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Short communication

Determination of irinotecan (CPT-11) and SN-38 in human whole blood and red blood cells by liquid chromatography with fluorescence detection

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Abstract

An analytical method was developed for the anticancer agent irinotecan (CPT-11) and its main metabolite SN-38 in human whole blood and in red blood cells (RBCs). Sample pretreatment involved deproteinization of whole blood or plasma-diluted RBCs isolated by MESED instruments, with a mixture of aqueous perchloric acid and methanol (1:1, v/v). Separation was carried out using isocratic elution on a Hypersil ODS stationary phase, with detection at excitation and emission wavelengths of 355 and 515 nm, respectively. The lower limit of quantitation (LLQ) in blood was established at 5.00 ng/ml for both compounds, with values for within-run precision (WRP) and between-run precision (BRP) of less than 10%. The method is currently being applied to investigate the blood distribution of CPT-11 and SN-38 in cancer patients.

Keywords: Irinotecan; SN-38; CPT-11

1. Introduction

The topoisomerase I inhibitor irinotecan (CPT-11) has been registered for the treatment of colorectal cancer in both first and second line therapy [1]. CPT-11 is a semisynthetic analogue of the naturally occurring alkaloid camptothecin, and it acts as a prodrug of SN-38, which is 100–1000-fold more cytotoxic than the parent drug. The disposition characteristics of CPT-11 are influenced by several drug-transporting proteins and enzyme systems, which are highly susceptible to environmental influences and genetic factors [2,3]. Moreover, both CPT-11 and its active metabolite are prone to metabolic interconversion between an active lactone form and an inactive carboxylate form on the terminal E-ring [4]. This reversible process is dependent on the pH and the presence of binding proteins in the systemic circulation [5,6]. Previously, it was shown that only the lactone form of camptothecin analogues

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is able to pass cellular membranes, including those of blood cells [7].

So far, pharmacokinetic studies of CPT-11 have involved assays based on high-performance liquid chromatography (HPLC) that only measured levels of CPT-11 and SN-38 in the plasma compartment [8,9]. As CPT-11 and SN-38 have differential binding affinity for several serum proteins [10–12], we hypothesized that the blood distribution and partitioning in red blood cells (RBCs) of CPT-11 and SN-38 might greatly differ. In order to test this hypothesis, we set out to develop and validate an analytical procedure based on high-performance liquid chromatography (HPLC) for the determination of total levels of CPT-11 and SN-38 in human whole blood and RBCs.

2. Experimental

2.1. Stock solutions and standards

CPT-11 (batch KO16; purity 99.6%) and SN-38 (batch LIE 783; purity 100%) were obtained from Aventis Pharma (Vitry-sur-Seine Cedex, France). Dimethyl sulfoxide (DMSO) was purchased from Rathburn (Walkerburn, UK) and methanol from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate, hydrochloric acid and perchloric acid were obtained from Baker (Deventer, The Netherlands), and sodium chloride from Merck (Darmstadt, Germany). Tetrabutylammonium sulfate was obtained from Serva (Heidelberg, Germany). All chemicals were of the highest purity available. Water was purified and deionized by the Milli-Q-UF system (Millipore, Milford, MA, USA) and was used throughout. Blank human blood was obtained from healthy volunteers, and unwashed RBCs were isolated from whole blood samples using MESED (MEasuring off a SEDiment) instruments (Fabre, Kelmis, Belgium) [13,14].

2.2. HPLC instrumentation and conditions

The chromatographic system consisted of a constaMetric 3200 pump (LDC Analytical, Rivera Beach, FL, USA), a Waters 717plus autosampler (Milford, MA, USA), a model SpH99 column oven (Spark Holland, Meppel, The Netherlands) and a FluoriMonitor 4100 fluorescence detector (LDC Analytical, Rivera Beach, USA). A stainless steel (100 mm × 4.6 mm i.d.) analytical column packed with 5 μm Hypersil ODS material (Alltech Applied Science, Breda, The Netherlands) was used for chromatographic separation, and was maintained at a temperature of 50 °C.

The mobile phase was composed of methanol–0.1 M ammonium acetate containing 0.01 M tetrabutylammonium sulfate (35:60, v/v), with the pH of the mobile phase adjusted to 5.2 with hydrochloric acid. Prior to use, the acetate buffer was filtered through a 0.22 μm cellulosic acetate filter (Millipore), and after addition of methanol to the buffer, the mobile phase was degassed by ultrasonication. The mobile phase was delivered isocratically at a flow-rate of 1.0 ml/min, and the column effluent was monitored at an excitation wavelength of 355 nm and an emission wavelength of 515 nm, with a bandwidth of 30 nm.

Calibration curves were generated in the concentration range of 5.00–200 ng/ml and constructed using the peak height of CPT-11 and SN-38 versus the nominal concentration (x), by using weighted ($1/x^2$) least-squares linear-regression analysis.

2.3. Sample pretreatment

All samples were stored frozen ($T < -70$ °C) until analysis. Before analysis, the samples were thawed in a water bath and homogenized by vortex-mixing. Prior to extraction of RBC samples, 100 μl volumes of RBCs were diluted with 150 μl of human plasma followed by vortex-mixing on a MS2 minishaker (IKA Works Inc., Wilmington, NL, USA). Aliquots of 150 μl of the plasma-diluted RBCs were further prepared as blood.

For determination of CPT-11 and SN-38 in (whole) blood, 50 μl of 6 M sodium chloride and 500 μl of perchloric acid–water–methanol (1:20:20, v/v/v) were added to 150 μl of blood in a 1.5 ml propylene tube and vortex-mixed for 10 min. After centrifugation for 10 min at 24,000 × g, 75 μl of phosphate-buffered saline (PBS) was added to 200 μl of clear supernatant. A volume of 200 μl was injected into the HPLC system. It was not possible to separately determine the lactone forms, because the complete sample pretreatment procedure takes approximately 20 min, in which time frame the physiologic equilibrium between the

lactone and carboxylate forms of irinotecan and SN-38 will be artificially altered.

2.4. Method validation

Method validation was performed according to the guidelines recorded elsewhere [15]. A validation run for CPT-11 and SN-38 in human whole blood and RBCs included a six point calibration curve in whole blood prepared in duplicate and determination of a lower limit of quantitation (LLQ) sample spiked at 5.00 ng/ml and quality control (QC) samples at four concentrations spiked at 20.0, 75.0, 150 and 750 ng/ml of both CPT-11 and SN-38, which were analyzed in quintuplicate on four runs on four consecutive days. The QC sample containing the highest concentration was used to investigate the effect of dilution.

QC samples for validation of CPT-11 and SN-38 in unwashed RBCs were prepared by incubation of approximately 20 ml of human blood with simultaneously CPT-11 and SN-38, each at a concentration of 200 ng/ml, for 30 min at 37 °C. Next, 20 fractions of RBCs were isolated using the MESED instruments [14]. The RBC fractions were stored frozen ($T < -70$ °C) until validation as described above. The plasma supernatant in the MESED instruments was collected and stored frozen upon analysis as described in detail elsewhere [15]. The remaining blood was used for determination of the CPT-11 and SN-38 concentration in whole blood and for hematocrit measurement.

2.5. Pharmacokinetics

Blood samples were obtained from a 68-year-old female (157 cm, 48.8 kg) with a cholangioma, who was treated with single-agent CPT-11 given as a 90 min intravenous infusion. The drug was given at a dose of 600 mg in a once every 3-week schedule. The blood sampling for the purpose of pharmacokinetic analysis was approved by the Erasmus MC Ethics Board, and the patient provided written informed consent. Blood samples of approximately 7 ml were collected during the first course at the following time points: immediately prior to infusion; at 30 min after the start of the infusion; at the end of infusion; and at 10, 20 and 30 min, and 1, 1.5, 2, 3.5, 5, 6.5, 23, 30.5, 47 and 54.5 h after the end of infusion. After collection, blood samples were placed on ice and within 10 min an aliquot of 600 μl whole blood was transferred to a 2.0 ml propylene tube. Plasma and RBC fractions were isolated as described elsewhere [14,15]. All fractions were stored frozen ($T < -70$ °C) until analysis. Concentrations of CPT-11 and SN-38 in whole blood and RBCs were determined as described above and concentrations in plasma were determined as described in detail elsewhere [15].

Concentration–time data of CPT-11 and SN-38 were analyzed by standard noncompartmental methods using the software package WinNonlin version 4.0 (Pharsight, Mountain View, CA, USA). The area under the concentration–time curve over the sampling interval in the various matrices was calculated by trapezoidal summation, and was extrapolated to infinity (AUC). Inversed weighting of the measured concentrations was used for all analyses. The clearance of CPT-11 was calculated as dose divided by AUC.

The whole blood (C_b) to plasma (C_p) concentration ratio ($K_{b/p}$) of CPT-11 and SN-38 was defined as $K_{b/p} = C_b/C_p$. The whole blood to RBC (C_r) concentration ratio was defined likewise ($K_{b/r} = C_b/C_r$).

3. Results and discussion

3.1. Chromatography and validation

Blank human blood samples from six different individuals showed no interfering peaks for both compounds (Fig. 1A). Typical chromatograms of whole blood and RBCs spiked with known amounts of CPT-11 and SN-38 are shown in Fig. 1B and C. The retention times of CPT-11 and SN-38 were 8.3 and 15.7 min, respectively, with a total run time of 20 min. The assay was found to be linear over a concentration range of 5.00–200 ng/ml for both CPT-11 and SN-38, with a mean regression correlation coefficient of 0.993 and 0.996 in four validation runs, respectively. At the spiked concentration of 5.00 ng/ml, 95% (CPT-11) and 85% (SN-38) of the interpolated concentrations of the assayed samples in human whole blood were found to be in the acceptable range of accuracy from 80 to 120% with between-run precision (BRP) and within-run precision (WRP) values less than 10% for both compounds and an average accuracy (ACC) of 103.4 and 105.0% for CPT-11 and SN-38,

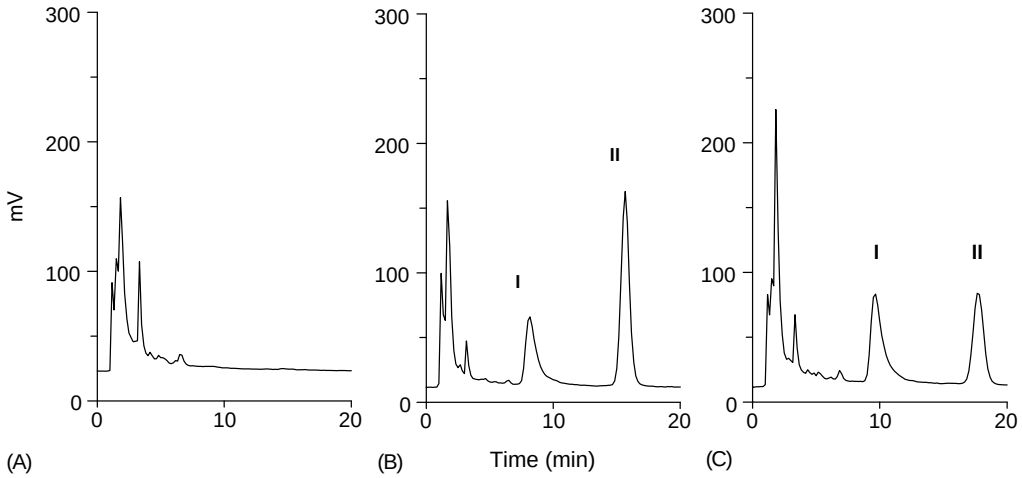

Fig. 1. HPLC chromatograms of (A) a blank human heparinized whole blood sample; (B) a whole blood sample spiked with CPT-11 (I) and SN-38 (II) at a concentration of 150 ng/ml; and (C) a RBC QC sample separated by a MESED instrument from human blood, which was incubated simultaneously with 200 ng/ml CPT-11 (I) and SN-38 (II).

respectively. Therefore this concentration was estimated to be the LLQ.

The WRPs and BRPs of the tested whole blood QC samples were <10% for both compounds, with an acceptable ACC range of 85.2–105.7%. At each spiked concentration, at least 80% of the QC samples was found to be in the acceptable range of accuracy from 80 to 120%. The mean overall extraction efficiencies were 66.3 ± 3.4% and 77.1 ± 6.9% for CPT-11 and SN-38, respectively, and were independent of the concentration. The results of the validation of the analytical method in terms of precision and accuracy in whole blood are listed in Table 1. In Table 2, the validation characteristics of the RBC QC samples are shown. Since we did not have a nominal value for the RBC QC samples, we established the acceptable range of accuracy from 80 to 120% on the basis of the average observed concentration of all analyzed samples, i.e. 266 ng/ml in case of CPT-11 and 118 ng/ml in case of SN-38. This resulted in a WRP of 3.6% and a BRP of

Table 1
Validation characteristics of LLQ and QC samples in human whole blood

Compound	Nominal concentration (ng/ml)	Observed concentration (ng/ml)	ACC (%)	WRP (%)	BRP (%)
CPT-11	5.00[a]	5.17	103.4	9.7	[b]
	20.0	17.1	85.2	3.3	1.7
	75.0	72.3	96.4	2.4	8.7
	150	147	97.9	3.7	8.9
	750	718	95.8	6.2	9.2
SN-38	5.00[a]	5.24	105.0	5.8	6.6
	20.0	17.6	87.8	5.0	2.2
	75.0	74.9	99.8	5.3	7.5
	150	146	98.4	6.8	6.6
	750	793	105.7	4.0	5.1

ACC, average accuracy; WRP, within-run precision; BRP, between-run precision.

[a] Lower limit of quantitation.

[b] No additional variation was observed as a result of performing the assay in different runs.

Table 2
Validation characteristics of RBC QC samples

Compound	Matrix	Observed concentration (ng/ml)	WRP (%)	BRP (%)
CPT-11	RBCs	266	3.6	11.4
	Blood	196		
	Plasma	124		
SN-38	RBCs	118	4.2	8.3
	Blood	217		
	Plasma	276		

WRP, within-run precision; BRP, between-run precision.

11.4% for CPT-11 and a WRP of 4.2% and a BRP of 8.3% for SN-38, using all analyzed samples.

Calculation of hypothetical whole blood CPT-11 and SN-38 concentrations, based upon the mean measured hematocrit value of 0.422 l/l, the observed RBC concentrations, and the observed plasma concentrations (Table 2), results in hypothetical concentrations of 184 ng/ml for CPT-11 (0.422 l/l × 266 ng/ml + (1.000 − 0.422) l/l × 124 ng/ml) and of 209 ng/ml for SN-38 (0.422 l/l × 118 ng/ml + (1.000 − 0.422) l/l × 276 ng/ml). These values differ only 6.1% (CPT-11) and 3.7% (SN-38) from the averaged observed concentrations in whole blood (Table 2), so no accumulation of CPT-11 or SN-38 affecting the kinetics is expected in platelets or white blood cells. Furthermore, the small contamination of RBC with plasma (approximately 3–4% [13]) can be considered negligible for the determination of total CPT-11 and SN-38 concentrations in RBCs.

3.2. Blood distribution in vitro

The mean CPT-11 whole blood (C_b) to plasma (C_p) concentration ratio ($K_{b/p}$) (calculated with the data presented in Table 2) was found to be 1.58, with a CPT-11 whole blood to RBC (C_r) concentration ratio ($K_{b/r}$) of 0.74. For SN-38, the values for $K_{b/p}$ and $K_{b/r}$ were 0.79 and 1.84, respectively. This suggests that CPT-11 tends to accumulate in RBCs, whereas SN-38 shows the tendency to localize in the plasma compartment, rather than crossing the RBC membrane.

3.3. Pharmacokinetic profile

The described method was applied to samples obtained from a patient treated with CPT-11 at a dose of 600 mg. Concentration–time profiles of CPT-11 and SN-38 in whole blood, RBCs and plasma are shown in Fig. 2. By comparing the whole blood and plasma

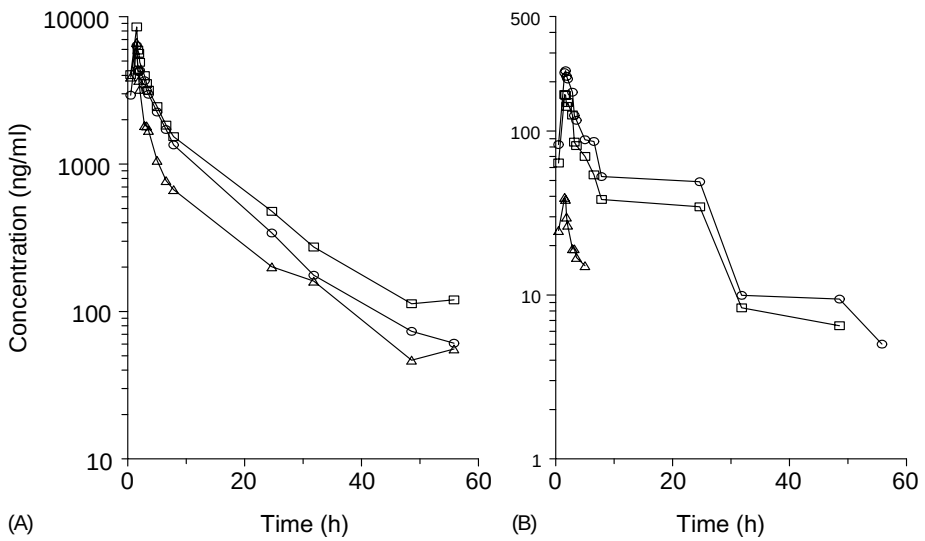

Fig. 2. Kinetic profiles of CPT-11 and SN-38 in a 68-year-old female patient receiving 600 mg CPT-11 as a 90 min infusion: (A) total form of CPT-11; and (B) SN-38 in the plasma compartment (○), in whole blood (□) and unwashed RBCs (△).

concentration–time profiles, we found that the systemic exposure to these compounds differs depending on the matrix used. While the AUC of CPT-11 in whole blood is slightly higher as compared to that observed in plasma (52.1 mg h/l versus 42.2 mg h/l), the opposite was noted for SN-38 AUCs (1.7 mg h/l versus 2.3 mg h/l). The clearance of CPT-11 in whole blood was found to be 11.5 l/h, 14.3 l/h in plasma, and 21.4 l/h in the RBC compartment.

Consistent with the in vitro observation, these data suggest that CPT-11 distributes to RBCs, whereas SN-38 mainly localizes in the plasma compartment. Indeed, the ratio of the AUC in the RBC compartment to the AUC in plasma is about six times higher for CPT-11 compared to SN-38. During infusion, about 38% of CPT-11 was localized in or bound to RBCs and this progressively decreased to 17% at 24 h after the start of infusion. In contrast, SN-38 levels in RBCs were three to nine-fold lower than in plasma, with only 16% of drug associated with RBCs during infusion, with a further decrease to 6% after infusion. In contrast to CPT-11, SN-38 mainly exists in the systemic circulation as the lactone form. It was also shown previously that only lactone forms of camptothecins can pass cellular membranes passively [7,16], and that SN-38 has a higher affinity for binding proteins in plasma as compared to CPT-11. Therefore, the difference in blood distribution may be the result of differences in passive diffusion of the unbound fraction of the lactone forms over the RBC membrane. However, the contribution of drug accumulation in other blood cells, including lymphocytes and platelets, to the differential distribution pattern of CPT-11 and SN-38 cannot be excluded.

Overall, these findings indicate a differential distribution pattern for CPT-11 and SN-38 in human blood, which may, in part, relate to differences in the extent of binding to serum proteins. RBCs were shown to be the principal carrier of CPT-11 in the systemic circulation as well as in vitro, whereas SN-38 is found in the plasma compartment primarily.

4. Conclusion

The presented method for the determination of total CPT-11 and SN-38 concentrations in human heparinized whole blood and in RBCs was shown to be specific, accurate and precise, and was found to be sensitive enough to be implemented in the analysis of samples from patients treated with CPT-11. The presented findings indicate a differential distribution pattern for CPT-11 and SN-38 in human blood. In part, this may relate to differences in the extent of binding to serum proteins. In the systemic circulation as well as in vitro, RBCs were shown to be the principal carrier of CPT-11, while SN-38 was found primarily in the plasma compartment. To further explore the role of RBCs in the pharmacokinetics of CPT-11 and its main metabolite SN-38 this method is currently being implemented in clinical studies.

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