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COMPLETE AUTOMATIZATION OF PEPTIDE MAPS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY USING *o*-PHTHALALDEHYDE PRE-COLUMN DERIVATIZATION*

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SUMMARY

The conventional automatic *o*-phthalaldehyde (OPA) pre-column labelling procedure used for routine amino acid analyses by high-performance liquid chromatography has been applied as a highly sensitive technique for peptide mapping. Tryptic peptide digests are first derivatized with OPA by using an automated pre-column derivatization system and then the fluorescent peptide mixture is automatically analyzed by reversed-phase HPLC. The complete automatization of peptide mapping is achieved in about 45 min with a high resolution and a routine sensitivity in the range of 10–30 picomoles. Peptide map analyses of dansylated tryptic peptides using the above automatic conditions and with an equivalent range of sensitivity are also described. The effectiveness of this method has been exemplified by using two proteins which are nearly identical in their amino acid sequences.

INTRODUCTION

o-Phthalaldehyde (OPA), which in the presence of 2-mercaptoethanol reacts rapidly with primary amino groups to form highly fluorescent thio-substituted isoindoles[1], has been used to detect amino acids in the picomole range by post-column derivatization in the classical ion-exchange chromatography amino acid analyzer[2–4]. Amino acid determinations with a similar sensitivity of detection but based on manual derivatization with OPA followed by reversed-phase high-performance liquid chromatography (RP-HPLC) have been also described[5–7]. In addition, an automatic

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device to derivatize amino acids with OPA[8], as well as a commercially available automatic pre-column derivatization equipment (Waters Auto. OPA), which are connected to on-line RP-HPLC systems have been also described[9].

Although OPA in the presence of 2-mercaptoethanol also reacts with peptides giving about 10–20 times less fluorescence than with amino acids[10], the reagent has been used to detect peptides after column chromatography[2,11–13]. In this paper we describe an automatic on-line pre-column derivatization procedure with OPA followed by RP-HPLC analysis for tryptic peptides. A similar RP-HPLC analysis for dansylated tryptic peptides is also described and compared.

MATERIALS AND METHODS

Reagents

Dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride, *o*-phthalaldehyde and 2-mercaptoethanol were obtained from Sigma. Acetic acid and acetonitrile were from Scharlau (Barcelona, Spain). Tetrahydrofuran, 1-tosylamino-2-phenylethyl chloromethyl ketone (TPCK)–trypsin and other compounds not specified were from Merck (Darmstadt, F.R.G.). Ultra-pure water for high-performance liquid chromatography (HPLC), generated by a Milli-R04 coupled to a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.), was used in the preparation of all buffers. The amino acid standards used were obtained by diluting a Beckman amino acid analysis standard (2.5 μM) to give a final concentration of 5 nM.

Proteins

Two cytotoxic proteins called mitogillin and restrictocin, referred in the text and figures as M and R respectively, were provided by Dr. D. Vazquez, Centro de Biologia Molecular, Madrid.

Trypsin digestion

Five hundred micrograms of reduced and carboxymethylated M and R proteins[14] were digested with 5 μg of TPCK–trypsin in 100 μl of 0.2 M N-methylmorpholine–acetate buffer pH 8.2 for 1 h at 37°C. After digestion the material wass freeze-dried, lyophilized and finally redissolved in water to give a concentration of 300 pmol per 100 μl.

Manual fluorescent labelling of peptides and peptide mixtures by the dansyl chloride method

Individual peptides (10 nmol) were dried, dissolved in 30 μl of 0.2 M sodium carbonate and incubated with 30 μl of dansyl chloride (2.5 mg/ml in acetone) for 1 h at 45°C. After incubation the reacted materials were dried and dissolved in water to give a concentration of 300 pmol per 100 μl. For the derivatization of tryptic digests (6.0 nmol), the incubation was carried out in 100 μl of 0.2 M sodium carbonate and 100 μl of the above dansyl chloride solution for 1 h at 45°C. A second 100-μl volume of dansyl chloride solution was added and incubated for another hour. After lyophilization the dried material was dissolved in water to give a concentration of 300 pmol per 100 μl.

Chromatography

(a) *Fluorometric detection.* The automated HPLC amino acid analysis system (Waters Associates, Milford, MA, U.S.A.) consisted of a Model 510 solvent-delivery pump, Model 6000 A solvent-delivery pump, a 420 AC fluorescence detector (8-μl flow cell) equipped with 338-nm excitation and 425-nm emission filters, a WISP 710 B sample processor, a 730 data module and a Model 721 system controller. The separation column was a Waters 5-μm Resolve C_{18} column (15 cm × 3.9 mm) protected by a guard column packed with μBondapak C_{18}/Corasil, both connected to another pre-column filled with glass beads (600 μm) (Ferro Corporation) to perform the automated OPA derivatization. The column and pre-column were thermostatted at 45°C using a Waters column-temperature controller.

(b) *UV detection.* The HPLC system consisted of a Waters 6000 A pump, a 510 pump, a Waters 680 automated gradient controller and a Waters 480 Lambda-Max variable wavelength absorbance detector. All sample injections were performed with a Waters U6K Universal injector. A C_{18} μBondapak column (30 cm × 3.9 mm fitted with a guard column volume 270 μl) packed with μBondapak C_{18}/Corasil was used. Further details of the chromatographic procedure are given in the figure legends.

Automatic o-phthalaldehyde pre-column derivatization procedure

The derivatizing solution consisted of 500 mg of *o*-phthalaldehyde in 10 ml methanol, diluted to 100 ml in 0.4 *M* sodium borate pH 10.0. Aliquots of the derivatizing solution (2.0 ml) and samples (0.1–0.2 ml) were placed in glass vials, stoppered and loaded on the carousel of the automated injector. In the first step, portions of the derivatization solution (1–50 μl) and sample solutions (1–50 μl) programmed with a microprocessor system controller were loaded in a needle as indicated in Fig. 1. The flow-rate was then set at 0.1 ml/min, the initial gradient condition was maintained (Table I) and the derivatization solution and samples were automatically mixed for 2 min (Fig. 1). The derivatized sample components were then automatically injected in the head of the column and analyzed according to the gradient program in Table I. Two buffers were employed in the HPLC separation of amino acids or peptides: A, methanol–tetrahydrofuran–water (2:2:96) containing 0.05 *M* dibasic sodium phosphate and 0.05 *M* sodium acetate, adjusted to pH 7.5 with acetic acid; B, methanol–water (65:35). Both buffers were filtered (0.45-μm Millipore filter) and degassed before use.

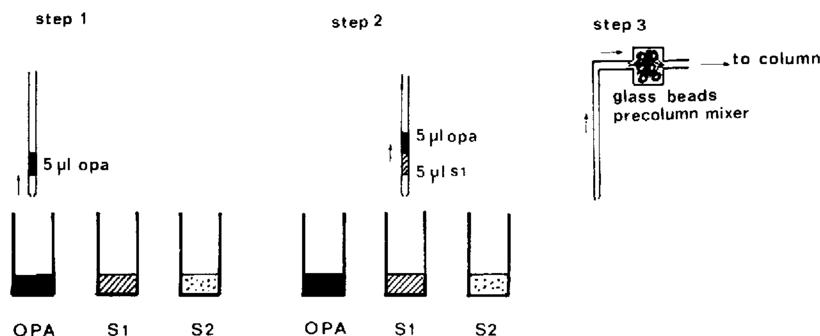

Fig. 1. Schematic representation of the Waters AUTO-TAG OPA pre-column derivatization system. OPA = Derivatizing solution; S1 and S2 = samples. OPA and samples are loaded into the WISP carousel.

TABLE I

CHROMATOGRAPHIC GRADIENT CONDITIONS FOR HPLC ANALYSIS OF OPA-DERIVATIZED PEPTIDES OR AMINO ACIDS (TOP) AND Dns-DERIVATIZED PEPTIDES (BOTTOM)

Time (min)	*Flow*	*%A*	*%B*	*Curve**
Initial	0	100	0	
2.0	0.1	100	0	1
2.5	1.5	100	0	6
40.0	1.5	0	100	5
50.0	1.5	100	0	11
57.0	0	100	0	11
Initial	1.5	100	0	
30	1.5	0	100	4
40	1.5	100	0	11

* Waters 680 gradient controller programmer.

RESULTS AND DISCUSSION

Peptide maps have traditionally been generated using one-dimentional paper or thin-layer electrophoresis in one direction followed by chromatography in the perpendicular direction[15,16]. Although the sensitivity of these maps is in the range of a few nanomoles or picomoles, depending on whether the peptides are stained with ninhydrin[17] or fluorescamine[18,19], the technique requires several manipulations and is therefore time-consuming and has low reproducibility. More recently, reversed-phase high-performance liquid chromatography (RP-HPLC) has been shown to be a powerful tool for the separation of peptides and is now extensively used for analytical peptide mapping[20–22]. Such HPLC analyses, which are sensitive and non-destructive when monitoring at 200–220 nm, are considerably more simple, reproducible and have a high degree of resolution in comparison with paper or on thin-layer chromatography.

Fig. 2 shows two peptide maps obtained by RP-HPLC monitored at 214 nm; these correspond to two virtually identical proteins M (Fig. 2A) and R (Fig. 2B), which differ at peptide 15 which is asparagine in M but serine in R[14]. The elution times for all the tryptic peptides, including peptide 15, are identical. Clearly Fig. 2 demonstrates the high reproducibility of the RP-HPLC system used in these two separate analyses.

Semipreparative peptide maps in RP-HPLC are routinely obtained by us from 10–25 nmol of protein digest, with a constant baseline for UV absorption at 200–220 nm. Moreover, the sensitivity of detection of peptides can be increased up to 0.5–1.0 nmol simply by use of the scale-expansion facility of the UV detector. This increase in sensitivity is only limited by the rise of the baseline at low wavelengths produced by the absorption of the organic solvents (acetonitrile, methanol, 1- or 2-propanol) during gradient elution.

In order to improve the sensitivity of peptide map analyses up to the picomole range, several pre-column derivatization techniques with different fluorogenic reagents, followed by RP-HPLC separation, have been tested. First, we chose the pre-

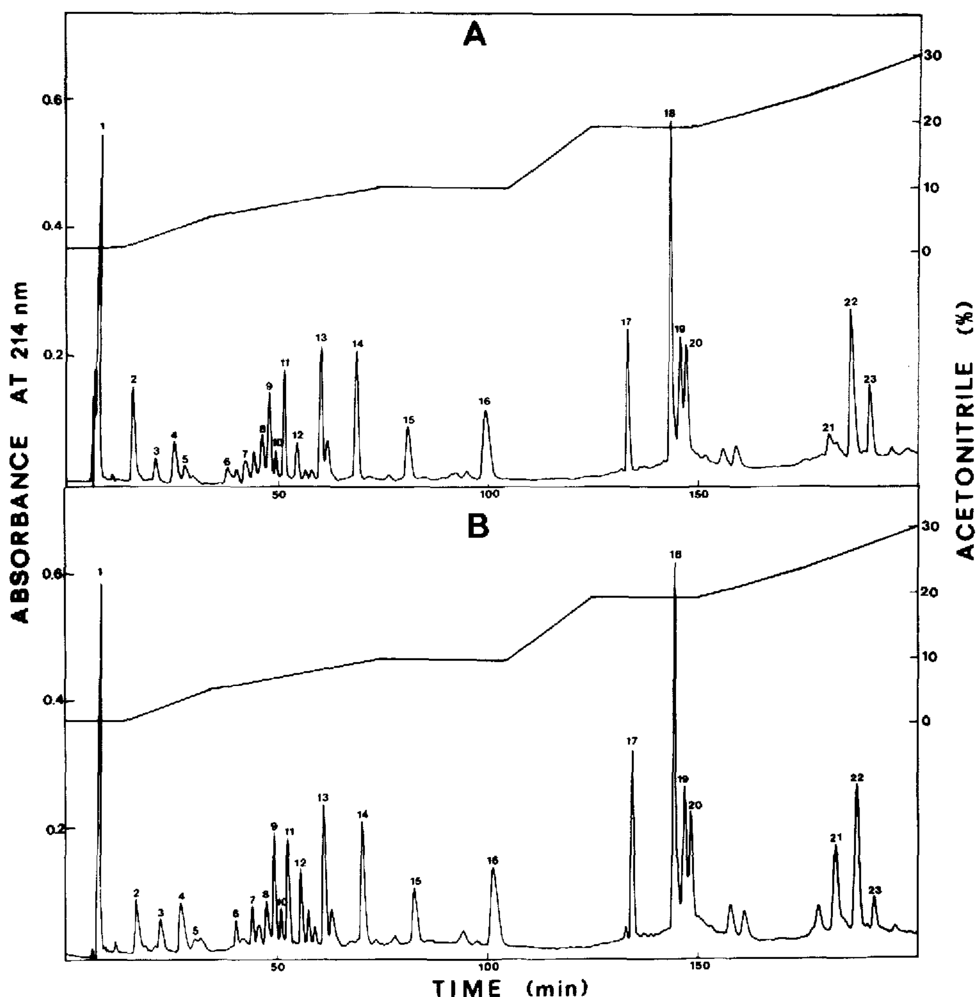

Fig. 2. Separation of tryptic digest of reduced and carboxymethylated proteins M (A) and R (B). Samples: 10 nmol. Range: 2.0. Column: μBondapak C_{18} (30 cm × 3.9 mm). Flow-rate: 0.5 ml/min. Peptides were eluted at room temperature with acetonitrile gradients containing 0.1% trifluoroacetic acid.

column derivatization of peptides with OPA/2-mercaptoethanol, followed by RP-HPLC analysis in a manner similar to that described for amino acids[5–7]. For this purpose we used a fully automated RP-HPLC method which included the automatic Waters AUTO-TAG pre-column derivatization system with OPA for analysis of primary amino acids in the picomole range[9]. A typical analysis of 50 pmol of each individual amino acid is shown in Fig. 3A and the chromatographic conditions are indicated in Table I.

In order to determine the above automated OPA system for amino acids could be used for peptide analysis, peptide mixtures were analyzed under exactly the same chromatographic conditions (Table I) including wavelengths of detection and column. Samples of tryptic digest from both proteins M and R were dissolved in water

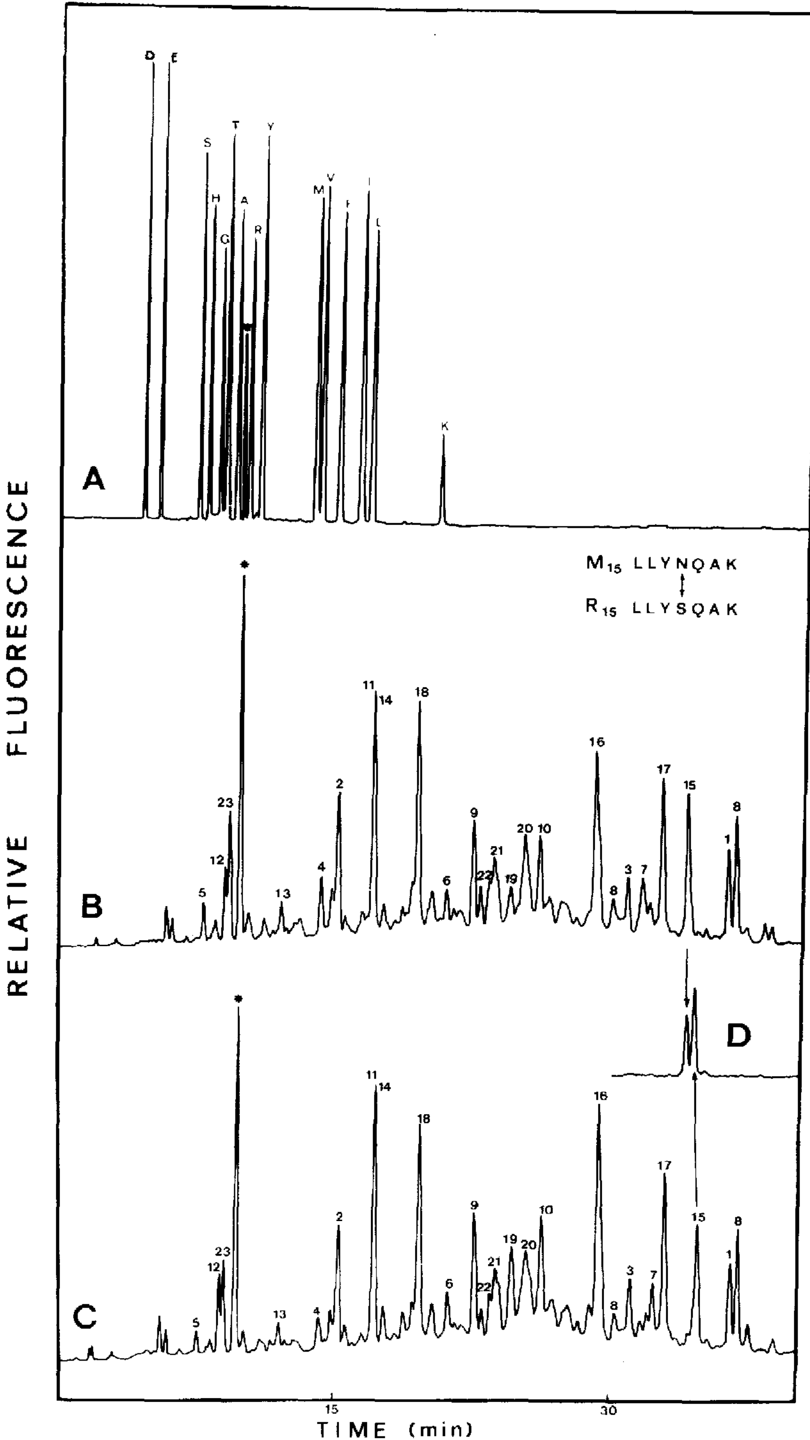

Fig. 3. Distributions of OPA-derivatized amino acids and peptides chromatographed by the automatic on-line OPA/2-mercaptoethanol system. A, 50 pmol of amino acid standard mixture; B and C, 50 pmol of tryptic peptide digests of proteins M and R respectively; D, co-chromatography of peaks M15 and R15 from Fig. 2. The peak marked with an asterisk is due to the derivatizing reagents. Column: 5-μm Resolve C_{18} (15 cm × 3.9 mm). Emission at 425 nm and excitation at 338 nm. A comparison between the sequences of peptides M15 and R15 is also shown.

and placed in the carousel of the automatic injector as indicated in Fig. 1. Aliquots of 3–10 μl containing 30–100 pmol of tryptic peptide digests were first automatically derivatized with OPA using a Waters automated pre-column derivatization system, and then injected automatically into a RP-HPLC system as indicated in Materials and Methods (Fig. 1). The chromatographic distributions of the fluorescent derivatives of peptides from proteins M and R are shown in Fig. 3B and C. As is seen, a satisfactory resolution was obtained for the peptide derivatives.

To determine whether the eluted fluorescent peptides obtained in these maps contained all tryptic peptides from both molecules, aliquots of 100–200 pmol of the isolated tryptic peptides (Fig. 2) were subjected individually to the same automated OPA pre-column derivatization system as in Fig. 3. Fig. 4 shows the identification of two tryptic peptides, 2 and 10 from the peptide maps of Fig. 2A, in the corresponding chromatogram of Fig. 3B. The remaining 23 tryptic peptides of each molecule were identified in the same way. This automatic OPA derivatization system can also be used, as shown in Fig. 4, as an additional and rapid criteria of peptide purity. In addition, the N-terminal amino acid of these OPA peptides, Figs. 3 and 4, can be determined simply by comparing the amino acid analysis of the original peptides with that from the fluorescent derivatives of the peptides[10].

Although the distributions in both maps (Fig. 3B and C) are apparently identical, only fluorescent peptides M15 and R15 corresponding to the two different peptides in both proteins present a slight difference in elution times. In order to demonstrate this slight difference, the corresponding unlabelled peptides M15 and

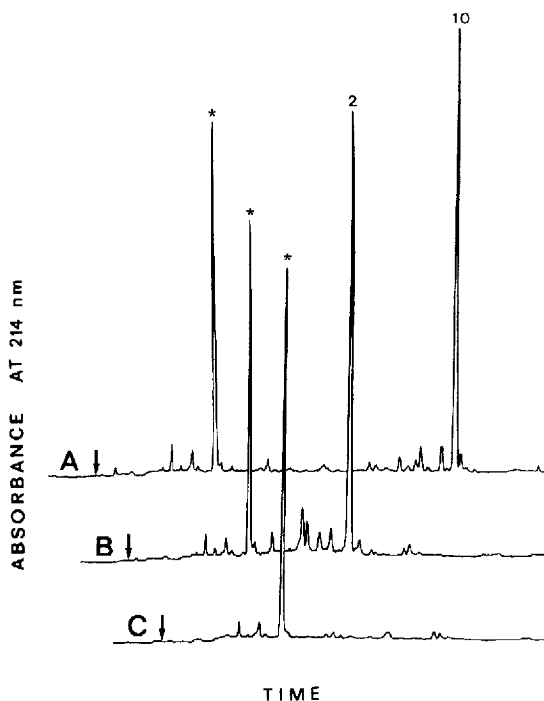

Fig. 4. Chromatography of peaks M2 (B) and M10 (A) from Fig. 2A analyzed as in Fig. 3; C, blank. Peaks marked with an asterisk are due to the derivatizing reagents.

R15 (Fig. 2A and B) were mixed and analyzed by the automated OPA pre-column system as well and the results shown in Fig. 3D. Fig. 3B and C demonstrate that this technique is able to differentiate between two proteins with a single difference in their amino acid sequence.

The results presented in Fig. 3B–D, as well as in Fig. 4, demonstrate the reproducibility of these chromatographic systems, peaks M2, M10, M15 and R15 appearing at positions identical to those in Fig. 3B and C. Such reproducibility has been maintained over a series of replicate peptide analyses performed in our laboratory under the same conditions. The detection limit with the automatic OPA pre-column chromatographic peptide map system, determined by serial dilutions of pep-

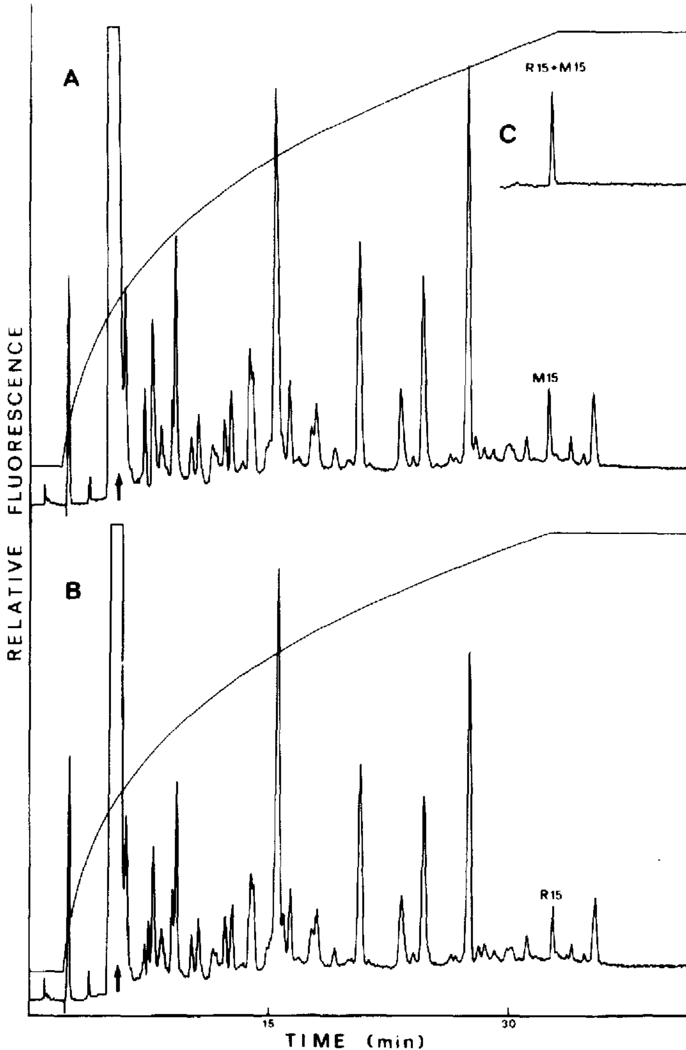

Fig. 5. Elution profiles of dansylated tryptic digests chromatographed on the 5-μm Resolve C_{18} column (15 cm × 3.9 mm). A and B, Tryptic digests of proteins M and R respectively; C, co-chromatography of dansylated peaks M15 and R15. The arrows indicate free dansyl chloride.

tide mixtures and/or by use of the scale-expansion facility of the fluorometer, was approximately 5–10 pmol with an acceptable baseline noise.

Dansyl chloride was another fluorogenic reagent tested in a similar way to the above. The digest of proteins M and R was manually derivatized as indicated in Materials and Methods, and the dansylated derivatives were loaded on the carousel of the automatic injector. The labelled mixture was injected automatically in the column and analyzed by a similar program to that with OPA, but omitting the pre-column derivatization step (Table I). Moreover, the buffers, wavelength filters and column described for OPA were also used. Aliquots of 5–10 μl containing 30–60 pmol were analyzed as well. The distributions of the dansylated peptides corresponding to proteins M and R are shown in Fig. 5A and B. These maps show a satisfactory resolution of the dansylated peptides, indicating that this system could also be applied to peptide mapping. The fact that both maps of the dansylated peptides from two different analyses of two almost identical proteins are the same demonstrates the reproducibility of this system, which has been maintained over a series of analyses. In this study only the two dansylated peptides corresponding to M15 and R15 have been identified in both maps by co-chromatography of both dansylated derivatives, and as is shown in Fig. 5C, both are eluted at the same position. Although the sensitivity of detection of these dansylated derivatives is in the same range as with OPA the sensitivity can be increased simply by using the appropriate filters for dansylated derivatives[23].

Finally another fluorogenic reagent fluorescamine[24] was been investigated in similar peptide map analyses. However, although the fluorescence of peptides labelled with fluorescamine is higher than with OPA[10,11], the resolution of the fluorescamine peptide digests analyzed under the same conditions as those used for OPA or dansyl was very poor (data not shown).

In conclusion we have shown that the same automated pre-column OPA derivatization procedure connected on-line with RP-HPLC used for amino acid analysis can also be applied for a full automatization of peptide mapping in the range of a few picomoles. The advantage of using an automatic injector with an automatic pre-column derivatization system is that it permits the derivatization of samples one by one, (to prevent the decay of the OPA derivatives) and also gives more precise control and reproducibility of sample mixing and reaction in comparison with the usual manual derivatization procedure.

In addition, the same equipment, including buffers, column and wavelength filters, has also been used for mapping of dansylated peptides with an equivalent degree of resolution, sensitivity and reproducibility as that for OPA.

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