Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1024 (2004) 87–94
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Quantitation of SU11248, an oral multi-target tyrosine kinase inhibitor, and its metabolite in monkey tissues by liquid chromatograph with tandem mass spectrometry following semi-automated liquid–liquid extraction☆

S. Barattè[a,*], S. Sarati[a], E. Frigerio[a], C.A. James[a], C. Ye[b], Q. Zhang[b]

[a] *Global Drug Metabolism, Pharmacia, Viale Pasteur 10, 20014 Nerviano, Italy*
[b] *Sugen Inc., South San Francisco, CA 94080-4811, USA*

Received 28 December 2002; received in revised form 1 October 2003; accepted 20 October 2003

Abstract

SU11248 is a potent inhibitor of PDGFR, VEGFR, KIT, and Flt3, and is currently under Phase I clinical evaluation as an anticancer drug. A sensitive and specific analytical method for the quantitation of SU11248 and its metabolite in several monkey tissues (liver, kidney, brain and white fat) using LC–MS–MS following semi-automated liquid–liquid extraction (LLE) was developed and validated. Amounts of 50 mg of tissue were homogenized using an ultrasonic processor. After addition of the stable labelled internal standard (IS) and ammonium hydroxide (0.3%), samples were extracted with 2.5 ml of *tert*-butyl methyl ether. Following centrifugation, aliquots of 1.8 ml of the organic phase were transferred into a 96-well plate. The Packard Multiprobe II robotic liquid handler was used to perform all steps mentioned above. The organic phase was dried and the residue was reconstituted with 800 μl of 15 mM ammonium formate buffer solution (pH 3.25) using a Tomtec Quadra 96 workstation. Aliquots of 10 μl of the resulting solution were injected into the LC–MS–MS system. A Symmetry Shield C_8 column (50 mm × 2.1 mm, 3.5 μm) was used to perform the chromatographic analysis. The mobile phase was 15 mM ammonium formate buffer solution (pH 3.25)–acetonitrile (74:26 (v/v)) with a flow-rate of 0.35 ml/min. Retention times of the metabolite and SU11248 were about 2.5 and 3.5 min, respectively. Total cycle time was 5 min. MS detection used the Applied Biosystems-MDS Sciex API 3000 with TurboIonSpray interface and multiple reaction monitoring (MRM) operated in positive ion mode. The method was validated for both compounds over the calibration range of about 2 and 2000 ng/g. The suitability and robustness of the method for in vivo samples were confirmed by analysis of monkey tissues from animals dosed with SU11248.

Keywords: SU11248; Enzyme inhibitors

1. Introduction

SU11248 (I) is an oral multi-targeted small molecule with potent inhibitory activity against PDGFR, Flk1/KDR, KIT, and Flt3 receptor tyrosine kinases. Preclinical studies suggest that I has powerful anti-tumour and anti-angiogenic activities [1]. I is currently under Phase I clinical evaluation as an anticancer drug.

Liquid chromatography with tandem mass spectrometry (LC–MS–MS) is nowadays the method of choice for analysis of drugs in biological fluids and tissues because of its specificity, sensitivity and high-throughput [2]. Plasma protein precipitation (PPP), liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are the sample preparation techniques most commonly used for processing plasma and tissue samples [3–5]. Protein precipitation has the potential to be significantly less time-consuming, especially when transfer of the organic phase and/or evaporation steps of LLE can be avoided. However, LLE for plasma and tissues analyses tends to give cleaner extracts than PPP, as evidenced by less matrix effects and less tendency for back-pressure build-up in the LC column as more samples are injected [6]. An analytical method for the determination of I and of its metabolite (II) in human plasma by LC–MS–MS following PPP by filtration in the 96-well format has been already developed and fully validated [7].

☆ Presented at the 19th (Montreux) Symposium on Liquid Chromatography–Mass Spectrometry, Montreux, 6–8 November 2002.

* Corresponding author. Tel.: +39-02-4838-3148; fax: +39-02-4838-3012.
E-mail address: simona.baratte@pharmacia.com (S. Barattè).

0021-9673/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2003.10.085

This paper describes a new LC–MS–MS method for the determination of I and II in several monkey tissues following homogenisation of samples by an ultrasonic processor and semi-automated LLE. The LLE procedure provided additional clean up given the diversity of the tissue samples, while automation was used to minimize the resource need for the more complex extraction. Given the limited availability of some tissue types, the validation approach had to be tailored to make the most of available tissues.

2. Experimental

2.1. Chemicals

I and II were supplied by Sugen, South San Francisco, CA, USA. Stable labelled SU11248, the internal standard (IS), was supplied by Global Drug Metabolism Department, Pharmacia, Nerviano, Italy. All other chemicals and solvents were of analytical reagent grade from Sigma–Aldrich, Seelze, Germany.

2.2. Equipment

The LC system consisted of an Agilent Technologies HP1100 series with degasser, binary pump and column oven (Waldbroom, Germany) and a Perkin-Elmer LC 200 autosampler (Norwalk, CT, USA). The detection system was an API 3000 triple quadrupole mass spectrometer (Applied Biosystems—MDS Sciex, Concord, Canada) equipped with a TurboIonSpray interface. Computer software used were: Applied Biosystems—MDS Sciex Mass Chrom 1.1 running LC2Tune 1.4, MultiView 1.4 and Sample Control 1.4. Robotic liquid handling systems used were: Multiprobe II from Packard (Meriden, CT, USA) and Tomtec Quadra 96-320 (Hamden, CT, USA). A High Intensity Ultrasonic Processor GEX 600 (Sonics & Materials, Newtown, CT, USA) equipped with a 3 mm diameter tapered microtip was used for tissue homogenisation. A refrigerated centrifuge (Multifuge 3, Heraeus) was used to separate the phases after LLE. A 96-well dry-down station for evaporation of the extracts (Stepbio, Italy) and a 96-well plate refrigerated centrifuge (Model PK120R, ALC, Italy) were also used. A laboratory information management system (Watson) was used to store concentration data and generate summary validation statistics.

2.3. LC–MS–MS conditions

The analytical separation was performed using a Symmetry Shield C_8 column (Waters, 50 mm × 2.1 mm, 3.5 mm), the mobile phase consisted of 15 mM ammonium formate buffer solution (adjusted to pH 3.25 with 1 M formic acid)–acetonitrile (74:26 (v/v)) with a flow-rate of 0.35 ml/min. The retention times of II and I were about 2.5 and 3.5 min, respectively. Total cycle time was 5 min. A mixture of acetonitrile and 0.05 M formic acid (50:50 (v/v)) was used as flushing solvent for the autosampler.

The TurboIonSpray interface was operated in the positive ion mode at 350 °C. Quantitation was performed by multiple reaction monitoring (MRM). The collision energy was 22 eV. The mass transitions (precursor to product) monitored were 399 → 326 *m/z* for I, 371 → 283 *m/z* for II and 409 → 326 *m/z* for IS. A dwell time of 500 ms was used for each transition. In order to obtain the 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

Primary standard stock solutions of I and II were prepared by dissolving 6.61 mg of I and 6.15 mg of II in methanol into two separate 25 ml volumetric flasks, to obtain final concentrations of 262 and 243 μg/ml, respectively, after adjusting for the purities of the compounds. From these primary stock solutions, six working solutions containing I and II were prepared, by diluting with methanol, to final concentrations of 10500, 5240, 2620, 524, 52.4 and 10.5 ng/ml for I, and 9720, 4860, 2430, 486, 48.6 and 9.72 ng/ml for II. Stock solutions of both compounds were stable for at least 4 months and working solutions for at least 1 week when stored at +4 °C in the dark. Due to the photo-instability of the compounds, all dilutions were made in brown-coloured volumetric glassware (Brand, Germany). Aliquots of 10 μl of these working standard solutions were spiked into 50 mg blank monkey tissue to prepare calibration standards with a concentrations range of 2.10, 10.5, 105, 524, 1050 and 2100 ng/g for I and 1.94, 9.72, 97.2, 486, 972 and 1940 ng/g for II. After adding 500 μl of 10 mM ammonium formate the samples were sonicated for 30 s with the ultrasonic processor.

2.5. Preparation of quality control (QC) samples

Primary standard stock solutions of I and II were prepared by dissolving in methanol into two separate 25 ml volumetric flasks 7.31 mg of I and 6.11 mg of II to obtain final concentrations of 290 and 241 μg/ml, respectively, after adjusting for the purities of the compounds. From these primary stock solutions, three working solutions containing I and II were prepared by diluting with methanol to give final concentrations of 29, 4060 and 7540 ng/ml for I and of 30.3, 4340 and 9640 ng/ml for II. Bulk blank control homogenate was prepared by sonication of 2.5 g of monkey liver with 25 ml of 10 mM ammonium formate solution. The monkey tissue quality control (QC) samples were prepared by spiking an aliquot of 10 μl of each working solution into a polypropylene tube containing 525 μl of monkey blank tissue homogenate corresponding to 50 mg of matrix to obtain final concentrations of 5.80 (low), 812 (mid), 1510 (high) and 20,800 ng/g (out of range) for I and 6.06 (low), 868

(mid), 1930 (high) and 19,300 ng/g (out of range) for II. QC samples were then frozen at −80 °C until analysis.

2.6. Preparation of internal standard solution

A primary standard stock solution of IS was prepared by dissolving 1.24 mg of the IS in 10 ml methanol to obtain a final concentration of 118 μg/ml after adjusting for the purity of this compound. An aliquot of 0.050 ml of this primary stock solution was diluted in 100 ml volumetric flask with methanol to obtain a working solution of 59.0 ng/ml. Stock and working solutions were stored at +4 °C.

2.7. Sample preparation

Fifty milligrams of each tissue sample were homogenised in a polypropylene tube kept in an ice-bath with 500 μl of 10 mM ammonium formate solution using an ultrasonic processor for 30 s. Homogenates were mixed with 50 μl of ammonium hydroxide (0.3%) in order to adjust the pH to approximately 9.2 and with 50 μl of IS working solution. The tubes were then capped and the samples were extracted with 2.5 ml of *tert*-butyl methyl ether by vortex mixing for 5 min. After centrifugation for 10 min at 1200*g* aliquots of 1.8 ml of the organic phase was transferred into a 96-well plate. The Packard Multiprobe II robotic liquid handler was used to perform all steps mentioned above. The organic phase was dried under nitrogen gas at 37 °C and the residue was re-constituted with 800 μl of 15 mM ammonium formate buffer solution (pH 3.25) using Tomtec Quadra 96 workstation. The plates were then capped, vortex mixed for 15 s and centrifuged at 4000*g* for 10 min. An aliquot of 10 μl of the resulting solution was injected into the LC–MS–MS system. Due to the photo-instability of the compounds all the procedures described above, including weighing of tissues, were performed under light protection conditions with an orange colored safe light (cutoff < 540 nm, Slimline A5ND4 T8 Safe Light, EncapSulite International, UK). "Windows" on the autosampler sample compartment were covered with an orange colour safety-light film (Rosco, USA).

2.8. Assay validation experiments

Extensive validation experiments were performed with liver tissues followed by limited 1-day validation experiments with the other matrices. Wherever possible, calibration and QC samples were prepared using the relevant control tissues in each case.

Linearity was evaluated from four calibration curves run on four different days over the concentration range of about 2–2000 ng/g for both the analytes. Each calibration curve included 12 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 ratio of the area of the compounds and the IS (y) against the analyte concentration (x). IS of I was used also for quantitation of II. A weighted linear regression function ($1/x^2$) was used to fit calibration lines and hence to calculate the concentrations of I and II in QC and unknown samples. The weighting factor was chosen to minimize deviation of back-calculated values from the 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) study was evaluated with QC samples above the high limit of standard level after 1:10 dilution with blank monkey tissue homogenate; five replicate samples were analysed on one day. "In process" stability was examined after storage in tissue homogenate for 4 h at room temperature and in final extracts at room temperature for 48 h. Each stability experiment included five replicate QC samples at three concentration levels (low, mid and high).

3. Results and discussion

The method used to determine I and II in different monkey tissues consisted of homogenisation of the tissue using an ultrasonic processor followed by a semi-automated LLE that provide cleaner extracts compare to other techniques. Two different tissue preparation techniques by an ultrasonic processor and using a micro-dismembrator (B. Braun, Germany) were tested prior the validation experiments. Although both provided adequate extraction, the ultrasonic probe was selected as faster and more practical considering the number of study samples anticipated. Preparation of the tissues before weighting was made by hand cutting avoiding the presence of fibers that make the samples difficult to be homogenized. The tapered microtip ultrasonic probe used minimized cross-contamination of the samples and helped in the washing procedures after each sample preparation. The extraction step was relatively rapid and conducted entirely with automated systems using disposable tips to avoid carry-over. The Packard Multiprobe II has the flexibility of pipetting samples from tubes to 96-well plates. On the other hand, the Tomtec Quadra 96 workstation is equipped with 96 tips and is capable of pipetting 96 samples simultaneously. In the current method, the Multiprobe II was programmed to add the IS and reagents and transfer the organic phase from the individual tubes to 96-well deep plates. After evaporation, the plates were moved to the Quadra 96 for reconstitution.

Adequate chromatographic separation is required for the quantitative determination of drugs in biological samples [8,9]. I and II were adequately retained on the column used with full baseline resolution. The narrow column avoided the use of a post column tee splitter and proved to be quite robust. No guard column was used. To avoid carry-over effects from the autosampler two pre- and two post-injection washes with 0.5 ml each of flushing solvent were made.

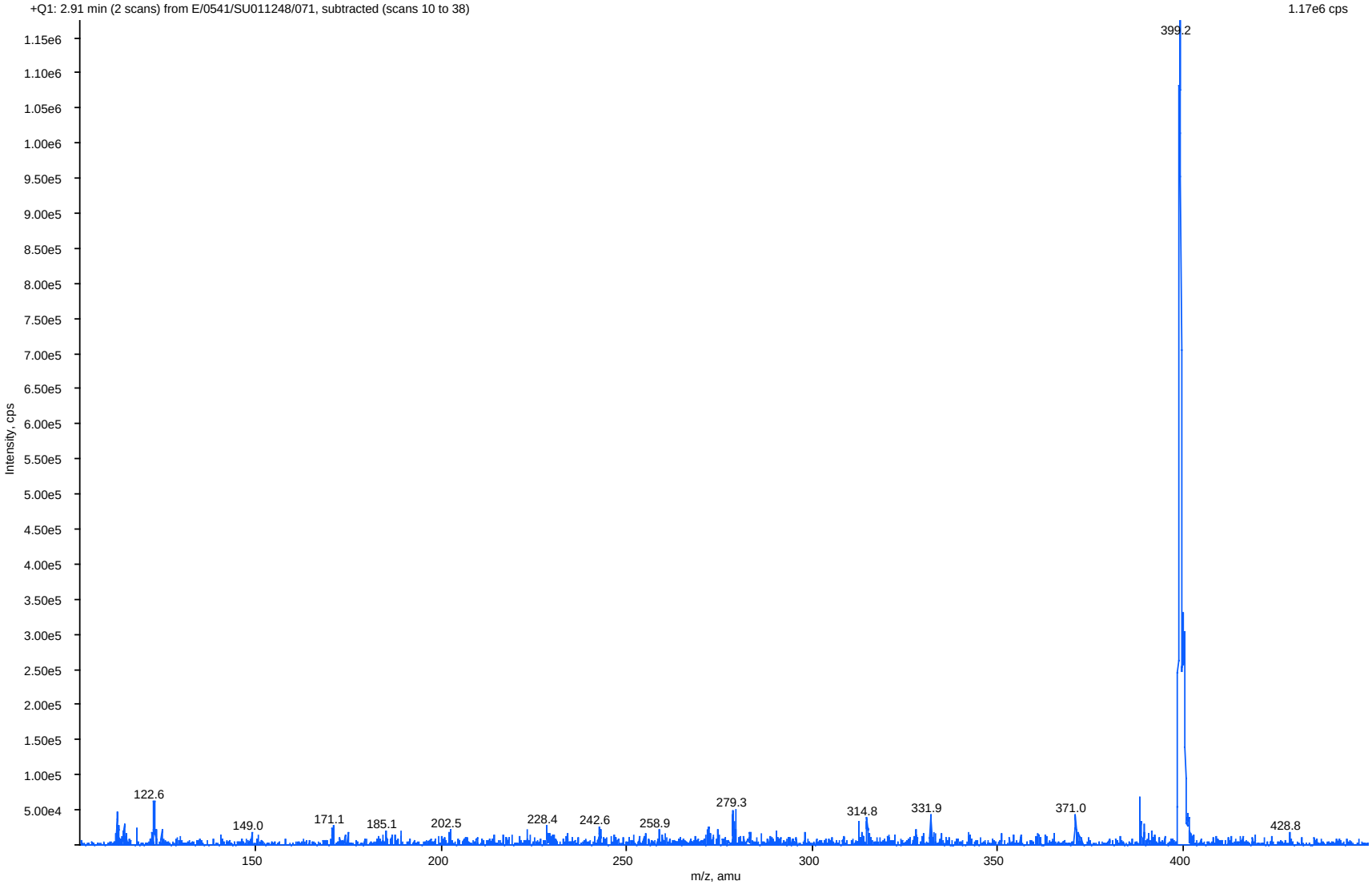

Fig. 1. Full scan mass spectrum of I.

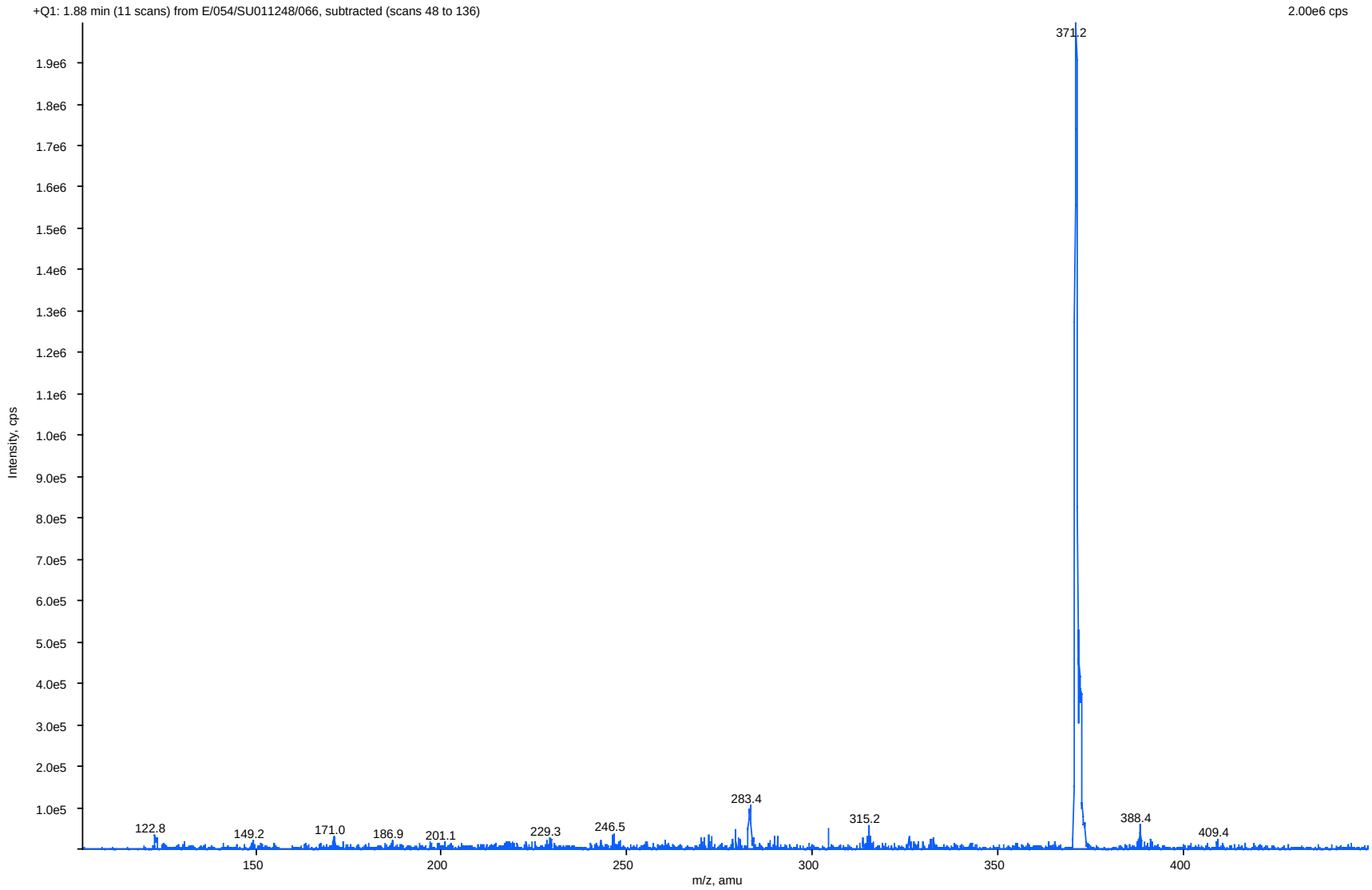

Fig. 2. Full scan mass spectrum of II.

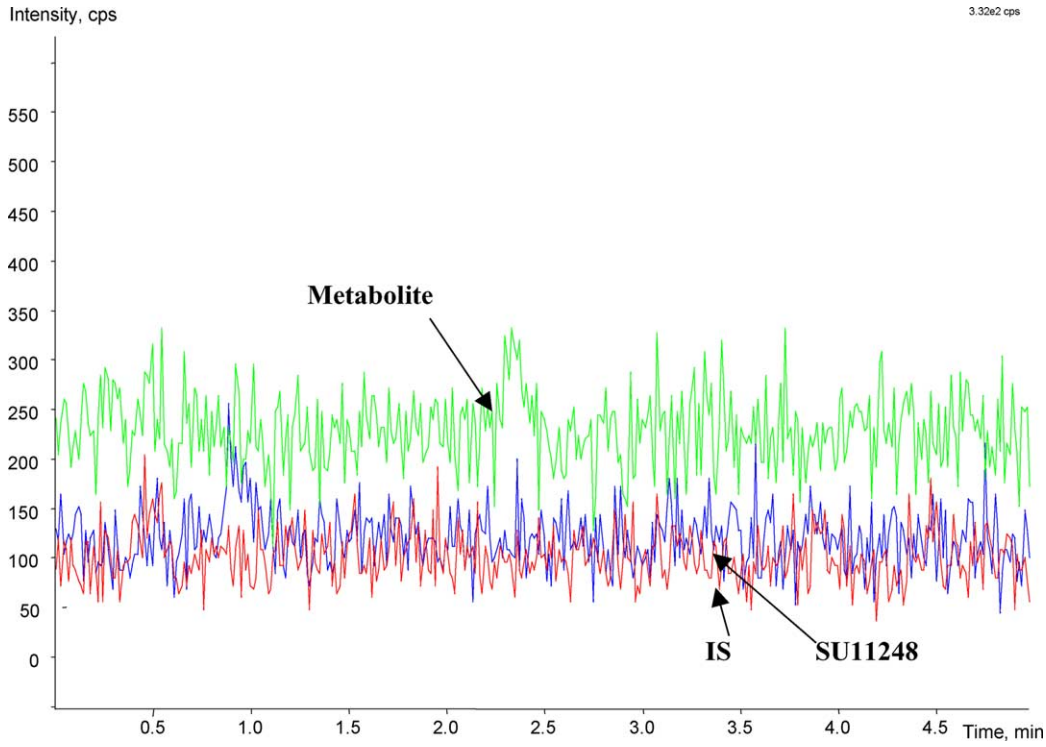

Fig. 3. Representative LC–MS–MS chromatograms of blank monkey liver.

The full scan spectrum of I and II (Figs. 1 and 2) revealed the protonated molecules to be in abundance with a mass-to-charge ratio (*m*/*z*) of 399 and 371, respectively. The product ion spectra of I and II using a collision energyof 22 eV showed the presence of the major fragments at *m*/*z* 326 and 283, respectively. The transition of 409 → 326 *m*/*z* was chosen to monitor the IS.

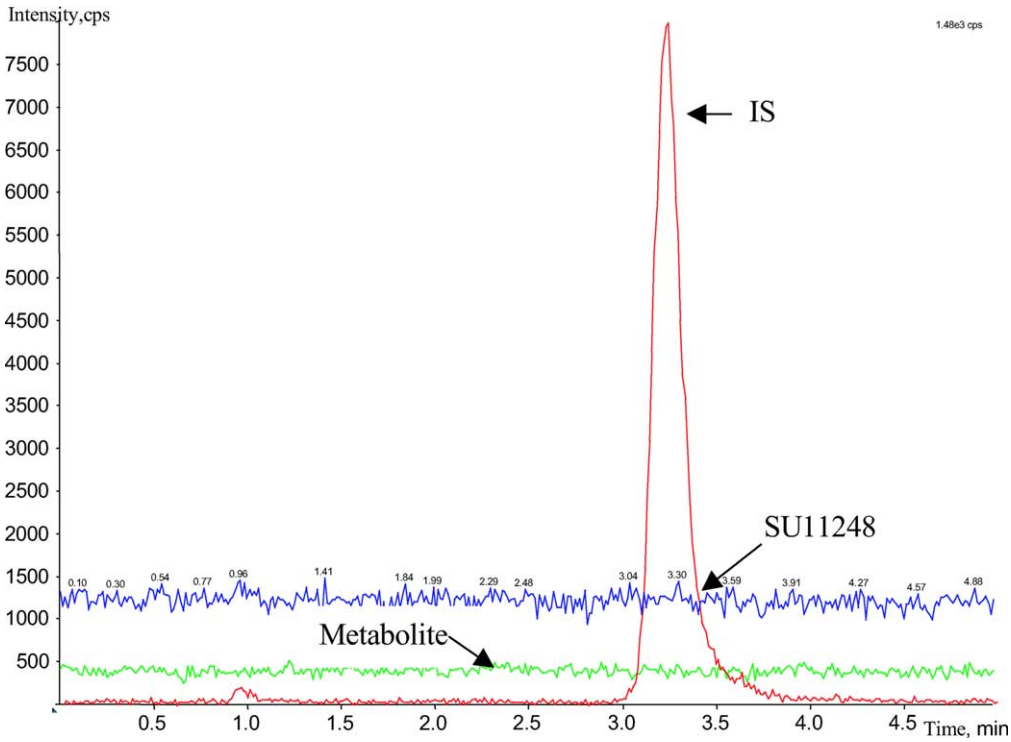

Fig. 4. Representative LC–MS–MS chromatograms of blank monkey liver spiked with IS.

For ethical reasons only one monkey was sacrificed to provide blank control tissues. Whilst no difficulties were met with the availability of the liver tissue, the other tissues were in limited supply. For this reason, extensive validation experiments were performed only in monkey liver. Chromatograms of blank monkey liver (Figs. 3 and 4) demonstrated the absence of interfering peaks in the transition channel of both analytes and also the IS. No peak 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 2–2000 ng/g. The linear correlation coefficients (r^2) ranged from 0.9904 to 0.9990 for I and from 0.9884 to 0.9985 for II. The mean calibration curves obtained were described by the following equations: $y = 0.0241 + 0.00504$ (slope CV = 1.1%, $n = 3$) for I and $y = 0.0366x + 0.0154$ (slope CV = 11.8%, $n = 3$) for II. The mean back-calculated concentration values for the calibration standards showed a bias ranging from −1.9 to 2.9% for I and from −3.9 to 3.9% for II. Coefficient of variations relative standard deviations (R.S.D.s) ranged from 2.1 to 11.2% for I and 4.9 to 7.7% for II. Typical chromatograms obtained for the LLOQ for I and II are shown in Fig. 5.

The intra-assay precision, evaluated with QC samples prepared in monkey liver and expressed as R.S.D., ranged from 1.4 to 3.6% for I and 5.4 to 8.6% for II. The intra-day accuracy (expressed as % bias) ranged from −1.0 to 7.9% for I and −7.1 to 0.3% for II. The inter-day precision evaluated at the same concentrations over three validation days ranged from 2.4 to 9.6% for I and 4.5 to 7.6% for II. The inter-day accuracy ranged from −1.3 to 2.2% for I and −9.4 to 2.1% for II.

Parallelism expressed by the intra-day precision with out of range QC samples was 12.4% for I and 10.5% for II. The intra-day bias was −13.0% for I and 0.0% for II.

There was no evidence of degradation of I or II after storage in liver homogenate at room temperature for 4 h in the dark. In this stability study, precision ranged from 0.8 to 13.3% for I and 6.3 to 15.4% for II and bias ranged from −1.8 to 7.6% for I and −4.1 to 2.8% for II. The stability of I and II from tissue extracts were also tested in the autosampler. After storage at room temperature for 48 h in the dark, precision of QCs ranged from 1.8 to 4.9% for I and 6.5 to 11.7% for II and accuracy ranged from −7.3 to 3.4% for I and 8.0 to 22.7% for II. These results suggest that there was no significant instability for both I and II under normal sample preparation and analysis conditions.

Intra-day precision and accuracy of I and II, evaluated with QC samples in monkey kidney, brain and white fat, met standard validation requirements [10]. The intra-assay precision ranged from 1.2 to 7.0% for I and 3.2 to 5.0% for II in the kidney, from 4.1 to 12.5% for I and 4.7 to 7.8% for II in the brain and from 3.6 to 20.8% for I and 4.5 to 19.6% for II in the white fat.

The intra-day accuracy ranged from −11.1 to −1.4% for I and −11.9 to −2.6% for II in the kidney, from −7.6 to 3.6% for I and −9.1 to 6.7% for II in the brain and from 9.9 to 11.7% for I and 4.7 to 13.0% for II in the white fat.

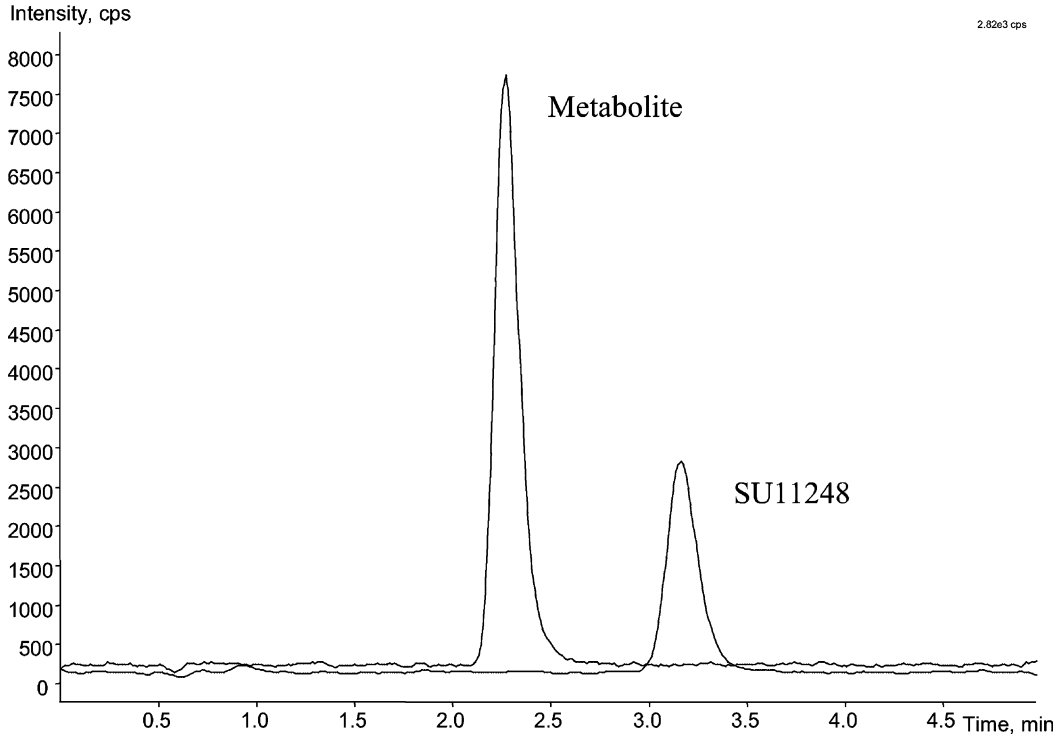

Fig. 5. Representative LC–MS–MS chromatograms of lower limit of quantitation for I (2.10 ng/g) and II (1.94 ng/g) in monkey liver.

The method was successfully applied to the determination of liver, kidney, white fat and brain levels of I and II in monkeys dosed with I in a toxicity study.

4. Conclusions

The method described here is sensitive and selective for the determination of I and II in monkey tissues. It proved to be precise and capable of accurately determining the two compounds in the 2–2000 ng/g concentration range. The method was extremely useful for monitoring tissue levels of I and II in a toxicity study in the monkey treated with I. The validated LLOQ of the method proved to be completely satisfactory for full evaluation of tissue exposure of I and II.

Acknowledgements

The authors thank Drs. E. Fontana and Mr. A. Pignatti (Isotope Labelling Group GDM-Italy) for their valuable contribution to this study for the synthesis of the stable isotope labelled internal standard.

References

[1] S.G. Louie, in: Proceedings of the 93rd Annual Meeting of AACR, South San Francisco, CA, USA, 6–10 April 2002.
[2] R. Bakhtiar, L. Ramos, F.L.S. Tse, L. Liq. Chromatogr. Rel. Technol. 25 (2002) 507.
[3] R.A. Biddlecombe, S. Pleasance, J. Chromatogr. B 734 (1999) 257.
[4] M. Jemal, D. Teitz, Z. Ouyang, S. Khan, J. Chromatogr. B 732 (1999) 501.
[5] D.T. Rossi, N. Zhang, J. Chromatogr. A 885 (2000) 97.
[6] M. Jemal, Biomed. Chromatogr. 14 (2000) 422.
[7] E. Frigerio, S. Sarati, S. Calderoli, C.A. James, Q. Zhang, in: Poster Presented at the 24th International Symposium on Chromatography, 15–20 September 2002, Leipzig, Germany. Abstract COUP-004.
[8] M. Jemal, Y.Q. Xia, Rapid Commun. Mass Spectrom. 13 (1999) 97.
[9] B.K. Matuszewsky, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.
[10] US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), May 2001, BP, Guidance for Industry, Bioanalytical Method Validation.