Journal of Chromatography, 477 (1989) 377–385
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 591

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC MONITORING OF TRANSPEPTIDATION REACTIONS IN ANALOGUES OF GONADOTROPIN RELEASING HORMONE CONTAINING ASPARTIC ACID DERIVATIVES IN POSITION SIX

ZSOLT VADÁSZ*, JÁNOS SEPRÖDI, JUDIT ÉRCHEGYI and ISTVÁN TEPLÁN
First Institute of Biochemistry, Semmelweis University Medical School, P.O. Box 260, H-1444 Budapest (Hungary)
and
ISTVÁN SCHÖN
Chemical Works of Gedeon Richter Ltd., P.O. Box 27, H-1475 Budapest (Hungary)
(First received January 9th, 1989; revised manuscript received April 24th, 1989)

SUMMARY

High-performance liquid chromatographic systems were used for monitoring the hydrolysis of five potent agonistic gonadotropin releasing hormone analogues containing aspartic acid derivatives in position six. To separate the closely related nonapeptides formed during the hydrolysis, columns with reversed-phase packings were used under isocratic conditions. The mobile phases were methanol containing ammonium acetate or triethylammonium phosphate buffers. Good separations of the hydrolysis products from the investigated peptides allowed the reaction rate constants for the transformations examined to be calculated.

INTRODUCTION

Recently we have reported the synthesis and *in vivo* biological activity of some potent agonistic analogues of gonadotropin releasing hormone (GnRH) containing L-aspartyl or L-β-aspartyl benzyl or methyl ester or an aminosuccinyl residue in position six[1,2].

In order to interpret the *in vivo* biological activities of these analogues, account must be taken of the fact that after the injection a rapid chemical transformation of these peptides can occur in the animal at physiological pH (*ca.* 7.3) and temperature (*ca.* 37°C).

The $\alpha \rightarrow \beta$ transpeptidation which may occur in peptides containing aspartic acid derivatives results in β-aspartyl peptide formation. This rearrangement involves an aminosuccinyl intermediate and is especially rapid at alkaline pH[3,4]. However, the same aminosuccinyl-peptide intermediate can be formed from β-aspartyl peptides as well (Scheme 1). A significant transpeptidation rate of a tetrapeptide with an -Asp(OCH_3)-Phe- sequence at pH 7.3 and 37°C was reported by McFadden and Clarke[5]. The formation of aminosuccinyl-peptides and transpeptidation is strongly sequence dependent[6].

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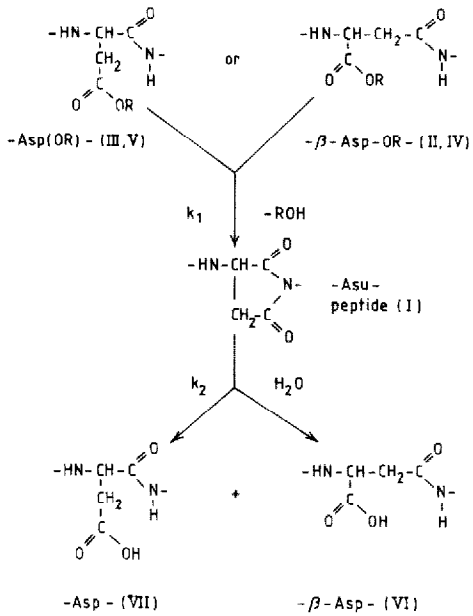


Scheme 1. The transformation of peptides containing an aspartyl- or β-aspartyl-ester moiety in aqueous media. The reactions follow the general scheme of consecutive reactions $A \xrightarrow{k_1} B \xrightarrow{k_2} C$ where (in this case) C represents two different end products. Roman numerals refer to peptides listed in Table I.

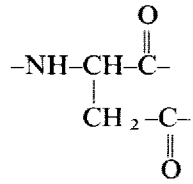
Numerous recent studies have documented the advantage of high-performance liquid chromatography (HPLC) in peptide analyses[7,8]. Its superior performance with reversed-phase (RP), chemically bonded packings has been utilized in the separation of several normal and isopeptides[9–11].

In this paper the usefulness of the RP-HPLC method for monitoring the transformations of our potent [aspartyl^6(ester)]-, [β-aspartyl^6-ester]- and [aminosuccinyl^6]-GnRH(1–9)-EA analogues is demonstrated. Several baseline separations achieved in isocratic systems enabled us to determine the kinetic parameters of the transformations and in one case a separation of epimer nonapeptides was achieved too.

EXPERIMENTAL

Materials

Peptides were synthesized by classical solution phase methods as described previously[1,2] and are listed in Table I. The abbreviations used follow the rules of the IUPAC-IUB commission on Biochemical Nomenclature[12]. Other abbreviations are:

Asu aminosuccinyl 

EA ethylamide $-NH-C_2H_5$

TABLE I
STRUCTURES OF PEPTIDES INVESTIGATED

	Peptide	*Structure*
	GnRH	Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
I	[Asu^6]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-Asu-Leu-Arg-Pro-EA
II	[β-Asp^6-OCH_3]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-Asp-OCH_3 | Leu-Arg-Pro-EA
III	[Asp^6(OCH_3)]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-Asp(OCH_3)-Leu-Arg-Pro-EA
IV	[β-Asp^6-OBzl]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-Asp-OBzl | Leu-Arg-Pro-EA
V	[Asp^6(OBzl)]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-Asp(OBzl)-Leu-Arg-Pro-EA
VI	[β-Asp^6]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-Asp-OH | Leu-Arg-Pro-EA
VII	[Asp^6]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-Asp-Leu-Arg-Pro-EA
VIII	[D-Asu^6]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-D-Asu-Leu-Arg-Pro-EA
IX	[D-β-Asp^6]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-D-Asp-OH | Leu-Arg-Pro-EA
X	[D-Asp^6]-GnRH(1–9)-EA	Glp-His-Trp-Ser-Tyr-D-Asp-Leu-Arg-Pro-EA

All amino acids are in the L-configuration and in normal (α) linkage if not stated otherwise.

HPLC

The HPLC analysis of samples was performed on a Waters chromatograph consisting of an M 6000A pump, an U6K injector, an M450 variable wavelength UV detector and a BBC Goerz 220 recorder. Samples were analysed on a Shandon ODS Hypersil 5-μm, 25 cm × 0.5 cm column or on a Shandon 25 cm × 0.46 cm column with the same packing material. The dimensions of the quard column were 2.3 cm × 0.4 cm.

The mobile phases used are listed in Table II. The chromatograph was operated isocratically at ambient temperature and the mobile phase flow-rates were between 0.6 and 1.2 ml/min. The absorbance of the column effluents was monitored at 280 nm.

Hydrolysis of peptides

A 1-mg amount of GnRH analogue (peptides I–V or VIII in Table I) was dissolved in 0.5 ml of water; then 0.5 ml of 0.1 *M* buffer solution (pH 7.3, sodium phosphate; pH 6.0, ammonium acetate; pH 4.5, sodium acetate) were added to achieve the required pH. The incubation mixtures were kept in sealed glass tubes at 2, 22 and 37°C respectively. At preselected times, 100-μl aliquots were withdrawn from each incubation mixture and the reactions stopped by the addition of 20 μl of acetic acid. The samples were stored at −20°C before being analysed by HPLC.

TABLE II
RETENTION DATA FOR THE GnRH ANALOGUES INVESTIGATED
Column: Shandon ODS-Hypersil 5 μm (25 cm × 0.5 cm) with guard column in experiments A, B and D; without guard column in experiment G and 25 cm × 0.46 cm in experiments C, E and F. Flow-rates: 0.8 (A, B, D); 0.6 (C, E, F) and 1.2 ml/min (G).

Experiment	*Peptides*	t_R	k'	*Eluent*
A	[β-Asp^6]-GnRH(1–9)-EA (VI)	11.6	1.58	Methanol–0.1 *M* ammonium
	[Asp^6]-GnRH(1–9)-EA (VII)	13.0	1.89	acetate (pH 4.0) (48:52, v/v)
	[Asu^6]-GnRH(1–9)-EA (I)	16.2	2.60	
B	VI	14.2	1.96	Methanol–0.1 *M* ammonium
	VII	16.4	2.42	acetate (pH 4.0) (47:53, v/v)
	[β-Asp^6-OCH$_3$]-GnRH(1–9)-EA (II)	20.4	3.25	
	I	21.0	3.38	
C	VI	14.4	1.77	Methanol–0.25 *M* TEAP
	VII	16.9	2.25	(pH 3.0) (47:53, v/v)
	II	19.4	2.73	
	I	20.6	2.96	
D	VI	12.0	1.61	Methanol–0.1 *M* ammonium
	VII	13.6	1.96	acetate (pH 4.0) (48:52, v/v)
	I	17.2	2.74	
	[Asp^6(OCH$_3$)]-GnRH(1–9)-EA (III)	18.0	2.91	
E	VI	7.6	0.46	Methanol–0.25 *M* TEAP
	VII	8.1	0.56	(pH 3.0) (62:38, v/v)
	I	8.1	0.56	
	[β-Asp^6-OBzl]-GnRH(1–9)-EA (IV)	14.5	1.79	
F	VI	7.4	0.42	Methanol–0.25 *M* TEAP
	VII	8.0	0.53	(pH 3.0) (62:38, v/v)
	I	8.0	0.53	
	[Asp^6(OBzl)]-GnRH(1–9)-EA (V)	13.4	1.58	
G	[D-β-Asp^6]-GnRH(1–9)-EA (IX)	27.4	11.2	Methanol–0.25 *M* TEAP
	[D-Asp^6]-GnRH(1–9)-EA (X)	30.7	12.5	(pH 3.0) (35:65, v/v)
	VI	32.0	13.2	
	VII	45.5	18.8	

Reaction kinetic calculations

The percentage of the peptides I–V (Table I) after hydrolysis for time t was determined by cutting and weighing the peak areas in the HPLC chromatograms. Supposing a pseudo-first order reaction for each transformation summarized in Scheme 1, the reaction rate constants were calculated according to

$$k = \frac{1}{t} \ln \frac{A_0}{A} \tag{1}$$

where A_0 represents the initial concentration of the investigated peptide chosen to be 100%, and A is equal to the percentage of the peptide after hydrolysis for time t (ref. 13). The extinction coefficients at 280 nm ε_{280}, of the peptides I–VII were calculated from data in the literature[14,15]. Though peptides with a benzyl ester group have

TABLE III
REACTION RATE CONSTANTS (h^{-1}) FOR TRANSFORMATIONS OF PEPTIDES I–V AT VARIOUS pH VALUES AND TEMPERATURES

Peptide	*37°C*	*22°C*			*2°C*		
	pH 7.3	*pH 7.3*	*6.0*	*4.5*	*pH 7.3*	*6.0*	*4.5*
I	0.41 ± 0.028	0.10 ± $6.1 \cdot 10^{-3}$	$9.3 \cdot 10^{-3}$ ± $3.4 \cdot 10^{-4}$	$4.6 \cdot 10^{-4}$ ± $2.4 \cdot 10^{-5}$	$6.8 \cdot 10^{-3}$ ± $5.3 \cdot 10^{-4}$	$5.9 \cdot 10^{-4}$ ± $4.2 \cdot 10^{-5}$	–
II	0.49 ± 0.046	–	–	–	–	–	–
III	0.25 ± 0.016	–	–	–	–	–	–
IV	0.94 ± 0.079	0.19 ± 0.012	$2.0 \cdot 10^{-2}$ ± $1.0 \cdot 10^{-3}$	$1.3 \cdot 10^{-3}$ ± $4.9 \cdot 10^{-5}$	$1.2 \cdot 10^{-2}$ ± $6.6 \cdot 10^{-4}$	$1.6 \cdot 10^{-3}$ ± $1.2 \cdot 10^{-4}$	$1.0 \cdot 10^{-4}$ ± $6.1 \cdot 10^{-6}$
V	0.39 ± 0.015	–	–	–	–	–	–

a slightly (by 1.7%) greater ε_{280} value than those without it, this effect seems to be negligible for our purposes. Each k value was determined from at least three different measurements at different times and is presented as mean ± standard deviation in Table III.

Knowing the experimentally determined k_2 and the k_1 values for the transformations of peptides II–V to peptide I (see Schema A and Table I), the percentage composition of any incubation mixture can be calculated at any time applying the well known equations[13] for consecutive reactions

$$B = \frac{k_1}{k_2 - k_1} \cdot A_0(e^{-k_1 t} - e^{-k_2 t}) \tag{2}$$

$$C = \frac{1}{k_2 - k_1} \cdot A_0 \left[k_2(1 - e^{-k_1 t}) - k_1(1 - e^{-k_2 t})\right] \tag{3}$$

where B represents the percentage amount of peptide I, C represents the sum of the percentage amounts of peptides VI and VII. To calculate the individual concentrations of VI and VII, it had to be taken into account that the ratio of VI to VII was 3.5:1 in our experiments.

RESULTS AND DISCUSSION

For the RP-HPLC chromatography of GnRH and its analogues the use of ammonium acetate buffers in combination with methanol, ethanol or acetonitrile as organic components has been reported several times[16,17]. The application of triethylammonium phosphate (TEAP) buffer for the RP-HPLC separation of peptides gave good results in the case of GnRH, too[18]. For the resolution of aspartyl- and β-aspartyl-peptides, methanol–water as the mobile phase containing acetic acid (2%), sodium acetate (pH 4.0) or ammonium acetate (pH 3.5) as a modifier was reported[9–11].

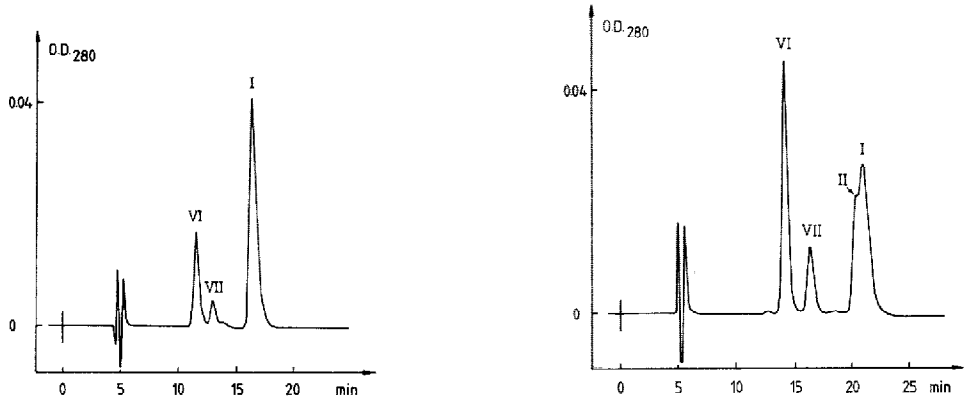


Fig. 1. HPLC analysis of the hydrolysis of [Asu^6]-GnRH(1–9)-EA (I) at pH 7.3 and 37°C for 40 min. VI = [β-Asp^6]-GnRH(1–9)-EA; VII = [Asp^6]-GnRH(1–9)-EA. Column: Shandon ODS Hypersil 5 μm (25 cm × 0.5 cm). Eluent: methanol–0.1 *M* ammonium acetate buffer (pH 4.0) (48:52, v/v); flow-rate 0.8 ml/min. Detection: 280 nm.

Fig. 2. HPLC chromatogram of the hydrolysis of [β-Asp^6–OCH_3]-GnRH(1–9)-EA (II) at pH 7.3 and 37°C for 180 min. Other peptides as in Fig. 1. Eluent: methanol–0.1 *M* ammonium acetate buffer (pH 4.0) (47:53, v/v). Other conditions as in Fig. 1.

In our experiments on the separation of GnRH analogues I–VII satisfactory results were obtained with mobile phase combinations of methanol–0.1 *M* ammonium acetate buffer (pH 4.0) or methanol–0.25 *M* TEAP buffer (pH 3.0). During the hydrolysis of [Asu^6]-GnRH(1–9)-EA (I) two products, [Asp^6]-GnRH(1–9)-EA (VII) and [β-Asp^6]-GnRH(1–9)-EA (VI), were formed (Fig. 1). Peptides I, VI, and VII can easily be separated presumably because they are differently ionized at pH 4.0.

After a short time of hydrolysis of [β-Asp^6-OCH_3]-GnRH(1–9)-EA (II), four peaks were detected by HPLC (Fig. 2). Peptides I and II were not well separated in this system, presumably because they have the same isoelectric points and can only have a slight difference in their hydrophobic characters. Changing the mobile phase to methanol–0.25 *M* TEAP (pH 3.0), almost baseline separations were achieved (Fig. 3).

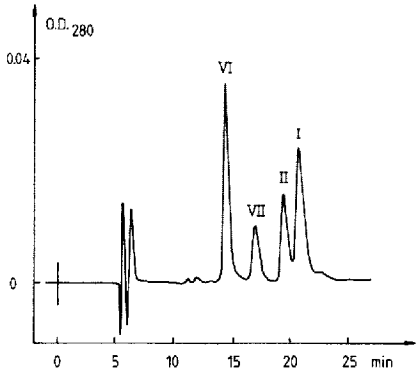


Fig. 3. HPLC analysis of the hydrolysis of [β-Asp^6-OCH_3]-GnRH(1–9)-EA (II) at pH 7.3 and 37°C for 180 min. Peptides as in Fig. 1. Column: Shandon ODS Hypersil 5 μm (25 cm × 0.46 cm). Eluent: methanol–0.25 *M* TEAP buffer (pH 3.0) (47:53, v/v); flow-rate 0.6 ml/min. Detection: 280 nm.

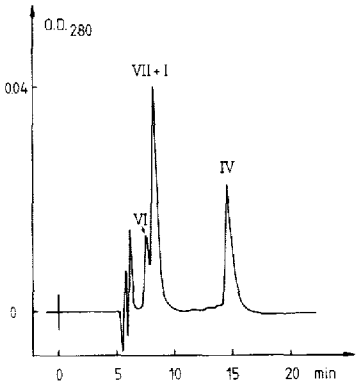


Fig. 4. HPLC diagram of the hydrolysis of [β-Asp^6-OBzl]-GnRH(1–9)-EA (IV) at pH 7.3 and 37°C for 60 min. Peptides as in Fig. 1. Conditions as in Fig. 3, except methanol–0.25 *M* TEAP buffer (pH 3.0) (62:38, v/v).

The best HPLC separation of [Asp^6 (OCH_3)]-GnRH(1–9)-EA (III) from its hydrolysis producs was obtained in methanol–0.1 *M* ammonium actate (pH 4.0) as the mobile phase. Retention times and k' value for these and all the other peptides investigated are listed in Table II.

Monitoring the hydrolysis of [β-Asp^6-OBzl]-GnRH(1–9)-EA (IV), a good separation of IV from I was observed which can be explained by the strong hydrophobic nature of the benzyl ester group in IV (Fig. 4). A coelution of peptides I and VII was observed in this HPLC system.

Satisfactory resolutions were also achieved by analysing the hydrolysis samples of [Asp^6(OBzl)]-GnRH(1–9)-EA (V.) For k' values and chromatographic conditions see Table II.

In each case, the separation of the starting peptides from the hydrolysis products allowed us to determine the percentages of these peptides after hydrolysis for time t, and thus to calculate the reaction rate constants of these transformations (Table III).

The values of the hydrolysis (pH 7.3, 37°C) reaction rate constants of peptides I (0.41 h^{-1}) and III (0.25 h^{-1}) are similar to those observed for two hexapeptides with -Asu-Phe- (0.37 h^{-1}) and -Asp(OCH_3)-Phe- (0.34 h^{-1}) sequences[5].

Knowing the reaction rate constants of all transformations examined, we can calculate the percentages of the peptides in any incubation mixture at any time (for detail see Experimental), *e.g.*, the incubation mixture (37°C, pH 7.3) of [β-Asp^6-OBzl]-GnRH(1–9)-EA (IV) (the most labile GnRH analogue examined in this work) contains 33.5% [Asu^6]-GnRH(1–9)-EA (I), 3.0% [β-Asp^6]-GnRH(1–9)-EA (VI), 0.9%[Asp^6]-GnRH(1–9)-EA (VII) and only 62.6% IV after hydrolysis for 30 min (Fig. 5).

We have suggested in an earlier paper that the striking biological activity of peptides II and IV may be attributed to their ability to form [Asu^6]-GnRH(1–9)-EA (I)[1]. Our recent findings indicate that there is no direct correlation between the order of the observed biological activities of I–V ($I \approx II > III > V \approx IV$) and their ability to form I at pH 7.3 and 37°C: $IV > II > V > III$ (Table III). The impact of the rapid chemical transformation of peptides I–V on the observed biological activities will be discussed in detail elsewhere[19].

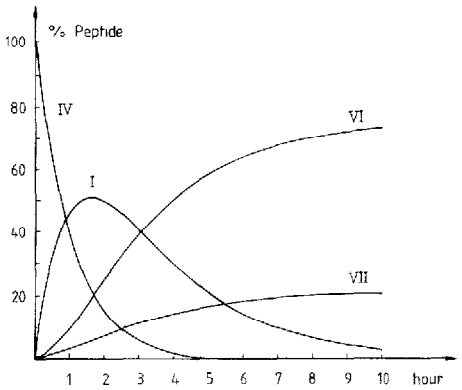


Fig. 5. The hydrolysis pattern of [β-Asp^6-OBzl]-GnRH(1–9)-EA (IV) at pH 7.3 and 37°C calculated from the experimentally determined reaction rate constants. Peptides as in Fig. 1.

In order to determine the pH and temperature range at which the purification of peptides I–V can be carried out without significant transformation, the reaction rate constants of the transformations examined were determined at different pH values and temperatures. From the k values listed in Table III the time required for minimum (1%) transformation of these peptides can be calculated. In the case of [β-Asp^6-OBzl]-GnRH(1–9)-EA (IV), the less stabile GnRH analogue examined in this work, 7.7 h are necessary for 1% transformation at pH 4.5 and 22°C which means that lower pH values and/or temperatures are required during the purification of these analogues.

As a result of the hydrolysis of an hexapeptide containing the -Asu-Gly- sequence, beside the L-aspartyl and L-β-aspartyl peptides the formation of the corresponding D-aspartyl and D-β-aspartyl peptides was also reported, but the amount

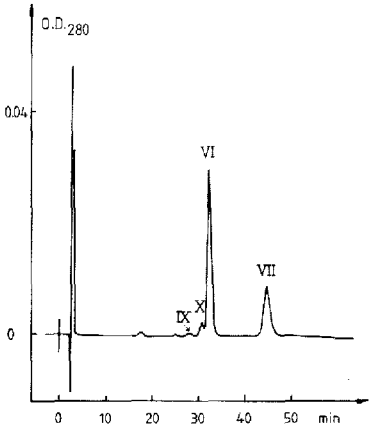


Fig. 6. HPLC analysis of the hydrolysis of [Asu^6]-GnRH(1–9)-EA (I) at pH 7.3 and 37°C for 19 h. Peptides as in Fig. 1, except IX = [D-β-Asp^6]-GnRH(1–9)-EA and X = [D-Asp^6]-GnRH(1–9)-EA. Column: Shandon ODS Hypersil 5 μm (25 cm × 0.5 cm). Eluent: methanol–0.25 *M* TEAP buffer (pH 3.0) (35:65, v/v); flow-rate 1.2 ml/min. Detection: 280 nm.

of D-epimers was less than 10%[20]. This epimerization side reaction can be attributed to the tendency of the amino succinimide part to racemization[21]. We were curious to know whether or not the unidentified small impurities in Figs. 1–4 are the result of a similar epimerization process during the hydrolysis of our peptides.

Recently we have synthesized [D-Asu^6]-GnRH(1–9)-EA (VIII)[19]. Its hydrolysis resulted in two materials as detected by HPLC (conditions were the same as in experiment C in Table II). We arbitrarily assigned the peak which eluted first from the HPLC column as [D-β-Asp^6]-GnRH(1–9)-EA (IX) and the other product as [D-Asp^6]-GnRH(1–9)-EA (X). After this, we mixed the hydrolysis samples of I and VIII in 1:1 ratio and tried to separate the epimer nonapeptides but failed under these conditions: VI and X coeluted and IX appeared as a shoulder of VI + X. Changing the mobile phase composition to 35% methanol–65% TEAP (0.25 *M*, pH 3.0), good separation was achieved on the same column. Then, on analysing the hydrolysis products of peptide I (pH 7.3, 37°C, 19 h), 0.7% IX and 4.4% X were detected beside VI and VII (Fig. 6).

ACKNOWLEDGEMENT

This work was supported by the Hungarian Academy of Sciences: the Research Fund for Basic Research in Biology No. 2.1.5.4.

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