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Journal of Chromatography B, 732 (1999) 277–285

JOURNAL OF CHROMATOGRAPHY B

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Development of a gradient elution high-performance liquid chromatography assay with ultraviolet detection for the determination in plasma of the anticancer peptide [Arg^{6}, D-Trp^{7,9}, mePhe^{8}]-substance P (6–11) (antagonist G), its major metabolites and a C-terminal pyrene-labelled conjugate

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Received 25 February 1999; received in revised form 15 June 1999; accepted 22 June 1999

Abstract

[Arg^{6}, D-Trp^{7,9}, mePhe^{8}]-substance P (6–11), code-named antagonist G, is a novel peptide currently undergoing early clinical trials as an anticancer drug. A sensitive, high efficiency high-performance liquid chromatography (HPLC) method is described for the determination in human plasma of antagonist G and its three major metabolites, deamidated-G (M1), G-minus Met^{11} (M2) and G[Met^{11}(O)] (M3). Gradient elution was employed using 40 m*M* ammonium acetate in 0.15% trifluoroacetic acid as buffer A and acetonitrile as solvent B, with a linear gradient increasing from 30 to 100% B over 15 min, together with a microbore analytical column (μBondapak C_{18}, 30 cm×2 mm I.D.). Detection was by UV at 280 nm and the column was maintained at 40°C. Retention times varied by <1% throughout the day and were as follows: G, 13.0 min; M1, 12.2 min; M2, 11.2 min; M3, 10.8 min, and 18.1 min for a pyrene conjugate of G (G–P). The limit of detection on column (LOD) was 2.5 ng for antagonist G, M1–3 and G–P and the limit of quantitation (LOQ) was 20 ng/ml for G and 100 ng/ml for M1–3. Sample clean-up by solid-phase extraction using C_2-bonded 40 μm silica particles (Bond Elut, 1 ml reservoirs) resulted in elimination of interference from plasma constituents. Within-day and between-day precision and accuracy over a broad range of concentrations (100 ng/ml–100 μg/ml) normally varied by <10%, although at the highest concentrations of M1 and M2 studied (50 μg/ml), increased variability and reduced recovery were observed. The new assay will aid in the clinical development of antagonist G.

Keywords: Peptides; [Arg^{6}, D-Trp^{7,9}, mePhe^{8}]-substance P (6–11); Antagonist G

1. Introduction

[Arg^{6}, D-Trp^{7,9}, mePhe^{8}]-substance P (6–11), code-named antagonist G, is the lead member of a novel series of chemically modified substance P analogues (SPA) [1] and is currently undergoing early clinical trials as an anticancer drug [2]. Antagonist G is believed to work by preventing receptor binding and subsequent mitogenic signalling [calcium mobilisation, protein kinase C (PKC) activation, mitogen-activated protein kinase (MAPK) acti-

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00294-7

vation, tyrosine phosphorylation] initiated by a broad spectrum of neuropeptide autocrine and paracrine growth factors constitutively expressed by small cell lung cancer (SCLC) [1,3,4]. In addition to inhibiting the growth of human SCLC cells in vitro, antagonist G displays in vivo antitumour activity against a panel of human xenografts including SCLC [5,6].

The molecular basis for the ability of antagonist G to compete with receptor binding of several structurally unrelated neuropeptides, such as vasopressin, gastrin-releasing peptide (GRP), bradykinin and neurotensin, remains to be resolved [3,4,7]. However, it is now clear that SPAs exhibit a more complex mechanism of action than simple competitive inhibition of ligand binding [8,9]. Their effect on cell proliferation extends beyond cytostasis and can involve the induction of programmed cell death, i.e., apoptosis [10,11]. Antagonist G can directly interact with G-proteins downstream of neuropeptide receptors and stimulate *c-jun*–N-terminal kinase (JNK) via a receptor-independent mechanism that is dependent on the generation of reactive oxygen species [8]. Recently, SPAs have even been shown to activate certain signal transduction pathways (extracellular regulated kinase (ERK) and stress activated protein (SAP) kinases, cytoskeletal changes) through preferential interactions with different activated conformational states of neuropeptide receptors [9]. This mechanism is termed biased agonism and can extend to non-neuropeptide receptors (interleukin-8-R; IL8-R). Thus, antagonist G exhibits a unique mechanism of action as a potential anticancer drug.

The introduction of D-amino acids and an N-methylated peptide bond in the structure of antagonist G confers increased stability against peptidase catabolism, yielding a half life of 28.8 min in mice, which is two orders of magnitude greater than that of naturally occurring substance P [12]. Nevertheless, antagonist G undergoes complex metabolism catalysed predominately by a cytosolic chymotrypsin-like serine carboxypeptidase/deamidase [13] to two major metabolites, which exhibit only a limited spectrum of neuropeptide antagonism [12,14]. Initially, antagonist G is deamidated (M1), followed by C-terminal removal of methionine^{11} (M2), in a sequential pathway that is pH dependent. In addition, antagonist G undergoes facile oxidation at methionine^{11} (M3) by a predominately non-enzymatic process that is accelerated in vivo [15,16]. G[Met^{11}(O)] also undergoes deamidation and evidence of further amino- and carboxypeptidase metabolism of M2 to yield M4 (metabolite 4) results in the detection of at least four major HPLC peaks in animal plasma, tissue and tumour specimens [12].

In the present report, a sensitive HPLC method is described for the determination of antagonist G and its major metabolites (M1–3) in plasma, where electrochemical detection [17] has been substituted with UV (photodiode array, PDA) detection. Increased sensitivity was achieved by the adoption of gradient elution and a microbore analytical column. Elimination of interference from plasma was accomplished by extensive sample clean-up using solid-phase extraction (SPE) sample preparation and the choice of 280 nm as the monitored wavelength. To provide insights into the nature of the molecular interactions between antagonist G and cellular components and neuropeptide receptors, a fluorescently labelled C-terminal derivative conjugated with pyrene was synthesised. In order to study the chemical and biological stability of this conjugate in tissue culture systems, it was included as a standard in the development of the method described.

2. Experimental

2.1. Materials

NH_2–Arg–D-Trp–mePhe–D-Trp–Leu–Met–NH_2 (M_r, 951; antagonist G, 99% peptide purity, 16.8% acetate content); NH_2–Arg–D-Trp–mePhe–D-Trp–Leu–Met–COOH (M_r, 952; M1) and NH_2–Arg–D-Trp–mePhe–D-Trp–Leu–COOH (M_r, 821; M2) were synthesised and provided as a kind gift by Peptech, Dee Why, NSW, Australia. The structures of these products were independently validated by HPLC amino acid analysis after acid-catalysed hydrolysis using the AccQ.Flour™ derivatisation reagent (Waters, Watford, UK) and by positive fast atom bombardment (FAB) mass spectrometry (MS) [14]. NH_2–Arg–D-Trp–mePhe–D-Trp–Leu–Met(O)–NH_2 (M_r, 967; M3) was synthesised in-house by treatment of antagonist G with hydrogen peroxide, as previously described [15], followed by purification

using SPE [17] and validation as above. NH_2–Arg–D-Trp–mePhe–D-Trp–Leu–Met–CO–NH–CH_2-Pyrene (M_r, 1165; G–P) was custom-synthesised by Dr Ram Sharma (Department of Biochemistry, University of Southampton, Southampton, UK). The structure of the conjugate was confirmed by FAB–MS and was estimated to be 94.7% pure by HPLC. 1-Pyrenemethanol (P–M, 98% purity) was obtained from Aldrich (Poole, UK); ammonium acetate (Aristar grade) was from Merck (Poole, UK), trifluoroacetic acid (TFA) was from Sigma (Poole, UK), acetonitrile and methanol were of HPLC reagent grade and from Rathburn Chemicals (Walkerburn, UK). Water was purified in a Millipore MilliQ water purification system (Millipore, Watford, UK). All other chemicals and reagents used were of the highest grade available commercially.

2.2. High-performance liquid chromatography

The liquid chromatograph used throughout consisted of an Alliance 2690 separations module and a 996 PDA detector with peak integration at 280 nm for quantitative analysis (Waters). Full system control, data collection, data analysis and data reporting were performed using Millennium Software (revision 2.21, Waters) operating on a Pentium processor personal computer. The stationary phase was μ-Bondapak C_{18}-bonded 10 μm silica particles packed in a 30 cm×2 mm I.D. stainless steel column (Waters). The final mobile phase comprised 40 m*M* ammonium acetate in 0.15% TFA as buffer A and acetonitrile as solvent B. Gradient elution was employed according to the following linear programme: time zero, 30% solvent B; 15 min, 100% solvent B; 20 min, 100% solvent B and 23 min, 30% solvent B. The flow-rate was 0.3 ml/min, the total run time was 25 min, the column was maintained at a temperature of 40°C and the autosampler at a temperature of 20°C. Mobile-phase components were filtered prior to use and vacuum degassed in situ during chromatography. The injection volume was 50 μl.

2.3. Sample preparation

Human plasma samples (or tissue culture media), up to 1 ml, were processed by SPE using C_2-bonded 40 μm silica particles packed in 1-ml-capacity mini-columns operating under negative pressure (Bond Elut C_2, Varian Associates, supplied by Phenomenex, Macclesfield, UK), as previously described [17]. The mini-columns were first activated with 1 ml of methanol, followed by conditioning with 1 ml of water prior to sample loading. The mini-columns were then washed sequentially with 1 ml of water, 1 ml of methanol–water (50:50, v/v) and 1 ml of acetonitrile prior to elution with 400 μl of methanol–l *M* ammonium acetate (90:10, v/v).

2.4. Assay validation

Antagonist G is highly water-soluble but exhibits maximum chemical stability at pH 4.2 [15,16]. Non-extracted standards of antagonist G and its major metabolites were dissolved and diluted in 90% methanol–10% 1 *M* ammonium acetate, the final eluting buffer in the SPE sample preparation technique, since diluent has been shown to have a major bearing on assay linearity (see below). For extracted standard curves and quality control samples, separate weighings of antagonist G and metabolites were performed. Stock solutions were prepared by dissolving antagonist G and metabolites in water. These concentrated stock solutions were initially diluted 1:9 (v/v) in plasma obtained from healthy volunteers and further diluted in control plasma to produce the range of concentrations described below. Extracted standard curves were prepared fresh at the following seven concentrations: 0.1, 0.5, 1, 5, 10, 50 and 100 μg/ml (12.5 ng–12.5 μg on-column). Quality control samples were prepared fresh at the following concentrations: 0.1, 0.5, 1, 10 and 100 μg/ml for antagonist G; 0.5, 1, 10 and 50 μg/ml for M1 and M2 and 0.5, 1, 10 and 100 μg/ml for M3.

Quality control samples were analysed for within-day ($n=5$) and between-day ($n=5$ on five–six separate days) precision and accuracy determination over the above concentration ranges. G–P and P–M standards were dissolved and diluted in dimethyl sulphoxide (DMSO) and were used as chromatographic standards and were not subjected to assay validation. The efficiency of the SPE method (i.e., recovery) was determined by taking the ratio of chromatographic peak areas of the extracted quality

control samples against those of a non-extracted standard curve.

2.5. Analysis of plasma samples from patients

Plasma samples were obtained from patients receiving antagonist G during a phase I trial performed in Edinburgh and were immediately frozen to −20°C. For longer-term storage, samples were maintained at −80°C prior to analysis. However, samples were normally analysed less than one month after collection, and long-term storage for several months (six months) did not result in significant loss of the peptide.

3. Results

3.1. Development of a gradient elution HPLC separation method for antagonist G, its major metabolites and a fluorescent analogue

The chemical structure of antagonist G consists of a highly hydrophobic (non-water soluble) tripeptide core consisting of D-Trp–mePhe–D-Trp, plus a hydrophobic C-terminal amide sequence (Leu–Met–NH_2) [15], yet, at physiological pH, it possesses a double-positive charge due to the presence of two basic residues at the N-terminal of the peptide (NH_2–Arg). This is likely to yield a high p*I* value for the peptide, of the order of 12.5, which is the pK_a for the guanidyl substituent of arginine [18]. The most difficult problem in the HPLC analysis of antagonist G is adsorption of the peptide onto the HPLC or SPE stationary phases (and storage container materials) resulting in loss of response and non-linearity followed by subsequent desorption, resulting in ghost peaks and over-estimation of the concentration [17]. This phenomenon is particularly troublesome at concentrations of antagonist G below 1 μg/ml and above 1 mg/ml. Incorporation of ammonium acetate into the HPLC mobile phase and SPE eluting solution significantly reduces secondary interactions and improves linearity [17]. In the present study, it has been established that diluent has a major bearing on the linearity of standard curves. Typical non-extracted calibration curves over the concentration range of 100 ng/ml–100 μg/ml deviated significantly from linearity ($y=383x$, $r^2=0.983$) when antagonist G was dissolved and diluted in water. Dissolving antagonist G in acetic acid improved the linearity of non-extracted standard curves ($y=417x$, $r^2=0.997$). However, dissolving and diluting antagonist G in the SPE eluting buffer, which comprised 90% methanol–10% 1 *M* ammonium acetate (EB), completely cured the problem of non-linearity of non-extracted standard curves ($y=582x$, $r^2=1.000$). At low concentrations of aqueous standards (50 ng and less), no antagonist G chromatographic peak was detectable. Subsequently, all standards of antagonist G and its metabolites were dissolved in EB, providing consistency with the SPE technique. It is likely that EB quenches the immediate adsorption of antagonist G onto the HPLC column after injection [19].

Optimisation of the resolution of antagonist G and its metabolites, together with the fluorescent conjugate G–P, was achieved by modifying the buffer ionic strength (ammonium acetate) of the mobile phase (Fig. 1). This had the net result of increasing retention and improving resolution without affecting sensitivity (peak width) while actually increasing peak symmetry. A typical chromatogram of standards of antagonist G, its three metabolites, G–P and P–M is illustrated in Fig. 2. Typical retention times (t_R) were as follows: antagonist G, 13.0 min; M1, 12.2 min; M2, 11.2 min; M3, 10.8 min; G–P, 18.1 min and P–M, 13.9 min. Within-day coefficients of variation in t_R of all six components were less than 1%. The LOD on-column at the signal-to-noise ratio of 3:1 was 2.5 ng for G, M1–3 and G–P and the LOQ yielding precision and accuracy of <20% was 20 ng/ml for G and 100 ng/ml for M1–3 (see below).

3.2. Assay validation

Assay validation was conducted using 1 ml samples of plasma from volunteers, spiked with a wide range of concentrations (100 ng/ml to 100 μg/ml for antagonist G; 500 ng/ml to 50 μg/ml for M1 and M2 and 500 ng/ml to 100 μg/ml for M3) encompassing those determined in patients during an ongoing phase I clinical trial of antagonist G performed in Edinburgh [2]. Antagonist G is not metabolised by human plasma but does undergo an

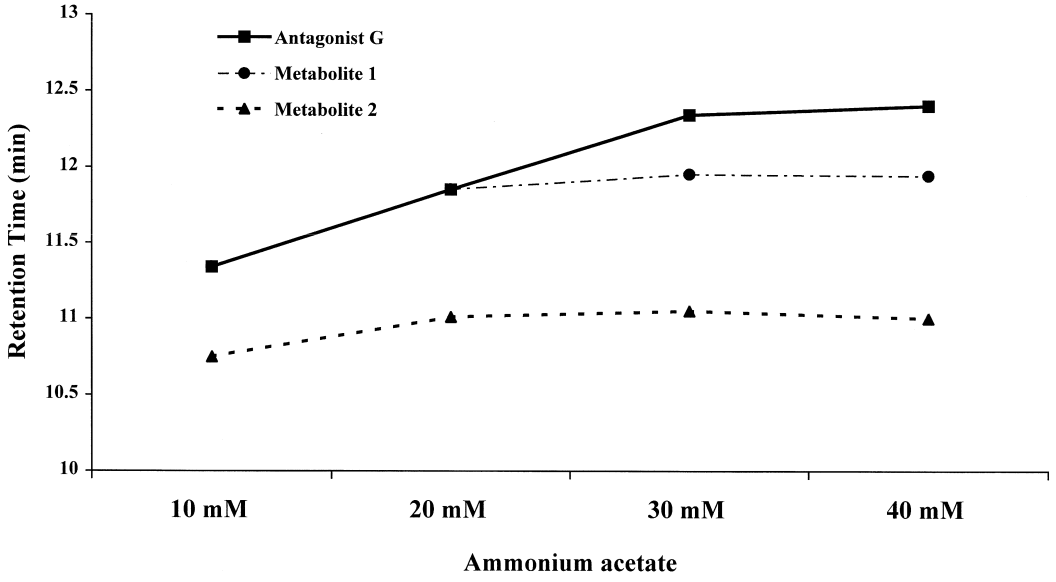

Fig. 1. Effect of buffer ionic strength on the resolution of antagonist G and its metabolites M1 (deamidated antagonist G) and M2 (G-minus Met[11]) by gradient elution reversed-phase HPLC using a microbore analytical column. For full details of the analytical conditions, see Section 2.2. –■–, Antagonist G; ·–●–·, M1 and --▲--, M2.

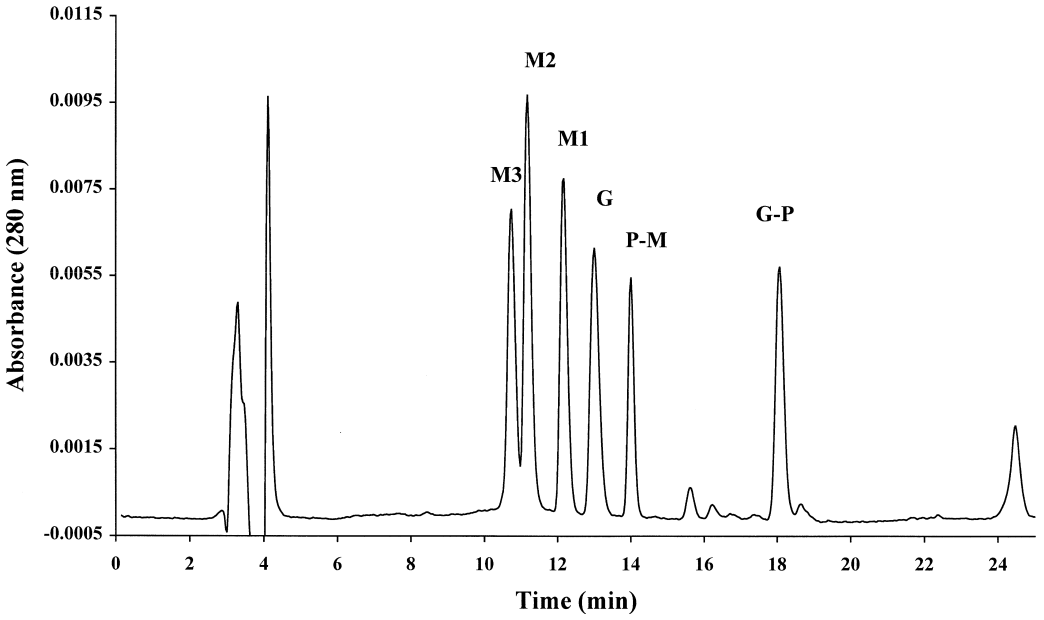

Fig. 2. Separation of antagonist G, it major metabolites M1–3, a 1-pyrene C-terminal conjugate of G (G–P) and 1-pyrenemethanol (P–M) by gradient elution reversed-phase HPLC using a microbore analytical column. Each peak represents 200 ng on-column, with the exception of P–M, at 50 ng.

Table 1
Within-day and between-day accuracy and precision and recovery for the determination of [Arg[6], D-Trp[7,9], mePhe[8]]-substance P (6–11), code-named antagonist G, in human plasma

Concentration G (μg/ml)	Within-day accuracy (μg/ml) (n=5)	Precision (±%)	Between-day[a] accuracy (μg/ml) (n=30)	Precision (±%)	Within-day recovery (%) (n=5)	SD
0.1	0.088	10.9	ND[b]	ND[b]	75.5	16.4
0.5	0.55	4.0	0.53	13.1	70.7	49.7
1	1.22	4.3	1.06	11.6	99.6	9.2
10	10.9	14.4	11.6	9.0	71.4	9.2
100	108.5	3.4	98.3	5.6	89.1	7.9

[a] Six separate days.
[b] ND=not determined.

accelerated rate of methionine[11] mono-oxidation (half life, 4.4 h at 100 ng/ml and 5.0 h at 10 μg/ml at 37°C) in comparison to complete stability over 24 h in water, 5% dextrose and 0.9% sodium chloride [15,20]. Recovery of antagonist G from plasma ranged from 70.7 to 99.6%, which is in accordance with recoveries determined using electrochemical (EC) detection and isocratic elution. Precision and accuracy for antagonist G normally varied by less than 10% from actual values (see Table 1). Occasionally, these values could vary in excess of 20%, which may reflect inherent instabilities due to reduced aqueous solubility in high ionic strength media [21], oxidation [17], tight binding to plasma proteins and adsorption onto materials.

A similar pattern of precision and accuracy was also observed with M1–3 (Table 2), although a clear trend towards reduced absolute recovery and increased variability was recorded specifically with M1 and M2 at the highest concentration studied (50 μg/ml). Thus, for M1 and M2, an upper limit of quantitation of 10 μg/ml is recommended. M1 and M2 differ from both antagonist G and M3 by the introduction of a negatively charged free acid at the

Table 2
Within-day and between-day accuracy and precision and recovery for the determination of the three major metabolites of [Arg[6], D-Trp[7,9], mePhe[8]]-substance P (6–ll) in human plasma[a]

Concentration (μg/ml)	M1			M2			M3[b]		
	Within-day accuracy ±precision (μg/ml) ±% (n=5)	Between-day[c] accuracy ±precision (μg/ml) ±% (n=25)	Recovery mean (%)±SD (n=5)	Within-day accuracy ±precision (μg/ml) ±% (n=25)	Between-day accuracy ±precision (μg/ml) ±% (n=5)	Recovery mean (%)±SD (n=5)	Within-day accuracy ±precision (μg/ml) ±% (n=25)	Between-day accuracy ±precision (μg/ml) ±% (n=25)	Recovery mean (%)±SD (n=5)
0.5	0.49± 10.4	0.46± 16.6	90.1± 20.1	0.46± 27.8	0.47± 23.1	52.1± 16.2	0.46± 10.8	0.49± 11.5	118.9± 7.7
1	1.09± 8.9	1.01± 19.0	115.0± 18.4	0.92± 4.8	1.0± 15.9	61.1± 14.5	0.87± 12.9	0.93± 10.1	101.1± 8.5
10	12.5± 11.5	10.2± 19.6	53.7± 11.0	11.8± 7.5	9.7± 27.9	33.7± 9.8	10.2± 5.9	11.0± 9.4	81.7± 12.9
50[b]	67.0± 17.1	56.1± 30.1	47.4± 15.3	45.4± 31.7	42.9± 46.5	28.9± 13.4	103.1 + 2.4	95.0± 4.4	93.6± 8.9

[a] For the amino acid sequence of metabolites, see Section 2.1.
[b] 100 μg/ml for M3.
[c] Five separate days.

C-terminal, and this may have a major bearing on analytical performance.

3.3. Analysis of plasma samples from patients

Substitution of EC detection [17] with UV detection raised the possible problem of reduced selectivity and increased interference from endogenous components present in plasma. Indeed, the use of SPE and gradient HPLC for related peptides has been demonstrated to preclude UV-detection at shorter wavelengths (200–220 nm) due to very large solvent fronts and interfering peaks, requiring instead fraction collection and radioimmunoassay (RIA) detection [22,23]. However, due to the presence of three aromatic residues in the structure of antagonist G, the peptide exhibits strong UV-adsorption centred at 275 nm (ε, 7690) [20]. A wavelength of 280 nm was chosen in the present study and yielded good sensitivity (LOD, 2.5 ng on-column) when coupled to the microbore column and produced plasma extracts that were free from interference from 10 to 16 min, encompassing antagonist G and its three major metabolites. See Fig. 3 for examples of a blank plasma extract and samples spiked with 50 and 100 ng/ml antagonist G. Fig. 4 illustrates the end of infusion blood samples (6 h) collected after administration of a lower (135 mg/m^2 as a 6-h infusion) and higher dose (300 mg/m^2 as a 6-h infusion) of antagonist G. In these typical specimens from patients, only low levels of metabolites 1–3 (<1 μg/ml) were detected, in contrast to the situation found in mouse plasma samples where parent peptide might only account for 40% of the total, and metabolites (M1–4) could account for up to 60% of the total [12].

4. Discussion

In this work, a gradient elution HPLC method with UV-detection and SPE sample preparation has been developed for the sensitive determination of the anticancer peptide [Arg^6, D-Trp^{7,9}, mePhe^8]-substance P (6–11) (antagonist G) and its major metabolites in human plasma. Previous HPLC techniques developed for the pharmaceutical analysis of antagonist G have lacked efficiency and full resolution of

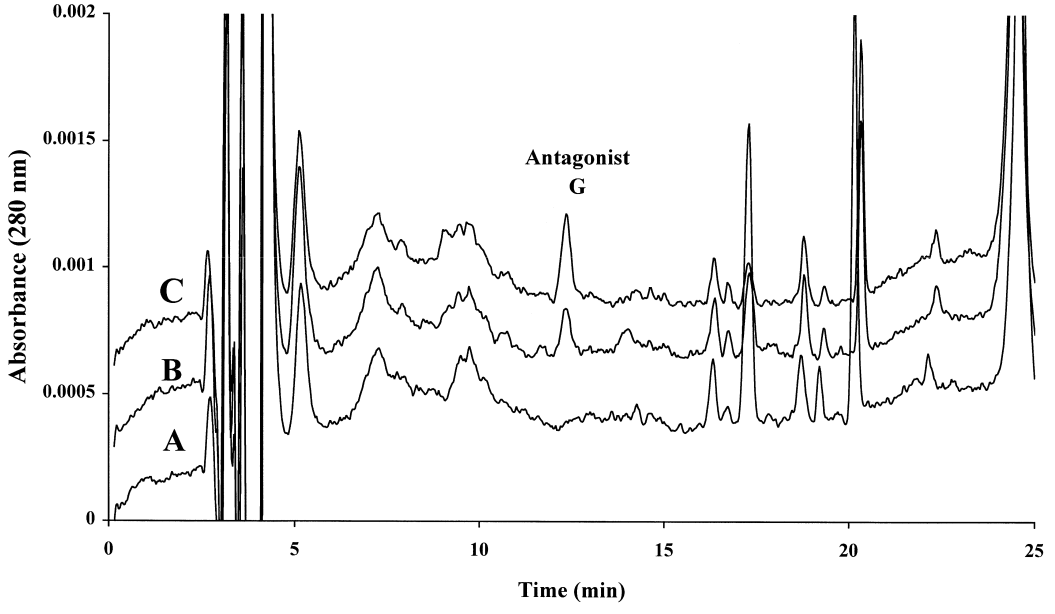

Fig. 3. Analysis of human plasma spiked with antagonist G using SPE and gradient elution HPLC with a microbore column. Analytical techniques are described in Experimental. Chromatogram A, human blank plasma; B, human plasma spiked with 50 ng/ml antagonist G and C, human plasma spiked with 100 ng/ml antagonist G.

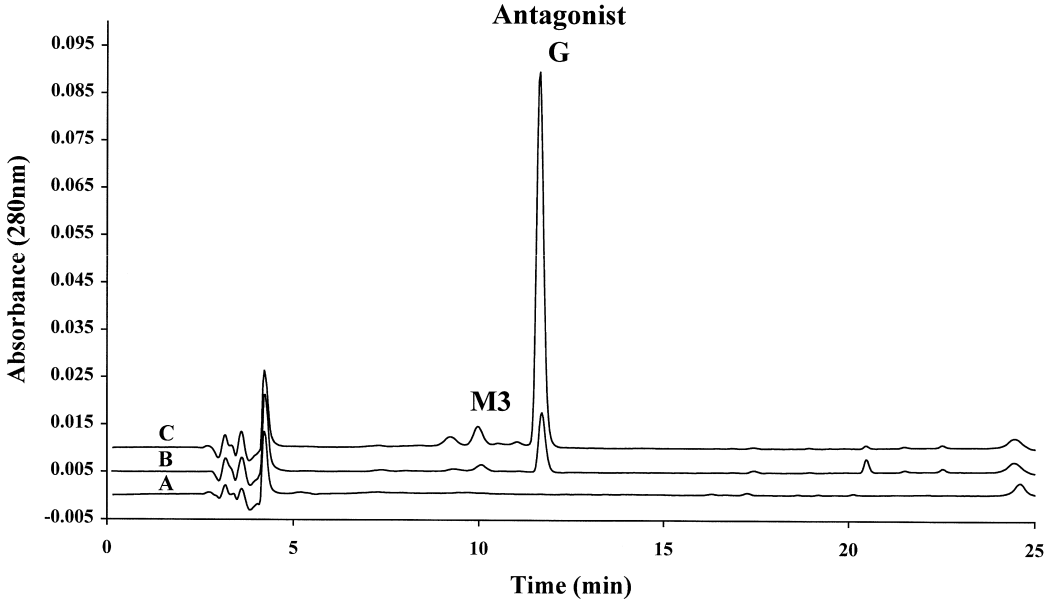

Fig. 4. Analysis of plasma from patients treated with antagonist G using SPE and gradient elution HPLC with a microbore column. Analytical techniques are described in Experimental. Chromatogram A, pre-dose plasma. Chromatogram B, plasma collected at the end of a 6-h i.v. infusion of 135 mg/m^2 antagonist G. The level of antagonist G was 2.82 μg/ml and of M3 was 0.34 μg/ml. Chromatogram C, plasma collected at the end of a 6-h i.v. infusion of 300 mg/m^2 antagonist G. The level of antagonist G was 18.4 μg/ml and that of M3 was 0.87 μg/ml.

degradation products and are not applicable to the determination of the peptide in biological matrices [16,18,20,24]. In these studies, which involved isocratic reversed-phase HPLC and gradient elution, antagonist G was monitored by UV detection at 214 nm and was not subjected to a sample preparation technique. Another HPLC technique published by our laboratory also lacks full resolution of metabolites [12,15,17]. In these studies, isocratic conditions were necessitated by the adoption of electrochemical (coulometric) detection, which was perceived to be necessary in order to provide the greater sensitivity required in bioanalysis and pharmacokinetic studies. However, present dosage levels being administered to patients are in excess of 300 mg/m^2, resulting in peak plasma levels in excess of 20 μg/ml. Thus, this sample analysis method does not require the increased sensitivity of EC detection [2], with its associated difficulties of variable electrode responses, a more limited detection range, the need to frequently clean electrodes and poor detector stability at higher sensitivity [17]. The present method achieves a limit of quantitation of 20 ng/ml for antagonist G by application of gradient elution and a microbore (2 mm I.D.) column that is five times more sensitive than the level of antagonist G measured in patient plasma at 24 h after a dose of 300 mg/m^2. Chromatograms are free from interference and the method achieves high efficiency baseline separation of antagonist G and its three major metabolites, together with a fluorescently labelled pyrene conjugate of the parent peptide.

In addition to the three major metabolites of antagonist G featured in the present study, seven degradation products have been identified by HPLC and partially characterised by a variety of spectroscopic techniques [16,18,20,24]. These products include deamidated antagonist G (otherwise known as M1 in the present work); oxidised antagonist G (M3); conversion of arginine[6] into ornithine (including loss of the guanidyl substituent) and racemization of L-methionine into D-methionine. However, these products are only formed under extreme conditions of temperature and pH and do not appear to

be formed in stability studies carried out at pH values of 4–7 and at 37°C, or in metabolism studies performed both in vitro and in vivo [12,14,17].

5. Conclusion

In conclusion, a new HPLC method, together with a SPE method, is presented for the determination of antagonist G and its major metabolites in human plasma. Adsorption and loss of peptide during HPLC is eliminated by the incorporation of 40 m*M* ammonium acetate in the mobile phase and by dissolving the peptide in methanol–1 *M* ammonium acetate (90:10, v/v). Increased sensitivity and high resolution is achieved by adoption of gradient elution and microbore column technology. Interference from endogenous plasma components is reduced by extensive sample clean-up and the choice of 280 nm as the monitor wavelength without compromising sensitivity, which was 20 ng/ml (LOQ) for antagonist G and 100 ng/ml for its metabolites.

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