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Review

Sample preparation, high-performance liquid chromatographic separation and determination of substance P-related peptides[1]

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Abstract

This review deals with the determination of low levels of substance P and peptide fragments derived from the undecapeptide, i.e. covers the whole amount of so-called substance P-like immunoreactivity (SPLI) in biological samples. First an overview of the most currently used sample pretreatment procedures is given, followed by a description of the most effective high-performance liquid chromatographic (HPLC) separation methods. Special attention is paid to the choice of the appropriate column and the possible pitfalls encountered in separation of fmol amounts of peptide material. Subsequently the most important techniques of detection are discussed. This section primarily focuses on the coupling of HPLC with radioimmunoassay (RIA), which is indispensable for detection of components in the fmol range at present. Finally, some aspects of preparation and chromatographic separation of radiolabelled antigens for use in RIA are discussed.

Contents

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[*] This paper is dedicated to Professor Gottfried Schill on the occasion of his 65th birthday.

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1. Introduction

1.1. Substance P undecapeptide as a member of the tachykinin family

Substance P (SP) was first discovered in 1931 by von Euler and Gaddum [1] in alcoholic extracts of equine brain. In 1971 the peptide was structurally defined as an undecapeptide [2] with the sequence

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

and synthesized using the solid-phase strategy [3]. SP is a member of a family of structurally related peptides called the tachykinins, with the common N-terminal sequence

-Phe-Xaa-Gly-Leu-Met-NH_2

where Xaa is the only variable amino acid. The most prominent members of this peptide family in addition to SP are the mammalian peptides neurokinin A (Neuromedin L, substance K), its N-terminally extended form neuropeptide K that contains 36 amino acids, neurokinin B (Neuromedin L) and the non-mammalian tachykinins of amphibian and fish origin such as kassinin, carassin, eledoisin, uperolein, physalaemin, phyllomedusin, the ranatachykinins A, B and C, scyliorhinin I and II [4–8] and others.

The amino acid sequences of some members of the tachykinin family are depicted in Fig. 1.

1.2. Pharmacological actions of substance P

Substance P, which is currently the best characterised of the tachykinin family, is unevenly distributed throughout the vertebrate brain and periphery and exerts a wide spectrum of physiological actions in the peripheral and central nervous system (CNS). Thus an assignment of a

Fig. 1. Amino acid sequences of representative members of the tachykinin family (from Ref. [6] with permission).

neurotransmitter role to SP, as already supposed by Lembeck in 1953 [9], may be justified.

The most salient pharmacological properties of SP in the periphery are its action on blood vessels (vasodilatation, lowering of blood pressure, extravasation), bradycardia, salivation [10,11], bronchoconstriction and mucus hypersecretion. The latter two activities imply a substantial role for SP in the development of asthma bronchiale [12–16]. In contrast, the actions of SP on the CNS are less well-understood and, for this reason, are the subject of numerous investigations. Nevertheless, its key role in pain perception is now well-established [11,17,18]. Furthermore, evidence is provided that SP may be involved in the regulation of extrapyramidal and cognitive functions (in common with substance K) and more recently it gains more and more interest as an important factor in the modulation of neuroimmunological responses [19]. The reader interested in the knowledge of the biological and pharmacological properties of SP is referred to the reviews of Pernow [20] and Maggio [21].

1.3. Enzymatic processing of substance P

Substance P is released from α-, β- and γ-preprotachykinin by tryptic cleavage. In 1983 Nawa et al. [22] isolated a cDNA coding for β-preprotachykinin consisting of 110 amino acids, which contains the sequence of SP at amino acid positions 58–68.

The undecapeptide is further processed in biological systems (tissue, body fluids) to smaller fragments by a multitude of enzymatic activities, which raises the question whether SP may be considered as an intermediate, i.e. a "precursor" for a variety of closely related peptides all exhibiting distinct but different biological and pharmacological actions. This view may be justified because the parent peptide consists of a hydrophilic (basic) N-terminal and a hydrophobic C-terminal region, which, after enzymatic

cleavage, provides fragments with a wide range of both polarity and basicity. Among the enzymes involved in the processing cascade of SP the most important ones may be briefly mentioned: Trypsin cleaves after Arg and Lys, dipeptidyl aminopeptidase IV (EC 3.4.14.5) sequentially releases the N-terminal Arg^1-Pro^2- and Lys^3-Pro^4- dipeptides generating the SP_{3-11} and SP_{5-11} fragments [23] and the so-called post-proline cleaving enzyme [24] leads to the formation of SP_{1-4} and SP_{5-11} by cleavage between Pro^4 and Gln^5. A neutral Zn^{2+}-metalloendopeptidase, also termed enkephalinase (EC 3.4.24.11) cleaves SP_{1-11} between Gln^6-Phe^7, Phe^7-Phe^8 and Gly^9-Leu^{10} [25] and dipeptidyl carboxypeptidase I, also termed angiotensin-converting enzyme (EC 3.4.15.1) between Phe^8-Gly^9 and Gly^9-Leu^{10} [26]. Further, the amidopeptidases calpain I and II are involved in the termination of the biological actions of SP by hydrolyzing the C-terminal carboxamide endgroup to the SP_{1-11} free acid [27]. Additionally some other "SP-degrading enzymes" preponderantly acting on SP and only to a minor extent on other mammalian tachykinins, play a substantial role but their nature has not been fully elucidated.

1.4. Importance of measurement of substance P in tissues and body fluids and aim of the review

Measurement of SP concentrations in tissues of intestine and brain as well as in body fluids may reflect either its metabolism or biological and pharmacological actions. Owing to the small concentrations in the lower fmol/ml range and the high activities of proteolytic enzymes, determination of SP levels in the plasma will be of only minor importance. Although SP levels in the CSF are of the same order of magnitude as in plasma, measurement of substance P-like immunoreactivity (SPLI) in this fluid may be of substantially greater importance for clinical research and diagnostics because the cerebrospinal fluid (CSF) is the only CNS compartment easily accessible to the monitoring of SP levels. No other body fluid comes in closer contact with the interior brain area and circumventricular tissues than CSF, which is adjacent to the extracellular fluid that surrounds brain tissues. Further it is the medium, where transport and metabolism of SP synthesized in the brain takes place and thus may reflect various pathophysiologies.

Despite the discovery of degrading enzymes in this compartment [28–32], SP seems to be stable for at least some days at 4°C probably due to their low concentration. However, in contrast, after incubation of CSF for 1 h at 37°C, a significant increase of SP concentrations was reported with respect to the non-incubated sample, which may be indicative for the release of SP from its precursor by in-vivo proteolytic activities [33].

In many cases the SP-undecapeptide represents only the minor amount of total SPLI. This observation is supported by the existence of various N- and C-terminal fragments in biological matrices, such as e.g. 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_{7-11}, SP_{8-11} etc., which thus may be considered as products of SP metabolism. For this reason the "splitting" of either precursors of SP_{1-11} or the undecapeptide itself into a multitude of SP-like peptides by enzymatic processing presents a tremendous challenge for the analyst in his continuous search for novel methods and improvements in experimental procedures.

The two main problems encountered in the measurement of SP-related peptides arise from their low concentration in tissues and biological fluids and the wide range in polarity and basicity of the different fragments, which require very efficient separation and detection systems.

Reversed-phase high-performance liquid chromatography (RP-HPLC) and octadecylsilyl-based silica gel columns are at present mainly used for the analysis of SP in tissues and body fluids, and the refinement of this method during the last 15 years has resulted in improved separation and lowering of detection limits. Its coupling to an appropriate sample pretreatment by e.g. liquid–liquid extraction, precipitation–extraction and solid-phase extraction or combined techniques can be used to minimize chromatographic interferences as well as to optimise

detection. Additionally, HPLC offers an attractive means for the separation of radiolabelled SP from either its by-products formed during the labelling procedure or by autoradiolysis, for its subsequent use in radioimmunoassay (RIA).

The following section starts with a compilation of commonly used sample preparation procedures for peptides, subsequently switches to the chromatographic separation techniques including the choice of appropriate columns and mobile phases (Section 3), then presents a survey of appropriate detection methods (Section 4) and finally considers some important aspects of preparation as well as purification of radiolabelled SP for use in HPLC-RIA coupling (Section 5). Due to the often extremely low sample amounts of SPLI in biological sources HPLC-RIA plays the dominant role.

2. Sample preparation and enrichment

2.1. Fundamental principles

For achieving the highest degree of (i) recovery, (ii) selectivity of chromatographic separation, (iii) specificity of detection, (iv) accuracy and (v) reproducibility of results an extensive sample clean-up procedure is generally required. In many cases large volumes of biological fluids of more than 10 ml (e.g. CSF, serum or plasma, perfusion liquids) have to be worked up in order to exceed the determination limit of the final quantitation step, e.g. by RIA. For this reason it is easy to understand that such volumes cannot be handled in a facile manner by analytical HPLC (at least without loss of separation efficiency and a concomitant decrease of sensitivity of detection) and makes sample enrichment a crucial step.

The following sections address the various aspects of sample preparation. Nevertheless it should be emphasised that often more than one method or a combination of at least two of them will be necessary (e.g. precipitation–extraction and subsequent solid-phase extraction).

This chapter does not include methods for the large-scale preparation of tissues, which often require some 100 g of material and are used to evaluate the structure of novel tachykinins as reported in Refs. [4–8], but refers to pre-purification and enrichment of biological samples for analytical application.

2.2. Sample preparation and enrichment procedures

2.2.1. Preliminary considerations

It is imperative that the biological tissue and fluid should be obtained as rapidly as possible to avoid production of artifacts via metabolism of either peptide precursors or peptides. For effective minimisation of artifact production, rapid sample preparation at low temperature (e.g. at +4°C) followed by immediate immersion into liquid nitrogen and storage at −70°C as well as boiling, microwave irradiation and addition of a cocktail of enzyme inhibitors have been applied successfully. Furthermore, the possibility of air oxidation of Met residues to the corresponding sulfoxide during sample extraction, as reported by Floor and Leeman [34], has to be taken into account and thus working-up under reducing conditions may be required. In a corresponding manner reduced gluthathione (GSH) autoxidises in biological samples yielding erroneously high levels of GSSG [35].

Another question focuses on the stability of SP in buffers that are usually used in studies of the peptide in a biological environment. Higa and Desiderio [36] investigated chemical degradation of SP with [^{3}H]SP as the probe by means of HPLC. They observed that Tris buffer at neutral pH provides formation of the Arg-Pro-Lys tripeptide and the Arg-Lys-Pro-Pro-Gln-Gln- hexapeptide at a relatively high rate, whereas no structural changes are observed with triethylammonium formate buffer at about pH 3. In the same study the influence of repeated freeze–thaw cycles as well as temperature on stability of [^{3}H]SP was checked, which revealed that repeated freezing and thawing did not substantially affect structural integrity. However, small amounts of the corresponding sulfoxide were found after the sixth cycle. In contrast, stability was much more influenced by incubation of

[^{3}H]SP at 37°C for about 3 h, whereas storage at 4°C did not invoke substantial structural changes of the peptide.

A very important point of view includes the adsorption of the peptide to the surface of glassware or plastic materials, in particular in that cases where only pmol or even fmol amounts are present. Admixture of bovine serum albumin to the buffer solutions at concentrations between 0.05 and about 0.25% will markedly reduce adsorption and concomitantly increase peptide recovery.

The following sections focus on the most commonly used procedures for sample preparation and enrichment.

2.2.2. Homogenisation and extraction of biological samples

This chapter considers some of the most important preliminary steps of sample preparation (see section 2.2.1), whereas, in particular, conversion of the resulting extracts into samples that are utilisable for subsequent HPLC separation is treated in more detail in section 2.2.4.

Homogenisation of tissue samples in an acid milieu provides precipitation of the larger (precursor)proteins, whereas in general, the N-terminally extended SP peptides, which do not exceed the $M_r \cong 5000$ Da as well as the smaller fragments of SP_{1-11} remain in the supernatant after centrifugation. A multitude of convenient approaches are available and a few of them will be mentioned briefly.

Tissue can be homogenised in cold 1 *M* acetic acid [37–40], hot 1 *M* acetic acid [41,42], 2 *M* acetic acid [43], boiling 2 *M* acetic acid in 4% EDTA [44], 0.1 *M* HCl [45,46], boiling 0.1 *M* HCl [47] and 1 *M* HCl containing 0.1% disodium-EDTA [48]. In a study of Igwe et al. [49] investigating different acids for homogenisation/extraction (e.g. formic acid, acetic acid, TFA, trichloroacetic acid at different molarities) optimum extraction yields for a variety of either N- or C-terminal SP fragments were obtained with ice-cold formic acid. Table 1 reveals the dependence of homogenisation/extraction yields on the experimental conditions. Very recently, Zhu and Desiderio [40] supported the findings of Igwe et al. Their study revealed that e.g. acetic acid at low temperature (4°C) can be used for homogenisation of bovine pituitary tissue without the use of any inhibitor. However, when homogenisation was performed at 4°C in Tris-HCl buffer of pH 7.5 and subsequently subjected

Table 1
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[a] (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.[b]
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.

[a] 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.

[b] N.D. = not detected.

(From Ref. [49] with permission.)

to incubation at 37°C (i.e the ideal conditions for enzymatic processing) for 1 h marked degradation of SP was observed irrespective if enzyme inhibitors have been added or not. Furthermore, the superiority of TFA over trichloroacetic acid (which is suspected to cause marked reduction in recovery due to co-precipitation of marked amounts of desired peptide in common with proteins) and improvement of extraction yield by addition of NaCl is reported [50]. On the other hand, for complete recovery of peptide precursors of relatively high molecular mass homogenisation should be performed in a neutral medium [51] at low temperature (0–4°C), to which an inhibitor cocktail should be added. Additionally, in order to avoid oxidation during extraction to the corresponding sulfoxides it is recommended that homogenisation and extraction be performed in the presence of 10 m*M* mercaptoethanol [34]. However, it should be kept in mind that mercaptoethanol reduces disulfide linkages to the corresponding sulfhydryl derivatives, which has to be taken into account when e.g. somatostatin, oxytocin, vasopressin and insulin etc. are analysed in common with SP.

The homogenates are centrifuged or further extracted with mixtures consisting of different compositions of 0.01 *M* HCl–acetone (20:80) [37,51], 0.2 *M* HCl–acetone (25:75) containing 0.2 mg/ml phenylsulfonylfluoride as an inhibitor of serine proteases [52], acetone alone [45] or are directly treated with ethanol–0.7 *M* HCl (3:1) [5] and centrifuged. The supernatants are then evaporated to dryness by lyophilisation or vacuum centrifugation and reconstituted in an appropriate solvent or buffer system for subsequent measurement, e.g. directly by HPLC or, more appropriately, subjected to further treatment by e.g. solid-phase extraction as reported in section 2.2.4.

In contrast, the corresponding treatment of body fluids (e.g. plasma, CSF, perfusion liquids) is less expensive. Deproteinisation of plasma with an equal volume of either 30% trifluoroacetic acid (TFA) [53] or extraction with 1% TFA [54], and 0.1% TFA [55] was reported, whereas Cummings et al. [39] used plasma and urine samples without prior extraction of proteins. CSF can be used either directly [31,32,39,59] or after acidification with 0.1% TFA [55] or microwave irradiation [56].

2.2.3. Liquid–liquid extraction (LLE) of tissue homogenates and body fluids

This alternative can be used after elimination of residual enzymatic activities by the pretreatment shortly reviewed in section 2.2.2. However, it should be emphasised that peptides are polar compounds and LLE into a water-immiscible organic medium can thus be achieved only with difficulty even in the case of the relatively hydrophobic C-terminal SP fragments. As a consequence, the extraction yields will be too low even after a time-consuming multiple extraction procedure. Igwe et al. [49] have tested the ability of 5 water-immiscible organic solvents with respect to their ability to extract fragments of SP with a wide range of polarity from the aqueous into the organic layer. Their study revealed that this alternative is not suited for extraction of peptides from an aqueous environment. Table 2 shows the percentage of SP-like peptides remaining in the aqueous phase after extraction with different organic solvents.

Although hydrophobicity, in particular of the basic N-terminal SP fragments, and thus extraction into a polar organic solvent (e.g. *n*-butanol) can be significantly increased by addition of suitable ion-pairing agents, it is often difficult to remove excessive agent from the peptide [57]. Therefore problems are encountered during the following quantitation step by e.g. HPLC–MS detection through association of the SP fragments with the ion-pairing agent, as well as marked suppression of biological and immunological responses when biological and immunological determination methods are applied. At least more or less marked decreases in either sensitivity or specificity have to be taken into account.

2.2.4. Solid-phase extraction of samples

During the past decade the liquid–solid extraction technique (LSE) or the more commonly used term solid-phase extraction (SPE) on small disposable cartridges has gained more and more

Table 2
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.
(From Ref. [49] with permission.)

interest in sample preparation and is now preferentially used for peptide extraction from biological sources. Numerous applications dealing with SPE of peptides have been published hitherto and the following short compilation of methods is thus far from complete. Nevertheless, the present paper at least attempts to give some insight into the peculiarities of this promising technique.

A variety of adsorbents similar to the materials applied in RP-HPLC (e.g. C_{18}, C_8, C_1, phenyl, cyclohexyl, CN, NH_2 etc., as well as ion-exchange matrices) are now available. The method offers the great advantage that even large amounts of liquid can be passed through the cartridges, resulting in an enormous sample concentration, which is often necessary to exceed the detection limit of the following determination step. Furthermore, large numbers of sample can be processed within a short time period by use of semi-automatised sampling devices. However, in contrast to the packing materials used for HPLC, the particle size is markedly larger (approx. 25–40 μm). This, in turn, allows simple handling of samples and elution from the cartridge by use of a small overpressure or under reduced pressure.

Prior to use the cartridges have to be preconditioned with ca. 2–5 ml of organic solvent (e.g. methanol, acetonitrile, acetone) as wetting agents, followed by the corresponding volume of the solvent or buffer system usually applied for sample dissolution. After adsorption of the sample the cartridge is rinsed with a washing solution that is suitable for elimination of substances, which may interfere with the following HPLC separation and detection process. Very efficient pre-purification is often achieved by a step-gradient with increasing percentages of organic solvent in order to remove unwanted material and thus facilitates the following HPLC separation. Octadecylsilyl functionalised silica (ODS) gel is up to now the preponderantly used adsorbent for peptide trapping from pretreated samples due to its hitherto unattainable adsorptive properties. For analyte desorption from the cartridge matrix a multitude of organic solvents as well as aqueous mixtures of organic solvents have been successfully used. Addition of small amounts of TFA (usually about 1%) to the eluent seems to play an essential role for good recovery. TFA ensures that all basic moieties of the peptides are completely protonated and acid–base interactions between solute and matrix (so-called silanophilic interactions, see section 3 below) are substantially suppressed. Although a variety of other acids have also been used, TFA seems to be by far the best choice. A further attempt to minimise silanophilic interactions is reported by Hermann et al. [47]. After preconditioning, the cartridge matrix was coated with a polypeptide solution (1%) followed by a second

preconditioning using the previously described conditions.

The sample concentration procedures by SPE are similar for either plasma or CSF and tissue and therefore are only referred to briefly.

Fluid samples are often directly loaded onto the SPE matrix without further pretreatment and eluted with methanol–0.1% TFA–1 *M* ammonium acetate (80:10:10, v/v) [39]. However, it is more convenient to use the supernatants of TFA-deproteinised plasma (e.g. one volume + one volume 30% TFA (see Ref. [53]) or plasma diluted with 1% TFA [33,54]. The cartridge is subsequently washed with 1% TFA or an appropriate buffer system and peptides desorbed with methanol–water–TFA (80:19:1, v/v) [53] or acetonitrile (ACN)–water–TFA (80:19:1, v/v) [54]. CSF is pretreated for subsequent HPLC–RIA similarly as described for plasma [53] and peptides eluted from ODS cartridges with methanol–water–formic acid (80:19:1, v/v) [58], methanol–water–TFA (80:19:1, v/v) [47], ACN–0.1% aqueous TFA (50:50, v/v) [33,59]. Further, plasma adjusted to pH 4.0 was extracted using silicic acid [52]. The adsorbent was washed with water and 1 *M* hydrochloric acid and peptide material eluted with 50% acetone.

In a corresponding way supernatants from tissue extraction were adsorbed on preconditioned ODS materials, washed with appropriate solvent or buffer systems and eluted with ACN–water (70:30, v/v) [5,8], ACN–water–TFA (80:19:1, v/v) [37], methanol–1 *M* ammonium acetate (90:10, v/v) [39], ACN–0.1 *M* phosphate buffer (pH 2.3) (7:3, v/v) [45], ethanol [48], methanol–water–TFA (80:19:1, v/v) [50], ACN–0.1% aqueous TFA (50:50, v/v) [60], 50% ACN without TFA [61] and pure acetonitrile [38,62]. In another approach the tissue extract is applied to an acetone–water preconditioned ODS matrix, the cartridge washed with 4% acetic acid and acetone–0.2 *M* HCl (75:25, v/v) used for peptide elution [52].

Pre-purification, i.e. pre-separation of radiolabelled peptides was carried out in a similar manner on ODS cartridges (see also section 5). Two techniques comparable to the ODS cartridge system but using cation-exchange [56] as well as anion-exchange [47,63] resins were also applied.

Several studies have reported optimisation strategies for SPE. The most comprehensive investigation with regard to the variety of SPE matrices and elution solvents used for the study of either N- or C-terminal SP fragments (SP_{1-4}, SP_{1-7}, SP_{1-9}, SP_{5-11}, SP_{2-11}, SP_{1-11}) was published in 1988 by Igwe et al. [49]. Tissue homogenates dissolved in 1 *M* formic acid spiked with a cocktail of SP fragments and applied to methanol-preconditioned ODS cartridges yielded recoveries ranging from 53 to 98% after elution with methanol (see Table 3). Surprisingly the

Table 3
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

Peptide	Recovery (mean ± S.D., $n = 6$) (%)			Coefficient of variation (%)
	0.5 μg/ml	2.5 μg/ml	5.0 μg/ml	
SP_{1-11}	53 ± 6	56 ± 5	72 ± 13	8–18
SP_{1-4}	63 ± 17	61 ± 1	77 ± 16	2–28
SP_{1-7}	62 ± 4	62 ± 3	80 ± 3	4–6
SP_{1-9}	61 ± 12	62 ± 3	66 ± 6	5–20
SP_{2-11}	98 ± 9	93 ± 3	95 ± 8	3–9
SP_{5-11}	74 ± 15	79 ± 3	88 ± 9	4–20

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.
(From Ref. [49] with permission.)

recovery of the very polar SP_{1-4} fragment, even by use of the highly hydrophobic matrix, approximately equals the value obtained for the substantially more hydrophobic undecapeptide. For desorption of the peptide material from the SPE matrix the following elution potencies have been found: methanol ≈ methanol–dichloromethane (1:1) > diethyl ether–methanol (1:1) > acetonitrile–methanol (1:1) > acetonitrile > diethyl ether > dichloromethane. Furthermore, recoveries were measured after application of tissue extracts onto a weak (carboxymethyl-) as well as a strong (sulfopropyl-) cation-exchanger. However, these materials are unsuitable matrices for extraction of C-terminal SP fragments, as can be reasonably expected due to their low basicity, and only the N-terminal fragments containing a strongly basic anchor group (Arg, Lys) are at least partially retained (e.g. SP_{1-4}, SP_{1-7}, SP_{1-9}).

Rissler et al. [64] investigated the influence of decreasing hydrophobicity of the cartridge matrix ($C_{18} > C_8 >$ phenyl > CN) on the recoveries of SP and its C-terminal SP fragments (SP_{1-11}, SP_{2-11}, SP_{3-11}, SP_{4-11}, SP_{5-11}, pyroglutamyl-SP_{5-11}) with acetonitrile as organic eluent modifier (ACN–water–TFA, 80:19:1, v/v). In the case of the CN material either acetonitrile or methanol were used. The study was further undertaken to evaluate the possible involvement of $\pi-\pi$ interactions in the adsorption process. In general, recoveries increased with increasing hydrophobicity of the peptide species and best results were obtained with a C_{18} matrix. Although the phenyl matrix is markedly less hydrophobic than the C_8 material, recoveries of the fragments exhibiting a high density of aromatic amino acid residues (SP_{4-11}, SP_{5-11}, pyroglutamyl-SP_{5-11}) are substantially better on the phenyl cartridge, which may be interpreted as due to a participation of $\pi-\pi$ interactions during adsorption of the sample. When using a CN matrix, recoveries, in particular of the more hydrophobic fragments with a high density of aromatic amino acids, increase with methanol compared with acetonitrile. This observation is somewhat surprising because $\pi-\pi$ interactions between the aromatic moieties and the triple bond of the CN groups of the matrix might be expected, which should be more markedly suppressed with acetonitrile than with methanol. Thus due to the presumed ability of acetonitrile to compete for the CN anchor groups on the surface of the cartridge matrix, a better recovery should have been realised with the aprotic modifier. Perhaps residual silanophilic interactions may be responsible for this effect, which however, are more substantially eliminated with methanol. The results are presented in more detail in Tables 4 and 5.

Table 4
Recoveries of synthetic SP_{1-11} and related fragments on different sorbents with acetonitrile as the organic modifier

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

Sample volume, 2 ml, containing 250 pg/ml.
(From Ref. [64] with permission.)

Higa and Desiderio [65] investigated the influence of the flow-rate of the sample when applied onto an ODS cartridge. In the case of tritiated SP they found that a slow elution was more important than a slow rate of sample application (see Table 6). In order to obtain reproducible application–elution conditions they

Table 5
Recoveries of synthetic SP_{1-11} and related fragments on a CN cartridge with methanol as the organic modifier

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

Sample volume, 2 ml, containing 250 pg/ml.
(From Ref. [64] with permission.)

Table 6
Recovery of ^{3}H-SP with different flow-rates in application and elution

	Flow-rate (ml/min)		Recovery of ^{3}H-SP (%)
	Application	Elution	
A	19.2	0.075	92.5 ± 1.3 (n = 6)
B	0.075	19.2	85.3 ± 2.2 (n = 6)[a]

^{3}H-SP (50 nCi, 1.5 pmol) was applied onto the ODS disposable cartridge. Sample A was obtained from the fast flow-rate of application (19.2 ml/min) and slow elution (0.075 ml/min). Sample B was from the slow flow-rate of application (0.075 ml/min) and fast elution (19.2 ml/min). The flow-rate of rinse time was the same (4.8 ml/min) for both samples.

[a] $P < 0.005$, compared for value of sample A. Values are mean ±S.E.

(From Ref. [65] with permission.)

recommend the use of a syringe infusion pump to maintain an exact flow-control.

A comprehensive scheme of the pretreatment of tissue samples for HPLC is presented in Fig. 2.

An important question focuses on the recoveries of peptides after having been subjected to an extensive sample preparation step in order to minimise interference with subsequent determination methods. Therefore it is logical that the interpretation of results from determinations of SP-related peptides requires measurement of their individual recoveries in biological matrices for correction of losses during extraction. Due to the wide range of polarities of SP fragments, more or less marked differences in individual recoveries have to be taken into account [49,64]. Despite the differences obviously encountered in peptide analytics, the use of an ODS matrix assures sample enrichment conditions, which at least could be successfully used to determine the C-terminal SP moieties without too large differences in individual recoveries. However, at variance with Ref. [49], it might be expected that the N-terminal (highly basic) SP fragments will be only weakly retained and thus would require more polar sorbents or even cation-exchange resins for "trapping".

Owing to the often extremely low concentrations of SPLI in body fluid samples high extraction yields are essential for the subsequent quantitation step, e.g. by RIA. Estimation of recovery can be done most conveniently by addition of the corresponding radioactive (usually the tritiated) compounds to the matrix prior to SPE. Ideally the structural integrity of the recovered radioactivity of the eluate should be checked by following HPLC. However, this method suffers from the drawback that every peptide fragment would require its own labelled analogue because recovery is essentially governed by the inherent structure-dependent hydrophobicity and thus will be only realisable in exceptional cases. For this reason calculation of

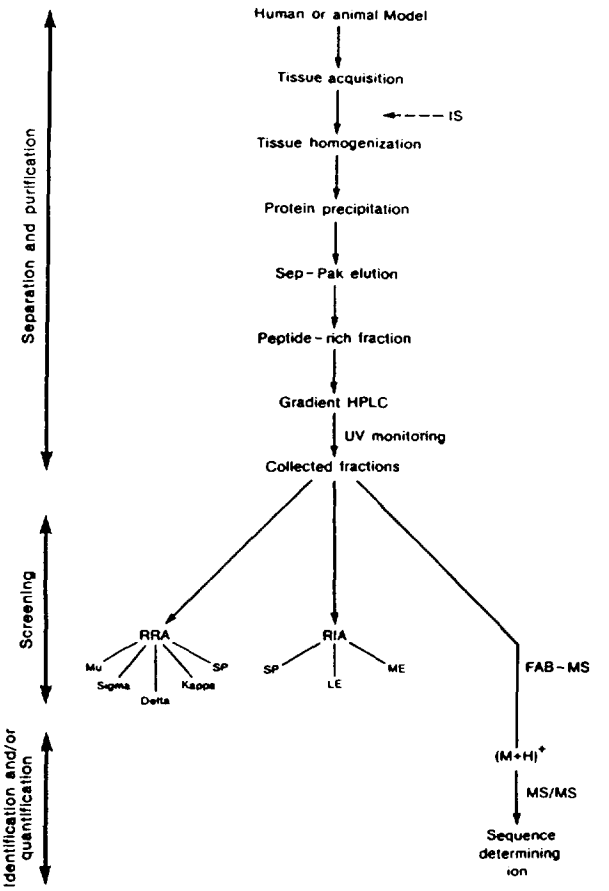

Fig. 2. Tissue extraction and analytical methodology for profiling and identifying neuropeptides (from Ref. [51] with permission).

recovery values has to be performed with the synthetic unlabelled peptide standards.

2.2.5. On-line sample preparation by pre-column/analytical column-switching

This technique, primarily applied by Roth et al. [66] can be used for on-line sample pre-purification and concentration of tissue and body-fluid extracts after prior liquid precipitation extraction (see section 2.2.2) and subsequent filtration to avoid clogging of the pre-column. As adsorbents the same materials as used for SPE (see section 2.1.4 above) can be applied and identical precautions have to be considered in the choice of the appropriate pre-column matrix. Very recently, novel stationary phases, the so-called "restricted access media" either based on silica [67–70] or polymer backbones [71] have been developed, which can also be used for sample concentration. The biological samples are applied onto these media directly after centrifugation without prior deproteinisation due to the inaccessibility of the micropores for larger proteins but not for smaller and medium-sized peptides. However, despite the obvious advantages in the use of this technique there exist some drawbacks, because one cannot ensure that all members of the SP family are focused as a small injection band at the pre-column head primarily due to the wide range of polarities, even when a completely aqueous transfer solvent is used. Thus back-flush of the peptide material to the analytical column can result in peak broadening or peak distortion and thus to a loss of sensitivity. Another important point of view arises from the possible incompatibility of the washing solution used to remove interfering components from the pre-column matrix and the final mobile phase for the back-flush and subsequent separation on the analytical column. For this reason the composition of either washing solution and eluent has to be carefully adjusted in order to minimise more or less marked solute–solvent and solvent–solvent incompatibility effects (see section 3.2.2 below). A review article concerning column-switching techniques for the preparation of biological samples has been published recently by Campins-Falco et al. [72].

3. High-performance liquid chromatographic separation

3.1. Fundamental considerations

Substance P undecapeptide contains a hydrophilic (basic) N- and a hydrophobic C-terminal region and enzymatic processing in the living organism generates peptide fragments with a wide range of polarity and thus in retention times. Therefore, application of a solvent gradient may be superior to isocratic elution because separation is achievable within a reasonable time range and peak broadening of later-eluting peaks can be reduced and thus sensitivity improved. A list of some SP-related peptides markedly differing in either chain length or polarity (basicity) is given in Table 7.

Table 7
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

(From Ref. [49] with permission.)

Although retention of small peptides with less than 20 amino acids can be predicted by summing the contribution to retention of each amino acid and end group [73], the peculiarities especially of polar, basic moieties due to either low retention on reversed-phase materials or problems attributable to irreversible adsorption onto residual matrix silanols are still present and well-known observations in RP-HPLC of peptides. These aspects have to be kept in mind, in particular in those cases where only fmol amounts of SPLI are to be separated and thus high recovery will be an indispensable prerequisite. Further, besides complete separation of the SP fragments from each other great attention has to be paid to possible interference arising from the biological matrix (which never can be quantitatively removed even after a thorough sample pretreatment as described in section 2). Their co-elution has to be either eliminated or at least extensively reduced to facilitate the subsequent determination step by e.g. mass spectrometric (MS) experiments or RIA with specific antisera.

Optimisation of chromatographic separation is determined by the appropriate choice of (i) the column matrix, (ii) the mobile phase and (iii) the temperature of separation. However, the item (iii) normally plays a minor role and is only of some importance when conformational equilibria are observed during separation, as is the case with larger peptides and proteins [74–76]. A large number of papers dealing with optimisation strategies, method development and improvement of HPLC of peptides as well as mechanistic aspects of separation has been published during the past 10–15 years and it is not the main goal of this review to give an overview. Instead, the interested reader is referred to either Ref. [77] or to the representative and exhaustive coverage by the group of Hearn in the Journal of Chromatography.

3.2. Separation materials

3.2.1. Choice of the column material

This chapter addresses the fundamental principles of stationary phases required for peptide separation to provide the user with sufficient knowledge for an optimal choice of the separation medium. Special interest is dedicated to column matrices with improved chromatographic performance of peptides containing basic amino acid residues on reversed-phase materials prepared from low acidity silica gels (see below) and includes the use of novel ligands, which may permit an extensive shielding of the silica gel matrix against deleterious influences exerted by the mobile phase. In the following chapter (section 3.2.3) the properties of mobile phases for HPLC of peptides are treated in more detail.

For separation of polypeptides particle sizes of 5 μm and pore diameters of 100–300 Å are preponderantly used. For shorter peptides such as SP and related fragments 5 μm particle size/ 100 Å pore columns are sufficient, whereas larger peptides and proteins require pore diameters of 300 Å. In particular marked size-exclusion effects have to be considered when larger peptides are chromatographed on materials with pore diameters <300 Å. Furthermore, matrices with pore diameters smaller than 80 Å should not be used due to the possible drawback of irreversible adsorption.

Most peptide separations are carried out on so-called reversed-phase materials, which fall into two general classes: fully polymeric materials such as divinyl benzene crosslinked polystyrene (PS-DVB) resins and silica gel to which alkyl chains of different chain length (e.g. C_{18}, C_8, C_6, C_4, C_3, C_1, phenyl, CN, diol, NH_2) have been bound covalently. The PS-DVB resins exhibit a wide range of pH stability and thus can be operated with solvents of either high acidity (pH 1) or basicity (up to pH 14) but unfortunately suffer from a relatively low pressure resistance and, in general, the pressure has to be kept below 200×10^5 Pa. Further, the physicochemical properties are extensively governed by a solvent-dependent swelling and shrinking of the organic matrix, which is associated with concomitant changes in pore diameters and, as a consequence, also in mass transfer characteristics. This effect may be encountered in particular during gradient elution starting with e.g. a pure aqueous phase (by which only little or no wetting

of the column matrix takes place) and termination with a pure organic solvent (e.g. methanol, acetonitrile, 2-propanol). Thus pore diameter will continuously change and may be often the cause of poor chromatographic performance. In contrast, silica gel as the starting material for subsequent alkyl silylation offers the advantage that a great number of different alkylsilica substituents can be bound to the silica surface and increased resistance of particles against pressure is obtained. Thus columns can be operated in a wide pressure range up to more than 400×10^5 Pa. In addition swelling and shrinking can be completely neglected and re-equilibration of the bonded stationary phases takes place rapidly, making the materials suitable for gradient elution, which up to now, is the most frequently used technique for HPLC of peptides.

During the last 5–6 years a number of researchers attempted to achieve a compromise between the high stability of polymer resins against solvents or buffers covering a wide pH range (e.g from pH 1–13) and the hitherto unattainable pressure resistance of silica gel based materials. For this reason either alkylsilyl silica gels coated with polymers of different hydrophobicities (obtained by polymerisation in the presence of the silica gel carrier) or polymers chemically bound to the silica surface (which has previously been reacted with e.g. vinyldimethylchlorosilane) via co-polymerisation with suitable monomers (e.g. acrylates with a wide range in hydrophobicity by the choice of appropriate side groups) have been prepared [78–81]. This procedure assures the prolonged use of the chromatographic columns even at slightly elevated pH (e.g. pH 7–9) without substantial stationary phase deterioration, and further markedly reduces deleterious influences arising from the incompletely reacted (free) silanol groups. Reduction of both effects is mainly attributable to the shielding efficiency of the polymer network. However, as can be concluded from the number of publications the use of these new stationary phases remains restricted to a few special applications. Thus it seems to be obvious that the superior mass-transfer characteristics of pure alkylsilyl silica gel-based matrices compared with the polymer coated analogues may be a crucial argument for their already preferable use.

Although a multitude of stationary phases containing polymer-coated alkyl silica gel or polymers covalently bound to silica gel are nowadays available, peptides are mainly chromatographed on reversed-phase materials of the alkylsilyl silica gel type despite the disadvantages, in particular their low hydrolytic stability and, as a consequence, an increase of the number of free silanol groups strongly interacting with basic solutes after prolonged use. Attempts to reduce these disadvantages are treated below in more detail.

The most pronounced deleterious effects of alkylsilyl silica gel matrices on peak shape and recovery are attributable to substantial participation of so-called silanophilic interactions [82–87]. This effect causes a more or less U-shaped curve of the values for the capacity factor k' when the aqueous eluent component in gradient chromatography was increasingly replaced by an organic modifier. They are invoked by the presence of incompletely reacted silanol groups on the silica gel surface giving rise to marked ionic (acid–base) interactions, in particular with basic solutes. For this reason, depending on their concentration, the separation, which is mostly governed by hydrophobic solute–matrix interactions, may be more or less superposed by an ion-exchange mechanism.

Minimisation or at least marked reduction of silanophilic interactions can be achieved in different manners by end-capping of column matrices with trimethylchlorosilane or application of novel ligands carrying voluminous alkyl substituents directly adjacent to the connecting point between ligand and silanol group. However, the presence of substantial amounts of so-called residual silanols must not absolutely be associated with bad chromatography (i.e. peak broadening and/or distortion, reduction and loss of recovery and/or resolution etc.). Indeed, despite the loss of over half of the bonded phase, in some cases adequate chromatographic performance has been reported [88] and despite a shift to lower retention times the separation

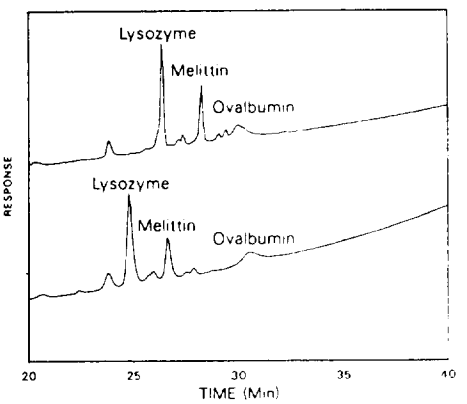

Fig. 3. Separation of lysozyme, melittin and ovalbumine on Ultrapore C_3 column. (Top) First gradient injection; (bottom) chromatogram after 6000 injections. (From Ref. [88] with permission).

efficiency often remains unchanged, which is depicted in Fig. 3. The reason for this at first sight unexpected and surprising observation can be ascribed to a so-called type B silica gel used for the derivatisation with the alkylsilyl substituent. Materials of this type are characterised by a high density of silanol groups on the silica surface, most of which are associated by hydrogen bonding, the so-called associated (bound) silanols [89] and only a relatively small amount of so-called free silanols. The following derivatisation reaction with the alkyl substituents preferably takes place at these bound silanols. The type B silicas are prepared by a special technique termed complete rehydroxylation of the silica gel and exhibit low acidity compared to the type A silica gels, which are substantially more acidic and thus after derivatisation show much more deleterious silanophilic interactions.

The continuous and sometimes even rapid loss of alkyl ligands by use of solvent systems with $pH < 3$ implies the ultimate use of materials prepared from type B silica and the analyst should address the manufacturer in order to ascertain that he uses a true type B based column matrix.

The other alternative is to provide stationary phases with increased resistance towards hydrolytic cleavage of the alkylsilyl substituents by aggressive mobile phases and successful efforts have been undertaken to apply alkylchlorosilanes carrying voluminous substituents, such as isopropyl, isobutyl and *tert.*-butyl groups [90,91], e.g. diisobutyloctadecylchloro-silane etc. The steric requirements of these bulky side chains makes access of the deleterious mobile phase to Si–O–Si bond more difficult as in the case of dimethylalkylsilanes. Synthesis of so-called bidentate ligands (X-$Si(R_2)$-Y-$Si(R_2)$-X, where X = Cl, OCH_3 and the spacer Y can be O, $-CH_2-$, $-CH_2-CH_2-$ or other bridges, offers a further improvement of the latter technique. With this approach, by selection of the dimensions of the spacer Y group in the silane, reactive X groups can be spaced to allow for a better match between these groups and the distance between silanols on the silica surface [90]. It seems reasonable that these bridged alkylsilyl silicas may offer a further gain in hydrolytic stability compared with the corresponding monomeric alkylsilylsilicas carrying bulky side chains. However, the synthesis of stationary phases of this type yielding optimum reproducibility may be a major problem and, at present, for this reason they are commercially available (whenever) only to a minor extent.

3.2.2. Appropriate mobile and stationary phases for the separation of SP and related peptides

In general, chromatography of peptides is carried out in the pH range between 2 and 4 assuring that ionisation of acidic Asp and Glu residues is extensively suppressed and basic amino acids like Arg, Lys and His remain as the cations, which in turn leads to marked reduction of silanophilic solute–matrix interactions.

Acetonitrile is the most widely applied organic mobile phase modifier primarily because of its excellent transparency at 210 nm. Additionally, the pressure of the mobile phase decreases approximately linearly with increasing percentage of the modifier. In contrast, aqueous mixtures of methanol, ethanol, 1-propanol and 2-propanol exhibit a more or less convex pressure dependence on the percentage of organic modifier, which, on average reaches maximum values between 40 and 70% of protic solvent and thus a maximum in viscosity. For this it may be reason-

able to assume consequences upon the mass-transfer characteristics, which in turn, may markedly influence separation efficiency. Further, in particular admixture of C3 alcohols yield unacceptably high column back-pressures so that their total percentage has to be reduced in the mobile phase or separation has to be performed at higher temperatures in order to reduce the column back-pressure. On the other hand silanophilic interactions are more markedly reduced with the alcoholic solvents than with the aprotic solvent acetonitrile.

Nevertheless improvement of selectivity by use of 1-propanol [92,93] and 2-propanol [94] compared with acetonitrile was reported. On the other hand Meek and Rossetti [73] observed substantial peak broadening when acetonitrile was replaced by 1-propanol, which may be associated with reduced mass transfer due to a concomitant increase in viscosity.

TFA is the most widely applied mobile phase additive for aqueous solutions of organic modifier. Normally 0.05 to 0.1% of TFA is added to the mobile phase and, at this concentration, it does not impair detection at 210 nm. It further acts as an ion-pairing agent, although it does not substantially influence retention of peptides containing basic amino acids due to its already relatively high hydrophilicity. However, retention can be shifted to higher values by use of more lipophilic TFA homologues like pentafluoropentanoic acid (PFPA), heptafluorobutyric acid (HFBA) and undecafluorocaproic acid (UFCA). All these additives are already sufficiently volatile and thus easily removable by vacuum centrifugation so that the residues can be isolated in pure form allowing further treatment without the drawbacks of residual mobile phase contaminants. Further, TFA can be used in gradient elution up to 100% of organic modifier. The advantages of TFA and analogues in RP-HPLC of peptides is reported by Bennett et al. [95]. Nevertheless it should be remarked that silica columns substituted with hydrocarbon chains undergo marked hydrolysis of the alkylsilyl silica bond after prolonged treatment of TFA-containing mobile phases, which may sometimes be the cause of severe deterioration of chromatographic performance [96].

Rivier [97] reports on the advantages of trialkyl ammonium phosphate (TAAP) buffer systems, which are compatible with in-vitro and in-vivo biological systems after elimination of the mobile phase. In contrast to acetate or formate buffers the TAAP systems can be operated at 210 nm even under gradient elution conditions. Optimisation of chromatographic performance is obtained by (i) a low pH (in most cases about 2–3 and in general approximately corresponding to the pK_a value of the peptide), (ii) a relatively low flow-rate (about 1 ml/min) and (iii) a hydrophobic column matrix (e.g. C_{18}). In contrast to inorganic phosphate and acetate buffers systems, TAAP mobile phases show better solubility in aqueous organic solvents due to the presence of an organic counter-ion even if the percentage of organic modifier is very high. This yields buffers with either high ionic strength favouring peak resolution [97] as well as post-separation column rinse with high amounts of organic solvent often necessary for complete removal of strongly retained sample constituents.

Usually, the smaller the number of amino acids constituting the peptide the higher the hydrophobicity of the stationary phase used for separation. For larger peptides, i.e for peptide precursors, due to their great and sometimes irreversible retention on highly hydrophobic materials less hydrophobic (e.g C_4) or even relatively polar matrices (e.g. CN) are preferred. On the other hand it was recently found that CN columns show excellent selectivities for peptides including short-chain species widely differing in hydrophobicity and chain length. In particular impurities arising from small organic molecules are often more effectively removed as e.g with a C_8 stationary phase [98].

SP, its fragments generated by enzymatic processing as well as its precursors are usually separated on highly hydrophobic stationary phases, such as C_{18} materials by either isocratic [34,36,37,39,43,63,99,101–106] or gradient elution [5–8,26,28–32,38,40,41,42,44–47,49,51,54–56,60,61,64,108–126,128], the gradient technique being by far the preferred alternative. In accordance with the virtual superiority of hydrophobic matrices for separation of small peptides several authors used a PS-DVB column in the isocratic

[60] and the gradient mode [33,59,61]. Nevertheless chromatography was also performed to some extent on the less hydrophobic C_8- stationary phase by either isocratic [100,105,106] or gradient [4,27,107] elution, whereas in contrast, only little work has focused on separation using less hydrophobic materials such as di-phenyl [6], C_6- (105),C_4- [5,7,8], phenyl- [4] or even more hydrophilic stationary phases such as a C_1- [4] material, all operated in the gradient mode and a CN- column [105] run isocratically. Although ion-exchange chromatography may be a suitable means for separation of SP-related peptides, in particular for those fragments containing at least one basic amino acid moiety, this technique seems to play only a minor role.

The preponderantly used eluent systems for SP-related peptides are mixtures of ACN–water–TFA [4–8,26,27,30,41,44,46,49,54–56,64,103, 107,109,112,115,117–123,126,128], methanol–water–TFA [28,29,42,63,99,101] and ACN–water–phosphoric acid [104,108,110,111,113]. Furthermore, aqueous solutions of the following buffer systems, all dissolved in acetonitrile, have been used: TEAP [24,100,102,128], TEAF [31–33,36,37,38,40,51,59–61,124,125,128] and inorganic phosphates [34,45,49]. Additional alternatives are ammonium acetate buffer (10 m*M*) in acetonitrile containing a final concentration of 0.15% TFA [39] and ammonium acetate buffer (10 m*M*) in methanol [47]. An isocratic mobile phase system with superior separation efficiency of tachykinins and SP-related peptides compared with TEAP is provided by tris(hydroxyaminomethyl)methane phosphate (Tris-phosphate) buffer in acetonitrile [106] and depicted in Figs. 4 and 5.

In all mobile phases the pH range is between 1.9 and 4.5. The concentration of acid has been adjusted between 0.04 and 0.1% (TFA) and 0.08 and 5% (H_3PO_4) and the concentration of buffer salt between 10 and 100 m*M*.

Volatile buffers, i.e. solvent systems from which the salts can be easily removed by simple vacuum centrifugation, play an important role for subsequent quantitation by RIA and radioreceptor assay (RRA). In these cases the peptides of interest exist in a carrier-free state and do not contain contaminants from the mobile phase, which may interfere in the subsequent RIA or RRA. The most commonly used agent of this type is TFA, but due to its often deleterious effects on the long-time chromatographic performance it may be replaced by the milder buffers TEAF or triethylammonium acetate (TEAA) in concentrations between 10 and 100 m*M*. It should be emphasised that column calibration with peptides can only be performed with difficulty when TEAF and TEAA are used

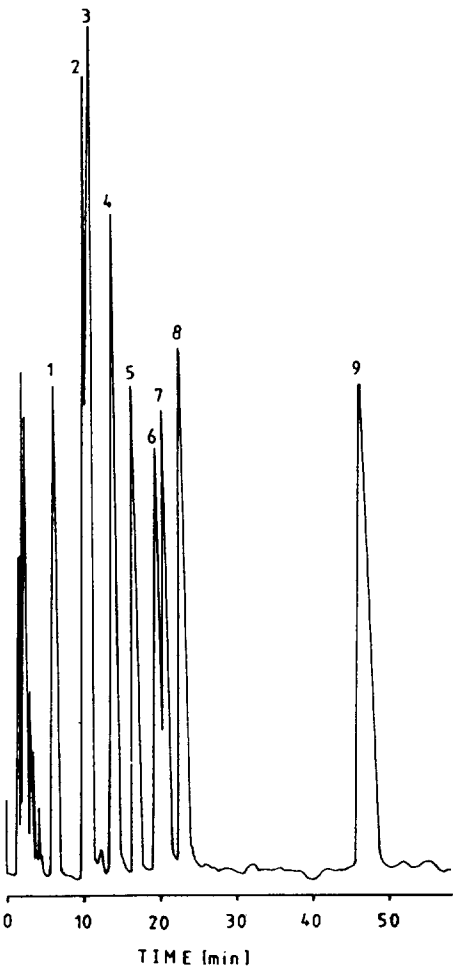

Fig. 4. HPLC of a mixture of tachykinin-like peptides on a Spherisorb ODS II column (125 × 4.6 mm I.D., 5 μm particle diameter). The mobile phase consisted of a 80:20 (v/v) mixture of 0.1 *M* TEAP in acetonitrile adjusted to an apparent pH of 2.8. Detection wavelength, 210 nm. Peaks: 1 = [Tyr^2]-SP_{1-11}; 2 = physalaemin; 3 = SP_{1-11}; 4 = SP-COOH (free acid); 5 = eledoisin; 6 = SP_{2-11}; 7 = SP_{4-11}; 8 = SP_{5-11}; 9 = pGlu-SP_{5-11}. (From Ref. [106] with permission).

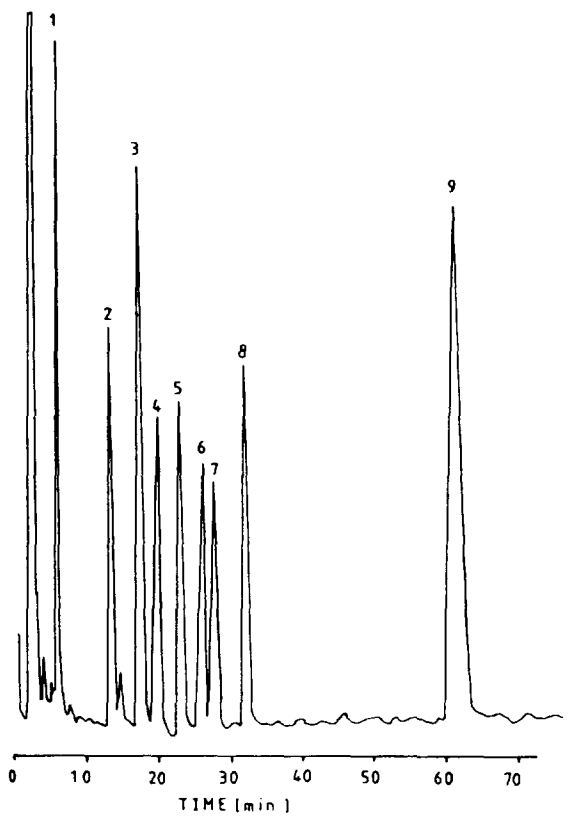

Fig. 5. HPLC of a mixture of tachykinin-like peptides on a Spherisorb ODS II column (125 × 4.6 mm I.D., 5 μm particle diameter). The mobile phase consisted of a 80:20 (v/v) mixture of 0.1 *M* Tris-phosphate in acetonitrile adjusted to an apparent pH of 2.8. Detection wavelength, 210 nm. Peaks as in Fig 5. (From Ref. [106] with permission).

at 210 nm due to the strong absorbance of the carboxylate group at this wavelength and therefore signal monitoring should more conveniently be done at 280 nm. Nevertheless flushing of the reference cell with mobile phase may compensate for the baseline drift invoked by the strongly UV-absorbing buffer ingredients but problems could occur when peptides are to be separated in the gradient mode. However, measurement at 280 nm is restricted to peptides containing aromatic amino acids and completely disregards fragments lacking them, like SP_{1-4} etc. In the latter case retention times of peptides that are invisible at 210–220 nm have to be ascertained by collection of fractions and measurement of the immunological elution profile with the RIA technique.

Sufficient separation of SP fragments largely differing in chain length as well as hydrophobicity was successfully performed. Isocratic separation of Arg-Pro, SP_{1-4}, SP_{1-11}, SP_{4-11}, SP_{5-11}, SP_{6-11}, SP_{7-11}, SP_{8-11}, SP_{9-11}, SP_{10-11} and Met–NH_2 on a C_8- column was reported by Blumberg et al. [24], and SP_{1-11}, SP_{3-11}, SP_{2-11}, SP_{6-11}, SP_{4-11} on a C_{18}- phase by Ben-Ari et al. [99] shown on Fig. 6. Gradient elution on C_{18}-columns was applied for separation of SP_{1-4}, SP_{1-7}, SP_{1-9}, SP_{3-11} by Kaneko et al. [32], SP_{1-7}, SP_{3-11}, SP_{5-11}, SP_{3-7} and SP_{1-11} by Sakurada et al. [42], SP_{1-4}, SP_{1-7}, SP_{1-9}, SP_{5-11}, SP_{2-11}, SP_{1-11}, $[Tyr^8]SP_{1-11}$ by Igwe et al. [49] shown on Fig. 7, SP_{1-4}, SP_{1-11}-sulfoxide, SP_{1-11}, SP_{2-11}, SP_{4-11}, SP_{5-11}, $pGluSP_{5-11}$, β-preprotachykinin$_{58-70}$ [1] by Higa et al. [59], SP_{1-11}, SP_{2-11}, SP_{3-11}, SP_{4-11}, SP_{1-11} and SP free acid (SP-COOH) by Fransson [105], SP_{1-11}, SP_{2-11}, SP_{5-11}, pyro-glutamyl SP_{5-11}, SP_{4-11} and SP-COOH by Rissler and Cramer [106] (Fig. 5), SP_{1-6}, SP_{1-7}, SP_{1-9}, SP_{7-11}, SP_{7-9}, SP_{8-9}, SP_{10-11} and SP_{1-11} by Matsas et al. [110], SP_{1-6}, SP_{1-7}, SP_{1-8}, SP_{4-11}, SP_{6-11}, SP_{7-11}, SP_{8-11}, SP_{9-11}, SP_{1-11} and SP_{1-11} sulfoxide by Lee et al. [108,111], SP_{1-6}, SP_{1-7}, SP_{1-9}, SP_{7-11}, SP_{8-11}, SP_{1-11}, SP_{1-11} sulfoxide by Nau et al. [112] shown on Fig. 8 and Table 8, SP_{1-5}, SP_{5-10}, SP_{7-8}, SP_{8-11}, SP_{7-11} by Endo et al. [113], SP_{1-7}, SP_{1-8}, SP_{3-7}, SP_{8-11}, SP_{9-11} and SP_{1-11} by Nyberg et al. [28,114], SP_{1-4}, SP_{1-7}, SP_{1-9}, SP_{3-11}, SP_{5-11}, SP_{1-11}, SP_{1-11}-sulfoxide by Toresson et al. [117,122], SP_{4-11}, SP_{5-11}, SP_{2-11}, SP_{1-11} and SP_{1-11}-sulfoxide by Rösler et al. [118], Jost et al. [123] and Cramer et al. [124].

To achieve an increase in retention of the polar and strongly basic N-terminal peptides SP_{1-4}, SP_{1-7} etc., in order to shift their signals markedly away from the injection peak, the gradient should be initialised with e.g. 100% of an aqueous solution of 0.1% TFA, in particular when hydrophobic stationary phases like C_{18} and C_8 materials (which yield optimum separation of

[1] This fragment corresponds to the SP_{1-11} peptide that contains a C-terminal extension by Gly-Lys.

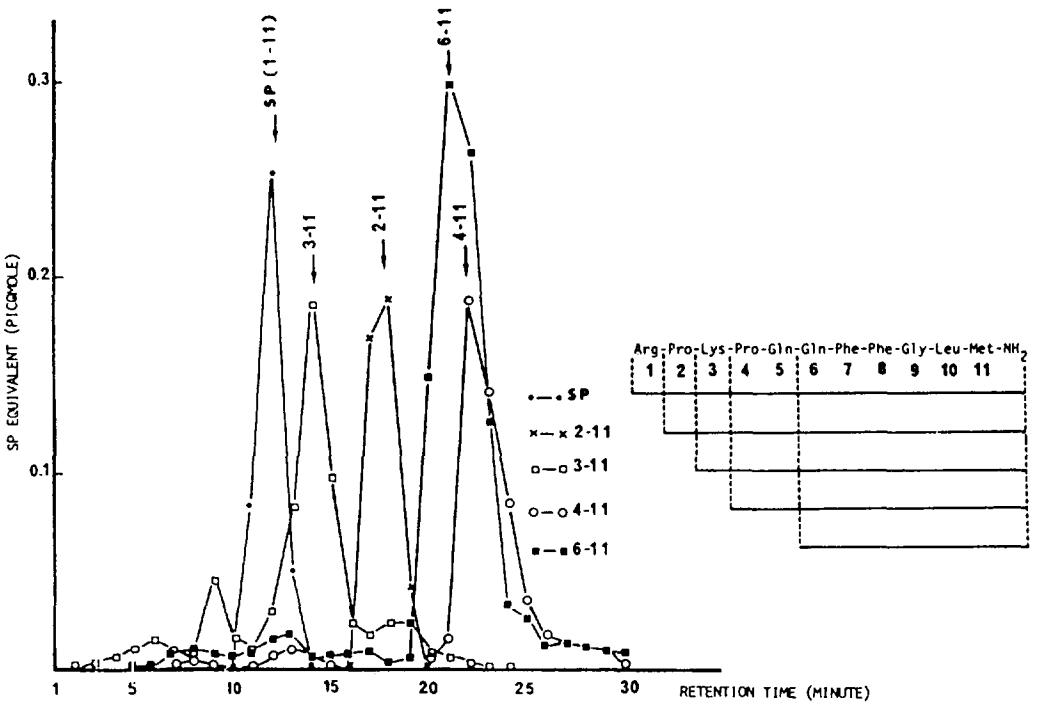

Fig. 6. Dissociation by a combined HPLC and RIA method of substance P and various related fragments. In brief the HPLC characteristics were as follows: packing μBondapak C_{18}; column, 30 cm × 3.9 mm I.D.; solvent, methanol–water–phosphoric acid (500:500:1, v/v); flow-rate, 0.5 ml/min; detection by RIA. (From Ref. [99] with permission).

the more hydrophobic C-terminal but only weakly retain the N-terminal fragments) are used [49,54].

In general, the whole family of SP-related peptides elutes in the range of their hydrophobicity at a given pH value when e.g. TFA, TEAP and inorganic buffers are added to the mobile phase, which is accordance with the investigations of Meek and Rosetti [73]. An increase of hydrophobicity of the peptides in the range $SP_{1-11} < SP_{2-11} < SP_{3-11} < SP_{4-11} < SP_{5-11} < SP_{6-11}$ due to the relative preponderance of more non-polar (aromatic) amino acids versus the hydrophilic residues located in the N-terminal region and, as a consequence, a concomitant shift to higher retention times is observed on reversed-phase materials. So far the elution range can be predicted; the most important problem remains to exploit the mutual differences in hydrophobicities of a pair of peptides differing in only one amino acid (e.g. SP_{1-11} and SP_{2-11}) by use of an efficient chromatographic system.

Besides RP-HPLC on a C_{18} matrix Kream et al. [115] applied chromatography on a Partisil SCX cation-exchanger with a gradient from 0.02 KH_2PO_4–0.05 *M* KCl to 0.2 *M* KH_2PO_4–0.5 *M* KCl in 25% acetonitrile containing 0.1% TFA.

An additional and efficient tool to influence retention in particular of basic components, is given by formation of hydrophobic ion-pairs between analyte and a suitable ion-pair reagent carrying a more or less extended alkyl chain in order to control hydrophobicity in a selective manner. Despite the fact that the more hydrophilic counter-ions, including TFA, triethylammonium salts, inorganic buffer systems etc., which, in the proper sense, are also ion-pairing agents, the term ion-pairing agent will be restricted in the following discussion to the strongly acidic alkylsulfonic acids of different chain length.

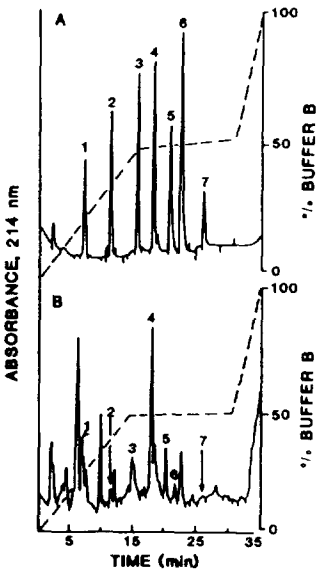

Fig. 7. (A and B) HPLC elution profiles of standard [0.5 μg synthetic peptide mixture per ml (150 μl injected)] SP_{1-11} and some of its metabolic fragments and spinal cord extracts, respectively, on Ultrasphere ODS column (150 × 4.6 mm, 5 μm particle size) at 35°C with buffer A as 50 m*M* $NaH_2PO_4 \cdot H_2O$, 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}. (From Ref. [49] with permission).

Spindel et al. [43] used 0.1% pentane sulfonic acid (PSA) in 35% aqueous acetonitrile containing 20 m*M* acetic acid for optimum separation of the undecapeptide from its analogues. Fransson [105] investigated separation of SP_{1-11}, SP_{2-11}, SP_{3-11}, SP_{4-11}, SP_{1-11} and SP-COOH on different RP materials (C_{18}, C_8, C_6, CN) with either ethanol or acetonitrile as organic modifier and sulfonic acid based ion-pairing agents markedly differing in the length of the alkyl chain (ethane sulfonic acid, 1-propane sulfonic acid, 1-butane sulfonic acid, 1-pentane sulfonic acid). His investigations revealed that retention on the C_{18}, C_8 and C_6 matrices was nearly identical when the same ion-pairing agent was used, which may be indicative for similar interactive surfaces with the peptides. Only in the case of more hydrophobic sulfonic acids a slight increase in retention was observed in the range $C_6 < C_8 < C_{18}$. In contrast to these findings only low values for the capacity factor k′ were found with the CN column. Virtually no differences in chromatographic efficiency were observed between ethanol and acetonitrile as organic component, but satisfactory results will only be achieved with end-capped column materials irrespective if the protic (in general more effective in masking residual silanols) or the aprotic modifier is used. Furthermore, it is not unexpected that the elution range of the SP fragments containing more basic amino acid moieties changes compared with e.g. phosphate buffer systems. The reason for this behaviour is that the basic sites are preferably attacked by the ion-pairing agent, and now exhibit substantially higher retention than the fragments lacking basic residues. Retention times of the latter remain almost unchanged and they still elute in accordance to their inherent hydrophobicity. The effect will of course be most prominent with extremely hydrophilic peptides [127] like SP_{1-4}, SP_{1-7} etc. containing two sites for ion-pairing (Arg^1 and Lys^3) within a short chain, for which acceptable retention is otherwise only achievable with a gradient starting with 100% of aqueous buffer (see Refs. [49,54]). Table 9 exhibits the effect of sulphonate addition to the eluent system compared with the non-sulphonate containing mobile phase. Recently Contreras Martinez and Vera-Avila [129] reported on their investigations of ion-pair RP-HPLC for the separation of small and hydro-

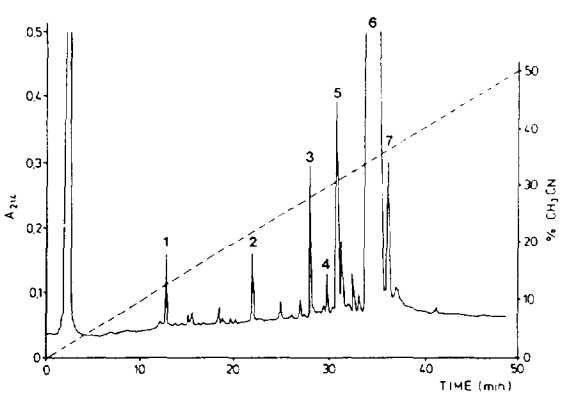

Fig. 8. Reversed-phase HPLC of the metabolites of substance P formed during a 3-min incubation of substance P with basolateral membranes. (- - -) gives the concentration of acetonitrile in the eluting solvent. Peaks 1–7 are identified in Table 8. (From Ref. [112] with permission).

Table 8
Identification by amino acid composition of the metabolites of substance P formed during incubations with basolateral membranes of pig small intestine

Chromatography peak	Fragment	Amino acid residue							
		Glx	Pro	Gly	Met	Leu	Phe	Lys	Arg
1	1–6	2.08	2.07	–	–	–	–	0.92	0.94
2	1–7	1.99	2.24	–	–	–	0.96	0.91	0.90
3	1–9	2.04	2.27	0.99	–	–	1.88	0.91	0.92
4	8–11	–	–	1.13	0.72	1.07	1.07	–	–
5	$1–11_{ox}$	2.13	1.01	1.01	–	0.97	1.98	0.90	0.86
6	1–11	2.22	1.03	1.03	0.81	0.98	1.97	0.97	0.99
7	7–11	–	1.12	1.12	0.64	1.09	2.16	–	–

(From Ref. [112] with permission.)

philic peptides (e.g. TRH and metabolites) by using sodium dodecyl sulphate and different buffer systems. However, despite its advantage in increasing retention of hydrophilic peptide moieties it should nevertheless be emphasised that removal of ion-pairing agent from the analyte is often very difficult, and can be associated in turn with severe problems when complete removal of the solvent system is required, such as e.g. in HPLC-RIA coupling experiments [43,57].

Finally an essential point in HPLC of biological samples is that the solvent composition for either analyte or eluent should be identical whenever possible, in order to reduce the probability of peak broadening and distortion due to solvent–solvent and analyte–solvent incompatibility. In the case of gradient elution the analyte should be dissolved in the solvent used at the starting conditions of the chromatography.

3.2.3. Recovery of SPLI from RP-HPLC columns

An almost complete recovery of SPLI after chromatographic separation is a fundamental prerequisite for the detection in the lower fmol range and requires careful pretreatment of the chromatographic column. Thus the problem is most crucial for detection of peptides in CSF, whereas it is not so critical (if at all) for tissues bearing high concentrations of SPLI. With the use of a non-prepared column matrix, often several injections of a biological sample are necessary before the endogenous amount of the

Table 9
Separation of substance P and four closely related derivatives by reversed-phase ion-pair chromatography with and without *n*-butanesulphonic acid present in the mobile phase using ethanol as organic modifier

Peptide	With sulphonate			Without sulphonate		
	H	k'	α	H	k'	α
SP	0.05	22.0	1.47	0.12	6.9	1.00
SP_{2-11}	0.04	18.7	1.25	0.09	9.3	1.35
SP_{3-11}	0.04	17.7	1.19	0.07	7.9	1.15
SP_{4-11}	0.04	14.9	1.00	0.07	10.2	1.49
SP-acid	0.05	25.1	1.68	0.10	8.7	1.26

SP = substance P; α = selectivity ratio.
(From Ref. [105] with permission.)

peptide of interest can be detected. Therefore the silanol-coating efficiency of the buffer ingredients even if they contain basic alkyl substituted ammonium counter ions is not sufficient for a complete blockade of all active sites on the HPLC columns in many cases at least at analyte concentrations in the fmol range.

Different approaches have been used to suppress unwanted adsorption of peptide material onto the column matrix, silanophilic interactions being the most prominent.

Liu and Desiderio [116] have undertaken extensive studies of the recovery of met-enkephalin (ME) from an RP-HPLC column. They performed three injections of pmol amounts of ME and measured an average recovery by radioreceptor assay (RRA) of only 34%. In contrast recovery of ME increased to 81% after repeated injection of nmol amounts of ME. Then the column was rinsed twice with the gradient but without sample in order to avoid memory effects and RRA showed only baseline activity. After the two washes, pmol amounts of ME again were injected and recovery measured, which then exhibited 67% yield of peptide. This study clearly demonstrates the need to saturate the active sites on the HPLC column with the target peptide. Henning et al. [55] and Wallasch et al. [56] supplemented eluent B of their gradient system with 350 pmol and 365 pmol of the basic amino acids lysine and arginine, respectively (which are also found at positions 1 and 3 in SP_{1-11}) to block the active centers on the column matrix.

Another problem is the possible observation of memory effects, in particular when a biological sample is injected directly after injection of a set of column calibrators, which are usually applied in nmol amounts when UV-calibration is used. In order to prevent any memory effects from either column calibration or a previously injected sample, a blank gradient without sample should be carried out followed by measurement of the baseline activity by RRA or RIA as performed by Zhu and Desiderio [40], Harajiri et al. [33], Rissler and Cramer [128], or an extensive column rinse with pure acetonitrile after the peptide material has been eluted as used by Kusmierz and Desiderio [31].

4. Detection and measurement of substance P

Whereas chromatographic separation of all fragments of SP is nowadays achievable with high efficiency by RP-HPLC using gradient as well as isocratic techniques and numerous buffer systems and mobile phase additives like acids and ion-pairing reagents (see section 3.2.2 above), on-line detection of the complex biological analytes, which often contain the members of the peptide family in only pmol or fmol amounts, still presents the main challenge for the analyst. Tremendous efforts have to be made in the future to achieve this goal, because at present, no on-line technique is available for detection of extremely low concentrations of biomolecules in the fmol range.

The following chapter gives a small survey of detection techniques for measurement of substance P and related peptides starting with the relatively insensitive UV detection and terminating with the extremely sensitive RIA procedure. Most attention is paid to HPLC–RIA coupling because up to now it still represents the most sensitive method to achieve detection in the fmol range.

4.1. Methods for determination of substance P

4.1.1. UV detection

Detection sensitivity at 210 nm (characteristic for the peptide bond) can be considered sufficient during the course of enzymatic degradation studies in tissues and body fluids, where nmol amounts of sample are usually applied and peak intensities of the released fragments exceed by far those invoked by the matrix. In general, pure peptides outside a biological environment can be measured down to the lower pmol range. Nevertheless, when embedded in a biological matrix, the limit of detection may increase about one or two orders of magnitude.

Monitoring of signal responses is further important for column calibration with synthetic samples of the peptides, which have to be detected in the biological matrix. This measure allows a precise assignment of retention times to individual peptide species and thus facilitates excision of the appropriate fractions out of the

chromatogram, e.g. for subsequent RIA investigations (see section 4.1.6). However, column calibration can only be done at 210–220 nm with buffers like TEAP, TFA and inorganic phosphates in aqueous acetonitrile, whereas TEAF, TEAA etc. and alcoholic modifiers require detection at 280 nm. This in turn is achievable only when substantial amounts of aromatic amino acids are present within the peptide sequence.

4.1.2. Fluorescence detection

As may be concluded from the relatively low sensitivity of UV detection, more selective and/or sensitive methods, such as fluorescence and electrochemical (ED) detection are required. However, due to the lack of either fluorescent or sufficiently fluorescent moieties in the native structure of peptides and proteins, derivatisation of the peptide substrates with so-called fluorogenic agents is necessary. Usually the drawbacks of this variant are that in most cases more than one reaction site is found in the peptide molecule, which often leads either to a multitude of reaction products or to decreases in the fluorescence response as a consequence of quenching effects invoked by introduction of more than one fluorogen into the same peptide.

Selective fluorescence-labelling of lysine-containing peptides, thus including SP, by derivatisation with either fluorescamine [130] and naphthalene-2,3-dicarboxaldehyde/cyanide [131] prior to HPLC with limits of detection in the lower pmol range have been applied. Furthermore, post-column on-line derivatisation of arginine-containing peptides to fluorescent derivatives with either ninhydrin [132] or benzoin [45] at the guanidine side-chain was reported. Another attractive alternative way to lower the detection limit of peptides is given by Engelhardt et al. [133]. Their method involves microwave-induced post-column alkaline hydrolysis in a special reactor coil and subsequent derivatisation with *o*-phthaldialdehyde (OPA) and fluorescence detection. Addition of single responses from the individual amino acids leads to signal amplification and increases in sensitivity. This method circumvents the relative instability often observed for OPA derivatives by pre-column derivatisation. Improvement of fluorescence responses and a concomitant lowering of the detection limits as reported for the ultrasensitive determination of primary amines [134] after reaction with 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde may be exploited for peptides in the post-column on-line technique involving hydrolytic cleavage according to Ref. [133].

A further decrease in detection limits of the pure peptide components down to the low fmol range can be achieved by measurement of laser induced fluorescence (LIF) of Pro-containing small peptides after derivatisation with 4-(N,N-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) as reported by Toyo'oka et al. [135]. In contrast, the sensitivity of detection of the DBD-derivatives with conventional fluorescence using a xenon lamp is limited to the subpmol level. Additionally selectivity is improved by the preferable attack of secondary amino and thiol groups, whereas the reaction rate of primary amino groups is substantially lower and that of hydroxy functions can be neglected. The method may thus be exploited for the determination of all fragments of SP containing Pro within their sequence.

Nevertheless, there still remains the principal drawback of all labels because a vast number of components within the biological matrix is simultaneously derivatised and thus can strongly interfere in the subsequent chromatographic separation procedure yielding both reduced selectivity and sensitivity.

4.1.3. Electrochemical detection (ED)

In contrast to the monitoring of fluorescence, measurement of responses from electrochemical oxidation/reduction would provide a more specific means of detection, because peptides often contain amino acids with a variety of functional groups which are susceptible to facile oxidation/reduction (e.g. tyrosine, tryptophan, methionine residues and disulfide linkages). Additionally the conditions of the oxidation/reduction can be carefully adjusted to the specific potential of the functional group in the peptide, so the peptides of particular interest are filtered out from the vast excess of matrix interferences. The methionine moiety of SP may at first sight

serve as a typical example of attack for ED with a low oxidation potential. However, the method cannot be applied for measurement of the N-terminal fragments of SP. On the other hand substantial amounts of oxidised peptide can be present in the biological matrix. In order to measure its concentrations the sample has first to be reduced and subsequently oxidised and the sum of both individual responses (due to different retention times) at the positive potential yields the total content of the corresponding SP species. Nevertheless, for a clear assignment of signals the individual retention times of the native peptides and their sulfoxides must be known.

A powerful electrochemical detection system was described by Cummings et al. [39] for the determination of two SP-derivatives containing Trp and used as growth-hormone antagonists. An increase of sensitivity was achieved by use of a cleaning potential (0.3 V) before the measuring cell, at which impurities arising from mobile phase and biological matrix but only negligible amounts of peptide are removed. Subsequent oxidation at a higher potential (0.7 V), which is sufficient for complete conversion of the Trp moiety then yields the full peptide response. Detection limits were within 4 and 8 ng/ml of plasma and 40 and 80 ng/g of tissue, respectively.

A survey of the determination of peptides and proteins by coupling of HPLC with ED including labelling of the substrates with easily oxidisable and reducible groups is reported by Dou et al. [136]. Another attractive alternative of ED was developed by Dou and Krull [137]. They observed a marked increase in either sensitivity (down to lower pmol range) or specificity after post-column irradiation of peptides and amino acids containing sulfur or aromatic moieties such as Met, Cys, Phe, Tyr and Trp in a UV reactor and subsequent ED of the photolysis products. Unfortunately the method presumably lacks a comparable detection sensitivity for N-terminal fragments of SP than would be expected for the C-terminal moieties due to the absence of amino acids that are susceptible to photolytic decomposition and subsequent electrochemical detection.

4.1.4. Nitrogen specific detection (CLND)

Some years ago Robbat Jr. et al. [138] and Fujinari and Courthaudon [139] described a detection principle derived from gas chromatography, which is based on chemiluminescence invoked by nitrogen-containing compounds and called chemiluminescence nitrogen specific detection (CLND). After sample separation the column effluent undergoes high temperature oxidation at about 1000°C and all chemically bound nitrogen components, except the N_2 molecule are converted to the nitrosyl radicals. In a reaction chamber NO· is mixed with ozone and nitrogen dioxide in the excited state (NO_2^*) is formed. As the NO_2^* molecule reaches the stabile ground state, light ($h\nu$) is emitted and detected by a photomultiplier tube. In a recent paper Fujinari and Manes [140] reported the use of CLND for the separation of small peptides and achieved a detection limit of 0.4 ng nitrogen (signal-to-noise ratio of 2:1), which would correspond to about 3 ng of substance P undecapeptide. The most important advantage of CLND is that all sample constituents in a biological matrix lacking nitrogen are invisible and do not contribute to the signal response. This fact renders CLND an excellent method for detection of biologically active components, which preponderantly consist of nitrogen-containing species. Nevertheless, due to the large amounts of such compounds in the highly complex biological matrices a thorough sample clean-up and pretreatment will be indispensable (see chapter 2 above). According to Refs. [139] and [140] limitation of the organic part of the mobile phase to nitrogen-free solvents is required and methanol is recommended as the organic modifier of choice. In contrast Robbat et al. [138] were able to use acetonitrile as organic eluent component (presumably due to an efficient removal of the organic solvent by a freezing-out process before generation of nitrosyl radicals) and found that the detection limit of CLND was the lower the higher the percentage of acetonitrile in the eluent mixture. Owing to this unique specificity, which further includes a sensitive measurement of peptides it is somewhat surprising that only a few reports deal with this promising technique.

The reason may eventually be found in the fact that sensitivity and reliability of available detectors has not yet reached an advanced and satisfactory state of development at present. Despite its relatively low detection limit it should be kept in mind that the method cannot be applied to the determination of SPLI in biological fluids but nevertheless should be applicable to the detection of SPLI in tissue samples.

4.1.5. Mass spectrometric detection (MS)

Great progress in on-line HPLC mass spectrometric (HPLC–MS) methods has been made during the last decade. At present the more common interface techniques to remove the vast excess of solvent from the analyte include moving belt [141], thermospray [142], continuous-flow fast-atom bombardment (FAB) [143] and particle beam [144]. The HPLC continuous-flow FAB-MS technique offers the advantage of higher mass capabilities (up to approx. 10 000 Da) and a gentle ionisation process, which preserves molecular information. Other soft ionisation methods are matrix assisted laser desorption ionisation (MALDI) [145] and ion evaporation ionisation via the electrospray method [146]. The latter technique as well as interfacing a liquid chromatograph with a time-of-flight mass spectrometer (TOF-MS) [147] are the methods of choice for the measurement of molecular masses >10 000 Da as required e.g. for precursors of SP. However, as a consequence of soft ionisation no structural information (i.e. the amino acid sequence) is obtained from these mass spectra, which is only available if extra internal energy is added to the ions, such as application of collision-induced dissociation (CID) or tandem mass spectrometry (MS–MS) [148,149]. This technique requires a selected amino acid sequence-determining fragment ion in the second mass spectrum of the peptide. Quantitative measurement is then easily achievable with a stable isotope-labelled peptide internal standard (e.g. by replacement of ^{1}H by ^{2}H or ^{16}O by ^{18}O at positions, which are inert against isotope exchange with the environment) added to the biological matrix before the sample extraction procedure (see Fig. 2).

Due to the huge excess of solvent, which precludes monitoring of mass spectrometric responses when HPLC is operated e.g. with 4.6-mm diameter columns, post-column flow-splitting (depending on the column dimensions and flow-rates and generally in the ratio of 1:100 to 1:1000) has to be applied. Alternatively micro-column and capillary HPLC (with or without flow-splitting) and concomitant signal monitoring by UV as well as MS also could be used. Despite the gain in sensitivity in particular with short and small internal diameter capillary columns (e.g. I.D. < 500 μm) this technique has limited application up to now and much effort is required to demonstrate ease of down-scaling to separation conditions developed by using conventional 4.6-mm or 1-mm internal diameter columns.

4.1.6. Detection by radioreceptor assay (RRA)

The RRA is based on the affinity of membrane receptors (which replace the antibody used in RIA, see section 4.1.7) for specific ligands. The careful choice of the radioligands competing for the binding sites with the corresponding unlabelled analogue in the sample determines which peptide or peptide family will be detected. RRA provides a semi-quantitative method because the measured values refer only to the displacement of the standard peptide (used for the calibration curve) from the receptor protein by the receptor-active component of the biological sample or fraction obtained by prior HPLC separation. Thus results are expressed in equivalents of the standard peptide. Only when the relative affinities of all peptides or peptide fragments relative to the standard are known, a more or less quantitative determination can be achieved. In general, despite its higher specificity the sensitivity of the RRA compared to the RIA procedure is ca. three orders of magnitude lower (pmol versus fmol amounts in the RIA) and thus approximates a similar concentration range as HPLC–MS [51,116]. It is further important to exclude any enzymatic degradation of either endogenous peptide material or exogenously added standards during the course of incubation by addition of an appropriate cocktail of enzyme

inhibitors and careful adjustment of experimental conditions.

4.1.7. Detection by radioimmunoassay (RIA)

The levels of neuropeptides in tissues and body fluids are often insufficiently low for their measurement by HPLC–MS or RRA, which in contrast, can be achieved with the RIA technique. Until highly efficient chromatographic separation was established by the development of HPLC in the seventies, RIA was the only method for the direct measurement of fmol amounts of peptides in biological sources without further pre-purification. However, despite the use of specific antibodies raised against the peptide or component of interest, the question arises whether the closely related peptides of the tachykinin family, which exhibit a marked degree of structural similarity show more or less cross-reactions with the antibody, by which molecular specificity is substantially decreased. Nevertheless, C-terminal fragments of different chain length would possibly be measurable at the same response ratio. Additionally substantial cross-reactions of the N-terminally extended forms containing the whole C-terminal region of SP_{1-11} with an antibody raised against the C-terminus of SP_{1-11} will be expected. Thus a highly sensitive measurement of a variety of peptide species on a molar basis will be achievable, which all derive from the undecapeptide by N-terminal extension and, as a consequence, a specific antibody for each member of SP-related peptides is not an ultimate prerequisite. However, when a higher degree of molecular specificity, especially for all peptides of the SP family is required, antibodies with different epitopes, i.e. raised against different structural sites of the peptide should be prepared. In practice this will be associated with extremely high costs for antibody production and thus unattractive. As stated above (section 1.4), SP splits into a hydrophilic N-terminal region containing the basic amino acids Arg^1, Lys^3 and a hydrophobic C-terminal part. Therefore, generation of specific antibodies against the N-terminal region [5,8,114,117,122] allows detection of e.g. SP_{1-4} and SP_{1-7}, which are not detectable with C-terminally directed antibodies, whereas the latter include detection of N-terminally extended species of SP, i.e. the whole amount of substance P-like immunoreactivity (SPLI)[2] within the biological matrix.

Despite the obviously highly sensitive measurement of SPLI there still remains some ambiguity in regard to hitherto unknown peptide material in a matrix, which may interfere in the RIA and thus can give rise to falsely high levels of the compound of interest. Thus insertion of a pre-chromatographic purification step and measurement of SPLI in distinct fractions after subsequent HPLC with either an N- or C-terminally directed antiserum permits a further gain in specificity by discrimination between N- and C-terminal fragments.

An important aspect of the measurement of SP levels by RIA is the possible oxidation of fragments containing the Met residue in their sequence. Although most of the antisera prepared against SP are also able to detect the corresponding sulfoxides, it should be considered that the cross-reactivities of the oxidation products are often substantially lower than those for the parent peptides and the measured values may be falsely low and do not reflect the true situation. As described above (see section 2.2.2), it would thus be advisable to use mmol or submmol amounts of a reducing agent [34] to stabilise the sample ingredients against unwanted oxidation. On the one hand this procedure may be feasible for SP-related peptides but, on the other hand, peptides containing disulfide bridges are extensively reduced to their sulfhydryl analogues, which often exhibit either decreased binding affinity towards an antibody or completely lack immunoreactivity. A detailed description of a reliable experimental procedure for RIA is given in Ref. [128].

Nowadays RIA is almost exclusively used after prior separation of biological extracts by HPLC, i.e. the so-called HPLC–RIA coupling procedure [5,7,8,28,30,33,38,40–44,46,47,49,55,56,

[2] The term "SPLI" takes into consideration that no amino acid sequence data are available.

59–61,64,99,114,115,117,118,121–126,128]. The first application of this technique for identification of SPLI in brain tissue was published in 1979 by Ben-Ari et al. [99]. Although excellent sensitivities down to the lower fmol range are obtained with HPLC–RIA coupling, the method suffers from the drawback that it is a typically time-consuming off-line sample preparation procedure, because the fractions obtained from the HPLC separation have to be evaporated to dryness and reconstituted in an appropriate buffer system for subsequent RIA. HPLC is a highly reproducible technique and, in general, only small changes in retention times of the calibrators are observed within a multitude of chromatographic runs. Thus for assignment of SPLI measured in individual fractions to distinct peptides or fragments on the basis of the retention times, it is sufficient to calibrate the column with the corresponding pure peptide standards only before each series of samples in order to assure the long-time reproducibility of reference-peptide retention values over the entire time range of separation when large numbers of samples are to be worked up.

For quantitative determinations of SPLI a calibration curve by use of a reference standard is required (usually the SP undecapeptide). However, this method yields only semi-quantitative results, unless the cross-reactions of the whole entity of SP-like peptides relative to the reference standard are known. This can be overcome by introduction of an individual correction factor for each peptide, which considers the differences in the cross-reactivities relative to the standard on a molar basis and thus permits more or less quantitative determination.

With respect to molecular specificity the HPLC–MS coupling method is substantially superior to the HPLC–RIA procedure, although an efficient separation step prior to RIA essentially improves molecular specificity of the latter. Despite this drawback, the HPLC–RIA procedure still remains the method of choice for the quantitation of small amounts of peptide material in a biological environment due to its otherwise unattainable sensitivity. This unique ability of the RIA permits detection of SPLI even when a total amount of SPLI in the lower fmol range is separated into multiple fractions by prior HPLC.

An important step to obtain optimal sensitivity consists in the use of volatile buffer systems (e.g. TFA, TEAF, TEAA as mobile phase additives), which, after evaporation retain the analyte without contaminations attributable to non-volatile mobile phase ingredients. The latter may interfere with the subsequent RIA and thus markedly impair sensitivity.

4.1.8. Heterogeneity of SPLI in biological samples

As can be reasonably expected from the presence of various SP-degrading enzymes in tissues and body fluids, marked heterogeneity of peptides related to the sequence of the undecapeptide was observed in biological matrices. SP_{1-11} is rapidly eliminated from brain tissue [24,25,150–153] and circulation [103,154,155], whereas in contrast, the concentration of enzymatic activity in CSF samples [28–32] seems to be too low to yield substantial amounts of degradation products. Thus fragments of the undecapeptide found in this compartment may be attributed to degradation in adjacent brain regions and subsequent secernation into the CSF. Nevertheless it cannot be excluded that at least minor amounts of SP fragments in the CSF may be attributable to the release from SP precursors in the living organism at the incubation conditions of body temperature by proteolytic cleavage as simulated by in-vitro experiments [33].

The investigations of Jessop et al. [121] in rat median eminence and paraventricular nucleus and of Ben-Ari et al. [99] in striatonigral and amygdaloid nuclei revealed that SPLI was solely present in the non-oxidised form of SP_{1-11}, whereas Spindel et al. [43] additionally discovered small quantities of the corresponding sulfoxide in rat striata. In contrast, Sakurada et al. [42] found SPLI co-eluting with synthetic SP_{1-11}, SP_{5-11} and SP_{1-7} in rat hypothalamus and spinal cord tissue and Igwe et al. [49] extracted SPLI co-eluting with SP_{1-11}, SP_{2-11} and SP_{5-11} from the spinal cord of mice. Le Grevés et al. [41] discovered SPLI attributable to synthetic SP_{1-11}

and SP_{1-7} in human hypothalamus and substantia nigra.

The existence of SPLI attributable to precursor forms of SP was demonstrated in human brain tissue by Nyberg et al. [114] and in hamster brain stem and spinal cord by Kream et al. [115]. Further, upon stimulation of superfused rat spinal cord slices with K^+ ions (50 m*M*) Toresson et al. [122] observed release of N-terminally extended SP as well as the undecapeptide itself.

In human CSF Toresson et al. [117] discovered an N-terminally extended form of SP_{1-11} but no immunoreactive material could be attributed to authentic SP_{1-11}. These results contrast markedly with the findings of Higa et al. [59] who found SPLI co-eluting with authentic SP_{1-4}, SP_{1-11}-sulfoxide, SP_{1-11}, SP_{2-11}, SP_{4-11}, SP_{5-11}, $pGluSP_{5-11}$, β-preprotachykinin$_{58-70}$ ($\cong SP_{1-11}$-Gly-Lys) in the CSF of patients suffering from lower back pain, Rösler et al. [118], Jost et al. [123] and Cramer et al. [124], who all discovered SPLI co-eluting with SP_{1-11}, SP_{2-11}, SP_{4-11}, SP_{5-11}, and the sulfoxide forms of SP_{5-11} and SP_{1-11} in the CSF of patients suffering from various neurological disease. In Figs. 9–11 the elution profiles of SPLI obtained from CSF of patients with different neurological diseases are shown.

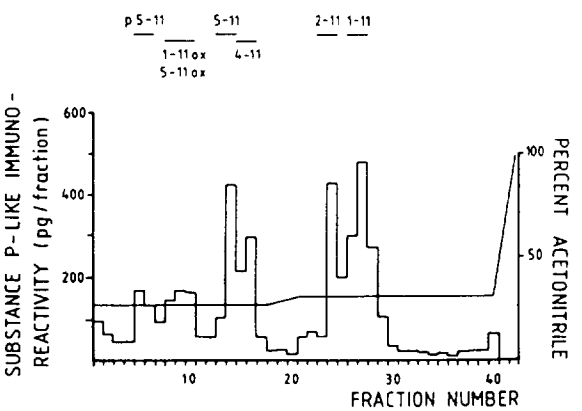

Fig. 9. HPLC separation of substance P-like immunoreactivity in CSF of patients with MS. Mean values of 3 pooles of 2–3 patients each. Fraction volume 1 ml. Horizontal bars indicate the elution volumes of authentic SP_{1-11}, SP_{2-11}, SP_{4-11}, SP_{5-11}, SP_{1-11} OX, SP_{5-11} OX and pyroglutamyl SP_{5-11} (p_{5-11}). (From Ref. [118] with permission).

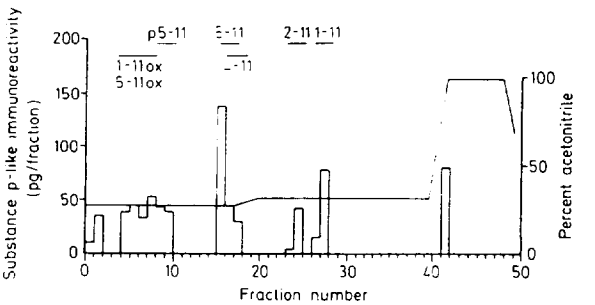

Fig. 10. HPLC of cerebrospinal fluid (CSF) coupled with radioimmunoassay of substance P-like immuno-reactivity. Pools of CSF from two or three patients with painful radiculopathy were lyophilized, resuspended in elution buffer and chromatographed. Mean values of three different pools. Horizontal bars indicate elution volumes of synthetic SP_{1-11}, SP_{2-11}, SP_{4-11}, SP_{5-11}, pyroglutamyl-SP_{5-11}, sulphoxidated SP_{1-11} and SP_{5-11}. (From Ref. [123] with permission).

5. Preparation of radiolabelled analogues of SP

This chapter addresses one-step iodination procedures via methods which assure the rapid incorporation of ^{125}I directly into biologically active peptides and which, in general, do not require expensive laboratory equipment. In contrast, preparation of tritiated SP is very often characterised by long-lasting and tedious synthetic ad initio procedures, which only can be carried out in laboratories with special and, for this reason, expensive equipment. Additionally, the relatively low specific activities limit application of tritiated peptides to the determination of SPLI concentrations in the lower fmol range, providing a further argument for the use of radio-iodinated derivatives exhibiting substantially higher specific activity.

5.1. Methods for incorporation of ^{125}I

An overview of the preponderantly used methods for radio-iodination of SP-related peptides is given, which also considers the advantages as

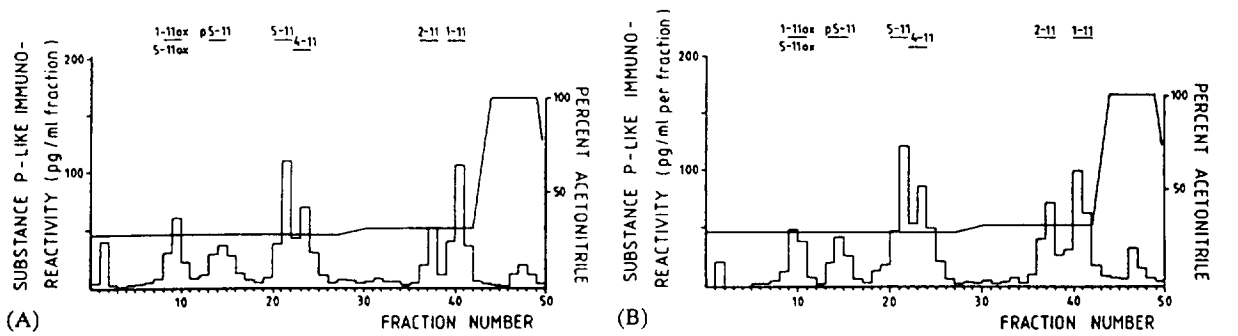

Fig. 11. HPLC of substance P-like immunoreactivity in ventricular CSF of patients with PD. Horizontal bars indicate the elution volumes of authentic SP_{1-11}, SP_{2-11}, SP_{4-11}, SP_{5-11}, pyroglutamyl-SP_{5-11} and SP_{1-11} OX. (A) CSF from lateral ventricle, means of nine patients, calculated for each fraction. (B) CSF from foramen Monro, means of four patients. (From Ref. [124] with permission).

well as the drawbacks of the different alternatives.

5.1.1. Labelling with the chloramine-T method

This technique, introduced in 1963 by Greenwood et al. [156] for the labelling of human growth hormone with ^{131}I, is still the method most often used for incorporation of radioactive isotopes of iodine into peptides. The great advantage of this procedure is that the reaction takes place instantaneously and generates peptides with high specific activity. However, it requires the presence of a tyrosine residue within the peptide sequence. Thus for peptides lacking a Tyr moiety, it may be reasonable to replace a Phe moiety by Tyr, as in the case of [Tyr^8]-SP. For preparation of the iodinated SP derivative an alkaline solution of carrier-free $Na^{125}I$ is oxidised by chloramine-T prior to addition of the peptide. Then the peptide is added and the molecular iodine formed is immediately trapped by the Tyr moiety yielding the mono- as well as the di-iodo-derivative at the ortho positions of the hydroxy group. An excess of peptide versus ^{125}I has to be applied because incorporation of a second iodine exhibits a higher velocity than mono-substitution [157]. An excellent incorporation yield of ca. 80% of radioactive iodine was achieved with this method [125]. The reaction is usually stopped after 20–30 s by addition of sodium metadisulfite or bovine serum albumin (BSA) to reduce excessive molecular iodine to the corresponding ions or to trap it by incorporation into Tyr moieties of the protein. For SP and derivatives both variants are applicable. Nevertheless it should be taken into account that disulfide linkages can be reduced to sulfhydryl groups in the presence of sodium metadisulfite, as for e.g. somatostatin, oxytocin, vasopressin and insulin etc.

In SP the Met^{11} residue is the most susceptible site for oxidation to the corresponding sulfoxide. Michelot et al. [158] ascribed the markedly reduced biological activity of ^{125}I-[Tyr^8]-SP prepared by the chloramine-T method to a presumptive formation of the Met^{11}-sulfoxide and Rissler and Cramer [125] provided evidence for complete oxidation of Met^{11} during radio-iodination of [Tyr^8]-SP with ^{125}I. Oxidation of Met residues of peptides under the conditions of radio-iodination with chloramine-T was reported in a multitude of papers [159–168]. Figs. 12 and 13 show the elution profiles of mercaptoethanol-treated chloramine-T/^{125}I-labelled [Tyr^8]-SP and its analogue after reoxidation with H_2O_2.

In contrast to the formation of the peptide sulfoxides further oxidation to the corresponding sulfones will take place very slowly and thus requires more drastic reaction conditions, such as the use of e.g. performic acid [169]. In most cases the labelled sulfoxides of SP can be used

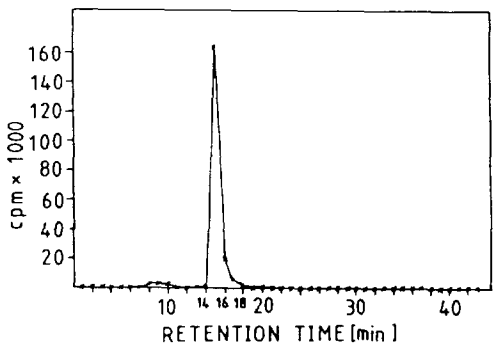

Fig. 12. RP-HPLC profile measured on a Spherisorb ODS II (125 × 4.6 mm I.D., 5 μm particle size) column of ^{125}I-labelled [Tyr^{8}]-SP (after reduction with mercaptoethanol). (From Ref. [125] with permission).

for RIA due to the sufficient cross-reactivity of the tracer towards the antibody, whereas receptor-binding is often completely abolished, as observed in our laboratory (unpublished results). For this reason it may be advisable to reduce the Met-sulfoxide to its native structure in order to achieve an extensive reconstitution of biological activity as well as receptor-binding.

Reduction of the sulfoxidised species can be successfully performed with thioglycolic acid or thioglycol, whereas catalytic hydrogenation in the presence of Pd or reaction with sodium meta disulfite or sodium dithionite yields unsatisfactory results [170]. Mercaptoethanol in sodium acetate buffer of pH 4 was used by Rissler and Cramer [125] and 92% yield of true radiolabel

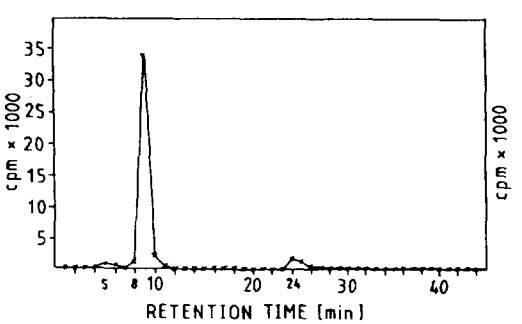

Fig. 13. RP-HPLC profile measured on a Spherisorb ODS II (125 × 4.5 mm I.D., 5 μm particle size) column of mercaptoethanol-reduced [^{125}I]-[Tyr^{8}]-SP (see Fig. 13) after subsequent reoxidation with hydrogen peroxide. (From Ref. [125] with permission).

was obtained as checked by RP-HPLC. This high recovery of the native tracer suggests that further oxidation to the sulfone is negligible because the reduction capability of thiol components for sulfone derivatives is not sufficient to obtain the thioether. In order to circumvent Met oxidation during radio-iodination Bienert et al. [171] synthesised the S-*tert.*-butyl sulfonium derivatives of Met-containing peptides, which could be easily deprotected by warming up the solution to 60°C for 2 h. Another attractive alternative, replacement of the Met^{11} residue by a Norleu moiety, was reported by Kerdelhue et al. [172] and 80% of the receptor-binding affinity observed with the corresponding labelled Bolton–Hunter-SP was achieved. However, this SP-derivative is not yet commercially available.

In contrast to the tritiated analogues substantial alterations in their secondary structure due to conformational changes may be expected for the iodinated peptides, which is attributable to the incorporation of the voluminous iodine substituent into the C-terminal tachykinin moiety of SP (at position Tyr^{8}). For this reason, even after reduction of the radio-iodinated (sulfoxidised) peptide to the native thioether derivative, there still remains the possible influence of the extended ^{125}I-[Tyr^{8}]-residue on the conformation and thus biological and receptor-binding activities. Surprisingly no substantial differences between receptor-binding of this label and its Bolton–Hunter derivative, where the iodine residue resides in the N-terminal region of the molecule, were observed (unpublished observations of our laboratory).

5.1.2. Labelling with Bolton–Hunter reagent

The so-called Bolton–Hunter derivatives of peptides [173], which do not contain an inherent Tyr moiety, are prepared by reaction of iodinated tyrosin succinimidyl ester and free amino groups of the target peptide. The coupling procedure, which in general is carried out in a weak alkaline medium (e.g. in borate buffer of pH 8.5–9) is rapid (approx. 10 min.) and can be stopped by addition of glycine in borate buffer [174]. Owing to the inherent synthetic procedure Met is preserved from oxidation. Despite its

undeniable advantages over the competing chloramine-T technique the method suffers from the drawback that more than one free amino group of comparable reactivity is often available in peptides and thus the possibility has to be considered that either di- or tri-labelled derivatives or mixtures of mono-labelled derivatives can be formed. This in turn, would involve tedious and expensive separation procedures in order to obtain the label of choice. Nevertheless, in the case of substance P the reaction seems preferably to take place at the ϵ-lysin moiety in position 3 of SP as shown by Michelot et al. [158]. Laufer et al. [63] prepared Bolton–Hunter SP_{6-11}, which only contains the free N-terminal amino group and thus yielded a radiolabel with a defined structure. The authors achieved an incorporation of 44% of radioactivity into the target peptide. This is in good accordance with our own investigations with SP_{1-11}, whereby 46% of incorporation yield was achieved (unpublished results).

Hitherto unpublished investigations performed some years ago in our laboratory provided evidence that labelling of SP with Bolton–Hunter reagent can also be effected with high yield in methanolic solution. In our scheme SP was reacted with "cold" Bolton–Hunter reagent in methanol without catalysis of an exogenously added base and the reaction course monitored by RP-HPLC. In a parallel experiment the coupling reagent alone was subjected to methanolysis. As could be seen, the latter reaction proceeded very slowly, whereas labelling at the amino function proceeded much more rapidly due to the considerably higher nucleophilicity of the basic NH_2 group compared with the hydroxy group of the solvent, despite its vast excess. Obviously the inherent basicity as well as nucleophilicity of the ϵ-amino group of Lys^3 is sufficient for a successful nucleophilic attack of the activated carboxyl group of the reagent.

5.1.3. Labelling with the lactoperoxidase method

In general, this method is known as a very gentle labelling procedure [175] of Tyr and His residues, because only mmol amounts of hydrogen peroxide are used at room temperature. Thus a realistic chance should exist to preserve the integrity of Met residues.

The reaction proceeds with substantially lower velocity compared with the chloramine-T variant but nevertheless high specific activities are obtained. If necessary, the rate of labelling can be further decreased by immobilisation of the enzyme to e.g. sepharose or another solid phase [176–180]. However, despite the advantages of the method it was reported that Met and Cys may interfere with peroxidase-catalysed reactions [175]. Indeed, labelling of $[Tyr^8]$-SP with the lactoperoxidase technique yielded a ratio of native radiopeptide and sulfoxide in approximately equal amounts as evidenced by RP-HPLC (unpublished results from our laboratory), which makes the method unsuitable for iodination of SP, whereas in contrast, a fully receptor-active labelled insulin was obtained after purification by RP-HPLC (unpublished results from our laboratory).

5.1.4. Labelling with the iodogen reagent

The iodogen™ reagent [166,181] the chemical name of which is 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril is a bicyclic chloramine derivative that is sparingly soluble in an aqueous medium. For practical purposes the reagent is coated as a thin layer onto the walls of the reaction vessel by evaporation of the solvent (normally chloroform) used for dissolution. The labelling reaction takes place at low velocity at the solid reagent–aqueous solution interface and the reaction can be stopped by simple removal of the aqueous solution from the reagent-coated vial. As compared with the procedures mentioned above, oxidative side reactions are markedly reduced and thus radiolabels with either high activity at the receptor or the antibody are obtained. The drawbacks arising from application of this procedure are the relatively long reaction times and the often low specific activities of the tracer molecules.

5.1.5. Labelling with the iodo beads method

The iodo beads™ reagent consists of N-chlorobenzenesulfonamide immobilised on nonporous polystyrene spheres [182] with a diameter of 2.8

mm and thus represents a further improvement of the iodogen labelling procedure. The reaction is stopped in an identical manner by removal of the aqueous solution from the beads, which remain on the bottom of the vessel. The advantages of this alternative with respect to the competing iodogen technique is the higher actual concentration of reagent due to the greater surface of the beads, which in particular, provides a more intimate contact with the active reagent site when the reaction mixture is stirred and/or gently shaken. For this reason it can be expected that the specific activities are much better compared with the iodogen reaction.

5.1.6. Comparison of the different labelling techniques

The preparation of ^{125}I-[Tyr^{8}]-SP with chloramine-T as oxidising agent proceeds rapidly (approx. 20–30 s) and provides satisfactory incorporation of radioactivity into the peptide. Thus a radiolabel with high specific activity is obtained. However, concomitant complete oxidation of the Met^{11} residue to the corresponding sulfoxide makes reduction to the native compound necessary (in particular in cases, where binding to the receptor is to be measured), which requires a further reaction step with e.g. mercaptoethanol. Nevertheless, the high yield of iodine incorporation (approx. 80%, see Ref. [125]) into the peptide as well as the ease of the reduction procedure, by which approx. 92% of applied radioactivity was recovered [125], compensates for the inconveniences of the side reaction. In this context it should be important to note that radio-iodination of [Tyr^{0}]-somatostatin-14 and [Tyr^{11}]-somatostatin-14 yielded tracers exhibiting markedly better immunoreactivities compared with those prepared by the iodogen and enzymobeads™ procedures [183], which implies superiority of this method, especially for small peptides.

In contrast, coupling of Bolton–Hunter reagent to SP preserves the thioether structure, but the coupling reaction provides an incorporation yield of radioactivity of only approx. 40–50% [63]. Its main advantage with respect to the chloramine-T variant is that conjugation of the mono-iodinated reagent (which is commercially available and can be easily removed from the di-iodinated species by prior HPLC) to peptides provides labels which do not require further removal of di-iodinated contaminants the latter giving rise to accelerated autoradiolysis and thus decreased stability. For this reason di-iodination as the inevitable side reaction in direct iodination as well as the noxious influence of ^{125}I-ions [173] can be excluded. However, the ^{125}I-labelled Bolton–Hunter reagent is much more expensive than the alkaline solution of Na^{125}I required for the chloramine-T reaction. Nevertheless, this method presents a valuable alternative to the latter procedure, in particular for SP, where formation of di-labelled as well as a mixture of mono-labelled derivatives can probably be excluded [158].

The labelling of [Tyr^{8}]-SP with ^{125}I in the presence of lactoperoxidase as the catalyst and mmol amounts of hydrogen peroxide partially converts Met to its sulfoxide, which makes reduction of the product indispensable, as with chloramine-T. However, in contrast to this method, the chloramine-T procedure (i) proceeds at substantially higher velocity, (ii) yields higher incorporation of radioactivity and concomitantly of specific activity and (iii) additionally is much easier to perform.

The labelling procedures of [Tyr^{8}]-SP involving iodogen™ and iodo beads™ are similar. The reaction rate is markedly decreased with respect to the methods described above due to the fact that it takes place at a solid–liquid interface. For this reason formation of products from side reactions as reported in sections 5.1.1 and 5.1.3 is markedly suppressed. Owing to these properties the field of application may mainly be restricted to the labelling of larger peptides or proteins containing a multitude of amino acids easily susceptible to chemical (oxidative) modification, such as Trp, Cys, Met, Cys–Cys bridges etc.

Taking together all the advantages and drawbacks of the chloramine-T method it is not surprising that it is still the most widely used procedure for radio-iodination of small peptides

such as [Tyr^8]-SP. For most RIA applications (at least in the case of tissue samples, where the concentrations are normally in the pmol range) it does not seem imperative in every case to reduce the labelled sulfoxide to the native thioether. However, when very low amounts of SPLI in body fluids (i.e. in the lower fmol range) are to be measured, reduction of the sulfoxide derivative with mercaptoethanol is recommended, affording a marked increase of the sensitivity of the RIA [125].

5.1.7. Purification of radiolabelled SP by chromatography

An efficient purification step is required either directly after radiolabelling to remove interfering components (which give rise to increased unspecific or decreased receptor- and antibody-binding, respectively) or, after a distinct time period has elapsed since preparation in order to eliminate detrimental radiolysis products. The chromatographic procedure should further be able to separate the radiopeptide from its unlabelled precursor because substantial quantities of the latter act as inherent competitor in receptor- and antibody-binding experiments and thus markedly decrease sensitivity.

Analogous to sample pre-preparation and/or enrichment of peptides in biological sources the SPE technique is a feasible and powerful tool to pre-separate the main contaminants from the crude iodination mixture. By the choice of appropriate cartridge matrices and elution conditions excessive inorganic iodine (formed by stopping the reaction with sodium meta bisulfite) as well as labelled BSA (when the protein was used to terminate the reaction) leave the cartridge unretained, whereas the peptide can be eluted by admixture of an appropriate amount of organic solvent (e.g. methanol, ethanol, 2-propanol, acetone, acetonitrile etc.) to the aqueous buffer system. Although sufficient separation of α-, β- and γ-endorphin as well as of enkephalins was achieved by application of a step gradient of 2-propanol in formate pyridine buffer [184], SPE should always be used in combination with RP-HPLC [157]. A three-step purification procedure providing ^{125}I-[Tyr^8]-SP with excellent quality, is reported in Ref. [125]. For this purpose the crude iodination mixture from the chloramine-T reaction was initially adsorbed onto microfine silica, desorbed from it with a mixture of water–acetone–acetic acid (39:40:1, v/v) and further purified by ion-exchange chromatography on carboxymethyl cellulose (step gradient: 6 m*M* ammonium acetate buffer, pH 4.6, followed 10 min later by the same buffer at 0.6 *M*). After treatment of the radiolabel with mercaptoethanol (see section 5.1.1) it was finally subjected to RP-HPLC on a C_{18} matrix (linear gradient of 20 to 35% of acetonitrile in 100 m*M* formate buffer, pH 3.5–4 in 30 min). The final HPLC separation is a crucial step which never should be omitted because removal of contaminants markedly decreases non-specific binding and thus improves assay sensitivity [125].

The recoveries of ^{125}I-labelled peptides from reversed-phase column materials are very high and thus losses can be neglected. Emanuel et al. [185] reported quantitative recovery of the ^{125}I derivatives of angotensin-II, bradykinin and LHRH, whereas in contrast VIP exhibits only 75 to 85% recovery. For SP similar values are observed extending from more than 80 to about 100%. Therefore RP-HPLC proves to be a suitable method for final purification of radiolabelled substrates.

6. Conclusions

A large number of methods for the measurement of substance P and related fragments in a wide variety of biological matrices (tissue, body fluids, perfusion and superfusion liquids etc.) at the analytical level is now available.

In general, the tissue or fluid is first subjected to thorough sample pretreatment in order to minimize perturbations arising from interferences of the matrix during the following analysis steps.

This aim is efficiently achieved by precipitation of larger proteins and/or solid-phase extraction of the supernatants by use of short cartridges filled with reversed-phase material, after which

the peptide fragments are additionally obtained within small volumes. The peptide concentrates are further purified and separated from co-extracted contaminants by a powerful chromatographic system, such as RP-HPLC. Octadecasilyl silica gel materials have still proven to be the optimal media either for use in SPE or HPLC separation. Trifluoroacetic acid (ca. 0.1%) in aqueous organic solvents like methanol and acetonitrile are the preferably used mobile phase systems. Nevertheless other more gentle buffer ingredients like trialkyl ammonium phosphates or their volatile formate or acetate analogues, which can be applied when complete removal of the mobile phase is required after chromatography (e.g. for the use in RRA and RIA), compete more and more successfully with the conventional aqueous TFA–acetonitrile systems.

The choice of the procedure for the subsequent determination of SP and its fragments depends on the expected concentration to be found in the biological sample. In a coarsely simplified manner sensitivity increases in the range UV- (nmol) < fluorescence- (pmol) < ED- (lower pmol) ≅ CLND (lower pmol) < MS- (lower pmol to upper fmol) < RRA- (upper fmol) < RIA- (lower fmol) detection. Up to now RRA and RIA are still the most used determination techniques of SPLI in tissue containing levels of SP in the upper fmol range, whereas only RIA is able to detect SPLI in body fluids, where concentrations in the lower fmol range are usually found. The main drawback of RRA and RIA compared with the MS method is their relatively low molecular specificity despite the fact that in most cases highly efficient and specific antibodies are used. Nevertheless, specificity is further improved by prior RP-HPLC separation and subsequent RIA with two and more different antibodies directed to different epitopes of the same peptide, which may be important for the distinction of N- and C-terminal SP fragments.

For introduction of ^{125}I into SP and its derivatives the chloramine-T method, after which, however, a further reaction step with a reducing agent is required and the Bolton–Hunter conjugation techniques are the most promising for radiolabelling. Both are nearly equivalent in their efficiency to prepare radiopeptides with either high specific activity or binding capacity to both membrane receptors and antibodies.

At present no application of the HPLC–LIA (luminescence immunoassay) coupling to SP, by which similar or even lower detection limits as in RIA can be expected [186], has been published. For this reason it is desirable that the future development of this technique as well as refinement of post-column MS detection may provide an analytical tool that will be able to replace the radioanalytical methods and the concomitant exposition of the laboratory personnel to detrimental radiation.

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