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Validation of the high performance liquid chromatography separation of GLF and related di- and tripeptides

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Abstract

This paper describes a simple and rapid procedure for the determination of the important tripeptide glycyl-leucyl-phenylalanine (GLF) and its homologues by reversed phase high-performance liquid chromatography. The eluent for convenient separation on a phenyl-bonded silica stationary phase was 15/85 (v/v) acetonitrile/0.1 M sodium citrate buffer pH 3.0, UV detection was set at 258 nm. The method is specific, linear, accurate (recovery mean = 99.6 ± 0.8%), and reliable (precision = 1.2%). Limit of detection is established for 0.05 mM and limit of quantification for 0.10 mM (precision = 1.5%). © 1998 Elsevier Science S.A. All rights reserved.

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1. Introduction

As a result of the increasing interest in peptides and proteins in academic research, pharmaceutical and food industry, the chromatographic separation of this class of compounds has become one of the prime interests. In this field, the first attempts to use reversed-phase liquid chromatography (which was introduced in the mid-1970s) were not very successful because of deficient column technology [1] that led to low efficiencies and poor peak shapes. However, these initial problems have been solved, and reversed-phase liquid chromatography (RP-LC) is currently used as a routine technique for separation of peptides and small proteins [2–4].

The nutritional interest for oligopeptides, specially those issued from casein hydrolysate have been largely demonstrated [5,6]. Indeed, amino-acid, di- and tri-peptides pass directly through the enterocyte membrane, without previous hydrolysis. In addition, some amino-acids like glycine and proline, which are absorbed with difficulty by the enterocyte are much more absorbed than dipeptide. Increasing the di- and tripeptides ratio in proteolysate increases their nutritional efficacy [7]. Official methods of analysis (Kjeldhal method, α-amino nitrogen assay) cannot conveniently evaluate this ratio. Studies on copper complexation on oligopeptides and 2nd order spectrophotometry allow an estimation of this ratio, but cannot identify the di- and tripeptides involved [8–11]; also gel permeation chromatography [12] is more difficult to use as a routine method.

In that context, this work was performed to develop and validate a simple methodology for the separation of the important peptide glycyl-leucyl-phenylalanine (GLF) from its related compounds through the use of an isocratic elution mode and a basic high-performance liquid chromatography (HPLC) device. GLF, which has been isolated from human milk proteins [13–15], possesses immunostimulating properties. To facilitate the ultimate step of HPLC analysis of the peptidic fraction yielding GLF, the elution of the latter with some related di- and tri-peptides was studied on a reversed phase column (phenyl-bonded silica). The eluent in order to separate GLF, glycyl-phenylalanyl-leucine (GFL), phenylalanyl-glycine (FG), glycyl-phenylalanine (GF), phenylalanyl-leucine (FL) and leucyl-phenylalanine (LF) was acetonitrile/0.1 M sodium citrate buffer pH 3.0, 15/85 (v/v).

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2. Experimental

2.1. Chemicals

All peptides were supplied by Bachem Biochimie Sarl (Voisins-le-Bretonneux, France). All were in levogyral (natural) form. All reagents were HPLC grade or laboratory quality. Water (Biosedra, Louviers, France) and acetonitrile (Merck, Nogent sur Marne, France) were HPLC grade.

2.2. Apparatus and method

The HPLC system used in this work consisted of an L-6000 Merck pump (Nogent sur Marne, France) with an automatic Wisp717 sample injector (Millipore, St. Quentin, France), and a Waters 486 UV/visible detector (Millipore, St. Quentin, France) set at 258 nm and connected to a Merck Hitachi interface (Nogent sur Marne, France). A supelcosil LCDP (Supelco, St. Quentin-Fallavier, France) (4.6 mm internal diameter, 25 cm length) packed with 5 μm particles of phenyl-bonded silica and 120 Å pore size was used. The mobile phase was acetonitrile/0.1 M sodium citrate buffer, pH 3.0 15/85 (v/v). The eluent was flushed at a flow rate of 1 ml/min at room temperature (24 ± 2°C). The injection volume was 100 μl. Raw data were processed with computer software, Merck HM® and Merck VMM® (Nogent sur Marne, France).

Peptides were dissolved in 0.1 M sodium citrate buffer, pH 3.0 at 10^{-3} M.

3. Results and discussion

The method to analyse a mixture of GLF and its homologues was validated according to the protocol established by SFSTP commission [16].

3.1. Specificity

Successive individual solutions of each peptide were injected in the system, and no interferences were shown. Fig. 1 shows a typical chromatogram of a standard mixture solution of the studied peptides (5×10^{-4} M each).

The retention times (min) and relative standard deviations (in brackets) determined during the validation period were, respectively, 4.9 (1%) and 5.8 (1%) for FG and GF, 13.7 (3%) and 14.8 (2%) for FL and LF, 21.4 (3%) and 23.0 (3%) for GFL and GLF. The resolution factors (R_S) show good separation, $R_S = 1.8$ between FG and GF, $R_S = 1.2$ between FL and LF, $R_S = 1.2$ between GFL and GLF.

3.2. Linearity

The calibration curves which give the peak area as a function of concentration are linear in the range 0.05–1.00 mM. Analyses were repeated on 3 days ($n = 15$). The statistical interpretation [17,18] was assessed for both the calibration curve ($y = b_2x + a_2$) and the sample curve ($y = b_1x + a_1$), as below:

1. equation of the straight line curve and correlation coefficient, by the least-square regression method,
2. analysis of variance by tests of Fisher, for both calibrations:
   - the existence of a significant slope, $F_{1\,\mathrm{exp}} = s_I^2/s_R^2 > F_1$ (95%); where s_I^2 is the variance of the regression, and s_R^2 is the residual variance.
   - validity of fit, $F_{2\,\mathrm{exp}} = s_L^2/s_E^2 < F_2$ (95%); where s_L^2 is the variance of regression error, and s_E^2 is the variance of experimental error.
   - comparison between slopes b_1 and b_2 by Student's test,

$$t_{\mathrm{exp}} = \frac{|b_1 - b_2|}{\sqrt{s_{b_1}^2 + s_{b_2}^2}} < t_{(0.05;\ 26)};$$

where $s_{b_1}^2$ and $s_{b_2}^2$ are the variances for sample and calibration curves, respectively.

   - comparison between a_1 and a_2 by Student's test,

$$t_{\mathrm{exp}} = \frac{|a_1 - a_2|}{\sqrt{s_{a_1}^2 + s_{a_2}^2}} < t_{(0.05;\ 26)};$$

where $s_{a_1}^2$ and $s_{a_2}^2$ are the variances of the intercept for sample and calibration curves, respectively.

Results are detailed in Table 1. In short, coefficients of correlation for each product were always superior to 0.999. Analysis of variance shows no differences between calibration and sample curves.

3.3. Accuracy

The calculated concentrations (C_C) (via calibration curves with standard solutions) in the samples were compared with their theoretical values (C_T). Recovery (%) was obtained by $C_C \times 100/C_T$ ($n = 15$). Table 2 gives raw values from which statistical calculations were issued and presented in Table 3. The statistical interpretation was performed according to the following tests:

- fitting of linked variances homogeneity, by Cochran's test

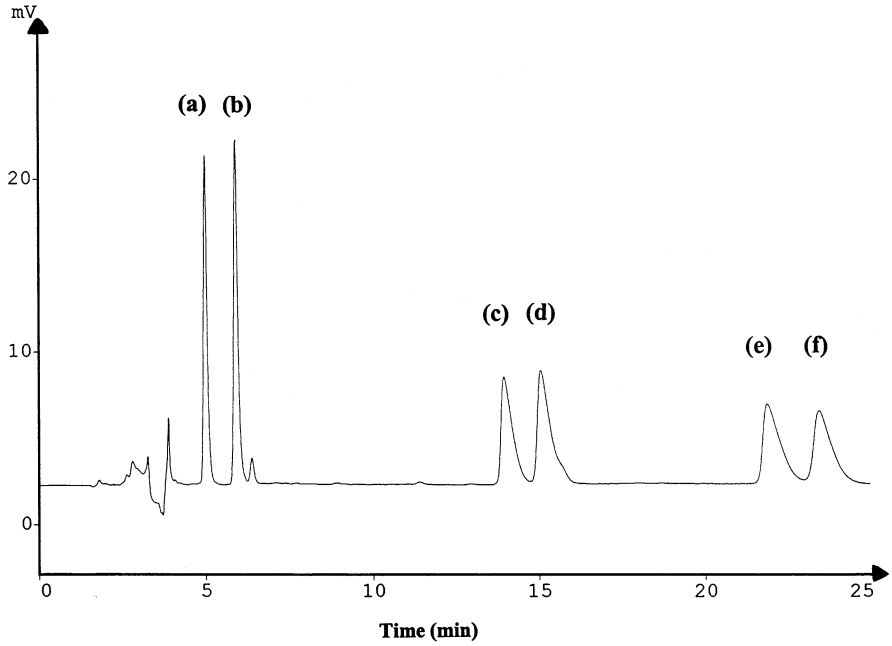

Fig. 1. Chromatogram of peptides (5×10^{-4} M) obtained with acetonitrile/0.1 M sodium citrate buffer pH 3.0, 15/85 (v/v, 1.0 ml/min), on a reversed phase column (Supelcosil LCDP, 5 μm, 120 Å, 4.6 × 25 cm) at 24 ± 2°C, UV-detection at 258 nm (injection = 100 μl, attenuation factor 2^4). (a) FG, (b) GF, (c) FL, (d) LF, (e) GFL, (f) GLF.

Table 1
Linearity—analysis of regression

	FG	GF	FL	LF	GFL	GLF
Slope of the straight line b	718 388	867 085	742 177	907 787	834 248	816 692
y-value for $x = 0$; a	3170	7794	2333	4102	2220	1052
Coefficient of correlation r	0.9999	0.9999	1.000	0.9994	0.9999	0.9998
Variance of the regression s_I^2	$956\,815 \times 10^6$	$1\,393\,905 \times 10^6$	$1\,021\,232 \times 10^6$	$1\,527\,841 \times 10^6$	$1\,290\,327 \times 10^6$	$1\,236\,591 \times 10^6$
Residual variance s_R^2	5 079 382	6 456 826	2 130 334	65 712 548	7 842 006	21 342 040
Variance of experimental error s_E^2	$74\,812 \times 10^6$	$108\,862 \times 10^6$	$79\,651 \times 10^6$	$117\,719 \times 10^6$	$100\,667 \times 10^6$	$96\,037 \times 10^6$
Variance of regression error s_L^2	$249\,163 \times 10^6$	$362\,983 \times 10^6$	$265\,928 \times 10^6$	$397\,980 \times 10^6$	$336\,002 \times 10^6$	$322\,042 \times 10^6$
Variance of b; s_b^2	8 219 065	10 447 939	3 932 579	106 330 984	12 689 329	34 534 045
Variance of a; s_a^2	481 871	612 547	230 561	6 234 022	743 956	2 024 678

$C_{exp} = s_{max}^2 / \Sigma s^2 < C_{(0.05;\ 5;\ 2)}$; where s_{max}^2 is the higher variance of all groups, and Σs^2 is the sum of variances of each group.

- test for validity of means, by Fisher's test
  $F_{exp} = s_C^2 / s_E^2 < F_{(0.05;\ 4;\ 10)}$; where s_C^2 is the inter-group variance, and s_E^2 is the intra-group variance
- estimation of mean recovery ($\bar{\bar{Y}}$) and of its confidence range (I)
  $\bar{\bar{Y}} = \Sigma Y/15$; where Y is the recovery; and $I = (t_{(0.05;\ 14)}\, s)/\sqrt{15}$; where s is the total standard deviation.

Results show satisfactory recoveries and valid statistical tests.

3.4. Reliability

Reliability was investigated by studying intra-day variation on one day ($n = 6$, 3 times) and inter-day variation on 3 days ($n = 18$), for the 0.5 mM equimolar solution of peptides.

Statistical calculations are described below:

$\bar{m}$	general mean
m_j	mean for each day
s_j	standard deviation for each day

3.5. Precision (repeatibility)

The variability of the method was investigated by performing variations as small as possible (same day, column, reagent batches, technician,...).

The variance of the precision (s_r^2) was defined as $s_r^2 = (\Sigma_{j=1}^{3} s_j^2)/3$, and the relative standard deviation of the precision as $CV_r = (s_r/\bar{m}) \times 100$.

Table 2
Accuracy—recovery means and standard deviation for each group of concentration

Recovery mean (σ) $C_C \times 100/C_T$	$C_T = 0.05$ mM	$C_T = 0.10$ mM	$C_T = 0.20$ mM	$C_T = 0.50$ mM	$C_T = 1.00$ mM
FG	100.0 (0.0)	100.0 (0.0)	101.7 (2.9)	101.3 (1.2)	100.5 (0.7)
GF	100.0 (0.0)	103.3 (5.8)	101.7 (2.9)	100.7 (1.2)	102.3 (3.2)
FL	100.0 (0.0)	100.0 (0.0)	100.0 (0.0)	100.7 (1.2)	100.0 (0.0)
LF	100.0 (0.0)	100.0 (0.0)	100.0 (5.0)	102.0 (0.0)	99.0 (0.0)
GFL	100.0 (0.0)	100.0 (0.0)	98.3 (2.9)	99.3 (1.2)	100.0 (0.0)
GLF	100.0 (0.0)	100.0 (0.0)	98.3 (2.9)	99.3 (1.2)	100.3 (1.2)

Table 3
Accuracy—statistical interpretation

	Homogeneity of linked variances		Validity of means		Mean recovery (%)
	C_{exp}	<0.68	F_{exp}	<3.48	$\pm \frac{t_{(0.05;\ 14)}s}{\sqrt{15}}$
FG	0.61	Pass	3.29	Pass	100.8 ± 0.9
GF	0.44	Pass	1.18	Pass	101.6 ± 1.7
FL	1.00	Pass	2.40	Pass	100.1 ± 0.3
LF	1.00	Pass	1.73	Pass	100.2 ± 1.2
GFL	0.71	Pass	1.99	Pass	99.5 ± 0.8
GLF	0.56	Pass	2.07	Pass	99.6 ± 0.8

Table 4
Reliability—statistical interpretation

	Mean area (CV%)			CV_r%	CV_R%
	Day 1	Day 2	Day 3		
FG	368912 (0.4)	366586 (0.8)	368049 (0.2)	0.6	1.0
GF	445775 (0.1)	440279 (0.2)	442316 (0.1)	0.1	0.6
FL	378005 (0.2)	373304 (0.1)	376250 (0.2)	0.2	0.6
LF	466263 (0.1)	465730 (0.4)	465654 (0.4)	0.3	0.3
GFL	414584 (0.3)	420384 (0.3)	411031 (0.6)	0.4	1.2
GLF	404391 (0.8)	412341 (0.4)	397833 (1.0)	1.2	1.8

3.6. Reproducibility

In this case, the variability of the method was investigated by performing variations as large as possible (different days, columns, reagent batches, technicians,…). The inter-group variance (s_g^2) was defined as

$$s_g^2 = \frac{\sum_{j=1}^{3} (m_j - \overline{m})^2}{2} - \frac{s_r^2}{6}$$

The variance of reproducibility was defined as $s_R^2 = s_r^2 + s_g^2$ and the relative standard deviation of the reproducibility as $CV_R = (s_R/\overline{m}) \times 100$.

Table 4 shows good reliability, with precision less than 1.2% and reproducibility less than 1.8%.

3.7. Limit of detection and limit of quantification

The limit of detection (LD) was determined from analysis of a blank, by the maximum amplitude (h_{max}) of the background signal measured over a period corresponding to the 20 half-width of the peak height. The definition is $LD = 3h_{max}R$, where R is the response factor (in height). The limit of quantification (LQ) is taken as the smaller concentration for which the precision is less than 3%. Results show $LD = 0.01$ mM and $LQ = 0.05$ mM for dipeptides, $LD = 0.05$ mM and $LQ = 0.10$ mM for tripeptides. As GLF is immunostimulating with quite a low dose such as 0.1 μM by intraveinous methods [19], these thresholds are relatively high and would need to be improved. Work is in

progress, studying a possible fluorimetric detection. Nevertheless, the significant advantage of the present work with regard to previously published methods [2–4,8–12] is to propose a simple method, easily set and permitting assays of the GLF content in the range 0.1–1.0 mM.

4. Conclusion

This work describes a simple methodology for the separation of an important peptide GLF from its related compounds through the use of an isocratic elution mode and a basic HPLC device.

Concerning GLF, the procedure is specific, linear, accurate (recovery mean = 99.6 ± 0.8%), reliable (precision = 1.2%). The limit of detection is established for 0.05 mM and limit of quantification for 0.10 mM (precision = 1.5%). Concerning the homologues, the method permits a good separation and assay. $LD =$ 0.01 mM and $LQ = 0.05$ mM for dipeptides, $LD = 0.05$ mM and $LQ = 0.10$ mM for tripeptides.

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References

[1] N. Lundell, K. Markides, Optimization strategy for reversed-phase liquid chromatography of peptides, J. Chromatogr. 639 (1993) 117–127.
[2] G.A. Guiochon, Chromatography, today and tomorrow, Anal. Chim. Acta 283 (1993) 309–319.
[3] N. Lundell, A Peptide in a haystack—strategies for a biochromatographer, LC–GC Int. 8 (1995) 636–647.
[4] K. Stulik, V. Pacakova, J. Suchankova, H.A. Claessens, Stationary phases for peptides analysis by high performance liquid chromatography: a review, Anal. Chim. Acta 352 (1997) 1–19.
[5] M. Hamon, Appréciation de la teneur en petits peptides de protéolysats enzymatiques à l'aide d'une méthode cuprimétrique: intérêt en médecine nutritionnelle, Bull. Acad. Natl. Med. 174 (1990) 469–477.
[6] H. Meisel, E. Schlimme, Bioactive peptides derived from milk proteins: ingredients for functional foods?, Kieler Milchw. Forsch. 48 (1996) 343–357.
[7] G.K. Grimble, P.P. Keohane, B.E. Higgins, M.V. Kaminsk, D.B.A. Silk, Effect of peptide chain on amino and nitrogen absorption from two lactalbumine hydrolysate in the normal human jejunum, Clin. Sci. 71 (1986) 65–69.
[8] E. Lati, C. Dauphin, M. Hamon, Comportement de quelques di-, tri- et tétrapeptides vis-à-vis du cuivre(II). Etude en potentiométrie (électrode de cuivre) et en spectrométrie infrarouge, Anal. Chim. Acta 254 (1991) 89–94.
[9] E. Lati, C. Dauphin, M. Hamon, M. Silvestre, Comportement de mélange d'acides aminés et de peptides vis-à-vis du cuivre(II), Anal. Chim. Acta 268 (1992) 163–164.
[10] M Silvestre, E. Lati, C. Dauphin, M. Hamon, Cuprimetric assay of casein hydrolysates, J. A.O.A.C. 76 (1993) 1295–1299.
[11] M Silvestre, C. Dauphin, M. Hamon, Application of UV absorbance and second-derivative spectrophotometry for analyzing casein hydrolysates, Anal. Chim. Acta 282 (1993) 603–612.
[12] F. Pellerin, D. Baylocq, C. Majcherczyk, M. Hamon, Fractionnement de proteines et de leurs hydrolysats par chromatographie d'exclusion sur gel Séphadex, Rev. Ind. Agroaliment. Tech. Lait 356 (1985) 9–13.
[13] J. Bethou, D. Migliore-Samour, A. Lifchitz, J. Delettre, F. Floc'h, P. Jolles, Immunostimulating properties and three-dimensional structure of two tripeptides from human and cow caseins, FEBS Lett. 218 (1987) 55–58.
[14] D. Migliore-Samour, M. Roch-Arveiller, M. Tissot, M. Jaziri, K. Keddad, J.P. Giroud, P. Jolles, Effects of tripeptides derived from milk proteins on polymorphonuclear oxidative and phosphoinositide metabolisms, Biochem. Pharmacol. 44 (1992) 673–680.
[15] M. Jaziri, D. Migliore-Samour, M.R. Casabianca-Pignede, K. Keddad, J.L. Morgat, P. Jolles, Specific binding sites on human phagocytic blood cells for Gly-Leu-Phe and Val-Glu-Pro-Ile-Pro-Tyr, immunostimulating peptides from human milk proteins, Biochim. Biophys. Acta 1160 (1992) 251–261.
[16] J. Caporal-Gautier, J.M. Nivet, Guide de validation analytique, rapport d'une commission SFSTP, STP Pharm. Prat. 2 (1992) 205–226.
[17] J. Fleury, Introduction à l'usage des méthodes statistiques en pharmacie, Medicine Hygiène (1987) 66.
[18] Norme Française NF ISO 5725, Décembre 87, Afnor-Paris.
[19] A.M. Fiat, D. Migliore-Samour, P. Jolles, L. Drouet, C. Balditsollier, J. Caen, Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and immunomodulating activities, J. Dairy Sci. 76 (1993) 301–310.