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Journal of Pharmaceutical and Biomedical Analysis
30 (2002) 377–389

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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LC-MS-MS determination of nemorubicin (methoxymorpholinyldoxorubicin, PNU-152243A) and its 13-OH metabolite (PNU-155051A) in human plasma

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Received 18 May 2001; received in revised form 5 September 2001; accepted 14 September 2001

Abstract

A selective and sensitive liquid chromatography-tandem mass spectrometry (LC-MS-MS) method for quantitative determination of nemorubicin, (PNU-152243A, 3′-deamino-3′[2(*S*)-methoxy-4-morpholinyl]doxorubicin) hydrocloride and its reduced metabolite PNU-155051 in human plasma has been developed and validated. The method involved solid phase extraction (SPE) in 96-well plates. Plasma samples (0.5 ml plasma, spiked with doxorubicin as internal standard and diluted with 0.5 ml of 0.01 M borate buffer, pH 8.4) were extracted using Oasis™ HLB SPE material. The elution of PNU-152243, PNU-155051 and of **IS** was performed with 1 ml of methanol:0.1 M formic acid mixture (90:10, v/v). The organic phase was reduced to dryness under a stream of nitrogen at 20 °C and the residue was reconstituted with 0.25 ml of 10 mM ammonium formate buffer pH 4.15:acetonitrile mixture (90:10, v/v). Aliquots of 60 μl of the resulting solution were injected onto the LC-MS-MS system. A Zorbax SB C18 column (2.1 × 150 mm, 3.5 μm) was used to perform the chromatographic analysis. The mobile phase consisted of ammonium formate buffer 10 mM pH 4.15:acetonitrile (73:27, v/v) with a flow-rate of 0.2 ml/min. Detection was achieved by a PE-SCIEX API 3000 with Turbo IonSpray interface, and multiple reaction monitoring (645 → 321 for PNU-152243, 647 → 363 for PNU-155051 and 545 → 345 *m/z* for doxorubicin) operated in positive ion mode. A weighted linear regression was used to calculate PNU-152243 and PNU-155051 concentrations in QC and unknown samples. Linearity, precision, accuracy and recovery of the method were evaluated over the concentration range of 0.1–5 ng/ml for both compounds. No interference from blank human plasma was observed. The suitability of the method for in vivo samples was assessed by the analysis of samples obtained from patients who had received a single intrahepatic artery dose of PNU-152243A. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Methoxymorpholinyldoxorubicin; Nemorubicin; Doxorubicin derivative; LC-MS-MS determination; Human plasma

1. Introduction

Nemorubicin, (3′-deamino-3′-[2-(*S*)-methoxy-4-morpholinyl]doxorubicin hydrocloride, PNU-152243A, FCE 23762, **I**), is a doxorubicin (**IS**) analogue bearing a methoxymorpholinyl group in position 3′ of the sugar moiety. **I** has displayed

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PII: S0731-7085(02)00222-4

a broad spectrum antitumor activity in experimental tumor models, including activity against multidrug resistant tumor cell lines and tumors, and tumor cell lines resistant to alkylating agents [1–3]. **I** is between 3- and 15-fold more potent than doxorubicin in vitro and 80- and 120-fold more potent in vivo [2]. Moreover **I** differs from doxorubicin and most of the anthracyclines in its mechanism of action [4–6], pattern of resistance [7], metabolism [8–12] and is not cardiotoxic at therapeutic doses in animals [12,13]. In previous phase **I** and **II** studies the drug was administered by i.v. bolus injection at 3–4 week intervals [14,15]. The maximum tolerated dose was about 1500 μg/m^2. The only metabolite detected both in patients and in animals [16,17] was PNU-155051 (FCE 26176, 13-dihydro-3′-deamino-3′-[2(*S*)-methoxy-4-morpholinyl]doxorubicin, **II**), which is less cytotoxic and less potent in vivo than the parent compound but equally effective. The original method for the determination of **I** and its reduced metabolite in human plasma used liquid-liquid extraction followed by HPLC with fluorimetric detection and had a lower limit of quantitation (LLOQ) of 0.5 ng/ml for both compounds [18]. Due to the low drug levels observed in humans an improved method with a LLOQ of 0.1 ng/ml using 1 ml of plasma was subsequently reported [19]. Herein, a new simpler method using solid phase extraction (SPE) in 96-well plates in conjunction with LC-MS-MS is presented. The method developed was fully validated over the concentration range of 0.1–5 ng/ml for both compounds using 0.5 ml of plasma. The suitability of the method for in vivo samples was assessed by the analysis of plasma samples obtained from a phase **I**–**II** clinical study after intrahepatic artery (IHA) dose.

2. Experimental

2.1. Chemicals and solutions

I, **II** and **IS** (all as hydrochloride salt, Fig. 1) were supplied by the Pharmaceutical Develop-

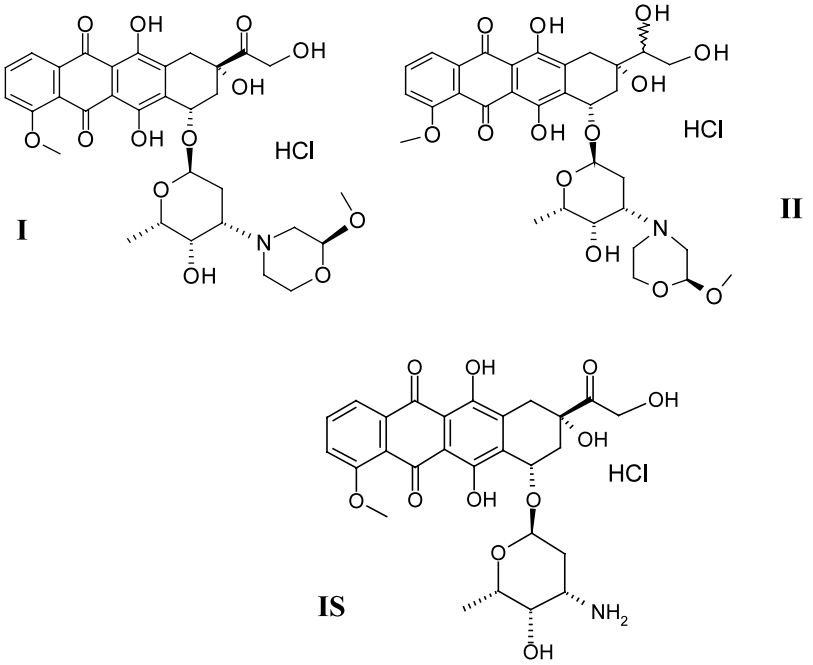

Fig. 1. Structure of PNU-152243 A (3′-deamino-3′[2(*S*)-methoxy-4-morpholinyl]-doxorubicin) hydrocloride (**I**), PNU-155051 A (13-dihydro-3′-deamino-3′[2(*S*)-methoxy-4-morpholinyl]-doxorubicin) hydrocloride (**II**) and doxorubicin hydrocloride (**IS**).

ment Department of Pharmacia (Milan, Italy). The purity for **I**, **II** and the **IS** was 96.08, 80.7 and 99%, respectively. All other chemicals and solvents were of HPLC or analytical reagent grade, and were obtained from Carlo Erba Reagents (Milan, Italy). Stock solutions were prepared by dissolving in distilled water accurately weighted amounts of **I**, **II** and **IS**. Suitable working solutions were prepared by further dilution of the appropriate stock solution with distilled water.

Separate stock and working solutions were used for the preparation of standard curves and quality control samples. All solutions were kept refrigerated when not in use. Stock solutions of **I**, **II** and **IS** were found to be stable for at least 5 months when stored at -20 °C. Acceptable stability for **I** and **II** working solutions was demonstrated following storage at -20 °C for 4.5 months.

To prevent losses of **I** which were seen with untreated glassware, silanised glassware and polypropylene tubes were used throughout the sample preparation procedure. The silanisation mixture used consisted of 7% dimethyldichlorosilane in toluene.

2.2. Equipment

The chromatographic system used in this study consisted of an HP 1100 HPLC (Hewlett Packard, Waldbronn, Germany) equipped with PE 200 autosampler (Perkin Elmer, Norwalk, USA) running at room temperature. MS detection used a PE-SCIEX API 3000 (Applied Biosystem/MDS Sciex, Thornhill, Ont., Canada). Data acquisition and evaluation were performed using the PE Sciex Mass Chrom 1.1 software running Sample Control version 1.4 and MacQuan, version 1.6 (Perkin Elmer).

2.3. Chromatographic conditions

The chromatographic separation was performed with a Zorbax SB C18 2.1 × 150 mm i.d., 3.5 μm particle size, reversed-phase column (Hewlett Packard). The column was kept thermostated at 25 °C. The mobile phase consisted of ammonium formate buffer 0.010 M (adjusted to pH 4.15 with 1 M formic acid):acetonitrile (73:27, v/v). The separation was performed at a flow-rate of 0.2 ml/min. Typical back-pressure was about 90 bar. The total chromatographic run and cycle time was 14 min.

2.4. Mass spectrometric conditions

Detection was in the positive ion mode using a Turbo IonSpray interface at a temperature of 300 °C, and multiple reaction monitoring. Nitrogen was used as curtain (setting 12), nebulizing (setting 12), and collision (setting 4) gas. The collision energy (Q0-R02) was set at 50 V. The orifice (OR) and ring (RNG) potentials were set at 70 and 380 V, respectively.

Molecular-to-product transitions of $645 \rightarrow 321$ m/z for **I**, $647 \rightarrow 363$ m/z for **II** and $545 \rightarrow z$ 345 m/z for **IS** were used. The dwell time for **I** and **II** was 500 ms whilst for the internal standard it was 200 ms.

2.5. Sample preparation

The method involved a solid phase extraction of **I**, **II** and **IS** from human plasma using Oasis™ HLB Extraction Plate (96-wells) with 30 mg of sorbent. The plate was first conditioned with methanol (1 ml) followed by 0.01 M borate buffer, pH 8.4 (1 ml). Plasma samples (0.5 ml plasma, spiked with **IS** and diluted with 0.5 ml of 0.01 M borate buffer, pH 8.4) were manually transferred and passed through the wells. The plate was then washed with water (1 ml), methanol:water mixture (5:95, v/v) (1 ml), and water (1 ml). The analytes were eluted into a polypropylene 96-deep well plate (1 ml) (Stepbio, Bologna, Italy) using methanol:0.1 M formic acid mixture (90:10, v/v) (1 ml). After each solution was added, vacuum was applied to gently pull all solutions through the cartridges.

The organic phase was dried under nitrogen stream at 20 °C using the Micro-DS96 (Porvair Sciences Ltd) evaporator equipped for 96-well plates and the residue was reconstituted with 0.25 ml of 10 mM ammonium formate buffer pH 4.15:acetonitrile mixture (90:10). The plates were capped using aluminum Unitek Heat Sealing Foils (Stepbio, Bologna, Italy) and hot sealed.

After 15 s vortex mixing (setting 5, on dial of 717 vortex mixer) and 10 min centrifugation at 1200 g the collection plate was transferred to the autosampler and aliquots of 60 μl of the resulting solution were injected onto the LC-MS system.

2.6. Assay validation experiments

Analyses of blank human plasma spiked with known amounts of **I** and **II** was carried out applying the above procedure. Linearity was evaluated from six (3 in duplicate) calibration curves prepared and run on three different days over the concentration ranges of 0.11–4.36 ng/ml and 0.11–4.58 ng/ml for **I** and **II**, respectively. Precision and accuracy were evaluated by repeated analyses of the compounds at three concentrations (low, mid and high) with five replicate samples analysed every day. All chromatograms obtained were evaluated by peak-area measurement. Calibration curves were constructed by plotting the ratio of peak-area of the compound and **IS** versus the concentration.

A weighted linear regression model (weighting factor $1/x^2$) was used to fit the calibration line and hence to calculate **I** and **II** concentrations in quality control and unknown samples. The concentrations of **I** and **II** were expressed in ng/ml (as free base).

In order to evaluate 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.

The possibility of ion suppression/enhancement was evaluated by comparing responses obtained from injection of standard solutions compared with identical concentrations of **I**, **II** and **IS** spiked into extracts of control human plasma. Stability of **I** and **II** was examined after storage in plasma at room temperature for 5 and 24 h and after storage of final extract at room temperature for 24 h. Thereafter, analytes stability was determined after three freeze/thaw cycles. Aliquots of QCs were stored at -80 °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 analysed on the third cycle. For each stability experiment, QC concentrations at 0.35, 1.94 and 3.9 ng/ml for **I** and 0.35, 1.97 and 3.93 for **II** were analysed in triplicate.

3. Results and discussion

The full scan spectra of **I** and **II** (Fig. 2A–Fig. 3A) revealed the protonated parent molecule $(M+H)^+$ to be in abundance with mass to charge ratio (m/z) of 645 and 647, respectively. The product ion spectra of the same compounds (Fig. 2B–Fig. 3B), using collision energy of 50 V, resulted in a major fragment at m/z 321 and 363, respectively.

For the internal standard, **IS**, the full scan spectrum (Fig. 4A) revealed the protonated parent molecule $(M+H)^+$ to be in abundance with m/z of 545, and the product ion spectrum (Fig. 4B), using the same collision energy as above, showed three fragments at m/z 320, 345 and 361. The transition ($545 \rightarrow 345$ m/z) was chosen to monitor the internal standard.

The chromatograms obtained from blank human plasma spiked with internal standard are shown in Fig. 5. Fig. 6 shows the chromatograms of calibration samples at the LLOQ for the method with **I** and **II**. Typical chromatograms obtained from a patient given an 800 $\mu g/m^2$ dose via IHA of **I** are shown in Fig. 7.

Sharp and symmetric peaks were obtained for **I** and **II** under the chromatographic isocratic conditions chosen. Under these conditions the analysis time (14 min) was sufficiently short to allow 96 samples/day to be assayed. The method was linear over the range of concentrations of 0.11–4.36 ng/ml for **I** and 0.11–4.58 for **II**. Correlation coefficients (r) for the regression were always better than 0.992 for both compounds. The back calculated calibration standard values for the compound showed a relative standard deviation (RSD) ranging from 5.1 to 13.5% for **I**, and from 0.5 to 11.8% for **II**. Results for the intra-day and inter-day precision and accuracy evaluated from QC samples are shown in Tables 1 and 2. The bias for all the concentrations was within $\pm 15\%$ of the target.

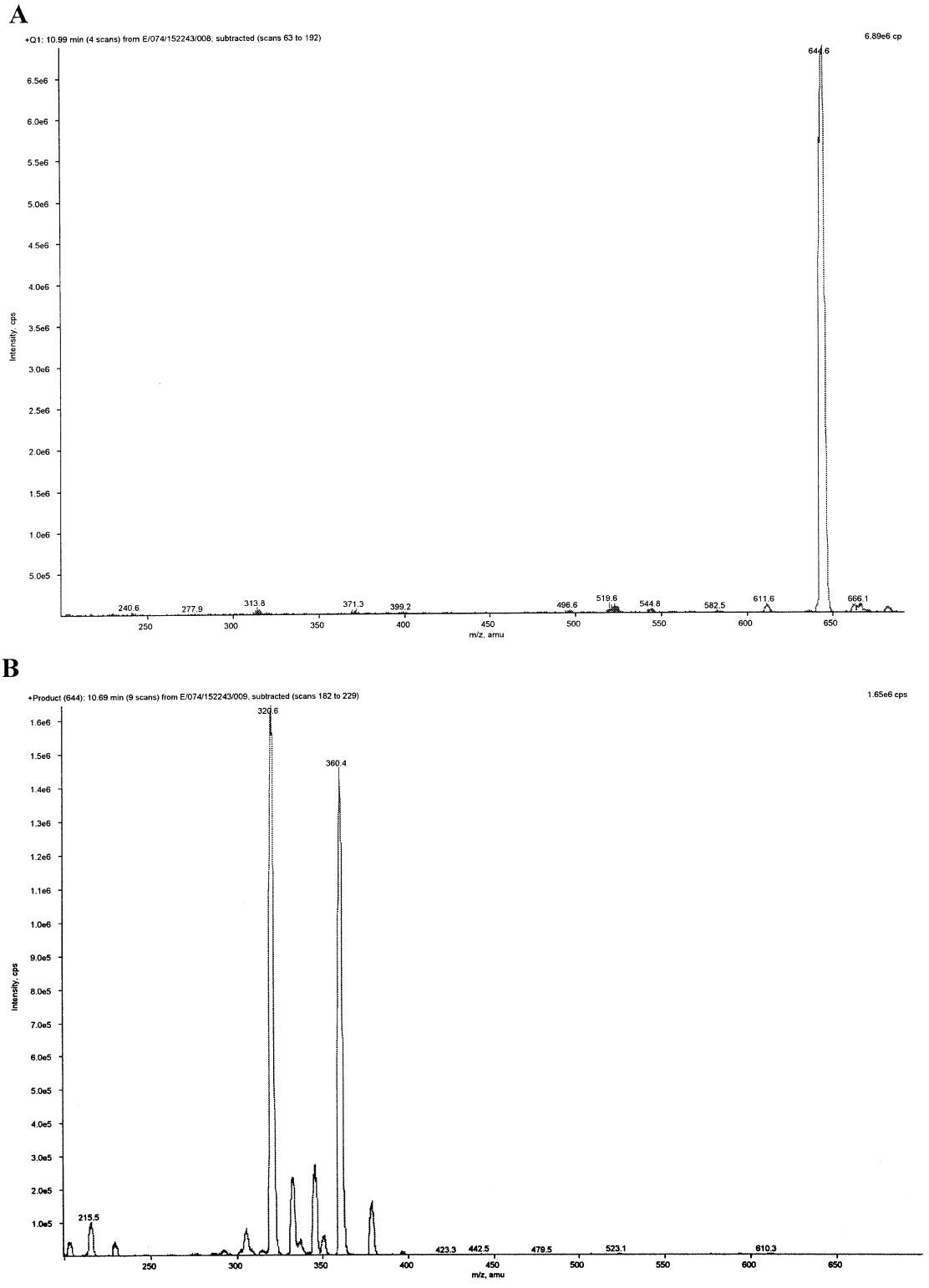

Fig. 2. Full scan spectrum (A) and product ion spectrum (B) of PNU-152243.

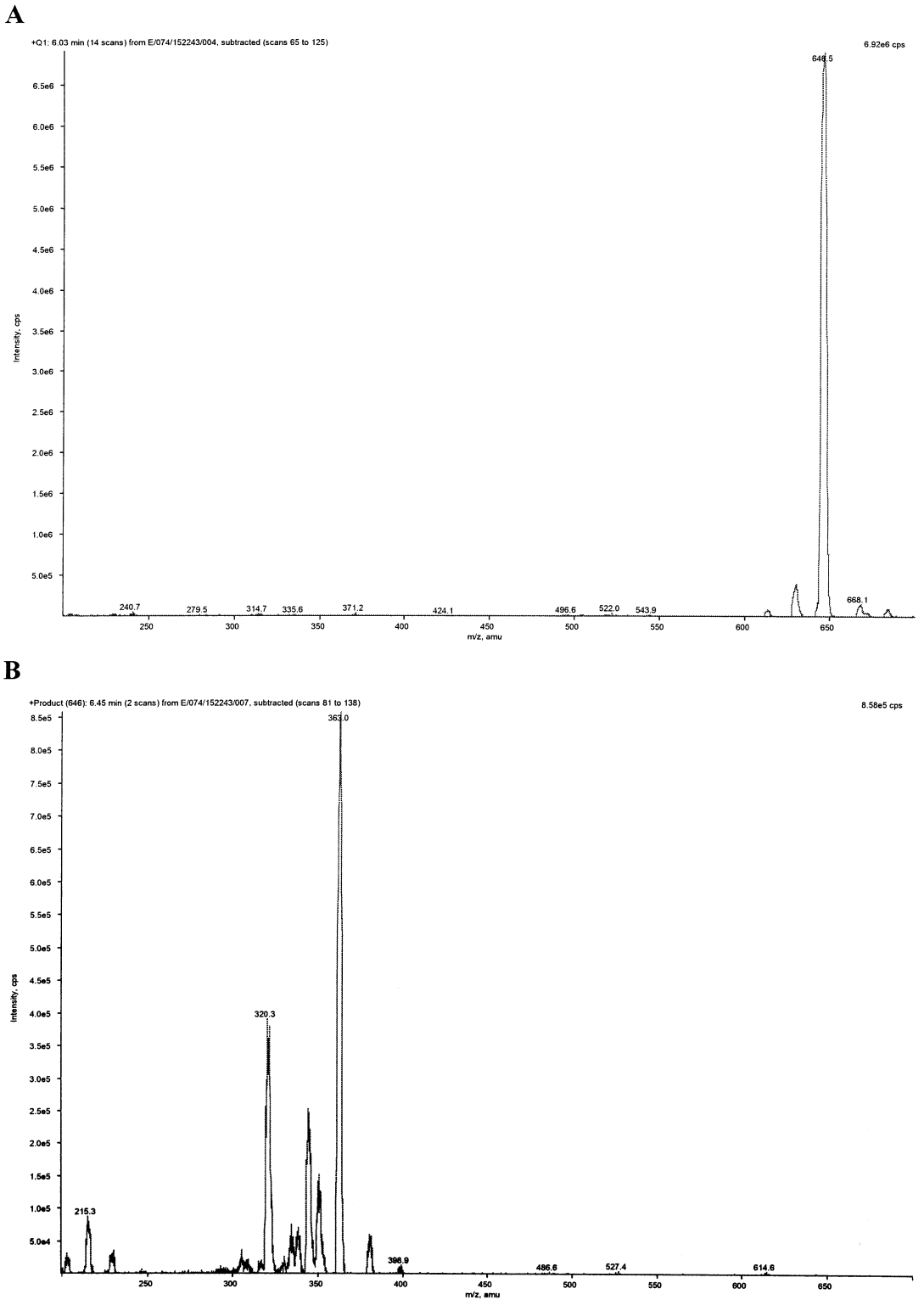

Fig. 3. Full scan spectrum (A) and product ion spectrum (B) of PNU-155051.

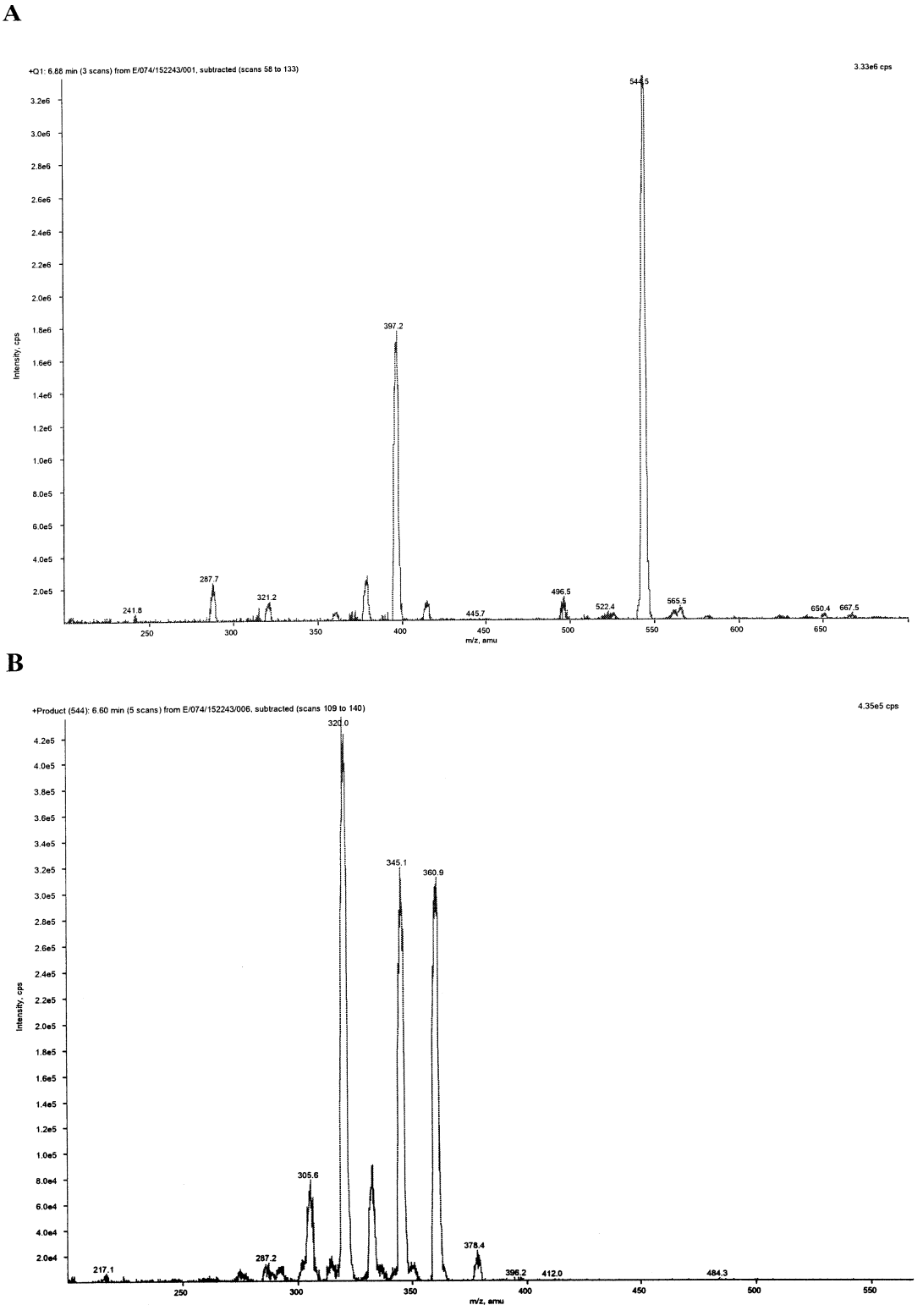

Fig. 4. Full scan spectrum (A) and product ion spectrum (B) of doxorubicin (internal standard).

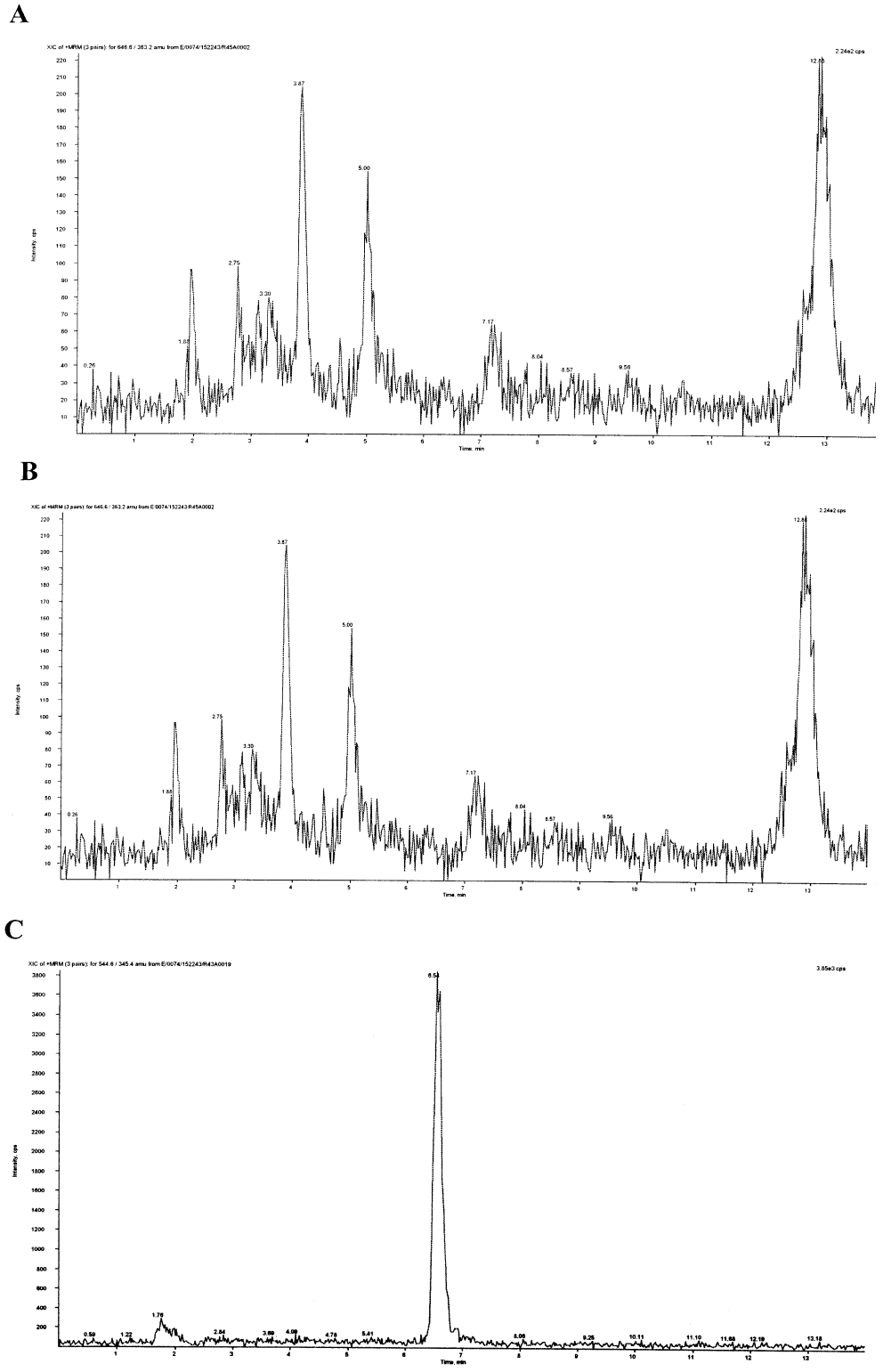

Fig. 5. Chromatograms of blank human plasma spiked with internal standard at the transitions 645 → 321 m/z for PNU-152243 (A), 647 → 363 m/z for PNU-155051 (B) and 545 → z 345 m/z for doxorubicin (**IS**) (C).

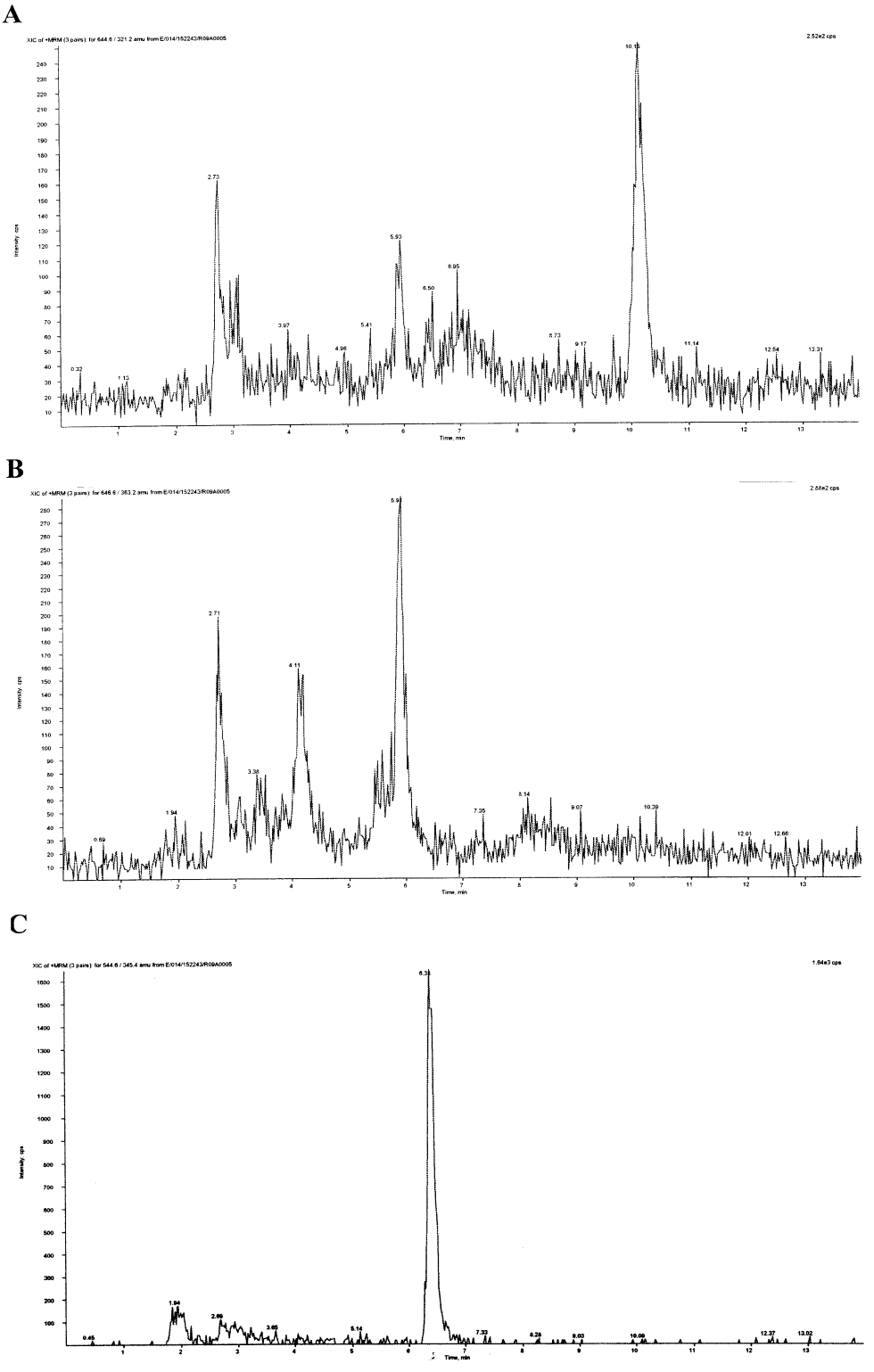

Fig. 6. Chromatograms of lower calibration standard (0.11 ng/ml) for PNU-152243 (A, Rt 10.15 min) and PNU-155051 (B, Rt 5.91 min) and the internal standard (C, Rt 6.36 min).

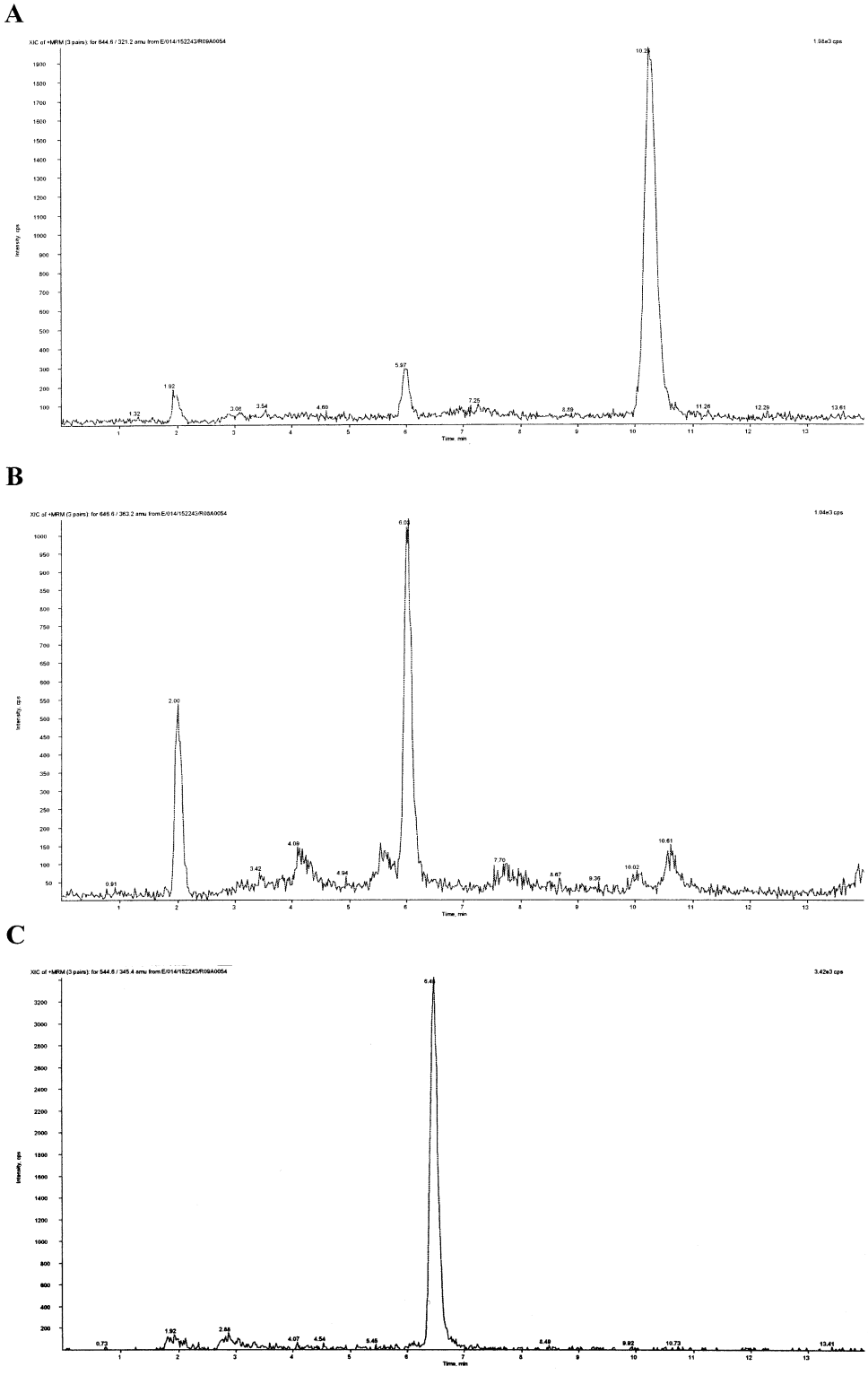

Fig. 7. Chromatograms of plasma from a patient given an 800 μg/m^2 dose via IHA of PNU-152243A, 6 h after the administration. The transitions for monitoring PNU-152243 (645 → 321 m/z) (A), PNU-155051 (647 → 363 m/z) (B) and the **IS** (545 → z 345 m/z) (C) are showed.

Table 1
Accuracy and precision data of the method for PNU-152243 determination in human plasma

Control sample (ng/ml)	Day	*n*	Accuracy			Precision		
			Mean found (ng/ml)	Mean (%) accuracy (intra-day)	Pooled (%) accuracy (inter-day)	RSD	%RSD (intra-day)	Pooled %RSD (inter-day)
0.35	1	5	0.35	100.4		0.02	5.3	
	2	5	0.34	97.5		0.01	4.1	
	3	5	0.35	99.0	99.0	0.01	3.3	4.2
1.94	1	5	2.11	108.9		0.13	6.0	
	2	5	2.09	107.5		0.12	5.5	
	3	5	1.98	102.2	106.2	0.05	2.8	4.8
3.90	1	5	4.27	109.5		0.20	4.7	
	2	5	4.38	112.4		0.15	3.5	
	3	5	3.17	95.1	105.7	0.10	2.7	3.6

Table 2
Accuracy and precision data of the method for PNU-155051 determination in human plasma

Control sample (ng/ml)	Day	*n*	Accuracy			Precision		
			Mean found (ng/ml)	Mean (%) accuracy (intra-day)	Pooled (%) accuracy (inter-day)	RSD	%RSD (intra-day)	Pooled %RSD (inter-day)
0.35	1	5	0.31	89.1		0.03	9.2	
	2	5	0.33	95.0		0.05	14.0	
	3	5	0.40	114.5	99.5	0.02	4.6	9.2
1.97	1	5	2.02	102.6		0.13	6.6	
	2	5	2.11	107.3		0.06	2.7	
	3	5	2.24	113.5	107.8	0.02	0.8	3.4
3.93	1	5	4.20	106.9		0.18	4.3	
	2	5	4.27	108.5		0.24	5.7	
	3	5	4.20	107.0	107.5	0.23	5.5	5.2

The extraction recovery of the method for the determination of **I**, **II** and **IS** following SPE in the 96-well plate format evaluated at three concentrations ranged from 71.2 to 105.7%.

Similar responses were seen following injections of standard solutions compared with identical concentrations of **I**, **II** and **IS** spiked into extracts of control human plasma, indicating that control extracts do not significantly suppress or enhance signal.

Stability data were obtained using spiked QC samples. There was some evidence of degradation of both **I** and **II** after storage in human plasma at room temperature for 24 h. Accuracy ranged from −36.8 to −32.0% for **I** and from −26.4 to −13.1% for **II**. After storage in human plasma at room temperature for 5 h results were obtained 'within specifications' (±20% of target) for both **I** and **II**, although some indication of minor degradation was still seen. Accuracy ranged from −19.0 to −15.2% for **I** and from −15.8 to 2.2% for **II**. In every case, the samples were never left on the bench more than what needed for defrosting (less than 1 h). After defrosting they were immediately extracted. Data obtained after storage in the autosampler of final plasma extracts at room temperature for 24 h and after three freeze/thaw cycles did no show evidence of degradation for both compounds.

The present method was developed in order to evaluate the pharmacokinetics of **I** when administered via hepatic artery in a Phase **I**–**II** study. On the basis of the low doses administered in the clinical study a calibration range of approximately 0.1–5 ng/ml for both compounds was considered suitable. If necessary, higher concentrations could be analysed after appropriate dilution of the samples.

4. Conclusions

A sensitive, selective and highly efficient method combining 96-well plate technology to the use of LC-MS-MS for the determination of **I** and **II** in human plasma has been developed and validated. With respect to the previous HPLC/FL method similar performance has been obtained with one-half of the plasma volume, and with a much simpler and faster sample preparation procedure. The proposed method proved to be specific and sensitive enough to be applied to a phase **I**–**II** study carried out to evaluate the antitumor efficacy and the pharmacokinetics and of **I** and **II** in cancer patients given **I** via IHA.

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