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High-performance liquid chromatographic analysis of the new antitumour drug *N*-benzoylstaurosporine (CGP 41 251) and four potential metabolites in micro-volumes of plasma ☆

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Abstract

A high-performance liquid chromatographic (HPLC) assay is described for the determination of the new antitumour drug *N*-benzoylstaurosporine (CGP 41 251; **I**) and four of its potential metabolites in micro-volumes (100 μl) of plasma. After addition of an internal standard, the compounds were isolated from plasma by liquid–liquid extraction with diisopropyl ether. Chromatography was carried out using a 5 μm LiChrospher C-18 end-capped column (125 × 4.0 mm i.d.) and binary gradient elution with acetonitrile and a triethylamine-containing phosphate buffer (pH 3.6) as solvents. Fluorimetric detection was performed with excitation and emission wavelengths set at 286 and 386 nm, respectively. The absolute recovery was more than 98% for all of the investigated compounds. The limit of detection (LOD) for **I** and three metabolites was 0.1 ng ml^{-1} and the lower limit of quantitation (LLQ) was 0.2 ng ml^{-1} in 100 μl of plasma. The LOD and LLQ for the fourth metabolite was 0.25 and 0.5 ng ml^{-1}, respectively. The between-day and within-day precisions were always < 15% for all the analytes. A limited pharmacokinetic study in mice treated and with **I** demonstrated that the method is appropriate for this purpose.

Keywords: CGP 41 251; *N*-benzoylstaurosporine; High-performance liquid chromatography; Bioanalysis; Metabolites

1. Introduction

The staurosporine derivative *N*-benzoylstaurosporine (CGP 41 251; **I**) (Fig. 1) has demonstrated profound antitumour activity in different, experimental model systems, both in vitro and in vivo [1–3]. The drug is an inhibitor of the protein kinase C (PKC) enzyme family and has recently been reported also to reverse P-glycoprotein (Pgp)-mediated multi-drug resistance (MDR) in vitro [4–6]. In comparison with the parent compound staurosporine, **I** displays a more selective

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inhibition of PKC [2]. In mice, **I** is better tolerated than staurosporine and shows an improved efficacy in tumour growth inhibition [2]. To elaborate further its favourable antitumour activity and its potential as a Pgp-MDR reversal agent, a study of the pharmacokinetics of **I** in animals (rats and mice) is planned. For this purpose, a sensitive assay using only micro-volumes of samples is needed. To support clinical phase 1 studies with **I** an HPLC method for the biodetermination of **I** in human plasma was developed [7]. However, this assay uses large sample volumes (0.5 ml) and is not suitable for the sensitive detection of low levels of **I** in micro-volumes of samples. Furthermore, this assay is not suitable for the determination of CGP 50 673, which is one of the expected metabolic products.

A novel assay, based on gradient elution, is presented for the determination of **I** and four potential metabolites, CGP 50 723 (**II**), CGP 50 750 (**III**), CGP 50 673 (**IV**) and CGP 52 421 (**V**) (Fig. 1) in micro-volumes (100 µl) of plasma. CGP 41 126 was used as the internal standard (IS). The method is now being used successfully for pharmacokinetic research on **I** given to mice.

2. Experimental

2.1. Chemicals

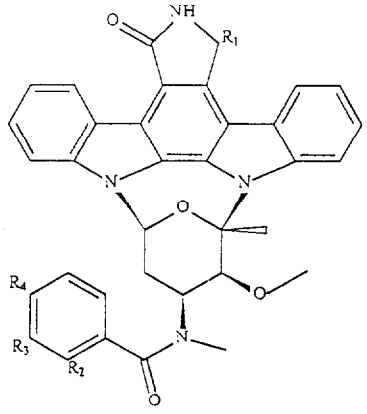
Compounds **I–V** and the IS originated from Ciba Geigy (Basle, Switzerland) and were used as supplied. HPLC-grade acetonitrile (ChromAr) was purchased from Mallinckrodt (Paris, KY, USA). Triethylamine (TEA), phosphoric acid (85%, w/w), dimethyl sulfoxide (DMSO), ethanol (all of analytical grade) and diisopropyl ether (LiChrosolv) originated from Merck (Darmstadt, Germany). Laboratory-prepared distilled water was used throughout. Drug-free heparinized human plasma was obtained from the Central Laboratory of the Netherlands Red Cross (Amsterdam, The Netherlands).

2.2. Chromatography

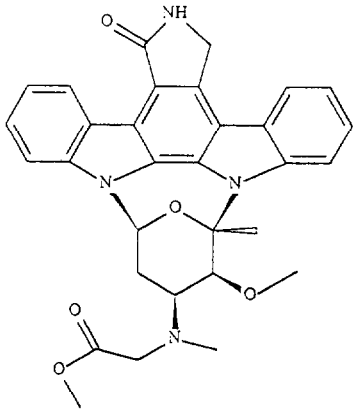
The chromatographic equipment consisted of an SP 8800 ternary gradient liquid pump, a Model SP 8880 autosampler (Spectra-Physics, Santa Clara, CA, USA), and an FP-920 fluorescence detector (Jasco, Tokyo, Japan) equipped with a 5 µl flow cell and with excitation and emission wavelengths set at 286 and 386 nm, respectively. Data collection was carried out on a Chromjet Integrator (Spectra-Physics) connected to an IBM 386 compatible computer running WINner autolab software (Spectra-Physics) for acquisition. Chromatographic separations were performed on a reversed-phase 5-µm LiChrospher C-18 end-capped column (125 × 4.0 mm i.d.) (Merck). The mobile phase was delivered at a flow rate of 0.6 ml min^{-1} using binary gradient elution with acetonitrile (100%; solvent A) and a triethylamine-containing phosphate buffer (1000 ml of distilled water +260 µl of phosphoric acid (85%, w/w) +445 µl of triethylamine, adjusted to pH 3.6 with potassium hydroxide (10%, w/w); solvent B). During the first 10 min of the gradient programme the mobile phase consisted of 40% of A and 60% of B, from 10 to 20 min there was a linear gradient to 60% of A and 40% of B, this phase composition was then held constant for 6 min, and was followed by a linear gradient (1 min) to return to the initial conditions of 40% of A and 60% of B. The equilibration time was established at 2 min.

2.3. Standard solutions

Stock solutions of **I–V** and the IS were prepared in DMSO–ethanol (1:9, v/v) at a concentration of 1 mg ml^{-1}. The stock solutions were stable for at least 2 months when stored at −30 °C [7]. Standard solutions were made by diluting the stock solutions (with the exception of the IS solution) with DMSO–ethanol at concentrations of 10^5, 10^4, 10^3 10^2 and 50 ng ml^{-1} and were stable for 1 week when stored in the dark at 4 °C. Standard mixtures containing 2000, 200, 20 and 4 ng ml^{-1} of each component were prepared daily. The stock solution of the IS was diluted with DMSO-ethanol to give a final concentration of 400 ng ml ^{1}. This solution was stable for at least 1 week when stored in the dark at 4 °C.



I - V



I.S.

COMPOUND	R_1	R_2	R_3	R_4
I (CGP 41 251)	H	H	H	H
II (CGP 50 723)	H	H	H	OH
III (CGP 50 750)	H	H	OH	H
IV (CGP 50 673)	H	OH	H	H
V (CGP 52 421)	OH	H	H	H

Fig. 1. Structures of CGP 41 251 (**I**), CGP 50 723 (**II**), CGP 50 750 (**III**), CGP 50 763 (**IV**), CGP 52 421 (**V**) and CGP 41 126 (IS).

2.4. Calibration and quality control samples

To cover the investigational calibration range (0.2–1000 ng ml^{-1}), 17 calibration samples were prepared in duplicate with each run by adding 0, 5, 10, 25 and 50 µl of each mixed standard solution to 100 µl of drug-free plasma. Next, to each sample 900 µl of distilled water were added and the total volume of each sample was supplemented to 1050 µl with DMSO–ethanol. Quality control (QC) samples were prepared by spiking plasma to a final concentration of 0.5, 5, 50 and 500 ng ml^{-1} of each component (**I–V**) and stored at −30 °C until analysis.

2.5. Unknown species

To a 100 µl sample, 900 µl of distilled water and 50 µl of DMSO were added.

2.6. Extraction procedure

To each sample (rat, calibration or QC), 10 µl of the IS solution and 5.00 ml of diisopropyl ether were added. The samples were vortex mixed for 1 min and centrifuged at 2500*g* for 10 min at room temperature. Next, the aqueous phase was frozen in a dry-ice–ethanol mixture and the supernatant was transferred into a clean tube. The

solvent was evaporated under a gentle stream of nitrogen at 40 °C. The residue was dissolved in 100 μl of DMSO and an aliquot of 20 μl of this solution was injected into the HPLC system.

2.7. Validation

A validation run in plasma included calibration lines constructed in plasma and calibration lines in DMSO (untreated standards) in duplicate and analysis of quality control samples for **I** and the metabolites replicated fivefold. This validation procedure was performed on three separate days. Calibration lines were calculated by unweighted and weighted ($1/X$, $1/X^2$) linear regression analysis. The precision and accuracy of the assay were assessed by calculating the between-day and within-day precision and percentage deviation (%Dev) from the nominal concentration, respectively. An estimate of the precision was obtained using one-way analysis of variance (ANOVA), by calculating values of variance for the the day mean square (DayMS), the error mean square (ErrMS) and the grand mean (GM). The between-day precision was calculated as

Compound of between-day precision

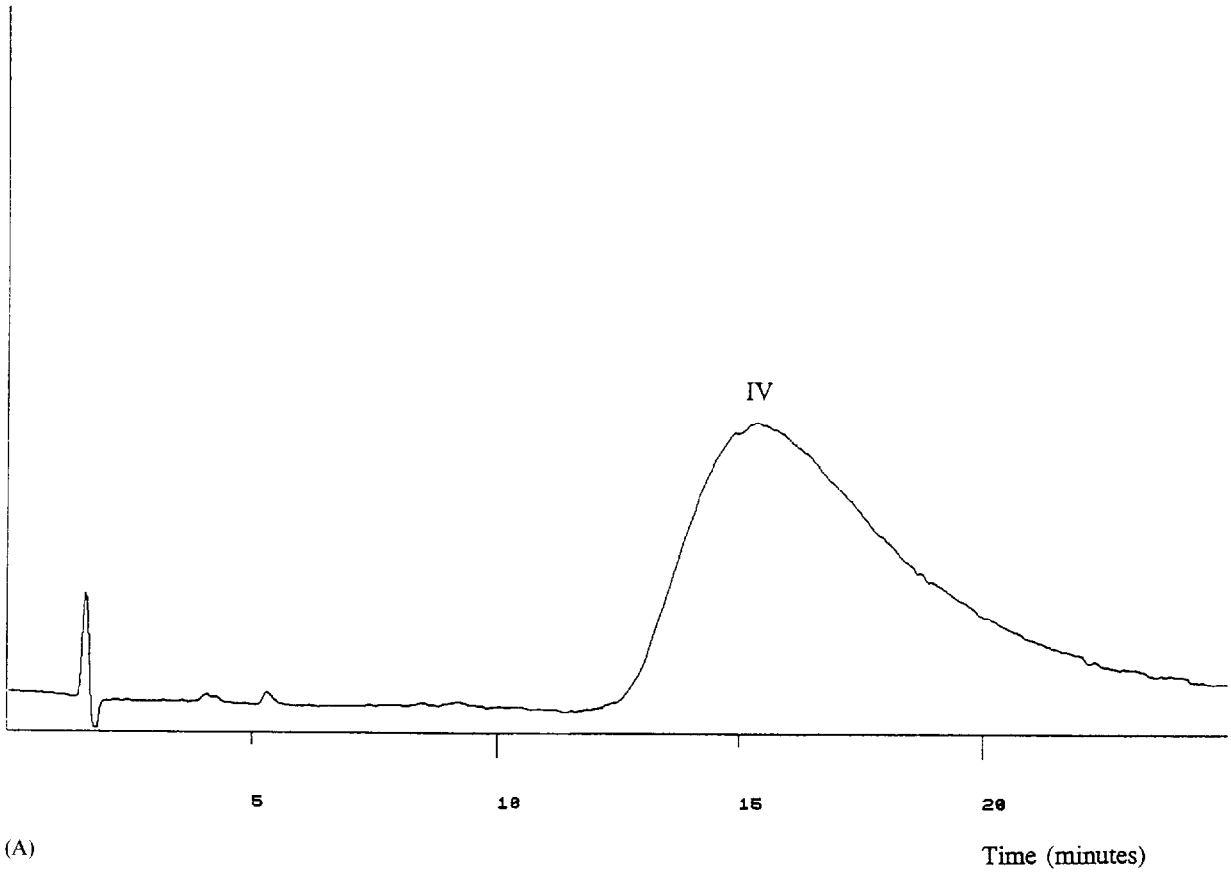
$$= \{[(\text{DayMS} - \text{ErrMS})/n]^{0.5}/\text{GM}\} \times 100 \quad (1)$$

where n represents the number of replicates within each run. The within-day precision was calculated as

Compound of within-day precision

$$= [(\text{ErrMS})^{0.5}/\text{GM}] \times 100 \quad (2)$$

Calculations were performed using a commercial software package (NCSS, Kaysville, UT,



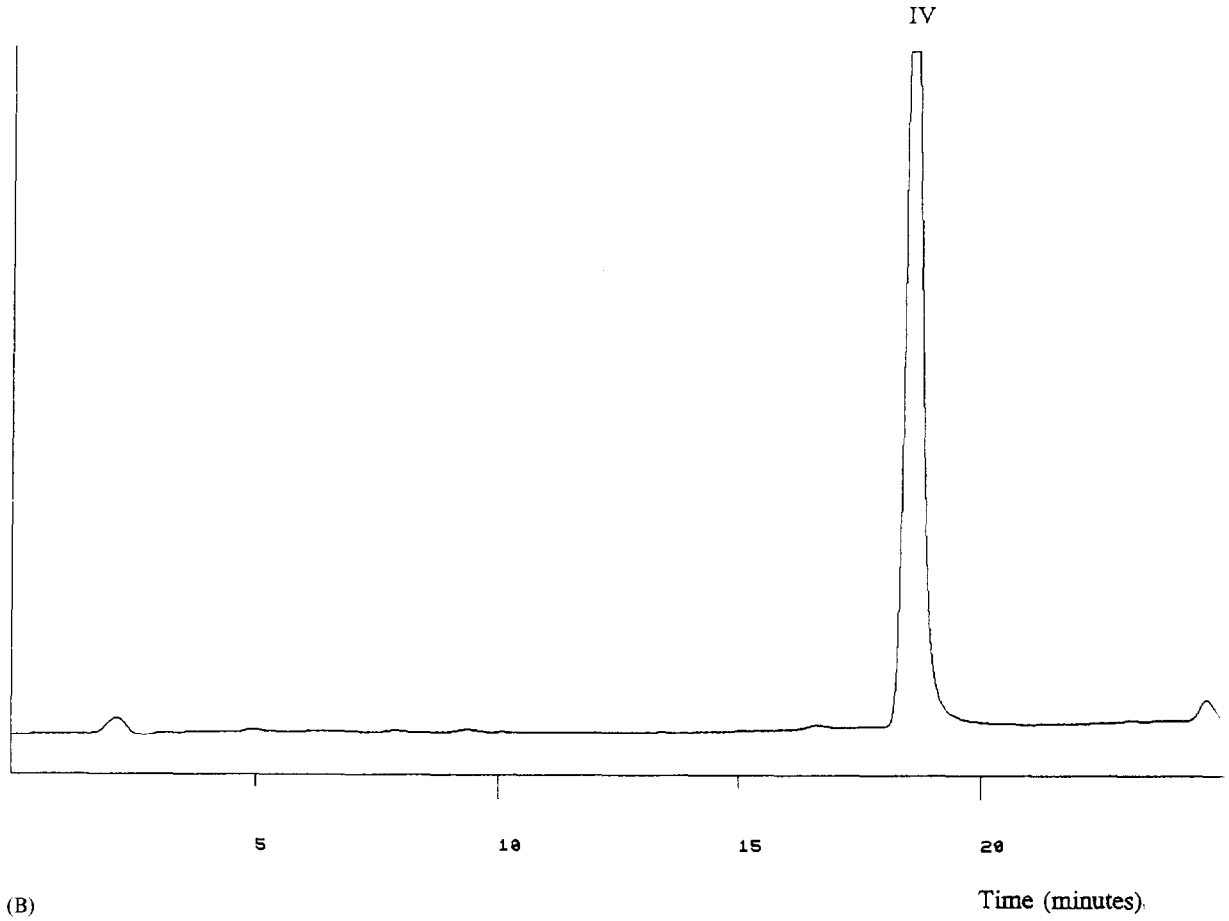
(A)

Fig. 2(A).

USA). The absolute recovery of the extraction procedure was calculated from the ratio of the slopes of the calibration line constructed in plasma and a calibration line constructed in DMSO (untreated standards). Ratios of the peak areas of **I**, **II**, **III**, **IV** or **V** and the IS were used for quantitative computations. The limit of detection (LOD), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, was estimated at a signal-to-noise ratio of three. The concentration of the lowest standard in the analytical run quantitated with a definite level of certainty, i.e. the lower limit of quantitation (LLQ), was accepted when the values for %Dev and within-day and between-day precisions were less than 20% [8–10].

2.8. Preliminary pharmacokinetic study

Male FVB mice (weight approximately 30 g) received 50 μg of **I** dissolved in 50 μl of DMSO by intravenous bolus injection into the tail vein. At specific time points the animals were killed and blood samples of 300–600 μl were obtained by heart puncture and collected in Eppendorf cups containing 50 I.U. of heparin. Plasma fractions were obtained by centrifugation (3 min at 2500*g*).



(B)

Fig. 2. (A) Chromatogram of **IV** obtained with the HPLC system described in Ref. [7]. Column, μBondapk C18; mobile phase, acetonitrile–ammonium acetate buffer (pH 4.0; 0.001 M) (45:55 v/v). (B) Chromatogram of **IV** obtained with the present HPLC system.

Table 1
Equations for the calibration lines (range 0.2–1000 ng ml^{-1}) for determination of **I–V** in human plasma (processed) and DMSO (non-processed)

Compound	Sample type	Equation[a,b]	r^2	n[c]
I	Processed	$y = 0.0299(\pm 0.0011)x + 0.0001(\pm 0.0006)$	0.9953(±0.0001)	3
	Non-processed	$y = 0.0294(\pm 0.0002)x + 0.0006(\pm 0.0005)$	0.9946(±0.0017)	3
II	Processed	$y = 0.0295(\pm 0.0021)x - 0.0017(\pm 0.0009)$	0.9958(±0.0015)	3
	Non-processed	$y = 0.0292(\pm 0.0007)x + 0.0010(\pm 0.003)$	0.9974(±0.0010)	3
III	Porcessed	$y = 0.0237(\pm 0.0017)x - 6.6 \times 10^{-5}(\pm 0.0005)$	0.9976(±0.0001)	3
	Non-processed	$y = 0.0239(\pm 5.6\text{E-}5)x - 0.0006(\pm 0.0010)$	0.9974(±0.0004)	3
IV	Processed	$y = 0.0299(\pm 0.0021)x + 1.5 \times 10^{-5}(\pm 7.7 \times 10^{-5})$	0.9966(±0.0016)	3
	Non-processed	$y = 0.0296(\pm 0.0006)x + 0.0006(\pm 0.0006)$	0.9976(±0.0004)	3
V[d]	Processed	$y = 0.0058(\pm 0.0002)x - 0.0002(\pm 0.0002)$	0.9943(±0.0003)	3
	Non-processed	$y = 0.0062(\pm 0.0002)x - 2.8 \times 10^{-5}(\pm 0.0010)$	0.9964(±0.0022)	3

[a] x is the concentration of the analyte in ng ml^{-1} and y is the ratio of integrator peak area units of **I**, **II**, **III**, **IV** or **V** and the integrator peak area units of the IS. The S.D. is given in parentheses.
[b] Calibration lines were calculated by "weighted" ($1/X^2$) least-squares linear regression analysis.
[c] n = Number of independent runs. For each run the calibration lines were constructed in duplicate and were performed on separate days.
[d] Range 0.5–1000 ng ml^{-1}.

Table 2
Accuracy and precision (RSD) for the biodetermination of **I–V** in human plasma

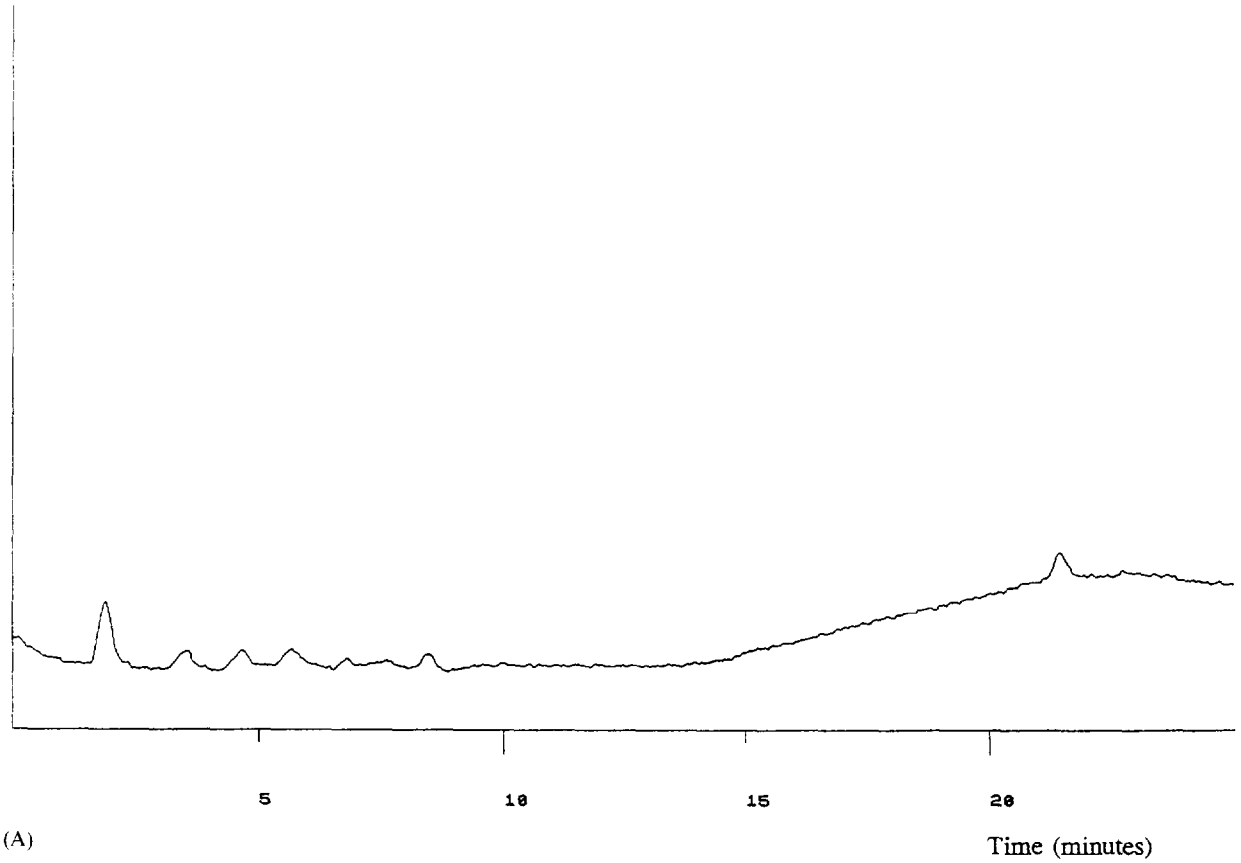
Compound	Nominal concentration (ng ml^{-1})	Intrapolated concentration (ng ml^{-1})	%Dev (%)	Precision[a]		
				Between-day (%)	Within-day (%)	n[b]
I	0.500	0.484	−3.2	–[c]	10.0	5
	5.00	5.43	8.6	1.7	3.1	5
	50.0	53.8	7.6	2.8	7.0	5
	500	490	−1.8	0.7	3.6	5
II	0.500	0.469	−6.2	2.0	7.5	5
	5.00	5.07	−5.6	–[c]	3.6	5
	50.0	49.3	−1.5	[c]	7.4	5
	500	482	−3.7	–[c]	5.0	5
III	0.500	0.455	−9.0	–[c]	10.5	5
	5.00	4.99	−0.3	1.5	2.6	5
	50.0	49.4	−1.2	4.0	5.5	5
	500	492	−1.5	1.7	5.3	5
IV	0.500	0.440	−12.0	3.5	14.9	5
	5.00	5.05	1.0	–[c]	7.9	5
	50.0	52.1	4.3	–[c]	6.6	5
	500	500	0.1	–[c]	5.3	5
V	0.500	0.484	−7.2	4.4	7.2	5
	5.00	5.13	2.7	–[c]	3.5	5
	50.00	49.6	−0.9	–[c]	3.8	5
	500	487.6	−2.5	4.3	6.6	5

[a] Between-day precision was calculated from three independent runs.
[b] n = Number of replicates.
[c] No significant additional variation was observed as a result of performing the assay in different runs.

3. Results and discussion

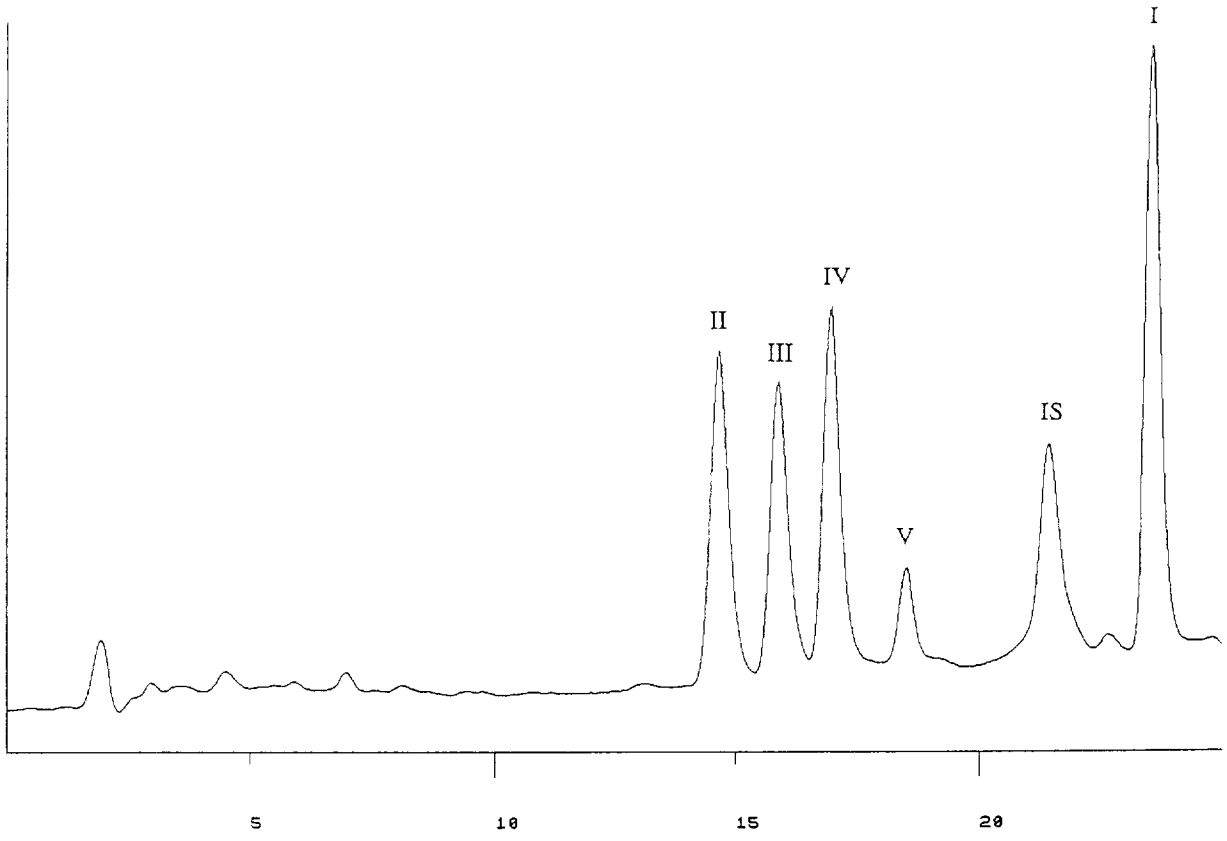
Compound **I** possesses a complex molecular structure (Fig. 1), which is likely to be subjected to extensive metabolism in animals and humans. Several potential metabolic products have been synthesized and are available as reference compounds. An HPLC method based on isocratic reversed-phase chromatography and fluorescence detection was described previously and was capable of separating **I** and three potential metabolites (**II**, **III** and **V**) from endogenous interferences. The method, however, was not suitable for the determination of **IV**, which is also one of the expected metabolic products. Further, the assay requires relatively large volumes (0.5 ml) of plasma, which limits its usefulness for pharmacokinetic investigations in small laboratory animals. The structure of **I** and the four possible metabolites differ only in the position of a hydroxyl function in the molecule (Fig. 1). The position of this group appear to be very important for the chromatographic behaviour of the investigated compounds.

Experiments with the analytes in the earlier reported system using a μBondapak C18 column in combination with a mobile phase consisting of acetonitrile and acetate buffer (pH 4) showed the separation of **I**, **II**, **III** and **V** and the internal standard [7]. Baseline separation between **II** and **III** could not be obtained, however. Also, the chromatography of **IV** in this system was poor and characterized by a broad tailing peak (Fig. 2(A)). By using gradient HPLC, the separation of **II** and **III** could be obtained. Several columns tested (C8, phenyl) did not show an improved



(A)

Fig. 3(A).



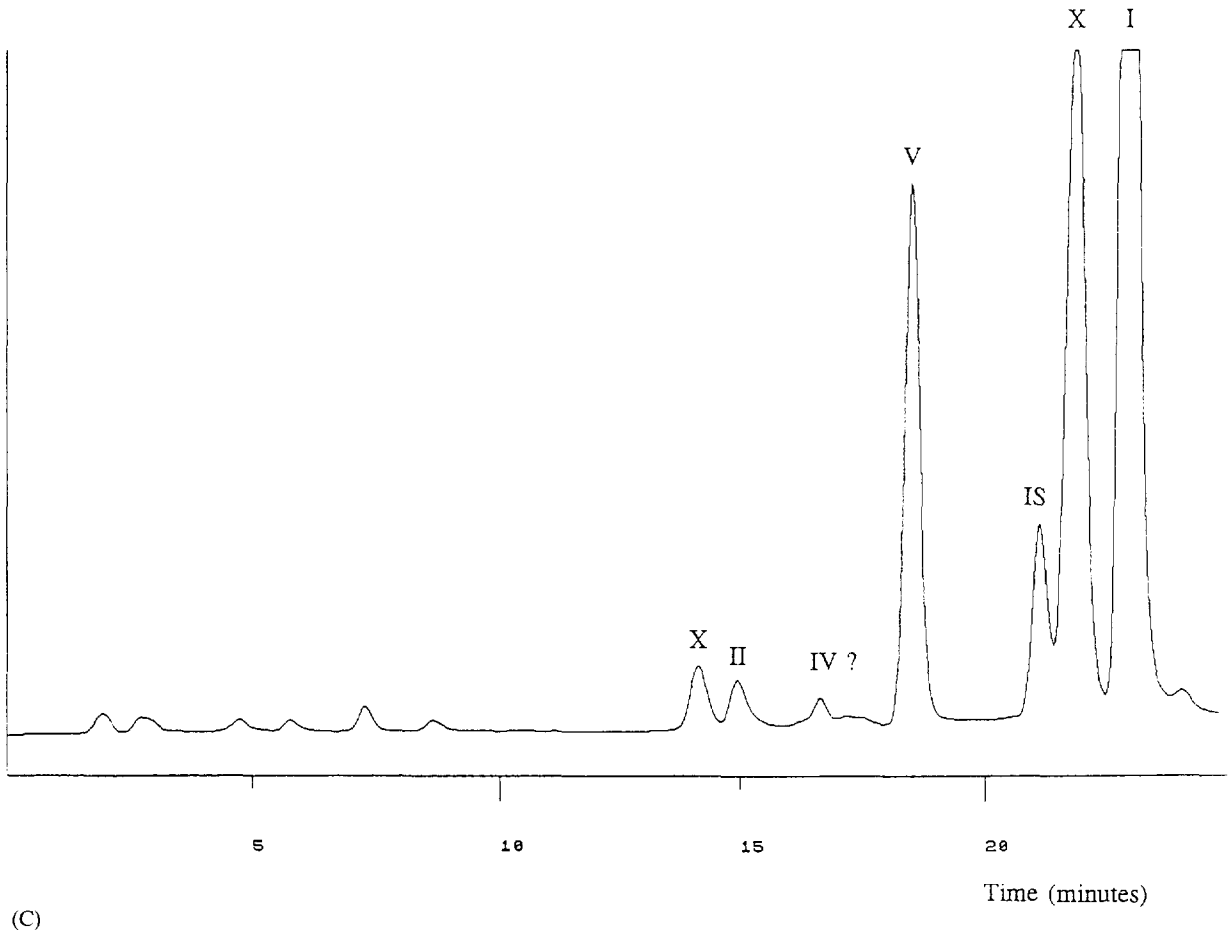
(B)

Fig. 3(B).

chromatography of **IV** and only end-capped C18 columns demonstrated potentially useful results. The addition of triethylamine to the mobile phase finally improved the chromatographic behaviour for all compounds tested, including **IV** (Fig. 2(B)). Increased sensitivity was obtained by replacing the LS-40 detector (Perkin-Elmer, Norwalk, CT, USA) with an FP 920 (Jasco). Changing the volume of the standard flow cell (16 μl) of the FP-920 into a flow cell with a volume of 5 μl resulted in higher resolution between **II**, **III** and **IV**. Further, with this detector the signal-to-noise ratio was also improved 20-fold, allowing the use of only 100 μl of plasma with adequate sensitivity (LLQ 0.2 ng ml^{-1}).

3.1. Validation

The absolute recovery of the liquid–liquid extraction procedure was over 98% (range 98.3–107.2%) for all of the compounds investigated. The chromatograms of blank plasma samples obtained from six different mice were free of any interfering substances. Linear calibration lines were obtained over the concentration ranges tested, i.e. 0.2–1000 ng ml^{-1} for **I–IV** and 0.5–1000 ng ml^{-1} for **V**, with regression correlation coefficients $\geqslant 0.995$ (Table 1). The best fit for the calibration line was obtained by using weighted $(1/X^2)$ linear regression analysis. Deviations of the interpolated concentrations of all standard



(C)

Fig. 3. HPLC of (A) blank mouse plasma, (B) a spiked mouse plasma (concentrations of **I**, **II**, **III**, **IV** and **V** = 50 ng ml^{-1}; concentration of IS = 40 ng ml^{-1}) and (C) a mouse plasma sample collected 40 min after administration of 50 μg of **I** as an intravenous bolus injection (concentrations: **I** = 750 ng ml^{-1}; **II** = 15 ng ml^{-1}; **V** = 405 ng ml^{-1}; IS = 40 ng ml^{-1}). Unknown metabolites are marked X.

samples were always within the acceptable 85–115% range [8]. The intercept values of the calibration lines were not significantly different from zero by Student's t-test ($p > 0.05$). The limit of detection (LOD) for **I**, **II**, **III** and **IV** was 0.1 ng ml^{-1} and the limit of quantitation (LLQ) was 0.2 ng ml^{-1} using 100 μl of plasma. For metabolite **V** the LOD and LOQ were 0.25 and 0.5 ng ml^{-1}, respectively. The between-day and within-day precisions were always < 15% for all the analytes (Table 2).

Several drugs, including acetylsalicylic acid, dexamethasone, acetaminophen, ibuprofen, megesterol acetate, morphine, procainamide and ranitidine, each in its own therapeutic concentration and dissolved in the mobile phase, were injected into the HPLC system. None of these compounds interfered with the compounds to be determined.

3.2. Applicability

The applicability of the method was tested in a pilot study with mice receiving 50 μg of **I** as a bolus injection. Plasma samples were obtained 40 min after drug administration; at this time drug metabolism should have been detectable [7]. Typical chromatograms of a blank, a spiked

plasma sample and plasma obtained from treated mice are depicted in Fig. 3(A), (B) and (C), respectively. In the sample, in addition to **I**, metabolites **II** and **V** were also present. Further, there was an indication of the presence of **IV**, although uncertain at this stage. It is clear that the drug, when given to mice, is metabolized extensively. The resolving power of the proposed assay also appears acceptable from the fact that other metabolites can be found with the method (X, Fig. 3(C)).

4. Conclusions

The proposed HPLC method provides adequate accuracy, reproducibility, selectivity and sensitivity for the quantitative determination of **I** and four potential metabolites in 100 µl of plasma. The small amount of plasma required makes this method suitable for use in pharmacokinetic studies in mice, e.g. in clarifying the role of **I** as a reversal agent of Pgp-mediated MDR and for its antitumour activity.

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