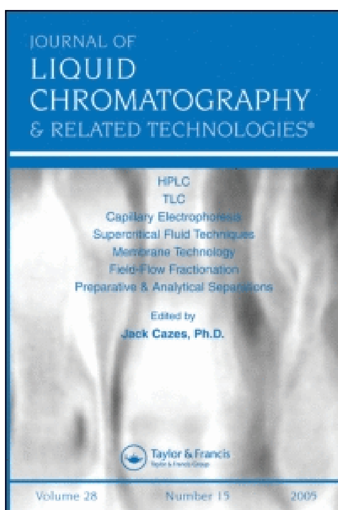
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Investigations on Radiolabeling Substance P Derivatives and Reversed-Phase Liquid Chromatographic Separation of the Products

Klaus Rissler[a]; Gerhard Friedrich[a]; Hinrich Cramer[b]
[a] Pharmbiodyn Institute of Contract Research, Germany [b] Department of Neurology Neurochemical Laboratory, Neurochemical Laboratory, Hansastrasse, Freiburg, Germany

To cite this Article Rissler, Klaus , Friedrich, Gerhard and Cramer, Hinrich(1998) 'Investigations on Radiolabeling Substance P Derivatives and Reversed-Phase Liquid Chromatographic Separation of the Products', Journal of Liquid Chromatography & Related Technologies, 21: 13, 1907 — 1923
To link to this Article: DOI: 10.1080/10826079808006599
URL: http://dx.doi.org/10.1080/10826079808006599

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

J. LIQ. CHROM. & REL. TECHNOL., 21(13), 1907–1923 (1998)

INVESTIGATIONS ON RADIOLABELING SUBSTANCE P DERIVATIVES AND REVERSED-PHASE LIQUID CHROMATOGRAPHIC SEPARATION OF THE PRODUCTS

Klaus Rissler,[1,†,*] Gerhard Friedrich,[1] Hinrich Cramer[2]

Pharmbiodyn
Institute of Contract Research
P.O. Box 1108
D-79211 Denzlingen, Germany

Department of Neurology
Neurochemical Laboratory
University of Freiburg
Hansastrasse 9
D-79104 Freiburg, Germany

Downloaded At: 10:55 24 January 2011

ABSTRACT

In investigations of radiolabeling substance $P_{1\text{-}11}$ (SP) with ^{125}I isotope, its [Tyr^8]-derivative was subjected to the lactoperoxidase (LPO) technique, whereas the native substance P undecapeptide was coupled with ^{125}I-tyrosin-N-hydroxy-succinimidyl ester to the corresponding ^{125}I-Bolton-Hunter SP derivative. For separation of the desired components from their by-products, labeled [Tyr^8]-SP was pre-purified by ion-exchange chromatography (IEC) on carboxymethyl cellulose and subsequently investigated by gradient high performance reversed-phase liquid chromatography (gRP-HPLC) with 0.1 M triethyl-ammonium phosphate

Copyright © 1998 by Marcel Dekker, Inc.

in acetonitrile (pH 2.5) on an octadecylsilyl silica gel (RP-18) stationary phase. When the total amount of labeled peptide affording a specific activity of approx. 2 x 10^6 Ci/M was checked by gRP-HPLC, the LPO technique yields about an equimolar mixture of ^{125}I-[Tyr^8]-(Met^{11})-SP and ^{125}I-[Tyr^8]-(Met^{11}→O)-SP. However, in contrast, substantial enrichment of ^{125}I-[Tyr^8]-(Met^{11})-SP was observed when more retained IEC fractions were analyzed.

The ^{125}I-Bolton-Hunter reagent was coupled in about 54 % yield to the SP undecapeptide at slightly alkaline conditions and separated from by-products only by gRP-HPLC yielding a specific activity of approx. 2 x 10^6 Ci/M. Both radiolabeled derivatives of SP are further characterized by their binding properties to a specific antibody raised in rabbits against synthetic SP_{1-11}. When individual fractions of either radioiodinated [Tyr^8]-SP or the corresponding ^{125}I-Bolton-Hunter derivative were investigated with respect to their binding properties to the antibody, only those fractions coinciding with the maximum amount of radioactivity showed a satisfactory ratio of specific vs. non-specific antibody-binding, especially in the case of the ^{125}I-Bolton-Hunter derivative. Therefore, the observed results are in good accordance with the existence of the desired target components.

Downloaded At: 10:55 24 January 2011

INTRODUCTION

The undecapeptide substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 = SP) is a member of a family of structurally related peptides, called the tachykinins, with the common C-terminal sequence -Phe-Xaa-Gly-Leu-Met-NH_2, Xaa being the only variable amino acid. SP was first discovered in 1931 by von Euler and Gaddum[1] in alcoholic extracts of equine brain and synthesized in 1971 using the solid-phase strategy.[2-3] The peptide is released from a 110 amino acid precursor protein[4] by tryptic cleavage and further processed in vivo to smaller fragments exerting various biological and pharmacological actions by a multitude of enzymes.[5-9]

Due to the widespread spectrum of biological and pharmacological activities of SP, a neurotransmitter role for the peptide was already proposed in 1953 by Lembeck.[10]

Some of the most salient pharmacological properties of SP include the action on blood vessels (vasodilatation, lowering of blood pressure, extravasation), bradycardia, salivation,[11,12] bronchoconstriction and mucus hypersecretion,[13-17] regulation of extrapyramidal and cognitive functions, pain perception[12,18,19] and its role as an important factor in the modulation of neuroimmunological responses.[20]

Radiolabeled derivatives of substance P are widely used in receptor-binding experiments and radioimmunoassay (RIA). Preparation and purification of ^{125}I-[Tyr^8]-substance P obtained by the chloramine-T technique for application in RIA and receptor-binding experiments was reported previously.[21-23] Two alternative procedures generally yielding tracer peptides with comparable high specific activities, such as radioiodination of [Tyr^8]-SP by use of lactoperoxidase and traces of hydrogen peroxide as well as reaction of SP undecapeptide with ^{125}I-Bolton-Hunter reagent are reported and compared with the chloramine-T procedure. In particular, the question arises if the lactoperoxidase technique could overcome the problems associated with the chloramine-T procedure, i.e., complete oxidation of Met^{11} to the corresponding sulfoxide, because of the more gentle reaction conditions, while still yielding sufficiently high specific activities of labeled SP.

Downloaded At: 10:55 24 January 2011

EXPERIMENTAL

Materials

Acetonitrile of HPLC grade was purchased from Rathburn (Walkerburn, UK) and water for use in HPLC was prepared with a Milli Q reagent water system™ from Millipore-Waters (Milford, MA, USA). Triethylamine, phosphoric acid, mercaptoethanol, hydrogen peroxide (30 %), sodium hydrogen phosphate, potassium hydrogen phosphate, sodium acetate, ammonium acetate, sodium tetraborate ($Na_4B_4O_7$), acetic acid, and trifluoroacetic acid (TFA), all of analytical grade, were obtained from Merck (Darmstadt, Germany). Substance P_{1-11} (SP_{1-11}), lactoperoxidase (LPO), and bovine serum albumin (BSA) were products from Sigma (Deisenhofen, Germany) and [Tyr^8]-SP_{1-11} was from Peninsula Laboratories (Belmont, CA, USA). Either sodium ^{125}iodide, purchased as a 0.1 M sodium hydroxide solution (1 μCi/μl, Code IMS 30), or ^{125}I-Bolton-Hunter reagent, i.e., mono- ^{125}iodo-tyrosin-N-hydroxy-succinimidyl ester (500 μCi) dissolved in benzene, were obtained from Amersham-Buchler (Braunschweig, Germany).

Downloaded At: 10:55 24 January 2011

Preparation of ^{125}I-[Tyr^8]-SP by the Lactoperoxidase/Hydrogen Peroxide Technique and Pre-Purification by Ion-Exchange Chromatography

To a solution of (i) 5 µl [Tyr^8]-SP (1mg/mL in 0.1 M acetic acid), (ii) 10 µL of 60 mM potassium phosphate buffer (pH 7.0) and (iii) 5 µL of Na ^{125}I (500 µCi), was added 5 µL of 0.8 mM hydrogen peroxide in 60 mM potassium phosphate buffer (pH 7.0). After gentle mixing, the labeling reaction was started by addition of 5 µL lactoperoxidase solution, 0.125 IU (1 U of LPO is defined as the amount of enzyme which will form 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 at 20°C.) in 60 mM potassium phosphate buffer (pH 7.0). After 30 min, the reaction was stopped by addition of 10 µL TFA in water (1 %, v/v) and the product subjected to ion-exchange chromatography (IEC) on a 60 x 10 mm carboxymethyl cellulose CM-52 column (Whatman, Springfield, UK). Step-wise elution was performed with 10 mL of 2 mM ammonium acetate followed by 0.2 M ammonium acetate. Fractions (2 mL) were collected in polystyrene tubes (containing 20 µL of 70 mg/mL BSA for minimization of surface adsorption) with a Frac 200 sample collector (Pharmacia, Freiburg, Germany).

For monitoring the elution profile, aliquots (20 µL) were withdrawn from each fraction and measured with a type LB 2104 multi-channel radioactivity detector (Berthold, Wildbad, Germany). Furthermore, individual fractions (diluted to about 10,000 cpm) were characterized by their binding to the specific antiserum SP 9-3a raised in rabbits against synthetic $SP_{1\text{-}11}$ at an antibody dilution of 1:50,000 corresponding to a final dilution of 1:350,000 in the assay system. Non-specific binding was determined in the presence of 5 µg non-radioactive $SP_{1\text{-}11}$. Details of the procedures were given in Ref. 22. Selected fractions were either directly subjected to gRP-HPLC or treated with mercaptoethanol prior to injection as described in Refs. 21 and 23. Pooled aliquots (20 µL) of IEC fractions (see Figure 1), as well as pooled fractions 22-24, 25-28 and 29-30 (10-20 µL, depending on the radioactivity measured), were analysed by gRP-HPLC for purity testing of ^{125}I-[Tyr^8]-SP.

Preparation and Purification of ^{125}I-Bolton-Hunter-SP

The ^{125}I-Bolton-Hunter reagent (500 µCi) was quantitatively transferred into a 1.5 mL polypropylene tube (1.5 mL) using a 100 µL rinse of benzene for complete recovery of radioactivity. After evaporation of the organic solvent by a gentle stream of nitrogen, 20 µL $SP_{1\text{-}11}$ (250 µg/mL) in 0.1 M sodium tetraborate buffer, adjusted to pH 8.5 with acetic acid, was added to the residue.

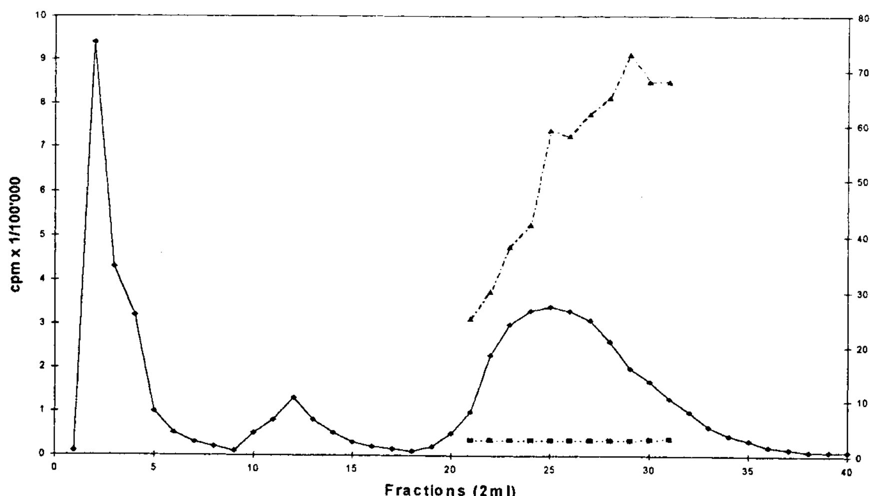


Figure 1. Ion-exchange chromatography of crude ^{125}I-[Tyr^8]-SP prepared with the lactoperoxidase method on a 60 x 10 mm CM-52 column (solid line). Aliquots (20 μl) of each fraction containing 2 mL of eluate were withdrawn and radioactivity measured. Specific (total binding - non-specific binding) as well as non-specific binding to the specific antibody SP 9-3a (scale on the right) were indicated by broken (upper curve) and dotted (lower curve) lines, respectively.

Downloaded At: 10:55 24 January 2011

The mixture was gently vortexed from time to time in order to afford complete reaction of the peptide, because the reagent is only sparingly soluble in the slightly alkaline medium (the reaction proceeds preponderantly at an heterogenous interface with only slow velocity). After 1 h of incubation, the reaction mixture was injected onto the HPLC column and eluted as described below. Fractions (1 mL) were collected with a Frac 200 sample collector and evaporated to dryness in a Speed Vac Concentrator™ (Savant, Hicksville, NY, USA).

Prior to evaporation, aliquots (10 μL) of each HPLC fraction were withdrawn for monitoring of the elution profile. Binding of selected fractions (diluted to about 10,000 cpm) to the specific antiserum SP 9-3a was performed at an antibody dilution of 1:100,000 corresponding to a final dilution of 1:700,000 in the assay system. Non-specific binding was measured after addition of 5 μg of unlabeled SP_{1-11}. The experimental details are referred in Ref. 22.

Downloaded At: 10:55 24 January 2011

Chromatographic Separation by gRP-HPLC

Chromatography was performed on a Spherisorb ODS II column (125 x 4.6 mm I.D., 5 μm particle size) obtained from Bischoff Analysentechnik (Leonberg, Germany) at a flow-rate of 1.0 mL/min using two pulse-dampened LC 410 pumps controlled by a Model 200 gradient controller (both from Kontron Analysentechnik, Munich, Germany). An injection valve equipped with a 100 μL sample loop (Valco Instruments, Schenkon, Switzerland) was used for sample injection. Selected fractions of ^{125}I-[Tyr^8]-SP from the IEC pre-purification step as well as the crude reaction mixture of ^{125}I-Bolton-Hunter SP_{1-11} were subjected to gRP-HPLC with solvent A [80:20 mixture of 0.1 M triethyl-ammonium phosphate (TEAP) - acetonitrile (v/v; apparent pH 2.5)] (the term "apparent" means that pH adjustment is done with the final aqueous-organic solution and not with the TEAP aqueous phase prior to addition of the organic modifier) and solvent B [50:50 mixture of 0.1 M TEAP - acetonitrile (v/v; apparent pH 2.5)]. The following gradient profile was applied: 0 % B to 50 % B in 30 min, 50 % B for 10 min followed by a drop to the starting conditions (0 % B) in 5 min and re-equilibration at these conditions for another 15 min until a new sample was injected. Aliquots (10 μL) of the column effluent were withdrawn and the elution profile measured with a type LB 2104 multi-channel radioactivity detector. Before separation of the ^{125}I-labeled peptides, two injections each of 5 μg of SP_{1-11} dissolved in 100 μL of 0.1 M acetic acid followed by a blank run with 0.1 M acetic acid were made in order to minimise "silanophilic interactions".

RESULTS

The elution profile obtained from IEC of the crude ^{125}I-[Tyr^8]-SP prepared by the lactoperoxidase technique is shown in Figure 1. A specific activity of about 2 x 10^6 Ci/M was evaluated for the radiolabeled SP derivative. When fractions exhibiting the highest amount of radioactivity (i.e., IEC fractions 21-32), were combined and subjected to RP-HPLC, ^{125}I-[Tyr^8]-(Met^{11})-SP and ^{125}I-[Tyr^8]-(Met^{11}→O)-SP were formed in 51 and 49 % yield, respectively, as calculated from the corresponding elution profile depicted in Figure 2. Nevertheless, a more close inspection of the data obtained from the binding experiments of individual IEC fractions to the specific antibody SP 9-3a revealed that the higher the fraction number, the better the affinity to the antibody, while non-specific binding remained approximately constant (see Figure 1). As reported elsewhere,[21] antibody-binding of ^{125}I-[Tyr^8]-SP prepared by the chloramine-T method and subsequently treated with mercaptoethanol increased compared with the untreated (native) radiopeptide. This observation is consistent with the presence of substantial amounts of ^{125}I-[Tyr^8]-SP

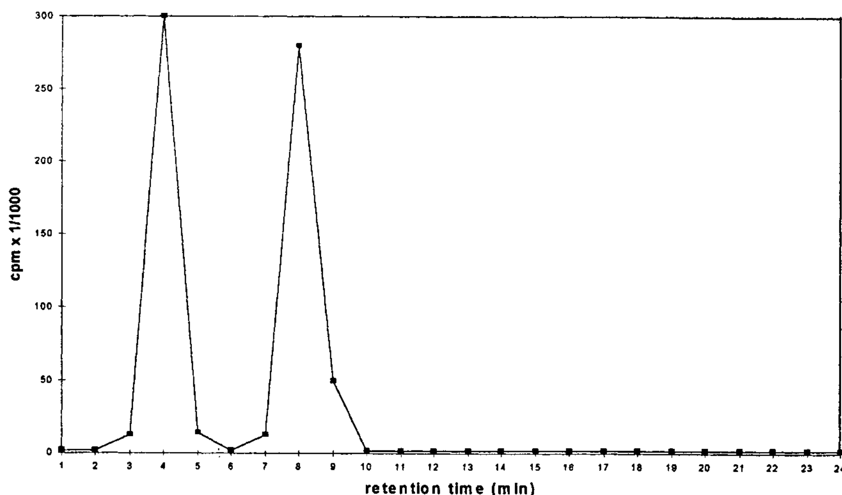


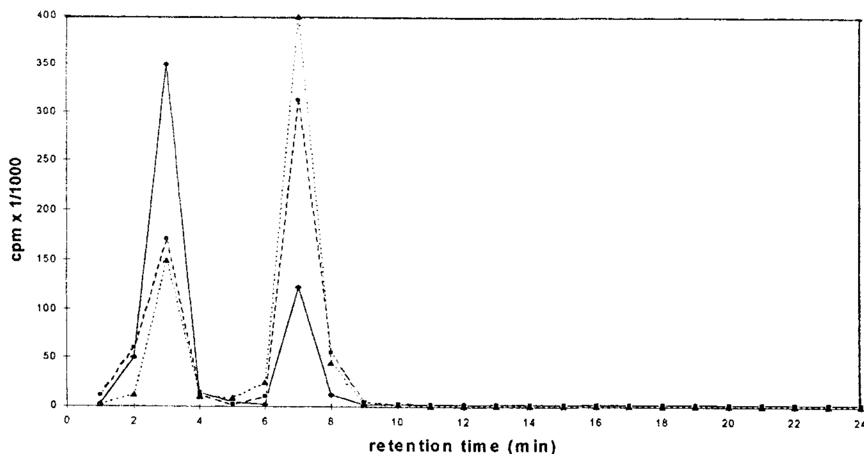
Figure 2. HPLC profile of pooled IEC fractions 21-32 (see figure 1) on a Spherisorb ODS II column (125 x 4.6 mm I.D., 5 µm particles). Aliquots (20 µl) of each fraction containing 1 mL of eluate were withdrawn and radioactivity measured; for chromatographic conditions see Experimental.

Downloaded At: 10:55 24 January 2011

possessing an intact Met^{11} moiety and thus can be ascribed to a substantial enrichment of ^{125}I-[Tyr^8]-(Met^{11})-SP at the trailing end of the IEC peak. This assumption was confirmed by gRP-HPLC. The ratio of ^{125}I-[Tyr^8]-(Met^{11})-SP to ^{125}I-[Tyr^8]-($Met^{11}\rightarrow O$)-SP in pooled fractions 22-24, 25-28, and 29-30 was 26/74, 58/42 and 73/27, respectively (Figure 3).

The peak exhibiting lower retention co-eluted with ^{125}I-[Tyr^8]-SP prepared by the chloramine-T method without mercaptoethanol treatment, whereas that showing higher retention co-eluted with a corresponding mercaptoethanol-treated sample. After treatment of fractions 22-24, 25-28, and 29-30 with mercaptoethanol (for experimental conditions see Refs. 21-23), the less retained peak completely disappeared and only the more retained peptide species was observed in the radiochromatogram (results not shown).

These results are in agreement with those from previous investigations,[21] providing proof that the more retained component formed during LPO-catalysed labeling consisted of the "true" ^{125}I-[Tyr^8]-(Met^{11})-SP derivative.



Downloaded At: 10:55 24 January 2011

Figure 3. HPLC profile of pooled IEC fractions 22-24 (solid line), 25-28 (broken line) and 29-30 (dotted line) on a Spherisorb ODS II column (125 x 4.6 mm I.D., 5 μm particles). Aliquots (10-20 μl, depending on the radioactivity) of each fraction containing 1 mL of eluate were withdrawn and radioactivity measured; for chromatographic conditions see Experimental.

Coupling between SP_{1-11} and ^{125}I-Bolton-Hunter reagent (i.e., mono-^{125}iodo-tyrosin-N-hydroxy-succinimidyl ester) afforded the corresponding ^{125}I-Bolton-Hunter-SP with a specific activity of about 2 x 10^6 Ci/g, corresponding to the value obtained with the LPO technique. Two main components in the ratio of approx. 3:1 and some peaks of minor intensity were observed in the radiochromatogram depicted in Figure 4. As calculated from the gRP-HPLC elution profile, about 54% of the radioactivity is recovered in the main component. Both major peaks of radioactivity exhibit significantly higher retention compared with ^{125}I-[Tyr^8]-(Met^{11})-SP, which is in accordance with incorporation of a more hydrophobic amino acid moiety and a concomitant decrease of the peptide's polarity. It is conspicuous that only fractions attributable to the main peak of radioactivity reveal an acceptable ratio of specific (i.e., total binding - non-specific binding) vs. non-specific binding, whereas in contrast, non-specific binding dramatically increases on either the leading or trailing end of the main peak. High values for specific binding of about 21, 24, and 18%, respectively were observed in fractions 26, 27, and 28. Comparable antibody-binding was also observed in fractions 29 and 30, but the unacceptable high non-specific binding precludes their use in RIA as well as receptor-binding experiments.

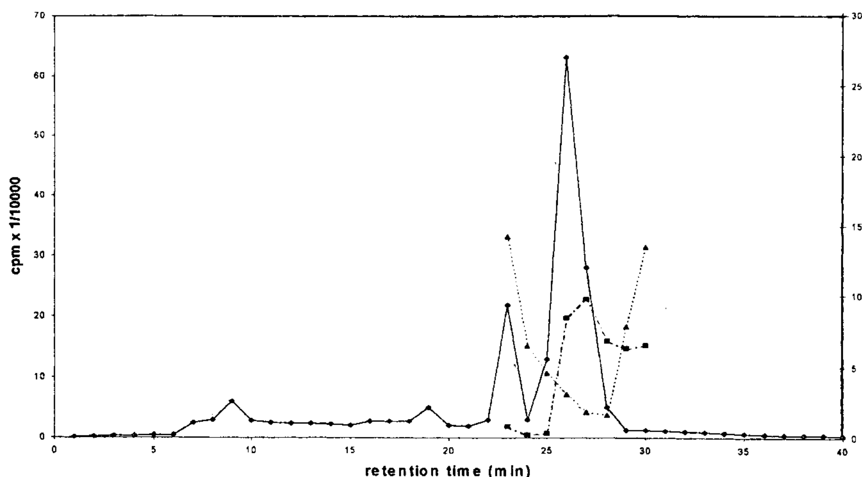


Figure 4. HPLC profile (solid line) of the crude reaction mixture obtained from coupling ^{125}I-Bolton-Hunter reagent to $SP_{1\text{-}11}$ on a Spherisorb ODS II column (125 x 4.6 mm I.D., 5 μm particles) Aliquots (10 μl) of each fraction containing 1 mL of eluate were withdrawn and radioactivity measured; for chromatographic conditions see Experimental. Specific (total binding - non-specific binding) as well as non-specific binding to the specific antibody SP 9-3a (scale on the right) were indicated by broken and dotted lines, respectively.

Downloaded At: 10:55 24 January 2011

DISCUSSION

There still exists a lack of structural information of radiolabeled compounds, which may be responsible for the widely differing biological and immunological properties of the tracer molecules prepared in different laboratories and supplied from different manufacturers. Furthermore, in most cases only very moderate information with concern to this aspect is available from producers of radiolabeled substances. In contrast to the purity assignment of unlabeled commercially available peptides on the basis of HPLC chromatograms, delivered by the manufacturers, radiochromatograms of the radiolabeled compounds are almost never available. However, in future investigations the modern chromatographic separation technology should be increasingly exploited for identification of by-products as well as structural characterization of radiolabeled compounds. In this paper, we report on the labeling of substance P derivatives using gentle labeling conditions, because this model substrate is highly susceptible to oxidation at its Met^{11} residue.

It is a well-known phenomenon that oxidation of Met residues under the conditions of radiolabeling takes place rapidly[24-37] and the poor biological activity of chloramine-T prepared ^{125}I-[Tyr^{8}]-SP was ascribed to formation of Met^{11}→O by Michelot et al.[36] It is noteworthy that generation of the unwanted ^{125}I-[Tyr^{8}]-(Met^{11}→O)-SP species seems to be extensively independent on the reaction time applied for labeling, because, as evidenced in our laboratory in a multitude of experiments, only the oxidised peptide was observed in the range of labeling times of 15 s up to 60 s. This finding raises the question, if sulfoxide generation and substitution of a proton in the aromatic ring of the Tyr^{6} moiety by ^{125}I takes place at the same time (i.e., in a so-called concertated reaction) or, as will be more probable, precedes the labeling reaction when chloramine-T was selected as the oxidizing agent.

As previously reported,[21-23] radiolabeling of [Tyr^{8}]-SP with ^{125}I by use of the chloramine-T procedure affords quantitative conversion of the Met^{11} residue to the corresponding sulfoxide. This observation is in accordance with the findings of Floor and Leeman.[37] The latter authors effected oxidation of non-radioactive SP undecapeptide to the corresponding sulfoxide, from which, after treatment with dithiothreitol, the starting material was quantitatively recovered. As evidenced by either Michelot et al.[36] or Liang and Cascieri,[38] marked reduction of biological activity is to be expected with chloramine-T as the oxidizing agent. Indeed, we have shown that the product of the chloramine-T method did not bind to rat brain membrane proteins,[23] although it still possessed sufficient immunoreactivity for the use in RIA experiments. However, when subjected to reductive treatment with mercaptoethanol, reconstitution of its biological activity took place and satisfactory receptor-binding was now achieved.

These findings are in agreement with reports that the chloramine-T/^{125}I system provides rather harsh labeling conditions presumably also being responsible for structural modifications of Cys, Cys-S-S-Cys, and Trp residues. As shown in the case of ring opening of the indole moiety of Trp-containing peptides,[39] iodine exerts a catalytic effect. This assumption is corroborated by the observation that chloramine-T alone, although being a rather strong oxidant, did not contribute to oxidative ring opening of the indole moiety.[39,40] The detrimental effect of the system chloramine-T/^{125}I plays an essential role during radioiodination of [Tyr^{0}]-somatostatin-14 (SST-14), where binding to a specific antibody substantially decreased when the peptide was subjected to the labeling conditions for more than 30 s.

However, in contrast to oxidation of the Met ^{11} moiety during labeling of [Tyr^{8}]-SP, incorporation of ^{125}I into Tyr^{0}-SST-14 seems to take place at significantly higher velocity than Trp^{8} oxidation. This assumption is

Downloaded At: 10:55 24 January 2011

substantiated by the observation that within a time interval of about 15-20 s, the target peptide was obtained in about 87% yield as evidenced by HPLC. Furthermore, it showed good binding to a specific antibody raised in rabbits against synthetic SST-14 (K. Rissler and H. Cramer, manuscript in preparation).

Despite being easily converted to the corresponding sulfoxide derivative by the chloramine-T technique, ^{125}I-[Tyr^8]-SP prepared by this latter procedure bound to receptors on dispersed pancreatic acinar cells from the guinea pig and on synaptic vesicles of rat brain, as reported by Sjödin et al.[41] and Mayer et al.,[42] respectively. In contrast, Michelot et al.[36] preferred the use of ^{125}I-Bolton-Hunter-SP (see below) compared with chloramine-T-prepared ^{125}I-[Tyr^8]-SP due to the unsatisfactory biological activity of the latter derivative, as also reported by Liang and Cascieri.[38]

Although interference of the lactoperoxidase procedure with Met and Cys moieties had been discussed,[43] we nevertheless attempted to overcome the complete oxidation of Met^{11} by selecting this more gentle alternative. In this respect, we were encouraged by the results obtained from LPO labeling of porcine insulin, affording a biologically active hormone.[44] Insulin does not contain any Met residues, but instead possesses three easily oxidisable disulfide linkages. However, these have apparently not been oxidized, since this would result in a complete loss of receptor-binding activity.

Despite the use of only traces of hydrogen peroxide (0.8 mM), substantial oxidation took place during the LPO procedure yielding an approximately equimolar ratio of [Tyr^8]-(Met^{11}→O)-SP and [Tyr^8]-(Met^{11})-SP. However, the IEC pre-purification step afforded at least substantial enrichment of the target compound in the more retained fractions. Although ^{125}I-[Tyr^8]-(Met^{11})-SP was enriched to about 75 % in fractions 29-30 (figure 1), the radioactive material has to be subjected to reductive treatment with mercaptoethanol in order to obtain sufficient receptor affinity. Further attempts to achieve labeling of [Tyr^8]-SP with ^{125}I via the LPO technique, to produce the desired radiopeptide without contaminants attributable to sulfoxide formation by application of different reaction conditions (i.e., concentration of substrate, catalyst, oxidising agent, buffer salt, pH), were not carried out. We believed that adjustment of "ideal" labeling conditions would be a rather laborious and time-consuming task primarily due to the multitude of variables influencing method development. This view is further supported by the observations of Greenwood et al.,[24] who hypothesized "noxious" influences of catalytically active iodide ions not only under the conditions of the chloramine-T but also of the LPO reaction. Nevertheless, ^{125}I-[Tyr^8]-(Met^{11})-SP prepared by the chloramine-T

Downloaded At: 10:55 24 January 2011

procedure and subsequent reduction of the sulfoxide derivative with mercaptoethanol represents an attractive alternative pathway. Additionally, the radiolabel can be easily purified from contaminants by gRP-HPLC and affords an overall yield of approx. 73 % as reported in Refs. 21 and 23.

For evaluating the potential advantages of ^{125}I-Bolton-Hunter SP_{1-11} with respect to the two-step procedure chloramine-T/^{125}I labeling - reductive treatment with mercaptoethanol, we decided to label SP_{1-11} with ^{125}I-Bolton-Hunter reagent.

Both, the profiles of specific and non-specific binding to the specific antibody SP 9-3a, as depicted in figure 4, revealed that radioactive material attributable to the main peak represents the target compound. However, compared with preparation of ^{125}I-[Tyr^{8}]-(Met^{11})-SP affording an overall yield of about 73 %,[21,23] formation of the ^{125}I-Bolton-Hunter SP_{1-11} yielded a substantially lower amount of radiolabeled peptide. Nevertheless, the yield of 54 % obtained in our laboratory is in satisfactory agreement with the findings of Liang and Cascieri,[45] who reported reproducible yields of about 60 %. However in contrast, Laufer et al.[46] obtained a yield of only 44 % of ^{125}I-Bolton-Hunter derivative by coupling of ^{125}I-Bolton-Hunter reagent to SP_{6-11}, which unlike the undecapeptide, contains only one free (N-terminal) amino group.

Downloaded At: 10:55 24 January 2011

Michelot et al.[36] reported that coupling of ^{125}I-Bolton-Hunter reagent preponderantly takes place at the ε-amino group of Lys^3 and only to a minor extent at both, the Lys^3 residue and the guanidine moiety of Arg^1, as was concluded from either dansylation or Edman degradation experiments. In contrast, as also shown by dansylation, Liang and Cascieri[45] provided evidence that only the N-terminal Arg^1 moiety is involved and no peptide reacted at the ε -amino group of Lys^3 was formed. The observation of different coupling positions of ^{125}I-Bolton-Hunter reagent by Michelot et al.[36] and Liang and Cascieri[45] can possibly be attributed to the use of different reaction conditions. This view is supported by the fact that Michelot et al.[36] have performed sequencing of non-radioactive ^{127}I-Bolton-Hunter SP, which was reacted with the peptide substrate in dimethylformide/aqueous borate buffer solution, whereas preparation of the ^{125}I-derivative was done in slightly alkaline aqueous borate buffer alone.

The authors discussed a substantial change in basicity of the N-terminal Arg^1 vs. the ε-amino group of Lys^3 by addition of the polar organic solvent. Obviously, despite the substantially higher basicity of Arg^1 (pK_b = 8.99) vs. the ε-amino group of Lys^3 (pK_b = 10.79), in buffered solutions containing dimethylformamide, coupling preferentially takes place at the Lys^3 position,

which was attributed to a substantial enhancement of the basicity of the reaction medium by the polar organic solvent. Liang and Cascieri[45] also used non-radioactive Bolton-Hunter SP for sequencing, but unfortunately no coupling conditions were given in their report. Nevertheless, it is assumed that the sites of SP's biological activity reside at the C-terminus, i.e., at amino acid positions 5-11 and thus, coupling of the reagent to either the N-terminal Arg^1 or the side-chain amino group of Lys^3 should be of only minor importance, because in both cases a radiolabel exhibiting sufficient immunoreactivity as well as receptor-binding activity may be generated.

With concern to specific activity, both ^{125}I-[Tyr^8]-SP prepared by the LPO technique and ^{125}I-Bolton-Hunter SP afford values of about 2×10^6 Ci/M and thus are in good agreement with the result obtained by use of the chloramine-T procedure of approximately 1.9×10^6 Ci/M.[21] Nevertheless, the LPO technique should be omitted due to either substantially longer reaction times and formation of marked amounts of ^{125}I-[Tyr^8]-($Met^{11}\rightarrow$O)-SP requiring subsequent treatment with a reducing agent, such as mercaptoethanol or dithiothreitol. When preparation of ^{125}I-Bolton-Hunter SP was compared with the two-step synthesis (chloramine-T/mercaptoethanol) affording ^{125}I-[Tyr^8]-(Met^{11})-SP, in our opinion the latter should be preferred, because each step runs in high individual yield and the final product is obtained in either high overall yield of about 73 % or sufficient purity. In addition, complete separation of by-products by gRP-HPLC possibly interfering with RIA as well as receptor-binding is unproblematic even in large scale preparations. However in contrast, the ^{125}I-Bolton-Hunter derivative is obtained in substantially lower yield (54 vs. 73 %, respectively) and, as shown in figure 4, separation of contaminants is much more difficult. For this reason, more than one chromatographic run may be required in order to obtain a tracer molecule with acceptable immuno- as well as biological activity, especially in large scale preparations.

CONCLUSIONS

During the search for alternative procedures for the preparation of radiolabeled substance P derivatives, we have applied the lactoperoxidase method for either direct introduction of ^{125}I into [Tyr^8]-SP or coupling of ^{125}I-Bolton-Hunter reagent to $SP_{1\text{-}11}$. Despite being described in the literature as a more gentle labeling technique compared with chloramine-T method, extensive conversion of the Met^{11} moiety to the corresponding sulfoxide took place when the system LPO/hydrogen peroxide is used for oxidation of iodine and reductive treatment with mercaptoethanol is required. Both, oxidised species and target

Downloaded At: 10:55 24 January 2011

compound were obtained in approximately equimolar amounts. Generation of ^{125}I-[Tyr^{8}]-(Met^{11}→O)-SP may be ascribed to the additional detrimental (catalytic) effect of the iodide ions. Although specific activity of the ^{125}I-Bolton-Hunter derivative was comparable with ^{125}I-[Tyr^{8}]-SP prepared by the chloramine-T technique, it suffers from either lower yields or formation of substantial amounts of by-products. In contrast to those formed by the two-step procedure with chloramine-T/mercaptoethanol they cannot be easily separated in large scale preparations and thus an additional chromatographic separation step may be required. Furthermore, ^{125}I-Bolton-Hunter reagent is much more expensive compared with Na^{125}I due to its preparation and purification.

In conclusion, the LPO technique is completely unsuited for generating ^{125}I-[Tyr^{8}]-(Met^{11})-SP, whereas in contrast, the ^{125}I-Bolton-Hunter derivative can be used as a more attractive alternative. Nevertheless, the two-step procedure for preparation ^{125}I-[Tyr^{8}]-(Met^{11})-SP offers some strong advantages primarily due to either high yields or ease of purification.

Downloaded At: 10:55 24 January 2011

REFERENCES

† Present address of Klaus Rissler: Ciba Specialty Chemicals, K-401, 2.08, CH-4002 Basel, Switzerland.

1. U. S. von Euler, J. H. Gaddum, J. Physiol. (London), **72**, 74 (1931).

2. M. M. Chang, S. E. Leeman, H. D. Niall, Nature (London), New Biol., **232**, 86 (1971).

3. G. W. Tregear, H. D. Niall, J. T. Potts Jr., S. E. Leeman, M. M. Chang, Nature (London), New Biol., **232**, 87 (1971).

4. H. Nawa, T. Hirose, H. Takashima, S. Inayama, S. Nakanishi, Nature (London), **306**, 32 (1983).

5. E. Heyman, R. Mentlein, FEBS Lett., **91**, 360 (1978).

6. S. Blumberg, V. I. Teichberg, J. L. Charli, L. B. Hersh, J. F. McKelvy, Brain Res., **192**, 477 (1980).

7. R. Matsas, M. Rattray, A. J. Kenny, A. J. Turner, Biochem. J., **228**, 487 (1985).

8. R. A. Skidgel, S. Engelbrecht, A. R. Johnson, E. G. Erdös, Peptides, **5**, 769 (1984) .

9. M. Hatanaka, T. Sasaki, T. Kikuchi, T. Murachi, Arch. Biochem. Biophys., **242**, 557 (1985).

10. F. Lembeck, Arch. Exper. Pathol. Pharmacol., **219**, 197 (1953).

11. B. Pernow, J. Immunol., **135**, 812s (1985).

12. P. Cesaro, Rev. Neurol. (Paris), **140**, 465 (1984).

13. J. Solway, A. R. Leff, J. Appl. Physiol., **71**, 2077 (1991).

14. P. J. Barnes, Int. Arch. Allergy Appl. Immunol., **94**, 303 (1991).

15. D. Stretton, Clin. Exp. Pharmacol. Physiol., **18**, 675 (1991).

16. J. G. Widdicombe, Am. Rev. Respir. Dis., **143**, S18 (1991).

17. P. J. Barnes, Am. Rev. Respir. Dis., **143**, S 28(1991).

18. J. L. Vaught, Life Sci., **43**, 1419 (1988).

19. M. Otsuka, M. Yanagisawa, Cell. Mol. Neurobiol., **10**, 293 (1990).

20. A. Eglezos, P. V. Andrews, R. L. Boyd, R. D. Helme, Immunol. Cell. Biol., **69**, 285 (1991).

21. K. Rissler, H. Cramer, J. Chromatogr., **564**, 67 (1991).

22. K. Rissler, H. Cramer, **Methods of Neuroscience: Neuropeptides, Analogs, Conjugates, and Fragments**, P. M. Conn (Editor), Academic Press, Vol. 13, San Diego, USA, pp. 360 (1993).

23. K. Rissler, H. Cramer, P. Engelmann, accepted for publication in J. Chromatogr., B (May, 1997).

24. F. C. Greenwood, W. M. Hunter, J. S. Glover, Biochem. J., **89**, 114 (1963).

25. J. J. Miller, G. S. Schultz, R. S. Levy, Int. J. Peptide Protein Res., **24**, 112 (1984).

Downloaded At: 10:55 24 January 2011

26. M. E. Koshland, F. M. Englberger, M. J. Erwin, S. M. Gaddone, J. Biol. Chem., **238**, 1343 (1963).

27. B. H. Stagg, J. M. Temperley, H. Rochman, J. S. Morley, Nature (London), **228**, 58 (1970).

28. C. Gros, P. Pradelles, C. Rouget, O. Bepoldin, F. Dray, M. C. Fournie-Zaluski, B. P. Roques, H. Pollard, C. Llorens-Cortes, J. C. Schwartz, J. Neurochem., **31**, 29 (1978).

29. R. A. Rosenberg, T. M. Murray, Biochim. Biophys. Acta, **584**, 261 (1979).

30. C. B. Heward, Y. C. S. Yang, J. F. Ormberg, M. E. Hadley, V. J. Hruby, Hoppe Seyler's Z. Physiol. Chem., **360**, 1851 (1979).

31. N. G. Seidah, M. Dennis, P. Corvol, J. Rochemont, M. Chrétien, Anal. Biochem., **109**, 185 (1980).

32. V. Clement-Jones, P. J. Lowry, L. H. Rees, G. M. Besser, Nature (London), **283**, 295 (1980).

33. W. G. Wood, C. Wachter, P. C. Scriba, Fresenius Z. Anal. Chem., **301**, 119 (1980).

34. U. Gether, H. V. Nielsen, T. W. Schwartz, J. Chromatogr., **447**, 341 (1988).

35. J. W. Leidy, Jr., J. Chromatogr., **483**, 253 (1989).

36. R. Michelot, H. Gozlan, J.-C. Beaujouan, M.-J. Besson, Y. Torrens, J. Glowinski, Biochem. Biophys. Res. Comm., **95**, 491 (1980).

37. E. Floor, S. E. Leeman, Anal. Biochem., **101**, 498 (1980).

38. T. Liang, M. A. Cascieri, J. Neurosci., **1**, 1133 (1981).

39. G. Mourier, L. Moroder, A. Previero, Z. Naturforsch., **39b**, 101 (1984).

40. N. M. Alexander, J. Biol. Chem., **249**, 1946 (1974).

41. L. Sjödin, E. Brodin, G. Nilsson, T. P. Conlon, Acta Physiol. Scand., **109**, 97 (1980).

Downloaded At: 10:55 24 January 2011

42. N. Mayer, F. Lembeck, A. Saria, R. Gamse, Naunyn Schmiedeberg's Arch. Pharmacol., **306**, 45 (1979).

43. M. Morrison, G. R. Schonbaum, Annu. Rev. Biochem., **45**, 861 (1976).

44. K. Rissler, P. Engelmann, J. Chromatogr. B, **679**, 21 (1996).

45. T. Liang, M. A. Cascieri, Biochem. Biophys. Res. Comm., **96**, 1793 (1980).

46. R. Laufer, U. Wormser, Z. Selinger, M. Chorev, C. Gilon, J. Chromatogr., **301**, 415 (1984).

Received August 15, 1997
Accepted October 17, 1997
Manuscript 4601

Downloaded At: 10:55 24 January 2011