Determination of vasoactive intestinal peptide in rat brain by high-performance capillary electrophoresis

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ABSTRACT

A high-performance capillary electrophoresis (HPCE) method for determining vasoactive intestinal peptide (VIP) in rat brain was developed. Cerebral cortex was first extracted by solid-phase extraction and purified by reversed-phase high-performance liquid chromatography. The VIP-rich fraction was further analysed by capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography using a commercial HPCE instrument with UV detection. The identity of the peak of endogenous VIP was confirmed by performing multiple CZE analyses at different pH values. This HPCE method allows VIP to be detected and measured with good molecular specificity and could represent a reference method to validate data obtained by radioimmunoassay.

INTRODUCTION

Vasoactive intestinal peptide (VIP) is a 28-amino acid polypeptide which was first isolated from porcine small intestine [1]. VIP was later shown also to be present in the central and peripheral nervous system where it may act as a neurotransmitter or neuromodulator [2]. Brain VIP appears to have important biological actions, being involved in cortical energy metabolism [3], the regulation of hormone secretions [4,5], the control of circadian rhythms [6] and the induction of sleep [7].

The determination of VIP in nervous tissue is mainly performed by radioimmunoassay (RIA), a highly sensitive technique that allows the quantification of neuropeptides contained in a complex bi-

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ological matrix. Nevertheless, RIA has limited molecular specificity as it is based on the interaction of the peptide with an antibody and therefore monitors a secondary structural parameter and not the amino acid sequence of the peptide [8]. Furthermore, interferences with divalent cations, and also with macromolecules and structural analogues of the peptide of interest, are difficult to evaluate and can hamper the selectivity of any RIA [9,10]. High-performance liquid chromatography (HPLC) followed by RIA improves the selectivity of the analysis, but such an approach is tedious and does not eliminate the ambiguity due to the detection by binding to an antibody. Consequently, it appears that more specific analytical methods in which primary structural parameters (amino acid composition and sequence) are monitored are needed in order to determine neuropeptides in biological samples. Recently, multi-dimensional HPLC followed by mass spectrometry (MS) was used to measure endogenous opioid peptides in animal and human

tissues [8,11,12]. Although such an approach has a high level of molecular specificity and can give unambiguous data, the MS analysis needs expensive instrumentation and therefore can only be carried out in a few laboratories.

High-performance capillary electrophoresis (HPCE) is a very powerful method for the analysis of biological samples [13–16]. This technique can be performed in most biochemistry laboratories, as commercial HPCE instruments are now available. Two modes of separation can be used to analyse the polypeptides by HPCE, namely capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MECC). In both of these modes, peptides are resolved according to their charge to mass ratio, while their hydrophobicity is also involved in MECC separations [13–17]. As these two parameters depend on the amino acid composition and sequence of the peptides, HPCE can be considered to monitor indirectly primary structural characteristics and therefore should allow the specific determination of peptides in biological samples.

HPCE is commonly used for the analysis of peptide maps and for the quality control of synthetic or recombinant polypeptides [18–20], but to our knowledge it has never been used to detect neuropeptides in brain tissue. The purpose of this study was to develop an HPCE method to detect VIP in rat cerebral regions with good molecular specificity. As a crude brain homogenate is too complex to be analysed directly by HPCE, a multi-dimensional approach including solid-phase extraction, reversed-phase HPLC (RP-HPLC) and finally HPCE was developed.

EXPERIMENTAL

Chemicals

Synthetic VIP was purchased from Sigma (St. Louis, MO, USA) and was used without further purification. Trifluoroacetic acid (TFA) was obtained from Pierce and HPLC-grade acetonitrile from Carlo Erba (Milan, Italy). Citric acid and trisodium citrate (Merck, Darmstadt, Germany), monobasic and dibasic sodium phosphate (Sigma) and sodium dodecyl sulphate (SDS) (Sigma) were used to prepare the electrolyte for HPCE. HPLC-grade water obtained with a Milli-Q (Millipore, Bedford, MA, USA) or a Spectrum (Elga, High

Wycombe, UK) purification system was used for making all the solutions.

Tissue acquisition and homogenization

Male rats (OFA strain) were killed by decapitation and the brain was rapidly removed and dissected over crushed ice. The cerebral cortex, hippocampus and cerebellum were weighed, placed in 10 volumes of cold 0.1 M hydrochloric acid and homogenized in a Potter-Elvehjem glass homogenizer. The homogenates were centrifuged (4000 g for 5 min at 4°C) and the supernatants were collected.

Solid-phase extraction

The supernatant was applied to an octadecylsilyl (ODS) disposable cartridge (Sep-Pak; Waters, Milford, MA, USA) that had been activated by eluting in sequence with methanol (2 ml), HPLC-grade water (5 ml) and 0.1% (w/v) TFA (10 ml). The supernatant was passed through the cartridge several times. Thereafter the cartridge was washed with 2 ml of a 0.1% TFA solution containing 24% of acetonitrile and the VIP-rich fraction was eluted with 1.6 ml of a 0.1% TFA solution containing 48% of acetonitrile. The collected effluent was evaporated to dryness (Vacuum Speed Vac, Savant). The residue was reconstituted in 1 ml of 0.1% TFA, filtered through a 0.45-μm Durapore filter (Millipore) and injected into the HPLC system.

Reversed-phase high-performance liquid chromatography

Gradient RP-HPLC separation was performed using the following instrument: two Waters Model 510 pumps controlled by a Waters Model 680 automated gradient controller (Millipore), a Waters static mixer, a six-port injection valve with a 1-ml sample loop (Rheodyne, Cotati, CA, USA), an SPD-6A spectrophotometric detector (Shimadzu, Kyoto, Japan) and a Model 330 recorder (Scientific Instruments, Basle, Switzerland). Separations were made at ambient temperature on a Brownlee Aquapore RP 300 C_8 column (220 × 4.6 mm I.D., particle size 7 μm, pore size 300 Å), which was protected by a Brownlee Aquapore RP 300 C_8 precolumn (30 × 4.6 mm I.D.) (both from Applied Biosystems, Foster City, CA, USA). The mobile phase for gradient elution was composed of 0.1% TFA (phase A) and 0.09% TFA–80% acetonitrile (phase B). The

monitoring wavelength was 214 nm and the flow-rate was 1.0 ml/min.

The column was equilibrated with a mixture of 95% A–5% B mobile phase, and the sample was then injected. The gradient was started when the absorbance returned to the baseline; this gradient was 5% phase B at 0 min, 65% at 60 min and 80% at 70 min. The elution time of synthetic VIP was determined in a separate experiment, after which the HPLC column was washed to avoid any memory effect. The VIP fraction was collected manually and evaporated to dryness. The residue was reconstituted in 10 μl of HPLC-grade water and analysed by HPCE.

High-performance capillary electrophoresis

HPCE separations were performed with a Model 270A capillary electrophoresis system (Applied Biosystems) and the electropherograms were recorded on a Chromjet integrator (Spectra-Physics, San Jose, CA, USA). Separations were carried out with an uncoated fused-silica capillary (72 cm × 50 μm I.D.) (Applied Biosystem) and on-column UV detection at 200 nm was carried out. The capillary was flushed with 0.1 M sodium hydroxide solution (2

min) and buffer (5 min) before each electrophoretic run. Injections were made by vacuum for a fixed period of time. HPCE analyses were carried out with two modes of separation *viz.*, CZE and MECC.

The electrolyte used for CZE was 20 mM sodium citrate buffer (pH ranging from 2.5 to 5.0). The capillary was maintained at 30°C and the running voltage was 25 kV. A low-pH mobility marker (Applied Biosystems) was simultaneously injected with the sample. The electrophoretic mobility (μ) was determined according to the equation $\mu = (L_dL_t/V)(1/t - 1/t_m) + \mu_m$ [17], were L_d is the length of the capillary from the injection end to the detector, L_t is the total length of the capillary, V is the voltage across the capillary, t and t_m are the migration times of the sample and of the mobility marker, respectively, and μ_m is the electrophoretic mobility of the mobility marker [$\mu_m = 3.95 \cdot 10^{-4}$ cm^2/V · s in 20 mM sodium citrate buffer (pH 2.5) at 30°C]. If μ_m was unknown, a relative electrophoretic mobility (μ_r) was determined according to the equation $\mu_r = (L_dL_t/V)(1/t - 1/t_m)$.

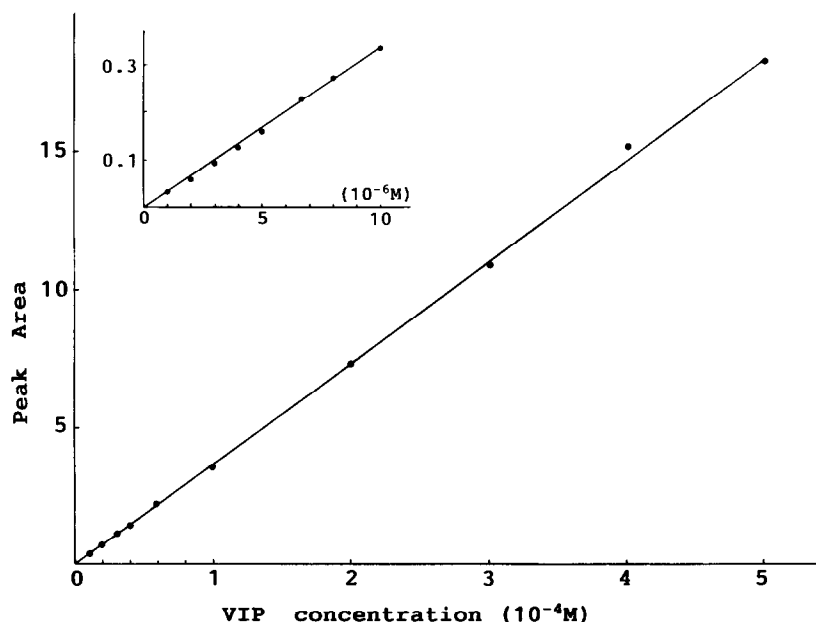The MECC separations were made with a 10 mM sodium phosphate buffer (pH 7.0) containing 20

Fig. 1. Plot of the peak area of synthetic VIP *vs.* the concentration of the standard solutions. Conditions: 20 mM sodium citrate buffer (pH 2.5); 30°C; 25 kV; vacuum injection for 1 s. The inset shows the plot for VIP concentrations between 10^{-6} and 10^{-5} M.

mM sodium dodecyl sulphate (SDS). A 0.2% mesityl oxide solution (used as a marker of electroosmotic flow) was injected simultaneously with the sample. The capillary was maintained at 35°C and the running voltage was 25 kV. As a marker of micelles migration was not injected, the capacity factor (k') [21] was not determined and only the migration time was expressed.

RESULTS

CZE analysis of synthetic VIP

In the first phase of this work, the characteristics of the electrophoretic migration of synthetic VIP were studied using 20 mM sodium citrate buffer (pH 2.5) as electrolyte. The electrophoretic mobility of VIP was found to be 2.42 ± 0.05 cm^2/V · s (mean ± standard deviation) and the limit of detection was about 10^{-6} M (for a 1-s injection). The peak

area varied linearly with synthetic VIP concentration between 1 · 10^{-6} and 5 · 10^{-4} M (r = 0.99975) (Fig. 1). The relative standard deviation was 1.8% for the migration time, 1.6% for the electrophoretic mobility and 4.6% for the peak area.

Detection of VIP in rat brain regions

A sample of cerebral cortex (2.97 g of wet tissue dissection from three rats) was homogenized and extracted and the VIP-rich extract was fractionated by RP-HPLC (Fig. 2). A 0.5-ml fraction was collected at the retention time of synthetic VIP and analysed by CZE. A peak exhibiting the same electrophoretic mobility (μ = 2.44 cm^2/V · s) as synthetic VIP was present on the electropherogram (Fig. 3B). The height of this peak increased when synthetic VIP was co-injected with this sample, whereas no additional peak appeared (Fig. 3C). Consequently, this peak of interest may correspond to endogenous VIP.
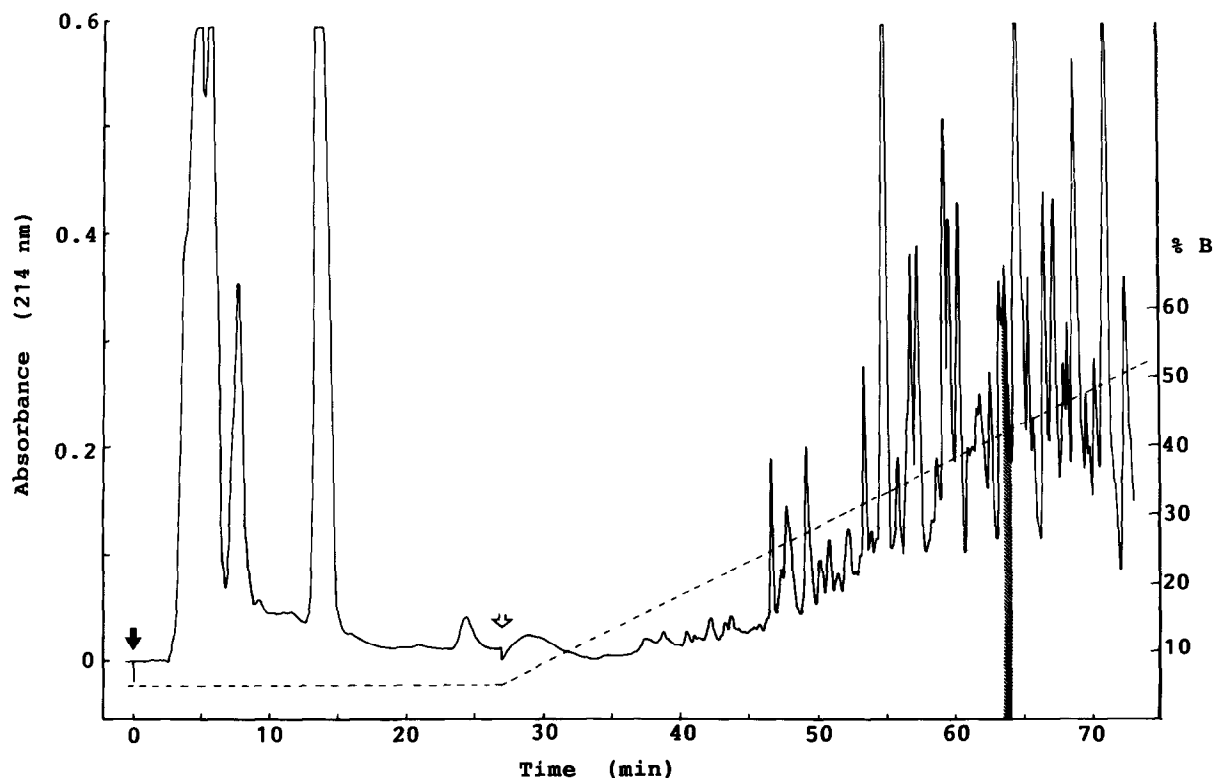

Fig. 2. Reversed-phase HPLC profile of the VIP-rich fraction obtained from the extraction of a cerebral cortex sample. The gradient profile is shown by the dotted line (expressed as % of mobile phase B). Closed arrow, injection; open arrow, beginning of the gradient. The fraction indicated by the shaded area was collected and analysed by HPCE. For chromatographic conditions, see text.
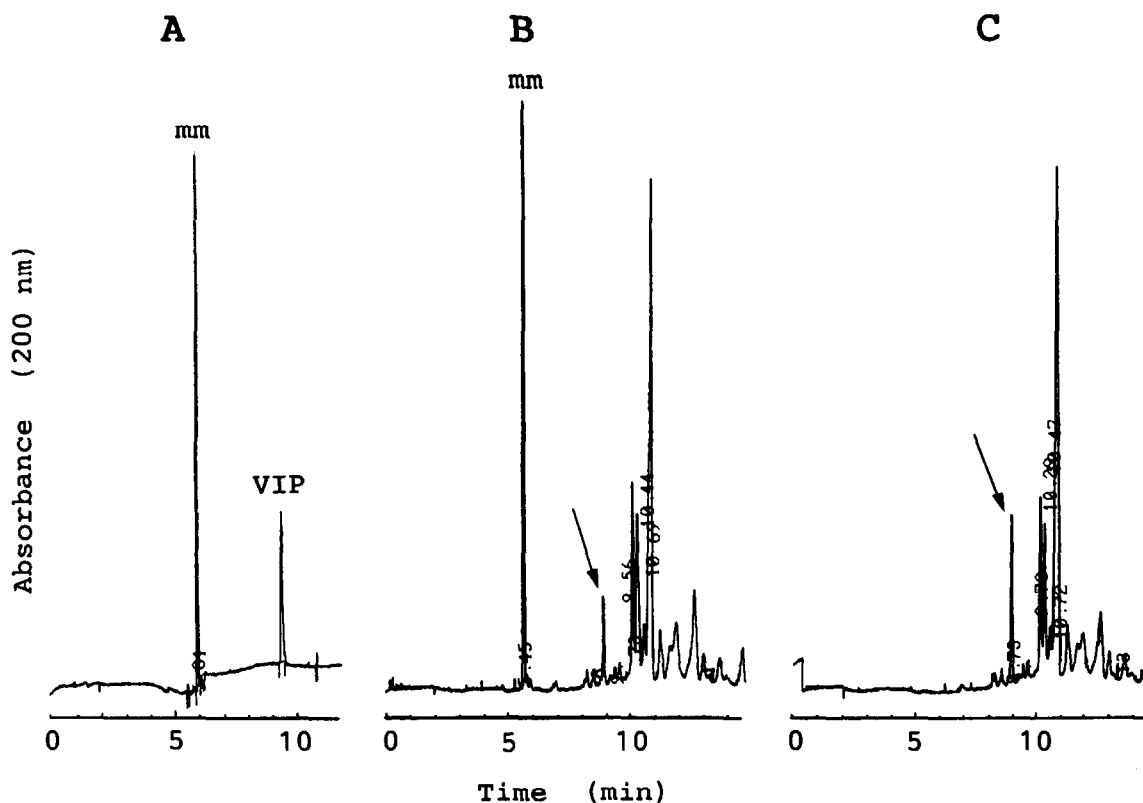
Fig. 3. CZE analysis of the sample of cerebral cortex. Conditions: 20 mM sodium citrate buffer (pH 2.5); 30°C; 25 kV. (A) Injection of $5 \cdot 10^{-6}$ M synthetic VIP and of mobility marker (mm). Each solution was injected for 2 s. (B) Injection of cerebral cortex sample (4 s) and of mobility marker (2 s). The arrow shows a peak with the same electrophoretic mobility as synthetic VIP (2.44 cm²/V · s). Sample corresponding to the RP-HPLC fraction indicated on Fig. 2. (C) Injection of the same sample (4 s) and of $5 \cdot 10^{-6}$ M synthetic VIP (2 s). The height of the peak shown by the arrow is increased as compared with (B).

A sample of hippocampus (0.99 g of wet tissue dissected from five rats) was analysed by the same protocol. The electropherogram showed a small peak with an electrophoretic mobility equal to 2.44 cm²/V · s. When synthetic VIP was co-injected with the sample, the height of this peak increased, suggesting that it may be due to endogenous VIP present in the hippocampus.

Finally, a sample of cerebellum (1.0 g of wet tissue) was analysed by the same protocol, but no peak with the same electrophoretic mobility as synthetic VIP was present on the electropherogram.

HPCE characterization of brain VIP

A sample of cerebral cortex was analysed by HPCE using different electrolytes in order to characterize better the peak which was detected as VIP.

TABLE I

ELECTROPHORETIC MOBILITY OF SYNTHETIC AND BRAIN VIP AT DIFFERENT pH VALUES

The mobility is expressed as μ_r (see text). Electrophoretic conditions: CZE, 20 mM sodium citrate buffer; 30°C; 25 kV. Sample: cerebral cortex.

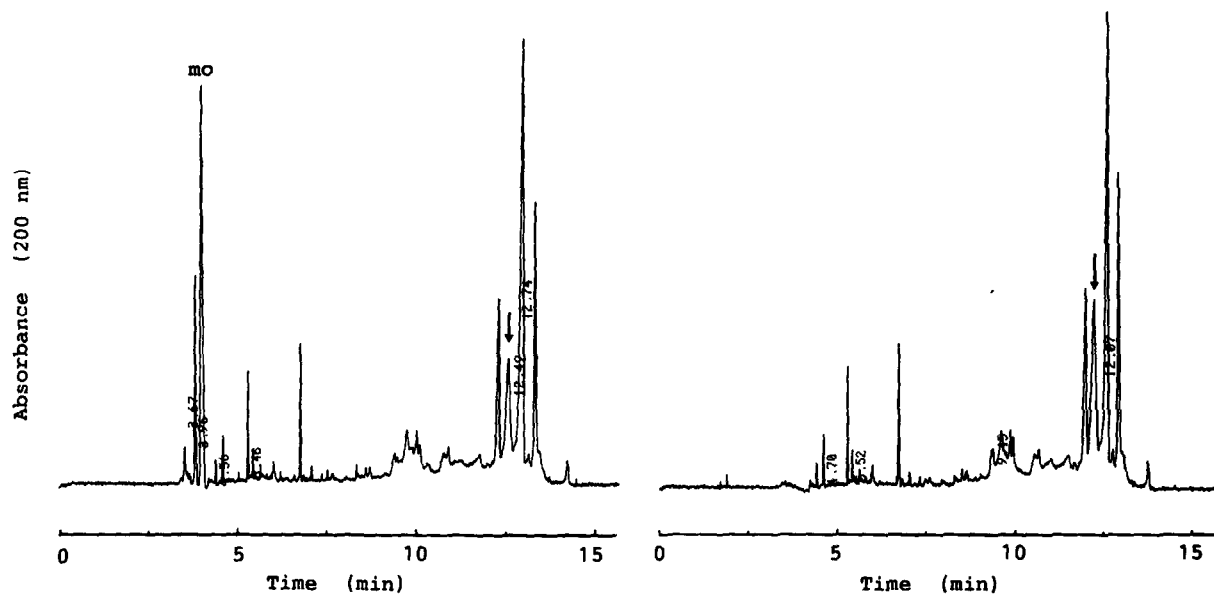| pH of electrolyte	μ_r (10^{-4} cm²/V · s)	
	Synthetic VIP	Brain VIP
2.5	−1.56	−1.61
3.0	−1.22	−1.24
4.0	−0.72	−0.75
4.5	−0.48	−0.48
5.0	−0.29	−0.31

Fig. 4. MECC analysis of the sample of cerebral cortex. Conditions: 10 m*M* sodium phosphate buffer (pH 7.0) containing 20 m*M* SDS; 35°C; 25 kV. Left: injection of cerebral cortex sample (2 s) and of 0.2% mesityl oxide (mo; 1 s). Right: injection of the same sample (2 s) and of 10^{-5} *M* VIP (1 s). The height of the peak shown by the arrow is increased.

Multiple CZE analyses of the cortex sample and of the synthetic VIP were made at various pH values. The values of the relative electrophoretic mobility (μ_r) of the peak from the brain extract and of the synthetic VIP were determined. As shown in Table I, these mobilities exhibited similar changes when the pH of the electrolyte was increased from 2.5 to 5.0.

Attempts were made to perform CZE separations at higher pH, but such conditions appeared unsuitable for analyses for VIP. Synthetic VIP did not migrate when the electrolyte was 10 m*M* sodium phosphate buffer (pH 7.0) or 20 m*M* borate buffer (pH 8.25) containing 20 m*M* sodium chloride. It was detected as a very broad peak when CZE was carried out with 20 m*M* CAPS buffer (pH 11.0).

Conversely, the cortex sample was analysed by MECC, as VIP migrates in this mode. The electropherogram showed a peak with the same migration time as synthetic VIP. The height of this peak increased when synthetic VIP was co-injected, whereas no additional peak appeared on the electropherogram (Fig. 4).

Determinations of VIP in cerebral cortex

Homogenates of cerebral cortex were divided in-

to two equal parts and 500 pmol of synthetic VIP were added to one part as an internal standard. The samples were subjected to the entire protocol and the areas of the peaks corresponding to VIP were measured during the CZE analysis. The concentration of VIP in rat cerebral cortex was found to be 25.5 ± 7.5 pmol per gram of tissue.

DISCUSSION

To our knowledge, this is the first work in which a commercial HPCE instrument with UV detection has been used to determine a neuropeptide in mammalian brain. The results presented in this paper illustrate the molecular specificity of the method. VIP was identified on the electropherograms as a peak which was well resolved from other endogenous compounds. Such a result was possible owing to the high separation power of HPCE and the orthogonal approach which has been developed, *i.e.*, gradient RP-HPLC separation followed by HPCE analysis.

The electropherograms of the CZE analysis of rat cerebral cortex showed a peak that seems to correspond to true endogenous VIP for several reasons. First, this peak was present in a fraction that was

collected at the retention time of synthetic VIP during the RP-HPLC step. Second, this peak had the same electrophoretic mobility as synthetic VIP under different conditions of CZE analysis, *i.e.*, with five sodium citrate buffers with pH values between 2.5 and 5.0. These data demonstrated that this peak corresponds to a compound that has the same charge to mass ratio as authentic VIP in this pH range. Third, a peak that has the same migration time as synthetic VIP was found during the MECC analysis of the same samples. These data show that the sample contains a compound that has both the same hydrophobicity and the same charge to mass ratio as synthetic VIP. Finally, this peak was found in samples from cerebral cortex and hippocampus, two brain regions known to contain VIP neurons, whereas it was absent from cerebellum, a part of the brain that is devoid of VIP innervation [22,23].

The marked specificity of the present method arises from the possibility of performing multiple HPCE analyses under various conditions of separation. Indeed, as only a small amount of material is injected into the HPCE instrument, the same sample can be used for many electrophoretic runs. In addition, most of these HPCE analyses can be performed within a day, as the run times are short and because the equilibration of the capillary seems to be rapid after each change of electrolyte. Although the selectivity of the present method seems good, it may be improved by including more HPCE analyses under different conditions of separations, such as CZE with an electrolyte other than citrate buffer, CZE at neutral or basic pH in a coated capillary and MECC in the presence of surfactants other than SDS. During this work, attempts to perform CZE analysis in an uncoated capillary at neutral or basic pH were unsuccessful. Under such conditions, synthetic VIP did not migrate or was eluted as a very broad peak, indicating strong interactions between the peptide and the capillary wall. VIP is a 28-amino acid polypeptide that contains two arginine and three lysine residues and therefore has a basic character. Consequently, VIP is still in a cationic form at pH 7.0–8.5 and interacts with the capillary wall, which is negatively charged owing to the dissociation of silanol groups. Such a phenomenom has been well described for basic proteins and peptides [18,24].

The present method seems to be quantitative, although this aspect has not been fully investigated. In CZE, the peak area, migration time and electrophoretic mobility were reproducible. Moreover, a plot of the peak area of synthetic VIP *vs.* sample concentration gave excellent linearity. The HPCE measurement of VIP in cerebral cortex gave values that are of the same order of magnitude as the concentrations determined by RIA in rats of the same strain [6].

One drawback of the present method is its lack of sensitivity, mainly due to the low sensitivity of the UV detection used in the HPCE analysis. More sensitive means of detection, such as laser-induced fluorescence [19,25], should decrease the limit of detection by several orders of magnitude. In addition, further studies are needed to improve the extraction and the RP-HPLC fractionation, in order to decrease the loss of material that occurs in these two steps.

In comparison with RIA, the present method has a higher molecular specificity, as HPCE separations monitor indirectly primary structural parameters. Nevertheless, it only allows the determination of VIP that is contained in grossly dissected brain regions and cannot determine the low level of VIP present in discrete brain areas dissected as "punches" [6], a measurement that can still only be made by RIA. Hence, the two techniques appear to be complementary, RIA allowing highly sensitive measurements but with a limited molecular specificity, whereas the proposed HPCE method can be either a reference method to validate results obtained by RIA, or can be used to study neuropeptides with good molecular selectivity when the amount of biological material is not as limited.

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