Biochem. J. (1977) **168**, 9–13
Printed in Great Britain

Use of Octadecasilyl-Silica for the Extraction and Purification of Peptides in Biological Samples

APPLICATION TO THE IDENTIFICATION OF CIRCULATING METABOLITES OF CORTICOTROPIN-(1–24)-TETRACOSAPEPTIDE AND SOMATOSTATIN *IN VIVO*

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(*Received* 17 *March* 1977)

Peptides can be adsorbed on octadecasilyl-silica from large volumes of aqueous solution and eluted with aqueous solvent mixtures containing methanol or acetonitrile. These properties may be used for the extraction and purification of peptide fragments in plasma samples collected from rats. After intravenous injection of Synacthen [corticotropin-(1–24)-tetracosapeptide], it was shown that within 2min the main circulating products were intact peptide and its sulphoxide. In addition, a number of fragments indicative of cleavage at the *N*- and *C*-termini were present. Most of the products formed from Synacthen have low biological activity. Somatostatin was rapidly cleaved *in vivo* and *in vitro* to a single product, which probably retains biological activity. The absence of other circulating products suggests that somatostatin is only inactivated once it leaves the circulation.

Although the metabolism of peptide hormones has attracted the attention of investigators for many years, detailed chemical information about the inactivation of most peptide hormones *in vivo* is still unavailable. It is important that studies should be carried out *in vivo*, since many peptides will not readily cross cell membranes, and studies with tissue homogenates, where compartmentalization of enzymes is destroyed, may have little bearing on the fate of circulating peptide. The present paper outlines a simple method for extracting, purifying and identifying peptide fragments in the circulation after administration of suitable doses of intact peptide.

Materials and Methods

Materials

Disposable syringes used for the extraction columns were from Gillette Surgical, Isleworth, Middx., U.K. Porous Teflon discs were cut from sheets obtained from Aerox Plastics, Stroud, Glos., U.K. Synacthen [corticotropin-(1–24)-tetracosapeptide] and somatostatin were kindly supplied by Dr. W. Rittel, CIBA-GEIGY, Basle, Switzerland. Prepacked Partisil-ODS (particle size 10μm) columns were obtained from Reeve Angel, London S.E.1, U.K. Spherisorb (particle size 10μm) was from Phase Separations, Queensferry, Clwyd, Wales, U.K. Porasil A (35–70μm mesh) was from Waters Associates, Northwich, Cheshire, U.K., and octadecatrichlorosilane was from Aldrich Chemical Co., Gillingham, Dorset, U.K. Where possible other reagents were analytical-reagent grade.

Methods

For investigation of the metabolism of Synacthen, heparin-treated Nembutal-anaesthetized male rats were exsanguinated through the dorsal aorta at various times after intravenous administration of a mixture of peptides labelled specifically with ^3H in residue 2, 7 or 23 (Brundish & Wade, 1973, 1976; Brundish *et al.*, 1976). Plasma (about 4ml/rat) was deproteinized with an equal volume of 30% (v/v) trifluoroacetic acid, and the supernatant was applied to an extraction column consisting of 0.5ml of octadecasilyl-Spherisorb (10μm) packed between two porous Teflon discs in a 1ml plastic syringe barrel. The syringe plunger was used to pass 0.5ml portions of sample through the bed, which was washed (3×0.5ml of 1% trifluoroacetic acid) and eluted with 0.5ml of methanol/water/trifluoroacetic acid (80:19:1, by vol.). The eluate contained 90% of the adsorbed radioactivity measured by liquid-scintillation spectrometry (Bennett *et al.*, 1974). Extraction of one plasma sample takes about 5min. Extracts can be film-dried and redissolved in a small volume of 1% trifluoroacetic acid for injection, or diluted with 3vol. of 1% trifluoroacetic acid and pumped directly on to octadecasilyl-silica high-pressure columns.

For somatostatin, the extraction procedure was modified since large losses of this peptide occurred during deproteinization with 15% trifluoroacetic

acid. Plasma was extracted directly by using a larger-particle-size octadecasilyl-silica [Porasil A (35–70 μm) treated with 10% (v/v) octadecyltrichlorosilane in chloroform and rinsed with chloroform followed by methanol]. A 1 ml bed volume was used for 4–5 ml of plasma, and 2 ml of eluate (with methanol/water/trifluoroacetic acid, 80:19:1, by vol.) was collected. Extracts were diluted and applied to high-pressure octadecasilyl-silica columns, which were monitored for A_{280} and for free thiol groups by the Ellman (1959) reaction. Recoveries of somatostatin (linear or cyclic) added to blood were about 90%. As shown in Fig. 3(*a*), when control plasma extracts are monitored in the u.v. several background peaks are observed.

Peptide fragments were identified by their amino acid content, determined by amino acid analysis after acid hydrolysis or after complete enzyme digestion (Bennett *et al.*, 1972). Enzyme digestion releases unchanged methionine sulphoxide, and this amino acid can be measured quantitatively, since it separates from methionine on the amino acid analyser column.

Results and Discussion

The methods for plasma extraction and high-resolution chromatography are based on the powerful adsorption of peptides to hydrophobic octadecasilyl-silica and their elution with an aqueous solvent mixture containing a comparatively hydrophobic component, such as methanol or acetonitrile, and a volatile electrolyte, such as 1% trifluoroacetic acid (to minimize ion-exchange effects). The method is similar in principle to silica extraction used in radioimmunoassay (Ratcliffe & Edwards, 1971), but (1) with methanol or acetonitrile instead of acetone undamaged peptide is recovered, (2) octadecasilyl-silica adsorbs more strongly and extends the adsorption range to smaller peptides, (3) peptide is quantitatively adsorbed and can be quantitatively eluted in small solvent volumes and (4) high-pressure columns of 10 μm octadecasilyl-silica give much better resolution than untreated silica, with quantitative recoveries. Fig. 1 shows the separation of a peptide mixture obtained by using a linear solvent gradient and an octadecasilyl-silica column. Equally sharply defined peaks are obtained when peptides are added to plasma and extracted.

The elution position of a peptide probably depends on its total content of hydrophobic residues and their distribution. Elution time is extremely sensitive to solvent composition, a few per cent decrease in organic-solvent concentration increasing the elution volume severalfold. Thus after dilution of an extract with water to decrease the concentration of organic solvents, peptides will strongly adsorb on octadecasilyl-silica. This allows extract diluted with water to be pumped directly on to a high-resolution octadecasilyl-silica column.

Fig. 2 shows a chromatogram obtained for plasma collected 2 min after intravenous injection of 1 mg of [^{3}H]Synacthen/rat (similar chromatograms were obtained with 3 μg/animal, indicating that the larger

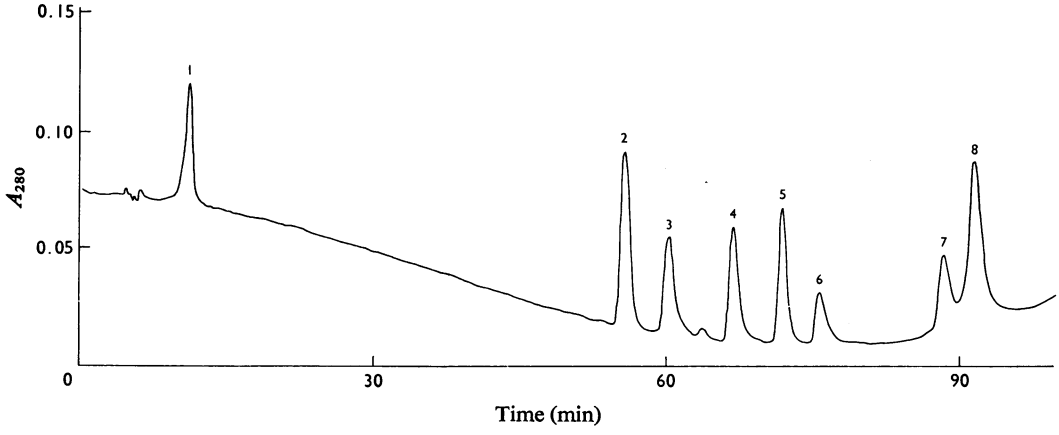

Fig. 1. *High-pressure liquid chromatography of a mixture of peptides*
Peptides dissolved in a few microlitres of 1% trifluoroacetic acid were injected on to a 4mm×250mm Partisil-ODS (10 μm) prepacked column (Reeve Angel), which was eluted at 0.7 ml/min with a linear gradient from 1% trifluoroacetic acid to methanol/water/trifluoroacetic acid (80:19:1, by vol.) in a total volume of 100 ml. Eluate was monitored with a 2.5 cm-pathlength cell in a Uvicord II spectrophotometer (Lowry & McMartin, 1974). The peptides were as follows: 1, D-Ser-Tyr (10 μg); 2, corticotropin-(4–10)-heptapeptide (8 μg); 3, [D-Ser^1,Lys^{17,18}]corticotropin-(1–18)-octadecapeptide amide (16 μg); 4, corticotropin-(1–24)-tetracosapeptide (Synacthen) (40 μg); 5, somatostatin (10 μg); 6, human corticotropin-(1–39)-nonatriacontapeptide (16 μg); 7, pig insulin (50 μg); 8, pig glucagon (50 μg).

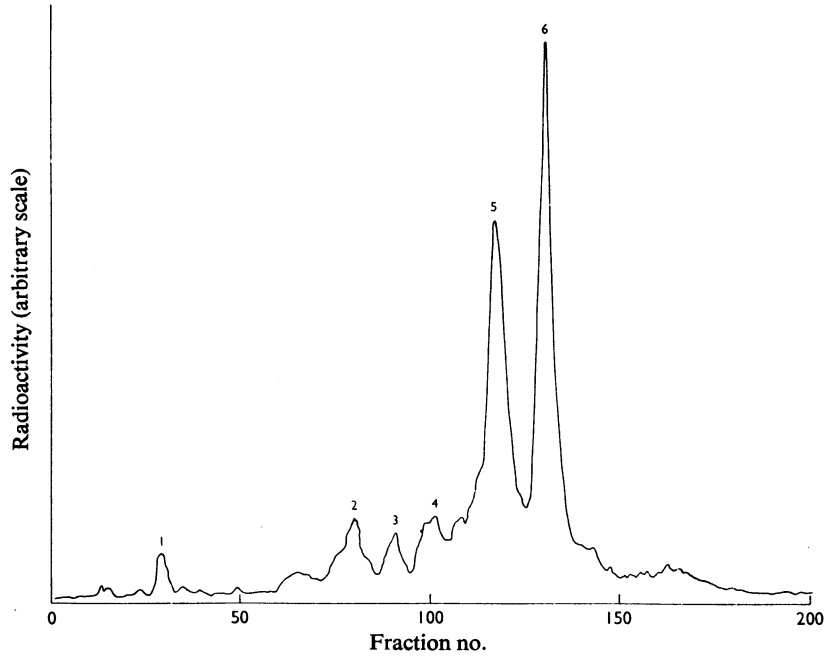

Fig. 2. *High-pressure liquid chromatography of ^3H-labelled peptides extracted from the circulation 2 min after intravenous injection of [^3H]Synacthen*

Plasma extracts from ten rats were applied to a column (4mm×250mm) of Partisil-ODS (10μm). A linear gradient from methanol/water/trifluoroacetic acid (20:79:1, by vol.) to methanol/water/trifluoroacetic acid (55:44:1, by vol.) (volume 86ml) at a flow rate of 0.7ml/min was used. The pressure was in the range 4.2–5.6MPa (600–800lbf/in^2). Radioactivity was determined in 10μl portions of 0.5min fractions. Products, further purified by CM-cellulose chromatography (Bennett *et al.*, 1974), were identified by amino acid analysis after acid hydrolysis or complete enzyme digestion (Bennett *et al.*, 1972). This allows unequivocal identification of methionine sulphoxide in peak 5. The amounts of sulphoxide found *in vivo* are much larger than those formed in control extractions of peptide added to blood. The peaks contained the following fragments of corticotropin: 1, (17–24)-octapeptide and (18–24)-heptapeptide; 2, (9–24)-hexadecapeptide; 3, (1–20)-eicosapeptide, (2–20)-nonadecapeptide and (3–20)-octadecapeptide; 4, (1–15)-quinquedecapeptide; 5, (1–24)-tetracosapeptide sulphoxide; 6, (1–24)-tetracosapeptide (Synacthen). The peptides in peak 1 separated on CM-cellulose chromatography and were individually identified; those in peak 3 partially separated on CM-cellulose chromatography and were deduced from a single amino acid analysis.

dose does not saturate inactivation mechanisms). After further purification by CM-cellulose chromatography (Bennett *et al.*, 1974), the products were identified as shown in the Fig. 2. It is generally assumed that peptide inactivation is a result of catabolism by peptidases, but this experiment shows that oxidation of methionine is also an important route for the inactivation of Synacthen (the sulphoxide has very low biological activity). Identification of peptide fragments in this and other experiments (Hudson *et al.*, 1977) revealed, in addition to oxidation, the cleavage sites shown in Fig. 3. Cleavages within the 1–18-portion of the molecule will produce fragments of low or insignificant potency (McMartin *et al.*, 1977).

Somatostatin is widely distributed throughout the body and has a multiplicity of inhibitory properties (Vale *et al.*, 1975). *In vivo* its duration of action is very short, and this has hampered experimental and clinical investigations.

At 1 min after intravenous administration to rats of linear (reduced) somatostatin, no u.v.-absorbing or thiol-containing peptides, apart from u.v.-absorbing products present also in control samples, were detectable in plasma. This indicates that less than 2% of the injected dose remained in the circulation. However, with cyclic somatostatin, in addition to the control peaks a single u.v.-absorbing peak corresponding in position to somatostatin was found in plasma collected 1 min after injection (Fig. 4). This peak normally accounted for 10–20% of the dose and contained no reduced peptide. After reduction with dithiothreitol at pH8.0, it chromatographed as a single peak corresponding in position to reduced

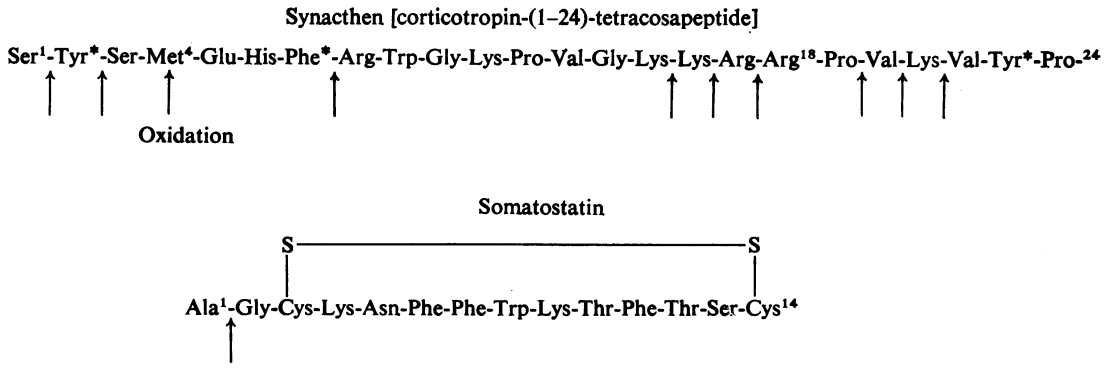

Fig. 3. *Sequences of Synacthen and somatostatin, showing points of cleavage and oxidation deduced from the identification of circulating fragments*

*, Positions specifically labelled with ^3H. Arrows show identified sites of cleavage. The formation of Synacthen sulphoxide is indicated by the arrow pointing to Met⁴.

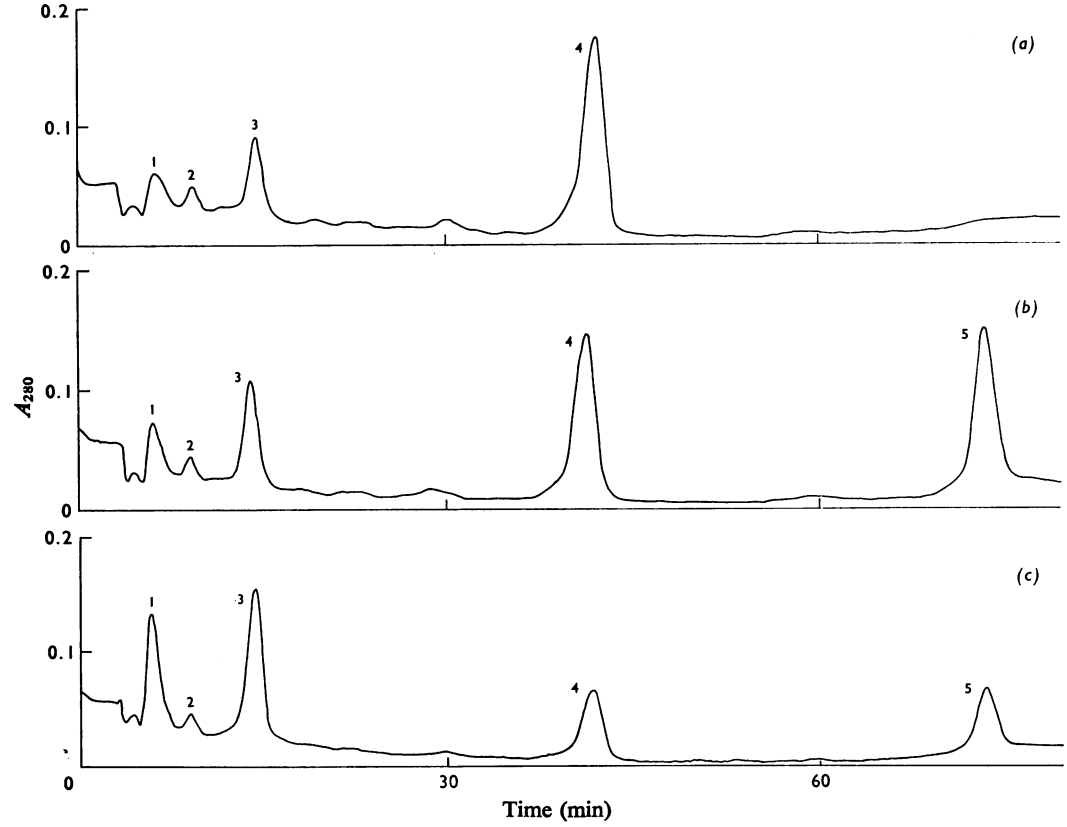

Fig. 4. *Detection of peptide(s) similar to somatostatin in the circulation after intravenous administration of somatostatin*

Plasma was extracted as described under 'Methods'. Chromatographic conditions and detection system were as in Fig. 1, except that octadecasilyl-Spherisorb (10 μm) was used. (*a*) Control plasma from an untreated rat. Peaks 1–4 are usually found in control samples and the amounts can vary appreciably. (*b*) Control plasma + 100 μg of somatostatin (cyclic). Peak 5 was somatostatin. (*c*) Plasma collected 1 min after intravenous administration of 1 mg of somatostatin to a rat. Amino acid analysis of peak 5 showed that it contained only 0.2 of a residue of alanine and therefore consisted of a 4:1 mixture of des-Ala¹-somatostatin and intact somatostatin.

somatostatin, suggesting absence of cleavage of any peptide bond lying between the cysteine residues at positions 3 and 14. Amino acid analysis confirmed that these residues were present, but the presence of only 0.1–0.2 of a residue of alanine showed that 80–90% of the peptide in the peak was des-Ala^1-somatostatin, the rest being intact somatostatin. Thus after 1 min only a small proportion of the dose was present in the circulation, and most of this had been rapidly converted into des-Ala^1-somatostatin. Structure–activity studies (Vale *et al.*, 1975) suggest that removal of the first two amino acids from the *N*-terminus does not alter potency. However, no studies have been reported on des-Ala^1-somatostatin as such, and the possibility remains that conversion into the product may have biological significance. In investigations of somatostatin *in vivo*, it is likely that the biological responses are in fact caused by the des-Ala^1 form (Fig. 3). It is evident that more information is needed about the forms of somatostatin acting in various test systems and acting locally when endogenous peptide is released.

In conclusion, the methods described in this paper have proved useful for studying the behaviour of circulating peptides in the first minutes after injection. For Synacthen [corticotropin-(1–24)-tetracosapeptide] this has given information about processes that inactivate the molecule, but it seems likely that a full understanding of the inactivation of somatostatin requires investigation of its fate once it has left the circulation.

We thank Dr. D. E. Brundish, Mr. J. R. Martin and Dr. R. Wade for the ^{3}H-labelled corticotropins, Dr. W. Rittel for somatostatin and corticotropin analogues and Mr. B. E. Evans for carrying out the amino acid analyses.

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