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Journal of Chromatography B, 656 (1994) 115–118

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short Communication

Synthesis and high-performance liquid chromatographic purification of tritiated thyrotrophin-releasing hormone-like peptides

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Abstract

A simple method is presented for the synthesis and RP-HPLC purification of tritium-labelled thyrotrophin-releasing hormone (TRH)-like tripeptides. These peptides differ from TRH (pGlu–His–Pro–amide) in that they possess a neutral or acidic residue in place of the histidine of TRH. The method involves the preparation of the appropriate dipeptide by a solid-phase peptide synthesis procedure using 9-fluorenylmethoxycarbonyl (Fmoc) protection. Very small amounts of tritiated glutamine are then converted into tritiated pyroglutamic acid, and coupling to the dipeptide is effected using a mixed anhydride derived from Fmoc–phenylalanine and the tritiated pyroglutamic acid. The required labelled product is then separated from unlabelled material by reversed-phase HPLC, as the hydrophobicity of the phenylalanine-containing product ensures that it is strongly retained. The availability of a series of tritium-labelled markers prepared by this method has permitted the unequivocal identification of certain naturally occurring TRH-like peptides.

1. Introduction

It has been reported that thyrotrophin-releasing hormone (TRH) is the major TRH-immunoreactive peptide in rat prostate [1], but recent studies have shown that the TRH immunoreactivity in the prostate is due principally to the presence of a series of pyroglutamyl tripeptides that differ from TRH. One of these has been identified as pGlu–Glu–Pro–amide [2] whereas others appear to have a neutral amino acid in place of the histidine of TRH [3]. In order to facilitate the identification of the new peptides in various tissues, we have developed a simple method for the synthesis of tritium-labelled pyroglutamyl tripeptides [4] which can be used as internal standards. The main advantage in the use of such a standard is that the elution positions of the endogenous peptides can be compared with the elution profile of the radioactive markers. In addition, the successful chromatography of the labelled peptides provides assurance that the unknown endogenous peptides present in the same mixture are also likely to be equally well resolved. The peptides extracted from the tissues are purified, together with the tritium-labelled markers, in separation systems based on minicolumn cation-exchange chromatography or

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0378-4347/94/$07.00
SSDI 0378-4347(94)00057-C

column gel permeation chromatography and gradient high-performance liquid chromatography (HPLC). Analysis of the effluents from these columns is carried out using TRH radioimmunoassay (RIA) [5,6].

The synthesis of the tritiated pyroglutamyl tripeptides involves the preparation of an appropriate dipeptide by a solid-phase procedure and coupling of this to ^{3}H-labelled pyroglutamic acid by a mixed anhydride method according to the following reaction scheme:

[^{3}H]pGLU + Fmoc–Phe

↓

[^{3}H]pGlu–CO–O–CO–Phe–Fmoc

+ Fmoc–X–Pro–amide

↓

[^{3}H]pGlu–X–Pro–amide + Phe–X–Pro–amide

where X is the amino acid required at position 2 of the ^{3}H-labelled tripeptide and Fmoc is 9-fluorenylmethoxycarbonyl.

The tritium-labelled product is separated readily from the expected by-product by reversed-phase HPLC.

2. Experimental

2.1. Conversion of [^{3}H]glutamine into [^{3}H]pyroglutamic acid

A 250-μCi amount of [^{3}H]glutamine (Amersham International, Amersham, UK) (5 nmol in 250 μl of 2% ethanol) was incubated overnight in 10 μl of 0.1 *M* sodium phosphate buffer (pH 5.3) at 37°C or for 4 h at 100°C.

2.2. Preparation of the asymmetric anhydride of [^{3}H]pyroglutamic acid and phenylalanine

Fmoc-Phe (25 μmol), [^{3}H]pyroglutamic acid (5 nmol) and dicyclohexylcarbodiimide (30 μmol) were dissolved in dichloromethane–dimethylformamide (9:1, v/v) and the mixture was allowed to stand at room temperature for 1 h. The dicyclohexylurea formed was removed by filtration and the resulting solution was evaporated to dryness *in vacuo*. The residue was taken up in dimethylformamide (1 ml) for storage at −20°C.

2.3. Synthesis of tritium-labelled tripeptides

The resin-linked dipeptides Fmoc–Phe–Pro–resin, Fmoc–Glu–(*tert.*-butyl)Pro–resin and Fmoc–Val–Pro–resin were prepared on 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy-resin (Novabiochem, Nottingham, UK) using Fmoc–amino acid pentafluorophenyl esters. This resin was selected for formation of peptide amides.

The N-terminal Fmoc group of the dipeptide resin (100 mg, 42 μmol) was cleaved with 20% piperidine in dimethylformamide for 10 min and washed with dimethylformamide and dichloromethane. The solution of the mixed anhydride of Fmoc–phenylalanine and tritiated pyroglutamic acid was then added. Coupling was allowed to take place overnight at room temperature. The resin carrying the synthesized tripeptide was washed with dimethylformamide, resuspended in 5 ml of dichloromethane–trifluoroacetic acid (98:2, v/v) and then shaken for 2 h at room temperature to liberate the peptide amide. The resin was removed by filtration and the remaining solution containing the labelled tripeptide was evaporated to dryness. The *tert.*-butyl group was removed in trifluoroacetic acid–water (95:5, v/v), the reaction requiring 2 h at room temperature. After removal of the solvent, the residue was taken up in 10 m*M* HCl.

The tissue was homogenized in dilute acetic acid or in acidified acetone, extracted in methanol and the peptides obtained were concentrated on a minicolumn cation-exchange resin (SP Sephadex C-25) or by column gel permeation chromatography (Sephadex G-25). Fractions exhibiting TRH-like immunoreactivity (TRH-LI) were separated by reversed-phase HPLC and fractions collected for subsequent RIA.

2.4. HPLC

HPLC was carried out on a μBondapak C_{18} stainless-steel column (300 × 3.9 mm I.D., 10-μm particle size) (Millipore–Waters, Milford, MA, USA) or on a Separon SGX C_{18} stainless-steel column (250 × 4.0 mm I.D., 10-μm particle size) (Tessek, Prague, Czech Republic) using a linear 10 m*M* HCl–methanol gradient (0.5% min/methanol for 6 min, followed by 2% min/methanol for 30 min) at a flow-rate of 1.5 ml/min. Fractions were collected and the elution profile of the tritiated tripeptide was monitored by scintillation counting of 20-μl aliquots.

2.5. Radioimmunoassay

RIA was performed using a sheep antiserum raised against synthetic TRH donated by Fraser and McNeilly as described previously [7]. The method employed has been reported [5,6]. The final dilution of antiserum was 1:96 000. The antibody exhibits a high specificity for the pyroglutamyl residue at the tripeptide position and for the C-terminal amide group; however, the antibody can accept a range of amino acids at position 2.

3. Results and discussion

The synthesis of tritiated TRH-like tripeptides could not be achieved by using nanomolar amounts of carrier-free tritiated pyroglutamic acid for its coupling to a resin dipeptide in the solid phase. Under these conditions the incorporation of tritiated pyroglutamic acid to the resin dipeptides did not take place to a significant extent, probably because of the difficulty of converting such a small amount into the symmetrical anhydride. Clearly, the direct coupling of small amounts of tritiated pyroglutamic acid to the dipeptides attached to the resin fails. Therefore, the acid was converted into its more reactive mixed anhydride with an excess of Phe as the other acid component to ensure quantitative formation of the intermediate. However, the preparation of a mixed anhydride of tritiated pyroglutamic acid and unlabelled Fmoc–Phe present in large excess was successful and in this instance more than 90% of the tritiated pyroglutamic acid was incorporated into the tripeptide. Tritiated pGlu was prepared from commercially available tritiated Gln and this was coupled in nanomolar amounts as a mixed anhydride with an unlabelled Fmoc–Phe. The latter was present in large excess to promote the formation of the anhydride. The removal of the phenylalanine-containing tripeptide, formed as a by-product, from the desired pyroglutamyl tripeptide was carried out without difficulty by reversed-phase HPLC using gradient elution with methanol–10 m*M* HCl. The aromaticity of the phenylalanine-containing peptide ensured that it was strongly retained on the stationary phase of the column.

The resolution of different TRH-like peptides, which were used as markers for the identification of TRH immunoreactivity in human prostate, is demonstrated in Fig. 1 and the corresponding experiments on the peptides in human testis are shown in Fig. 2. Owing to the use of these radiolabelled markers, identification of the corresponding peptides that occur in mammalian tissues has been markedly facilitated. From the chromatogram depicted in Fig. 1 it is evident that a major portion of TRH-LI in human prostate is attributable to fractions co-eluting with the pGlu–Glu–Pro–amide. In contrast, extracts from human testis after tripsin digestion possess considerable amounts of TRH-LI, which

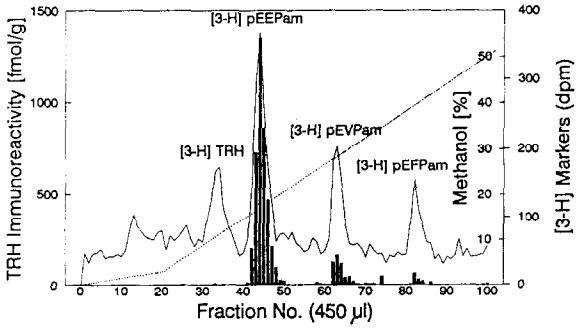

Fig. 1. Elution profile of tissue extracts using tritiated TRH-like peptides as internal standards in the RP-HPLC determination of TRH-LI in human prostate. ■ = TRH immunoreactivity; solid line = ^3H markers (dpm); dotted line = methanol (%).

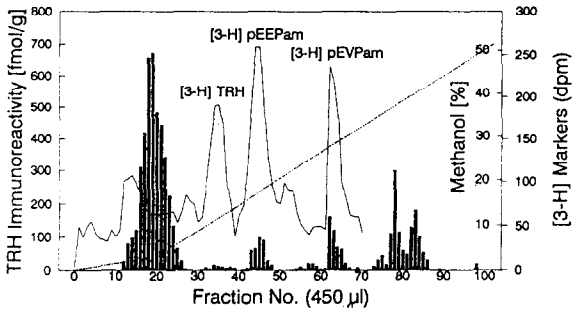

Fig. 2. Elution profile of TRH-LI from a tryptic digest of human testis using tritiated TRH-like peptides as internal standards. Identification as in Fig. 1.

do not co-elute with the synthetic radiolabelled TRH or TRH-like peptides and thus imply different amino acid sequences. From these experiments, it is clear that the main TRH immunoreactivity in human prostate is accounted for by pGlu–Glu–Pro–amide, as the TRH immunoreactivity co-chromatographed with ^{3}H-labelled marker peptide. In the experiments on human testis, TRH immunoreactivity was observed only after trypsin digestion, which indicates that the TRH immunoreactive fragment forms the C-terminal end of a larger peptide and is released only after cleavage at a trypsin-sensitive site on the N-terminal side of the tripeptide. It is of particular interest that the TRH immunoreactive peptide released from the larger forms in the testis did not possess the same retention parameters as pGlu–Glu–Pro–amide and thus represents a different TRH-like peptide from that present in the prostate.

4. Acknowledgements

We are grateful to Dr. H. Fraser, Edinburgh University, for generously donating the TRH antiserum. This work was funded by the Wellcome Trust, by the Internal Grant Agency of the Czech Ministry of Health (No. 0849-3) and by the Grant Agency of the Czech Republic (No. 0994), who provided a research fellowship for R.B., and by EC Grant (No. SC1-CT92-0762) of D.G.S.

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