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Automated amino acid analysis using precolumn derivatization with dansylchloride and reversed-phase high-performance liquid chromatography[a]

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SUMMARY

An automated procedure for the precolumn derivatization of amino acids with dansylchloride and a liquid chromatographic method for separation of the derivatives with fluorimetric detection in the picomole range are reported. The method involves a simple solvent preparation, which does not require the inclusion of non-volatile buffers of low ionic strength and delicate pH adjustments. The procedure was also utilized for the identification of COOH-amidated amino acids released from peptides after digestion with carboxypeptidase.

INTRODUCTION

Classical ion-exchange chromatography with subsequent post-column ninhydrin derivatization still remains the most reliable methodology for routine amino acid analysis in the nanomole range. For the detection of amino acids in the picomole range, a number of procedures have been described which require precolumn derivatization with reagents such as phenyl isothiocyanate (PITC), 4-dimethylaminoazobenzene-4-sulphonyl chloride (DABS-Cl), dimethylaminoazobenzene 4-isothiocyanate (DABITC), *o*-phthalaldehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC-Cl) or dimethylaminonaphthalene-1-sulphonyl (dansyl) chloride[1–7], followed by separation and identification of the derivatives by reversed-phase high-performance liquid chromatography (RP-HPLC). The state-of-the art of these techniques, and in particular of amino acid analysis using phenylisothiocyanate derivatization, was recently reviewed[8]. The success of the different procedures reported in the literature depends on a multiplicity of factors, such as the possibility of finding reaction conditions adequate for all protein amino acids, the ease of automation of the procedure, the stability and spectroscopic properties of the derivatives and the effi-

[a] Dedicated to Professor G. B. Marini Bettolo on the occasion of his 75th birthday.

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ciency of the chromatographic method proposed for their separation and identification.

These methods often prescribe the inclusion of non-volatile buffers of low ionic strength and with pH values in the range 3.5–6.5 in the formula of at least one of the solvents to be used for the chromatographic separation. Slight alterations of these parameters can modify significantly the retention times of several amino acids. Further, salts adversely affect the maintenance of the hydraulic components of the apparatus and the lifetime of the column.

Using an automated version of the precolumn derivatization procedure with dansyl chloride, we developed an HPLC procedure capable of separating dansylamino acids and derivatives using a system of simply prepared volatile solvents. The entire procedure is flexible enough to permit both an adequately sensitive amino acid analysis of protein and peptide hydrolysates and the detection of some post-translationally modified forms of amino acids, *e.g.*, COOH-amidated residues.

EXPERIMENTAL

Acetonitrile and 2-propanol (HPLC grade) were obtained from Carlo Erba, trifluoroacetic acid, sodium hydrogencarbonate and sodium hydroxide from Merck, dansyl chloride from Fluka, amidated amino acids, dansylamino acid standards and N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES) from Sigma, L-[U-^{14}C]phenylalanine from New England Nuclear and carboxypeptidase Y (CPY) from Boehringer. A standard solution of amino acids (Pierce) was diluted in water (0.1 m*M*) and then stored at −20°C. Deionized water was first glass-distilled and then passed through a Sep-Pak C_{18} cartridge (Millipore).

Samples of peptides to be hydrolysed (25–250 pmol) were dried in recently pyrolysed glass microtubes (40 × 6 mm I.D.) after addition of 2 nmol of norleucine as internal standard, and then enclosed in a Pyrex container with a 1-ml layer of 5.7 *M* hydrochloric acid plus 0.1% phenol at the bottom. The container was evacuated, flushed with nitrogen, evacuated again and sealed. Hydrolysis was performed in the vapour phase for 20–24 h at 110°C. After the hydrolysis, the tubes were dried *in vacuo* at 40°C over sodium hydroxide pellets for 30 min. Automated dansylation of amino acid mixtures was achieved by using a Gilson auto-sampling injector consisting of two modules, a Model 231, sample injector equipped with a code 31 rack thermostated at 35°C and a 20-μl sample loop, and a Model 401 diluter, both controlled by the sample controller keypad. The rack positions 18/2, 18/4 and 18/6 were modified to accommodate glass vials (Pierce, 32 × 12 mm I.D.) containing the reagents. The tubes with dried samples were placed on the rack of the auto-sampling injector, which automatically dissolved the amino acid mixture in 10 μl of 0.1 *M* $NaHCO_3$–5 m*M* EDTA (pH 9.0), and then performed the derivatization by adding 10 μl of the dansylchloride solution (2.5 mg/ml in acetonitrile) followed by incubation for 30 min. One minute before loading onto the column, 10 μl of 0.4 *M* sodium hydroxide solution were added to the tubes to hydrolyse the excess of unreacted dansylchloride; 20 μl of this mixture were loaded onto the column.

For the chromatographic analysis, the apparatus consisted of two Waters Model 510 pumps (Millipore); gradient formation, quantification of chromatographic peaks and data treatment were effected by an IBM Model 286 PC XT computer using a Baseline 810 program (Millipore).

Separation of dansylamino acids was carried out on a Beckman Ultrasphere column (RP-18, 5 μm, 250 × 4.6 mm I.D.), thermostated at 48°C. The column was protected by a 2-μm stainless-steel filter (Rheodyne).

For the chromatographic separation a gradient was formed with two mobile phases: solvent A, 0.05% trifluoroacetic acid; and solvent B, acetonitrile–2-propanol (4:1, v/v). The flow-rate was 1.2 ml/min. The column effluent was mixed before the detector with 0.05 *M* sodium hydroxide in 20% acetonitrile using a Beckman Model 110A pump with the flow-rate set at 200 μl/min. This procedure permits the optimization of the fluorescence response of dansyl derivatives[9].

Dansylamino acids were detected using a Fluorichrom fluorescence detector for liquid chromatography (Varian), equipped with an excitation band filter at 280–340 nm and sharp emission cut-off filter at 430 nm.

Conventional amino acid analysis by ion-exchange chromatography (IEC) and post-column ninhydrin derivatization was performed using an LKB 2131 Alpha Plus instrument.

Digestion of peptides (1 nmol or less) with carboxypeptidase Y was performed at 35°C in 100 μl of 20 m*M* HEPES (pH 8.0) containing 2 nmol of norleucine as internal standard; the enzyme–substrate ratio was 1:50 (w/w). Aliquots of 20 μl, usually drawn at 1, 5, 10, 20 and 30 min, were immediately lyophilized and then derivatized with dansylchloride. Half of each sample was loaded onto the column for analysis; the remainder was dried, subjected to acid hydrolysis and then analysed.

RESULTS AND DISCUSSION

A simple program for the automated dansylation of amino acids and their derivatives was prepared for the Gilson auto sample injector and is reported in Table I.

The dansylation reaction yields stable, highly fluorescent derivatives with both primary and secondary amino acids[3]; no troublesome extraction of the products is required before analysis. According to the present version of the method, the samples are processed on-line under conditions which permit a complete dansylation also of the slowly reacting amino acids. The efficiency of dansylation and possible losses of the sample during automated derivatization were controlled by counting the radioactivity of an aliquot of a labelled phenylalanine solution before and after derivatization, and by counting the radioactivity of the dansylphenylalanine peak collected from the HPLC effluent, after automated injection of an aliquot of the labelled sample onto the column. The recovery was always ⩾95%. The automated addition of dilute sodium hydroxide solution to the samples after the derivatization reaction effects the complete hydrolysis of unreacted dansyl chloride, with a consequent improvement of the baseline quality.

All dansyl derivatives of amino acids obtainable from protein hydrolysates can be separated in 30 min by using a system of volatile solvents of very simple preparation. The total running time, including column recycling, is about 34 min. The conditions for the chromatographic analysis are given in Table II and a typical chromatographic separation is shown in Fig. 1. The percentage of trifluoroacetic acid in solvent A is critical for the separation of Asp–Glu and Ala–Arg derivatives. Only slight adjustments of the gradient parameters are occasionally required to ensure prolonged

TABLE I

PROGRAM FOR AUTOMATED DANSYLATION WITH THE GILSON AUTO SAMPLER INJECTOR

```
1  RACK CODE 31
2  AUXIL 6/1
3  INPUT C/1
4  INPUT C0/41
5  INPUT C6/23
6  C1=0
7  FOR C2=1/10
8  FOR C3=1/6
9  C1=C1+1
10 PRINT/80
11 TUBE 18/4
12 HEIGHT
13 ASPIR 0/50/2
14 HEIGHT
15 ASPIR 0/12/0
16 HEIGHT
17 TUBE C2/C3
18 HEIGHT -1
19 DIS 0/18/0
20 TUBE 18/6
21 HEIGHT +7
22 ASPIR 0/12/0
23 TUBE C2/C3
24 HEIGHT -1
25 DIS 0/18/0
26 FOR C4=1/4
27 ASPIR 0/10/0
28 HEIGHT 0
29 DIS 0/11/0
30 HEIGHT -1
31 NEXT C4
32 HEIGHT
33 RINSE
34 DIS 0/1000/9
35 C5=0
36 FOR C5=1/C0
37 PRINT C5/25
38 WAIT 1
39 NEXT C5
40 PRINT/75
41 TUBE 18/2
42 HEIGHT
43 ASPIR 0/50/2
44 HEIGHT +8
45 ASPIR 0/10/1
46 TUBE C2/C3
47 HEIGHT -1
48 DIS 0/15/1
49 FOR C5=1/3
50 ASPIR 0/15/0
51 HEIGHT 0
52 DIS 0/17/0
53 HEIGHT -1
54 NEXT C5
55 HEIGTH
56 RINSE
57 DIS 0/1000/9
58 WAIT 30
59 PRINT C1/1
60 TUBE C2/C3
61 IF C6=0
62 GO TO 75
63 DIS 0/C6/0
64 HEIGHT
65 ASPIR 0/50/0
66 HEIGHT 0
67 FOR C5=1/3
68 ASPIR 0/20/0
69 HEIGHT 0
70 DIS 0/22/0
71 HEIGHT -1
72 NEXT C5
73 HEIGHT
74 WAIT 30
75 HEIGHT -1
76 ASPIR 0/25/0
77 TUBE 0/0
78 DIS 0/27/0
79 WAIT 2
80 INJECT 1
81 AUXIL 7/2
82 WAIT /3/1
83 WAIT 100
84 INJECT 0
85 DIS 0/500/4
86 RINSE
87 DIS 0/1000/9
88 IF C1=>C
89 GO TO 92
90 NEXT C3
91 NEXT C2
92 WAIT C0
93 AUXIL 6/0
94 HOME
```

efficacy of the chromatographic performance. Under these conditions the column has a long lifetime (about 4000 analyses).

The reproducibility of the retention time of each derivative is shown in Table III. The coefficient of variation (C.V.) for each retention time was within 0.2% in six successive runs.

TABLE II

GRADIENT PARAMETERS

Time (min)	*Composition*[a] *A (%)*	*B (%)*	*Curve*[b] *(type)*
0.0	93.0	7.0	
1.0	93.0	7.0	0
9.0	86.0	14.0	+1
24.0	57.0	43.0	+1
28.0	30.0	70.0	+7
28.2	0.0	100.0	+1
30.0	0.0	100.0	0
30.7	93.0	7.0	+1

[a] Solvents: A, 0.05% trifluoroacetic acid; B, acetonitrile–2-propanol (4:1, v/v).
[b] Waters gradient curve: 0 = isocratic; +1 = linear; +7 = convex.

For fluorescence detection we used a broad transmission band filter (280–340 nm) and an emission filter with a cut-off at 430 nm, as the nature of our solvent system affects both the fluorescence spectra and the intensity of the various dansyl-amino acids differently. The linearity of response of the method of derivatization was tested for amino acid standards at different concentration levels; the results are shown in Fig. 2. Peak area was proportional to the concentration of amino acid in the dansylation tubes from 0.3 to 50 μM. Inclusion of EDTA in the derivatization reaction mixture is essential in order to obtain good yields with those amino acids whose solubility is impaired by the formation of salts with cations extracted from glass during the hydrolysis[10].

All these features render this procedure an attractive alternative to other derivatization reactions for amino acid analysis at the picomole level. Moreover, the liquid

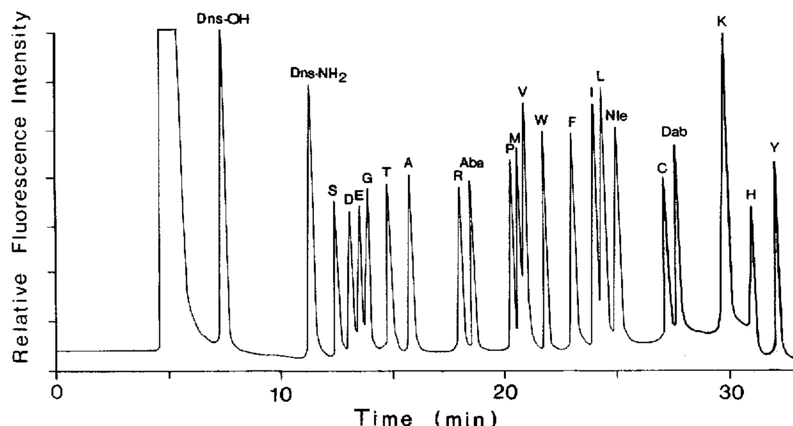

Fig. 1. Separation of an amino acid mixture after automated derivatization with dansylchloride. Sample size, 100 pmol of each amino acid. The peaks are labeled by one-letter abbreviations for the usual protein amino acids; Aba = α-aminobutyric acid, Nle = norleucine, Dab = 2,4-diaminobutyric acid, Dns-OH = 1-dimethylaminonaphthalene-5-sulphonic acid, $Dns\text{-}NH_2$ = 1-dimethylaminonaphthalene-5-sulphonamide.

TABLE III

REPRODUCIBILITY OF RETENTION TIMES OF DANSYLAMINO ACIDS

Compound	*Retention time (min)*	*C.V. (%)*
Dansyl-OH	7.21	0.18
Dansyl-NH_2	11.31	0.15
Dansyl-Ser	12.52	0.21
Dansyl-Asp	13.09	0.21
Dansyl-Glu	13.53	0.15
Dansyl-Gly	13.85	0.28
Dansyl-Thr	14.84	0.13
Dansyl-Ala	15.80	0.08
Dansyl-Arg	18.04	0.30
Dansyl-Aba	18.10	0.18
Dansyl-Pro	20.34	0.17
Dansyl-Met	20.70	0.08
Dansyl-Val	20.92	0.13
Dansyl-Phe	22.06	0.09
Dansyl-Ile	24.05	0.16
Dansyl-Leu	24.42	0.16
Dansyl-Nle	25.03	0.17
Di-dansyl-Cys	27.21	0.19
Di-dansyl-Dab	28.08	0.22
Di-dansyl-Lys	29.78	0.21
Di-dansyl-His	30.99	0.27
Di-dansyl-Tyr	32.09	0.20

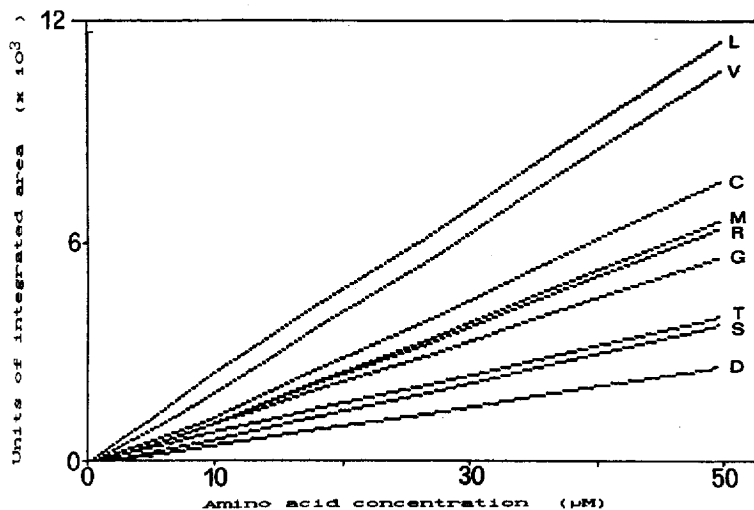

Fig. 2. Relationship between different concentrations (0.3, 1, 10, 50 μM) of amino acids and peak area of the corresponding derivatives after automated dansylation and chromatographic separation. For the sake of clarity only data related to some representative amino acids are reported.

chromatographic separation of dansyl derivatives can be used in the course of sequence determinations of peptides according to the manual dansyl–Edman procedure[11]. In fact, this procedure is still widely used not only because of its simplicity and low cost, but also because in some instances it gives more sequence information than expensive automated machines. The different scheme of the extraction steps may be very convenient for the analysis of hydrophobic peptides.

It should be noted that the vapour-phase hydrolysis of peptides, as described here, is both easy to perform and necessary in order to minimize contamination from various sources when analysing micro amounts of samples.

A comparison of the amino acid compositions of some bioactive peptides determined by the present method and by conventional IEC is reported in Table IV.

Finally, we adopted the automatic dansylation procedure for the analysis of amidated amino acid residues, which are often found at the C-terminus of biologically active peptides. The peptide of interest is digested with carboxypeptidase Y under conditions (see Experimental) that minimize the amidase activity. In fact, it has been shown that the salt composition and pH of the solvent affect to different extents the various enzymatic activities of carboxypeptidase Y, and in particular the amidase and the peptidyl amino acid amide hydrolase activities[12,13]. The time course of the release of amino acids from the C-terminus is monitored by analysing appropriate aliquots of the digestion mixture by the procedure described above. Under these conditions, at least a portion of the C-terminal amidated residue is released intact. It can be identified by direct HPLC analysis of its dansyl derivative. The identification can be confirmed by identifying in a second aliquot, after acid hydrolysis of the dansylamide, the corresponding dansylamino acid. Alternatively, the peptide can be subjected to the appropriate number of cycles of the Edman degradation; having subtracted the penultimate residue, the tube containing the putatively amidated C-terminal residue is

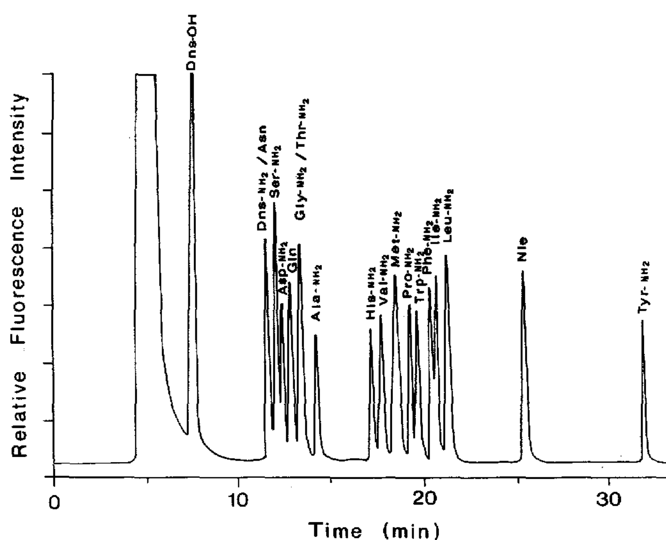

Fig. 3. Separation of an amidated amino acid mixture after automated derivatization with dansylchloride. Sample size, 200 pmol of each compound.

TABLE IV

AMINO ACID COMPOSITION OF BIOACTIVE PEPTIDES DETERMINED BY THE PRESENT METHOD[a] (Dns-Cl) AND BY CONVENTIONAL ION-EXCHANGE CHROMATOGRAPHY (IEC)

	HP1[b]			*HP2*[b]			*SP*[c]			*K*[c]		
Amino acid	*Dns-Cl*	*IEC*	*Sequence*[d]	*Dns-Cl*	*IEC*	*Sequence*[d]	*Dns-Cl*	*IEC*	*Sequence*[d]	*Dns-Cl*	*IEC*	*Sequence*[d]
Asp							1.8	1.7	2	0.9	0.9	1
Ser	1.0	1.1	1									
Glu	0.9	1.0	1				2.0	2.0	2	2.0	1.9	2
Pro	1.2	1.2	1	1.1	1.2	1	2.1	1.8	2	2.3	1.7	2
Gly	0.9	1.0	1	2.0	1.8	2	1.0	1.0	1	1.0	1.0	1
Ala	1.8	2.0	2	1.2	1.0	1						
Val										1.1	0.9	1
Met							1.1	0.9	1	1.0	1.0	1
Ile	1.9	2.1	2	1.1	1.0	1						
Leu	3.0	3.8	3	5.2	4.9	5	0.9	1.0	1	1.0	0.9	1
Phe	2.2	2.5	2	2.0	2.4	2	1.9	1.7	2	0.7	0.9	1
His										0.7	0.8	1
Lys				1.1	1.0	1						

[a] For hydrolysis and derivatization, see Experimental.
[b] Haemolytic peptides (HP) from the skin of *Rana esculenta*.
[c] Substance P-like (SP) and kassinin-like (K) peptides from the skin of *Pseudophryne güntheri*.
[d] Sequence was determined with a gas-phase sequencer (unpublished data from our laboratory).

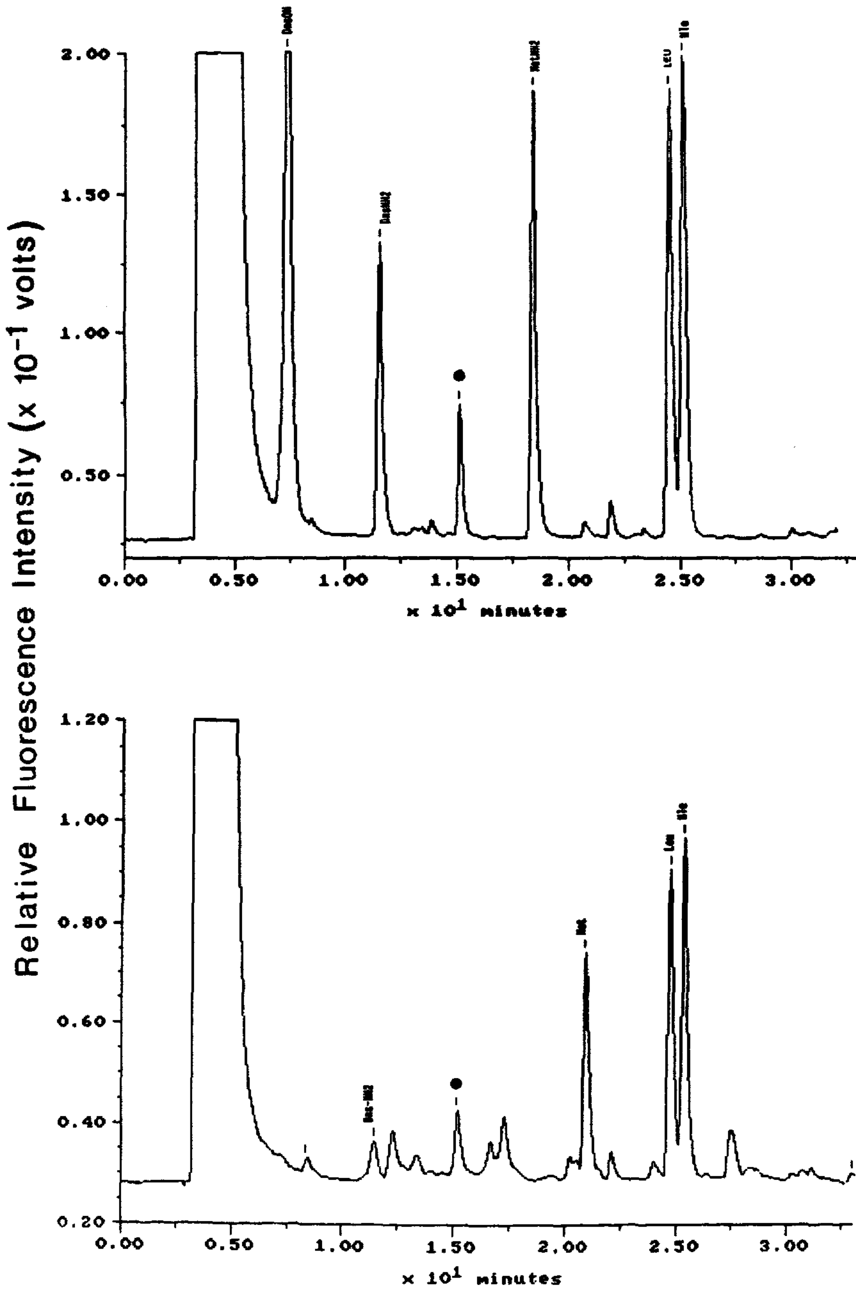

Fig. 4. Determination of the α-amide group on the carboxyl terminus of a kassinin-like peptide isolated from the skin of *Pseudophryne güntheri*, p-Glu–Pro–His–Pro–Asp–Glu–Phe–Val–Gly–Leu–MetNH$_2$ (unpublished data). Top, carboxypeptidase Y, 10 min; bottom, 6 *M* hydrochloric acid, 110°C, 2 h. ●, Buffer impurity.

reacted with dansylchloride and analysed as above. The separation of dansyl derivatives of amidated amino acids is shown in Fig. 3. In Fig. 4 the determination of the C-terminal amidated residue of a kassinin-like peptide isolated from the skin of *Pseudophryne güntheri* is shown. In conclusion, automated precolumn derivatization with dansylchloride is a convenient way to study amino acid compositions and to determine the COOH-terminal structure of peptides in the picomol range.

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