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Determination of 4-demethoxy-3′-deamino-3′-aziridinyl-4′-methylsulphonyldaunorubicin and its 13-hydroxy metabolite by direct injection of human plasma into a column-switching liquid chromatography system with mass spectrometric detection

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Abstract

A selective, sensitive and fully automated column-switching LC system using direct injection of human plasma followed by mass spectrometry (MS) detection was developed and validated to determine the concentrations of 4-demethoxy-3′-deamino-3′-aziridinyl-4′-methylsulphonyldaunorubicin (PNU-159548) and its 13-hydroxy metabolite (PNU-169884). A 50-μl human plasma sample was directly introduced into a C_4-alkyl-diol silica clean-up column separating analytes from proteins and polar endogenous compounds using water and methanol as the mobile phase. The fraction containing PNU-159548 and its metabolite was back-flushed and transferred to the analytical column. The compounds were separated using a Zorbax SB C_8 column (150×4.6 mm, 5 μm) under gradient conditions with the mobile phase containing acetonitrile and 2 m*M* ammonium formate, pH 3.5. MS detection was by atmospheric pressure ionisation with multiple reaction monitoring in positive ion mode. Linearity was demonstrated over the calibration range of 0.051–10.291 ng/ml for PNU-159548 and 0.104–10.434 ng/ml for PNU-169884. The assay was validated with respect to accuracy, precision and analyte stability. On the basis of the validation data, the developed analytical method was found to be suitable for use in Phase I clinical studies.

Keywords: Column switching; Demethoxydeaminoaziridinylmethylsulfonyldaunorubicin; PNU-169884; PNU-159548

1. Introduction

Anthracycline antibiotics are a class of therapeutic agents widely used as anticancer drugs in current cancer chemotherapy. The toxicity of this class of compounds and the emergence of multidrug resistance after treatment with anthracyclines prompted a search for new more potent and less toxic analogues. PNU-159548 (4-demethoxy-3′-deamino-3′-aziridinyl-4′-methylsulphonyldaunorubicin, Fig. 2) is the lead of a new class of antitumor drugs (alkycyclines) whose interaction with DNA differs from those of previously identified DNA interacting drugs [1]. PNU-159548 showed wide spectrum antitumor activity in animal models [2] and minimal cardiotoxicity in comparison with doxorubicin in a long-term toxicity study in rats [2].

The existing method, based on HPLC with fluorescence detection, had limits of quantitation in the region of 0.5–1 ng/ml [3]. The objective of the current study was to develop and validate an LC–MS–MS method for the determination of PNU-159548 and its 13-hydroxy metabolite (PNU-

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169884, Fig. 3) after direct injection of human plasma to obtain higher sensitivity and to reduce the manipulation of the samples.

2. Materials and methods

2.1. Chemicals and solutions

PNU-159548 (purity 94.5%) and PNU-169884 (purity 88.5%) were supplied by Pharmacia and Upjohn [Nerviano (Mi), Italy]. All other chemicals and solvents were of analytical grade and were obtained from Carlo Erba Reagents (Milan, Italy). Stock solutions of PNU-159548 and PNU-169884 were prepared by dissolving a weighed amount of these two compounds in acetonitrile in silanized volumetric flasks.

2.2. HPLC conditions

The HPLC apparatus consisted of a HP 1100 system (pump B) (Hewlett–Packard, Waldbronn, Germany), a TSP Model P1000 isocratic pump (pump A) (Thermo Separation Products, Santa Clara, CA, USA), a Waters Model 717 plus temperature controlled autosampler (Waters, Milford, MA, USA) maintained at +4°C, an electrically driven multifunction HPLC valve (Thar Design, Pittsburgh, PA, USA) and a universal valve switching module (Anachem, Charles St., Luton, UK). A clean-up column (25×4.6 mm I.D.) packed with LiChrospher RP-4 ADS (25 μm particle size, Merck, Darmstadt, Germany) was placed between pump A and the first switching valve (Fig. 1). The sample (injection volume 50 μl) is loaded into the clean-up column by pump A, which delivers mobile phase A (water–methanol, 95:5, v/v) at a flow-rate of 2 ml/min. A 2-μm filter (Alltech, Deerfield, IL, USA) was placed between the injector and the clean-up column. The analytical separation was performed on a Zorbax SB C_8, 150×4.6 mm column (5 μm, Hewlett–Packard) with a Zorbax SB C_8 12.5×4.6 mm guard column (5 μm, Hewlett–Packard), maintained at 45°C. The compounds were eluted under gradient conditions (Table 1) using mobile phase B (delivered by pump B) containing acetonitrile and 2 m*M* ammonium formate (adjusted to pH 3.5 with formic acid) at a flow-rate of 1 ml/min.

Table 1
Gradient elution program for mobile phase B delivered by pump B

Time (min)	Flow-rate (ml/min)	2 m*M* Ammonium formate, pH 3.5	CH_3CN (%)
0–6.5	1	40	60
6.5–7.5	1	10	90
7.5–9.5	1	10	90
9.5–12	1	40	60
12–14	1	40	60

2.3. Mass spectrometry conditions

The LC–MS–MS analysis was performed using a Perkin-Elmer Sciex API III+ triple quadrupole mass spectrometer (PE Sciex, Beaconsfield, UK). The instrument was operated with atmospheric pressure

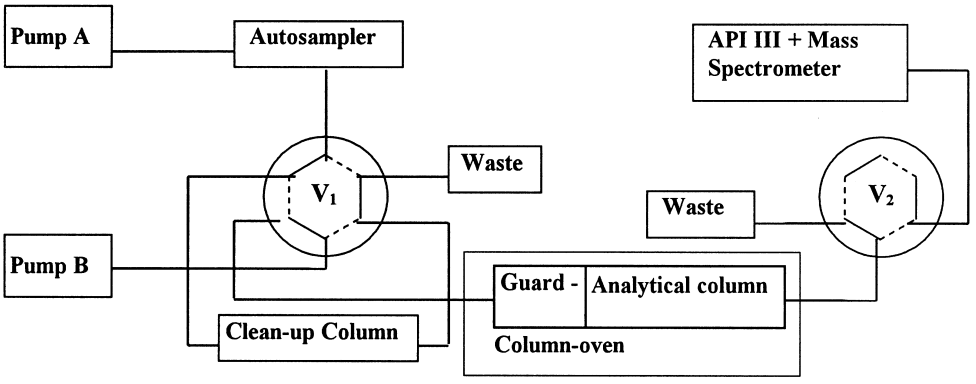

Fig. 1. Scheme of chromatographic apparatus used for automatic on-line clean-up of sample and HPLC with column switching system.

ionisation (API) utilising the PE Sciex IonSpray interface. Curtain gas (nitrogen) flow was 1.4 l/min, while the nebulizer gas (air) pressure was set at $4 \cdot 10^5$ Pa. Multiple reaction monitoring (MRM) detection was employed using argon as the collision gas, set at a thickness of approximately $300 \cdot 10^{12}$ molecules cm^{-2} with a collision energy of 35 eV. Precursor-to-product ion transitions of m/z 602 to 291 for PNU-159548 and m/z 604 to 291 for PNU-169884 were used. The dwell time for each transition was 200 ms. The peak area for the selected ions was determined automatically using the PE Sciex software package MacQuan (version 1.5).

2.4. Sample preparation

Plasma samples were thawed, and after centrifugation at 4°C for 10 min at 21 000 *g*, were pipetted into polypropylene autosampler vials (0.7 ml, Waters). It was necessary to centrifuge again at 4°C for 10 min at 2300 *g*, as without this step the back pressure of the clean-up column can increase.

2.5. Analysis cycle

The coupled-column analysis cycle for the determination of PNU-159548 and PNU-169884 can be subdivided into three different phases (Fig. 1).

2.5.1. Sample loading (0–6 min)

The plasma sample is loaded with mobile phase A, delivered by pump A, at a flow-rate of 2 ml/min via the autosampler through valve positions 6–1 into the RP-4 ADS precolumn. During this clean-up sample processing step, the analytical column is equilibrated with mobile phase B, delivered by pump B, at a flow-rate of 1 ml/min.

2.5.2. Analyte(s) transfer (6–8 min)

Switching the automatic valve (V_1) to the inject position, mobile phase B is delivered through valve connections 3–4 to the clean-up column (backflush mode) at a flow-rate of 1 ml/min. The retained anthracyclines are eluted from the clean-up column and transferred in a narrow elution band through valve positions 1–2 to the analytical column. After 2 min the valve is switched back to the load position; the precolumn is reconditioned with mobile phase A at a flow-rate of 2 ml/min for the next sample injection.

2.5.3. Separation (8–14 min)

The analytes are separated on the analytical column under gradient conditions by mobile phase B at a flow-rate of 1 ml/min. After 0.5 min the second automatic valve (V_2) switches to the inject position; so the mobile phase B is transferred to the mass spectrometer. At 12 min the valve is switched to the original position excluding the MS instrument. The two last minutes are utilised to requilibrate the system.

2.6. Assay validation experiments

The linearity of the method was evaluated from five calibration curves prepared and run on five different days in the concentration range 0.051–10.291 ng/ml for PNU-159548 and 0.104–10.434 ng/ml for PNU-169884. The precision and accuracy were evaluated by repeated analyses of quality control (QC) samples at four different concentrations (0.157, 4.220, 8.440 and 25.320 ng/ml for the unchanged drug and 0.248, 4.140, 8.050 and 24.160 ng/ml for the metabolite) in six replicated samples analysed on the first day of validation (intra-assay) and in three replicated samples analysed on the other four days (inter-assay). The QC samples at a concentration above the calibration range were analysed after dilution (1:10) with blank human plasma. All chromatograms obtained were evaluated by peak-area measurement. The quality control and unknown samples were calculated with the calibration graph generated on each day by least-squares linear regression of the analytes peak area against their concentration. Precision and accuracy at the lower limit of quantitation (LLOQ) at 0.051 and 0.104 ng/ml for PNU-159548 and PNU-169884, respectively, were assessed by analysis of six spiked plasma samples in one analytical run. The stability of the organic (acetonitrile) standard solutions at three concentrations, stored at +4°C was assessed by repeated injections over a period of one month. The stability of the plasma was determined by analysis of the QC samples after they had been left for 4 h at room temperature, 24 h in an autosampler at +4°C and after 3 freeze/thaw cycles.

The recovery of PNU-159548 and PNU-169884 from spiked plasma samples was calculated by comparing the RF (response factor=ratio between the area of the analyte and its concentration) in the chromatograms of the samples with those obtained after injections of the acetonitrile solutions of these two compounds ($N=5$).

3. Results

A Q1 scan of PNU 159 548 (Fig. 2) and PNU-169884 (Fig. 3) revealed the protonated parent molecule $(M+H)^+$ to be in abundance with a mass to charge ratio (m/z) of 602 and 604, respectively. Impurities were detected both in PNU-159548 and PNU-169884 standard compounds at lower molecular mass. The peaks with a m/z higher than 602 and 604 were cluster ions due to the addition of ammonium and/or acetonitrile. The product ion spectrum of PNU-159548 and PNU-169884 using a collision energy of 35 eV resulted in a major fragment at m/z 291 for both compounds. The resulting product ions were in high abundance. The spectra were in agreement with the structures of these compounds. A proposed fragmentation pathway is shown in Figs. 4 and 5 for the unchanged drug and its metabolite, respectively.

Under the chromatographic conditions utilised in this study, the retention time was about 9.6 min for PNU-169884 and 10 min for PNU-159548 (Fig. 6).

No interfering endogenous peaks were detected in any extracts of control human plasma and no interfering peaks were noted in human study predose samples.

The calibration curves for PNU 159 548 were linear over the range of 0.051 to 10.291 ng/ml and for PNU-169884 over the range of 0.104 to 10.434 ng/ml. The linear correlation coefficients (r^2) ranged from 0.9955 to 0.9980 for PNU-159548 and from 0.9939 to 0.9978 for PNU 169-884, using linear regression with a $1/X^2$ weighting. The weighting factor was chosen to minimise deviation of back-calculated values from theoretical concentrations. The back-calculated calibration standard points showed an RSD ranging from 3.1 to 7.9% for PNU-159548 and from 5.5 to 10.1% for PNU-169884.

A LLOQ of 0.051 ng/ml for PNU-159548 and of 0.104 ng/ml for PNU-169884 was achieved following direct injection of 50 μl of human plasma. This represents approximately 2.5 and 5 pg on column for the parent compound and the metabolite, respectively with a S/N better than 3. The accuracy at these levels was better than ±5% and the intra-day RSD was <10% for both compounds.

The results of intra-day and inter-day precision (expressed as %RSD) and accuracy (expressed as %Bias) are described in Table 2.

Stock solutions when stored in the dark at +4°C until use are stable for at least one month. There was no evidence of degradation of PNU-159548 and PNU-169884 after storage in plasma at room temperature for 4 h, during storage in plasma at +4°C for 24 h and after 3 freeze/thaw cycles (Table 3).

Recovery from plasma samples ranged from 84.7 to 92.9% for PNU-159548 and from 82.5 to 87.5% for PNU-169884.

The method was used to determine PNU-159548 and its metabolite levels from cancer patients in first Phase I clinical studies. Typical chromatograms are shown in Fig. 7.

4. Discussion

Initially, the PE Sciex Turbo IonSpray interface was used to achieve maximum sensitivity for the analytes without the need to split the eluent from the analytical column. However, although sensitivity was extremely high, the method showed poor reproducibility. The reasons for this were not clear, but may be related to the thermolability of the two analytes, as reverting to conventional IonSpray interface resolved the problem. Sensitivity was reduced by a factor of approximately three, and analytical conditions had to be carefully optimised to maximise the sensitivity that could be achieved with the IonSpray interface.

The optimum IonSpray current was dependent upon several factors, including mobile phase composition. In our experiments, decreasing the molarity of the buffer (from 20 mM to 2 mM) gave an increase in sensitivity, while lowering the pH below 3.5 gave no improvement. The use of acetonitrile as an eluent under gradient conditions enhanced the

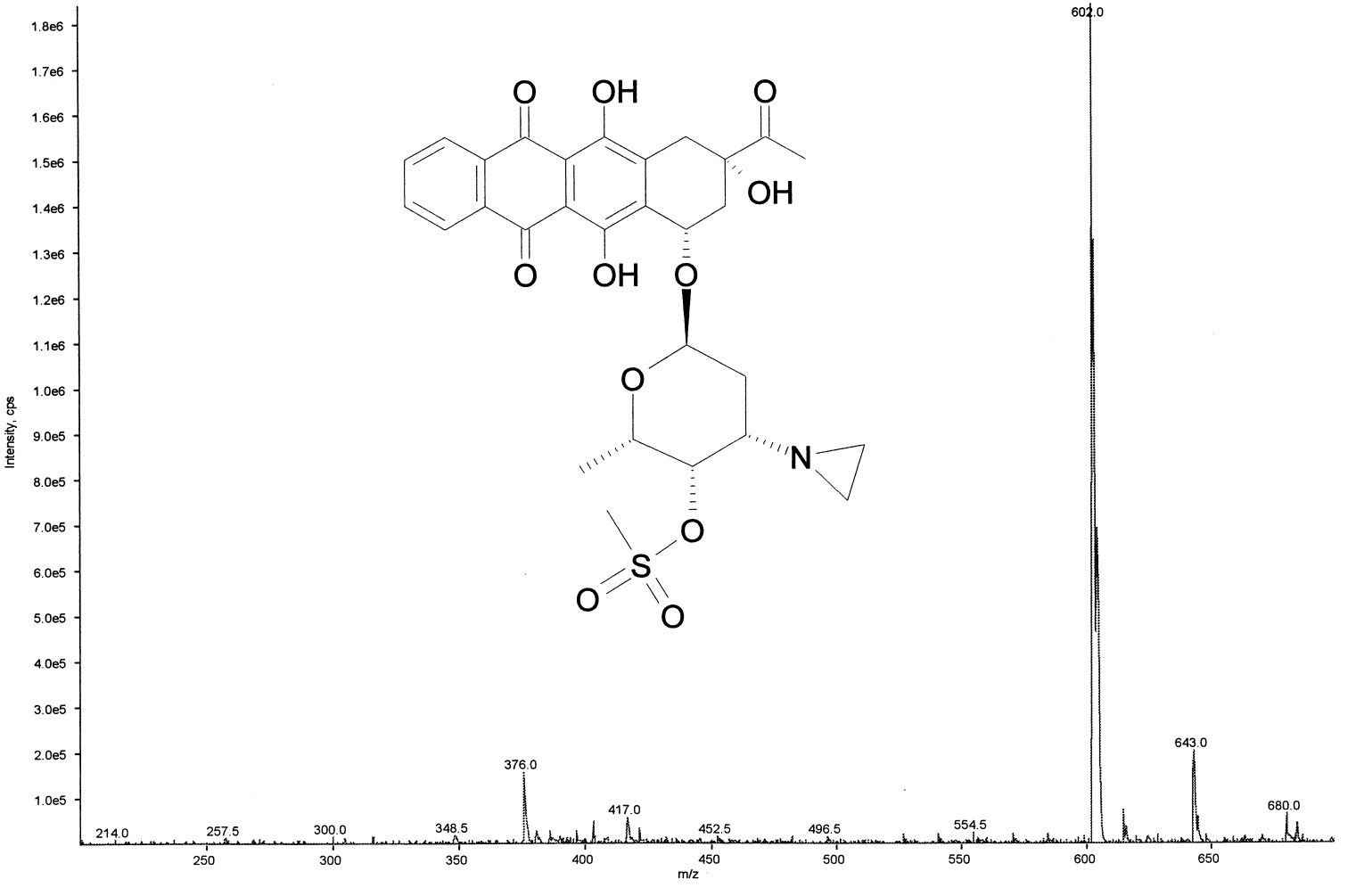

Fig. 2. IonSpray scan mass spectrum and structure of PNU-159548.

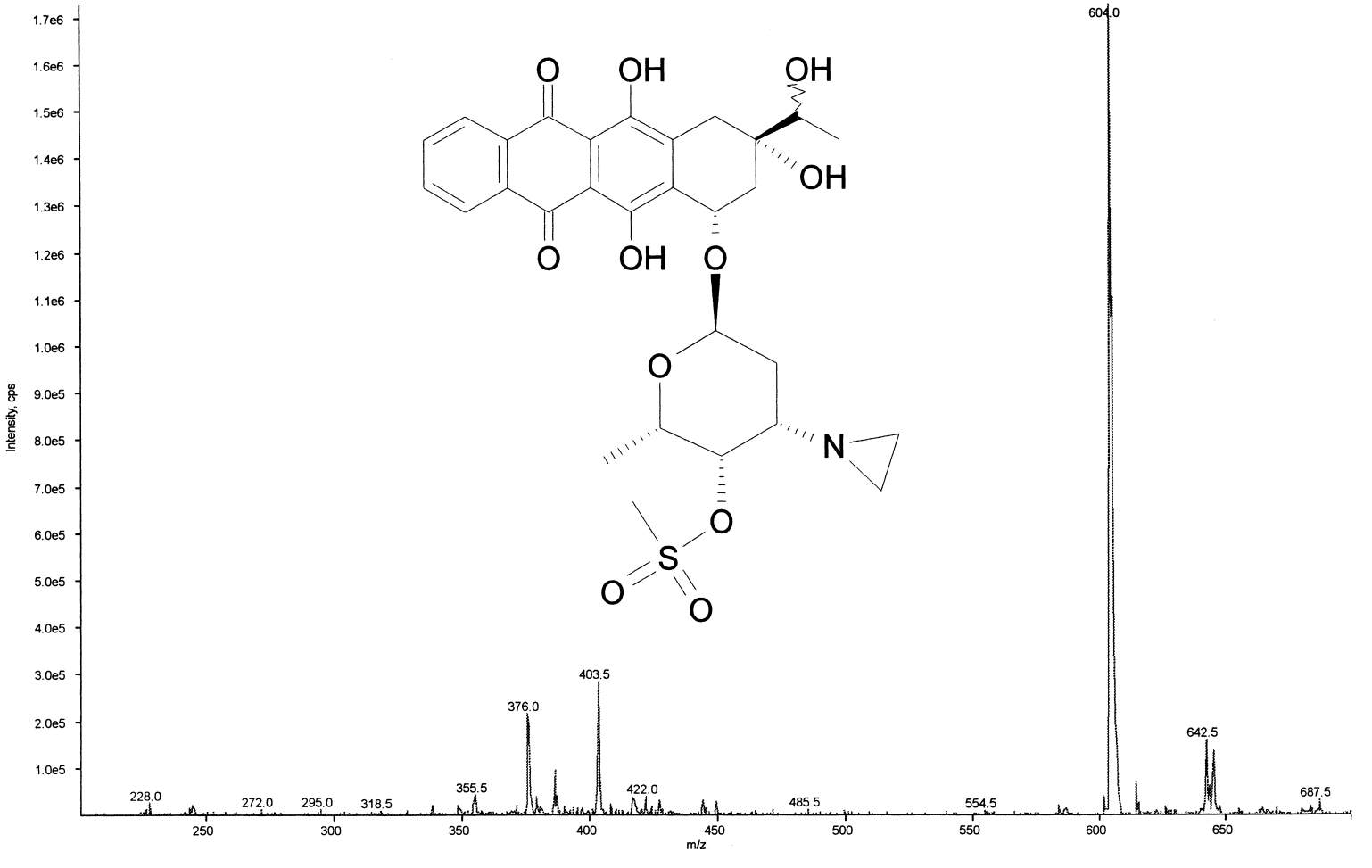

Fig. 3. IonSpray scan mass spectrum and structure of PNU-169884.

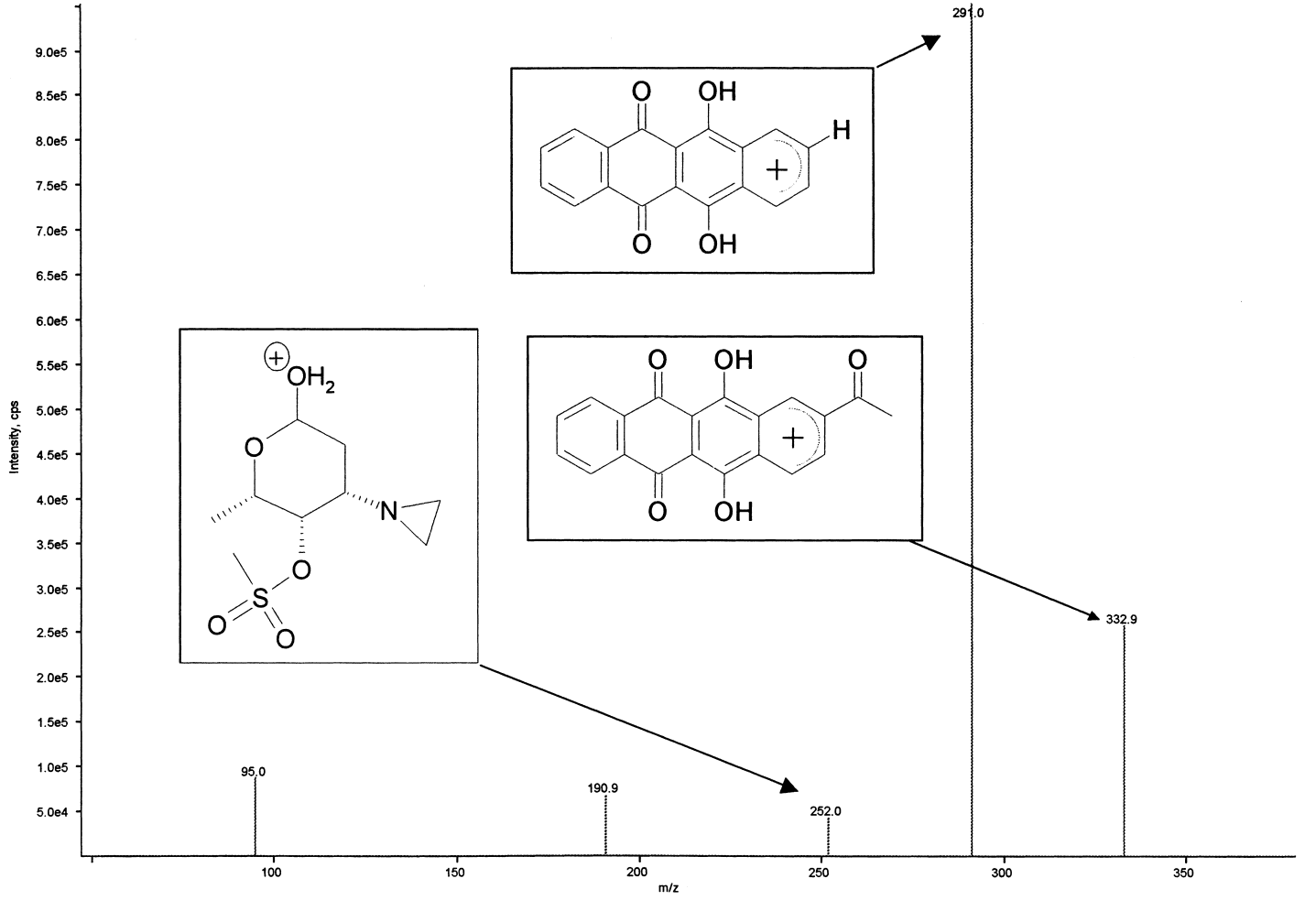

Fig. 4. Product ion mass spectrum and the structure of the main fragment ions of PNU·159548.

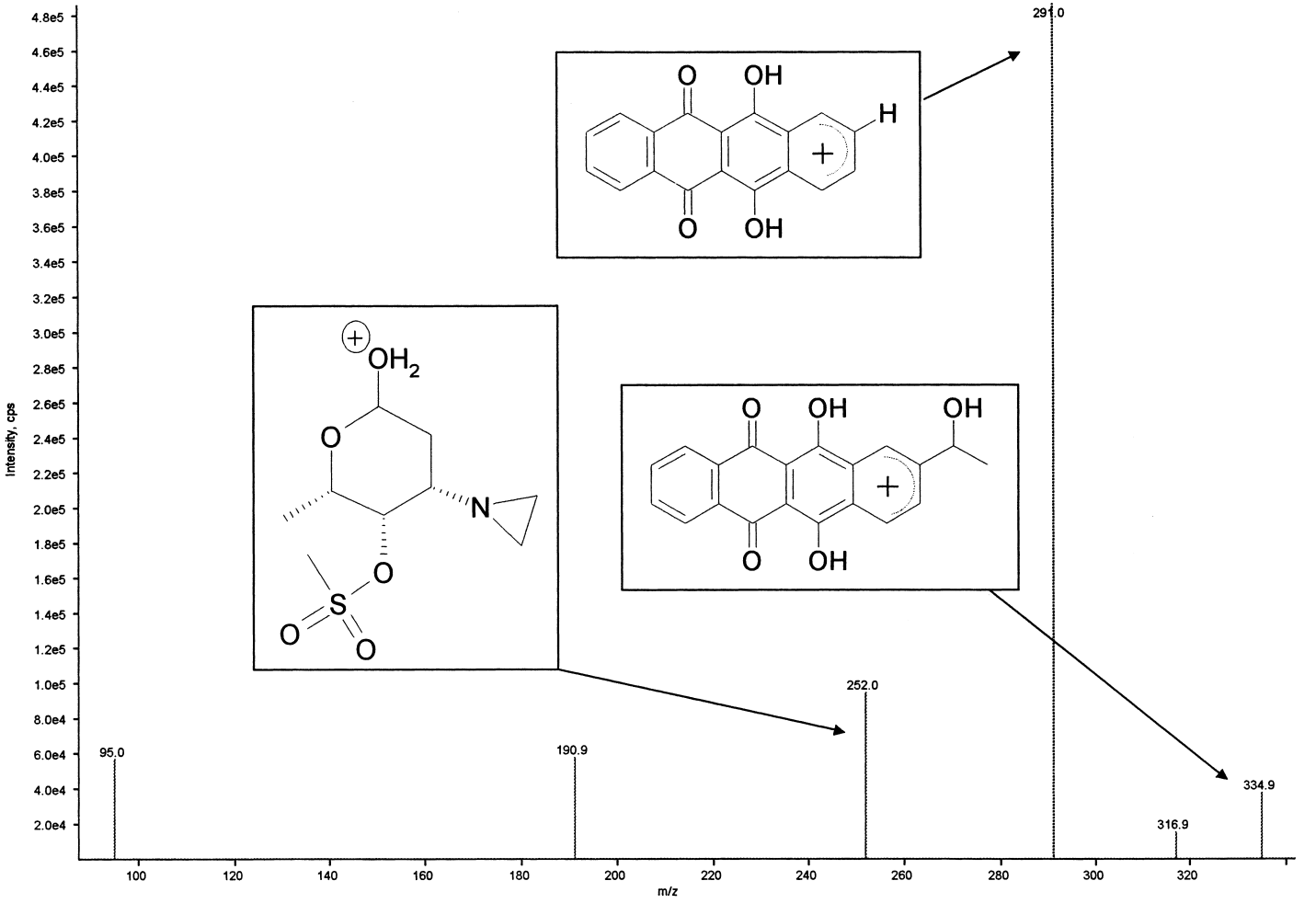

Fig. 5. Product ion mass spectrum and the structure of the main fragment ions of PNU·169884.

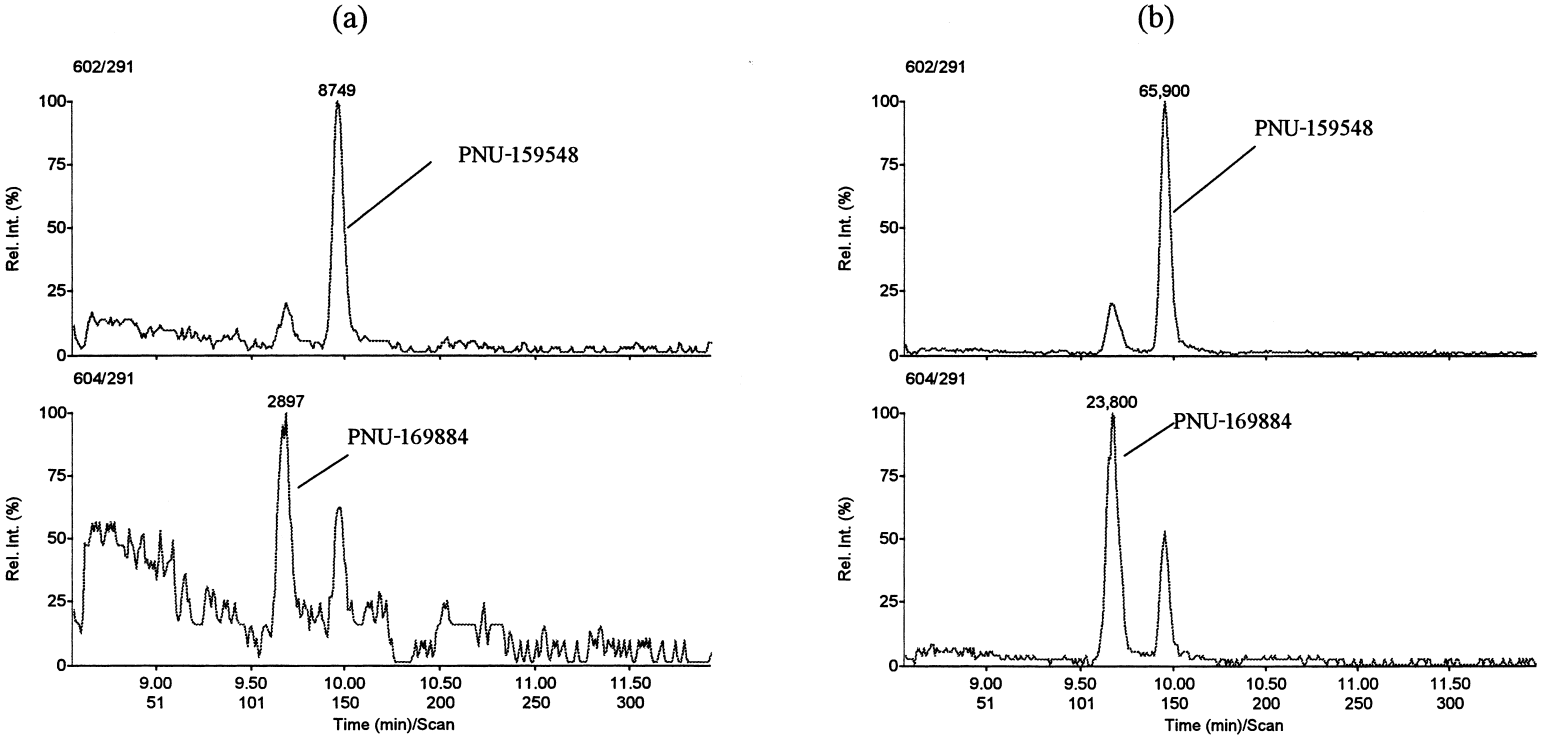

Fig. 6. LC–MS–MS multiple reaction monitoring chromatogram of (a) plasma spiked with 0.309 ng/ml of PNU·159548 (m/z 602→291) and 0.313 ng/ml of PNU·169884 (m/z 604→291) and (b) plasma spiked with 2.573 ng/ml of PNU·159548 and 2.609 ng/ml of PNU·169884 (the labels on the peak indicate the area).

Table 2
Accuracy and precision of PNU-159548 and PNU-169884 determination in human plasma

Compound	Concentration (ng/ml)	Intra-assay precision (%RSD, $N=6$)	Inter-assay precision (%RSD, $N=18$)	Intra-assay accuracy (%Bias, $N=6$)	Inter-assay accuracy (%Bias, $N=18$)
PNU-159548	0.157	7.6	10.8	−10.1	−0.3
	4.220	3.7	8.8	7.2	2.8
	8.440	2.3	8.2	11.6	5.8
	25.300	3.1	7.2	7.4	1.6
PNU-169884	0.248	8.9	7.3	3.8	3.6
	4.140	3.2	8.4	7.0	1.8
	8.050	4.3	11.0	6.0	2.3
	24.200	2.4	8.8	6.5	0.4

peak response and shape whilst maintaining a good chromatographic resolution between the two peaks. The optimum flow-rate for use with the IonSpray interface was found to be 100 μl/min, this being achieved by use of a split. A second switch valve between the HPLC and MS devirted the eluent from the analytical column to waste for the 8.5 min of each injection, significantly increasing the time intervals between cleaning the interface.

PNU-159548 and PNU-169884 peaks were separated with good chromatographic resolution. However, due to the low mass resolution, the small difference (2 u) between the molecular masses of the two compounds and the same product ion selected for the quantitative assay, a small peak corresponding to the retention time of the metabolite was present in the MRM chromatogram of the transition m/z 602→291 produced by the unchanged drug. For the same reason, a small peak at the retention time of the unchanged drug was detected in the MRM chromatogram of the transition m/z 604→291 produced by the metabolite. The potential overlap of the peaks, during quantitation measurements, was easily avoided through a specific filter procedure of MacQuan software. This allowed us to easily distinguish between the total ion current produced by the same product ion of the two different transitions. Quantitative determination was then performed by measuring the area of the peak corresponding to the total ion current produced by m/z 291 from m/z 602 (Fig. 6,

Table 3
Stability of PNU·159548 and PNU·169884 in human plasma

Storage conditions	PNU·159548		PNU·169884	
	Concentration (ng/ml)	% Bias	Concentration (ng/ml)	% Bias
4 h at room temperature	0.157	−5.9	0.248	−10.5
	4.220	−9.1	4.140	−9.3
	8.440	2.8	8.050	−0.3
	25.300	8.5	24.200	5.6
24 h at 4°C	0.157	0.2	0.248	2.4
	4.220	−6.4	4.140	0.6
	8.440	−4.9	8.050	11.7
After 3 freeze/thaw cycles	0.157	3.6	0.248	−0.1
	4.220	−4.8	4.140	−11.5
	8.440	11.5	8.050	0.3
	25.300	7.5	24.200	−1.9

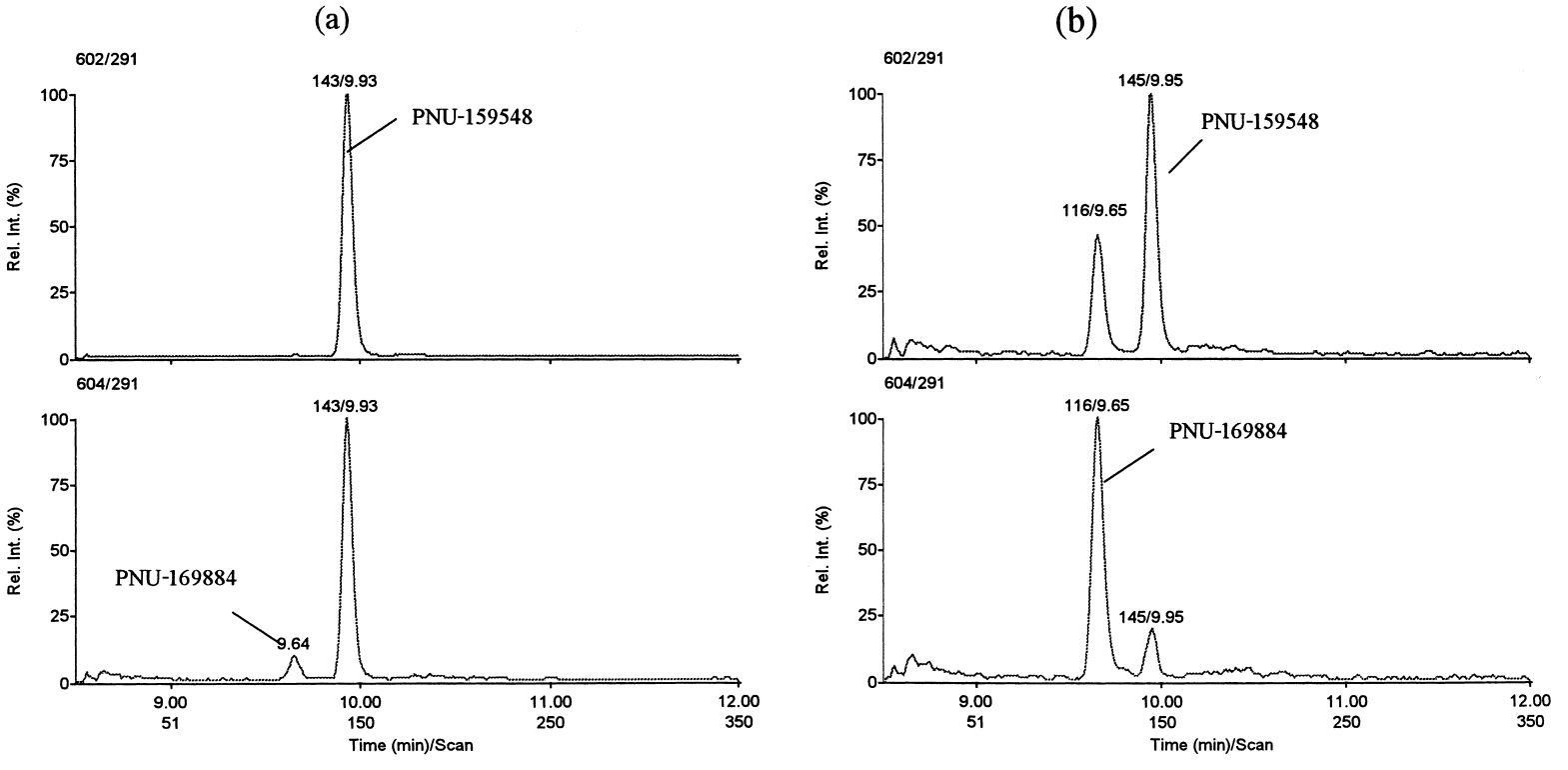

Fig. 7. LC–MS–MS multiple reaction monitoring chromatogram obtained after direct injection of 50 μl plasma samples collected (a) 5 min (10.4 ng/ml of PNU·159548 and 0.4 ng/ml of PNU·169884) and (b) 1 h (0.8 ng/ml of PNU·159548 and 2 ng/ml of PNU·169884) from a cancer patient treated with an infusion of PNU·159548 (1 mg/m^2).

upper traces) and m/z 604 (Fig. 6, lower traces) by PNU-159548 and PNU-169884, respectively.

HPLC integrated processing, i.e. extraction and fractionation of complex biological fluids, can be achieved by coupling a special clean-up precolumn to a conventional analytical column. Such a pre-column extracts the analytes of interest and removes the macromolecular matrix components that could otherwise deteriorate an analytical column. These special packing materials are known as restricted access material (RAM) (for review see [4]). The packing of the clean-up column, used in this work, was based on alkyl-diol silica (ADS). This family of restricted-access materials, has glyceropropyl-(diol-) groups as hydrophilic electroneutral ligands on the external surface and *n*-alkyl esters as a hydrophobic phase exclusively located on the internal surface [5]. The use of this type of clean-up column for the determination of related anthracyclines [6] and in combination with LC–MS [7] was already described. On-line coupling of solid-phase extraction (SPE) with HPLC allowed the direct and repetitive injection of biological samples as well as the separation of drugs and their metabolites on a routine basis. No manual sample pre-treatment steps except for centrifugation were required. This procedure was used in our method due to limited aqueous and solvent solubility of the analytes. Optimisation of the use of this clean-up column was investigated. The two compounds were injected into columns with different percentages of methanol and water in a simple LC system to test the hydrophobicities of the restricted access support materials. Similarly, to evaluate the effect of the ADS precolumn on sample clean up, blank plasma was injected into the column-switching system. The C_4 material best retained the analytes and provided the cleanest chromatogram after injecting blank plasma. The use of a replaceable in-line filter of at least 2 μm was necessary to obtain a stable backpressure and to enhance the life of the clean-up column.

5. Conclusions

A simple and reliable LC–MS–MS method for the simultaneous determination of PNU 159 548 and its metabolite PNU 169 884 in human plasma was developed and validated. The simple and rapid pre-treatment plus the direct injection of plasma by use of a tailor-made SPE packing and a column-switching technique allowed these two compounds to be well separated from endogenous interfering substances and to be detected at low concentrations with high specificity. The procedure is well suited for measuring therapeutic plasma levels of PNU 159 548 and its metabolite in cancer patients.

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