Journal of Chromatography, 612 (1993) 150–155
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6613

Short Communication

Recovery of substance P and related C-terminal fragments on solid-phase extraction cartridges for subsequent high-performance liquid chromatographic separation and radioimmunoassay

Klaus Rissler, Renate Katlein and Hinrich Cramer

Neurochemical Laboratory, Department of Neurology, University of Freiburg, Hansastrasse 9, D-7800 Freiburg (Germany)

(First received June 30th, 1992; revised manuscript received September 28th, 1992)

ABSTRACT

The recoveries of substance P (SP) and five related peptides were evaluated on different types of solid-phase extraction sorbent. Best results were obtained by use of a C_{18} silica gel cartridge. Marked differences of extraction yields occurred for the different peptide fragments and, in general, recovery increased with increasing hydrophobicity of the peptide when reversed-phase materials like C_{18} and C_8 cartridges were used. This observation is indicative of a sorption–desorption mechanism by prevailing solvophobic interactions. A similar trend was found when phenylpropyl silica gel (C_{Phenyl}), generally known as a reversed-phase adsorbent of lower hydrophobicity, was used. It was concluded that a substantial participation of analyte–matrix π–π interactions has to be taken into account when extraction yields are compared with corresponding values obtained by use of a C_8 cartridge. With CN silica gel cartridges, marked differences in extraction yields were obtained by use of acetonitrile or methanol as the organic modifier. As an attempt to explain this observation, conformational effects were assumed for the sorption–desorption behaviour of the peptides on the polar matrix.

INTRODUCTION

The undecapeptide amide substance P (SP) is a member of the tachykinin family, with the sequence Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2. It is one of the most investigated neuropeptides and plays a key role in pain perception. Furthermore, it seems to be involved in the regulation of the extrapyramidal system, in common with substance K, and in neuroimmunological pathways. SP is rapidly degraded in tissue by different enzymic activities [1–11], generating a multitude of fragments.

The low concentrations of SP and related peptides in biological samples require extremely sensitive and specific detection methods. For this reason samples are usually subjected to high-performance liquid chromatography (HPLC) and fractions which coeluted with the synthetic peptides are collected. These are subsequently measured by radioimmunoassay (RIA) by use of a specific antibody raised in rabbits against a conjugate of synthetic SP_{1-11} and bovine serum al-

Correspondence to: Dr. K. Rissler, Ciba-Geigy AG, K-401.208, CH-4002 Basle, Switzerland (present address).

0378-4347/93/$06.00

bumin. The antibody exhibits no (or only negligible) cross-reactions towards a variety of other members of the tachykinin family, which share the common C-terminal part Phe-X-Gly-Leu-Met-NH_2 where X represents the only variable amino acid. ^{125}I-[Tyr^8]-SP_{1-11} was used as the radiolabel. Additionally, in order to minimize possible interferences with both HPLC separation and RIA, it may be considered as a reasonable assumption that reliable and reproducible results can be obtained only by application of a very efficient sample pretreatment.

During the past decade, the solid-phase extraction (SPE) technique [12–24] has proved to be preferentially used for peptide extraction and offers a rapid and highly valuable tool for sample enrichment from biological sources. The undecapeptide amide SP possesses a hydrophilic N-terminal region containing the basic Arg^1 and Lys^3 residues and a hydrophobic C-terminal part containing the Phe^{7,8} amino acids. For this reason, enzymic cleavage leads to formation of fragments with a wide range of both polarity and basicity. Therefore, interpretation of results from determinations of SP-related peptides requires measurement of their individual recoveries in biological matrices in order to correct for extraction treatment. Owing to the marked variation in their polarities, the chosen examples provided a suitable means for the study of recovery values as a function of either the hydrophobicity of the SPE adsorbents or of the SP fragments.

To our knowledge, no data are yet available on the extraction yields of different peptides all associated with the sequence of SP by use of different types of SPE material. For this purpose, the recoveries of synthetic SP_{1-11}, SP_{2-11}, SP_{3-11}, SP_{4-11}, SP_{5-11} and pyroGlu-SP_{5-11} (pGlu-SP_{5-11}) were determined by use of reversed-phase [*e.g.* octadecylsilyl (C_{18}), octylsilyl (C_8), phenylpropylsilyl (C_{phenyl}) silica gel], as well as bonded-phase [*e.g.* cyanopropylsilyl (CN) silica gel] SPE cartridges. Results were evaluated after HPLC separation of samples and subsequent RIA of appropriate fractions (HPLC–RIA coupling) as described elsewhere [10,11,19,20,25–28]. This study was restricted to the measurement of SP fragments containing at least the C-terminal SP_{6-11} sequence, which also permits measurement of N-terminally extended precursor forms of SP, *i.e.* the total amount of the so-called "C-terminal" substance P-like immunoreactivity (SPLI).

EXPERIMENTAL

Materials and chemicals

SPE was performed by using a type spe-10 multiple extraction system from Baker (Gross-Gerau, Germany). The velocity of either sample application or rinse or elution was adjusted to 2 ml/min by a slight vacuum. As adsorbents Baker-bond SPE cartridges (Baker) containing C_{18}, C_8, C_{Phenyl} and CN silica gel were used. SP_{1-11}, SP_{2-11}, SP_{3-11}, SP_{4-11}, SP_{5-11} and pGlu-SP_{5-11} were obtained in HPLC quality from Peninsula Labs. (Belmont, CA, USA). As the radiolabeled antigen for RIA ^{125}I-[Tyr^8]-SP_{1-11} from Peninsula Labs. was chosen. Methanol and acetonitrile (both HPLC grade) and trifluoroacetic acid (TFA, for synthesis) were purchased from Merck (Darmstadt, Germany). Water was purified for use in HPLC with a Seralpur Pro 90 C system (Seral, Ransbach-Baumbach, Germany). Peptides were dissolved in a special buffer system (assay buffer) described elsewhere [28–31], which was also used for the RIA quantification and mimicking the environment in cerebrospinal fluid (CSF). In brief, the buffer system consisted of 0.1 *M* sodium phosphate (pH 7.5) containing 0.025 *M* EDTA, 0.15 *M* sodium chloride, 0.005 *M* (0.02%) sodium azide, and 0.25% bovine serum albumin. For HPLC separation a Spherisorb ODS II column (125 mm × 4.6 mm I.D.) (Bischoff Analysentechnik, Leonberg, Germany) was used.

Solid-phase extraction procedure

SPE cartridges were rinsed with 2 ml of methanol followed by 3 ml of water, at a flow-rate of 2 ml/min. Then 250 pg of each peptide, dissolved in 2 ml of assay buffer, were passed through the cartridge at slightly reduced pressure. After a rinse with 1 ml of 0.25 *M* aqueous acetic acid (w/v) and

2 ml of water, the peptide material was eluted with two 1-ml portions of 80:20 (v/v) acetonitrile–water supplemented with 0.1% (v/v) of TFA. For estimation of the RIA blank value, a buffer aliquot used for sample dissolution was processed identically and the value was subtracted from those obtained from measurement of a sample spiked with SP or its fragments. The eluate was evaporated to dryness in a Univapo SVC concentrator centrifuge (UniEquip, Martinsried, Germany) at 30°C and reduced pressure. The residues from both samples and blanks were dissolved in 200 μl of methanol containing 0.1% of TFA and subjected to HPLC separation.

Chromatographic system

HPLC was performed with a gradient system consisting of two pulse-dampened one-piston LC T 414 pumps controlled by a Model 200 gradient controller (both from Kontron Analysentechnik, Munich, Germany) at a flow-rate of 1 ml/min. For this purpose, aqueous acetonitrile containing 0.1% TFA was used. First an initial isocratic step with 25% of acetonitrile was applied for 17 min. The percentage of acetonitrile was linearly raised to 31% from 17 to 31 min, and elution continued isocratically up to 40 min. The acetonitrile content was then raised to 100% within 2 min, and elution performed in this manner for 10 min. Re-establishment of initial conditions was caried out within 2 min followed by reequilibration of the column for 15 min. The column was rinsed with an equimolar aqueous solution of arginine and lysine (5 m*M*) for 30 min, either before or after each injection of a sample, in order to minimize peptide adsorption on the column matrix via residual silanol groups, and followed by a switch to the starting conditions and reequilibration for 20 min before injection of another sample. Injection of samples was done by use of a type 7125 switching valve (Rheodyne, Cotati, CA, USA) equipped with a 250-μl sample loop. Fractions (2 ml) were collected with a Frac 200 sample collector (Pharmacia, Uppsala, Sweden) and evaporated to dryness as described above. The residues were dissolved in 200 μl of assay buffer and subjected to the RIA quantification procedure (limit of determination 20 pg, limit of detection 5 pg) as described elsewhere [28–31].

TABLE I

RECOVERIES OF SYNTHETIC SP_{1-11} AND RELATED FRAGMENTS ON DIFFERENT SORBENTS WITH ACETONITRILE AS THE ORGANIC MODIFIER

Sample volume, 2 ml, containing 250 pg/ml.

Peptide	Recovery (mean ± S.D., n = 6) (%)			
	C_{18}	C_8	C_{Phenyl}	CN
SP_{1-11}	50 ± 9	26 ± 5	42 ± 5	47 ± 12
SP_{2-11}	81 ± 9	36 ± 4	61 ± 6	64 ± 3
SP_{3-11}	92 ± 9	54 ± 9	90 ± 8	70 ± 2
SP_{4-11}	99 ± 4	82 ± 6	91 ± 16	59 ± 6
SP_{5-11}	86 ± 10	73 ± 8	76 ± 16	47 ± 7
pGlu-SP_{5-11}	84 ± 3	91 ± 6	95 ± 7	39 ± 2

RESULTS AND DISCUSSION

The results of the extraction procedures with acetonitrile as the organic modifier are summarized in Table I. A general trend towards an increase of extraction yields with increasing hydrophobicity of SP fragments was observed with C_{18} and C_8 materials. Nevertheless, use of the C_{18} cartridge with SP_{3-11} and SP_{4-11} produced a plateau of recovery values virtually exhibiting an inverse U-shaped dependence of extraction yields as a function of increasing retention time (and thus reflecting a concomitant increase in hydrophobicity). In the same direction a deviation from the gradual recovery increase was observed with SP_{5-11} on the C_8 matrix. In comparison with the C_{18} material, the extraction yield is substantially lower with the less hydrophobic C_8 matrix. These observations suggest sorption–desorption characteristics on the basis of prevailing hydrophobic analyte–matrix interactions. The extent of these effects depends markedly on the length of the alkyl chain, and, as a consequence, on the cartridge hydrophobicity.

With C_{phenyl} cartridges, which are more polar adsorbents than C_8 materials, a substantial increase of recovery values was obtained for the investigated peptides instead of the expected de-

crease. For this reason, it seems that the sorption–desorption behaviour is influenced not only by solvophobic solute–matrix interactions but also by attractive forces based on π–π interactions [32–34] between the Phe side-groups of the peptide backbone and the aromatic substituents of the cartridge matrix. This view is further supported by observation of a marked increase of k' values of the Z-Phe-Phe dipeptide, which exhibits a high density of π-electrons with respect to other "low-π-electron-density" peptides on π-electron-rich stationary phases (*e.g.* copolymers of styrene and divinyl benzene) in comparison with alkyl-bonded silica [35].

Unlike the results obtained by use of "pure" reversed-phase materials, no general trend towards improved recovery as a function of the peptide hydrophobicity was observed on the CN material. Recovery data exhibited a maximum value (70%) for SP_{3-11}, which is a fragment of intermediate hydrophobicity in the range of peptides investigated. On the other hand, the more polar as well as the more hydrophobic fragments exhibited a substantial decrease of recoveries. However, a markedly higher extraction yield would have been expected for SP_{1-11}, owing to its presumed ability to increase polar interactions with the sorbent. Nevertheless, the reduced extraction yields for SP_{5-11} and pGlu-SP_{5-11} are in accordance with decreased polar interactions due to the hydrophobicity of these peptide fragments.

Currently we cannot give a comprehensive explanation of the underlying sorption mechanism. However, it should be emphasized that the CN matrix is able to undergo hydrogen bonding with the amide linkages as well as with functional groups of peptides and proteins (*e.g.* hydroxy, amino, carboxy, imino, mercapto and guanidino residues), which all contain exchangeable hydrogens. In general, as the hydrogen bonds become stronger, the efficiency of acetonitrile to break them decreases, and the peptide will be displaced with more difficulty from the sorbent [36]. In such cases, methanol is usually recommended as the more suitable elution solvent [36]. In contrast, however, when methanol was used as organic modifier, extraction yields increased with increasing peptide hydrophobicity, except for SP_{5-11} (see Table II). This effect is surprising because it is assumed that SP_{1-11} and SP_{2-11} might exhibit the most polar and SP_{5-11} and pGlu-SP_{5-11} the least polar interactions with the CN matrix compared with the polarity of the other investigated peptides. For this reason the more polar peptides should be displaced more easily from the cartridge with methanol than with acetonitrile. Nevertheless, it should be remarked that "binding" interactions of peptides with appropriate sorbents may also depend markedly on the peptide's actual "hydrophobic surface", *i.e.* on its conformation. For this reason, conformational effects as described for the unusual chromatographic behaviour of crown ethers on C_{18} columns [36,37] and their possible consequences towards interactions of peptides with CN materials is one way to interpret the results.

In this respect, it should be noted that a corresponding effect may bring about the plateau in recovery values, which was observed for SP_{3-11} and SP_{4-11} on the C_{18} cartridge (see above).

Sequential cleavage of amino acids from the N-terminus of SP produces fragments with increasing hydrophobicity in the sequence $SP_{1-11} < SP_{2-11} < SP_{3-11} < SP_{4-11} < SP_{5-11} \ll$ pGlu-SP_{5-11} owing to the gradual loss of hydrophilic (*e.g.* the basic amino acids Arg^1 and Lys^3) and the concomitant preponderance of hydrophobic amino acids (*e.g.* $Phe^{7,8}$). This fact was shown by HPLC of different peptide species of

TABLE II

RECOVERIES OF SYNTHETIC SP_{1-11} AND RELATED FRAGMENTS ON A CN CARTRIDGE WITH METHANOL AS THE ORGANIC MODIFIER

Sample volume, 2 ml, containing 250 pg/ml.

Peptide	Recovery (mean ± S.D., n = 6) (%)
SP_{1-11}	38 ± 5
SP_{2-11}	43 ± 5
SP_{3-11}	65 ± 5
SP_{4-11}	67 ± 7
SP_{5-11}	61 ± 9
pGlu-SP_{5-11}	72 ± 6

the tachykinin family, which exhibited a substantial hydrophobicity-dependent shift of retention time values [38]. This effect was extremely marked in the case of pGlu-SP_{5-11} [37]. In order to approximate peptide extraction under real-sample conditions (*e.g.* a sample of CSF), the peptides were applied in a buffer solution mimicking their environment in CSF [28–31]. Recoveries of peptides directly subjected to HPLC were quantitative (96–104%) and, therefore, results from the present study permit a reliable comparison of extraction yields. Owing to the vast discrepancies in recoveries often observed by use of SPE materials from different suppliers [39], sorbents from only one producer were chosen. Furthermore, in order to minimize interactions of polar peptide sites with residual silanol groups of the cartridge matrix "end-capped" sorbents were applied.

The study is in accordance with similar investigations exhibiting marked phase-to-phase variations during SPE extraction of pharmaceutical substances [40]. It can be concluded that the more hydrophobic the peptide the more marked is its adsorption on C_{18} and C_8 materials, whereas a likely participation of π–π interactions must also be taken into account when C_{Phenyl} matrices are used. Best results were obtained with C_{18} cartridges.

No general trend was found when CN cartridges were used with acetonitrile as the organic elution component. In contrast, methanol favoured recoveries of more hydrophobic peptide fragments, although an increase in the extraction yields of the more polar fragments was expected. This observation may be attributed to different inherent conformational effects rather than to a preferential cleavage of hydrogen bonds (formed between polar sites of the peptides and residual silanols of the cartridge matrix) by methanol.

Nevertheless, the sorption–desorption mechanism should be further investigated in order to optimize extraction yields of peptides from biological sources.

We do not claim that this method is generally applicable to the SPE extraction of a wide variety of peptides, because marked batch-to-batch variations [41] are always present, as well as phase-to-phase and manufacturer-to-manufacturer variations (see above). Furthermore, the choice of an appropriate sample enrichment procedure by use of SPE can also depend on the type of biological matrix from which the peptide is extracted [41]. Despite these restrictions, the results may be useful in the planning of similar experiments.

REFERENCES

1 E. Heymann and R. Mentlein, *FEBS Lett.*, 91 (1978) 360.
2 S. Blumberg, V. I. Teichberg, J. L. Charli, L. B. Hersh and J. F. McKelvy, *Brain Res.*, 192 (1980) 477.
3 R. Matsas, M. Rattray, A. J. Kenny and A. J. Turner, *Biochem. J.*, 228 (1985) 487.
4 R. Nau, G. Schäfer, C. F. Deacon, T. Cole, D. V. Agoston and J. M. Conlon, *J. Neurochem.*, 47 (1986) 856.
5 M. Hatanaka, T. Sasaki, T. Kikuchi and T. Murachi, *Arch. Biochem. Biophys.*, 242 (1985) 557.
6 R. Rimón, P. Le Grevés, F. Nyberg, L. Heikkilä, L. Salmela and L. Terenius, *Biol. Psychiatry.*, 19 (1984) 509.
7 I. Lantz and L. Terenius, *FEBS Lett.*, 193 (1985) 31.
8 H. Vaeroy, F. Nyberg, H. Franzen and L. Terenius, *Biochem. Biophys. Res. Commun.*, 148 (1987) 24.
9 J. J. Kusmierz and D. Desiderio, *J. Chromatogr.*, 574 (1992) 189.
10 Y. Ben-Ari, P. Pradelles, C. Gros and F. Dray, *Brain Res.*, 173 (1979) 360.
11 G. Toresson, E. Brodin, A. Wahlström and L. Bertilsson, *J. Neurochem.*, 50 (1988) 1701.
12 H. P. J. Bennett, A. M. Hudson, C. McMartin and G. E. Purdon, *Biochem. J.*, 168 (1977) 9.
13 C. McMartin and G. E. Purdon, *J. Endocrinol.*, 77 (1978) 67.
14 J. M. Conlon and B. Göke, *J. Chromatogr.*, 296 (1984) 241.
15 J. M. Conlon and L. Sheehan, *Regul. Pept.*, 7 (1983) 35.
16 K. Hermann, R. E. Land, T. Unger, C. Bayer and D. Ganten, *J. Chromatogr.*, 312 (1984) 273.
17 R. Laufer, U. Wormser and Z. Selinger, *J. Chromatogr.*, 301 (1984) 415.
18 M. Ohno, M. Kai and Y. Ohkura, *J. Chromatogr.*, 490 (1989) 301.
19 T. M. Wallasch, K. Henning, U. Lange, W. Kuhn, K. Eckart-Wallasch and H. Pruntzek, *J. Chromatogr.*, 425 (1988) 175.
20 O. J. Igwe, L. J. Felice, V. S. Seybold and A. A. Larson, *J. Chromatogr.*, 432 (1988) 113.
21 T. Higa and D. M. Desiderio, *Int. J. Peptide Protein Res.*, 33 (1989) 250.
22 J. J. Miller, G. S. Schultz and R. S. Levy, *Int. J. Peptide Protein Res.*, 24 (1984) 112.
23 J. O. Naim, D. M. Desiderio, J. Trimble and J. R. Hinshaw, *Anal. Biochem.*, 164 (1987) 221.

24 P. Angwin and J. D. Barchas, *J. Chromatogr.*, 231 (1982) 173.
25 P. Le Greves, C. Lundqvist and F. Nyberg, *Prep. Biochem.*, 20 (1990) 145.
26 N. Rösler, C. Reuner, J. Geiger, K. Rissler and H. Cramer, *Peptides*, 11 (1990) 181.
27 H. Cramer, S. Jost, C. Reuner, E. Milios, J. Geiger and F. Mundinger, *Neuropeptides*, 18 (1991) 69.
28 K. Rissler and H. Cramer, in M. J. Conn (Editor), *Methods in Neuroscience*, Vol. 13, Academic Press, San Diego, CA, 1992, in press.
29 H. Cramer, D. Schaudt, K. Rissler, D. Strubel, J.-M. Warter and F. Kuntzmann, *J. Neurol.*, 232 (1985) 346.
30 H. Cramer, K. Rissler, N. Rösler, D. Strubel, D. Schaudt and F. Kuntzmann, *Eur. Neurol.*, 29 (1989) 1.
31 K. Rissler, H. Cramer, D. Schaudt, D. Strubel and W. F. Gattaz, *Neurosci. Res.*, 3 (1986) 213.
32 N. Tanaka, T. Tokuda, K. Iwaguchi and M. Araki, *J. Chromatogr.*, 239 (1982) 761.
33 S. A. Matlin, W. J. Lough and D. G. Bryan, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 3 (1980) 33.
34 Y. Saito, M. Mifune, J. Odo, Y. Otsuki, M. Mitsuhashi, Y. Mori, A. E. H. Gassim and J. Haginaka, *Anal. Sci.*, 7 (1991) 805.
35 E. Krause and M. Bienert, *J. Liq. Chromatogr.*, 15 (1992) 1773.
36 K. E. Bij, C. Horvath, W. R. Melander and A. Nahum, *J. Chromatogr.*, 203 (1981) 65.
37 W. R. Melander, A. Nahum and C. Horvath, *J. Chromatogr.*, 185 (1979) 129.
38 K. Rissler and H. Cramer, *J. Chromatogr.*, 533 (1990) 179.
39 V. Marko and K. Radova, *J. Liq. Chromatogr.*, 14 (1991) 1645.
40 V. Marko and K. Radova, *J. Liq. Chromatogr.*, 14 (1991) 1671.
41 V. Marko and K. Radova, *J. Liq. Chromatogr.*, 14 (1991) 1659.