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Short Communication

Densitometry for analysis of protein and peptide hydrolysates: application to luteinizing hormone releasing hormone

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ABSTRACT

Densitometric analysis has been performed on various standard protein and peptide solutions and on luteinizing hormone releasing hormone hydrolysate. After thin-layer chromatographic separation using two-dimensional separation on cellulose plates, the plates were mapped and each amino acid was assayed individually in order to obtain a specific integration profile of the hydrolysate. Densitometry proved to be a useful method for the screening of protein hydrolysates and is accurate enough for the assay of peptide hydrolysates. The hormone was hydrolysed with hydrochloric acid–trifluoroacetic acid (1:1, v/v) in the presence of thioglycolic acid. This limits the loss of tryptophan and does not interfere with the analysis. Results were sufficiently reliable, accurate and reproducible for routine analysis.

INTRODUCTION

Human peptides and proteins, obtained by peptide synthesis or genetic engineering, are used increasingly in therapy. The amino acid sequence has to be checked after each production run, and this is done either with classical sequencers or by reversed-phase high-performance liquid chromatography (HPLC). The latter method has disadvantages, however, derivatization is often difficult when the amino acids are at low concentrations in complex mixtures, and the derivatives formed have poor stability. Also, when the number of amino acids is high and their proportions vary widely, the analysis becomes too lengthy for routine work. Hence we looked for a simple and rapid method for routine batch screening, where HPLC could provide complementary precision analysis when the need arose. Thin-layer chromatographic (TLC) separation of the amino acids, followed by densitometric assay, proved promising.

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EXPERIMENTAL

Chemicals

Analytical-grade reagents and HPLC solvents were obtained from Merck (Darmstadt, Germany). Amino acids and hydrolysates were purchased from Sigma (La Verpillère, France). Luteinizing hormone releasing hormone (LH-RH) was kindly donated by Pr. A. V. Schally (Tulane University, New Orleans, LA, USA).

Materials

HPLC analyses were performed using a Waters chromatographic system consisting of two Model 510 pumps, a Model U6K injector, a Model 680 gradient programmer, a Model 490 programmable multi-wavelength spectrophotometer and a D2000 (Merck) recorder–integrator.

Densitometric analyses were carried out with a GS-9000 dual-wavelength flying-spot scanner (Shimadzu, Tokyo, Japan) and with a TLC Scanner II (Camag, Muttenz, Switzerland) fitted with CATS 3 software. Samples were applied with a Linomat IV TLC spotter from Camag.

High-performance liquid chromatography

Amino acids were derivatized using 4-dimethylaminobenzene-4′-sulphonyl chloride (DABS), previously recrystallized from acetone as described by Chang [1]. DABS-amino acids were then separated using the method of Stocchi *et al.* [2] on a Spherisorb ODS2 column (5 μm particle size, 250 mm × 4.6 mm I.D.). The mobile phase consisted of two eluents: solvent A, potassium dihydrogenphosphate (0.1 *M*, pH 6.8); and solvent B, acetonitrile–2-propanol (80:20, v/v). Conditions were as follows: 1 min at 20% B; 6 min up to 23% B; hold for 16 min; 1 min to 27% B; hold for 16 min; 20 min up to 70% B; hold for 10 min. The initial conditions were restored in 8 min. The detection wavelength was 436 nm, and the sensitivity 0.05 a.u.f.s.

Thin-layer chromatography

TLC separations (ascending development mode, unsaturated chamber) were performed with cellulose high-performance TLC (HPTLC) plates, 10 cm × 10 cm (Merck 15 035) previously washed with methanol as described by Fournier [3].

Elution in the first dimension was performed with solvent C: 2-propanol–2-butanone–1 *M* HCl (60:15:25, v/v). The migration distance was 80 mm. Plates were dried for 15 min at room temperature, for 2 min under a nitrogen flow, and for 5 min at 40°C. The second dimension was carried out with *tert.*-butanol–2-butanone–acetone–distilled water–28% NH_4OH–methanol (40:20:20:14:5:1, v/v). Solvents were prepared immediately before use. Plates were sprayed using the ninhydrin–collidin reagent [4] or the ninhydrin–cadmium acetate reagent [5],

followed by 15 min heating at 60°C. The TLC analysis times were 90 and 60 min, respectively.

LH-RH acid hydrolysis

LH-RH (5 mg) was hydrolysed, as described by Fournier [3], in sealed tubes at 155 ± 0.5°C for 30 min containing 10 ml of HCl–trifluoroacetic acid (TFA) (2:1, v/v) containing 5% thioglycolic acid.

RESULTS AND DISCUSSION

In order to determine if the densitometric assay of protein hydrolysates was sufficiently reliable, accurate and reproducible for routine use, we compared the results from densitometry with those obtained by HPLC for various hydrolysates.

HPLC method

We chose pre-column derivatization with dabsyl chloride by the method of Stocchi *et al.* [2]. The analysis took 65 min and afforded perfect separation of 23 amino acids, including Trp and Orn (Fig. 1). The coefficient of variation (C.V.) measured on five assays was always less than 4.1%, indicating excellent reproducibility.

TLC method

Various methods were tried, in particular using reversed-phase plates [6–8]. The most satisfactory was the two-dimensional separation on cellulose HPTLC plates described by Fournier [3], which resolved 23 amino acids within 3 h without sample pretreatment. Two methods were tried for visualization: ninhydrin–collidin, with a detection limit of *ca.* 0.15 μg, and ninhydrin–cadmium acetate, which is more sensitive (detection limit 0.1 μg) but has the disadvantage of being

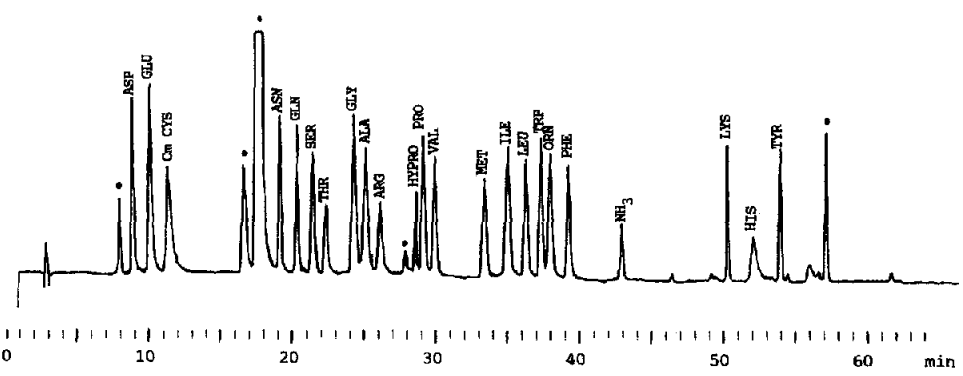

Fig. 1. HPLC separation of DABS-amino acids as described in Experimental; injection volume, 10 μl. (●) By-products originating from the excess of reagent.

polychromatic. This property is very useful when mere visualization of amino acids is sought, but it makes densitometric assay complicated because each spot has to be read at its own absorption maximum. Ninhydrin–collidin was therefore preferred.

Calibration curves

A seven-point calibration curve was plotted for each amino acid for concentrations between 0.5 and 5 μg. To limit diffusion, the sample volume has to be as small as possible, preferably less than 2.5 μl. However, with a loading speed of 15 s/μl and a 10-s pause every 0.5 μl, up to 15 μl can be loaded. For each concentrations, the same sample volume has to be loaded, and the concentration read with the same light-beam width. Under these conditions, the linearity was satisfactory between 0.5 and 3 μg, and excellent between 0.75 and 2 μg.

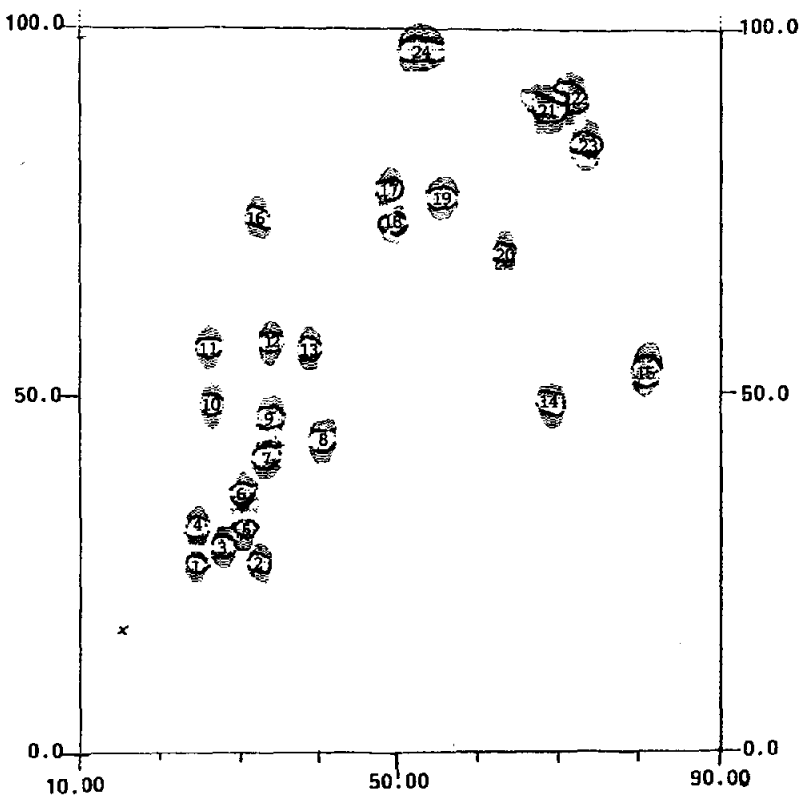

Fig. 2. Mapping of standard hydrolysate: dump volume, 10 μl; contour map mode; detection wavelength, 577 nm; threshold level, 0.40; interval, 1 mm. Spots: 1 = Orn; 2 = His; 3 = Lys; 4 = Arg; 5 = Asn; 6 = Gln; 7 = Gly; 8 = Ser; 9 = OH-Pro; 10 = Asp; 11 = Glu; 12 = Ala; 13 = Pro; 14 = Thr; 15 = Cys; 16 = $(Cys)_2$; 17 = Val; 18 = Tyr; 19 = Met; 20 = Trp; 21 = Ile; 22 = Leu; 23 = Phe; 24 = thioglycolic acid.

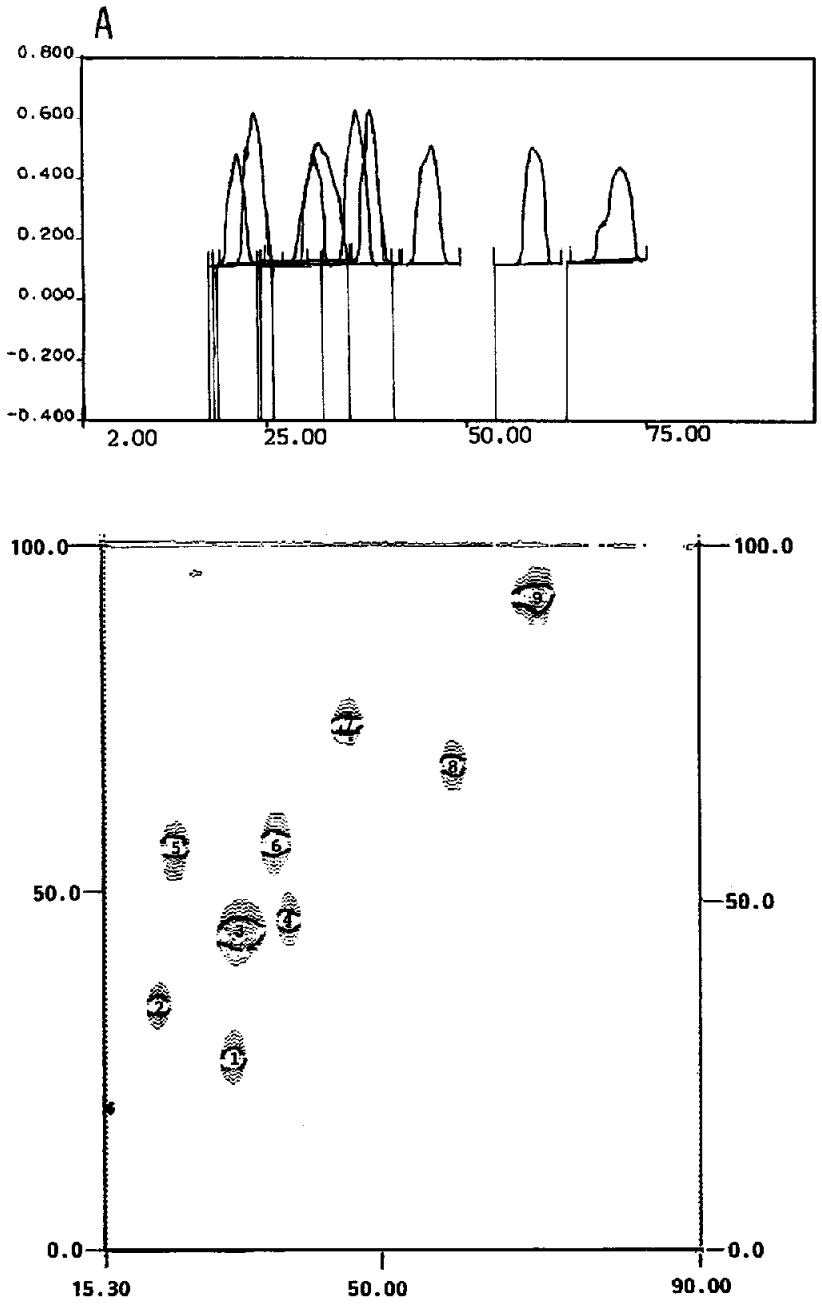

Fig. 3.

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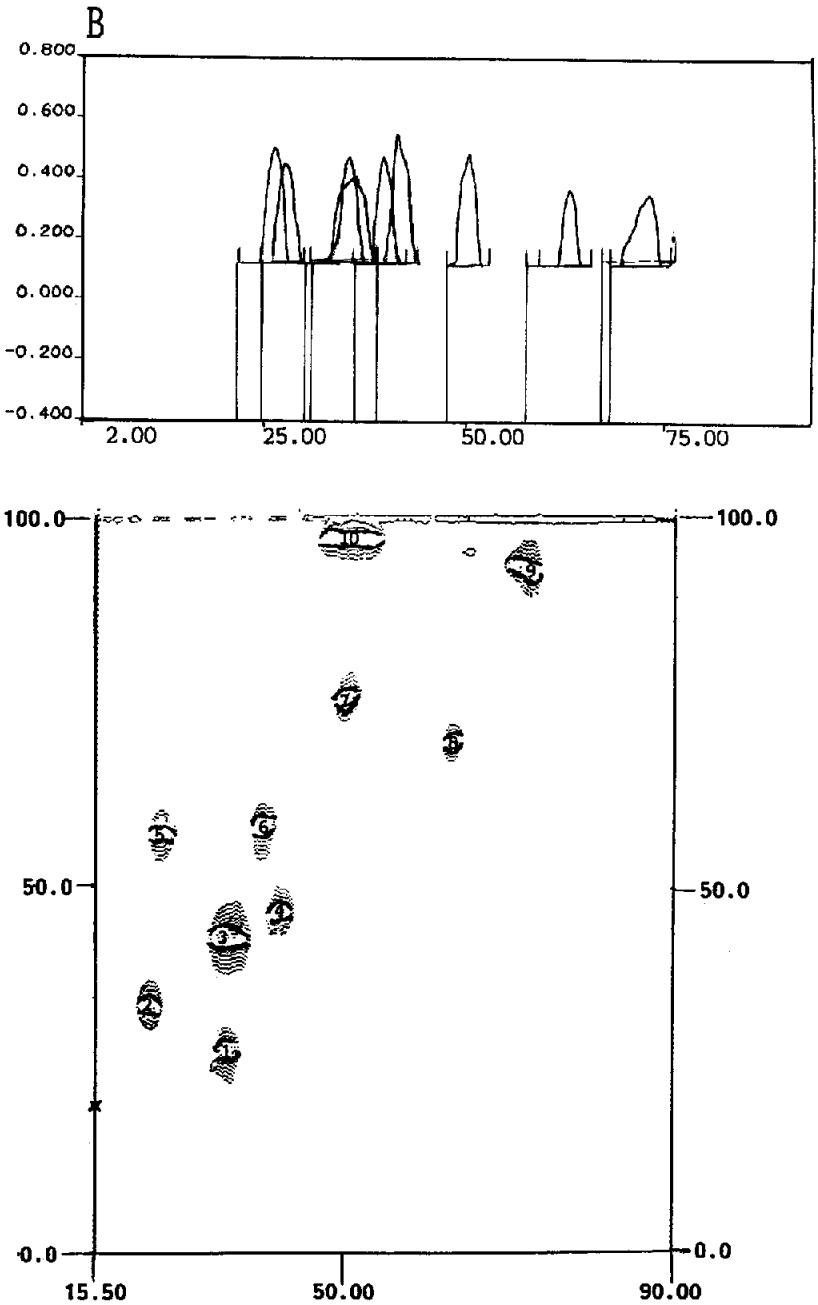

Fig. 3. Densitograms and mappings of LH-RH hydrolysate: dump volume, 15 μl; contour map mode; detection wavelength, 577 nm; threshold level, 0.25; intervals, 1 mm. (A) Synthetic solution; (B) assay. Spots: 1 = His; 2 = Arg; 3 = Gly; 3 = Ser; 5 = Glu; 6 = Pro; 7 = Tyr; 8 = Trp; 9 = Leu; 10 = thioglycolic acid.

Protein hydrolysate analysis

The reliability of the method was assessed by carrying out a preliminary run using a solution designed for calibrating sequencers (Sigma A216) enriched in tryptophan and hydroxyproline (2.5 μmol/ml). Separation was qualitatively excellent (Fig. 2) but quantitatively disappointing.

As the instruments used did not allow automatic assay of samples, the reading coordinates had to be determined manually. When the amino acids are close together, it would seem desirable to take a reading of the intensity at the centre of the spot in order to minimize interference, but the difficulty of operating under perfectly reproducible conditions resulted in a high C.V. (10–25%). An assay performed over the whole spot gave good results when the amino acids were well resolved (Trp), but is liable to be seriously inaccurate when the amino acids are very close together (Orn, His, Lys, Arg, Asn, Gln, Leu and Ile). The method proposed is thus not accurate enough to replace HPLC. However, it affords a means for rapid routine screening of samples that may subsequently be analysed by HPLC.

A control plate was prepared, visualized with ninhydrin–collidin and detected at 577 nm. It is preferable to use the system commercialized by Shimadzu, which provides for two-dimensional reading of the plates. The plate was mapped and each amino acid assayed individually using a beam width appropriate for each spot. Mapping was made in the contour map mode, which provides a good densitometric profile of the hydrolysate. The threshold value is chosen in order to discriminate the background noise. The interval value is chose in order to visualize the diffusion of the spot. For each amino acid, a set of values (threshold, threshold plus one interval, threshold plus two intervals, etc.) is printed, each mark being used as a threshold. Quantitation was made in the zigzag scanning mode using the flying spot method. This offers much better quantitation than linear scanning, owing to the homogeneity of the light intensity throughout the beam area. A specific integration profile of the protein to be analysed was thereby obtained. Under these conditions, the C.V. measured for five analyses ranged from 1.7 to 5.1%, except for the amino acids that migrate extensively in both directions (Ile, Leu and Phe) for which it was between 8.7 and 10.3%. However, this is still adequate for screening purposes. The C.V. for Trp was less than 3%.

When the ratios of the different amino acids are very different, two or more control plates should be prepared with appropriate volumes of amino acids, in order to increase the accuracy of the assay. Trials with test mixtures corresponding to h-GH (human growth hormone) and t-PA tissue (tissular plasminogen activator) showed that the accuracy is sufficient to specify the exact ratio of each amino acid.

Hence densitometry is of obvious interest, though it needs further development for fine work. The software currently provided with the instrument does not exploit all its possibilities fully: a wider choice of beam widths would be an advantage when widely ranging amino acid concentrations are encountered, in

TABLE I

ANALYSIS OF SOME BIOACTIVE PEPTIDES

T = theoritical concentration; D = densitometric analysis; ND = not determined.

Amino acid	Human angiotensin I standard			Human angiotensin II standard			Bradykinin standard			Leu-enkephalin standard			Met-enkephalin standard			LH-RH				
																Standard			Assay	
	T	HPLC	D	T	HPLC	D	T	HPLC	D	T	HPLC	D	T	HPLC	D	T	HPLC	D	HPLC	D
Asp	1	0.952	0.973	1	0.969	0.949	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ser	–	–	–	–	–	–	1	ND	0.946	–	–	–	–	–	–	1	0.962	0.948	0.856	0.849
Gly	–	–	–	–	–	–	1	ND	0.965	2	ND	1.973	2	ND	1.968	2	1.910	1.893	1.831	1.787
Arg	1	0.957	0.934	1	0.971	0.939	2	ND	1.967	–	–	–	–	–	–	1	0.978	0.948	0.895	0.917
Pro	1	0.912	0.956	1	0.971	0.947	3	ND	2.953	–	–	–	–	–	–	1	0.957	0.932	0.905	0.904
Val	1	0.961	0.964	1	0.964	0.953	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ile	1	0.973	0.911	1	0.972	0.943	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Leu	1	0.966	0.904	–	–	–	–	–	–	1	ND	0.923	–	–	–	1	0.968	0.913	0.921	0.879
Phe	1	0.965	0.921	1	0.966	0.929	2	ND	1.987	1	ND	0.954	1	ND	0.957	–	–	–	–	–
Tyr	1	0.972	0.965	1	0.959	0.957	–	–	–	1	ND	0.958	1	ND	0.949	1	0.972	0.897	0.969	0.953
His	2	1.961	1.955	1	0.951	0.943	–	–	–	–	–	–	–	–	–	1	0.974	0.935	0.814	0.787
Met	–	–	–	–	–	–	–	–	–	–	–	–	1	ND	0.987	–	–	–	–	–
Glu	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	0.978	0.954	0.899	0.921
Trp	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	0.965	0.972	0.821	0.839

order to obtain greater accuracy and to program the integration of the spots from the reading co-ordinates. It would also be convenient to be able to store control hydrolysate data, enlarge it, and subtract or add it to the data from an analysis. In this way, specific amino acids could be singled out for assay. A three-dimensional integration profile would be valuable for the analyses of proteins containing a large number of different amino acids.

Peptide hydrolysate analysis

We first prepared six standard solutions corresponding to the hydrolysates of six biologically active peptides. The number of amino acids ranged from 5 to 10. The solutions were assayed by HPLC and densitometry. The results (Table I) show the densitometric detector to be sufficiently accurate for routine work. We then carried out a trial on a true hydrolysate; we chose LH-RH as it contains tryptophan, a particularly fragile amino acid, and because it is widely used. The hydrolysis was done on a 0.5% solution in the presence of thioglycolic acid (Fig. 3). This limits the loss of trytophan and does not interfere with the analysis. The results obtained (Table I) are satisfactory, showing that densitometry can be used for routine analysis.

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