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Determination of two neuropeptide growth factor antagonists, [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]-substance P and [Arg^{6}, D-Trp^{7,9},N-MePhe^{8}]-substance P(6–11), by high-performance liquid chromatography with electrochemical detection

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Abstract

N-MePhe^{8}]-substance P(6–11) (G) are currently undergoing preclinical evaluation as potential anticancer agents and clinical trials are planned for G in the near future. A reversed-phase high-performance liquid chromatographic separation has been developed which is both sensitive (limit of detection 250 pg/263 fmol for G; 500 pg/330 fmol for D) and selective, based on electrochemical detection of the two tryptophan residues present in each peptide. Two ion-pairing agents were included in the isocratic mobile phase to eliminate adsorption of the peptides onto the analytical column. Extensive sample clean-up procedures have been developed for plasma, tissue and tumour based on solid-phase extraction. Precision and accuracy of each assay was 91.3 ± 16.9% (between-day) for G and 99.3 ± 16.9% (between-day) for D. The assays were able to detect the intact peptides and a number of their metabolites in plasma, liver and the WX 322 SCLC human xenograft in nude mice for at least 6 hr after administration of therapeutic and pharmacological doses.

1. Introduction

Lung cancer remains the commonest form of fatal malignancy in the western world. Small cell lung cancer (SCLC, or oat cell lung cancer) accounts for 25% of the total and, despite an initial high rate of responsiveness to combination chemotherapy, survival at two years can be as low as 13% [1]. Human SCLC cells produce and secrete a number of hormones and neuropeptides including bombesin/gastrin releasing peptide (GRP), neurotensin, cholecystokinin and vasopressin [2–4]. Evidence that bombesin/GRP can function as an autocrine growth factor in SCLC was finally provided by specific antagonism with a monoclonal antibody (2A11) which neutralises the C terminus of the peptide important for receptor binding [5]. These results lead to the promotion of the concept of growth factor antagonism as a new therapeutic strategy

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Fig. 1. Amino acid sequence of [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]-substance P and [Arg[6],D-Trp[7,9],N-MePhe[8]]-substance P(6–11), known as antagonists D and G respectively.

in SCLC [6]. Despite initial enthusiasm for bombesin/GRP as a target, confirmation of its widespread role in SCLC was not achieved in other cell lines [7] and results from early clinical trials with 2A11 have proved disappointing [8].

In search for more potent bombesin/GRP antagonists two substance P analogues have been identified: [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]-substance P [9] and [Arg[6],D-Trp[7,9],N-MePhe[8]]-substance P(6–11) [10] (codenamed antagonists D and G respectively; for full amino acid sequences see Fig. 1). Apart from inhibiting the binding of ^{125}I-labelled GRP to its receptor, they block receptor binding and subsequent mitogenic signalling (Ca^{2+} mobilization) induced by vasopressin, bradykinin, cholecystokinin, galanin and neurotensin with each showing different degrees of selectivity [10]. Recently antagonists D and G have been shown to alter significantly the growth of human SCLC xenografts WX 322 and H69 *in vivo* [11–12]. Consequently, these agents are now under consideration for clinical evaluation.

As part of a larger programme of preclinical pharmacology studies we have developed a high-performance liquid chromatographic (HPLC) assay for antagonists D and G. The substitution of two tryptophan residues into the structure of substance P in the case of both antagonists permitted the novel use of an electrochemical detector (ED), and this enabled a high degree of selectivity and sensitivity to be achieved.

2. Experimental

2.1. Materials

Neuropeptide antagonists D and G were purchased from Peninsula Laboratories (Belmont, CA, USA), product number 7492 and 7498 respectively. Standard solutions of D and G were made up in 1 *M* acetic acid in siliconised coated glassware (Sigmacote, Sigma, 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, UK) and trifluoroacetic acid (TFA) was from Sigma. All other chemicals were of the highest grade available commercially and were used as received. Water was de-ionised and bi-distilled in a quartz glass still. Human plasma for control experiments was a gift from the Department of Haematology, Western General Hospital, Edinburgh and liver and WX 322 human SCLC xenograft were obtained in house (see below).

2.2. High-performance liquid chromatography

The liquid chromatograph consisted of a Model 510 solvent delivery system, a Model 680 controller, a Wisp autosampler (set to inject 50 μl, all from Waters, Northwich, UK); a Model 5100A Coulochem electrochemical detector with a pre-column Model 5020 guard cell (GC) and a twin electrode (D1 and D2) Model 5011 high sensitivity analytical cell (ESA, Inc. Bedford, MA, USA) and a Hewlett-Packard 3396A computing integrator (Hewlett-Packard, Walborn, Germany). Quantitation was by the external standard method. After optimisation (see results section) detector voltages were fixed at +0.7 V for GC, +0.3 V for D1 and +0.7 V for D2. The maximum operational sensitivity of the EC detector was 100 nA full scale deflection (FSD) and this dictated the limit of detection.

The stationary phase was μ-Bondapak C_{18} packed in a 30 cm × 3.9 mm I.D. stainless steel column (Waters) and the mobile phase consisted of 0.15% TFA in 10 m*M* ammonium acetate (pH 2.75)–acetonitrile: 54:46 (v/v) for antagonist D and 60:40 (v/v) for antagonist G. Mobile phase components were passed through a 0.22-μm filter, vacuum degassed before use (Waters) and kept constantly free from dissolved oxygen by continuous sparging with helium gas during chromatography. Elution was isocratic at a flowrate of 1 ml/min at ambient room temperature.

2.3. Sample preparation

The sample preparation technique is shown schematically in Fig. 2. A different solid phase was necessary for each peptide: C_2 (100 mg) for antagonist G and C_8 (100 mg) for antagonist D (Bond Elut 1-ml reservoirs, Varian Sample preparation products, Harbour City, CA, USA). Up to 1 ml of biological sample could be applied on to the mini-columns and for the activation step and all 4 subsequent washes a 1-ml volume was

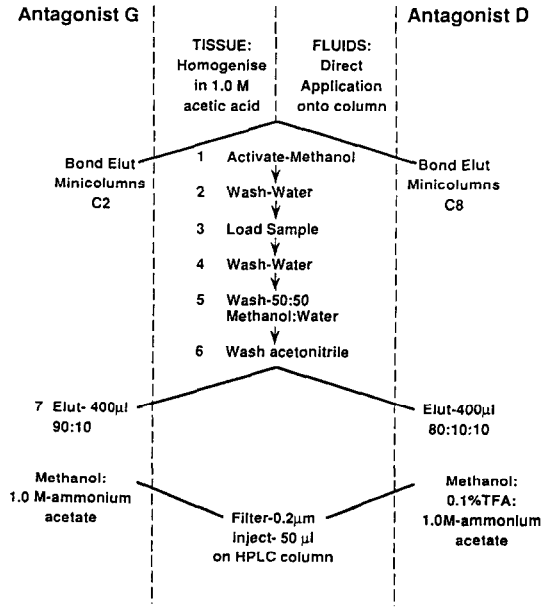

Fig. 2. Schematic representation of the solid-phase sample preparation techniques for the isolation of antagonists D and G from biological fluids (plasma and urine, up to 1 ml) and tissue (normal tissues and solid tumour material, up to 0.1 g).

employed (see Fig. 2). Peptides 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. All glassware used in sample preparation was siliconised with Sigmacote (Sigma).

2.4. Analysis of biological specimens

Animals were dosed with antagonists D and G and plasma, liver and tumour specimens were collected at several different time points to act as pilot studies for future proposed large scale pharmacokinetic trials. The animal model was the nude mouse (*nu/nu*): these were purchased from OLAC (Oxford, UK) and were maintained in negative pressure isolators (La Calhene, Cambridge, UK). The tumour model was the human WX 322 SCLC xenograft which was originally derived from a sub-cutaneous (s.c.) metastasis of an untreated SCLC tumour [13] and it was grown as a s.c. tumour in the flank of the animals. Both antagonists were administered as aqueous solutions i.v. or i.p. at a dose level of 45 mg/kg for G and 12 mg/kg for D. This i.p. dose can be growth inhibitory against WX 322 for antagonist G [14] and is the maximum tolerated dose for antagonist D in the nude mouse. Samples were collected (in the case of blood, plasma was first separated) and immediately placed in liquid nitrogen and stored at −80°C prior to analysis. Tissue/tumour was homogenised in 1 *M* acetic acid (0.1 g in 0.9 ml) when still only partially thawed and all biological samples were kept on ice until solid-phase extraction.

3. Results and discussion

3.1. High-performance liquid chromatography

The aim of the present study has been to develop an HPLC method for the neuropeptide antagonists D and G (see Fig. 1) which would be both sensitive and efficient. To achieve high sensitivity we exploited the fact that both pep-

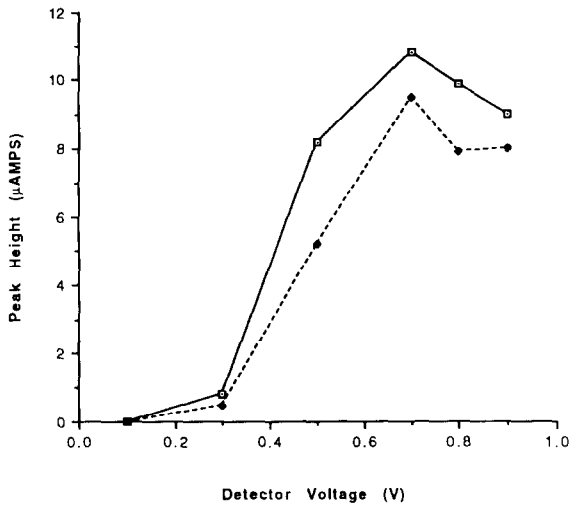

Fig. 3. Hydrodynamic voltammogram of antagonists D and G. A constant amount of D (2 μg; ——) and G (1 μg; -----) was repeatedly injected on to the HPLC column, the electrochemical detector voltage (D2, x-axis) was varied and the detector response in the form of the peak height in μAmp (y-axis) was recorded.

tides contain two tryptophan residues and employed electrochemical detection. Fig. 3 shows a hydrodynamic voltammogram (a plot of peak height *versus* detector voltage) for antagonists D and G in the oxidation mode. Neither D nor G underwent reduction. Maximum detector responses were reached with an electrode potential of +0.7 V (D2). A voltage of +0.3 V (D1) was chosen as the pre-screen potential for the analytical cells' first electrode since this results in minimal oxidation of both peptides.

Isocratic elution was utilized instead of the preferred method for peptides of gradient elution, since the latter cannot be run at high sensitivity with an EC detector due to baseline fluctuations [15]. In preliminary studies the main problems encountered with isocratic elution were: (1) poor peak symmetry; (2) non-linear detector response where higher than expected levels were recorded at low peptide concentrations (especially below 1 ng); (3) ghost peaks appearing in blanks; and (4) far greater than 100% extraction efficiency being recorded during sample preparation. Although retention mechanisms for peptides on reversed-phase supports are complex and poorly understood [15], these problems were eventually ascribed to variable secondary interactions with unreacted silanols [16] and adsorption on to the HPLC column followed by rogue elution during a subsequent analysis. Optimal efficiency was achieved using a μ-Bondapak C_{18} stationary phase, and incorporation of ammonium acetate (10 mM) into the mobile phase improved greatly reproducibility.

Full details of HPLC of antagonists D and G are shown in Table 1. Over 4 orders of magnitude calibration curve linearity remained good. Limit of detection was 250 pg (263 fmoles) for G and 500 pg (330 fmoles) for D. The factor restricting detection limits remained adsorption and slow release of peptide from the HPLC column and as a consequence the maximum workable range on the detector was 100 nA (FSD) which is ten fold less than the Coulochem 5011A's theoretical limit. A blank chromatogram (50 μl acetic acid) run at 100 nA is shown in Fig. 4a together with chromatograms of standards of antagonist D (Fig. 6b) and antagonist G (Fig. 4c).

Table 1
High-performance liquid chromatography of neuropeptide antagonists D and G

Neuropeptide antagonist	Retention time		k'	Efficiency (plates/m)	Calibration curve linearity over the range 1 ng–10 μg (r^2)	Detection limit on column (pg)	Detection limit after extraction	
	min	C.V.[a] (%)					Plasma (ng/ml)	Tissue (ng/g)
G	15.9	4	8.93	17 600	0.936	250	4	40
D	8.85	2	4.53	12 490	0.982	500	8	80

[a] Within-day coefficient of variation, ten separate runs.

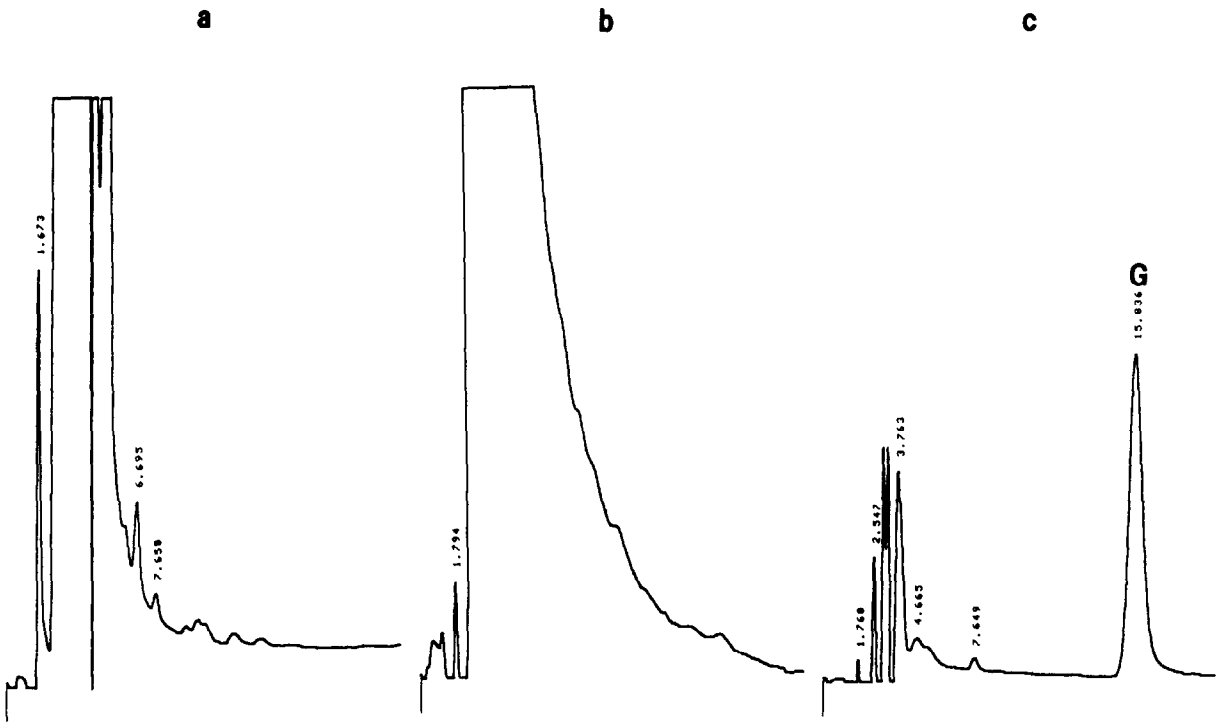

Fig. 4. Reversed-phase HPLC chromatograms of a standard solution (made up in 1 *M* acetic acid) of antagonist G. Chromatographic details are reported in Experimental. In each case the time frame for the *x*-axis is 20 min and the retention times of all significant peaks have been marked by the integrator. Chromatogram a, blank injection of 50 μl of 1 *M* acetic acid: electrochemical detector sensitivity is 100 nA full scale deflection (FSD). Chromatogram b, WX 322 tumour extract (0.1 g) from non-peptide treated nude mouse, FSD = 100 nA; Chromatogram c, 50 ng/53 pmoles antagonist G (t_R, 15.84 min), FSD = 400 nA.

3.2. Sample preparation technique (SPT)

In order to eliminate spurious 'ghost' chromatographic peaks being eluted from the HPLC analytical column, it is recommended that 50 μl of appropriate SPT eluting solution (see Fig. 2) is injected three times at the start of the day prior to analysis of biological samples. For standards made up in water or acetic acid the mean efficiency of the SPT over the concentration range 0.01–10 μg/ml was 99.9% for antagonist G and 89.2% for antagonist D. Complete data on extraction efficiency, assay accuracy and precision for biological specimens are shown in Table 2.

Although much simpler solid-phase sample preparation methods (Bond Elut phenyl) have been described for related peptides which report higher recoveries than in the present study (90–100%), these rely on radioimmunoassay (RIA) for detection [17,18]. Direct detection by UV-absorbance was impossible in these studies due to the size of the solvent front which remained off scale for at least 15 min. We have concentrated on developing a SPT which allows direct determination of antagonists D and G even at very high detector sensitivities and have avoided RIA. Considering the fact that the intact peptide may only represent 20–30% of the total peptide related material present in plasma, tissue and tumour specimens (see Fig. 5a–c), and these metabolites are likely to retain cross-immunoreactivity to some extent, this decision appears merited.

Table 2
Extraction efficiency and assay accuracy and precision for biological specimens

Concentration (μg/ml g)	Extraction efficiency (mean ± S.D.)[a] (%)			Between-day accuracy[b] (%)	Between-day precision[b] (%)
	Plasma	Liver	WX 322 SCLC xenograft		
Antagonist G					
10	70.9 ± 5.2	50.6 ± 6.6	67.1 ± 3.6	91.3	16.9
0.1	80.3 ± 21	94.7 ± 15	69.6 ± 7.4	106	21.9
Antagonist D					
10	48.6 ± 4.8	37.0 ± 4.1	50.1 ± 4.3	99.3	16.9
0.1	77.6 ± 6.6	49.4 ± 1.9	38.9 ± 12	90.9	8.2

[a] $n = 10$ replicates for each individual entry.
[b] Accuracy and precision refer to data pooled from plasma, liver and tumour specimens; $n = 12$–20 separate determinations per concentration for each peptide where analyses were performed over a 4–6 month period.

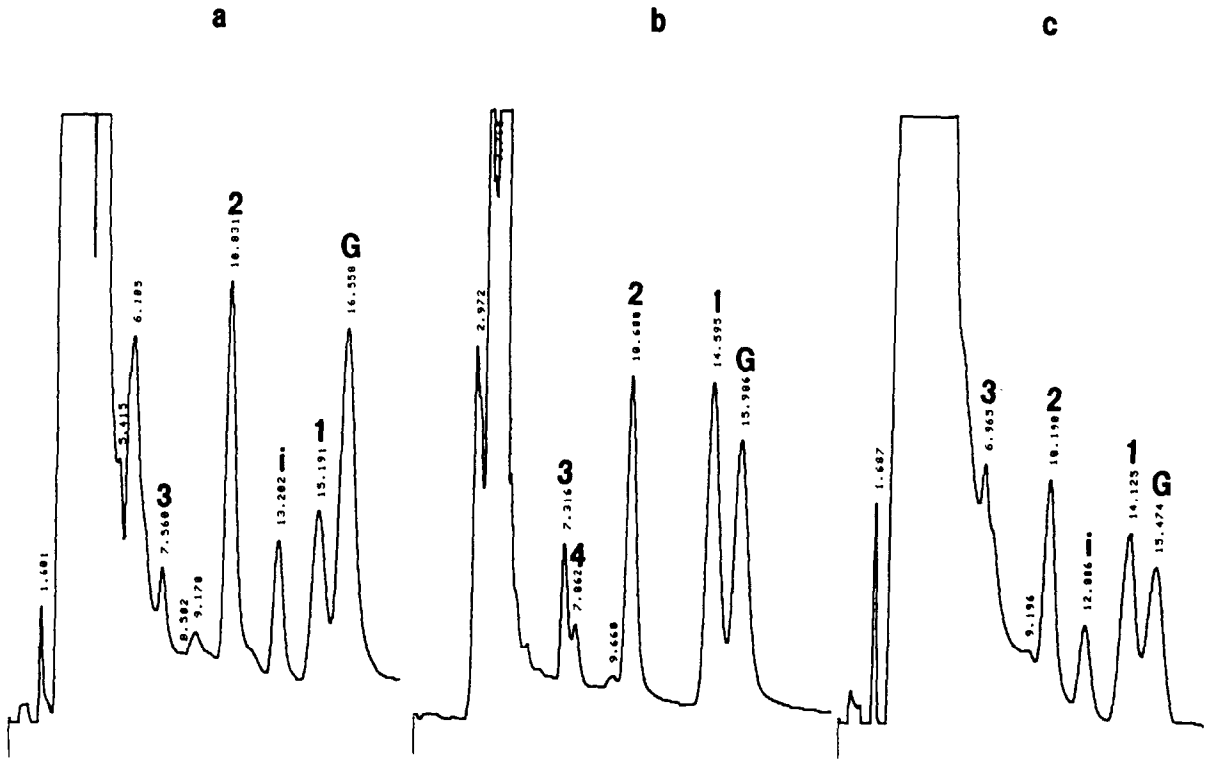

Fig. 5. Analysis of samples collected from nude mice treated with 45 mg/kg antagonist G. Chromatographic details as in Fig. 4. Chromatogram a, plasma (approximately 100 μl) collected 2 h after i.v. treatment, FSD = 400 nA. The concentration of intact peptide was 1.06 μM. Peak i is an interference introduced during sample preparation and peaks 1–3 are metabolites. Chromatogram b, liver (approximately 0.1 g) collected 2 h after i.p. treatment, FSD = 1400 nA. The concentration of G was 17.8 μM and 4 metabolite peaks were detected. Chromatogram c, tumour (approximately 0.1 g) collected 45 min after i.p. treatment, FSD 200 nA. Concentration of antagonist G was 0.41 μM and 3 metabolites were identified.

3.3. Analysis of biological specimens from nude mice bearing the WX 322 SCLC xenograft treated with either antagonist D or G

Figs. 4–6 show a selection of HPLC traces which illustrate the full range of chromatographic profiles encountered during the analysis of biological specimens collected from treated animals. These chromatograms are compared against examples of the same biological specimens taken from control, non-peptide treated animals (Fig. 4b and Fig. 6a). As well as measuring the parent peptide a number of clearly identifiable new chromatographic peaks related to drug administration were evident. In the case of antagonist G at least four new peaks (see Fig. 5: peak 1, t_R 14.1–15.1 min; peak 2, t_R 10.1–10.6 min; peak 3, t_R 7.0–7.5 min, and peak 4, t_R 7.8 min) were resolved and consistently appeared in plasma, liver and tumour specimens. No additional peaks were resolved from antagonist D in liver and tumour specimens and only one separate peak was identified in plasma (see Fig. 6: peak 1, t_R 5.3 min). At present these peaks are being purified for amino acid sequencing and for characterization of their biological properties. Due to the highly selective nature of the EC detection system, it can be concluded that these peaks represent sequences which contain either one or both D-tryptophan residues derived from the intact peptide. D-Tryptophan itself had a t_R of 3.5 min. As a consequence, their quantitation, on the basis of moles of tryptophan, can be achieved using a standard curve of intact peptide

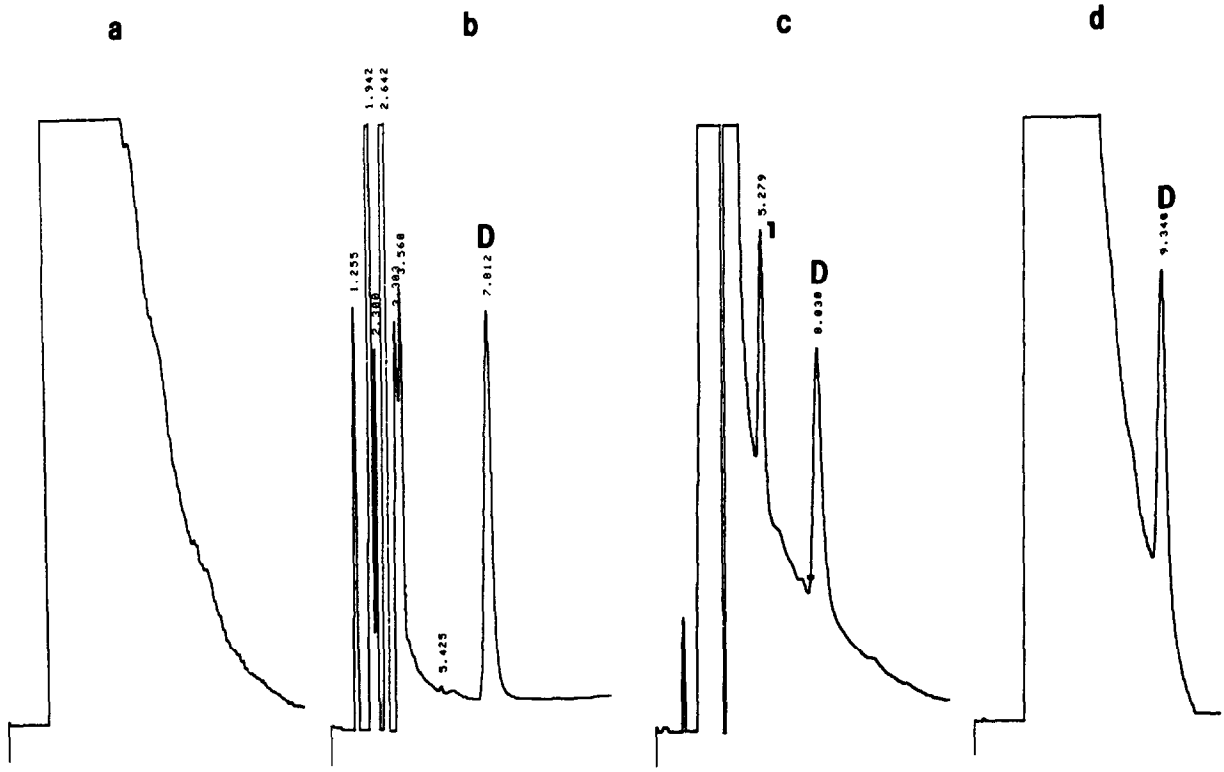

Fig. 6. Analysis of samples collected from nude mice treated with 12 mg/kg antagonist D. Chromatographic details as in Fig. 4. Chromatogram a, liver (approx 0.1 g) from non-peptide treated nude mouse, FSD = 100 nA. Chromatogram b, 50 ng/33 pmoles of a standard 1 *M* acetic acid solution of antagonist D (t_R, 7.81 min), FSD 200 nA. Chromatogram c, plasma (approximately 100 μl) collected 3 h after i.p. treatment. The concentration of antagonist D was 1.5 μM and peak 1 was a metabolite. Chromatogram d, liver (approx 0.1 g) collected 30 min after i.p. treatment with antagonist D, FSD = 100 nA. The concentration of antagonist D was 3.7 μM.

Table 3
Concentrations (μM) of intact peptide after administration of 12 mg/kg antagonist D and 45 mg/kg antagonist G to nude mice bearing the WX 322 SCLC xenograft

Sample	Concentration (μM)							
	30 min				6 h			
	Intravenous		Intraperitoneal		Intravenous		Intraperitoneal	
	D	G	D	G	D	G	D	G
Plasma	1.5	1.83	1.7	10.7	0.61	0.13	0.53	0.10
Liver	<0.10	5.23	3.7	34.6	0.25	0.18	0.27	0.21
Tumour	0.49	0.10	<0.10	0.46	0.49	<0.10	0.39	0.10

despite the fact that no information is known about their molecular weights.

Levels of intact antagonists D and G measured *in vivo* 30 min and 6 h after i.v. and i.p. peptide administration are contained in Table 3 as μmol equivalents (for tissue specimens 1 g is taken as representing 1 ml). The 30-min time point represents the peak levels achieved after i.p. administration. From *in vivo* studies with SCLC cell lines it has been shown that continuous exposure to concentrations above 10 μM are required for inhibition of growth [10]. In the present work, at the two key time points studied, the concentration of native peptide in the tumour never exceeded 1 μM (see Table 3). These preliminary pharmacokinetic data, therefore, suggest that doses well in excess of those employed here (or continuous administration schedules) are necessary to reach these target peptide concentrations in the tumour. Indeed, it has been shown with antagonist G that 45 mg/kg i.p. has to be injected on 14 consecutive days in order to produce marginal activity against WX 322 [14].

4. Conclusion

We have developed a selective HPLC method for direct determination of two substance P analogues which are under consideration for clinical trials as broad spectrum antagonists of neuropeptide growth factors in SCLC. The methodology is sensitive and can detect the intact peptides at least 6 h after their administration to animals at pharmacological and therapeutic doses. It is also efficient and identifies a number of interesting *in vivo* derived metabolites which probably arise from the action of plasma and tissue peptidases.

5. References

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