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Sensitive high-performance liquid chromatographic fluorescence assay for the quantitation of topotecan (SKF 104864-A) and its lactone ring-opened product (hydroxy acid) in human plasma and urine

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Abstract

A sensitive reversed-phase high-performance liquid chromatographic fluorescence method is described for the simultaneous determination of topotecan (I) and the hydrolysed lactone ring-opened product hydroxy acid (II) in plasma and for the determination of I in urine. To 250 μl of plasma, a 750-μl volume of cold methanol was added to stabilize the pH-dependent conversion of I into II. In plasma, the lower limit of quantitation (LLQ) for both compounds was 0.10 ng/ml. The between-day variation for I at the LLQ was 7.1% and for II was 5.5%. Prior to injection, urine samples were acidified with orthophosphoric acid and diluted with phosphate-buffered saline (PBS). In urine, the calibration curve for I was linear in the range of 10 to 250 ng/ml and the LLQ was 10 ng/ml. The assay was developed to enable pharmacological analysis of I, in on-going phase I and II studies, in patients with solid tumors.

Keywords: Topotecan

1. Introduction

Compound I [(*S*)-9-dimethylaminomethyl-10-hydroxy-camptothecin, SKF 104864-A] is a semi-synthetic water-soluble analogue of camptothecin, presently being evaluated in clinical phase I and II trials. Compound I is an inhibitor of the nuclear enzyme topoisomerase I. It stabilizes the cleavable complex between DNA and topoisomerase I, resulting in single-strand breaks of the DNA and finally in cell death. Antitumor activity has been demonstrated in pre-clinical models and in phase I and II studies [1–6]. The results of pre-clinical and clinical studies indicate enhanced antineoplastic activity of I when administered daily for prolonged periods of time [2,5–9].

Compound I is not stable at physiological pH in an aqueous solution. It is reversibly hydrolysed from the closed-ring lactone (I) to an open-ring form (II) in aqueous solution (Fig. 1). Compound II is not pharmacologically active [10,11].

An HPLC assay for the analysis of I and II in human plasma has previously been developed by Beijnen et al. [11] with a lower limit of quantitation (LLQ), for both compounds, of 1 ng/ml. The present methodology was developed because blank plasma

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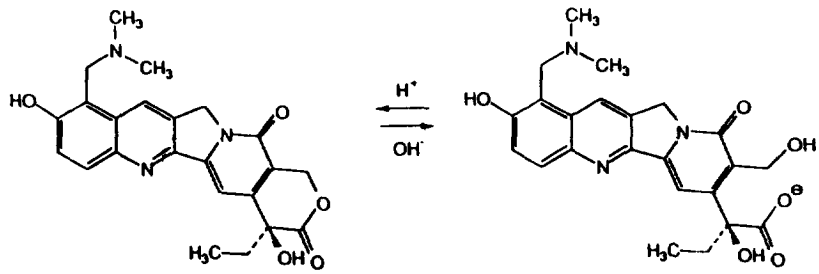

I II

Fig. 1. pH-Dependent interconversion of I and II.

samples revealed an interfering peak at almost the same retention time as II. In addition, the plasma concentrations in our clinical study, where I is administered orally for prolonged periods of time, were anticipated to be much lower than the LLQ of the previously developed methodology. Furthermore, an assay for I in urine was developed to determine the magnitude of the renal clearance of I in studies after i.v. administration.

2. Experimental

2.1. Chemicals and reagents

Compound I was obtained from Smith Kline Beecham Pharmaceuticals (King of Prussia, PA, USA). Methanol (HPLC-grade) was obtained from Rathburn and supplied by Brunschwig (Amsterdam, Netherlands). Triethylamine, potassium dihydrogenphosphate, sodium hydroxide and acetic acid (all analytical grade) were obtained from Baker (Deventer, Netherlands). Orthophosphoric acid (analytical grade) was obtained from Merck (Amsterdam, Netherlands). Phosphate-buffered saline (PBS) was obtained from Oxoid and supplied by Boom (Meppel, Netherlands). PBS consisted of sodium chloride (8.0 g/l), potassium chloride (0.2 g/l), disodium hydrogenphosphate (1.15 g/l) and potassium dihydrogenphosphate (0.2 g/l) and was supplied in tablet form. One tablet was dissolved in 100 ml of purified water. The water was purified with a Milli-Q-UF system (Millipore, Ettenleur, Netherlands).

A stock solution containing 1.0 mg/ml of I was made by dissolving 50.0 mg of I in 50.00 ml of purified water. To 5.00 ml of the stock solution in water, 45.00 ml of a 0.10% acetic acid solution were added. This solution contained 0.10 mg/ml of I. To another 5.00 ml of the stock solution, 45.00 ml of a 0.10 *M* sodium hydroxide solution were added. This solution contained 0.10 mg/ml of II.

2.2. Chromatographic system

The HPLC system consisted of a constaMetric 4100 pump (Thermo Separations), a Rheodyne 7125 injection port and a fluoriMonitor 4100 fluorescence detector (LDC Analytical). The data were analyzed by the Chrom-Card data analysis system (Fisons). These apparatuses were obtained from Interscience (Breda, Netherlands). The separation was achieved on a Shandon Hypersil BDS C_{18} column (100 mm × 3 mm I.D., 3 μm particle size), delivered by LC Service (Emmen, Netherlands). A Model SpH 99 column oven, delivered by Spark Holland (Meppel, Netherlands), was set at 35°C for the plasma assay and at 60°C for the urine assay. The excitation wavelength was set at 381 nm and the emission wavelength was set at 525 nm.

For the assay in plasma, the mobile phase consisted of 10 m*M* potassium dihydrogenphosphate (filtered through a 0.45-μm HA Millipore filter, Millipore) containing 25% methanol and 0.2% triethylamine. The pH was adjusted to pH 6.0 by the addition of orthophosphoric acid. The mobile phase

was degassed using ultrasound and helium. The flow-rate was set at 0.70 ml/min.

For the assay in urine, the mobile phase consisted of 20% methanol instead of 25%. The flow-rate was set at 1.00 ml/min.

2.3. Sample preparation and calibration curves in plasma

Immediately after preparing the standards, 250 μl of plasma were added to 750 μl of cold methanol of −20°C, according to the method of Beijnen et al. [11]. After mixing on a vortex-mixer for 10 s, the samples were centrifuged for 5 min at 4000 *g*, at 4°C, and stored at −80°C. Plasma samples from patients were stored, after mixing, at −80°C and centrifuged on the day of analysis. Prior to analysis, 250 μl of the supernatant were added to 750 μl of PBS and mixed on a vortex-mixer for 10 s. A 200-μl volume was injected into the HPLC.

For the validation of the assay in plasma, a nine-point calibration curve was processed, in duplicate (Table 1), and analyzed on three occasions. For the determination of the LLQ, six plasma samples from six independent individuals were taken and spiked with 0.10 ng/ml of both compounds. Also, four pools of quality control (QC) samples were prepared. Plasma pools were spiked with 0.50, 2.00, 4.00 and 20.00 ng/ml of both compounds. The QC sample containing 20.00 ng/ml was used for subsequent dilutions. On each run, the QC samples were analyzed five times.

The recovery of I and II was determined at concentrations of 2.00 and 4.00 ng/ml in plasma. The peak heights of five analyzed plasma samples were compared with the peak heights of two spiked concentrations, of 2.00 and 4.00 ng/ml in PBS.

Calibration curves were made by linear regression analysis of peak heights versus concentration. For the concentration accepted as the LLQ, the percentage deviation (% DEV) of at least 80% of the samples assayed should be ≤ 20%. The average within- and between-run precision (%C.V.) for each concentration, excluding the LLQ, should be ≤ 15% and should be ≤ 20% for the LLQ. The average accuracy (%) for each concentration, including the LLQ, should be within 85–115%.

2.4. Urine sample preparation and calibration curve

In urine, the total concentration of I was measured after conversion of II into I. To 250 μl of urine, 250 μl of a 100-fold dilution of pure orthophosphoric acid were added. After mixing on a vortex-mixer for 10 s, the mixture was incubated for at least 10 min at room temperature. Prior to injection, 50 μl of this mixture were added to 950 μl of PBS and mixed on a vortex-mixer for 10 s. A 20-μl volume was injected into the HPLC.

For the validation of the assay in urine, a seven-point calibration curve was prepared (Table 2). The calibration curves were made in duplicate and analyzed on three occasions. For the determination of the LLQ, ten urine samples from ten independent individuals were taken and spiked with 10 ng/ml of I. Pools of QC samples were spiked with 25, 100, 200 and 1000 ng/ml of I. The QC sample of 1000 ng/ml was used for all subsequent dilutions. In each run, the QC samples were processed five times.

The recovery of I was determined at concen-

Table 1
Preparation of the calibration curves in plasma

	Final concentration of I and II (ng/ml)								
	5.00	3.00	2.00	1.00	0.50	0.30	0.20	0.15	0.10
Plasma added (μl)	500	700	800	1800	500	700	800	850	900
10.0 ng/ml I and II added (μl)	500	300	200	200					
1.00 ng/ml I and II added (μl)					500	300	200	150	100

Solutions containing 0.10 mg/ml of I and 0.10 mg/ml of II were separately diluted 100-fold with PBS and again 10-fold with plasma. A-200 μl volume of both solutions was added to 1600 μl of plasma. This solution contained 10.0 ng/ml of I and II.

Table 2
Preparation of the calibration curve in urine

	Final concentration of I (ng/ml)						
	250	200	150	100	50	25	10
Urine added (μl)	750	800	850	900	500	500	900
1000 ng/ml of I added (μl)	250	200	150	100			
100 ng/ml of I added (μl)					500		100
50 ng/ml of I						500	

A solution containing 0.10 mg/ml of I was diluted 100-fold in urine. This results in a 1000 ng/ml solution of I.

trations of 100 and 200 ng/ml in urine. The procedure is the same as the procedure described for plasma.

Calibration curves were constructed by linear regression analysis of peak heights versus concentration. The same acceptance criteria were applied as those described for plasma samples.

2.5. Stability of I and II in plasma and urine

The stability of I and II was tested in plasma extracts and in urine at different temperatures.

In plasma, the stability of both compounds was tested by incubating plasma with extracts containing either I alone or II alone, for 24 h at room temperature (22°C), 4°C and at −20°C. The stability at −80°C, the storage temperature for patients' samples, was tested with methanolic plasma mixtures containing both compounds.

In urine, the stability of I was tested by incubation of urine with I for 24 h at 4°C, 22°C and at 37°C and also in urine that was diluted one-fold with orthophosphoric acid (1:100, v/v), at 22°C. Also, the stability of I in urine, at −80°C, was tested.

2.6. Human experiments

In an oral phase I study, the starting dose was 0.15 mg/m^2. On days 1 and 8, blood samples were collected for up to 12 h. One of the first patients was treated with a dose of 0.4 mg. Immediately after sampling, the blood was centrifuged for 5 min at 3500 *g* and the plasma was treated as outlined.

In another study, where I is administered intravenously at a low daily dose of 0.5 mg/m^2, urine samples were collected also.

3. Results

3.1. Assay in plasma

The calibration curves of I and II in plasma were linear in the range of 0.10 to 5.00 ng/ml, with correlation coefficients of at least 0.9986. The retention time of II is 2.5 min and that of I is 6.5 min (Fig. 2). No significant interfering peaks were found in six independent blank plasma samples. The LLQ for both compounds in plasma was 0.10 ng/ml. The mean recovery of I in plasma was 99.3% and of II was 100.6%. The within-run precision of the LLQ samples of I was 4.4% and of II was 9.7%. The accuracies were 93.2% and 106.6%, respectively. The between-run precision of the LLQ was calculated using the lowest concentration of the individual calibration curves used for the validation of the assay. The between-run precisions were 7.1 and 5.5%, respectively. The average values for the accuracy, within-run precision and the between-run precision of the QC samples are given in Table 3.

3.2. Assay in urine

The calibration curves of I in urine were linear in the range of 10 to 250 ng/ml with correlation coefficients of at least 0.9984. Also for the assay in urine no significant interfering peaks for I were found. The LLQ was established at 10 ng/ml (concentrations in the clinical studies were not expected to be lower than 10 ng/ml). The mean recovery of I in urine was 101.9%. The within-run precision of the LLQ samples was 7.2%, the between-run precision was 5.4% and the accuracy of the LLQ was 97.6%. The average values of the accuracy, the within-run precision and the between-

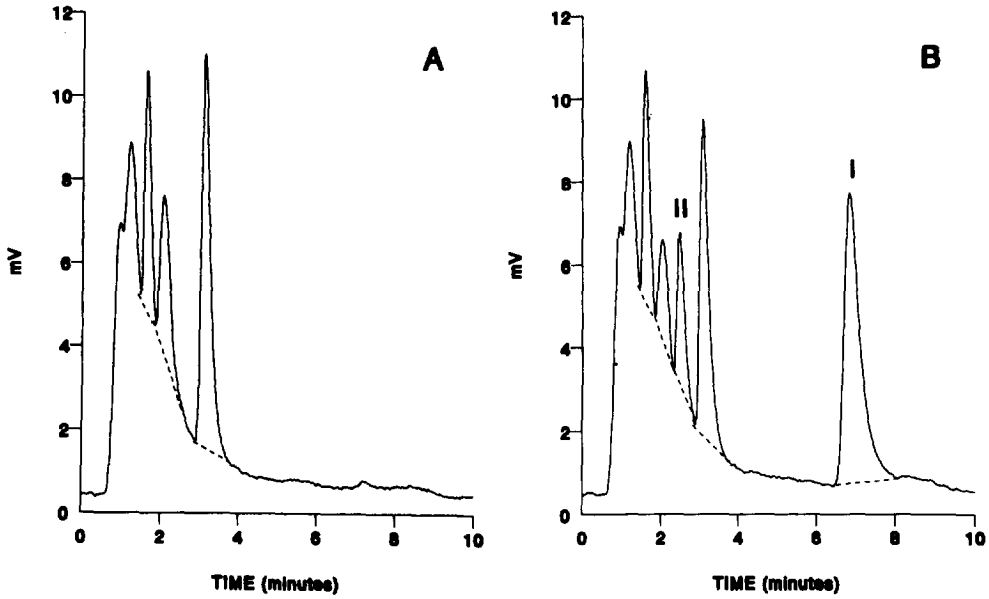

Fig. 2. Chromatograms of a blank blood sample (A) and of a blood sample containing 0.78 ng/ml of I and 0.24 ng/ml of II (B).

run precision of the QC samples are given in Table 4.

3.3. Stability of I and II in plasma and of I in urine

The reversible hydrolysis of I in plasma extracts is dependent on the temperature. Compounds I and II were found to be unstable at 4°C and at 22°C. There was no hydrolysis at −20°C (Fig. 3). Compounds I and II were stable in methanolic plasma mixtures for at least 4 months at −80°C.

The stability of I in urine was also dependent on the temperature. At 37°C, I was found to be unstable, at 22°C it was moderately stable and at 4°C, I was stable for 24 h. Compound I was stable at 22°C after dilution with orthophosphoric acid (Fig. 4). At −80°C, I was stable for more than 3 months.

3.4. Human experiment

The plasma concentration–time curves of I and II from the patient treated with 0.4 mg of I are given in Fig. 5.

The concentration of I in the urine samples of

Table 3
The average accuracy, within-run precision and between-run precision of I and II in the QC samples in plasma

QC sample (ng/ml)	Average accuracy (%)		Precision (%)			
	I	II	Within-run (mean)		Between-run	
			I	II	I	II
0.50	102.7	101.3	2.8	1.5	6.9	6.3
2.00	108.4	106.5	3.8	1.3	8.6	5.2
4.00	102.8	102.6	3.1	1.0	5.0	5.5
20.00	103.3	102.3	3.9	3.7	3.0	3.5

Table 4
The average accuracy, within-run precision and between-run precision of I in the QC samples in urine

QC sample (ng/ml)	Average accuracy (%)	Precision (%)	
		Within-run (mean)	Between-run
25	99.7	4.4	3.3
100	97.5	4.1	0.6
200	97.8	5.3	1.8
1000	98.9	3.2	2.0

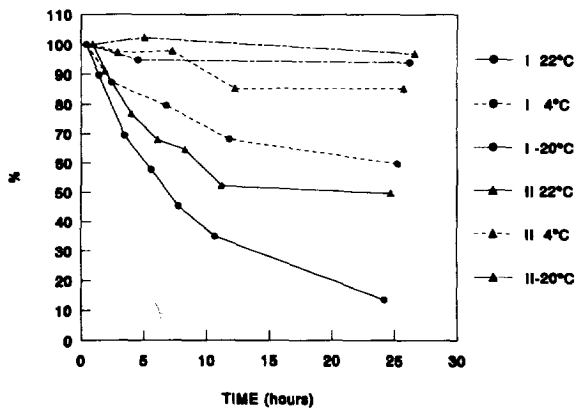

Fig. 3. Stability of I and II in plasma extracts, at different temperatures for 24 h.

patients who were treated using the intravenous protocol were all > 10 ng/ml (data not shown).

4. Discussion

The described methodology for the assay in plasma with an LLQ of 0.10 ng/ml for I and II is appropriate for the measurement of plasma samples in ongoing clinical studies, where low daily doses are administered. For the assay in urine, the LLQ of 10 ng/ml was also satisfactory. Both compounds were unstable in plasma extracts at 4°C and at 22°C. In urine, I was found to be unstable at 37°C and moderately stable at 22°C. Methanolic plasma mixtures and urine stored at −80°C were found to be stable for at least 4 and 3 months, respectively.

In urine, only I was measured after acidification, to ensure total conversion of II into I.

5. Conclusion

A sensitive, selective, accurate and reproducible isocratic reversed-phase HPLC method has been developed for the simultaneous analyses of I and II in plasma and for the analysis of I in urine. Plasma sample pretreatment was carried out immediately after sample collection by deproteinizing the sample with cold methanol, as previously described [11]. Prior to injection, the sample was diluted with PBS. The urine samples were analyzed after acidification

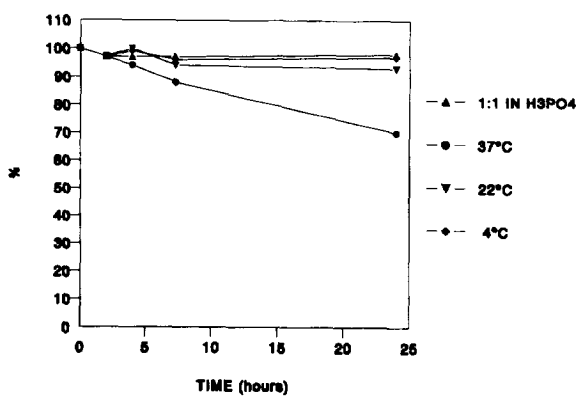

Fig. 4. Stability of I in urine at different temperatures and when diluted one-fold with orthophosphoric acid.

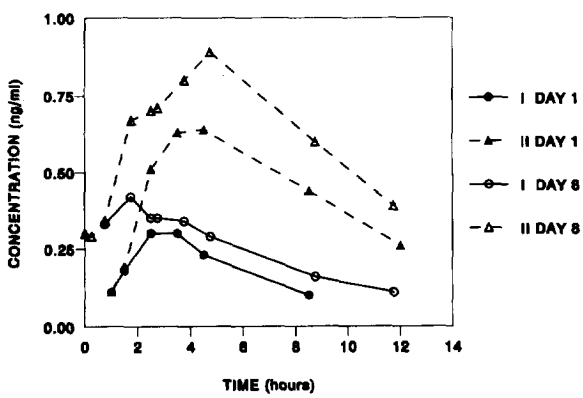

Fig. 5. Plasma concentration–time curve of I and II after oral administration of 0.4 mg on days 1 and 8.

with orthophosphoric acid and dilution with PBS. The methodology described for the measurement of plasma concentrations of both compounds and of urine concentrations of I can be used to determine the pharmacokinetics in clinical studies when I is administered at low doses.

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