Journal of Chromatography, 296 (1984) 241–247
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 365

METABOLISM OF SUBSTANCE P IN HUMAN PLASMA AND IN THE RAT CIRCULATION

J. MICHAEL CONLON*
Clinical Research Unit for Gastrointestinal Endocrinology of the Max-Planck-Gesellschaft, Humboldtallee, 1, D-3400 Göttingen (F.R.G.)
and
BURKHARD GÖKE
Division of Gastroenterology and Metabolism, Department of Medicine, University of Göttingen, D-3400 Göttingen (F.R.G.)

SUMMARY

Incubation of substance P in human plasma at 37°C resulted in rapid conversion to des (Arg[1]-Pro[2]) substance P (fragment 3–11) and to des (Arg[1]-Pro[2]-Lys[3]-Pro[4]) substance P (fragment 5–11). The metabolites were purified by high-performance liquid chromatography and identified by sequence analysis. These data are consistent with the hypothesis that substance P is metabolized by enzyme(s) with the specifity of dipeptidyl aminopeptidase IV (EC 3.1.14.5). Analysis by high-performance liquid chromatography of plasma extracts following intravenous infusion of Substance P (300–350 nmoles) into anaesthetized rats showed that the peptide was cleared from the circulation within 1–2 minutes. No circulating metabolites could be identified.

INTRODUCTION

Although regarded primarily as a neurotransmitter or neuromodulator, substance P is released into blood in response to nutritional[1] and other[2] stimuli. Studies in animals have identified the intestine as a major source of circulating substance P[3] and elevated concentrations have been reported to be present in the blood of patients with intestinal carcinoid tumours[4]. Indirect evidence for rapid clearance *in vivo* was obtained by Lembeck *et al.*[5] in experiments where the salivary response of the rat was used to assess the amount of substance P eliminated when the peptide was infused at different vascular sites. The liver, kidney and peripheral vascular beds were important sites of degradation. Studies *in vitro* have also shown rapid degradation. Studies *in vitro* have also rapid degradation of substance P in plasma[6,7] but the mechanisms of degradation are not known. In this study, high-performance liquid chromatography (HPLC) was used to identify metabolites of substance P produced during incubations in human plasma and in the circulation of the rat following intravenous infusion.

0021-9673/84/$03.00

EXPERIMENTAL

Substance P triacetate salt was purchased from Sigma, substance P (1–4 fragment) and substance P free acid from Peninsular Laboratories, ODS-silica-Hypersil (5 μm) from Shandon, Sep-Pek C_{18} cartridges and a μBondapak C_{18} (10 μm) column from Waters Assoc. Blood from healthy non-fasted subjects was collected in heparinised tubes and from rats into tubes containing (ethylenediaminetetraacetic acid (EDTA) (1 ml of a 500 m*M* solution) and Trasylol (10,000 K.I.U.). Blood was centrifuged immediately and the plasma extracted as described.

Isolation of metabolites of substance P formed in human plasma

Substance P (33–66 nmole) was incubated with plasma (2 ml) at 37°C. After 2, 5, 10 and 15 min, reaction was stopped by the addition of trifluoroacetic acid (TFA) (20 μl) and the mixture centrifuged at 1600 *g* for 15 min. The supernatant was diluted to 10 ml with 1% TFA and passed through a Sep-Pak C_{18} cartridge at a flow-rate of 2 ml/min. The cartridge was washed with 1% TFA (10 ml) and bound material was eluted with acetonitrile–water–TFA (80:19:1). Solvent was removed under reduced pressure, the residue was dissolved in 0.1% TFA and the solution was filterd through a 0.45-μm filter. The sample (300–500 μl) was filtered through a 0.45-μm filter. The sample (300–500 μl) was applied a column (25 × 0.46 cm I.D.) of Shandon ODS-Hypersil maintained at 30°C and eluted (1.5 ml/min) with a linear gradient (67.5 ml total volume), formed from acetonitrile–water–TFA (10.5:89.4:0.1) and acetonitrile–water–TFA (38.5:61.4:0.1). The eluate was monitored at 214 nm and peaks of UV-absorbing material with retention times between 36 and 43 min were collected manually. Solvent was removed under reduced pressure and the N-terminal peptide sequences were determined by a modified Edman degradation procedure using a 4-N,N-dimethylaminoazobenzene 4′-isothiocyanate–phenylisothiocyanate double-coupling method[8]. The 4-N,N-diumethylaminoazobenzene 4′-thiohydantoin amino acids were identified by two-dimensional thin-layer chromatography (TLC) on polyamide plates. Substance P (1–4 fragment) was not bound to the ODS-Hypersil column under the previous conditions of chromatography. In order to investigate the possible formation of this metabolite, the column was eluted at 30°C and 1.5 ml/min with a linear gradient (45 ml) formed from water–TFA (99.9:0.1) and acetonitrile–water–TFA (10.5:89.4:0.1). The ability of metabolites of substance P to bind to an antiserum raised against substance P was determined at appropriate dilutions under radioimmunoassay conditions, as decribed in a previous report[9]. The antiserum P4 is directed against the residues 6–11 region of substance P and requires the presence of a C-terminal methionine amide for binding[9].

Metabolism of substance P in the rat circulation

Male Wistar rats (300–350 g) (n = 6) were anaesthetized with intraperitoneal nembutal (60 mg/kg) and cannulae inserted into their jugular vein and carotid artery. Substance P (300–350 nmole) in 0.9% sodium chloride solution (1 ml) was infused via the jugular vein at a rate of 2 ml/min. After 1 min, blood samples (3–5 ml) were drawn over 30 sec from the carotid artery. Plasma was prepared and extracted as previously described. In control infusions (n = 3), 0.9% saline (1 ml) alone was infused and blood collected. In order to measure the recovery of substance P from

rat blood in the extraction procedure, substance P (6.5 nmole) and [^{125}I](Tyr^8) substance P (*ca.* 10,000 cpm) were added to freshly drawn rat blood and extracted using a Sep-Pak cartridge. HPLC was carried out on a Waters μBondapak C_{18} column (30 × 0.39 cm I.D.) under the elution conditions described previously.

RESULTS

In a control experiment, substance P, added to human plasma at 4°C and immediately extracted using a Sep-Pak C_{18} cartridge, was eluted from the ODS-Hypersil column as a sharp peak with retention time 39.2 ± 0.3 min together with minor peaks with retention times 41.2 and 42.6 min. (Fig. 1A). The recovery of substance P during this procedure was always greater than 90%. After incubation for 2 min, an additional peak with retention time 38.0 ± 0.1 min was observed in the chromatogram (Fig. 1B). The eight amino acid residues at the N-terminus of this peptide were unambiguously identified as Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu, equivalent to substance P (3–10 fragment). The formation of the 4-N,N-dimethylaminoazobenzene 4′-thiohydantoin derivative of the last residue proceeded in poor yield but the presence of a C-terminal methionine amide in the molecule was inferred from the fact that the metabolite was bound to antiserum P4 under radioimmunoassay conditions. After incubation for 5 min in plasma, a metabolite with retention time 37.1 ± 0.1 min was observed and subsequently identified as Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, equivalent to substance P (5–11 fragment). The height of these peaks, relative to substance P, increased after incubation for 10 min. After 15 min, a third metabolite with retention time 38.6 ± 0.2 min was observed (Fig. 1C) but complete resolution of this peak was not accomplished. The nature of the peaks eluted after substance P in Fig. 1 is unclear. Treatment of these components with the DABITC reagent did not result in the formation of a thiohydantoin derivative, suggesting that the components lack a free amino terminal group. Thus, these components may

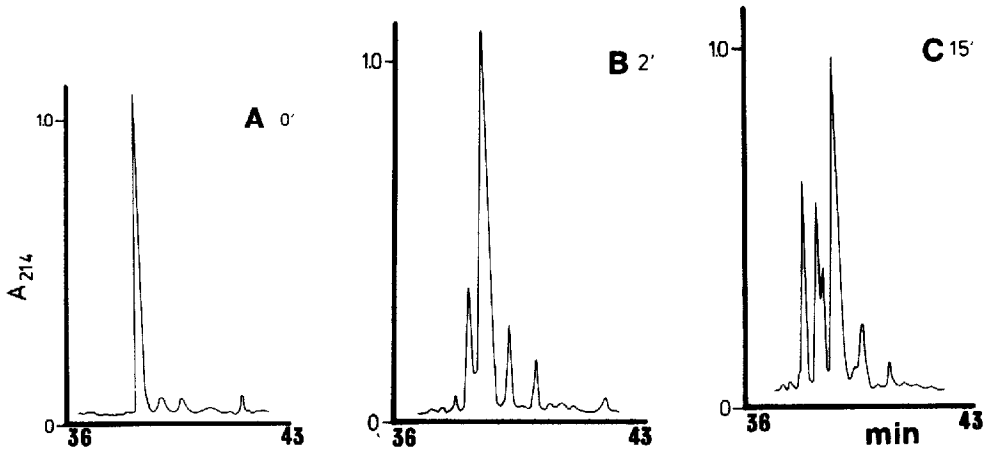

Fig. 1. HPLC of extracts of human plasma after incubation with substance P. Substance P (66 nmole) was incubated with plasma (2 ml) at 37°C for (A), zero time; (B) 2 min; (C) 15 min. Metabolites were extracted using Sep-Pak C_{18} cartridges. The chromatograms show only material that was eluted from the ODS-Hypersil column with retention times of 36–43 min.

represent post-cleavage modifications of metabolites, such as pyroglutamyl formation.

Synthetic substance P free acid was eluted from the ODS-Hypersil column with a retention time of 40.4 ± 0.2 min. Addition of this component to extracts of the incubation mixtures before HPLC resulted in the appearance of a completely new peak in the chromatograms, indicating that substance P did not undergo appreciable C-terminal deamidation in plasma. The N-terminal tetrapeptide of substance P (Arg-Pro-Lys-Pro) was eluted from the column at an acetonitrile concentration of 6.7% (retention time 12.7 min). Analysis of the extracts of the incubation mixtures after 5, 10 and 15 min showed that a peak with this retention time was not present in the chromatograms. Similarly, addition of synthetic substance P (fragment 1–4) to the extracts before HPLC resulted in the appearance of a new peak. At the sensitivity range used, 1 nmole of the tetrapeptide, representing less than 2% of the substance P substrate, was clearly detectable by absorbance at 214 nm. It is probable, therefore, that the formation of the 5–11 fragment proceeds sequentially from the 3–11 fragment rather than by an independent cleavage at Pro^4Gln^5. Under the conditions of chromatography, the dipeptides (Arg-Pro) and (Lys-Pro) did not bind to the column.

Metabolism of substance P in the rat circulation

Intravenous infusion of substance P at the pharmacological concentrations used in this study produced marked vasodilation in the rats. In the control infusions of saline alone, 8–10 ml of blood was obtained, but after substance P infusion only 3–5 ml was possible. After addition of [^{125}I](Tyr^8) substance P (*ca.* 10,000 cpm) to rat blood and immediate centrifugation (1600 *g* for 15 min at 4°C), 89.5 ± 0.4% of the radioactivity (mean ± standard deviation; $n = 12$) was associated with the plasma fraction. The elution profiles on a Waters μBondapak C_{18} column of plasma extracts after saline infusion (Fig. 2A) and after infusion of substance P (Fig. 2B)

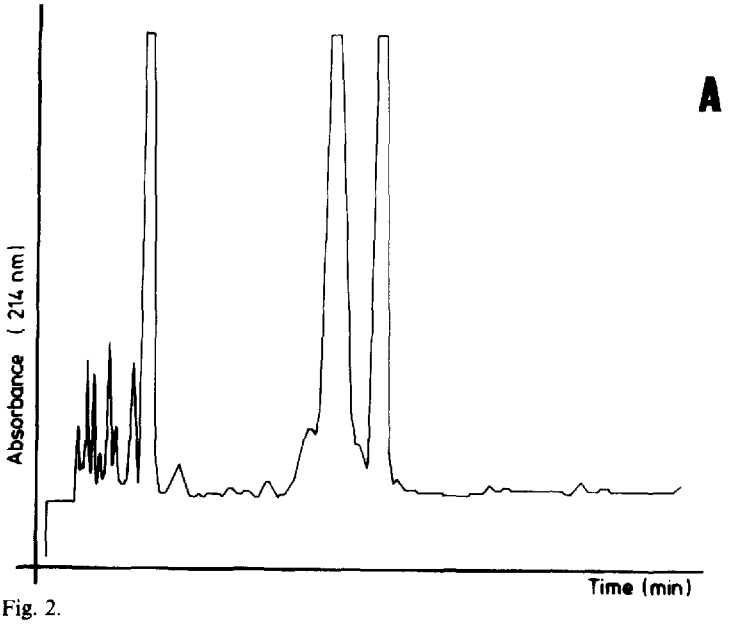

Fig. 2.

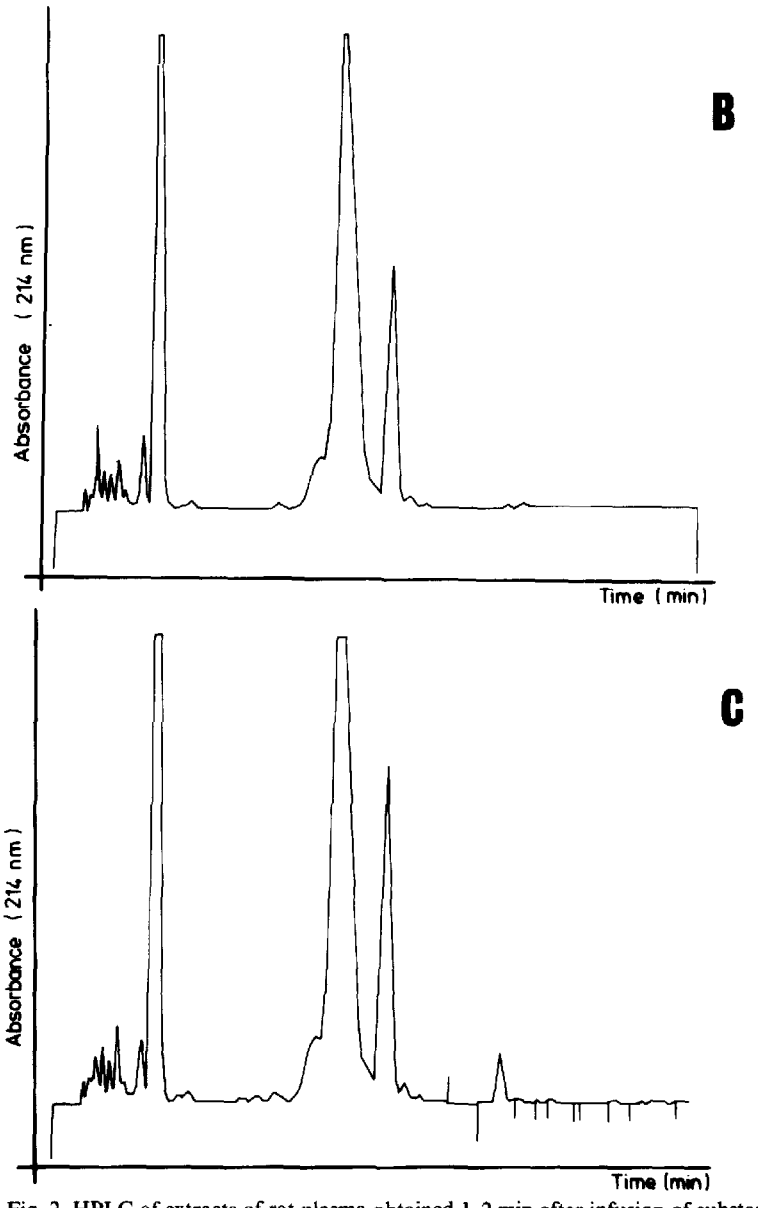

Fig. 2. HPLC of extracts of rat plasma obtained 1–2 min after infusion of substance P, (A) after infusion of saline alone; (B) after infusion of substance P (350 nmole); (C) after infusion of saline alone and saline to which substance P (6.5 nmole) has been added. The chromatograms show material that was eluted from the Waters μBondapak C_{18} column with retention times of 0–40 min.

were not significantly different. Under these conditions of chromatography, substance P had a retention time of 31.7 ± 0.2 min and substance P (5–11 fragment) 29.9 min. In all the experiments, no peaks corresponding to substance P or to its C-terminal fragments were detected in the chromatograms. At the sensitivity range employed, a peak corresponding to 2 nmole of substance P could be reproducibly

detected. Assuming a total volume of blood in the rat of approximately 25 ml, it is apparent that more than 96% of the substance P was eliminated from the circulation within 1–2 min. In order to demonstrate that substance P was not lost during the extraction procedure, the peptide (6.5 nmole) was added to rat blood and subjected to extraction and chromatography. As shown in Fig. 2C, this material was eluted from the columns as a resolved peak with the retention time of substance P. These data provide direct evidence that substance P is cleared very rapidly from the circulation of the rat. No evidence was obtained for the production of circulating metabolites.

DISCUSSION

The present study has demonstrated that substance P is converted in human plasma to C-terminal fragments by successive removal of Arg^1-Pro^2 and Lys^3-Pro^4 from the N-terminus of the molecule. This observation suggests that substance P is a substrate for dipeptidyl aminopeptidase IV. This enzyme, isolated from rat liver[10] and human submaxillary gland[11] and present in the vascular surface membranes of hog mesenteric artery and aorta[12], has been shown to cleave substance P to give the same fragments[10–12]. A post-proline cleavage enzyme from bovine brain[13] has been shown to cleave specifically substance P at Pro^4-Gln^5, but evidence has been provided that this enzyme is not important in the degradation by human plasma. Similarly, a neutral metalloendopeptidase from human brain[14] cleaving between Gln^6 and Phe^7, Phe^7 and Phe^8 and between Phe^8 and Gly^9 is probably not involved in degradation of substance P in plasma. Both the 3–11 fragment and the 5–11 fragment show equal or increased potency relative to substance P in several bioassay systems[15] and it has been demonstrated that nerve terminals and glial cells, although lacking an active uptake system for substance P, possess a high affinity transport system for the 5–11 fragment[16].

The metabolism of other regulatory peptides by enzymes in human plasma has been described. For example, the conversions of somatostatin to des (Ala^1) somatostatin[17], neurotensin to fragment (1–11) and to fragment (1–8)[18] and of TRH to histidine and dipeptide[19] have been reported. Consistent with the present observations, no C-terminal deamidation of TRH in plasma was observed. The present study has confirmed, by direct measurement, the observations of Lembeck *et al.*[5] that exogenous substance P is cleared very rapidly from the circulation of the rat. Although neither study may be relevant to the mechanism of clearance of endogenous substance P released from the intestine, the results suggest that a classical endocrine role for substance P is improbable. The absence of C-terminal fragments in the circulation suggests that inactivation of substance P takes place in tissues rather than in blood. The very efficient clearance of substance P is reminiscent of the comparable rate of clearance of linear somatostatin (more than 98% in 1 min)[20].

ACKNOWLEDGEMENT

We wish to thank the Volkswagen-Stiftung for financial support.

REFERENCES

1 B. Akande, P. Reilly, I. M. Modlin and B. M. Jaffe, *Surgery*, 89 (1981) 378.
2 B. M. Jaffe, B. Akande, I. M. Modlin, P. Reilly and D. Albert, *Dig. Dis. Sci.*, 27 (1982) 28.
3 R. Gamse, E. Mroz, S. Leeman and F. Lembeck, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 305 (1978) 17.
4 E. Wilander, L. Grimelius, G. Portela-Gomes, G. Lundqvist, V. Skoog and P. Westermark, *Scand. J. Gastroenterol.*, 14 Suppl. (1979) 19.
5 F. Lembeck, P. Holzer, M. Schweditsch and R. Gamse, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 305 (1978) 9.
6 H. Berger, K. Fechner, E. Albrecht and H. Niedrich, *Chem. Pharmacol.*, 28 (1979) 3173.
7 R. Couture and D. Regoli, *Can. J. Physiol. Pharmacol.*, 59 (1980) 621.
8 J. Y. Chang, D. Brauer and B. Wittmann- Liebold, *FEBS Letters*, 93 (1978) 205.
9 J. M. Conlon and L. Sheehan, *Regulatory Peptides*, 7 (1983) 335.
10 E. Heymann and R. Mentlein, *FEBS Letters*, 91 (1978) 360.
11 T. Kato, T. Nagatsu, K. Fukasawa, M. Harada, I. Nagatsu and S. Sakakibara, *Biochim. Biophys. Acta*, 525 (1978) 417.
12 F. E. Palmieri and P. E. Ward, *Biochim. Biophys. Acta*, 755 (1983) 522.
13 S. Blumberg, V. I. Teichberg, J. L. Charli, L. B. Hirsch and J. F. McKelvy, *Brain Res.*, 192 (1980) 477.
14 C.-M. Lee, B. E. B. Sandberg, M. R. Hanley and L. L. Iversen, *Eur. J. Biochem.*, 114 (1981) 315.
15 V. I. Teichberg and S. Blumberg, *Prog. Biochem. Pharmacol.*, 16 (1980) 84.
16 Y. Nakata, Y. Kusaka, H. Yajima and T. Segawa, *J. Neurochem.*, 378 (1981) 1529.
17 F. Märki, L. Schenkel, B. Petrack, A. J. Czernik, J. Ansell, M. Allen, D. E. Brundish, J. R. Martin, C. McMartin, G. E. Peters and R. Wade, *FEBS Letters*, 127 (1981) 22.
18 R. A. Hammer, R. E. Carraway and S. E. Leeman, *J. Clin. Invest.*, 70 (1982) 74.
19 S. Aratan-Spire and P. Czernichow, *J. Clin. Endocrinol. Metab.*, 50 (1980) 88.
20 C. McMartin and G. E. Purdon, *J. Endocrinol.*, 77 (1978) 67.