Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 987 (2003) 249–256
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of PNU-248686A, a novel matrix metalloproteinase inhibitor, in human plasma by liquid chromatography–tandem mass spectrometry, following protein precipitation in the 96-well plate format

E. Frigerio*, V. Cenacchi, C.A. James
Global Drug Metabolism, Pharmacia, Viale Pasteur 10, 20014 Nerviano, Milan, Italy

Abstract

A sensitive, specific and high-throughput analytical method for the quantitation of PNU-248686A (*I*), in human plasma has been developed. *I*, sodium (2*R*)-3-{[(4′-chloro(1,1′-biphenyl)-4-yl]sulfonyl}-2-hydroxy-2-[(phenylsulfanyl)methyl] propanoate, is an orally active matrix metalloproteinase (MMP) inhibitor developed for the treatment of solid tumors over-expressing MMPs. Concentrations of *I*, as free acid, were determined in human plasma by LC–MS–MS after plasma protein precipitation in the 96-well plate format. Aliquots of plasma (50 μl) were placed into the plates and 0.2 ml of methanol was added. The plates were shaken for 5 min and centrifuged at 1500 *g* for 10 min. Aliquots of 10 μl of the supernatants were then directly injected into the LC–MS–MS system. A Symmetry Shield C_8 column (50×2.1 mm, 3.5 μm) was used to perform the chromatographic analysis. The mobile phase was 5 m*M* ammonium formate buffer solution pH 5.0–acetonitrile (60:40, v/v) with a flow-rate of 0.3 ml/min. Retention time of *I* was about 1.2 min. Total cycle time was 2.5 min. MS detection used the Applied Biosystems–MDS Sciex API 3000 with TurboIonSpray interface and single reaction monitoring (461→251 *m*/*z* transition) operated in negative ion mode. Calibration curves were constructed by plotting the area of the compound (*y*) against its concentration (*x*). A weighed linear regression (weighting factor $1/x^2$) was used to calculate *I* concentrations in quality control and unknown samples. The method was fully validated over the range of 5.0–5000 ng/ml. The suitability and robustness of the method for in vivo samples was confirmed by analysis of plasma samples from a pilot clinical study.

Keywords: PNU-248686A; Matrix metalloproteinase inhibitors; Enzyme inhibitors; Sodium (2*R*)-3-{[(4′-chloro(1,1′-biphenyl)-4-yl]sulfonyl}-2-hydroxy-2-[(phenylsulfanyl)methyl]propanoate

1. Introduction

Extracellular matrix (ECM) and basal membranes must be degraded by cancer cells to grow within the host tissue and to migrate to distant sites to form metastases. This process is partly mediated by matrix metalloproteinases (MMPs), proteolytic enzymes over-expressed by cancer cells directed against components of the ECM [1]. Moreover, MMPs are also produced by endothelial cells during tumor-dependent neoangiogenesis, thus contributing to new vessel formation [2]. Given the documented role of MMPs in cancer invasion and angiogenesis, inhibition of these enzymes may represent a novel ap-

*Corresponding author. Tel.: +39-02-4838-3197; fax: +39-02-4838-3012.
E-mail address: enrico.frigerio@pharmacia.com (E. Frigerio).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)01907-6

proach for cancer therapy to prevent disease progression and metastasis [3–6]. The MMP family currently consists of more than 20 members. Gelatinases (e.g. MMP-2 and MMP-9) are the most relevant MMPs involved in tumor progression and metastatic spread. In particular, the role of MMP-2 in tumor progression, metastases and angiogenesis has been demonstrated both in in vitro and in vivo models. On the other hand, a possible association of collagenase (e.g. MMP-1) inhibition with muscoloskeletal toxicity has been suggested, as MMP-1 is expressed in joints.

In a research program aimed at developing pharmacological agents able to inhibit MMPs by oral route (2*R*)-3-{[(4′-chloro(1,1′-biphenyl)-4-yl]sulfonyl}-2-hydroxy-2-[(phenylsulfanyl)methyl] propanoate sodium salt (laboratory code: PNU-248686A, *I*, see Fig. 1) was synthesized. The inhibition profile (K_i) of *I* against a panel of recombinant MMPs was evaluated by a biochemical method based on the hydrolysis of a fluorescent peptidic substrate [7]. The highly specific MMP-inhibition profile observed suggested the possibility of obtaining antitumor efficacy without inducing joint toxicity. The in vivo antitumor activity of this compound was confirmed using DU145 human prostate carcinoma and HCT116 human colon carcinoma xenografts in nude mice.

Several methodologies have been developed for the determination of MMPs inhibitors in biofluids [8]. In recent years, liquid chromatography with tandem mass spectrometry (LC–MS–MS) has become the method of choice for analysis of drugs in biological samples because of its near universality, specificity, sensitivity and speed. Many sample preparation techniques have been developed for processing plasma samples. Among them, protein precipitation, liquid–liquid and solid-phase extraction are most commonly used. This paper describes a new LC–MS–MS method for the determination of *I* in human plasma after a simple and rapid protein precipitation step in the 96-well plate format. The method developed was fully validated down to a plasma concentration of 5 ng/ml. The suitability of the method for in vivo samples was checked by analysis of plasma samples obtained from a pilot clinical study where a single dose of 30 mg of *I* was administered to six healthy volunteers.

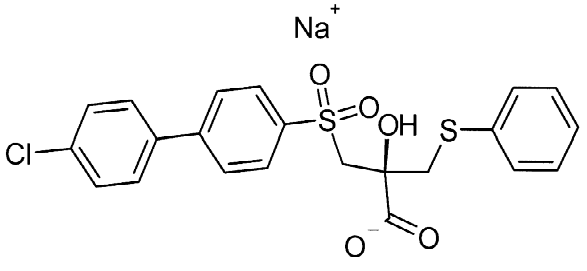

Fig. 1. Chemical structure of *I* (PNU248686A).

2. Experimental

2.1. Chemicals

I (as sodium salt) was supplied by the Chemistry Department of Pharmacia, Nerviano, Italy. All other chemicals and solvents were of analytical reagent grade from Carlo Erba Reagents (Milan, Italy).

2.2. Equipment

The LC system consisted of Agilent Technologies 1100 series degasser, binary pump and column oven (Waldbronn, Germany) and Perkin-Elmer LC 200 autosampler (Norwalk, CT, USA). The detection was by a PE Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems–MDS Sciex, Concord, Canada) equipped with a TurboIonSpray interface. Computer software: Applied Biosystems–MDS Sciex Mass Chrom 1.1 running LC2Tune 1.4, MultiView 1.4 and Sample Control 1.4.

2.3. LC–MS–MS conditions

The chromatographic separation was performed with a 50×2.1 mm I.D. Symmetry Shield C_8 reversed-phase column (particle size 3.5 μm) (Waters, Milford, MA, USA). Column temperature was maintained at 35 °C. The mobile phase consisted of 5 m*M* ammonium formate buffer solution pH 5.0–acetonitrile (60:40, v/v). The flow-rate was 0.3 ml/min. The flushing solvent of the autosampler was a methanol–water mixture (80:20, v/v).

The TurboIonSpray interface was operated in the negative ion mode at −4000 V and 375 °C and was

supplied by an auxiliary gas 2 flow-rate of about 7000 ml/min. The nebulizer gas 1 (NEB), curtain gas (CUR) and collision gas (CAD) were set to values of 9, 12 and 4 of the state file parameters, respectively. Nitrogen gas was used for CUR, CAD and auxiliary gas 2. Compressed air was used for NEB. Orifice plate (OR) and focusing ring (RNG) lens voltages were set at −40 and −240 V, respectively. The collision energy (defined as the difference between the Q0 and RO2 lens voltages) was −20 V. Quantitation was performed by single reaction monitoring (SRM, dwell time of 500 ms) of the depronated precursor ion and the related product ion. The mass transition of 461→251 m/z was used. The channel electron multiplier (CEM) was set at 2200 V. In order to achieve maximum sensitivity, the quadrupoles Q1 and Q3 were set on low resolution by lowering of 0.08 units the resolution offset parameters.

2.4. Preparation of stock and working solutions and plasma calibration standards

A stock solution of *I* was prepared by dissolving 13.65 mg of *I* in 10 ml dimethyl sulfoxide (DMSO) to obtain a solution of 1.303 mg/ml as free acid. From this primary stock solution, six working solutions were prepared by dilution with methanol at final concentrations of 50,166, 20,080, 5212, 1303, 261 and 50.2 ng/ml. Aliquots of 20 μl of these working solutions were spiked into blank human plasma (180 μl) to prepare six calibration standards with concentrations of 5.02, 26.1, 130.3, 521.2, 2006.6 and 5016.6 ng/ml. Stock and working solutions were stable for at least 1 month when stored at +4 °C.

2.5. Preparation of plasma quality control (QC) samples

A separate stock solution of *I* was prepared by dissolving 20.92 mg of I in 2 ml of DMSO. From this primary stock solution, three working solutions were prepared by dilution with methanol at final concentrations of 1997.2, 499.3 and 19.97 μg/ml. Aliquots ranging from 20 to 50 μl of these working solutions were spiked into volumetric flasks (25 or 10 ml for out of range QC) and brought to volume with blank human plasma to prepare samples with concentrations of 15.6, 999, 3994 and 7989 ng/ml for low, mid, high and out of range QCs, respectively. Aliquots of 0.2 ml of each QC level were transferred into Eppendorf tubes and frozen at −20 °C until analysis.

2.6. Sample extraction procedure

Aliquots of 50 μl of human plasma were pipetted into 96-well plates, mixed with 0.2 ml of methanol, shaken for 5 min and centrifuged at 1500 *g* for 10 min to remove any precipitated material. Aliquots of 10 μl were then directly injected into the LC–MS–MS system.

2.7. Assay validation experiments

The specificity of the assay was assessed by analysing six aliquots of blank human plasma obtained from six different subjects. Linearity was evaluated from three calibration curves run on three different days over the concentration range of about 5.0–5000 ng/ml. Each calibration curve included twelve calibration points (six concentration levels in duplicate; six were run at the beginning and six at the end of each analytical batch). Calibration curves were constructed by plotting the peak area of the compound (y) against its concentration (x). A weighed linear regression (weighting factor $1/x^2$) was used to fit the calibration line and hence to calculate *I* concentrations in QC and unknown samples. The weighting factor was chosen to minimize deviation of back-calculated values from theoretical concentrations. Intra and inter-day precision and accuracy were evaluated by repeated analyses of QCs at three concentrations (low, mid and high) with five replicate samples analysed every day. Parallelism (accuracy and precision of dilution of samples) was evaluated with out of range QC samples after 1:10 dilution with blank human plasma; five replicate samples were analysed on 1 day. Data analysis was performed using MacQuan software, version 1.6.

Stability of *I* was examined after storage in plasma and in final extracts at room temperature for 24 h. Moreover, stability was determined after three freeze/thaw cycles. Aliquots of QCs were stored at −20 °C for 24 h and thawed unassisted at room

temperature. When completely thawed the samples were refrozen for 24 h under the same conditions. The freeze/thaw cycle was repeated two more times and the samples were analyzed on the third cycle. Each stability experiment included five replicate QC samples at three concentration levels (low, mid and high).

Ion suppression and/or enhancement effects were checked by injections of pure standard solutions and samples spiked with *I* into extracts of blank human plasma.

In order to evaluate the extraction recovery, the mean peak area of the extracted QC samples was compared to the mean peak area of the extracted blanks spiked with the corresponding neat solutions.

3. Results and discussion

To determine the optimal parameters for the mass spectrometer, tuning solutions of 1000 ng/ml of *I* were infused. The ion efficiency was tested in the positive and negative ion mode of the TurboIonSpray source. The best results were obtained operating in

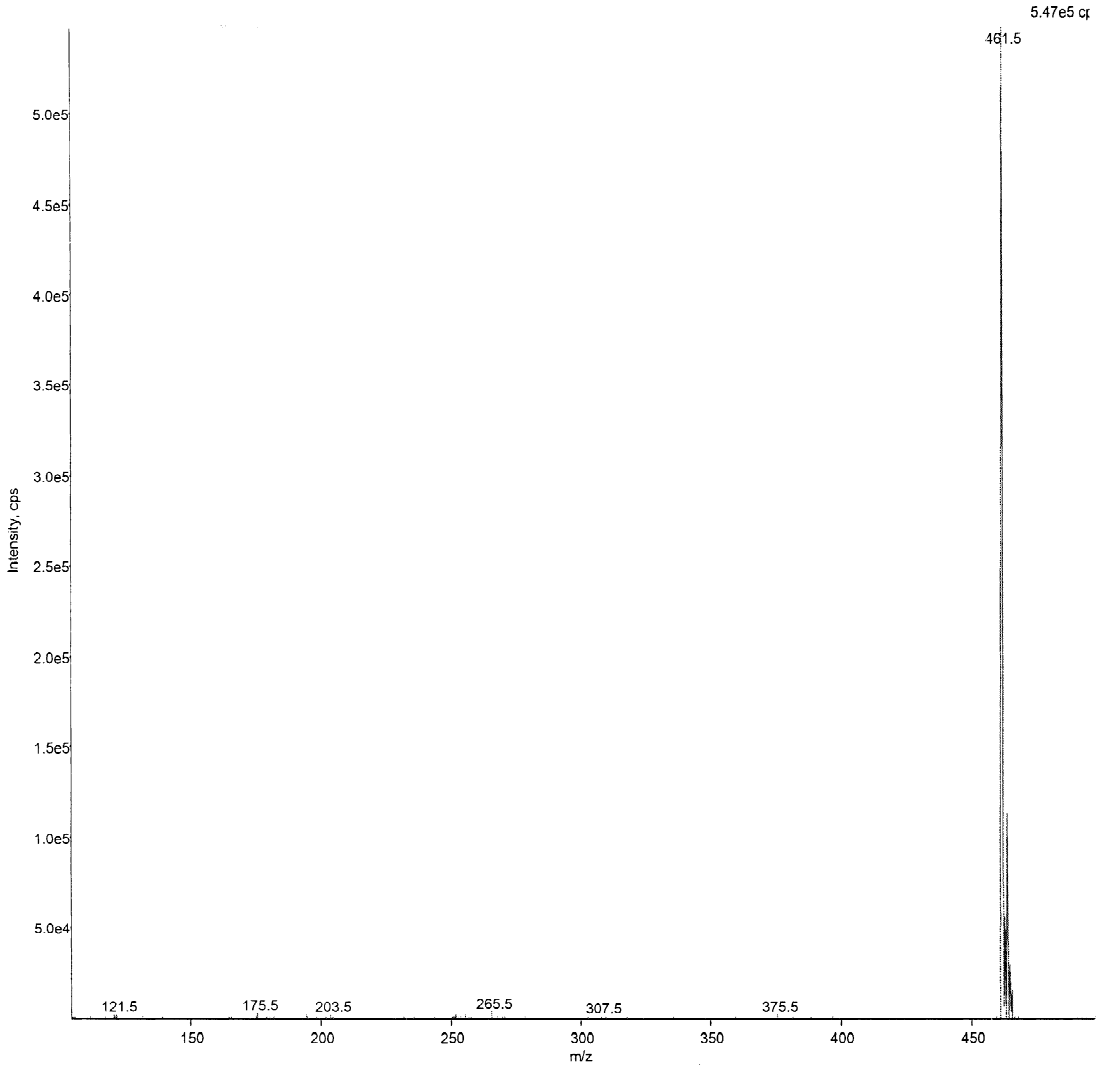

Fig. 2. Full scan spectrum of *I*.

negative ion mode. A Q1 scan of *I* (see Fig. 2) revealed the deprotonated parent molecule $[M–H]^-$ to be in abundance with a mass to charge ratio (m/z) of 461. The product ion spectrum of *I* (see Fig. 3) resulted in a major fragment at m/z 251.

Under the isocratic chromatographic conditions chosen the retention time of *I* was about 1.2 min. The total cycle time was set at 2.5 min. The narrow bore column employed (2.1 mm I.D.) avoided the use of a post column tee splitter. The column proved to be quite robust showing only a slight increase of back-pressure after about five hundred samples had been injected. No guard column was used. All precipitated material was left on the bottom of the 96-well plates. In order to avoid blocking, the needle level (%) of the autosampler was set at 2. The sample preparation step and the analysis cycle time were sufficiently short to allow three to four batches (of 96 samples) to be assayed per day. To avoid carry-over effects from the autosampler two pre and two post injection washes with 0.5 ml of flushing solvent were made.

A typical chromatogram obtained by analysis of blank human plasma is shown in Fig. 4. No peak

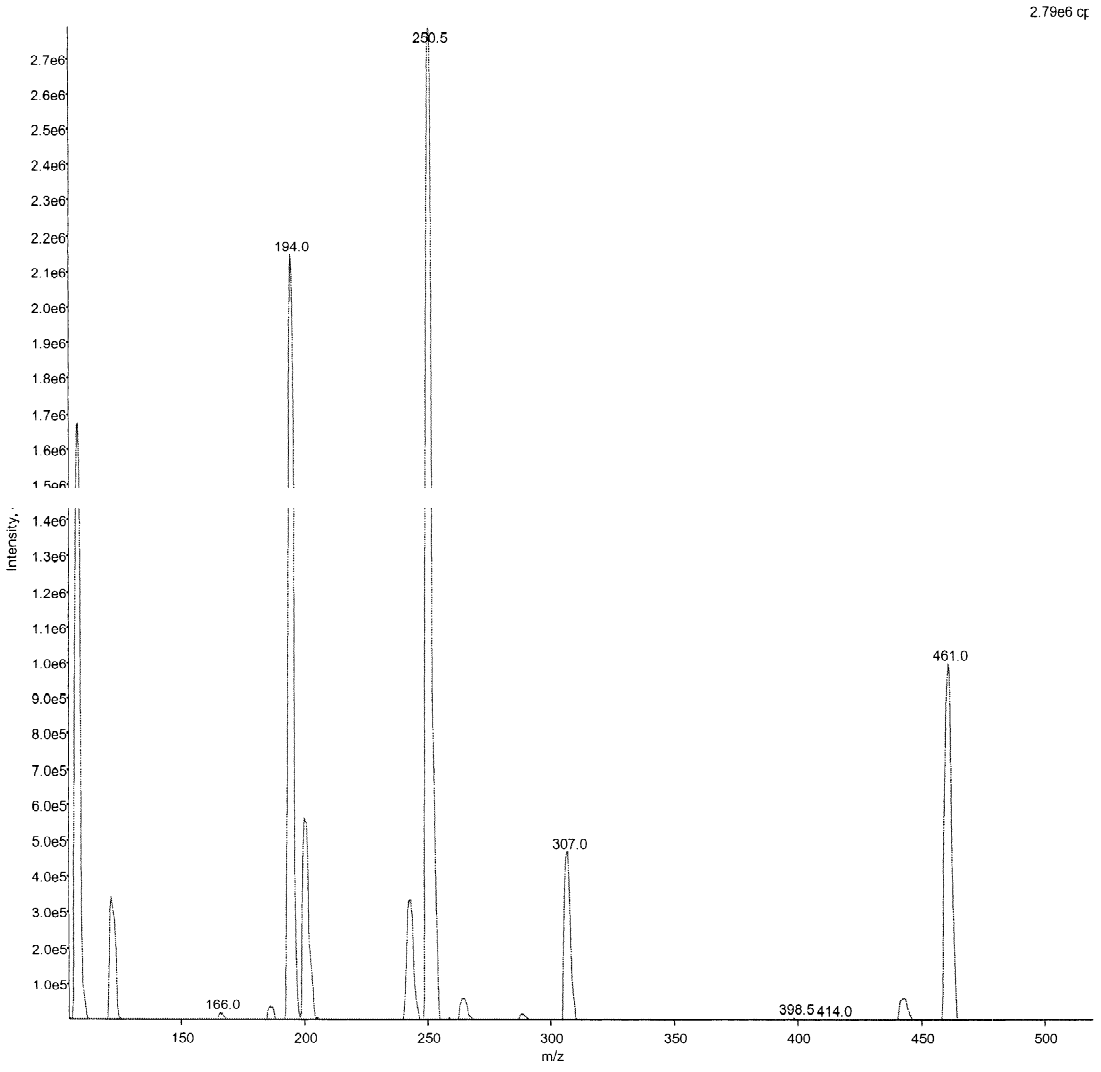

Fig. 3. Product ion scan of *I*.

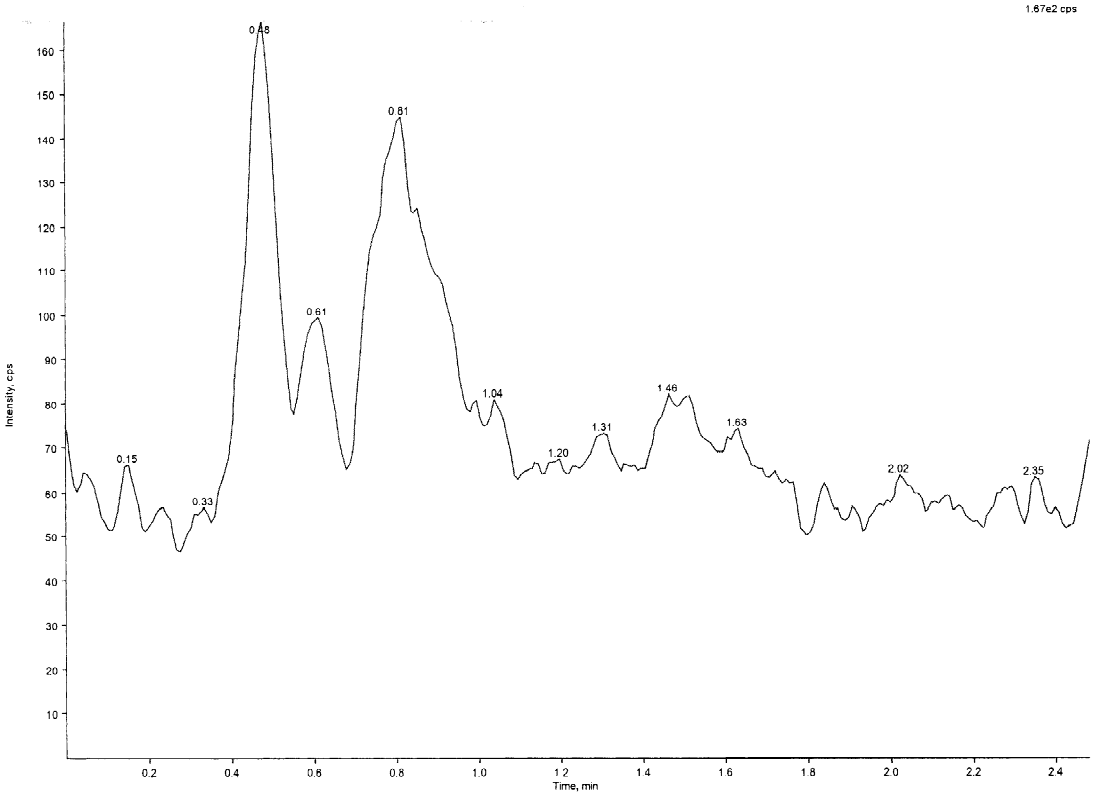

Fig. 4. Representative LC–MS–MS chromatogram of blank human plasma.

higher than 20% of the analyte peak area at lower limit of quantitation (LLOQ) level was detected.

The method was linear over the concentration range of about 5.0–5000 ng/ml. The linear correlation coefficients (r) ranged from 0.9937 to 0.9958. The back-calculated calibration standard points showed a RSD ranging from 4.3 to 11.3%. The mean calibration curve obtained was described by the equation $y = 438x + 325$ (slope RSD = 6.5%, $n = 3$). The LLOQ was 5.0 ng/ml. At this level, precision was 6.1% ($n = 5$) and accuracy was +1.3%. A typical chromatogram obtained is shown in Fig. 5.

The intra-day precision (expressed as RSD, $n = 5$) ranged from 3.2 to 11.2%. The intra-day accuracy (expressed as bias) ranged from −10.3 to 16.7%. The inter-day precision ($n = 15$) ranged from 6.9 to 11.1%. The inter-day accuracy ranged from −2.5 to +6.6%. Full details are shown in Table 1.

There was no evidence of degradation of *I* after storage in human plasma at room temperature for 24 h. Precision and accuracy ranged from 2.0 to 16.2% and from −5.9 to −3.3%, respectively. After storage of final extracts at room temperature for 24 h precision and accuracy ranged from 2.7 to 17.6% and from −4.0 to +0.4%, respectively. Results obtained after 3 freeze/thaw cycles did not show evidence of degradation. Precision and accuracy ranged from 1.0 to 6.8% and from −12.1 to −2.1%, respectively.

Similar responses were seen following injections of standard solutions compared with identical concentrations of *I* spiked into extracts of control human plasma indicating that control extracts do not significantly suppress or enhance signal. The extraction recovery of *I* ranged from 63.7 to 88.3%.

The method was applied to the determination of plasma levels of *I* in six healthy volunteers who had received a single oral 30 mg non-pharmacological dose of *I* during a pilot clinical study. The procedure described in this report was also extensively and successfully employed to measure *I* in phar-

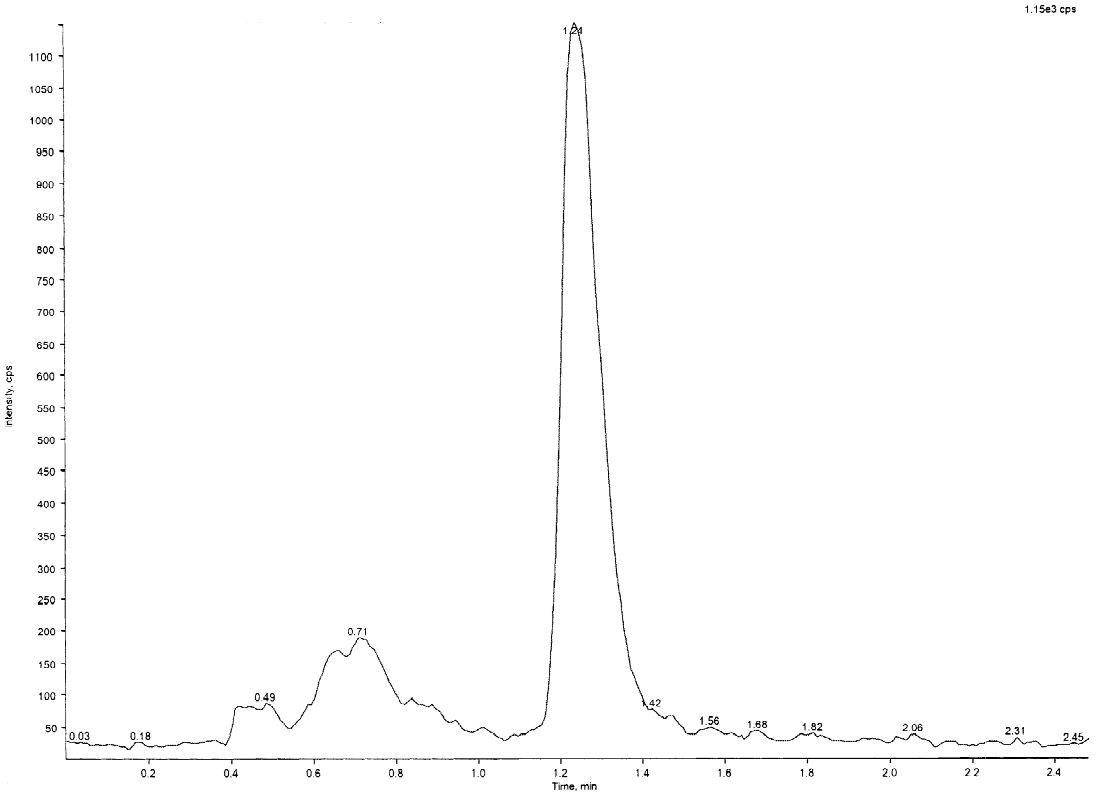

Fig. 5. Representative LC–MS–MS chromatogram of lower limit of quantitation (5.0 ng/ml).

Table 1
Precision and accuracy of the method for the determination of I in human plasma

Quality control	Conc. (ng/ml)	Day	*n*	Dilution factor	Accuracy			Precision		
					Mean found (ng/ml)	Intra-day (% bias, $n=5$)	Inter-day (% bias, $n=15$)	SD	Intra-day (% RSD, $n=5$)	Inter-day (% RSD, $n=15$)
Low	15.6	1	5	1	15.4	−1.0		0.49	3.2	
		2	5	1	15.4	−1.5		1.57	10.2	
		3	5	1	14.8	−4.9	−2.5	0.96	6.5	6.9
Mid	999	1	5	1	938	−6.0		105	11.2	
		2	5	1	1165	16.7		51.2	4.4	
		3	5	1	1089	9.0	6.6	46.0	4.2	11.1
High	3994	1	5	1	3713	−7.0		206	5.6	
		2	5	1	3873	−3.0		163	4.2	
		3	5	1	4306	7.8	−0.8	344	8.0	8.8
Out of range	7989	1	5	10	7169	−10.3		635	8.9	

macokinetic and toxicokinetic studies carried out in rats and monkeys.

4. Conclusions

The method described here is sensitive and selective for the determination of *I* in human plasma. It proved to be linear, precise and capable of accurately quantitating the analyte in the concentration range of 5.0–5000 ng/ml. The suitability of this method was demonstrated in a clinical pilot study of *I* in humans. Considering the relatively high plasma levels and the long half-life observed the validated LLOQ of the method proved to be completely satisfactory for full definition of the pharmacokinetics of *I*.

References

[1] C. Chang, Z. Werb, Trends Cell Biol. 11 (2001) 537.
[2] W.G. Stetler-Stevenson, J. Clin. Invest. 103 (1999) 1237.
[3] R. Hoekstra, F.A. Eskens, J. Verweij, Oncologist 6 (2001) 415.
[4] R. Giavazzi, G. Taraboletti, Crit. Rev. Oncol. Hematol. 37 (2001) 53.
[5] L.M. Coussens, B. Fingleton, L.M. Matrisian, Science 295 (2002) 2387.
[6] S. Zucker, J. Cao, W.T. Chen, Oncogene 19 (2000) 6642.
[7] C.G. Knight, F. Willenbrock, G. Murphy, FEBS Lett. 296 (1992) 263.
[8] S.X. Peng, J. Chromatogr. B 764 (2001) 59.