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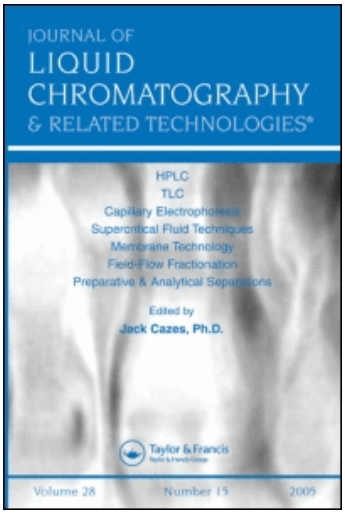

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Bioavailability Prediction of Amino Acids and Peptides in Nutritive Mixtures by Separation on Non-Polar Stationary Phases by High-Performance Liquid Chromatography and Direct UV Detection at 210 NM

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BIOAVAILABILITY PREDICTION OF AMINO ACIDS AND PEPTIDES IN NUTRITIVE MIXTURES BY SEPARATION ON NON-POLAR STATIONARY PHASES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND DIRECT UV DETECTION AT 210 NM

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ABSTRACT

A reversed phase high-performance liquid chromatography method was developed to separate amino acids contents in total parenteral nutrition solution and peptides in enteral mixtures. Retention is attributed to hydrophobic interactions between the solutes and the hydrocarbonaceous functions covalently bound to the stationary phase surface. Consequently, the analytes were eluted in the order of their increasing hydrophobicity. The mixtures were analyzed by using gradient elution with increasing content and monitoring the column efluent at 210 nm.

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INTRODUCTION

Analysis of nitrogen content in nutritional mixtures for parenteral or enteral administration implies separation and determination of free amino acids and peptides.

Over the past decade, numerous procedures involving high-performance liquid chromatography (HPLC) or gas-chromatography (GC) have been developed for the determination of amino acids and peptides (1-4).

In the liquid chromatographic area, the development of ion-exchange chromatogaphy (5) involved commercially available amino acid analyzers equipped with ion-exchange resin columns and a ninhydrin reactor widely used, not only for the analysis of amino acids, but also for that of the small peptides. Large peptides have usually been separated on weak ion exchangers such as carboxymethylcellulose (6), by gel filtration (5,7) or bio affinity chromatography (8), and now by capillary zone electrophoresis (CZE) (9,10).

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Recent progress in HPLC has revired the interest in non-polar stationary phase. In fact, the presently most widely used chromatographic technique, which is often referred to as a "reversed-phase" chromatography (RP) employs bound silica-gel and ion-pairing between amino acid and an alkyl sulfonate salt as a mobile phase conter-ion (5, 11-17).

Nevertheless, for all these techniques, pre-column or post-column derivatization was necessary used for detection of the compounds.

This paper describes two new procedures of analysis of underivatized amino acids and peptides by RP-HPLC and one application for the control of amino acids composition in

parenteral nutrition mixtures. Indeed, derivatization involves the substitution of the amino or/and carboxyl fraction. Therefore, with such a procedure in parenteral nutrition mixtures it becomes impossible to distinguish between free and complexed amino acid which is of importance for the bioavailability of the mixture. As an example, a complexe between selenium (IV) and cysteine has been identified by a HPLC method involving direct UV detection at 210 nm when cysteine and selenite are mixed in 4/1 molar ratio (18,19).

MATERIALS AND METHODS

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Chemicals and reagents

Acetonitrile (HPLC grade), trifluoroacetic acid (TFA) and sodium sulfate (p.a) were obtained from MERCK (Darmstadt, RFA), sodium hydroxyde (analytical grade) was obtained from LABOSI (Paris, France), sulfuric acid (analytical grade) was obtained from UCB (Leuven, Belgium), amino acids (standards), peptides (standards) and sodium heptane sulfonate were purchased from SIGMA (Saint Louis, Mo, USA). Water was desionized on an ion exchange resin AQUADEM (France).

Apparatus

All chromatographic procedures were performed with a HEWLETT PACKARD HP 1090A liquid chromatograph (USA) coupled to a Rheodyne model 7125 injector and a diode array detector monitored at 210 nm. All measurements were recorded with a HEWLETT PACKARD HP 9000 / 300 computing integrator (USA). Reversed-phase chromatography was carried out with a Rosil® octadecylsilica or octylsilica stationary phase (ALTECH, Eke, Belgium) both with a nominal particle diameter of 5 µm packed into 250 x 4.6 mm I.D. columns. All injections were made with 50 µl syringes and pH measurements were performed with an ORION model SA 520 pH meter (ORION, Cambridge, Ma, USA).

Chromatographic procedures

Mobile phases were filtered and degased under vacuum.

• For peptide separation, mobile phase, eluent A was 0.1% (v/v) TFA in water and eluent B was 0.1% (v/v) TFA in acetonitrile. Linear gradient rate was 0.5% acetonitrile/min in 40 minutes.

• For amino acid separation, gradient was carried out with 4 g sodium heptane sulfonate and 5 g sodium sulfate per liter of water (eluent A) and the same components in 50% aqueous acetonitrile (eluent B). The gradient is described in figure 2.

Apparent pH of eluents A and B are adjusted at 2.5 for peptide separation and 2.3 for amino acid separation with concentrated sulfuric acid.

The flow rate and column temperature were respectively 0.7 mL/min and 25°C in the two procedures.

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Standard amino acids and peptides solutions were prepared by dissolving the compounds of interest in the eluent A at a concentration of 0.05 to 1.80 mmol/L each.

Parenteral nutrition mixtures (composition presented in table 1) are directly injected.

For amino acids analysis, a standard mixture was done in order to range from 66% to 133% of nominal concentrations of the parenteral solution to be determined. Then this standard is diluted in desionized water before injection, in order to obtain an appropriate calibration curve.

RESULTS AND DISCUSSION

1 - Choice of analytical parameters

RP-HPLC is now the most widely used technique for the analytical, micro and semi-preparative separation of amino acids and peptides. This is due to important reasons including :

Table 1 :
Composition of total parenteral nutrition solution (for 1000 ml of solution)

Glucose	150 g
Amino acids (Primene®)	20 g
Electrolytes :	
Sodium	690 mg
Potassium	977 mg
Calcium	160 mg
Magnesium	24.31 mg
Phosphore	248 mg
Trace elements :	
Iron	1.50 mg
Zinc	7.50 mg
Copper	0.300 mg
Manganese	0.150 mg
Chromium	0.030 mg
Selenium	0.045 mg
Fluoride	0.450 mg
Iodine	0.075 mg

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(i) the excellent resolution that can be achieved for homologous, as well as structurally dissimilar compounds under a wide range of chromatographic conditions ; (ii) the high recovery of solutes (even at microanalytical levels) ; (iii) the reproducibility of the separation ; (iv) the ability to use RP-HPLC to evaluate various physicochemical parameters associated with peptid surface interactions and folding (conformational hierachies) (20).

In the separation and analysis of peptides and amino acids, the molecular processes that rules the interaction between the non-polar stationary phase, the mobile phase and the solute have been extensively described (20-25). Retention process of solutes in RP-HPLC are due to the expulsion of the solute from the polar mobile phase with concomitant adsorption onto the non-polar

stationary phase. The differential elution of the solute species is dependent upon its intrinsic hydrophobicity, the eluotropicity of the mobile phase and the nature of the hydrocarbonaceous stationary phase. Once the stationary phase ligand and the mobile phase have been selected, the other chromatographic parameters such as flow rate and column length have only a marginal effect (21). As cited in the litterature, a decrease in the alkyl chain length fo the support from 18 to 8 carbon atoms produced a statistically significant variation of the so called amino acid group retention coefficient that measure the structure-retention dependencies (20) for the following amino acids when they are included in peptidic bound : Alanine (Ala), Aspartic acid (Asp), Methionine (Met), Glutamine (Gln), Tryptophan (Trp), Leucine (Leu), Phenylalanine (Phe). Furthermore, the strongly hydrophobic amino acids Phe, Leu and Trp, all exhibit increasing retention time when interacting with the octyl silica column rather than octadecyl silica. This strongly suggest the classic hydrophobic interaction that rules the partition of solute between the eluent and the RP support is not, in this case, the only factor involved conversly to free amino-acids mixtures. According to Wilce et al. (20), the amino acids, when present in the peptide contact area, all exhibit an increased apparent hydrophobicity.

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For all these reasons, we selected octyl silica column for the separation of amino acids solutions rather than octadecyl silica which was prefered for peptides separation.

Related to amino acids, several studies showed that the longer the alkyl sulfonate hydrophobic chain length was, the longer the gradient time was (14). We choiced heptane sulfonate (C_7) permiting an acceptable analysis time and an effective resolution. The same results were obtained with octane sulfonate (C_8) as ionpair-ion.

Addition of a neutral sodium salt in the mobile phase favors a better exchange between the two phases (21).

Related to peptides, high resolution mapping is achieved by using column 150-250 mm long, packed with 5 µm low-silanophilic materials operating at low flow rate and long gradient time. High sensitivity is achieved with sensitive detectors operating at 210 nm and by using the balanced absorbance TFA mobile phase (23).

In our experiment, in order to separate amino acids and peptides, it was necessary to optimize the pH value of the eluent at 2.3 - 2.5 to enhance the capacity factor of charged elutes permitting the ion-pair formation with the counterion in the mobile phase and a higher capacity factor of the ion-pair complexe.

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2 - Results

The chromatogram in Fig 1 illustrates the separation of 13 small peptides contained in a standard solution. Free amino acid contents in total parenteral nutrition solution have been separated as shown in Fig 2. Both Glycine and Serine have the same retention time even using a pregradient of solvent A which has the weaker elution strength.

The chromatographic results and statistical evaluation were summarized in Table 2. It can be considered as a selective method due to the absence of interaction between amino acids and the others components in these mixtures

We observed the absence of cysteine wheareas cystine (Cys-Cys) is present (normally absent in these parenteral nutrition solutions) and the presence of not yet described complexes between trace elements (Zinc, Selenium (IV)) and cysteine. For

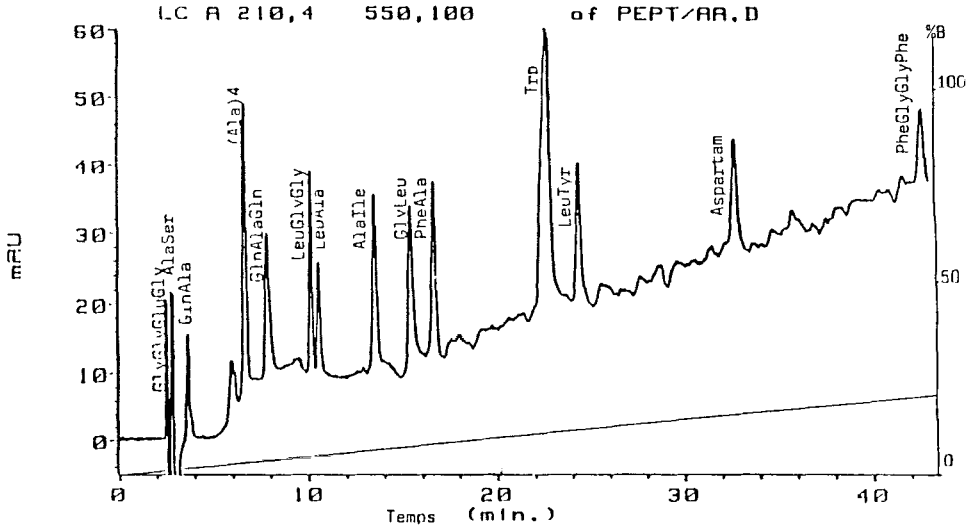

Figure 1 :
Chromatogram of 13 small peptides-column dimensions : 4.6 x 250 mm, flow rate : 0.7 ml/min, column eluted at 25°C with a linear AB gradient (0.5% B/min. during 50 min) where A is 0.1% aqueous TFA and B is 0.1% TFA in acetonitrile, at pH = 2.5

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instance, selenio-cystine, which proceeds from selenium (IV) and cysteine, has a retention time of 33 min. (18). This compound is not stable and releases cystine and selenium on lower oxidation state (II); which can explain the lower bioavailability of selenium yet reported in the litterature(19). The identification of such complexed species is one the main justification of the choice of the direct UV detection.

Despite the increasing performances of RP-HPLC for the separation and analysis of peptides and amino acids, the selectivity of this method was not sufficient enough to separate all amino acids and small peptides present in commercial products. From our own experience, it seems that the elution of

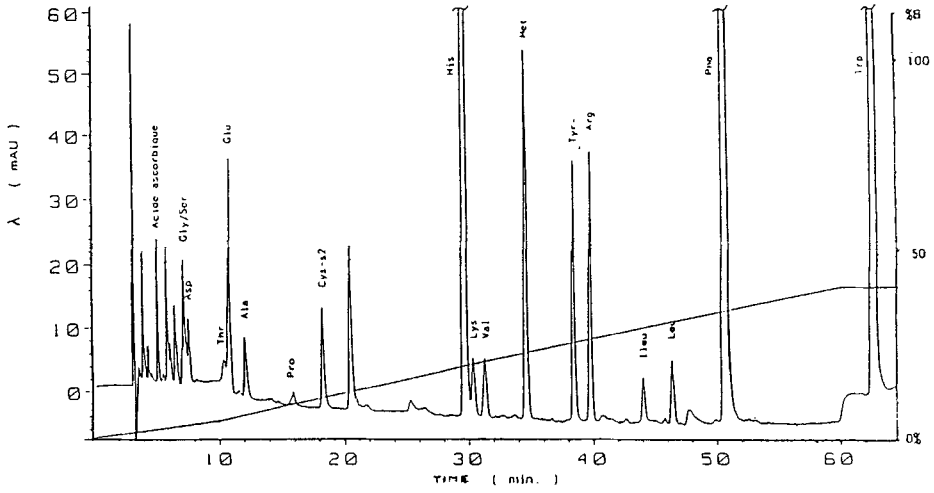

Figure 2 :
Chromatogram of amino acid contents in total parenteral nutrition solution.-column dimensions : 4.6 x 250 mm, flow rate : 0.7 ml/min, column eluted at 25°C with a AB gradient (0-5% B/min. during 10 min, 5-40 % B/min. at 10 to 60 min, 40% B/min. after 60 min) where A is an aqueous solution of sodium heptane sulfonate (4 g per liter) and sodium sulfate (5 g per liter) and B the same components in 50% aqueous acetonitrile.

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a peptide is not only ruled by the polarity of their constituting amino acids, and others factors, as steric parameters or charge of the solvated spheres, certainly play a crucial role towards the interaction with the stationary phase. From our experiment and more precisely, Leu-Gly-Gly, Gly-Leu and Phe-Ala were eluted at the respectives retention times, 10 min., 15 min., 17 min., when Trp was eluted at 23 min. by a linear gradient elution (buffer A = 0.1% aqueous TFA, buffer B = 0.1% TFA in acetonitrile with 0.5% acetonitrile/min.) and a flow rate of 0.7 mL/min. So peptides are sometimes eluted faster than predicted. Conversly to previous works suggesting that the chromatographic behaviour of small peptides depends only of their aminoacidic

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Table 2 :
Results of method validation (coefficient of correlation is calculated by least square method)

Amino acids		Concentration (mmol/l)	Mean retention time	Reproducibility		Linearity	
				Mean area ± σ (n=10)	CV(%)	Coefficient of correlation	Regression line parameters
Glycine/Serine	GLY/SER	1.82	8.10	131 ± 6,2	4.7	0.9986	9.7 X + 0.16
Aspartic acid	ASP	0.90	8.40	136 ± 3.2	2.3	0.9983	11.9 X + 0,38
Threonine	THR	0.62	10.30	64.4 ± 1.78	2.7	0.9983	11.4 X - 0.14
Glutamic acid	GLU	1.36	10.60	246 ± 9.1	3.7	0.9989	16.6 X + 0.30
Alanine	ALA	1.80	11.80	160 ± 4.8	3.0	0.9987	5.6 X + 0.15
Cysteine	CYS	0.314	13.00	171 ± 5.5	3.2	0.9991	36 X + 0.10
Proline	PRO	0.520	15.30	42 ± 3.5	8.5	0.9885	4.4 X + 0.14
Cystine	CYS-S2	0.157	16.85	590 ± 11.2	1.9	0.9982	266 X + 2.10
Lysine	LYS	0.490	30.00	5687 ± 136	2.4	0.9995	716 X + 3.70
Histidine	HIS	1.50	30.95	148 ± 8.5	5.7	0.9857	4.4 X + 0.09
Valine	VAL	1.30	31.90	158 ± 5.7	3.6	0.9967	5.9 X + 0.26
Methionine	MET	0.322	35.40	978 ± 26.6	2.7	0.9993	165 X + 0.96
Tyrosine	TYR	0.050	40.65	565 ± 15.5	2.7	0.9994	676 X + 0.81
Arginine	ARG	0.964	43.15	609 ± 21,5	3.5	0.9996	35 X + 1.40
Isoleucine	ILE	1.02	47.20	128 ± 6.7	5.3	0.9966	5.6 X + 0.32
Leucine	LEU	1.52	49.70	190 ± 9.8	5.2	0.9860	6.1 X + 0.34
Phenylalanine	PHE	0.508	53.70	15205 ± 357	2.3	0.9992	1561 X + 24.6
Tryptophan	TRP	0.196	65.40	10386 ± 305	2.9	0.9990	3279 X + 2.70

composition (23), it seems that peptidic bound plays a crucial role in the retention of peptides.

In order to solve the cited separation problems, il seems that the high selectivity of the ligand exchange procedure combined with the high efficiency obtained with the silica microparticles made ligand exchange copper (II) chromatography offers an interesting alternative. Further studies on this field are being carried out.

CONCLUSION

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A rapid and simple method was developed using RP-HPLC for the determination of free amino acids contents in total parenteral nutrition solutions and small peptides content in enteral mixtures. The separation was performed on a reversed-phase column using the ion-pair formation. With direct detection at 210 nm, post derivatization is not necessary. The complete separation of 17 amino acids and 13 peptides was performed with a mobile phase gradient, and trace elements/amino acids complexes could have been detected.

Becaus of the role of peptidic bound in the retention of peptides, other new selective method has to be choisen as ligand-exchange procedure.

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