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Identification of substance P metabolites using a combination of reversed-phase high-performance liquid chromatography and capillary electrophoresis

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Abstract

Gradient elution reversed-phase high-performance liquid chromatographic and capillary electrophoretic separations were optimised to separate substance P (SP) and twelve of its fragments. The methods were applied to a study of the in vivo metabolism of substance P in the rat after intrastriatal injection of the peptide (10 nmol). SP and significant amounts of its N-terminal fragments, SP(1-7) and SP(1-4), were detected but no major C-terminal fragments could be identified. At the concentration studied, the metabolism of SP was shown to follow zero order elimination kinetics with a rate of decay of 0.2 nmol/min. As we have shown that SP(1–4) and SP(1–7) can be produced in vivo in the striatum in relatively large amounts, it is conceivable that these fragments contribute to the overall pharmacological pattern of activity of the parent peptide.

Keywords: Substance P; Peptides

1. Introduction

Substance P (SP), Arg^{1}–Pro^{2}–Lys^{3}–Pro^{4}–Gln^{5}–Gln^{6}–Phe^{7}–Phe^{8}–Gly^{9}–Leu^{10}–Met^{11}–NH_2, a peptide of the tachykinin family, is present in the central and peripheral nervous systems and induces a wide range of biological effects [1,2]. More recently, SP fragments have been shown to possess biological activity in various experimental models [3–6]. N- and C-terminal fragments may induce effects which are similar or opposite to those induced by the parent peptide. We have shown that both N- and C-terminal SP fragments increase dopamine release in rat striatum [7,8], suggesting that the metabolites may have modulator effects alongside SP in the basal ganglia.

To elucidate the role of SP and the contribution to its modulatory effects made by its metabolites, it is necessary to determine not only the concentration of the parent peptide but also to identify and quantify which SP fragments are present under physiological conditions. Functional studies with various fragments gain relevance if the active fragments are formed in vivo. Because of the low concentrations of peptides in the nervous system, the tissue stores and peptide outflow in the extracellular fluid can only be mea-

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sured by radioimmunoassay (RIA). However, because of the lack of specificity of RIA, peptides must be separated from their metabolites prior to quantification. Furthermore, N- and C-terminal directed antibodies must be used in order to measure fragments corresponding to the two domains of a peptide. The fact that even such antibodies do not significantly cross-react with all the potential metabolites limits the number of fragments that can be detected. As some fragments may be missed, this approach may lead to a completely erroneous conclusion about the pattern of metabolism. Sakurada et al. [9] identified SP(1–7) and SP(5–11) as major fragments in rat central nervous system. Igwe et al. [10], using a C-terminal directed antibody on fractions collected after gradient elution high-performance liquid chromatography (HPLC), detected SP(2–11) and SP(5–11) in significant concentrations in mouse spinal cord. However, when the concentrations were determined by UV detection at 214 nm they were several orders of magnitude greater [10]. The problem of high background signals when low wavelength UV detection is used can be overcome by subjecting HPLC fractions to capillary electrophoresis (CE) [11]. We report here the use of CE as a method complementary to HPLC in the study of SP metabolism in rat striatum.

2. Experimental

2.1. Materials

Substance P (SP), SP(1–4), SP(1–7), SP(1–9), SP(2–11), SP(3–11), SP(4–11), SP(5–11), SP(6–11), SP(8–11), SP(9–11), leucine and hydrogen peroxide (3% w/v) were purchased from Sigma (Poole, UK). SP(10–11) and SP(1–2) were from Bachem (Saffron Walden, UK). Oxidized forms of the peptides (sulphoxides) were obtained by oxidation with 0.03% H_2O_2 for 60 min at 40°C [12]. [4,5-^{3}H-Leu^{10}]SP (176 Ci/mmol) was purchased from Zeneca (Northwich, UK). Trifluoroacetic acid (TFA) and acetonitrile (far UV grade for HPLC) were from BDH (Poole, UK). Optiphase HiSafe 3 scintillation fluid was obtained from Wallac (Loughborough, UK). Isolute C_{18} extraction columns (International Sorbent Technology, Hengoed, UK) were a gift from Jones Chromatography (Hengoed, UK).

2.2. Liquid chromatography

The peptides were separated by gradient elution chromatography using two Model 110B pumps controlled by an NEC PC8300 computer and a SYSTEM GOLD 406 analogue interface module (Beckman, High Wycombe, UK). Samples were injected via a Rheodyne 7725 injection valve fitted with a 100-μl loop. A precolumn (10×2 mm I.D.) packed with Co:Pell ODS (Whatman, Maidstone, UK) was inserted between the valve and the Ultrasphere 5 μm ODS analytical column (250×4.6 mm I.D., Beckman). The gradient was formed by using 0.1% TFA in water (solution A) and 0.09% TFA in acetonitrile–water, 1+1 (solution B). High pressure mixing was achieved with a dynamic mixer (Beckman). Separations were performed using a gradient run from a starting composition of 10% solution B to 60% solution B in 20 min and then held at 60% for 10 min before being returned to 10% over 2 min. The column was equilibrated for 10 min before each injection.

Peaks were detected at 214 nm using an SA6504 variable wavelength absorbance detector (Severn Analytical, Shefford, UK) connected in parallel to a Servogor 220 analogue recorder (Recorderlab Services, Sutton, UK) and a JCL 6000 16 bit A/D converter board (Jones Chromatography) fitted in an Elonex PC88M computer. Fractions (0.5 ml) of HPLC eluent, for radioactivity determination or subsequent CE analysis, were collected using a Gilson FC204 fraction collector (Anachem, Luton, UK).

Calibration curves were generated by injecting mixtures of SP, SP(1–4), SP(1–7), SP(1–9), SP(8–11), SP(6–11) and SP(5–11) at 1.25, 2.5, 5, 10 and 20 μ*M* in 1 *M* HCOOH. Estimates of precision were obtained from replicate (6) injections at 2.5 and 10 μ*M*. The percentage recovery was determined by homogenising control striata (4) in a mixture of the peptides (10 μ*M*) in 1 *M* HCOOH.

2.3. Capillary electrophoresis

The capillary electrophoretic system was a Beckman P/ACE 5510 fitted with a fixed wavelength (200 nm) UV detector. A fused-silica capillary (Composite Metal Services, Hallow, UK) 57 cm (50 cm to the detector window)×75 μm I.D., was used. The

peptides were separated using 0.05 *M* phosphate, pH 2.44, as the run buffer. The capillary was washed with 0.1 *M* NaOH for 10 min and then run buffer for 10 min prior to electrophoresis at applied potentials between 15–20 kV. The capillary was maintained at 20°C and samples were applied by pressure or electrokinetic injection. When using the migration time to identify a fragment, two known peptides or amino acids were injected as migration time markers.

2.4. Solid-phase extraction

Isolute solid-phase extraction (SPE) columns with a reservoir volume of 1 ml and a sorbent bed of 100 mg were used to evaluate three C_{18} phases: a trifunctional phase (trifunctional), an end-capped trifunctional phase (end-capped) and a monofunctional phase (monofunctional). Initially, wash, sample and eluent solutions were drawn through the columns by centrifugation (30 s at 400 *g*) and collected for analysis to determine the optimum conditions for the retention of the peptides. To determine the elution profile of the peptides, trifunctional and monofunctional columns were prepared by sequentially drawing 1 ml volumes of methanol, water, 1% TFA in 80% CH_3CN, water and 1 *M* formic acid, through them under reduced pressure. The sample, a mixture of SP and SP fragments (10 μ*M*) in 1 *M* formic acid (0.4 ml), was drawn through the columns which were washed with water (1 ml) prior to elution with 1% TFA in 80% CH_3CN (5×0.2 ml). The fractions were evaporated to dryness under nitrogen and the residues reconstituted in water (0.4 ml) for analysis by HPLC.

A slightly modified method was used for SPE of biological extracts. Trifunctional columns were prepared as described above and the supernatant solution from striatal homogenates in 0.1 ml 1 *M* formic acid was applied and allowed to run through slowly under gravity or by the application of slight positive pressure to initiate the flow. The columns were washed with water (1 ml) and eluted with 1% TFA in 80% CH_3CN (2×0.25 ml) under gravity into pointed glass tubes. After evaporation under nitrogen, the residue was dissolved in water (0.1 ml) and 50 μl injected into the chromatograph.

SPE was used to concentrate pooled fractions collected from the HPLC eluent. Samples (50 μl) were pipetted into a 1.5 ml Eppendorf tube, and the CH_3CN evaporated under a gentle stream of nitrogen. The aqueous residue was applied to trifunctional SPE columns, prepared and eluted as described above. After evaporation under nitrogen, the residue was dissolved in water (10 μl) prior to CE.

2.5. In vivo metabolism of substance P

Nine male Wistar rats (250–300 g, Charles River, Margate, UK) were sedated with midazolam (4 mg/kg, i.p.) prior to anaesthesia with Hypnorm (1 ml/kg, i.p., Janssen, High Wycombe, UK). A mixture of substance P (10 nmol) and [^{3}H]SP (1 pmol), dissolved in 1 μl saline, was injected into the right striatum (A/P=+0.3 vs. bregma, *L*=3, *D*=6 according to the stereotaxic atlas of Paxinos and Watson [13]) over 30 s. The needle was left in place for 60 s and withdrawn over 30 s. The animals were sacrificed 2, 10 or 20 min after withdrawal of the needle. A coronal slice of 2 mm thickness containing the injection site was dissected. The region corresponding to the striatum was dissected out, the contralateral side being used as a control. The tissue was homogenised manually (12 up and down strokes) in ice-cold formic acid (1 *M*, 0.4 ml) using a glass/glass homogeniser. The homogenates were centrifuged at 7000 *g* for 30 min at 4°C. The supernatant layers were transferred to clean tubes and were stored at either 4°C or −20°C, prior to analysis.

2.6. Radioactive counting

The radioactivity in collected fractions was determined using either a Wallac Model 1211 Minibeta or a Model 1410 liquid scintillation spectrometer. Counting efficiencies, determined by the channels ratio method (1211) or external standardization (1411), were in the range 49–51%.

3. Results and discussion

3.1. HPLC separation

Reversed-phase separations of SP and SP related peptides have often employed gradients formed from TFA and acetonitrile or acidic phosphate buffers and acetonitrile [14]. Igwe et al. [10] used a gradient of

0% to 60% acetonitrile in 0.1% TFA over 40 min to separate SP and four of its fragments. The elution order was SP(1–4), SP(1–7), SP(1–9), SP and SP(5–11) with retention times relative to SP of 0.46, 0.63, 0.80, 1.00 and 1.05, respectively. By starting our gradient at 5% acetonitrile we were able to elute the shorter N-terminal fragments, SP(1–4) and SP(1–7), earlier and ensure they were resolved from SP(10–11) and SP(9–11), respectively (Table 1, Fig. 1A). However, under these conditions six of the C-terminal metabolites had relative retention times in the range 0.99–1.11 and several pairs of compounds were not resolved. Furthermore, SP is known to be readily oxidized to its sulphoxide and the possible presence in biological samples of other oxidized SP fragments could not be ignored. The reference sulphoxides eluted earlier than their corresponding sulphides (Fig. 1B). The sulphoxide peaks were slightly distorted and on closer inspection it was clear that this was due to partial resolution of two peaks. As the sulphoxides are chiral, the pairs of peaks presumably are due to the formation of diastereoisomers. It was important to define the retention characteristics of the sulphoxides in order to prevent peaks being incorrectly assigned. In particular, SP(8–11) could be confused with SP sulphoxide, and leucine with SP(9–11) sulphoxide (Table 1).

Table 1
Relative HPLC retention times (RRT) and electrophoretic migration times (RMT) of the reference compounds

No.	Compound	RRT	RRT^{a}_{ox}	RMT
1	SP	1.00	0.86	1.00
2	SPOH	1.03	0.88	–
3	Arginine	–	–	0.76
4	SP(1–2)	0.17	–	0.67
5	SP(1–4)	0.31	–	0.65
6	SP(1–7)	0.55	–	0.86
7	SP(1–9)	0.77	–	0.93
8	SP(2–11)	1.07	0.88	1.21
9	SP(3–11)	0.99	0.86	1.17
10	SP(4–11)	1.05	0.89	1.71
11	SP(5–11)	1.04	0.88	1.64
12	SP(6–11)	1.10	0.91	1.53
13	SP(7–11)	1.11	0.93	1.42
14	SP(8–11)	0.85	0.66	1.29
15	SP(9–11)	0.53	0.27	1.09
16	SP(10–11)	0.37	0.13	1.02
17	Leucine	0.28	–	–

[a] RRT_{ox}, relative retention time of oxidised fragments.

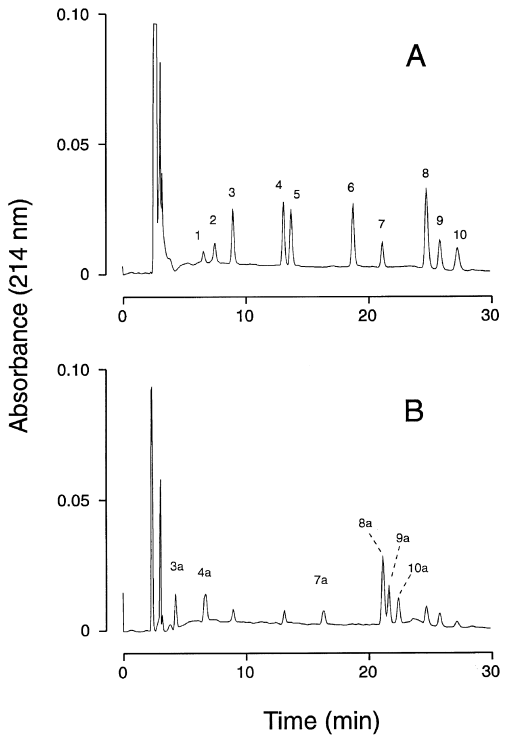

Fig. 1. HPLC separation of selected reference compounds. (A) Leucine, SP and a mixture of C- and N-terminal fragments. (B) Mixture of SP and C-terminal fragments after treatment with 0.03% H_2O_2. Peaks: 1=leucine; 2=SP(1–4); 3=SP(10–11); 4=SP(9–11); 5=SP(1–7); 6=SP(1–9); 7=SP(8–11); 8=SP; 9=SP(5–11); 10=SP(6–11); the corresponding sulphoxides are denoted as 3a, 4a, etc. Concentrations: all 10 μ*M* except SP(9–11) and SP(10–11) (100 μ*M*) and leucine (0.1 m*M*). Injection volume 50 μl.

3.2. Electrophoretic separation

The electrophoretic migration order of the SP fragments was as predicted by the Offord equation [15], with the exception of SP(10–11) which migrated more slowly than predicted. By using a longer capillary than that used by Nyberg et al. [16] we were able to resolve more fragments (Fig. 2). In all, SP and 12 fragments were resolved in 25 min. Kostel et al. [17] used a complex run buffer containing phytic acid and a sulphonated β-cyclodextrin to separate SP and eight fragments at neutral pH.

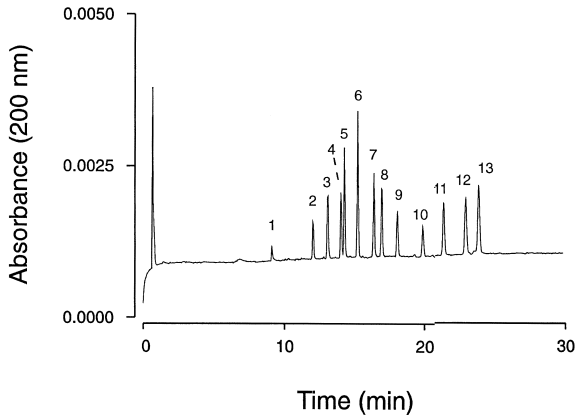

Fig. 2. Electrophoretic separation of SP and its fragments. Peaks: 1=SP(1–4); 2=SP(1–7); 3=SP(1–9); 4=SP; 5=SP(10–11); 6=SP(9–11); 7=SP(3–11); 8=SP(2–11); 9=SP(8–11); 10=SP(7–11); 11=SP(6–11); 12=SP(5–11); 13=SP(4–11). Concentrations: all 2 μ*M* except SP(9–11) and SP(10–11) (20 μ*M*). Conditions: 300 V/cm (17.1 kV) for 30 min. Injection: 10 s high pressure.

Adsorption of peptides onto the walls of the capillary can result in a reduction of electroosmotic flow and a consequent increase in migration times. Coated capillaries may be used in an attempt to reduce the effect, but we found thorough washing with 0.1 *M* NaOH and re-equilibration with buffer between runs to be satisfactory, although there was a tendency for the migration times to increase at the start of a run. For example, in a batch of 12 samples the migration times increased by 15% between the first and fourth injection, after which they were stable, the relative standard deviations (R.S.D.s) of the migration times for the remaining 8 injections being <1%.

3.3. Solid-phase extraction

Retention on the end-capped phase was found to be poor, with some 30% of the applied SP not being retained. Consequently, these columns were not investigated further. Retention was better with the other two phases, and the elution profiles of SP and selected SP fragments were investigated to determine which column offered the best combination of retention and elution. Fractions of the eluent were collected to determine the smallest volume that was required to retrieve the peptides. Only 14% of the applied SP(1–4) was recovered from the trifunctional column (Fig. 3), which probably reflects poor retention of this small polar molecule on this phase. The recoveries of other peptides tested were >70%, with the highest recovery being 86% for SP(5–11). With the monofunctional column 75% of the applied SP(1–4) was recovered in an elution volume of 0.6 ml (Fig. 4). Generally, the elution volumes required with the monofunctional column were greater than those for the trifunctional. This was particularly noticeable for the parent peptide, the volume required for elution increasing from 0.4 to 0.8 ml. Furthermore, the total recovery fell from 77% to 55% (compare the appropriate panels in Figs. 3 and 4).

Application of SPE to supernatant solutions from striatal homogenates resulted in only a marginal improvement of the chromatograms (Fig. 5). Peaks at the retention time of SP could be seen in samples from both treated and control (contralateral) striata, however the peaks from the treated side were clearly larger. The signal from control samples was not due to diffusion of SP from the injection site into the opposite hemisphere, as in the experiments in which [^{3}H]SP was injected, the radioactivity in the samples from the contralateral side was not significantly different from the background counts (data not shown). Neither were the peaks due to endogenous SP, as shown by CE of the appropriate fractions (see below). With this level of background signal, it is extremely unlikely that HPLC with UV detection could ever be used to determine endogenous concentrations of SP.

Because of the limited utility of SPE and the low recoveries of the smaller more polar fragments, in subsequent experiments supernatant solutions were injected directly onto the chromatographic column. This is not to conclude that SPE has no place in the analysis of SP and related peptides, but at the concentrations used in our study and the fact that minimum volumes of homogenising solutions were used, it offered no clear advantage. In studies using larger volumes of homogenising solution or when it is necessary to remove interfering substances prior to immunoassay, then SPE may be of value. However, it was a convenient way of concentrating pooled fractions of HPLC eluent.

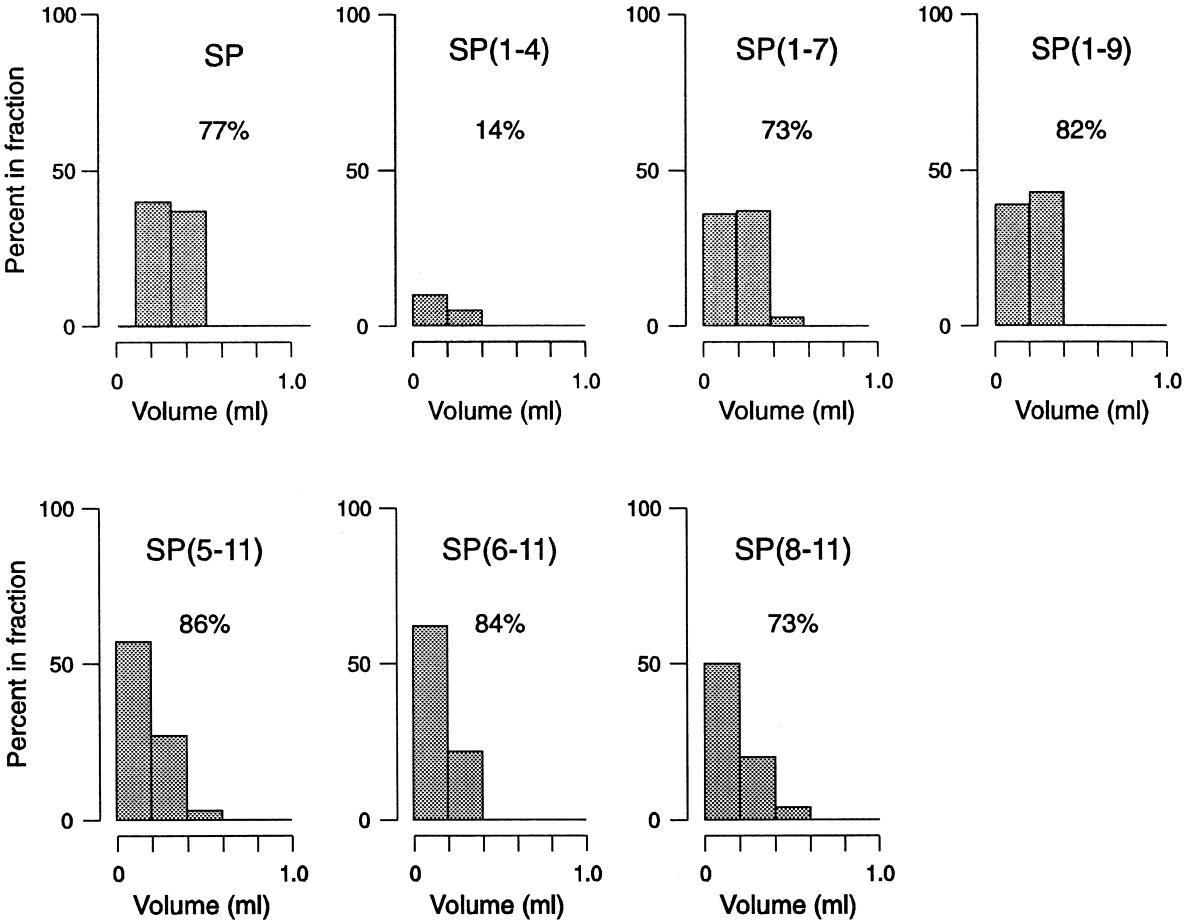

Fig. 3. Elution of SP and six of its fragments from trifunctionally bonded C_{18} extraction columns. The columns were eluted with 0.2 ml volumes of 1% TFA in 80% CH_3CN. The data are the mean of two determinations. The figures refer to the total percentage recovered.

3.4. Combination of HPLC and CE

Because 'free solution' CE separates on the basis of mass to charge it is an ideal complementary technique to reversed-phase HPLC, but separating compounds with similar mass/charge ratios is difficult. In the case of SP and its fragments, sulphoxide metabolites, which only differ from the parent peptides by 16 mass units, and deaminated metabolites, which only differ by one unit, could not be resolved. However, as the sulphoxide metabolites are well resolved from their nonoxidized forms by HPLC, the poor electrophoretic separation is not a problem if the sample is subjected to HPLC prior to CE. The converse was also true, i.e. compounds which co-eluted from the HPLC column could be separated by CE. Thus, we were able to show that the increase in the size of peaks at the retention times of SP and SP(1–7) after treatment with SP (Figs. 5 and 6) were due to the presence of these peptides (Fig. 7). Equally importantly, we were able to demonstrate that in control samples, peaks eluting at the same retention times as SP and SP(1–7) were not due to high endogenous concentrations of these peptides but due to some other unknown material, since electropherograms of HPLC fractions collected at the retention times of SP and SP(1–7) showed the presence of UV absorbing material, but not at the

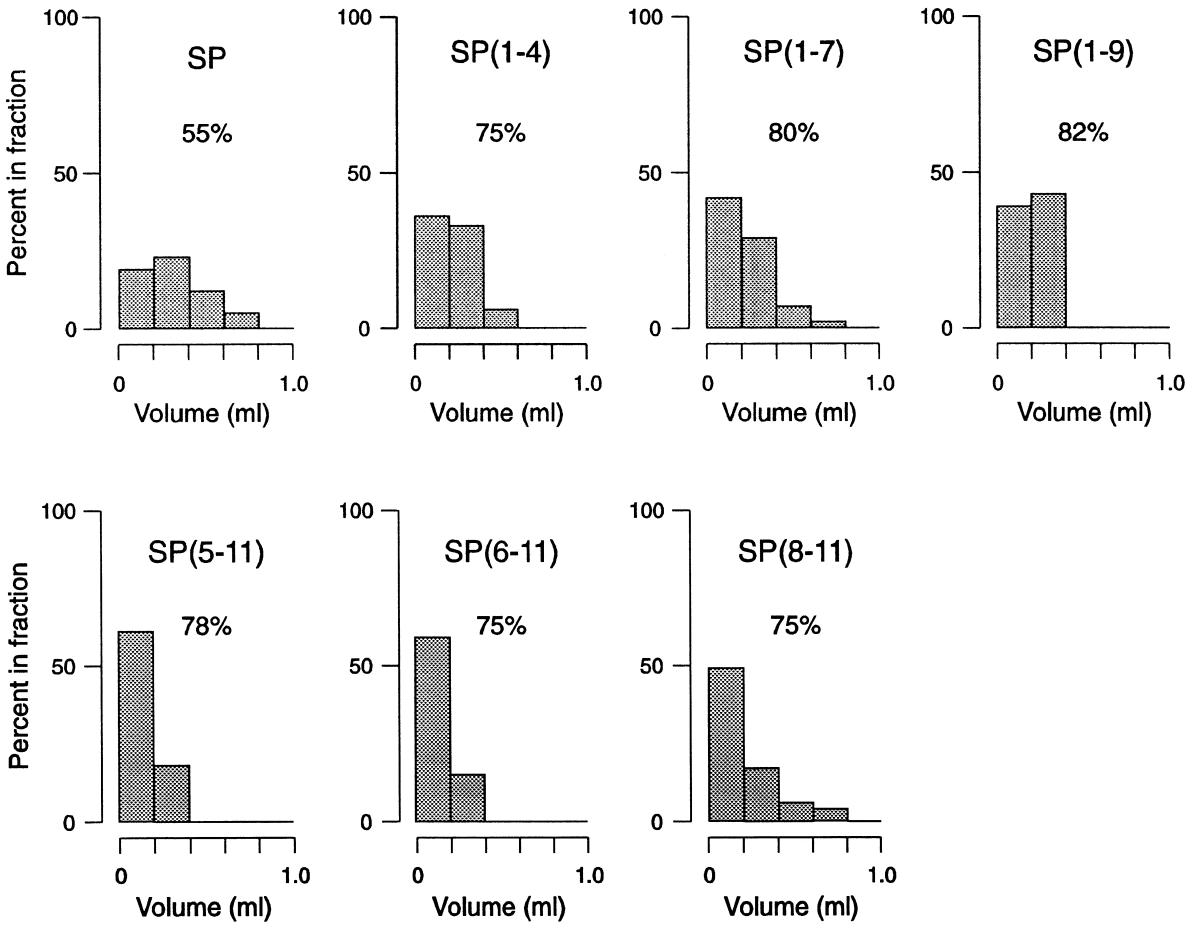

Fig. 4. Elution of SP and six of its fragments from monofunctionally bonded C_{18} extraction columns. The columns were eluted with 0.2 ml volumes of 1% TFA in 80% CH_3CN. The data are the mean of two determinations. The figures refer to the total percentage recovered.

migration times of SP or SP(1–7) (Fig. 7). By comparing the areas of the CE peaks with those for known standard solutions injected under the same conditions, the amount of SP(1–7) was estimated to be approximately 10% of the amount of SP.

Because of the high background signal in the chromatograms, it was not possible to identify other peaks which might be peptide related in the treated samples so HPLC fractions corresponding to the retention times of the other fragments were systematically examined by CE. When fractions collected at the retention volume of SP(1–4) were subjected to CE, a peak with the same electrophoretic mobility as SP(1–4) was observed (Fig. 8). The amount of SP(1–4), estimated as described for SP(1–7), was approximately 15% of the amount of SP. No SP(1–9) was detected in the HPLC fraction collected at the retention time of this fragment.

When HPLC fractions corresponding to the retention times of SP(5–11), SP(6–11) and SP(8–11), were examined by CE, peaks at the migration times corresponding to these three peptide fragments were not observed, even after concentration by evaporation under nitrogen. SPE was applied to pooled HPLC fractions collected between 25 and 30 min (the interval in which SP(5–11) and SP(6–11) normally elute) and the concentrated extract examined by CE, and again no peaks corresponding to

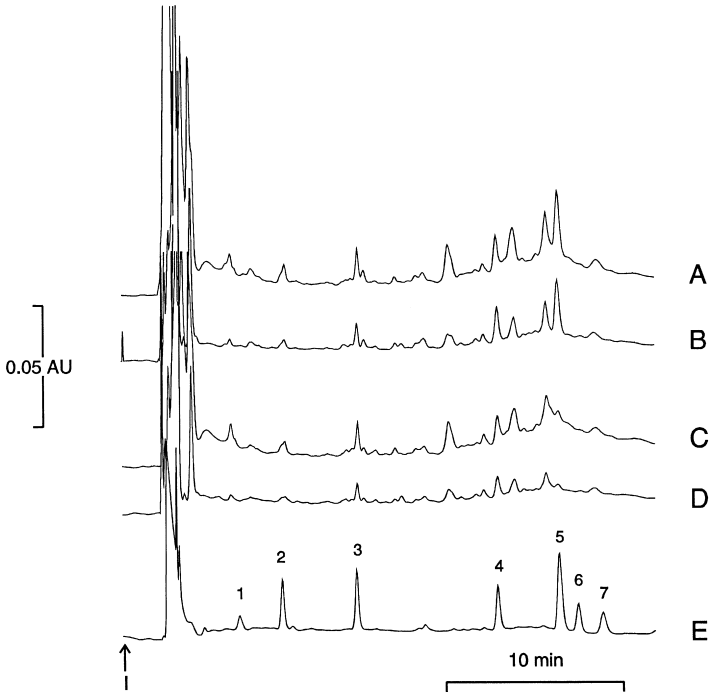

Fig. 5. HPLC of supernatant solutions from treated and control striata prepared 2 min after intrastriatal injection of SP (10 nmol). (A) treated striatum (B) treated after SPE (C) control striatum (D) control striatum after SPE and (E) solution of reference compounds in 1 *M* HCOOH. Peaks: 1=leucine; 2=SP(10–11); 3=SP(9–11); 4=SP(8–11); 5=SP; 6=SP(5–11); 7=SP(6–11). Concentrations and injection volume as in Fig. 1.

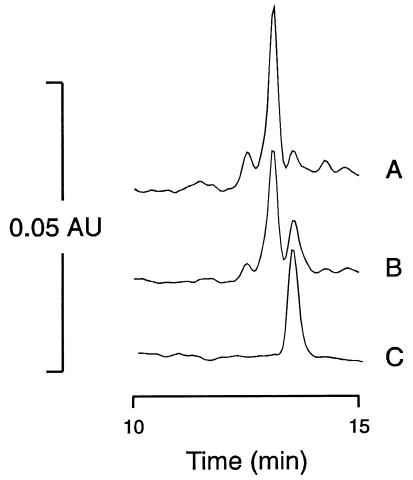

Fig. 6. Chromatograms of (A) untreated striatum, (B) striatum 10 min after injection of SP (10 nmol) and (C) a standard solution of SP(1–7) (50 μl, 10 μ*M* in 1 *M* HCOOH).

these C-terminal fragments could be detected. However, peaks were clearly visible when a control solution containing SP(5–11) and SP(6–11) at 10^{-6} *M* was subjected to SPE and CE. In the case of SP(5–11) and SP(6–11) these findings are in keeping with our experiments using [^{3}H-Leu^{10}]SP, when no radioactive peaks at the appropriate retention times were observed. The situation with regard to SP(8–11) is a little more complex, as this peptide coelutes with SP sulphoxide. However, it is clearly not present at high concentrations, since we found no attributable CE peak. The percentage recoveries (mean±R.S.D., $n=4$) of SP(5–11), SP(6–11) and SP(8–11), were 80.6±13.5%, 92.6±9.7% and 116±8.1%, respectively, so their absence cannot be explained by poor recovery from the homogenate. A small radioactive peak at the retention time of SP(9–11) was observed, but the concentration was too low for us to be able to confirm the presence of this fragment by CE.

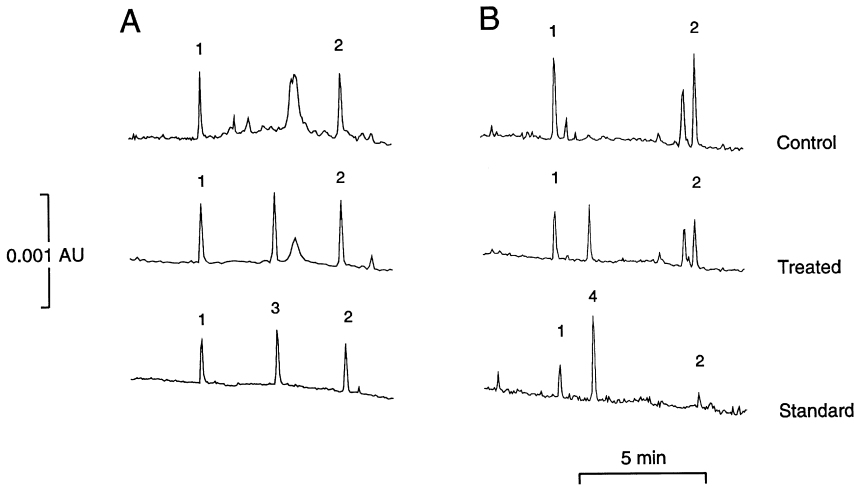

Fig. 7. (A) Electropherograms of HPLC fractions collected at the retention time of SP, after injection of control and treated supernatant solutions and standard solution of SP (1 μ*M* in HPLC eluent). The HPLC eluate was applied by electrokinetic injection (10 kV for 10 s) without further treatment. SP(1–4) and SP(2–11) were coinjected as migration time markers. (B) Electropherograms of HPLC fractions collected at the retention time of SP(1–7), after injection of control and treated supernatant solutions and standard solution of SP(1–7) (1 μ*M* in water). The sample was concentrated 5-fold by evaporating the HPLC eluate under nitrogen and redissolving in water. Electrokinetic injection (10 kV for 10 s). Separation conditions: 350 V/cm (20 kV) for 30 min. Peaks: 1=SP(1–4); 2=SP(2–11); 3=SP; 4=SP(1–7).

3.5. Kinetics of disappearance of substance P

Having established that the increase in size of the chromatographic peak in treated samples was due to the presence of SP and that the peak at the same retention time in chromatograms of control samples was not endogenous SP, the difference in peak areas was used to quantify the amount of SP present, by comparison with standard solutions. The calibration curve was linear over the range tested (Table 2). The R.S.D. values of replicate analyses (6) at 10 μ*M* and 2.5 μ*M* were 4.1 and 14%, respectively and the recovery (±R.S.D.) from 'spiked' homogenates was 90.9±10.6%. The rate of disappearance of SP, calculated from a plot of SP concentrations versus time (Fig. 9) was 0.2 nmol/min, which explains why, in preliminary investigations using an injection of only 1 pmol of [^{3}H]SP, we could detect no parent peptide at our earliest time point (2 min). By increasing the amount injected to 10 nmol, as reported here, it was possible to monitor the disappearance of SP for 20 min, over which time the kinetics were zero order, reflecting the fact that at these concentrations the metabolising enzymes are saturated. Because the metabolism of SP in the striatum is so rapid, it would appear that monitoring the metabolism of SP at concentrations approaching those in vivo will prove to be difficult.

3.6. Comparison with other studies

There are few reports on the metabolism of SP in vivo, and until recently most of these measured tissues levels. Sakurada et al. [9] using preparative electrophoresis combined with RIA, found 1.3 pmol/g of SP(1–7) which represented some 2% of the concentration of the parent peptide in rat striatum. The C–terminal fragments, believed to be principally SP(5–11) and a small amount of SP(3–11), were present at approximately 1% of the parent. The concentration of SP(1–7) was greater in the rat spinal cord, 5.8 pmol/g in the dorsal part and 3.1 pmol/g in the ventral part, representing 10 and 18% of the concentration of the parent peptide, respectively. The concentrations of SP fragments in mouse spinal cord reported by Igwe et al. [10] using HPLC with UV detection at 214 nm, were several orders of magnitude higher (nmol/g protein): SP 136, SP(1–4) 577, SP(1–7) 82, SP(1–9) 135, SP(2–11) 86, and

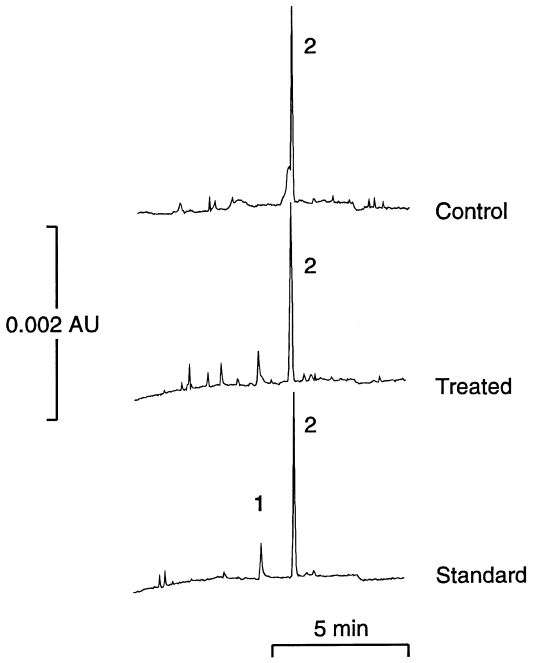

Fig. 8. Electropherograms of HPLC fractions collected at the retention time of SP(1–4), after injection of control and treated supernatant solutions and standard solution of SP(1–4) (1 μ*M* in water). The sample was concentrated 5-fold by evaporating the HPLC eluate under nitrogen and redissolving in water. Pressure injection for 20 s. Separation conditions: 300 V/cm (17.1 kV) for 30 min. Peaks: 1=SP(1–4); 2=histidine (marker).

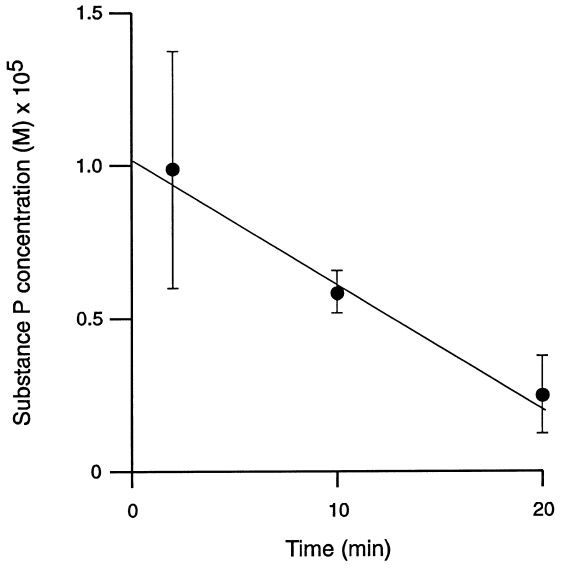

Fig. 9. Disappearance of SP as a function of time after injection of 10 nmol in rat striatum. The data are the mean of three determinations (±S.D.). The solid line represents the least squares fit to the data (r=0.993).

SP(5–11) 319. However, the concentrations determined by RIA (pmol/g protein): SP 190, SP(2–11) 20 and SP(5–11) 38, were in keeping with the results of Sakurada et al. [9]. The chromatograms of control tissues (Figs. 5 and 6), illustrate how endogenous material might be erroneously quantified as being SP or one of its fragments, if only HPLC with UV detection is used.

Table 2
HPLC calibration data for SP and five of its fragments

Compound	Intercept	Gradient	r
SP	−245	1275	0.998
SP(1–4)	−690	457	0.999
SP(1–7)	28	1211	0.998
SP(1–9)	−60	1344	0.999
SP(8–11)	−230	571	0.997
SP(6–11)	−455	932	0.997
SP(5–11)	−507	993	0.999

A study in which SP was administered to rat striatum, is that reported by Andrén and Caprioli [18] who infused SP (15 pmol/min) via a microdialysis probe. SP fragments were detected by LC–electrospray MS, but unfortunately the absolute quantities of the fragments were not quoted but presented as a percentage of the largest fragment detected, SP(1–9). After correction for differences in the proportion of peptide ionized in the electrospray and recovery through the dialysis membrane, the most abundant fragments were SP(6–11) and SP(5–11). The ratio of SP(6–11) to SP(1–7) increased from 6 to 25, between 30 min and 2 h of infusion. In our experiments we have not been able to detect appreciable amounts of SP(6–11) or SP(5–11). If either of these fragments were present at concentrations greater than those of SP(1–7) we would have expected to see an increase in signal at the appropriate retention times. Furthermore, these C-terminal fragments were not detected after injection of [^{3}H-Leu]SP. Indeed, our results are more in

keeping of those of Kostel and Lunte, who when they infused 90 pmol/min via a microdialysis probe, showed greater quantities of SP(1–4) and SP(1–7) than SP(1–9) [19].

Because of our experimental design the rate of disappearance of SP was constant (zero order) and, presumably, the rate of production of primary fragments constant. It is to be expected, therefore, that the concentration of a fragment will increase until the rate of disappearance is equal to the rate of production — a kind of steady-state. At this point the concentration will remain fairly constant and the concentration of a fragment will be highly dependent, not only on the rate of synthesis, but on the rate of subsequent metabolism. That SP(1–4) and SP(1–7) were found in concentrations higher than those of the complementary fragments, SP(5–11) and SP(8–11), may indicate that these N-terminal fragments have longer elimination half-lives than the C-terminal fragments. If the N-terminal fragments arise solely from primary Pro^4–Gln^5 and Phe^7–Phe^8 cleavages, then this must be the case.

Differences in the relative proportions of SP fragments could be explained, in part, by differences in experimental design. Because the exogenously administered SP is being metabolised while the concentrations of the fragments are relatively steady, then the ratio of product to SP will increase with time and cognisance must be taken of this fact when comparing our results with those of others. Sakurada et al. [9] clearly showed the presence of SP(1–7) in rat striatum, but the C-terminal fragments were assayed as a single fraction, and the concentration of any one fragment might not have been as high as reported. SP(1–4) is probably too small to cross-react with an N-terminal directed antibody, and so it is unlikely that this fragment will be detected by RIA. The N-terminal directed antibodies used by Igwe et al. [10] would not react with SP(1–7), so it was not possible to compare the relative concentrations of N- and C-terminal fragments in their study. Other differences, such as the concentrations of SP(2–11) and SP(5–11) reported in mouse spinal cord, may be due to species differences. The microdialysis results of Andrén and Caprioli [18] are more difficult to explain. Some discrepancies may arise because the recovery experiments were conducted in vitro and do not take account of differential loss of fragments by diffusion and uptake in vivo. It would be interesting to conduct a microdialysis experiment using radiolabelled SP, when all the radioactive fragments must arise from the exogenously perfused peptide, and to assay the surrounding tissues for radioactive fragments at the end of the perfusion.

Without the combined use of HPLC and CE it would not have been possible to show that SP is metabolised in the striatum in vivo to yield significant amounts of SP(1–4) and SP(1–7). In view of the fact that these fragments have been shown to have pharmacological activity similar to that of the parent peptide in a striatal slice preparation [7,8], it is conceivable that they contribute to the overall pharmacology of the parent peptide in vivo.

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