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Stability and *in vitro* metabolism of the mitogenic neuropeptide antagonists [D-Arg^1,D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P and [Arg^6, D-Trp^{7,9}, MePhe^8]-substance P (6–11) characterized by high-performance liquid chromatography

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Abstract: The substance P (SP) analogues [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-SP and [Arg^6, D-Trp^{7,9}, MePhe^8]-SP (6–11) (antagonists D and G, respectively) are under consideration as new anticancer drugs. In this report, the stability and *in vitro* metabolism of both antagonists in up to seven different media (water, 1 M acetic acid, human plasma, nude mouse liver and WX 322 human SCLC xenograft homogenized in either 1 M acetic acid or phosphate buffered saline (PBS), pH 7.4) have been characterized by both isocratic and gradient elution reversed-phase HPLC. Antagonist D was stable (never >13% degradation over 24 h, at 37°C) in water, 1 M acetic acid and plasma but was metabolized by PBS liver homogenates (10%, w/v) sequentially to two stable metabolites with a half life of 0.98 h at a concentration of 500 μg ml^{-1}. The major pathway of degradation of antagonist G appeared to be C-terminal methionine oxidation (particularly in plasma) as well as hydrolysis, with even aqueous solutions being significantly affected at low concentrations of peptide (0.1 μg ml^{-1}, half life 20.9 h at 37°C). Stable metabolites of antagonist G were also detected in incubations with PBS liver homogenates (half life 1.53 h at 500 μg ml^{-1}, 37°C). Overall, the data presented indicate that the modifications made to SP have been relatively successful in preserving chemical and biological stability.

Keywords: *Substance P analogues; peptide stability;* in vitro *metabolism; reversed-phase HPLC; gradient/isocratic elution; electrochemical detection.*

Introduction

The high *in vitro* biologic potency of many natural products is often not converted into satisfactory therapeutic efficacy when these molecules are administered systemically as pharmacological agents [1]. This is especially true in cancer chemotherapy where cytokines, anti-sense oligonucleotides, antibodies and peptide growth factor antagonists are being considered, and will increasingly feature, as new drug treatments. As large, endogenous molecules, they are perfect substrates for rapid enzyme catalysed catabolism and inactivation, and this remains a constant problem in natural product therapy.

Two substance P (SP) analogues [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P and [Arg^6, D-Trp^{7,9}, MePhe^8]-substance P (6–11) (codenamed antagonists D and G, respectively; see Fig. 1) have recently been identified as antagonists of a broad spectrum of neuropeptides believed to act as growth factors in the maintenance of small cell lung cancer (SCLC) cell lines *in vitro* [2–4]. Antagonists G and D retain antitumour activity *in vivo* against human SCLC xenografts WX 322 and H69 grown in nude mice (nu/nu), but in the case of the latter only when constantly infused through sub-cutaneous osmotic pumps [4, 5]. Antagonist D has also been shown to exhibit antitumour activity against the HC12 SCLC xenograft [6]. Here again only when administered by continuous 14-day sub-cutaneous infusion through osmotic mini-pumps implanted adjacent to the tumour [6]. As a consequence, these two peptides are now under serious consideration for clinical evaluation in human cancer, and antagonist G is presently progressing through toxicology with a view towards a pilot clinical trial being performed in Edinburgh.

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Amino acid		1	2	3	4	5	6	7	8	9	10	11	
	-0.4	1.1	3.6	-0.2	3.6	1.1	1.1	8.7	8.7	0.5	6.5	5.5	3.0
Substance P	NH_2 -	Arg -	Pro -	Lys -	Pro -	Gln	- Gln -	Phe -	Phe	- Gly	- Leu -	Met -	NH_2
	-0.4	1.1	3.6	-0.2	3.6	8.7	1.1	10.6	8.7	10.6	6.5	6.5	3.0
Antagonist D	NH_2 -	DArg -	Pro -	Lys -	Pro -	DPhe -	Gln -	DTrp -	Phe	- DTrp -	Leu -	Leu -	NH_2
						-0.4	1.1	10.6	8.7?	10.6	6.5	5.5	3.0
Antagonist G						NH_2 -	Arg -	DTrp -	MePhe	- DTrp -	Leu -	Met -	NH_2

Figure 1
Amino acid sequence of SP, antagonists D and G. The numbers above each amino acid are their retention coefficients (RC, [19]) which relates to hydrophobicity and their relative contribution to the retention time of the peptide as a whole on reversed-phase columns after gradient elution.

A major part of the philosophy behind the design of antagonists D and G was the introduction of D-amino acids into the sequence of SP and *N*-methylation of peptide bonds, both of which have been shown to protect SP from the action of plasma and tissue peptidases [7, 8]. In this paper, the stability and *in vitro* metabolism of antagonists D and G have been characterized by reversed-phase high-performance liquid chromatography (HPLC) with electrochemical (EC) detection.

Experimental

Materials

Neuropeptide antagonists D and G were purchased from Peninsula Laboratories, Inc (611 Taylor Way, Belmont, CA, USA) product numbers 7492 and 7498, respectively. Standard solutions of D and G were made up in 1 M acetic acid in siliconized coated glassware (Sigmacote, Sigma Chemical Co. Ltd, Poole, Dorset, UK), were stored refrigerated at 4°C and were renewed every week. Acetic acid (AnalaR grade) and ammonium acetate (Aristar grade) were from BDH chemicals (Poole, Dorset, UK); all methanol and acetonitrile were HPLC reagent grade (Rathburns chemicals, Walkerburn, Scotland, UK); triethylamine (TEA), trifluoroacetic acid (TFA) and hydrogen peroxide (30% solution) were from Sigma; and orthophosphoric acid (PRONALYS* AR) was from May and Baker Ltd (Dagenham, UK). All other chemicals were of the highest grade available commercially and were used as received. Water was deionized and bi-distilled in a quartz glass still.

Human plasma was a gift from the Department of Haematology, Western General Hospital, Edinburgh. Livers and WX 322 human SCLC xenografts were obtained in house from nude mice (nu/nu) which were maintained in negative pressure isolators (La Calhene, Cambridge, UK).

High-performance liquid chromatography

The liquid chromatograph consisted of two model 501 solvent delivery systems, a model 680 gradient controller, a Wisp autosampler (set to inject 50 μl, all from Waters Chromatography Division, Milford, MA, USA) and a model 5100A Coulochem electrochemical detector with a pre-column model 5020 guard cell (G) and a twin electrode (D1 and D2) model 5011 high sensitivity analytical cell (ESA, Inc. Bedford, MA, USA). Output from D2 (the last electrode in the series) was connected to a Hewlett–Packard 3396A computing integrator through a 1 V line and the integrator was set at an attenuation of 1 V in order that peak heights could be measured in absolute current values (Hewlett–Packard, Analytical Walborn, Germany). Electrode voltages were set at +0.7 V for G, +0.3 V for D1 and +0.7 V for D2.

Quantitation was by the external standard method referring to calibration curves and extracted standards run on the same day. This process was necessary since detector responses could vary from day to day due to a build up of deposit on the electrodes, especially after the analysis of tissue extracts. Electrodes were passivated with 6 N nitric acid routinely once a week, or more frequently if required. The maximum operational sensitivity of the EC detector was 100 nA full scale deflection (FSD) and this dictated limit of detection.

Isocratic elution

The stationary phase was μ-Bondapak C18 packed in a 30 cm by 3.8 mm i.d. stainless steel column (Waters) and the mobile phase consisted of 0.15% TFA in ammonium acetate (pH 2.75; 10 mM)–acetonitrile (54:46, v/v).

Elution was at a flow rate of 1 ml min^{-1} at ambient room temperature. Mobile phase components for isocratic and gradient elution were passed through a 0.22 μm filter, vacuum degassed before use and kept constantly free from dissolved oxygen by continuous sparging with helium gas during chromatography.

Gradient elution

Gradient elution was based on the method described by Wang *et al.* [9] for the separation of SP metabolites. The stationary phase was as above. Solvent A was orthophosphoric acid (pH 2.5, adjusted with TEA, 0.1 M) and solvent B was acetonitrile. The composition of the mobile phase at time zero was A–B (95:5, v/v) and the following linear gradient programme was employed: 5% B–55% B (by 25 min); 55% B–5% B (by 30 min). Total run time was 35 min and flow rate was 2 ml min^{-1} at ambient room temperature.

Sample preparation

Both antagonists were extracted from plasma, liver and tumour homogenates by solid-phase extraction using Bond Elut C_2 (100 mg sorbent, 1 ml reservoir) for antagonist G and Bond Elut C_8 (100 mg sorbent, 1 ml reservoir) for antagonist D (Varian Sample Preparation Products, Harbour City, CA, USA). Mini-columns were eluted under negative pressure using an SS-24 sample preparation station (Varian) connected to a small vacuum pump (Waters). Columns were first activated with 1 ml methanol, then washed with 1 ml water. Next the sample was loaded (1 ml plasma or 1 ml homogenate) and then the columns were washed sequentially with 1 ml water; 1 ml methanol–water (50:50, v/v) and 1 ml acetonitrile. Finally antagonist G was eluted with 400 μl of methanol–ammonium acetate (1 M; 90:10, v/v) and antagonist D with 400 μl of methanol–TFA (0.1%)–ammonium acetate (1 M; 80:10:10, v/v/v). After elution, samples were filtered (0.2 μm HPLC filters, Waters) and then 50 μl was injected directly on to the HPLC column. Antagonists D and G were shown to be stable in their eluting solutions for at least 24 h at 4°C. Drying down samples was avoided as this resulted in significant loss (possibly due to degradation) of each peptide. No internal standard was included. Aqueous solutions and 1 M acetic acid solutions of antagonists D and G were injected directly onto the HPLC column without sample preparation.

Precision and accuracy of the assay for 10 μg of either antagonist added to 1 ml of either plasma, liver or tumour homogenate was 91.3 ± 16.9% (between day) for antagonist G and 99.3 ± 16.9% (between day) for antagonist D [10].

Incubation conditions

All incubations (stability studies and *in vitro* metabolism) were performed at 37°C in a water bath over a time frame of 24 h in siliconized glassware and in the dark. For stability studies the following media were investigated: distilled water; 1 M acetic acid; human plasma; nude mouse liver homogenized in either 1 M acetic acid or phosphate buffered saline (PBS; pH 7.4; 10% solution w/v); and WX322 human SCLC xenograft homogenized in either 1 M acetic acid or PBS (pH 7.4; 10% solution, w/v). The range of antagonist concentrations used are to be found in the Results section. Samples generated from stability studies were analysed by the isocratic HPLC method. *In vitro* metabolism was performed with a fixed peptide concentration (500 μg ml^{-1}) in the following media: human plasma and nude mouse liver homogenized in either 1 M acetic acid or PBS (pH 7.4). Samples were analysed by the gradient elution method. HPLC determinations were normally carried out at time zero, 1, 2, 5 and 24 h and experiments were normally repeated on three separate occasions.

The oxidized form of antagonist G (S = O, at the C-terminal methionine group, see Fig. 1) was synthesized by incubating G (100 μg ml^{-1}) with 0.2% hydrogen peroxide (v/v) for 24 h at 4°C as previously described [11].

Results

Kinetics of degradation/metabolism of antagonists D and G

Stability of antagonists D and G has been investigated in seven different media. Results from these studies are summarized in Table 1 where degradation kinetics are represented by two separate parameters: (a) per cent of parent peptide remaining after 24 h and (b) initial half life for peptide degradation. The need for two parameters was that in certain media (tumour and liver homogenized in 1 M acetic acid, see Table 1) concentration/time decay profiles

Table 1
Degradation kinetics of antagonists D and G in different solvents and biologic media

Initial concentration	Antagonist D				Antagonist G			
	0.1 μg ml^{-1}		10 μg ml^{-1}		0.1 μg ml^{-1}		10 μg ml^{-1}	
Stability parameters	%*	$t_{½}$†	%	$t_{½}$†	%	$t_{½}$†	%	$t_{½}$†
Water	94	NE	87	NE	46	20.9	92	NE
1 M Acetic acid	89	NE	93	NE	60	>24	84	NE
Plasma	96	NE	97	NE	0	4.4	2	5.0
Liver homogenate (10% w/v in 1 M acetic acid)	31	9.0	36	4.6	35	14.6	35	4.8
WX 322 SCLC tumour homogenate (10% w/v in 1 M acetic acid)	29	11.8	67	>24	47	22.8	52	>24
Liver homogenate (PBS)	ND		0.0	<0.05	ND		0.0	<0.05
Tumour homogenate (PBS)	ND		0.0	<0.05	ND		0.0	<0.05

* Percentage intact peptide remaining after 24 h incubation at 37°C.
† $t_{½}$, hours (determined by non-linear regression curve fitting to the initial first-order phase decline in concentration–time profiles).
NE, not evaluable; ND, not determined. Data derived from curves where $n = 3$ for each time point.

deviated from expected first-order kinetics and therefore the % term was incorporated.

Antagonists D and G were relatively stable (never more than 13% degradation over 24 h) in water and acetic acid with the sole exception of low concentrations of G (0.1 $\mu g\ ml^{-1}$). Here a single degradation product appeared on chromatograms with a retention time identical to that of the oxidized form of antagonist G. Reducing conditions (i.e. 1 M acetic acid) offered some protection to low concentrations of antagonist G against degradation. Taken together these data indicate that antagonist G probably undergoes spontaneous oxidation in solution by reacting with dissolved oxygen, an effect which will only manifest itself at low peptide concentrations. Antagonist D lacks a C-terminal methionine (see Fig. 1) and this may explain why it was not affected by this degradation pathway.

Antagonist D was also stable in human plasma (Table 1) and this was confirmed by gradient elution reversed-phase HPLC (see below). However, antagonist G degraded with a half life of 4.4–5.0 h but again only one degradation product was evident: a chromatographic peak with a retention time identical to the oxidized form of antagonist G. Based on integrated peak areas which in turn are based on the number of moles of tryptophan present in each peak, it was estimated that this degradation product accounted almost completely for the disappearance of the parent peptide. To distinguish between metabolism and chemical oxidation, antagonist G was incubated with plasma that had either been boiled or heated to 80°C in order to inactivate peptidase activity. In both experiments degradation kinetics were unaffected (data not shown) suggesting that breakdown is: (a) through chemical oxidation, and (b) is operating in plasma at a much higher level than in simple aqueous solutions (see Table 1).

Both antagonists were very rapidly metabolized by tumours and livers homogenized in PBS where significant peptidase enzyme activity was presumed to be present. A number of transient degradation products were detected in chromatograms (see Table 2) and these appeared to correspond to the same metabolite peaks previously identified in the same samples after *in vivo* administration of antagonists D and G [10]. Homogenization of tumour and tissue in 1 M acetic acid greatly slowed down metabolism (especially in the tumour) and this was confirmed in gradient elution studies. Therefore, 1 M acetic acid is considered an ideal solvent for sample preparation of tissues and tumours collected from pharmacokinetic studies in order to avoid *in situ* degradation and the introduction of artefactual metabolite peaks.

In vitro *metabolism of antagonists D and G*

To further characterize the *in vitro* metabolism of antagonists D and G gradient elution reversed-phase HPLC was employed and to slow down the rate of metabolism to a measurable level high concentrations of both antagonists (500 $\mu g\ ml^{-1}$) were added to incubations. In these preliminary experiments, investigations were restricted to human plasma and mouse liver homogenates. Typical chromatograms from these studies are shown in Fig. 2 for D and Fig. 3 for G and the rate of disappearance of the parent peptide versus the formation of putative metabolites/degradation products is shown graphically in Fig. 4 (plasma) and Fig. 5 (liver) for D, and Fig. 6 (plasma) and Fig. 7 (liver) for G. Again,

Table 2
Chromatographic characterization of the degradation products/metabolites of antagonists D and G

	Antagonist D				Antagonist G			
Peak	Trp	1	2	D	1	2	3	G
Isocratic elution (retention times, min) [10]	3.5	5.5	—	8.5	5.5†	7.1	9.0	10.0
Gradient elution (retention times, min)	—	20.5	22.0	24.3	21.0	22.7	—	23.8
Retention* coefficients* [19]	—	55.2	59.2	65.4	42.0	45.4	—	47.6

*Retention coefficients (RC) were first calculated from the parent peptide using the values quoted in Fig. 1 above individual amino acids. RCs for putative metabolites were then proportionated based on their retention time relative to the parent peptide.

†Retention time of the oxidized form of antagonist G.

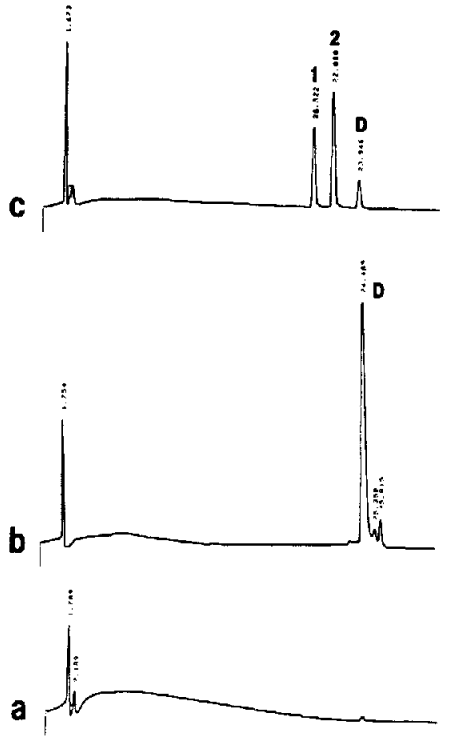

Figure 2
Typical chromatograms of antagonist D (500 μg ml^{-1}) incubated at 37°C with phosphate buffered saline–liver homogenates (10% w/v). Solid-phase extraction and gradient elution reversed-phase HPLC are as described in the Experimental section. Peaks were quantitated by selective coulometric detection of the tryptophan content of each peak. Chromatogram (a), blank liver extract; chromatogram (b), liver incubation at time zero; and chromatogram (c), liver incubation after 2 h. Peak D is antagonist D and peaks 1 and 2 are putative metabolites.

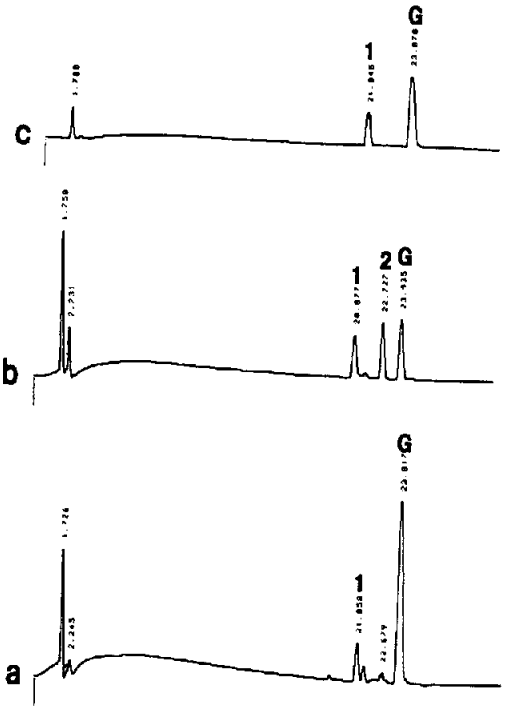

Figure 3
Typical chromatograms of antagonist G (500 μg ml^{-1}) incubated at 37°C. Experimental conditions as in Figure 2. Chromatogram (a), liver incubation at time zero; chromatogram (b), liver incubation after 2 h; and chromatogram (c), human plasma incubation after 5 h. Peak G is antagonist G and peaks 1 and 2 are putative metabolites.

quantitation is based on the selective electrochemical detection of the tryptophan content of each chromatographic peak.

Gradient elution confirmed that antagonist D is stable in plasma and that antagonist G is converted into a single product. The poor aqueous solubility of G in plasma resulted in a mis-match between parent peptide disappearance and degradation product appearance (see Fig. 6), and is indicated chromatographically by poor peak symmetry (see Fig. 3). In liver homogenates, antagonist D was metabolized (half life 0.98 h) to two distinct products which accounted for approximately 90% of the tryptophan content of the native peptide. Their kinetics indicated sequential formation with the peak at 22.0 min (peak 2) appearing first and then being converted to the peak at 20.5 min (peak 1). Both these products were not apparent in isocratic determinations (see Table 2). Interestingly, gradient elution of antagonist

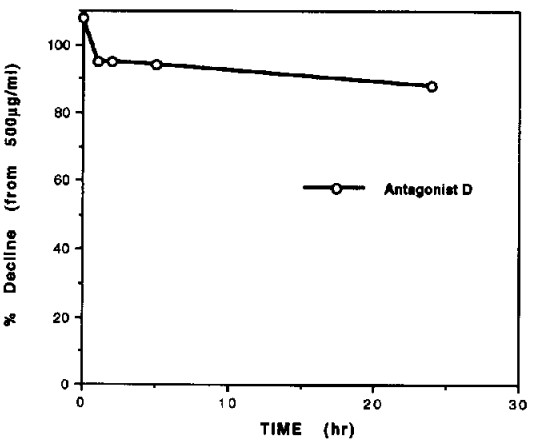

Figure 4
Typical concentration–time decay profile for antagonist D incubated at 37°C with human plasma. Experimental conditions as in Fig. 2.

G resolved fewer metabolites/degradation products than isocratic elution (Table 2). Again a sequential formation of two products is suggested with the peak at 22.7 min (peak 2) appearing first and then being converted into

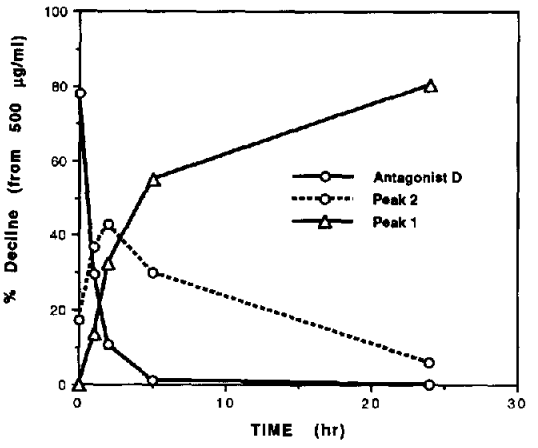

Figure 5
Typical concentration–time decay profile for antagonist D (500 µg ml^{-1}) incubated at 37°C with nude mouse liver homogenate (10% w/v in phosphate buffered saline). Experimental conditions as in Fig. 2. Curves are as follows: O——O, antagonist D; O----O, chromatographic peak 2 (see Fig. 2, Table 2 and Discussion) with retention time of 22.0 min; Δ——Δ, chromatographic peak 1 (see Fig. 2, Table 2 and Discussion) with retention time of 20.5 min.

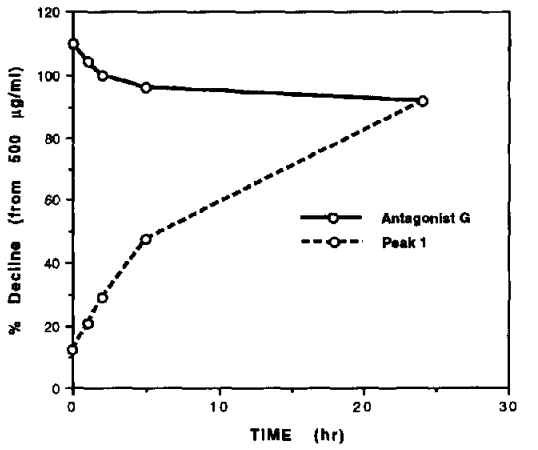

Figure 6
Typical concentration–time decay profile for antagonist G (500 µg ml^{-1}) incubated at 37°C with human plasma. Experimental conditions as in Fig. 2. Curves are as follows: O——O, antagonist G; O----O, chromatographic peak 1 (see Fig. 3, Table 2 and Discussion) with retention time of 21.0 min.

the final product with a retention time of 21.0 min (peak 1). Antagonist G disappeared with a half life of 1.53 h, indicating that it is significantly more stable than antagonist D against the action of tissue peptidases.

Discussion

The data reported herein indicate that, with the exception of oxidation of the C-terminal

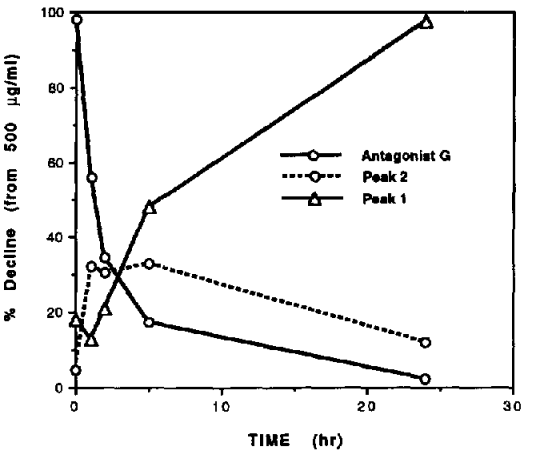

Figure 7
Typical concentration–time decay profile for antagonist G (500 µg ml^{-1}) incubated at 37°C with nude mouse liver homogenate (10% w/v in PBS). Experimental conditions as in Fig. 2. Curves are as follows: O——O, antagonist G; O---O, chromatographic peak 2 (see Fig. 3, Table 2 and Discussion) with retention time of 22.7 min; Δ——Δ, chromatographic peak 1 (see Fig. 3, Table 2 and Discussion) with retention time of 21.0 min.

methionine of antagonist G, both the SP analogues studied (see Fig. 1) are relatively stable in water, 1 M acetic acid and human plasma. Thus, the design features introduced into the structure of SP (namely D-amino acids and *N*-methylation [7, 8]) would appear to have succeeded in enhancing biologic stability. Rapid hydrolysis of SP in plasma (64% of all activity) is catalysed by dipeptidyl(amino)peptidase IV (DAP IV; EC 3.4.14.5) producing sequentially SP (3–11) and SP (5–11), the latter then being rapidly metabolized by aminopeptidase M (EC 3.4.11.2) [9, 12]. Angiotensin converting enzyme [ACE, EC 3.4.15.1, which converts SP to SP (1–9)] also contributes significantly to SP inactivation (19% of activity) in plasma. Spantide [D-Arg^1, D-$Trp^{7,9}$, Leu^{11}]SP, the SP antagonist closely related in structure to antagonist D, is also hydrolysed predominantly to spantide (5–11) by plasma (and aqueous humour) with a half life of approximately 15 min [13]. Lack of significant degradation of antagonist D by plasma may then reflect the fact that it is a poor substrate for DAP IV; presumably due to the introduction of the DPhe residue at position 5 in SP, the only difference in structure between antagonist D and spantide. Antagonist G is unlikely to act as a substrate for DAP IV since it lacks the structural requirements of NH_2-Xaa-Pro present in SP, spantide and antagonist D.

In peripheral tissues and the central nervous system a different picture of SP catabolism has emerged. Here predominantly *N*-terminal metabolites are formed [SP (1–6); SP (1–7); SP (1–8) and SP (1–9)] by a large number of plasma membrane peptidases including: ACE; neutral endopeptidase-24.11 (enkephalinase, EC 3.4.24.11); substance P degrading enzyme (EC 3.4.24); bacitracin-sensitive endopeptidase; acetylcholinesterase (EC 3.1.1.7); and the mitochondrial membrane substance P degrading endopeptidase [14–16]. The above list is by no means exhaustive [17]. Both antagonists D and G were rapidly degraded by active tissue preparations (10% w/v, liver and tumour PBS homogenates), although this effect was concentration dependent indicating enzyme saturability at peptide concentrations which may be realistically achieved *in vivo* (albeit for only a short time after administration). Indeed, intact antagonists D and G and a number of stable metabolites (particularly of G) can be detected *in vivo* in tissues and WX 322 tumour for up to 6 h after i.v. or i.p. administration to nude mice [10].

Retention times (t_R) of peptides on reversed-phase HPLC columns (after gradient elution) are known to correlate with their amino acid composition [18, 19]. In fact, it is possible to mathematically model and accurately predict the t_R of a peptide by summation of an individual contribution from each constitutive amino acid. These contributions are derived experimentally by following the behaviour of a large series of peptides of known composition, they are called retention coefficients (RC), and are based on the relative hydrophobicity of each amino acid. The converse also holds: HPLC retention times can be utilized to obtain information about the amino acid composition of an unknown peptide. RC values are additionally useful (especially in the present study) in predicting whether or not it is theoretically possible to resolve a potential metabolite from the parent peptide. Typical examples of RC values for the amino acids present in SP and both antagonists D and G are shown in Fig. 1. These data are from a recent extensive study of 104 peptides, which not only took into account amino acid hydrophobicity, but also modelled for peptide length and secondary structure [19]. RC for MePhe is assumed to be identical in value to Phe (which is probably an underestimation). Table 2 shows the overall retention coefficients for antagonists D and G together with RCs for their proposed metabolites, which were calculated from their retention times relative to that of the parent peptide.

The comparatively small differences in RC values between antagonist D (RC 65.4) and its two putative stable metabolites are consistent with sequential conversion to D (3–11; RC 61.1 versus 59.2 for chromatographic peak 2 with t_R 22.0 min) followed by D (5–11; RC 58.5 versus 55.2 for chromatographic peak 1 with t_R 20.5 min) analogous to the metabolism of both SP and spantide (see above). The only possible alternative pathway, based on the above chromatographic considerations, is C-terminal deamidation followed by hydrolysis of the leu_{10}—leu_{11} bond. To the best of the author's knowledge, no specific SP degrading enzyme has been reported which is capable of hydrolysing this particular bond [14–17]. SP (5–11) has been shown to exhibit SP biologic activities (bronchoconstriction) and, unlike SP (1–11), is taken up into nerve endings [14]. D (5–11) is, therefore, likely to express biologic activity and contribute significantly to the pharmacology of antagonist D. It contains the essential SP receptor binding domain of a hydrophobic core between residues 7–10 [20]. It also retains D-Trp residues at positions 7 and 9 present in a number of SP antagonists [21], and an intact C-terminal amide essential for activity [22]. However, whilst the full length SP antagonists spantide I and II activate histamine release from mast cells, some truncated analogues (D-Pro^4. D-$Trp^{7,9,10}$) SP (4–11) do not, and this could suggest that metabolite D (5–11) may actually be less toxic than its parent peptide [23]. The issue of the true identity of metabolites of both antagonists D and G is currently being addressed by purification of these species using preparative HPLC and elucidation of amino acid composition and peptide sequence.

RC values for antagonist G illustrate the highly hydrophobic nature of this peptide. In a recent report, the metabolism of a series of *N*-methylated SP (6–11) analogues has been studied in two different SP target tissue systems: rat parotid gland and rat hypothalamus [8]. The *N*-methylated analogues were protected from accelerated metabolism but, curiously, underwent C-terminal methionine oxidation more readily than (pGlu) SP (6–11). Two major peptidase cleavage sites were identified: at positions 7–8 and 9–10. If cleav-

age occurred with antagonist G at either of these sites the resultant metabolites would have retention times considerably different from the parent peptide, which was not the case with the chromatographic peaks detected in the present study. Out of the two putative metabolites of antagonist G identified here, the major species (peak 1) probably represents an oxidation product rather than the result of enzyme action. It could, therefore, be concluded that antagonist G is relatively resistant to peptidase catabolism. However, cleavage at the Arg^6—D-Trp^7 bond in antagonist G (and D-Arg^1-Pro^2 in antagonist D) is liable to produce metabolites that would co-elute with their parent peptides and would therefore not be identified by the HPLC methods utilized.

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