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Separation and identification of neuropeptide Y, two of its fragments and their degradation products using capillary electrophoresis–mass spectrometry

Kees Ensing*, Theo de Boer, Nanno Schreuder, Rokus A. de Zeeuw

Groningen Institute for Drug Studies, Department of Analytical Chemistry and Toxicology, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

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Abstract

This paper describes the development of an analytical method for the separation and identification of neuropeptide Y (NPY) and two important NPY fragments by capillary electrophoresis (CE) and mass spectrometry (MS). A satisfactory separation and the highest sensitivity were obtained with formic acid at high concentrations (250 m*M*, pH 2.75). The addition of 25 or 50 m*M* triethylamine (TEA) improved the separation. When applying full scan CE–MS, the separated peptides could be detected and identified using the spectra of each peak. The use of TEA as an additive to the formic acid slightly decreased the sensitivity but was compensated by the improved efficiency. The best compromise for optimal separation and MS detection was found to be 50 m*M* formic acid to which 50 m*M* TEA was added. CE–MS could be used for identification of the decomposition products of NPY. Decomposition products with one amino acid difference, which could not be distinguished with CE–UV, could be distinguished with CE–MS.

Keywords: Neuropeptide Y

1. Introduction

Neuropeptide Y (NPY) was first isolated in 1982 by Tatemoto et al. [1,2] from porcine brain. It is present in large parts of the brain [3] and the sympathetic perivascular nerves [4]. NPY acts as a vasopressor agent [4] and affects ingestive behavior, depression and anxiety [5,6]. The complete amino acid sequence of NPY along with the ribbon illustration of NPY that is obtained by molecular modeling are given in Fig. 1 [7]. Table 1 shows some chemical data for NPY and its fragments that are of importance for this project.

Capillary electrophoresis (CE) is a separation technique that can offer high efficiency and that has been used successfully for the separation of biomolecules [8–15]. The mass spectrometer (MS) as a detector for CE offers several advantages over other detection methods. Analytes varying from small inorganic species to large proteins can be detected, and mass spectrometry can provide high sensitivity as well as information on the analyte in terms of

*Corresponding author. Tel.: +31-50-363-3345; fax: +31-50-363-7582.
E-mail address: k.ensing@farm.rug.nl (K. Ensing)

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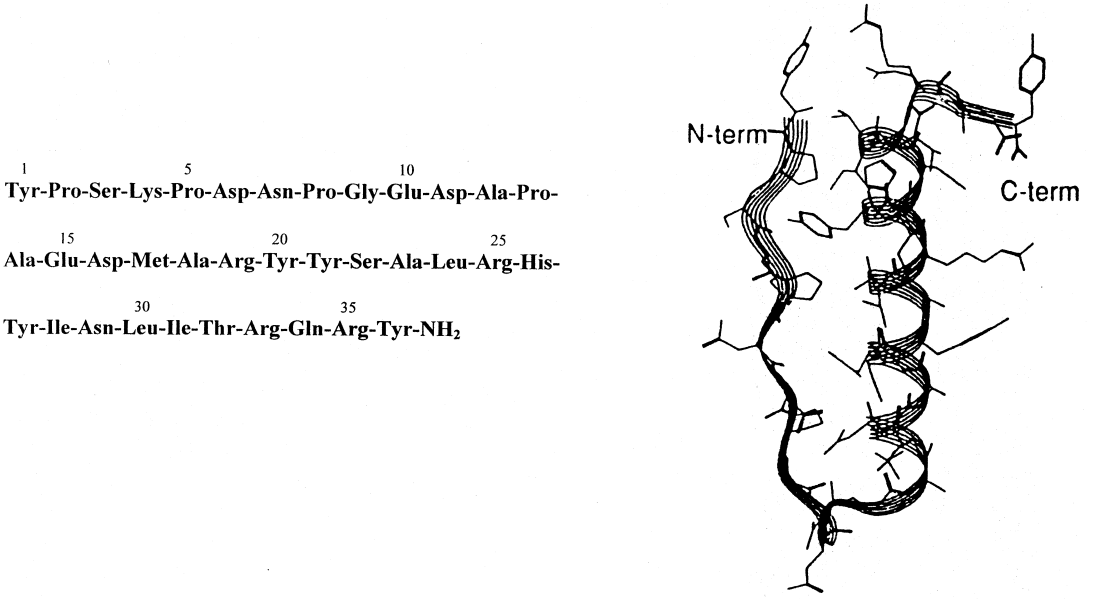

Fig. 1. (a) Amino acid sequence of NPY and (b) ribbon illustration of NPY obtained by modeling of NPY by homology to X-ray crystallographic structure of avian pancreatic polypeptide and tethered minimization to relieve side-chain stretch [7,35].

relative molecular mass. In the first paper on CE–MS, the use of the CE capillary as the electrospray ionization (ESI) emitter was reported [16], and the liquid flow necessary to support the electrospray process was delivered by the electroosmotic flow alone. In an improved CE–MS interface, a make-up liquid was used to make the liquid flow-rate into the ESI interface compatible with the commonly used 5 μl/min flow-rate of the electrospray process [17]. A further modification comprises pneumatic assistance of electrospray (IonSpray®) [18]. The coupling of CE with fast atom bombardment (FAB) and ESI mass spectrometry sources has been reviewed by Cai and Henion [19]. The on-line coupling of CE with ESI–MS has become an important technique in the analysis of peptides and proteins [20–24]. The method offers high sensitivity and mild ionization conditions [25]. ESI mass spectra do not show fragment ions of peptides and can be used for the molecular mass determination of proteins [26].

Table 1
Some physico-chemical data for NPY and its fragments

	M_r	pI	λ_{max}
NPY (human)[a]	4272.79	7.9	192.5
NPY (porcine)	4254.72	7.9	192.5
NPY(13–36)(porcine)	2983.41	10.6	192.5
NPY(18–36)	2457.85	11.3	192.5

[a] Human NPY and human NPY(13–36) contain methionine as residue 17, which makes these compounds very unstable, due to quick oxidation of methionine [36]. The more stable porcine compounds, containing leucine as residue 17, were used. The isoelectric points (pI) of human NPY and porcine NPY are the same.

In the combination of CE with MS, several problems arise. Cluster ions from buffer components may generate an abundant background spectrum that interferes with sample ions in a mass spectrum. A high concentration of buffer ions decreases the ionization efficiency of sample molecules in ESI–MS. In most papers on CE–MS, the running buffer in the CE column and the electrolyte present in the make-up liquid are made up from volatile components such as ammonium acetate, ammonium formate, acetic acid and formic acid. Separation in a CE column filled with such volatile ‘buffers’ is usually inferior to separation in phosphate, citrate, borate and other buffers containing non-volatile components. Mass spectrometer ion sources are not supposed to tolerate the introduction of non-volatile materials over an extended period of time. This is indeed true for FAB sources. ESI sources from some

manufacturers can handle non-volatile materials. In particular, ESI sources equipped with a nitrogen gas curtain originally developed by Sciex [27,28] are protected against blockage of the ion-sampling orifice that separates the atmospheric pressure ESI source from the vacuum of the mass analyzer.

The combination of a phosphate buffer in the CE column and a volatile acid such as acetic acid in the make-up liquid might seem a good compromise. However, the back-migration of electrolyte anions from the make-up liquid into the CE column may give rise to a serious change in separation conditions inside the CE column if the make-up and running buffer electrolytes do not match [20]. Combinations that can be used safely are acetate buffer with acetic acid, formate buffer with formic acid, and phosphate buffer with phosphoric acid.

The aim of the present study is the development of analytical methods for the quantitation and identification of peptides by CE–MS. Because of our interest in neuropeptide Y and two of its fragments, we used these as test compounds.

2. Experimental

2.1. Reagents and samples

Glacial acetic acid of gradient grade (GR), benzyl alcohol, formic acid GR (98%), methanol GR (99.8%), *ortho*-phosphoric acid GR (85%), sodium chloride GR, sodium dihydrogen phosphate monohydrate GR and sodium hydroxide pellets GR were obtained from Merck (Darmstadt, Germany). Triethylamine (TEA) (99.5%) was obtained from Fluka (Buchs, Switzerland). Water was taken from a MilliQ system (Millipore, Bedford, MA, USA)

Neuropeptide Y (porcine) (≥99%), neuropeptide Y (13–36) (porcine)(≥97%) and neuropeptide Y (18–36) (≥99%) were purchased from Saxon Biochemicals (Hannover, Germany). All peptides were supplied as the trifluoroacetate salt. Stock solutions of each peptide were made in water (1 mg/ml) and were stored at −18°C.

All solutions of samples, buffers and other reagents were filtered (0.45 μm) and degassed in an ultra-sonic bath immediately prior to use.

2.2. Capillary electrophoresis

A Prince programmable injector system (Lauer Labs, Emmen, The Netherlands), with a 30-kV high voltage power supply (Spellman, Plainview, TX, USA) and a Lambda 1000 variable wavelength UV–Vis detector (Bischoff, Leonberg, Germany) was used.

The CE columns used had a total length of 70.0 cm and an effective length of 55.0 cm, and were bare fused-silica capillaries with an outer polyimide coating (Polymicro Technologies, Phoenix, AZ, USA), 50 or 75 μm I.D., 375 μm O.D.

UV absorbance detection at 210 nm took place through a window created by removal of 1 cm of polyimide coating. The first 2 mm of the capillary were also stripped, in order to prevent adsorption of proteins to the outside of the capillary and, thereby, excluding a cause for peak-tailing [29]. A new capillary was rinsed with 0.2 *M* NaOH for 2 h at 105 Pa. The voltage applied over the capillary during CE analyses was 30 kV; the capillary was kept at 30°C. Before each run, the capillary was flushed with buffer for 1 min at 210 Pa. When the buffer system was changed, the capillary was flushed with 0.2 *M* NaOH and with the new buffer for 5 min at 210 Pa, with 0.2 *M* NaOH for 10 min at 105 Pa and, finally, with a 20-min flush of the new buffer at 21 Pa. Injections were made by pressure injection over 0.1 min at 21 Pa, producing an injection volume of approximately 100 nl and an injection plug of 12 mm long in a 50-μm capillary.

Data acquisition was performed by the Maclab 2E system (ADInstruments, Castle Hill, NSW, Australia) using the Chart program (ADInstruments) to collect the signals of the UV absorbance detector and the CE current monitor. Processing of the CE–UV data was performed with the Peaks program (ADInstruments). The detection limit was defined at a signal-to-noise ratio of three [30].

2.3. Mass spectrometer and CE–MS interface

The mass spectrometer was an R 3010 (Nermag, Argenteuil, France) equipped with a laboratory built atmospheric pressure ionization source [31] and pneumatically assisted ESI (ionspray) interface [18] operated at +4 kV. Nitrogen (99.8%, Air Liquide,

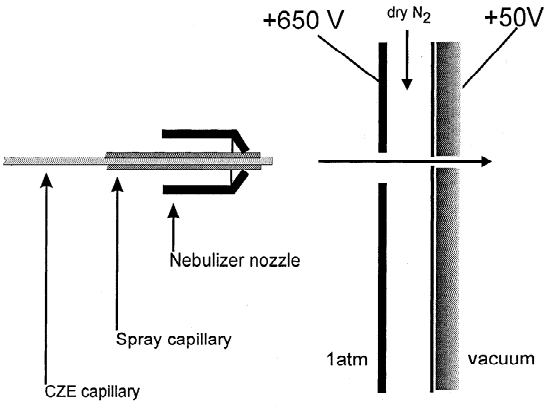

Fig. 2. Coaxial ionspray interface for CE–MS. The spray capillary (stainless steel, 0.4 mm I.D.×0.7 mm O.D.) is connected to the HV power supply.

Eindhoven, Netherlands) was used as the curtain gas and the nebulizer gas. Ions released from electrically charged droplets pass through the curtain gas and are drawn into the ion-sampling nozzle of the mass spectrometer. Uncharged sample components and neutral contaminants are swept away by the curtain gas and are prevented from reaching the ion-sampling nozzle [32]. The mass spectrometer was controlled by the Nermag Sidar data system and was scanned from m/z 100 to 1999 in 1 Da steps with a cycle time of 1.353 s. A dwell time of 0.163 s was used for each ion in the selected ion monitoring mode. The ion-sampling nozzle was held at 170 V.

The coaxial, pneumatically assisted electrospray interface is shown in Fig. 2. The fused-silica CE capillary was inserted all the way through to the tip of the coaxial CE–MS interface. The make-up flow (5 μl/min) was provided by a Brownlee Micro Gradient syringe pump (Applied Biosystems, Foster City, CA, USA). The make-up solution consisted of 10% water with electrolyte and 90% methanol. For off-line determination of the molecular masses of the samples, 1 μl of the analytes dissolved in the make-up solution was introduced by loop injection using the Brownlee Micro Gradient syringe pump.

3. Results and discussion

Several buffer (i.e. phosphate, cyclohexylaminopropane sulfonic acid (CAPS), formic acid, ammonium acetate) systems were tested in order to obtain a separation by CE–UV. Preliminary experiments revealed that run buffers with a pH between four and ten did not result in detection of the analytes within 3 h at 30 kV, which can be explained by adsorption of the peptides to deprotonated silanol groups on the wall [33]. Since volatile buffers appear to be more convenient for CE–MS, the use of nonvolatile buffers can lead to clogging of vacuum orifices and probe tips and can form adduct ions that effectively decrease the sensitivity of the instrument [25]. We also tested different concentrations of formic acid (50 to 500 m*M*, pH 2.75).

With a formic acid system, it was possible to obtain a separation, but with relatively low efficiencies, ranging from 1500 to 12 000 theoretical plates for formic acid concentrations of 50 and 500 m*M*, respectively. We decided to use triethylamine (TEA) as an additive to reduce solute–silica wall interactions. With 50 m*M* TEA added to a 50-m*M* formic acid solution, pH 2.75, a 260-fold increase in plate number was obtained (N=~400 000), which makes separation in this CE buffer comparable with separation in phosphate buffers. Addition of up to 50 m*M* TEA to the phosphate buffers did not result in higher plate numbers (data not shown). The limit of detection of NPY dissolved in run buffer for CE–UV in a 50-μm I.D. column with an injection plug length of 12 mm and without stacking is 1 μg/ml ($2.4 \cdot 10^{-7}$ *M* NPY). The limit of detection with an enlarged injection plug length of 117 mm and with stacking of a sample dissolved in a tenfold dilution of the run buffer is 0.1 μg/ml ($2.4 \cdot 10^{-8}$ *M* NPY).

A full-scan mass spectrum of a sample mixture of NPY and two of its fragments was obtained off-line by direct flow injection. The multiply charged ions observed for each compound and decomposition product are given in Table 2. The following fragments were identified: NPY(25–36), NPY(24–36), NPY(22–36), NPY(21–36), NPY(17–36) and NPY(14–36). The decomposition products NPY(17–36) and NPY(14–36) show peaks that coincide with the peaks of the starting compounds, NPY(18–36) and NPY(13–36), respectively. This latter observation shows that, despite the high separation power of CE, mass spectrometric detection is required to identify compounds with one amino acid difference, which cannot be distinguished with CE–UV.

There were also some other ions visible in the

Table 2
Multiply charged ions of NPY, two of its fragments and decomposition products

	Compound m/z of multiply charged ions, with charge states:				
	2	3	4	5	6
NPY			1064	851	710
NPY (13–36)		995	746		
NPY (18–36)		820	615		
NPY (25–36)	817	545			
NPY (24–36)	873	582			
NPY (22–36)		635	477		
NPY (21–36)		689	517		
NPY (17–36)		857	643		
NPY (14–36)			722		

mass spectra, which could be ascribed to decomposition of the samples. Coupling of CE with MS was tested using different buffer solutions and using small adaptations to the interface. One of the problems that occurred was interference of the sprayer with the injection process. The pneumatic nebulizer of the coaxial ionspray CE–MS interface acts as an aspirator, drawing liquid from the inlet vial through the CE column. When the buffer and sample vials are interchanged, a small air bubble is drawn into the capillary. This results in ghost peaks in the MS signal and to interruption of the CE current. The high voltage applied to the interface creates another problem during injection of a sample. When a +4-kV voltage is applied to the interface, while the CE voltage is switched off, an electric field is created that partially prevents a positively charged sample from being introduced into the column. This resulted in irreproducible peak areas, and in discrimination against the more highly charged and more mobile sample components. In order to avoid these problems, the nebulizer gas and the voltage on the interface were switched off during injection.

Another problem is shown in Fig. 3a. It shows the total ion electropherogram of the three analytes separated in formic acid. It was anticipated that TEA would affect the ionization efficiency, therefore, initial experiments were completed with formic acid only. Separation and sensitivity were reasonably good, but the noise was unacceptable. The noise might have been the result of irregularity in the spray, but readjustments of the CE capillary inside the sprayer and cutting off of a few centimeters of the CE capillary did not eliminate the noise on the peaks. One of the characteristics of the outer polyimide capillary coating is that it can bind peptides and proteins. We removed 5 mm of the polyimide coating, which assesses the possibility of binding of the peptides to the coating, but also has a large influence on the spray. In Fig. 3b, it can be seen that the total ion electropherogram of the three analytes with the stripped capillary now has sharp peaks.

Using NPY as a test compound and full-scan MS as the detection method with direct flow injection, the sensitivity of detection was investigated. Because sodium phosphate buffer and formic acid gave good separations in previous CE–UV experiments, the MS-injections were made in the make-up solutions compatible with these CE buffers, namely 10% 0.1 *M* phosphoric acid–90% methanol and 10% 0.1 *M* formic acid–90% methanol. The sensitivity, obtained for NPY at m/z 710, was much lower for a solution containing phosphoric acid than for the corresponding solution containing formic acid (about 500 times using a make-up flow of 5 μl/min). This is due to the formation of phosphoric acid adducts to the $[M+6H]^{6+}$ ion, which is a well known phenomenon in ESI mass spectrometry of peptides and proteins [34]. When adducts are formed, the abundance of the $[M+6H]^{6+}$ ion is, in part, converted into the abundance of a group of $[M+6H]^{6+}\cdot(H_3PO_4)_n$ ions, thereby reducing sensitivity. This effect may be obviated by the use of formic acid. When the CE was coupled to the mass spectrometer, we observed a 500-fold higher sensitivity with formic acid in the run buffer and in the make-up flow, compared to the use of a sodium phosphate buffer and phosphoric acid in the make-up flow.

It was shown that the addition of TEA to formic acid, creating triethylammonium ions that block negatively charged silanol groups on the capillary wall, can improve the separation. The only disadvantage of this additive is a slight decrease (<30%) in sensitivity, caused by the formation of protonated TEA ions that compete with sample ions during the process of the release of ions from charged droplets created by electrospray. Fig. 4 shows the results of CE–MS with selected ion monitoring using formic acid with or without 50 m*M* TEA, which is in line with our previous observations in CE–UV experiments.

NPY and its fragments appeared to be relatively unstable. During our experiments, the first decompo-

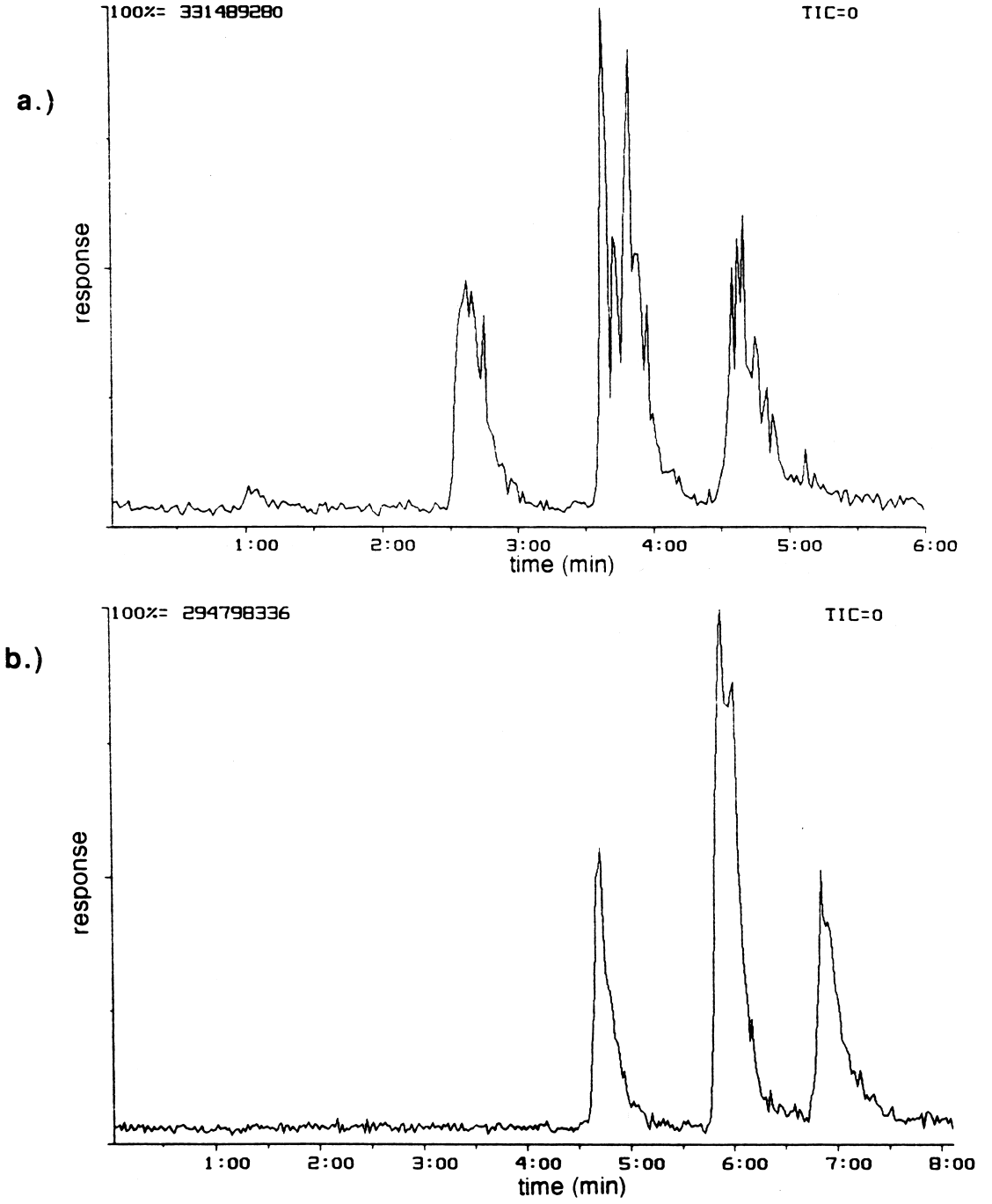

Fig. 3. Electropherogram (CE–MS, total ion current) obtained after separation of NPY, NPY(13–36) and NPY(18–36) in 250 m*M* formic acid, pH 2.75; (a) normal capillary end; (b) stripped capillary end.

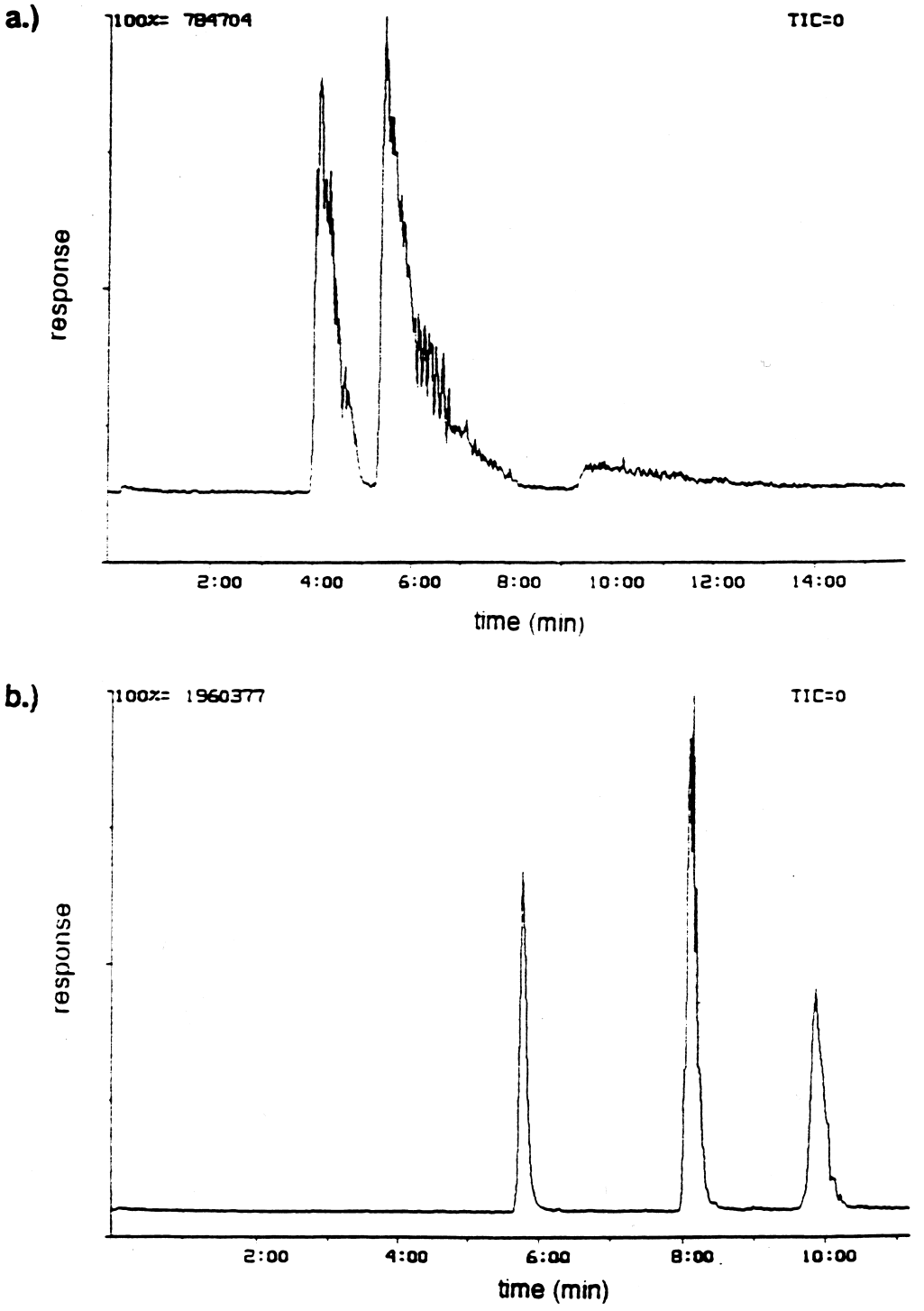

Fig. 4. Effect of TEA on the separation of NPY, NPY(13–36) and NPY(18–36) in 50 m*M* formic acid, pH 2.75, with mass spectrometric detection (total ion current); (a) without TEA and (b) with 50 m*M* TEA.

sition products could be detected by CE–UV after using the peptide solution at room temperature for 8 h. The peptides were stable for several months, as long as they were kept at −18°C. One sample mixture, used for the first experiments, was deliberately kept at +4°C. After several months, a mixture of decomposition products was obtained. A full scan CE–MS run was made, using 250 m*M* formic acid.

4. Conclusions

Using 50 m*M* TEA added to 50 m*M* formic acid, pH 2.75, as a volatile buffer, NPY and two of its fragments, can be separated readily by CE with UV absorbance detection, warranting an efficiency of 400 000 theoretical plates. The limit of detection in this matter could be set to 0.1 μg/ml in a tenfold diluted run buffer. In CE–MS, the binding of peptides and proteins to the outer polyimide coating of the capillary results in a decrease in sensitivity and efficiency. Removal of 5 mm from the coating prevents this binding, resulting in sharper peaks. Optimal separation and identification of NPY, its fragments and their degradation products by on-line coupling of CE with ESI–MS is accomplished using 50 m*M* TEA–50 m*M* formic acid, pH 2.75, as the run buffer and 10% 0.1 m*M* formic acid–90% MeOH as the make-up solution. Using these conditions, a 500-fold increase in sensitivity, in comparison with a phosphate buffer and a make-up solution containing phosphoric acid, could be accomplished and peptides with one amino acid difference could be distinguished.

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