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Marked improvement of a substance P radioimmunoassay by reduction of ^{125}I-labelled [Tyr^{8}]-substance P prepared by the chloramine-T method with mercaptoethanol and subsequent purification by reversed-phase liquid chromatography[a]

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ABSTRACT

[Tyr^{8}]-substance P was radiolabelled with ^{125}I by the application of the chloramine-T method. Due to the high oxidative potential of the ^{125}I–chloramine-T system the purified reaction product was converted into a derivative, which presumably had been oxidized to the corresponding sulphoxide at Met^{11}. This conversion was shown by reversed-phase high-performance liquid chromatography after consecutive reduction–oxidation experiments of the freshly prepared radiopeptide. The oxidized derivative exhibited only negligible binding to substance P receptors isolated from rat brain homogenates. However, in contrast, it showed marked cross-reaction to the antibody raised in rabbits against synthetic SP(1–11). The variance in the quantification of identical samples was marked, and the measurement of concentrations in the lower pg/ml range was not sensitive enough to determine levels of substance P-like immunoreactivity in human cerebrospinal fluid. Assay sensitivity could be substantially improved and variance significantly decreased by the use of a radiopeptide, which had been labelled by the chloramine-T method and which had been subsequently reduced with mercaptoethanol and purified by liquid chromatography.

INTRODUCTION

Substance P (SP), discovered in 1931 [1] and identified as an undecapeptide in 1970 [2], is widely distributed throughout the brain and spinal cord in a similar way to various other neuropeptides [3–6]. SP is a candidate for a neurotransmitter function in the brainstem [3] and in the spinal cord [4,7]. High concentrations of SP are found in the dorsal horn and in cell bodies of primary sensory afferents

[a] This paper is dedicated to Professor Gottfried Schill on the occasion of his 60th birthday.

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[5,6]. Somatostatin (SS), another neuropeptide discovered in 1968 in hypothalamic tissue [8] and identified as a tetradecapeptide in 1973 [9] is also present in the aforementioned brain structures. Both SP and SS seem to be involved in nociception [10] and seem to exhibit antagonistic properties towards each other [11,12]. Whereas pain was elicited by intrathecally applied SP in rats [13], a contrary effect was observed by intrathecally administered SS in animals and in man [14,15]. These and other findings raise the question of whether neurological disorders are associated with marked changes of neuropeptide concentrations in lumbar or ventricular cerebrospinal fluid (CSF) (for a review see refs. 16 and 17).

Because it is often assumed that the CSF (which represents a unique compartment in humans for the measurement of biologically active substances) reflects the metabolism of neuropeptides in the central nervous system [18], a great deal of interest has been focused on the determination of neuropeptides in the CSF. Furthermore, because it is a well known fact that levels of substance P-like immunoreactivity (SPLI) in the CSF are in the lower pg/ml range, with average levels below 20 pg/ml [19–21], sensitive detection methods are required for the quantification of SP concentrations by a direct measurement of the biological sample. Most radioimmunoassays (RIAs) based on the use of a radiopeptide prepared by the chloramine-T technique are not sensitive enough to detect concentrations of SPLI in samples of human CSF. Additionally, a tedious and time-consuming concentration step involving the lyophilization of the samples and reconstitution of the lyophilizate in a small volume of water has to be carried out [21] prior to the quantification by RIA.

We report our investigation into the preparation of ^{125}I-labelled [Tyr^8]-SP by the chloramine-T labeling procedure, the subsequent reduction of the expectedly formed sulphoxide derivative with mercaptoethanol and the purification of the product by reversed-phase high-performance liquid chromatography (RP-HPLC). To our knowledge, this paper is the first report concerning the preparation of monoiodo-[^{125}I]-[Tyr^8]-SP, which presumably contains an intact Met^{11} for use in radioimmunoassay.

EXPERIMENTAL

Preparation of [^{125}I]-[Tyr^8]-substance P

For radiolabelling 2.5 μg of [Tyr^8]-SP (HPLC grade, Peninsula Labs., Belmont, CA, U.S.A.) diluted in 5 μl of 0.1 *M* acetic acid was iodinated by the chloramine-T procedure [22]. First, 10 μl of 0.5 *M* phosphate buffer (pH 7.5) and 5 μl (0.5 mCi, *i.e.* $4.35 \cdot 10^6$ Ci/mol) of Na^{125}I (IMS 30, purchased as a 0.1 *M* NaOH solution, Amersham-Buchler, Braunschweig, Germany) was added to the peptide. The reaction was initiated by the addition of 10 μl of a chloramine-T solution (1 μg/μl) and stopped after 30 s by the addition of 500 μl of bovine serum albumin (7%). Both reagents were dissolved in 0.5 *M* phosphate buffer (pH 7.5). Microfine silica (10 mg) was added for prepurification. The suspension was mixed

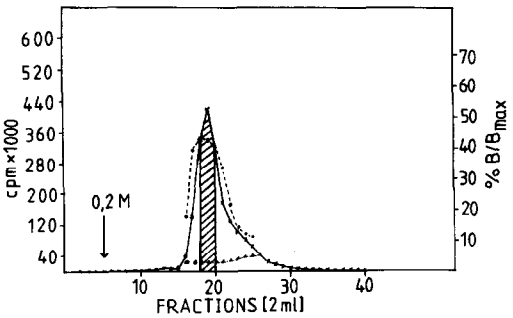

Fig. 1. Elution profile of the crude ^{125}I iodination product of [Tyr^8]-SP obtained by the use of the chloramine-T method (solid line) on a 6 cm × 1 cm I.D. CM-52 column. Aliquots (10 μl) of each fraction containing 2 ml of eluate were withdrawn and radioactivity was measured. First 10 ml of 2 m*M* ammonium acetate buffer (pH 4.6) were applied, followed by a switch to a 0.2 *M* ammonium acetate buffer of pH 4.6 (indicated by the arrow). The binding profile (specific binding) of the radiopeptide (broken line) was determined with an antibody dilution of 1:50 000. The maximum value of antibody binding coincides with the maximum value of radioactivity. Non-specific binding (dotted line) was performed by omitting the antibody from the incubation medium.

thoroughly, and the mixture was centrifuged (9800 *g*). After the supernatant had been discarded, the residue was washed by three consecutive portions of 500 μl of water. The radiopeptide was desorbed with 1 ml of water–acetone–acetic acid (40:39:1, v/v) and further purified by ion-exchange chromatography on a 6 cm × 1 cm I.D. carboxymethylcellulose column (CM-52, Whatman, Springfield, U.K.) with a stepwise elution with 10 ml of 2 m*M* ammonium acetate (pH 4.6) and 60 ml of 0.2 *M* ammonium acetate (pH 4.6). Fractions were collected in polystyrene tubes that contained 20 μl of bovine serum albumin (7%) to minimize adsorption to the vial surface.

Binding was determined at an antibody dilution of 1:50 000 (*i.e.* a final dilution of 1:350 000 in the assay system). Fractions 18–20 exhibiting a maximum in binding to the antibody and specific activity, respectively (see Fig. 1, hatched area) were pooled and stored at −40°C.

The purity of the radioiodinated antigen was checked by thin-layer chromatography (TLC) on cellulose (Eastman Kodak, Rochester, NY, U.S.A.) with *n*-butanol–water–acetic acid (6:2:1, v/v) and by HPLC using a gradient system; solution A, 80:20 mixture of 0.1 *M* triethylammonium phosphate (TEAP) in acetonitrile (v/v; pH 2.5); solution B, 50:50 mixture of 0.1 *M* TEAP in acetonitrile (v/v; pH 2.5)], from 0% B to 50% B in 30 min. A mobile phase composition of 50% B was maintained for 10 min, followed by a drop to the starting conditions (0% B) in 5 min. After a reequilibration time of 15 min another sample was injected. A Spherisorb ODS II column (125 mm × 4.6 mm I.D., 5-μm particles; Bischoff Analysentechnik, Leonberg, Germany) was used for separation. The gradient system, which consisted of two pulse-dampened LC 410 pumps, was

controlled by a Model 200 gradient controller (both from Kontron Analysentechnik, Munich, Germany). The flow-rate was 1.0 ml/min. The radioactivity profile was monitored with a radiochromatogram scanner LB 503 (Berthold, Wildbad, Germany), or alternatively by withdrawing 100-μl aliquots from collected fractions (Frac 200, Pharmacia, Uppsala, Sweden) and radioactivity was determined with a multi-channel detector LB 2104 (Berthold, Wildbad, Germany). Non-radioactive peptides were detected at 210 nm (0.05 a.u.f.s.) by a Uvikon 820 detector (Kontron, Munich, Germany). An injection valve equipped with a 150-μl sample loop (Valco Instruments, Schenkon, Switzerland) was used for sample injection. Aliquots (10 μl) of the iodinated peptide (see above) with cpm values ranging from 180 000 to 340 000, were injected onto the column. A volatile buffer system, which consisted of triethylammonium formate (TEAF) and acetonitrile [solution A: 80:20 mixture of 0.1 *M* TEAF in acetonitrile (v/v, pH 3.5); solution B: 50:50 mixture of 0.1 *M* TEAF in acetonitrile (v/v, pH 4.0)] was used for the purification of the crude mercaptoethanol-treated radiopeptide before its subsequent use in RIA experiments. The gradient used for the separation was identical to that described above. Fractions (1 ml) were collected with a Frac 200 sample collector and subsequently evaporated at room temperature in a Speed Vac concentrator (Savant Instruments, Hicksville, NY, U.S.A.).

In order to reduce the radiopeptide, 100 μl of mercaptoethanol (Merck, Darmstadt, Germany) and 1000 μl of 0.25 *M* sodium acetate buffer (pH 4.0) were added to an aliquot of 500 μl of the freshly iodinated peptide (mercaptoethanol–radiopeptide = $4 \cdot 10^5$), and the mixture was kept at 80°C for 2 h. The excess mercaptoethanol was evaporated in a gentle stream of nitrogen at 60°C for 1 h. The oxidation of [^{125}I]-[Tyr^8]-SP (approximately 100 000 cpm, *i.e.* 28 fmol) was performed by the addition of 100 μl of 0.01 *M* hydrogen peroxide in 0.25 *M* sodium acetate buffer (pH 4.0). The reaction mixture was kept at 80°C for 30 min, and the products were separated by HPLC. For comparative purposes approximately 5 μg of SP(1–11) and SP(5–11), respectively, were subjected to the same reaction conditions.

Radioimmunoassay of substance P

RIA of SP was performed as described elsewhere [21,23]. We used the antiserum 9–3a (kindly donated by Dr. R. Jakisch, Department of Pharmacology, University of Freiburg, Germany). For this purpose rabbits were immunized with a bovine serum albumin–synthetic SP(1–11) conjugate, which was suspended in complete Freund's adjuvant. This treatment was repeated three times at intervals of two, six and twelve weeks. It shows negligible cross-reactions (< 1%) with SP(1–4), SP(1–7), SP-COOH (free acid), eledoisin, substance K, neurotensin, bombesin and SS-14. Two members of the tachykinin family (kassinin and physalaemin) showed little to moderate cross-reactivity: 3% and 10%, respectively. Substantial cross-reactions were observed with SP sulphoxide (49%), SP(5–11) sulphoxide (60%), [Tyr^8]-SP sulphoxide (55%), [^{125}I]-[Tyr^8]-SP sulphoxide

(60%), [^{125}I]-[Tyr^8]-SP thioether (90%), whereas SP(2–11), SP(3–11), SP(4–11), [Tyr^8]-SP and SP(5–11) all exhibited a cross-reactivity of approximately 100%. All tachykinins were purchased from Peninsula Labs. (HPLC grade). The recognition site of the antibody is directed towards the C-terminal region (responsible for the typical tachykinin properties) of the peptide. Furthermore a serial dilution was prepared from a concentrate of lyophilized human CSF and from synthetic SP(1–11) in the concentration range 40–320 pg/ml (see Fig. 6) in order to check the linearity of SPLI concentrations in the CSF sample. The incubation of the samples was performed in polystyrene tubes (Greiner Labortechnik, Nuertingen, Germany). The incubation buffer was supplemented with bovine serum albumin (0.25%) to minimize the adsorption of the peptide to the vial's surfaces.

RESULTS

Approximately 80% of the amount of ^{125}I was incorporated into [Tyr^8]-SP and a specific activity of $1.9 \cdot 10^6$ Ci/M was achieved. The substitution takes place at the *o*-position of the hydroxyl group of Tyr^8. As calculated from the specific activity, 0.73 atoms of ^{125}I per mol of peptide were introduced into the *o*-position of the Tyr^8. The purity of fractions 18–20 (see Fig. 1) was estimated to be greater than 95% by HPLC and TLC (R_F = 0.58). A minor impurity (approximately 20%) occurred only in fraction 21 and eluted at a slightly increased retention time (t_R). The percentage of this derivative was approximately 20% relative to the total amount of radiolabelled peptide material. It showed significantly increased specific activity and could presumably be assigned to a derivative, which had been iodinated at both *o*-positions of Tyr^8 (results not shown) [24]. The retention time of the iodinated peptide prepared by the chloramine-T method was approximately 9 min.

HPLC and TLC investigations of a tracer stored for more than six months at −40°C established that no significant degradation took place during this time

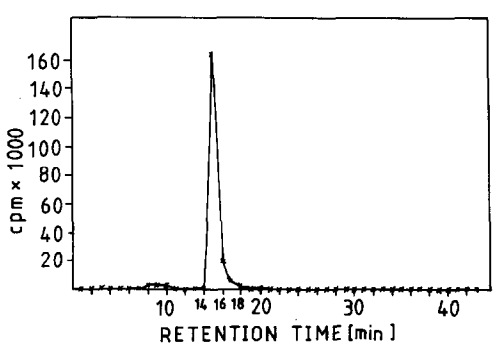

Fig. 2. RP-HPLC profile measured on a Spherisorb ODS II (125 mm × 4.6 mm I.D., 5-μm particles) column of ^{125}I-labelled [Tyr^8]-SP (after reduction with mercaptoethanol).

period. However, when this radiopeptide was used for binding experiments at substance P receptors isolated from rat brain homogenates, only negligible binding was observed. Yet, after the reduction of the label with mercaptoethanol, receptor-binding increased dramatically [25]. Such data raise the question as to whether the radiopeptide prepared by the chloramine-T method contains an oxidized Met^{11} residue (presumably a sulphoxide) as a consequence of the oxidative potency of the used agent. The reduced radiopeptide (presumably consisting of the thioether) was obtained in 92% yield. HPLC investigations showed that the peak of the radioactivity was shifted to approximately 15 min ("thioether") *versus* 9 min of the native tracer (Fig. 2).

After the subsequent re-oxidation of the mercaptoethanol-reduced tracer with hydrogen peroxide, an extensive conversion to the genuine radiolabel was shown by HPLC (Fig. 3). Two minor peaks of radioactivity were observed at t_R = 5 and 24 min, respectively. Their structures remain unknown and no antibody-binding was observed. It must be emphasized that the reaction conditions for the preparation of the reduced radiolabel, as described above, should be closely adhered to; if not, a by-product is formed in significant amounts (about 25% at 90°C), which leaves the column after a slightly longer retention time and might, therefore, be assignable to a more hydrophobic peptide. No further efforts were made to identify its structure. When synthetic samples of non-radioactive SP(1–11) and SP(5–11) were subjected to oxidation–reduction experiments, the HPLC of the oxidized samples showed that t_R decreased significantly, whereas subsequent reduction caused an increase in t_R and both peaks coelute with those of the starting material. This observation is in accordance with corresponding experiments performed with ^{125}I-radiolabelled [Tyr^8]-SP (see above). The probably formed SP(1–11) sulphoxide eluted at 9.9 min *versus* 14.9 min for SP(1–11), whereas the probably formed SP(5–11) sulphoxide eluted at 11.9 min *versus* 17.7 min for SP(5–11) (Figs. 4 and 5). A dilution curve of SPLI, prepared from a concentrate of lyophilized human CSF, showed parallelism with the dilution curve obtained

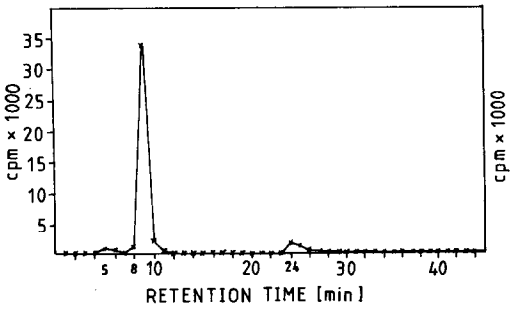

Fig. 3. RP-HPLC profile measured on a Spherisorb ODS II (125 mm × 4.6 mm I.D., 5-μm particles) column of mercaptoethanol-reduced [^{125}I]-[Tyr^8]-SP (see Fig. 2) after subsequent reoxidation with hydrogen peroxide.

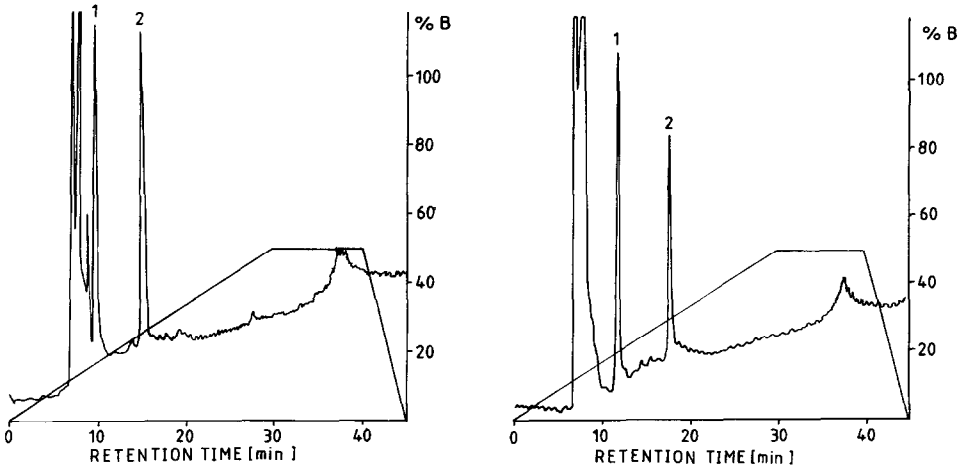

Fig. 4. RP-HPLC profile measured on a Spherisorb ODS II (125 mm × 4.6 mm I.D., 5-μm particles) column of SP(1–11) sulphoxide (peak 1) and SP(1–11) (peak 2). Detection wavelength was 210 nm.

Fig. 5. RP-HPLC profile measured on a Spherisorb ODS II (125 mm × 4.6 mm I.D., 5-μm particles) column of SP(5–11) sulphoxide (peak 1) and SP(5–11) (peak 2). Detection wavelength was 210 nm.

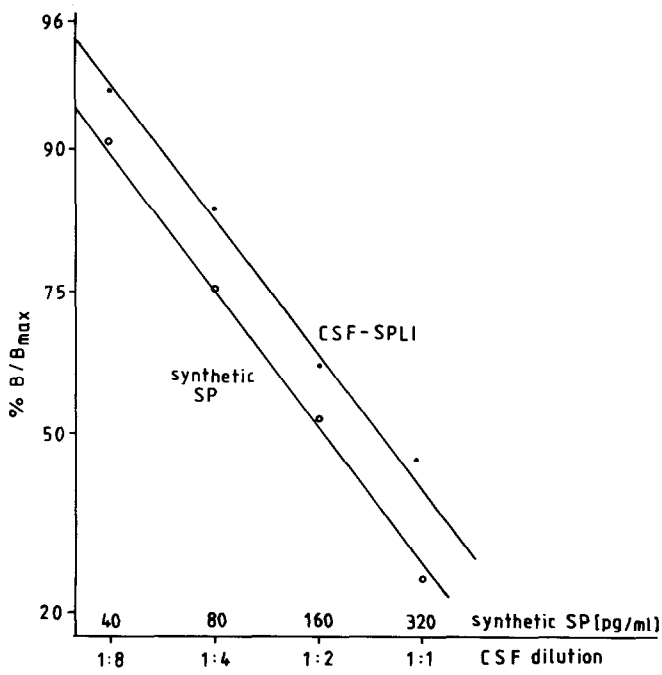

Fig. 6. Logit–log plot showing parallelism of both curves obtained from serial dilutions of synthetic SP(1–11) and SPLI (derived from a concentrate of human CSF), respectively.

TABLE I

INTER-ASSAY PARAMETERS OBTAINED WITH ^{125}I-LABELLED [Tyr^8]-SP, WHICH WAS PREPARED BY THE USE OF THE CHLORAMINE-T METHOD

Nominal concentration (pg/ml)	Concentration found (mean ± S.D.) (pg/ml)	C.V. (%)	Accuracy (%)	*n*
10	13.7 ± 5.8	42.3	137	8
20	30.9 ± 9.6	31.1	155	8
40	52.1 ± 8.8	19.1	130	8

TABLE II

INTER-ASSAY PARAMETERS OBTAINED WITH ^{125}I-LABELLED [Tyr^8]-SP, WHICH WAS PREPARED BY THE USE OF THE CHLORAMINE-T METHOD AND THE SUBSEQUENTLY PERFORMED REDUCTION WITH MERCAPTOETHANOL

Nominal concentration (pg/ml)	Concentration found (mean ± S.D.) (pg/ml)	C.V. (%)	Accuracy (%)	*n*
10	9.2 ± 1.1	12.0	92	8
20	20.4 ± 4.0	19.6	102	8
40	42.0 ± 2.5	6.0	105	8

TABLE III

INTRA-ASSAY PARAMETERS OBTAINED WITH ^{125}I-LABELLED [Tyr^8]-SP, WHICH WAS PREPARED BY THE USE OF THE CHLORAMINE-T METHOD

Nominal concentration (pg/ml)	Concentration found (mean ± S.D.) (pg/ml)	C.V. (%)	Accuracy (%)	*n*
10	12.1 ± 12.8	21.8	121	4
20	34.3 ± 1.5	5.0	172	4
20	52.8 ± 4.4	8.3	132	4

from synthetic SP(1–11) in the RIA (Fig. 6). This concentration dependency indicates that unspecific binding to the antibody can presumably be ruled out and allows us to interpret that we measured immunoreactive SP. This was further confirmed by the chromatographic identification of SPLI [26].

The inter-assay variance of analytical data could be substantially lowered (and thus reproducibility improved 150–350%) if the mercaptoethanol-reduced radiopeptide was used for RIA, whereas the inter-assay variance of the unreacted label

TABLE IV

INTRA-ASSAY PARAMETERS OBTAINED WITH ^{125}I-LABELLED [Tyr^8]-SP, WHICH WAS PREPARED BY THE USE OF THE CHLORAMINE-T METHOD AND THE SUBSEQUENTLY PERFORMED REDUCTION WITH MERCAPTOETHANOL

Nominal concentration (pg/ml)	Concentration found (mean ± S.D.) (pg/ml)	C.V. (%)	Accuracy (%)	*n*
10	9.2 ± 0.8	8.7	92	4
20	16.8 ± 0.3	1.8	84	4
40	43.1 ± 1.6	3.7	108	4

was significantly increased and, therefore, the reproducibility of data was decreased (see Tables I and II).

In contrast to these findings, intra-assay variance was similar and sufficient in both cases (see Tables III and IV).

If assay sensitivity is defined as that concentration of synthetic SP necessary to inhibit the binding of [^{125}I]-[Tyr^8]-SP to a mean value at least two standard deviations below the mean value obtained in the absence of SP, then 5 pg/ml could be calculated for the reduced radiolabel, and 15 pg/ml for the corresponding untreated derivative. Binding to the antibody [raised in rabbits against synthetic SP(1–11)] of the mercaptoethanol-treated label increased significantly when compared to the sulphoxide derivative. Therefore, antibody dilution had to be increased in this case. To obtain reliable and reproducible results for the maximal binding to the antibody, its dilution was chosen to be 1:300 000 for the non-mercaptoethanol-treated radiopeptide and 1:500 000 for the mercaptoethanol-treated derivative, which presumably consists of the thioether. Maximal binding was approximately 28% in both cases. Non-specific-binding was approximately 3%. Each determination was done in duplicate. No improvement of assay data was observed, when the chloramine-T prepared (genuine) radiopeptide was purified by HPLC without first being reduced with mercaptoethanol.

DISCUSSION

[^{125}I]-[Tyr^8]-SP labelled by the chloramine-T method is predominantly used as the radioactive antigen in RIA. However, when it is used for receptor-binding experiments [25] no binding is observed and a further purification of the peptide by HPLC did not improve its binding characteristics. Therefore, we concluded that a quantitative conversion of [Tyr^8]-SP thioether to the corresponding sulphoxide (Met^{11}) took place during radioiodination as a result of the high oxidative potential of the chloramine-T–iodine system [27,28].

When the products from the oxidization of the synthetic samples of SP(1–11)

and SP(5–11) with hydrogen peroxide were subsequently reduced with mercaptoethanol and were analyzed by HPLC, both peaks coincided with those of the starting peptides. A similar interconversion was observed with [^{125}I]-[Tyr^{8}]-SP. Thus it seems likely that reduction with mercaptoethanol indeed provided an intact thioether derivative, although no additional testing with amino acid sequencing was performed. It may be noted that the rather harsh reaction conditions were also responsible for the oxidation of Trp^{8} in [Tyr^{0}]–SS-14 [29]. In that case the chloramine-T method produces a labeled peptide with an almost complete loss of antibody binding, if the iodination time exceeds 30 s [24]. No oxidative side-effects were observed during the synthesis of tritiated SP and Bolton–Hunter SP [30] (the latter formed by coupling [^{125}I]-Tyr to the ε-amino group of Lys [31] and both being successfully used in receptor-binding studies with rat brain homogenates). These labeled SP derivatives carry an intact Met^{11} due to the lack of oxidative side-reactions during their preparation. Recently, [^{125}I]-[Tyr^{8}-Norleu^{11}]-SP, which contains a non-oxidizable Norleu^{11} instead of the weakly oxidizable Met^{11}, was described for use in receptor-binding studies. Approximately 80% of the binding affinity, compared to the ^{125}I-Bolton-Hunter SP, was recovered [32]. However, this attractive alternative is not yet commercially available. Two other alternative means of preparing iodinated peptides and proteins, without contamination elicited by side-effects such as sulphoxide formation are the iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) and the iodo beads variants [33–36]. However, disadvantage of these two labeling procedures are the extended reaction time and the usually low specific activities of the labelled peptides. Generally, both procedures are recommended in order to prepare radiolabelled derivatives of proteins which often contain a rather large number of oxidizable and cleavable groups such as Met residues and disulphide linkages, respectively. The use of tritiated SP results in high costs as the label must be used in micromolar amounts compared to ^{125}I-labelled SP (where femtomolar amounts are sufficient). Thus tritium's low specific activity does not allow determinations in the lower pg/ml range. The preparation of Bolton–Hunter-SP requires a coupling step with an expensive reagent in comparison to the low costs of sodium ^{125}I necessary for the direct introduction of iodine into Tyr^{8}. Additionally, losses in yield have to be taken into consideration because in our experiments only about 50% of the radiolabelled [^{125}I]-Tyr was coupled to SP, in comparison to the significantly higher yield of [^{125}I]-[Tyr^{8}]-SP when using the chloramine-T procedure. Moreover, Bolton–Hunter SP exhibited a significantly lower antibody binding, presumably because of its different physical and chemical properties, when identical conditions have been chosen. Preliminary attempts to avoid the oxidation of [^{125}I]-[Tyr^{8}]-SP by the use of the lactoperoxidase method failed. HPLC investigations of the reaction product revealed that a 1:1 mixture of [^{125}I]-[Tyr^{8}]-SP and its oxidized derivative (results not shown) was obtained, even though the method is known to preserve the thioether in Met^{11} [26]. However, substantial conversion of Met^{11} to the corresponding sulphoxide has been reported [28,37].

Although the mercaptoethanol-reduced peptide was obtained in 92% yield (related to the recovered radioactivity), purification by HPLC is a crucial step for obtaining optimal reliability and reproducibility of RIA. Otherwise, increased non-specific binding will occur at the cost of assay sensitivity. As far as we know, no efforts have been made yet by the users of RIAs to obtain a tracer by the reduction of chloramine-T–^{125}I-labelled [Tyr^{8}]-SP with mercaptoethanol. Recently, Wallasch *et al.* [38] described the use of [^{125}I]-[Tyr^{8}]-SP (prepared by the chloramine-T method without treatment with mercaptoethanol) for the determination of SPLI after HPLC separation of CSF samples. Igwe [39] used this radiopeptide for the detection of SPLI in tissue samples after chromatographic purification by HPLC. He presumably used the chloramine-T technique, though no description of the labelling procedure was given. Usually, the purity of the radiopeptide prepared by the chloramine-T method after ion-exchange chromatography (see Experimental, item 1) of the thus formed oxidized peptide was sufficient, and subsequent RP-HPLC neither improved its binding to the antibody nor the properties of the RIA. Normally, when determining the sensitivity of SP and its related peptides in tissue samples it is sufficient to use a tracer prepared by the chloramine-T procedure. However, the concentrations of SPLI in CSF and blood are approximately 100-fold lower; thus more sensitive methods are required. Therefore, the question is still open, as to whether assay sensitivity could be improved by the use of a labelled antigen containing an intact Met^{11}. A marked increase in assay sensitivity and reproducibility was achieved by the reduction of the label with mercaptoethanol and the purification of the presumably formed thioether by HPLC. If the chromatographic properties are considered, then the shift of the peak of the reduced peptide to a higher retention time (15 *versus* 9 min) is in accordance with the existence of a more hydrophobic structure in the unoxidized form. The introduction of a voluminous iodine residue will certainly cause significant changes in (secondary) structures and concommitantly in binding properties because the iodine resides in the C-terminal tachykinin moiety, which is responsible for antibody binding as well as receptor binding of the peptide. However, the oxidation of Met^{11} seems to affect receptor binding of the peptide rather than its avidity for the antibody.

The significant increase of reproducibility and reliability in RIA by use of the ^{125}I-labelled thioether will permit more sensitive determinations of SPLI in the CSF samples from patients with neurological diseases [26].

The linearity of the dilution series of SPLI prepared from a lyophilizate of concentrated human CSF indicates that we detected SPLI and influences elicited by unspecific binding to the antibody can be neglected. HPLC–RIA coupling experiments revealed marked molecular heterogeneity with peaks of SPLI coeluting with synthetic samples of SP(1–11), SP(2–11), SP(5–11) and pyroGlu-SP(5–11) [26]. In conclusion, reduction with mercaptoethanol of [Tyr^{8}]-SP, which had been radiolabelled by using the chloramine-T method and subsequent purification by RP-HPLC provided a derivative which probably consists of [^{125}I]-[Tyr^{8}]-

thioether. When this radiolabel is used for the RIA of SP, a marked improvement in the sensitivity and reproducibility of the assay was observed. Therefore, it can be successfully used for the quantification of SPLI in CSF samples either by the direct measurement of SPLI in the biological sample or after first using HPLC separation (a HPLC–RIA coupling technique).

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