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OPTIMIZATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–RADIOIMMUNOASSAY PROTOCOLS FOR THE ANALYSES OF SUBSTANCE P AND SOME OF ITS METABOLIC FRAGMENTS

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SUMMARY

A reversed-phase high-performance liquid chromatographic procedure combined with radioimmunoassay (HPLC–RIA) was developed and optimized for the concomitant quantitation of substance P (SP) and some of its C- and N-terminal fragments in the extracts of the spinal cord of mice. A selective and efficient solid-phase extraction protocol was used for preparative purification of sample homogenates prior to analyses. The sensitivity of the HPLC assay was 18.75 ng for SP and some of its fragments of interest. Recoveries of peptides were calculated from spiked aqueous standards carried through the experimental protocol and ranged from 53 to 98%. The precision of the peptide recoveries from aqueous-based standards, expressed as coefficient of variation, ranged from 2 to 28%. The sensitivities for the RIA procedure using SP antiserum were 1.5, 3.4 and 4.6 fmol $SP_{1\text{-}11}$, $SP_{2\text{-}11}$ and $SP_{5\text{-}11}$, respectively. The percentage cross-reactivity of $SP_{1\text{-}11}$ antiserum with the C-terminal fragments was complete whereas the cross-reactivities of the N-terminal fragments were essentially zero. The molar limits of detectability of SP and some of its C-terminal fragments determined by HPLC alone were several orders of magnitude greater than those determined from the same spinal cord samples using RIA after HPLC fractionation.

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INTRODUCTION

Substance P (SP) is an undecapeptide (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) that occurs in neuronal pathways of the peripheral and central nervous systems (CNS) [1–3]. In the primary afferent neurons, SP is a putative neurotransmitter/neuromodulator of unmyelinated C-fibers and is associated with the primary sensory pathway in the spinal cord [3,4]. Tissue levels of SP have been measured in health and in diseased states with the aim of identifying its primary role [5]. Because the concentrations of a neuropeptide in the CNS are a complex function of its biosynthesis, transport, release and enzymic degradation, such measurements give little useful information, especially if the releasable pool in a particular system is small. Extracellularly, SP is hydrolysed by cell-surface endopeptidases through several metabolic routes including cleavage at Pro^2–Lys^3, Pro^4–Gln^5, Gln^6–Phe^7, Phe^7–Phe^8 and Gly^9–Leu^{10} bonds generating carboxy- and amino-terminal fragments [6–8]. Some of the C- and N-terminal fragments of SP have been found to exhibit some of the biological properties of the parent molecule, both centrally and peripherally [9–12]. It has even been suggested that SP itself might be a precursor from which the active fragments could be released by the action of proteolytic enzymes [13]. Considering the dynamic state of the SP system in vivo, a reasonable approach to monitoring the concentration of SP should involve a concomitant measurement of all its pool of biologically active metabolites. This approach would yield a more complete information concerning the status of SP in the tissue of interest. A number of methods are available in the literature for the analyses of SP [14,15], but there is no standardized method, hence different laboratories report widely differing values for the peptide [14,16]. In addition, there is no literature on the concurrent monitoring of the parent peptide with some of its metabolic fragments which may be biologically active. The aim of the present work was to develop and optimize a simple and sensitive high-performance liquid chromatography–radioimmunoassay (HPLC–RIA) procedure for concomitantly quantifying SP and some of its C- and N-terminal fragments in the spinal cord.

EXPERIMENTAL

Peptides

The following synthetic peptides purified by thin-layer chromatography (TLC) and HPLC were used as standards: SP_{1-11}, SP_{1-4}, SP_{1-7}, SP_{1-9}, SP_{2-11}, SP_{1-11} sulfoxide, SP_{5-11} and [Tyr^8]SP_{1-11} and were purchased from Peninsula Labs. (Belmont, CA, U.S.A.). Peptide dilutions were made in 50 m*M* sodium dihydrogenphosphate (pH 3.0) to 200 μg peptide per ml and stored frozen in aliquots at –20 °C.

Animals

Male Swiss-Webster mice (Biolabs., White Bear, MN, U.S.A.) weighing 35–40 g were used. Animals were maintained in a 12-h light–dark cycle, with food and water available ad libitum. The animals were sacrificed by decapitation. Their

backbones were immediately cut at the cauda equina and the spinal cords rapidly flushed out by injecting ice-cold normal saline into the caudal cord canal using a 45.72-cm gauge needle. The spinal cords were frozen in liquid nitrogen within seconds of sacrifice and the tissue was subsequently prepared for analyses on the same day.

Tissue extraction

Each spinal cord was weighed frozen and rapidly homogenized for 3 min in a Fisher Scientific glass/glass manual tissue homogenizer immersed in ice and containing 5 ml of ice-cold 1 *M* formic acid. Homogenized tissue was left to stand in ice for 30 min to ensure completeness of organelle disintegration and peptide solubilization. The homogenizing tube was rinsed with an additional 5 ml of ice-cold 1 *M* formic acid. The combined initial homogenate plus the washings were centrifuged in a Beckman J21B centrifuge at 2500 *g* for 30 min at 4°C. This manual method of tissue homogenization was compared with the automatic method using the Biospec handmixer at a full setting for 3 min under the same conditions as specified above.

The supernatant was passed through a C_{18} Sep-Pak® column (cartridge) (Waters Assoc., Milford, MA, U.S.A.) which had been previously conditioned by washing with 10 ml methanol followed by 20 ml of distilled water. The retained portion of the sample was washed with 10 ml of 1 *M* formic acid to remove interfering materials and then eluted with 4 ml of methanol. The effluent was evaporated in vacuo at room temperature in a Savant Vac® concentrator (Hicksville, NY, U.S.A.). The residue was reconstituted in 400 μl of 50 m*M* phosphate buffer (pH 3.0) containing 5 ng $[Tyr^8]SP_{1\text{-}11}$ per μl as an internal standard (I.S.) and filtered through a 3-mm membrane, 0.45 μm pore size, HPLC nylon filter (Micron Separations, Honeoye Falls, NY, U.S.A.). A 150-μl volume of this filtered extract was separated by HPLC.

In one experiment, 1 *M* formic acid was supplemented during tissue homogenization with a cocktail of peptidase inhibitors achieving the following concentrations: leupeptin (2 μg/ml), phosphoramidon (4 μg/ml), chymostatin (4 μg/ml) and Bacitracin (40 μg/ml). The rationale for the inclusion of the peptidase inhibitor "cocktail" in the homogenization medium was to evaluate the influence of hydrolytic enzymes on SP recovery from tissue homogenization/extraction protocols.

Characterization of tissue extraction methods

The efficiency of various extraction methods for SP and some of its fragments from spinal cord samples was evaluated. The samples were homogenized in aqueous solutions (10 ml per spinal cord) as listed in Table I. Frozen spinal cord samples were homogenized either in (1) ice-cold 1 *M* formic acid; (2) 1 *M* formic acid after heating to 90°C for 10 min and cooling in ice for 5 min; (3) 1 *M* ice-cold acetic acid; (4) 1 *M* acetic acid after heating to 90°C for 10 min and cooling in ice for 5 min; (5) 10% ice-cold trichloroacetic acid (TCA); (6) ice-cold 1.5% trifluoroacetic acid (TFA); or (7) ice-cold 0.1% TFA. The homogenates were centrifuged at 8000 *g* for 30 min at 4°C. SP and some of its fragments were then

TABLE I

PEPTIDE STRUCTURES

Peptide	Amino acid sequence
SP_{1-11}	Arg^1-Pro^2-Lys^3-Pro^4-Gln^5-Gln^6-Phe^7-Phe^8-Gly^9-Leu^{10}-Met^{11}-NH_2
SP_{1-11} sulfoxide	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met(=O)-NH_2
SP_{1-4} COOH	Arg-Pro-Lys-Pro
SP_{1-7} COOH	Arg-Pro-Lys-Pro-Gln-Gln-Phe
SP_{1-9} COOH	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly
SP_{2-11}	Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
SP_{5-11}	Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
[Tyr^8]SP_{1-11}	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Tyr-Gly-Leu-Met-NH_2

extracted from the supernatants and the residues subjected to the experimental protocol as described above. The residue in each case was used to determine the total protein concentration using an assay kit procedure, TPRO-562, from Sigma (St. Louis, MO, U.S.A.).

Efficiency of sorbents to adsorb SP and its fragments

To determine the most suitable solid-phase extraction protocol as a preliminary purification prior to HPLC–RIA analysis, three different types of silica-bonded sorbents were studied: non-polar C_{18} Sep-Pak column and cation exchangers, sulfonylpropyl (PRS) and carboxymethyl (CBA) 3-ml Bond Elut® columns (Analytichem International, Harbor City, CA, U.S.A.).

A stock solution of the synthetic peptide mixture (SP_{1-11}, SP_{1-4}, SP_{1-7}, SP_{1-9}, SP_{2-11} and SP_{5-11}) was used at 1 μg/ml in 0.1 *M* formic acid (pH 6.5). The cation-exchange columns were washed with 10 ml of methanol and conditioned with 20 ml of 0.1 *M* formate–0.018 *M* pyridine (pH 8.2) before passing 4 ml of the peptide mixture through them. Columns were then washed with 10 ml of conditioning buffer followed by sample elution using 5 ml of 0.8 *M* formate–0.8 *M* pyridine buffer (pH 8.2) containing 40% methanol. The C_{18} column was washed and conditioned as described earlier and 4 ml of the peptide mixture were applied. Retained samples were then eluted with 4 ml of pure methanol.

Eluents were evaporated in vacuo and the residue was dissolved in phosphate buffer (pH 3.0) containing 5 μg [Tyr^8]SP_{1-11} per ml as an I.S. Aliquots of filtered samples were fractionated on the C_{18} column by HPLC.

Partitioning of SP and some of its metabolic fragments into organic solvents

The possibility of using organic solvent extraction (liquid–liquid) to reduce interference from lipids or other biological matrices derived from spinal cord supernatants prior to C_{18} Sep-Pak sample extraction (liquid–solid) was explored. The effectiveness of different organic solvents to remove extraneous materials (e.g. lipids) without removing the analytes of interest was compared. A 5-ml volume of a stock solution of the peptide mixture at 1 μg of each peptide of interest per ml of 1 *M* formic acid was used each time. The aqueous peptide mixture was extracted twice, each time with 4 ml of either diethyl ether, methylene chlo-

ride, hexane or ethyl acetate on a Rotorack® at moderate speed for 15 min. Samples were centrifuged at 4000 *g* at room temperature, the organic fractions removed by Pasteur pipette and evaporated in vacuo in a Savant evaporator. The aqueous layer was passed through a conditioned C_{18} column, eluted with methanol and dried in vacuo as described earlier. The residues from both the organic and aqueous layers were redissolved in phosphate buffer (pH 3.0) and analyzed by HPLC. Three experiments were carried out per solvent type.

Efficiency of organic solvents to elute peptides from C_{18} Sep-Pak columns

The efficiency of different organic solvents to quantitatively elute C_{18}-adsorbed samples of interest was explored using (1) methylene chloride–methanol (1:1), (2) methanol, (3) methylene chloride, (4) diethyl ether, (5) diethyl ether–methanol (1:1), (6) acetonitrile or (7) acetonitrile–methanol (1:1). A 4-ml volume of 1 *M* formic acid was spiked with the peptide mixture at 1 μg/ml and passed through conditioned C_{18} Sep-Pak cartridges (five cartridges per sample). Adsorbed peptides were subsequently eluted with each test solvent and the solvent was evaporated in vacuo. In these experiments, sorbent activation was done with solvents under study. The residues were carried through the experimental protocol as described earlier. Two to three trials were conducted per solvent.

Recovery and precision studies

Recovery studies of SP and some of its fragments from the analytical extraction process were conducted using aqueous-based (1 *M* formic acid) standards. The standards at concentrations of 0.5, 2.5 and 5 μg peptide per ml were extracted and treated as indicated earlier. Appropriate amounts of internal standard (1.0–5 μg/ml) were added to each sample during reconstitution of the vacuum-dried extracts.

HPLC conditions

All samples (150–200 μl aliquots per sample) were fractionated by reversed-phase HPLC using an Ultrasphere ODS column (150 mm×4.6 mm I.D., 5 μm particle size, Beckman, Woburn, MA, U.S.A.) in a Hewlett-Packard HP1090A liquid chromatograph with an automatic injector and HP3392A signal integrator. The column was preequilibrated at 35 °C in 50 m*M* sodium dihydrogenphosphate buffer adjusted to pH 3 with phosphoric acid (85%, v/v) (buffer A). The organic modifier (buffer B) was 60% of acetonitrile in buffer A. The column was always preceded by a guard column (40 mm×4.6 mm I.D., 5 μm particle size) dry-packed with the same solid support and thermostated at the same temperature as the main column. The peptide peaks were resolved by using the following combined linear gradients: 0–45% buffer B, 0 to 15 min; 45–50% buffer B, 15 to 30 min; 50–100% buffer B, 30 to 35 min, flow-rate, 1.0 ml/min. The second solvent system used in the HPLC separation was: solvent A as 0.1% TFA in HPLC-grade water and solvent B as 60% of acetonitrile in solvent A. The linear gradient system was as follows: 0–100% buffer B, 0 to 40 min at a flow-rate of 1.0 ml/min. The retention times for this buffer system were as follows: $SP_{1\text{-}4}$ = 11.7 min, $SP_{1\text{-}7}$ = 15.83

min, SP_{1-9} = 20.2 min, SP_{1-11} = 25.3 min and SP_{5-11} = 26.5 min. Products were detected by UV at 214 nm using a Hewlett-Packard diode array UV–VIS detector.

Preparation of calibration curves

The concentrations of SP and some of its fragments in spinal cord extracts were calculated from standard curves prepared by adding known amounts of these peptides plus the I.S. to buffer A and injecting 150 μl of the mixture per concentration in triplicate. Thereafter, at least, partial calibration was done in every series of experiments.

With a linear dynamic range of 0.25–5 μg/ml for each peptide of interest, least-squares regression lines of best fit were calculated for ratios of peak area to internal standard. Six determinations were performed per concentration level. The equation of the resulting sample regression line was determined for each peptide. The y-intercept was not significantly different from zero in each case. The correlation coefficient was not less than 0.997 in each case.

Radioimmunoassay

The I.S., [Tyr^8]SP_{1-11}, was not added to spinal cord samples used in RIA determination, because it cross-reacts with the SP_{1-11} antiserum. Otherwise samples were treated identically. For each sample, 1-ml fractions of the mobile phase were collected over a 40-min period. Samples were taken to dryness in a Savant evaporator, the residues redissolved in 50 m*M* phosphate-buffered saline (pH 7.4) with 0.1% Triton X-100 and aliquots were analyzed by RIA. The RIA procedure for SP was carried out using RIA kit from Peninsula Labs. according to the manufacturer's specification. The radioligand ^{125}I-[Tyr^8]SP_{1-11} was used as a tracer. The antiserum, raised against the peptide-thyroglobulin conjugates in rabbits, cross-reacted with SP_{2-11} and SP_{5-11} and was therefore used to monitor these C-terminal fragments. The second antibody used was goat anti-rabbit immunoglobulin and the reacted materials were completely precipitated with excess rabbit serum. For the C-terminal cross-reactivity, synthetic SP_{2-11} and SP_{5-11} at appropriate graded dilutions (2–1024 pg per assay tube) in the RIA buffer as SP_{1-11} were used as standards.

Several unsuccessful attempts were made to quantify N-terminal fragments by RIA using the same radiotracer ^{125}I-[Tyr^8]SP_{1-11} and different dilutions of SP amino terminus antiserum [i.e., antiserum supposedly directed towards the amino terminus of SP (Arg^1-Pro^2-Lys^3--)] raised in sheep (Peninsula Labs.). The second antibody used here was rabbit antisheep immunoglobulins (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.). This antiserum, however, recognized only the intact SP_{1-11} molecule but none of the N- or C-terminal fragments we examined.

RESULTS

Characterization of tissue homogenization/extraction methods

The effectiveness of different homogenization methods to optimally extract SP and some of its fragments from cold spinal cord samples is shown in Table II. No

TABLE II

COMPARATIVE HOMOGENIZATION/EXTRACTION METHODS FOR SUBSTANCE P AND SOME OF ITS C- AND N-TERMINAL FRAGMENTS FROM THE MOUSE SPINAL CORD

Homogenization/extraction conditions	Peptide content* (pmol per mg total protein)					
	SP_{1-11}	SP_{1-4}	SP_{1-7}	SP_{1-9}	SP_{2-11}	SP_{5-11}
1.0 *M* Formic acid (0°C)	136±30	577±66	82±14	135±23	86±11	319±51
1.0 *M* Formic acid (90°C)	69±11	336±40	6±3	69±15	29±8	N.D.**
1.0 *M* Acetic acid (0°C)	108±24	436±62	54±12	130±44	70±23	219±60
1.0 *M* Acetic acid (90°C)	54±14	225±87	6±2	77±27	17±6	N.D.
10% Trichloroacetic acid (0°C)	19±4	32±4	4±3	9±3	N.D.	N.D.
1.5% Trifluoroacetic acid (0°C)	22±6	36±8	N.D.	24±10	10±4	N.D.
0.1% Trifluoroacetic acid (0°C)	43±5	135±22	N.D.	43±8	33±14	N.D.

*Peptide content was normalized with total spinal cord protein content. Total protein content used for peptide normalization was determined using the spinal cord homogenate residue. This normalization method avoids concealing the absolute quantities of SP and/or fragments recovered in the extraction process. Values are expressed as mean ±S.D., three spinal cords per extraction method.
**N.D. = not detected.

quantitative differences in the recoveries of SP and some of its fragments were observed when tissues were homogenized with the automatic homogenizer (Biospec handmixer) at a full setting as compared with the manual tissue homogenizer under the same experimental conditions. The superiority of cold 1 *M* formic acid over the other methods is demonstrated. Although the use of cold acetic and formic acids of the same molarity were comparable in some cases, the use of the former for tissue homogenization gave higher signal-to-noise ratios (peak height-to-baseline ratio) on subsequent HPLC analyses at the UV absorbance of interest. Tissue homogenization in heated acids yielded considerably smaller amounts of SP and some of its metabolic fragments. The use of cold TFA and TCA also resulted in significant losses of the peptides on subsequent HPLC analyses.

Efficiency of adsorption and recovery of peptides by different sorbents

The rank order of the efficiency of recovery of SP and some of its metabolic fragments from spiked 0.1 *M* formic acid (pH 6.5) using different solid-phase sorbents was: C_{18} > PRS > CBA (Table III). Also, in terms of ease of elution of adsorbed peptides, the non-polar C_{18} sorbent was superior to both ion exchangers, PRS and CBA. Quantitative recovery was greater than 60% for individual peptides in the peptide mixture when the C_{18} column was used. For PRS-packed columns, a recovery of approximately 40% was consistently obtained for SP and some of its N-terminal fragments tested whereas the recoveries for the C-terminal fragments were much lower. However, no appreciable sorbent–peptide interaction occurred with CBA-packed columns as the peptide standards were consistently recovered intact in the aqueous "waste" generated prior to the elution of adsorbed materials with the indicated buffer (data not shown). Examination of the effects of various absorbents and elution solvents was also performed on a pool of spinal cord extracts spiked with the synthetic peptide mixtures.

TABLE III

SOLID-PHASE EXTRACTION EFFICIENCY OF SUBSTANCE P AND SOME OF ITS FRAGMENTS USING DIFFERENT BONDED SILICA SORBENTS FOR COLUMN PACKING

Sorbent type and structure	Peptide recovered in eluate* (%)					
	SP_{1-11}	SP_{1-4}	SP_{1-7}	SP_{1-9}	SP_{2-11}	SP_{5-11}
C_{18}: [-Si-$C_{18}H_{37}$]	75±8	62±7	69±11	65±10	92±15	68±9
PRS: [-Si-$(CH_2)_3$-SO_3^-]	39±8	32±6	44±10	35±8	10±4	N.D.**
CBA: [-Si-CH_2COO^-]	12±4	8±2	9±2	10±4	N.D.	N.D.

*Values are mean ±S.D. of three determinations of solid-phase extractions performed per sorbent type with 4 ml of peptide mixture in 0.1 *M* formic acid, pH 6.5 (1 μg of each peptide in the mixture per ml) passed through the sorbent bed.
**N.D. = not detected.

TABLE IV

PARTITIONING OF PEPTIDES IN ORGANIC SOLVENTS

Extraction solvent	Percentage of added peptide in the aqueous phase (mean ±S.D., $n=5$)					
	SP_{1-11}	SP_{1-4}	SP_{1-7}	SP_{1-9}	SP_{2-11}	SP_{5-11}
Diethyl ether	62 ± 4^a	20 ± 8^b	22 ± 6^b	30 ± 4^b	89 ± 9^a	75 ± 10^a
Ethyl acetate	40 ± 13^b	34 ± 7^b	28 ± 5^b	25 ± 14^b	39 ± 12^b	38 ± 16^b
Methylene chloride	66 ± 6^a	63 ± 11^a	62 ± 7^a	70 ± 15^a	90 ± 8^a	80 ± 8^a
Hexane	20 ± 5^b	30 ± 6^b	33 ± 5^b	42 ± 11^b	44 ± 7^b	42 ± 5^b
None[c]	65±10	68±8	65±8	68±5	92±9	77±11

[a]Peptide concentration in the aqueous phase was not significantly affected by the organic solvent used (Student *t*-test, $p \leq 0.05$) when compared with corresponding peptide value in *c*.
[b]Peptide concentration was significantly reduced in the aqueous layer (Student *t*-test, $p \leq 0.05$) when compared with corresponding values in *c*.
[c]Peptide in aqueous buffer was carried through the experimental protocol without organic solvent extraction.

Partitioning of peptides in organic solvents

The study of the effectiveness of using different organic solvents (diethyl ether, hexane, methylene chloride and ethyl acetate) to clean-up aqueous sample supernatants prior to solid-phase extraction with a C_{18} Sep-Pak column showed that the use of these solvents was of little value in the experimental procedure (Table IV). With no prior solvent extraction, adsorption of aqueous samples on conditioned C_{18} column followed by methanol elution gave a recovery of 65–92% (Table IV). Prior extraction of aqueous peptide mixture with methylene chloride did not affect subsequent recoveries of individual peptides from the aqueous phase to any significant extent. With diethyl ether, the N-terminal fragments were significantly (Student *t*-test, $p \leq 0.05$) reduced in the aqueous layer with no effect on SP and the C-terminal fragments. However, with ethyl acetate and hexane there was a total partitioning of SP and its fragments into the organic layer.

When methylene chloride was used for a prior clean-up (to remove possible

contaminating lipids) of spinal cord supernatants prior to C_{18} solid-phase extraction, the resulting sample chromatographic profiles were not different from non-methylene chloride extracted samples. Hence sample supernatants were extracted by solid-phase method only without prior clean-up with an organic solvent.

Efficiency of organic solvents in the elution of C_{18}-adsorbed peptides

The efficiencies of different organic solvent combinations to quantitatively elute C_{18}-adsorbed SP and some of its metabolic fragments were shown to be generally in the following order: methanol = methanol–methylene chloride (1:1) > diethyl ether–methanol (1:1) > acetonitrile–methanol (1:1) > acetonitrile > diethyl ether > methylene chloride.

Recovery studies

In the precision studies (Table V), a recovery range of 53–98% was obtained, showing that SP and some of its metabolic fragments were efficiently extracted from the spinal cords. The precision, expressed as percentage coefficient of variation (C.V.), is indicated for each peptide through the concentrations studied.

Radioimmunoassay

The immunoreactivities for $SP_{1\text{-}11}$, $SP_{2\text{-}11}$ and $SP_{5\text{-}11}$ corresponded with their HPLC elution profiles (Fig. 1). In the RIA protocol, the limit of detection for $SP_{1\text{-}11}$, $SP_{2\text{-}11}$ and $SP_{5\text{-}11}$ was 1.5, 3.4 and 4.6 fmol peptide, respectively. The linear portions of the percentage radiotracer bound versus log peptide concentration for the standard curves ranged from 4 to 22, 5 to 25 and 5 to 23 fmol for $SP_{1\text{-}11}$, $SP_{2\text{-}11}$ and $SP_{5\text{-}11}$, respectively. The mean range of the intra-assay coefficients of variation for the linear portions of these curves were 5–9% for $SP_{1\text{-}11}$, 1–2% for $SP_{2\text{-}11}$ and 1–4% for $SP_{5\text{-}11}$. The mean inter-assay coefficients of variation were 7% for $SP_{1\text{-}11}$, 2% for $SP_{2\text{-}11}$ and 3% for $SP_{5\text{-}11}$.

TABLE V

RECOVERIES AND PRECISION STUDIES OF 1.0 *M* FORMIC ACID BASED STANDARDS OF SUBSTANCE P AND ITS METABOLIC FRAGMENTS FROM C_{18} SEP-PAK CARTRIDGES

Corrections for losses for the sample values of each peptide were made based on the mean values of the percentage recoveries within the concentration range studied.

Peptide	Recovery (mean ± S.D., $n=6$) (%)			Coefficient of variation (%)
	0.5 μg/ml	2.5 μg/ml	5.0 μg/ml	
$SP_{1\text{-}11}$	53 ± 6	56 ± 5	73 ± 13	8–18
$SP_{1\text{-}4}$	63 ± 17	61 ± 1	77 ± 16	2–28
$SP_{1\text{-}7}$	62 ± 4	62 ± 3	80 ± 3	4–6
$SP_{1\text{-}9}$	61 ± 12	62 ± 3	66 ± 6	5–20
$SP_{2\text{-}11}$	98 ± 9	93 ± 3	95 ± 8	3–9
$SP_{5\text{-}11}$	74 ± 15	79 ± 3	88 ± 9	4–20

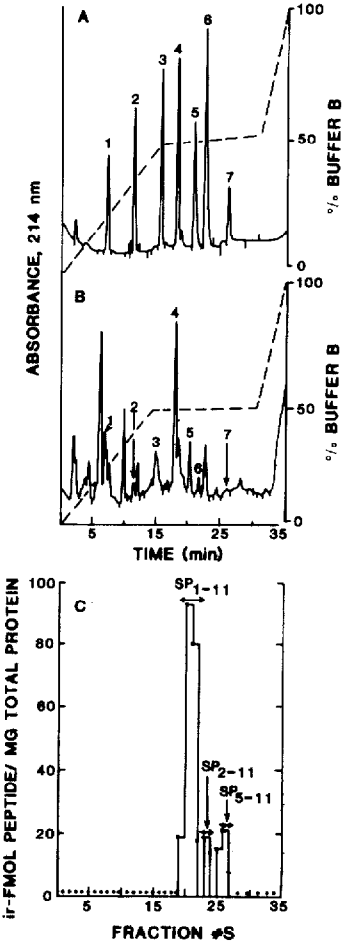

Fig. 1. (A and B) HPLC elution profiles of standard [0.5 μg synthetic peptide mixture per ml (150 μl injected)] SP_{1-11} and some of its metabolic fragments and spinal cord extracts, respectively, on Ultrasphere ODS column (150 mm×4.6 mm, 5 μm particle size) at 35°C with buffer A as 50 m*M* $NaH_2PO_4 \cdot H_2O$, pH 3.0 and buffer B as 60% of acetonitrile in buffer A. The flow-rate was 1 ml/min. Peaks: 1 = SP_{1-4}; 2 = SP_{1-7}; 3 = SP_{1-9}; 4 = [Tyr^8]SP_{1-11} (internal standard); 5 = SP_{1-11}; 6 = SP_{2-11}; 7 = SP_{5-11}. (C) Mean RIA profile for SP_{1-11} and its C-terminal fragments (n = 3). The fraction numbers approximate the HPLC elution time for each analyte. For each sample, fractions of 1.0 ml were collected from the HPLC separation at 1.0 ml/min over a 40-min period and each vacuum-dried fraction was analyzed by RIA. Panels B and C were generated from the same spinal cord samples but no internal standard was added to samples for panel C before HPLC separation (see text). Note that peaks 2 and 7 exceeded by only small amounts their signal-to-noise ratios in conventional chromatographic term. However, when the same spinal cord samples were spiked with 100 ng of synthetic SP_{1-7} and SP_{5-11}, the area under the curves of the peaks of these fragments consistently increased by the amounts suggested by their actual tissue signals.

DISCUSSION

The determination of neuropeptides in the pico- and nanomole range using HPLC–RIA has been described [17,18]. A number of reversed-phase HPLC sys-

tems has been introduced for the analysis of SP [19–21]. The present series of experiments have optimized a simple and sensitive HPLC–RIA procedure for concomitant determination of SP and some of its C- and N-terminal fragments in a biological system. A solid-phase extraction protocol was used to efficiently obtain trace quantities of the peptides of interest (Table I) from the spinal cord. Subsequent analysis of these in acidic low-ionic-strength phosphate buffer pH 3, 50 m*M* as a mobile phase and acetonitrile as an organic modifier, greatly improved selectivity [22] and sensitivity [23] of the HPLC assay.

In the optimized gradient system used, all the synthetic and endogenous SP-related peptides studied seemed fully resolved and eluted in the order SP_{1-4}, SP_{1-7}, SP_{1-9}, SP_{1-11}, SP_{2-11} and finally SP_{5-11} (Fig. 1). Peptides having a terminal basic amino acid residue were eluted earlier than the corresponding shorter peptides (SP_{2-11} versus SP_{5-11}), in agreement with Rekker's fragmental constants in liquid–liquid partition systems [24]. The formic acid extracts of endogenous SP and some of its fragments from the spinal cords behaved as the standard synthetic peptides with respect to retention times in the HPLC–RIA assays (Fig. 1). However, tissue extraction in heated acids yielded considerably smaller amounts of SP and some of its metabolic fragments and an SP-like immunoreactive material (with a broad peak and a relative retention time of about 28 min) in the RIA protocol. The exact nature of this component is unknown, but it may represent a protein precursor in the biosynthesis of SP [25,26]. The losses of endogenous SP and some of its fragments resulting from the use of ice-cold TFA and TCA in the tissue homogenization process could be due to the precipitation of the peptides by these acids. The critical nature of the extraction procedures in the quantitative measurement of tissue levels of SP is thus demonstrated.

The accurate quantitation of a neuropeptide from tissue samples requires rapid separation of the desired peptide from proteolytic enzymes in the tissue before degradation of the peptide occurs. Under the conditions of the present assay, the levels of SP and some of its metabolic fragments were found to be high in the mouse spinal cord (Table II) compared to determinations made in other laboratories [14,16]. The use of cold formic acid (low pH) supplemented with a cocktail of peptidase inhibitors did not significantly influence these values [8,27]. This shows that enzymic degradation of SP probably did not occur during the homogenization/extraction procedure. The levels of endogenous SP and some of its metabolic fragments quantified in the spinal cord point to the possibility that SP exists in a dynamic state in vivo, at least post-synaptically, with its metabolic fragments. The enzymic hydrolyses of SP have been demonstrated [6–8]. The existence of a post-proline cleaving enzyme capable of cleaving SP into SP_{1-4} and SP_{5-11} and the presence of SP_{1-7} and SP_{5-11} in the CNS have also been demonstrated [7,10]. Furthermore, a high-affinity uptake mechanism of SP_{5-11} but not SP_{1-11} into nerve terminals has been shown to play an important part in terminating the synaptic action of SP [28].

For accurate quantitation of the peptides of interest, it was necessary to control within narrow limits or eliminate factors affecting detector sensitivity. This control was achieved by using a suitable compensation technique, namely the internal standardization technique, in the HPLC assay. Thus, ordinary fluctuations

in instrument parameters affect the analytes of interest and the I.S. in the same way from one experiment to another. The use of $[Tyr^8]SP_{1-11}$ satisfies some of the criteria for I.S. [29]. For example, it elutes close to the peaks of interest (Fig. 1) and has structural similarities to SP. In the experimental protocol, it was necessary to add the I.S. to the sample during reconstitution in the mobile phase of the vacuum-dried extracts. This minimized the possibility of its proteolytic hydrolysis which might occur if $[Tyr^8]SP_{1-11}$ was added to the tissue homogenate/supernatant prior to the solid-phase extraction step. At least, theoretically, enzymic degradation of I.S. would yield the same array of N-terminal fragments (SP_{1-4} and SP_{1-7}) as SP and this would result in erroneously high values for these fragments in the assay. Under the conditions of the assay, the oxidation products of SP and/or I.S. were probably below their detection limits. No extraneous peaks corresponding to SP sulfoxide could be detected on analyses of a mixture of SP and some of its carboxy terminus metabolic fragments plus I.S. reconstituted in the mobile phase (pH 3.0) and left covered tightly on the laboratory bench for four days at room temperature. However, the same samples reconstituted in 0.1% TFA and treated in the same way gave multiple peaks, one with the same retention time as SP sulfoxide, in the same time period.

SP is a strongly basic, hydrophobic carboxy terminus peptide with three positive charges (at acidic pH), thus making it strongly adsorptive to anionic and hydrophobic packings. The rank order of adsorption efficiency for SP and some of its C- and N-terminal metabolic fragments is: hydrophobicity>>>positive charge. With the non-polar C_{18}, the peptides, even the shortest ones, were recovered with good efficiency (Table V). SP and some of its fragments have different affinities for the cation exchangers due to the differences in the number of positive charges each molecule carries (at acidic pH) and the relative acidity and alkyl chain length of the sorbent bed. Because the degree of dissociation and hence the extent to which ionic exchange sorbent bed is charged is dependent of the pH, basic conditioning of the cation exchangers is very important. Hence, for the strongly acidic cation exchanger with C_8-bonded silica, PRS, the number of positive charges on the peptides at pH 6.5 was important for interaction with and subsequent desorption from the sorbent bed. This is because SP and its N-terminal fragments each of which carries three positive charges (at pH 6.5) were recovered/eluted at uniform efficiency (Table III). The SP_{2-11} fragment with one positive charge (pH 6.5) was recovered poorly even though its C-terminus hydrophobic end was intact and SP_{5-11} fragment with no positive charge was not recovered at all. On the other hand, with the weakly acidic cation exchanger with C_1-bonded silica, CBA, recovery for all the peptides of interest was very poor. Desorption of bonded fragments required a mixture of basic high-ionic-strength buffer and methanol while elution of adsorbed peptides from the C_{18} bed required a polar solvent of which methanol was found to be the most efficient.

The filtration of reconstituted samples prior to HPLC fractionation resulted in reduced clogging of column materials. This extended column half-life and permitted the analyses of a large number of samples under essentially identical conditions.

The cross-reactivity experiments indicate that SP antiserum recognizes a de-

terminant located in the carboxy-terminal portion of the SP molecule. Complete cross-reactivity with SP antiserum was obtained with SP_{2-11} and SP_{5-11} fragments while the N-terminal fragments gave no cross-reactivity in the assay.

Quantitatively, individual concentrations of SP, SP_{2-11} and SP_{5-11} as determined by HPLC alone under our experimental conditions were always several orders of magnitude greater (pmol versus fmol) than their corresponding RIA values determined from the same spinal cord extracts after reversed-phase HPLC fractionation. This was a puzzling phenomenon for which a plausible explanation could be due to an undefined effect of the organic modifier (acetonitrile) of the mobile phase on SP and some of its C-terminal fragments [30,31] which may reduce their interactions with the SP antiserum. In an attempt to explain this apparent discrepancy, two series of experiments were performed. (1) Known amounts of SP_{1-11} (100, 200 and 400 ng per 200 μl of injection, two injections per concentration) were injected onto the HPLC system, using the conditions described earlier, and 1.0 ml/min aliquots were collected over a 35-min period. (2) The same amounts of SP_{1-11} in 200-μl volumes from the same dilution as in (1) were added to 800 μl of 80% acetonitrile in buffer A. In both cases, the HPLC buffer was evaporated, residues were redissolved in RIA buffer and analyzed by RIA. In all cases, the concentrations of SP_{1-11} determined by RIA were two or three orders of magnitude greater in samples that had not been fractionated by HPLC before analysis by RIA compared to those which had prior to RIA. Therefore, the presence of acetonitrile in the HPLC buffer does not interfere with the analysis of SP by RIA. A strong adsorption of SP on the C_{18} column is a doubtful possibility. This could not be proven since nothing that would react with RIA antiserum eluted when the HPLC column was washed with 100% solvent B or a combination of 80% methanol and 20% water for 20 min at 1.5 ml/min. These results emphasize the importance of processing standards used for RIA through the HPLC system in an identical fashion as the samples.

As far as we are aware, this is the first time that a concomitant quantitation of SP and some of its possible metabolic fragments has been developed and optimized by HPLC–RIA systems. The simplicity, selectivity and efficiency of the analytical protocols have been demonstrated by using cold formic acid for tissue homogenization, followed by a non-polar C_{18} solid-phase extraction of the supernatant. Working rapidly at low temperature and pH, the influence of proteolytic enzymes on the relative values of the recovered peptides of interest was minimal. Under the conditions of the assay, the array of SP fragments identified concurrently with SP lends support to the speculation that the parent molecule exists in a dynamic equilibrium with these fragments and that these metabolites may modulate the action of SP in the spinal cord or elsewhere.

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