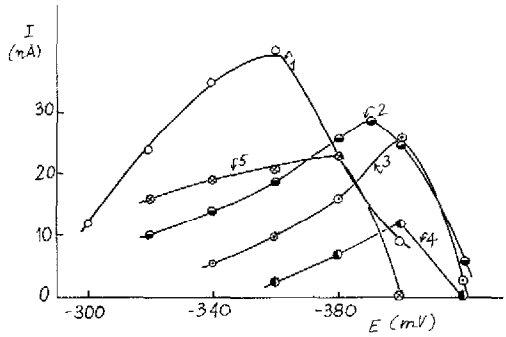

Fig. 6. Dependence of the tensammetric signal on the applied potential. Mobile phase, 0.1 *M* $NaClO_4$; flow-rate, 0.3 ml/min; pulse amplitude, 30 mV; drop time, 3 s. 1 = Gly–Gly; 2 = Leu–Gly–Gly; 3 = Gly–Gly–Gly; 4 = insulin; 5 = Glu–His–$ProNH_2$.

amplitude of the applied a.c. voltage. The maximum response was attained at a d.c. potential of −0.38 V, a drop time of 3 s and an a.c. voltage amplitude of 30 mV_r.

The dependence of the tensammetric response on the mobile phase pH is shown in Fig. 7. The optimum pH is 5–6, where the solutes are mostly uncharged, which supports their adsorption at the electrode surface. The tensammetric signal decreases with increasing flow-rate (Fig. 8), similarly to the amperometric signal at a copper electrode; the reason is the same in both instances, *i.e.*, a slow interaction (complexation reaction or adsorption) between the solutes and the electrode.

The tensammetric detection parameters are given in Table III. It can be seen that the detection sensitivity is comparable to that with amperometry at a copper electrode. On the other hand, tensammetric detection does not depend on the length of the peptide chain (*e.g.*, in the series Gly–Gly, Gly–Gly–Gly, and Gly–Gly–Gly–Gly). As can be expected, the sensitivity of tensammetric detection depends strongly

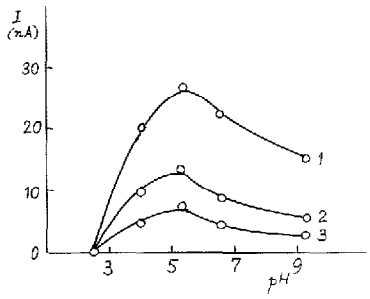

Fig. 7. Dependence of the tensammetric signal on the mobile phase pH. Applied potential, −380 mV; other conditions as in Fig. 6. 1 = Gly–Gly; 2 = glycine; 3 = insulin.

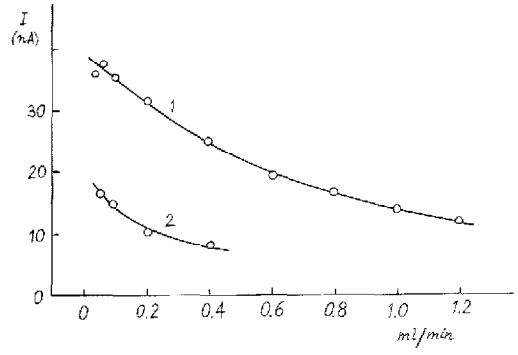

Fig. 8. Dependence of the tensammetric signal on the mobile phase flow-rate. Applied potential, −380 mV; flow-rate, 0.3 ml/min; other conditions as in Fig. 6. 1 = Gly–Gly; 2 = Gly–Gly–Gly–Gly.

on the mobile phase composition; mobile phases that do not contain surface-active components are generally preferable. In this work, measurement in the 0.1 *M* sodium perchlorate mobile phase yielded a signal twice as high as those in 0.025 *M* phosphate and acetate.

The reproducibilities of all the three detection techniques, *i.e.*, UV photometry, amperometry with a copper electrode and tensammetry, are similar, provided that each method operates under its particular optimum conditions. The relative standard deviations of the peak height were 1–2% at medium solute concentrations and increased to 5% close to the limit of detection.

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