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Determination of the lactone and lactone plus carboxylate forms of 9-aminocamptothecin in human plasma by sensitive high-performance liquid chromatography with fluorescence detection

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Abstract

Two sensitive reversed-phase high-performance liquid chromatographic fluorescence methods, with simple sample handling at the site of the patient, are described for the determination of the lactone and lactone plus carboxylate forms of 9-aminocamptothecin (9AC). For 9AC lactone, the sample preparation was a liquid–liquid extraction with acetonitrile–*n*-butyl chloride (1:4, v/v), whereas the sample preparation for 9AC total (lactone plus carboxylate) was a simple deproteinization with 5% perchloric acid–methanol (1:1, v/v), which results in the conversion of the carboxylate into the lactone form. The lower limits of quantitation were 50 pg/ml and 100 pg/ml for 9AC lactone and 9AC total, respectively. The within-run precisions at four tested concentrations were ≤6.3% for 9AC lactone and ≤5.3% for 9AC total. The between-run precisions were ≤8.9% and ≤5.6%, respectively. The assays were developed to enable pharmacological analysis of 9AC in a bioavailability and oral phase I study in patients with solid tumors. © 1997 Elsevier Science B.V.

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1. Introduction

20(*S*)-Camptothecin (CPT; NSC 94600; Fig. 1) is a cytotoxic plant alkaloid, that was first extracted from the wood and bark of the oriental tree *Camptotheca acuminata*, Decaisne (*Nyssaceae*) [1,2]. In spite of promising antitumor activity in vitro and in animal tumor models, severe and unpredictable toxicity, including diarrhea and dose-limiting myelosuppression precluded further clinical testing (reviewed in Ref. [3]). After the identification of DNA topoisomerase I as the molecular target of CPT [4,5], interest in new structure development was accelerated, with various derivatives of CPT (semi)synthesized to enhance the aqueous solubility. Two compounds of this type, viz. topotecan and irinotecan have shown impressive antitumor activity and were recently registered for clinical use in various countries, for treatment of ovarian and colorectal cancer, respectively [6]. Another derivative that is not soluble in water, 9-amino-20(*S*)-camptothecin (9AC; NSC 603071; Fig. 1) was

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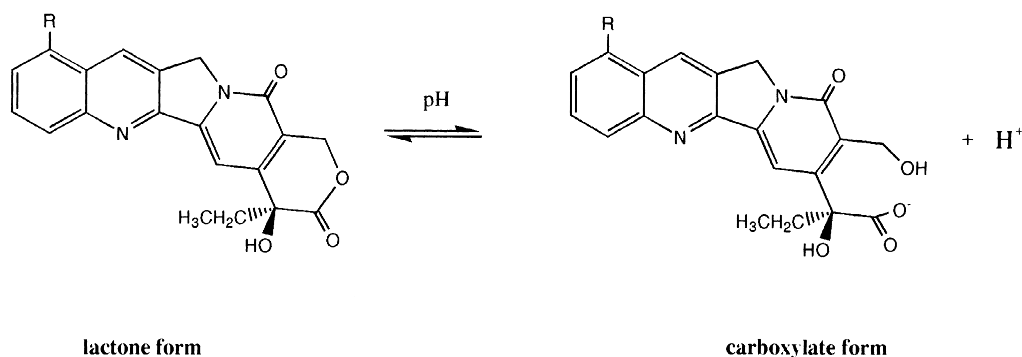

Fig. 1. Chemical structures and pH-dependent interconversion of the lactone and carboxylate forms of 9AC ($R=NH_2$) and CPT (R=H).

recently also introduced into clinical trials because of its unprecedented antitumor activity against solid tumor xenografts [7,8].

Pharmacokinetic studies of the camptothecins, including 9AC, are complicated by a chemical, pH-dependent instability of the lactone ring (Fig. 1) [9], generating an open-ring carboxylate, which is over 1000-fold less active as an inhibitor of DNA topoisomerase I [10]. The importance of this nonenzymatic hydrolysis reaction of the lactone moiety in the pharmacology and toxicology of 9AC is largely unknown. To address this question, development of analytical methodologies enabling the quantitation of both forms are required. Various authors recently described assays for the determination of 9AC lactone and/or total drug levels (lactone plus carboxylate; 9AC total) applicable to plasma samples obtained from patients [11–13]. Major drawbacks of these procedures are the necessity for immediate analysis by high-performance liquid chromatography (HPLC), postcolumn acidification and/or laborious solid-phase extraction techniques.

We now describe novel assays for the analysis of 9AC lactone and 9AC total in human plasma, which only require a one-step solvent extraction for sample clean-up prior to reversed-phase HPLC with fluorescence detection. The presented methods have been thoroughly validated in terms of accuracy and precision, and are now successfully implemented in studies on the pharmacology of 9AC in patients with solid tumors.

2. Experimental

2.1. Chemicals and reagents

9AC (Lot No. 93L07A, containing the base with a purity of 100%) and CPT (Lot No. 93K05A, containing the base with a purity of 100%) were obtained from Pharmacia (New Mexico, USA). Methanol, acetonitrile (both HPLC-grade), dimethyl sulfoxide (DMSO) and *n*-butyl chloride (both analytical grade) were purchased from Rathburn (Walkerburn, UK). Perchloric acid (70–72%, v/v; analytical grade) was obtained from Baker (Deventer, Netherlands). The water was filtered and deionized with a Milli-Q-UF system (Milford, MA, USA) and was used in all aqueous solutions. Drug-free human plasma originated from healthy donors and was delivered by the Central Laboratory of the Blood Transfusion Service (Amsterdam, Netherlands).

2.2. Stock solutions and standards

Separate stock solutions of 9AC and CPT were made by dissolving 10.0 mg of 9AC or CPT in 10.00 ml of DMSO. A volume of 5.00 ml of these solutions was accurately transferred to 50 ml volumetric flasks and diluted to the mark with DMSO, resulting in solutions containing 100 μg/ml of 9AC or CPT, respectively. Working solutions of 9AC lactone were prepared daily at 1, 2, 10, 20, 30 and 40

ng/ml by serial dilution in a mixture of methanol–water–perchloric acid (500:500:1, v/v/v) from the primary stock solution. Similarly, working solutions of 9AC total were made in methanol–water (1:1, v/v) at concentrations of 2, 4, 20, 100, 200 and 300 ng/ml. Spiked plasma samples used as calibrations standards were prepared daily by addition of 50 μl of the working solutions to 950 μl of drug-free human plasma, resulting in calibration standards of 50, 100, 500, 1000, 1500 and 2000 pg/ml for 9AC lactone and of 100, 200, 1000, 5000, 10 000, 15 000 pg/ml for 9AC total.

Four pools of quality control (QC) samples were prepared in human plasma in the concentrations of 250, 900, 1600 and 150 000 pg/ml for 9AC lactone and of 500, 7500, 12 500 and 250 000 pg/ml for 9AC total. The QC samples containing the highest concentration were used as diluted control and/or for low volume injection. To minimize differences in the composition between clinical and QC samples, also the carboxylate form of 9AC was added to the QC samples of 9AC lactone. The 9AC carboxylate was spiked at the same concentrations as 9AC lactone and was prepared by adding 9AC in methanol–water (1:1, v/v) to plasma, followed by incubation at 37°C for 20 h.

2.3. Sample preparation for the lactone form of 9AC

To 1.00 ml of human plasma a volume of 100 μl of 2.5 ng/ml of CPT in methanol–water–perchloric acid (500:500:1, v/v/v) and 0.8 g of NaCl were added in a glass tube supplied with PTFE-covered screw cap. For the extraction, 7.5 ml of acetonitrile–*n*-butyl chloride (1:4, v/v) was added and the sample was vortex-mixed for 5 min, which resulted in the formation of a gel. Subsequently, the sample was centrifuged for 2 min at 4000 *g* at ambient temperature. The sample was shaken once to break the gel and centrifuged for 5 min at 4000 *g*. The supernatant was collected in a glass tube containing 50 μl of DMSO and evaporated at 50°C under a gentle stream of nitrogen, until a residue of approximately 50 μl was left over. To the residue 50 μl of methanol and 150 μl of perchloric acid–water (1:500, v/v) was added. The sample was transferred to a low volume insert of glass, and a volume of 150 μl was injected into the HPLC system.

2.4. Sample preparation for total 9AC

A volume of 250 μl of 5% perchloric acid–methanol (1:1, v/v) was added to 250 μl of human plasma in a 1.5 ml polypropylene-microcentrifuge tube, followed by vortex-mixing for 10 min. Subsequently, the sample was centrifuged for 5 min at 24 000 *g* at ambient temperature. The clear supernatant was transferred to a low volume insert of glass and 200 μl were injected into the HPLC system.

2.5. Chromatographic system

The HPLC system consisted of a constaMetric 3200 pump from LDC Analytical, a subsidiary of Thermo Instruments Systems (Riviera Beach, USA). The samples were injected by a Waters 717plus autosampler (division of Millipore, Milford, MA, USA). A fluoriMonitor 4100 fluorescence detector from LDC Analytical was used for the detection. Chromatographic separations were achieved on an Inertsil ODS-80A column (150×4.6 mm I.D., 5 μm particle size) from GL Science (Tokyo, Japan), protected by a Lichrospher 100 RP-18 endcapped guard column (4×4 mm I.D., 5 μm particle size) obtained from Merck (Darmstadt, Germany).

For the assay of the lactone form of 9AC in plasma, the mobile phase was a mixture of methanol–water (40:60, v/v). The pH was adjusted to 2.20 by addition of perchloric acid. The mobile phase for the assay of 9AC total in plasma was composed of methanol–water (32.5:67.5, v/v), with the pH adjusted to 2.10 using perchloric acid. The mobile phases were degassed by ultrasonication and were delivered at a flow-rate set at 1.00 ml/min for both assays. The column was maintained at 40°C, also for both assays, using a model SpH99 column oven (Spark Holland, Meppel, Netherlands), and the eluents were monitored at an excitation wavelength of 370 nm and an emission wavelength of 450 nm, with a bandwidth of 40 nm.

2.6. Calibration

Acquisition and integration of chromatographic data was performed with the Chrom-Card data analysis system (Fisons, Milan, Italy) running on an ICW workstation. Calibration curves were made by linear regression analysis of peak heights versus 1/concentration using the software package Lotus 2.4 (Lotus Development Corporations, New York, NY, USA).

2.7. Validation

For the validation of the assays of 9AC lactone and 9AC total in plasma, the six-points calibration curves were processed in duplicate and analyzed on four occasions with the lower limit of quantitation (LLQ) and the QC samples in quintuplicate. For the determination of the LLQ, plasma samples of five independent individuals were taken and spiked to contain 50 pg/ml for 9AC lactone and 100 pg/ml for 9AC total. For the concentration accepted as the LLQ, the percent deviation of at least 80% of the samples assayed should be ≤20%. The mean within-run and between-run precision (expressed as the coefficient of variation (%C.V.) for each concentration, excluding the LLQ, should be ≤15% and should be ≤20% for the LLQ. The mean accuracy (%) for each concentration, including the LLQ, should be within 85–115% of the nominal values.

The absolute recoveries were calculated on the basis of the concentrations of the calibration curves. For 9AC lactone the peak heights of processed plasma samples were compared with the peak heights of two samples with a concentration equivalent to 1000 pg/ml 9AC and 250 pg/ml CPT in plasma extracts with 100% recovery. For this purpose, 50 μl of 20 ng/ml of 9AC and 100 μl of 2.5 ng/ml of CPT in methanol–water–perchloric acid (500:500:1, v/v/v) were evaporated in a tube containing 50 μl of DMSO, and were dissolved as plasma extracts. For 9AC total, the peak heights of processed plasma samples were compared with the peak heights of two samples with a concentration equal to 5000 pg/ml of 9AC in plasma extracts with 100% recovery; 25 μl of 100 ng/ml of 9AC in methanol–water (1:1, v/v) was added to 975 μl blank plasma extract.

The stability of 9AC total was tested by incubation of the four QC samples at 37°C for 20 h and during three freeze–thaw cycles. At the freeze–thaw cycles the QC samples were put at room temperature for 1 h before freezing again. The stability of 9AC lactone was only tested during three quick freeze–thaw cycles. The samples were thawed quickly in cold water and restored at −80°C within 5 to 10 min. The long term stability of 9AC total and 9AC lactone at −80°C was also tested at the concentrations of the QC samples over a four-month period.

The following potentially co-administered drugs were tested for interference with the analytical methods: paracetamol at a concentration of 0.50 mg/ml; alizapride, codeine, domperidon, morphine and ranitidine at a concentration of 0.10 mg/ml; and dexamethasone and metoclopramide at concentrations of 0.05 and 0.01 mg/ml, respectively.

2.8. Human experiments

To demonstrate the applicability of the analytical methods, blood samples from a patient were collected at 0, 20 and 40 min, and at 1, 1.5, 2, 3, 5, 7.5, 11, 24, 28, 31, 35, 48, 52 and 55 h after oral administration of 2.5 mg of 9AC. Immediately after sampling, the blood was centrifuged, at the site of the patient, for 5 min at 3000 *g* at 4°C. The plasma was collected and directly placed on dry-ice at −20°C. Within a few hours the samples were stored at −80°C.

3. Results and discussion

Calibration curves of the lactone and total (lactone plus carboxylate forms) of 9AC in human plasma were linear in the range of 50 to 2000 pg/ml and 100 to 15 000 pg/ml, respectively. The regression correlation coefficients were ≥0.992 for 9AC lactone and ≥0.997 for 9AC total, by using weighted (1/concentration) linear least-squares regression analysis to prevent domination of the highest concentrations. Intercept values of the standard curves for both 9AC lactone and 9AC total were not significantly different from zero by Student's *t*-test ($P>0.05$). The retention times of 9AC and CPT in the assay of the lactone form were 7.5 and 22 min (Fig. 2), while the

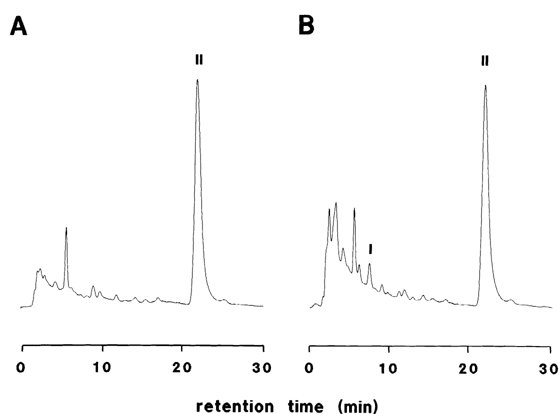

Fig. 2. Chromatograms of a blank human plasma sample containing 250 pg/ml of CPT, used as internal standard (A), and a human plasma sample containing 171 pg/ml of 9AC and 250 pg/ml of CPT (B) in the assay for 9AC lactone. Peaks labeled I and II correspond to 9AC and CPT, respectively.

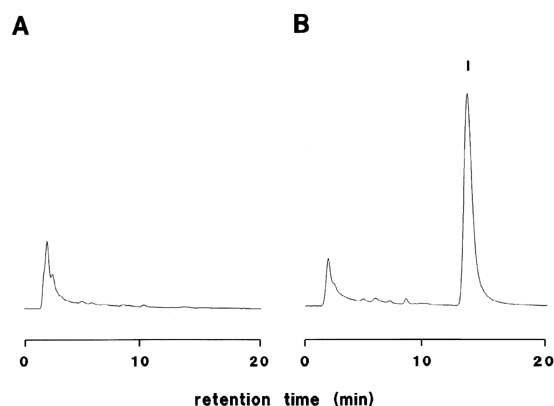

Fig. 3. Chromatograms of a blank human plasma sample (A), and a 10-fold diluted human plasma sample containing 89 264 pg/ml of 9AC (B) in the assay for 9AC total. The peak labeled I corresponds to 9AC.

retention time of 9AC in the assay of 9AC total was 12.5 min (Fig. 3), with overall chromatographic run times of 30 and 20 min, respectively. The selectivity for the analytes is shown by the sharp resolution of the peaks and no significant interfering peaks for both assays in blank plasma samples from five independent donors. All the tested potentially co-administered drugs did not give interfering peaks for 9AC and CPT in the assay for 9AC lactone, and for 9AC in the assay for the 9AC total.

The LLQ for 9AC in the assays for the lactone and total form were 50 and 100 pg/ml, respectively. The lower value of the LLQ for the determination of the lactone was a consequence of concentration of the sample as compared to dilution of the sample in the assay for 9AC total. The extraction recoveries of 9AC and CPT in the assay for 9AC lactone were 78.2±7.98% and 92.4±7.96%, and the recovery for 9AC in the total assay was found to be 89.6±6.87%. The extraction procedure was shown to be highly selective for 9-AC lactone, since less than 0.1% of the 9-AC caboxylate form present in the samples was extracted concomitantly. In both assays, extraction recoveries were independent of the spiked concentration. The within-run precisions of the LLQ samples of 9AC lactone and 9AC total were ≤4.2% and ≤10.2%, respectively. The range of accuracy for 9AC lactone was 95.6% to 112.0%. For 9AC total, the accuracy ranged from 95.2% to 107.6%. The average accuracy for QC samples at the four tested concentrations in both assays showed values ranging within 10% of the nominal values (Tables 1 and 2). The average within-run and between-run precisions for 9AC lactone and 9AC total in human plasma varied upto 8.9% and 5.6%, respectively (Tables 1 and 2).

9AC total was found to be stable for 20 h at 37°C,

Table 1
The mean accuracy, the mean within-run precision and the between-run precision of the QC samples (n=5) in human plasma of 9AC lactone

QC sample (pg/ml)	Accuracy (mean) (%)	Precision (%)	
		Within-run (mean)	Between-run
250	101.6	1.0	3.5
900	91.5	2.4	2.9
1600	90.3	1.7	5.2
150 000	98.9	4.1	8.9

Table 2
The mean accuracy, the mean within-run precision and the between-run precision of the QC samples ($n=5$) in human plasma of 9AC total

QC sample (pg/ml)	Accuracy (mean) (%)	Precision (%)	
		Within-run (mean)	Between-run
500	102.6	2.5	3.6
7500	103.1	1.4	5.6
12 500	103.5	1.5	5.0
250 000	98.4	2.4	2.2

and during three freeze–thaw cycles. However, 9AC lactone was not stable during three quick freeze–thaw cycles; there was a loss of the amount of 9AC lactone of approximately 10% in comparison with the mean of a set of QC samples analyzed at the same time. This instability necessitates quick freezing of the samples after blood sampling in order to stabilize the ratio between 9AC lactone and 9AC carboxylate. 9AC total and lactone forms were stable for at least four months when stored at −80°C. Processed plasma samples were also found to be stable at ambient temperature upon standing in the autosampler tray for at least 20 h.

Plasma collected from a patient prior to the drug administration did not reveal the presence of interfering endogenous peaks. Concentrations of 9AC lactone could be readily estimated from protein-free extracts, whereas acidification of plasma samples, re-converting the carboxylate into the corresponding lactone, enabled determination of 9AC total. The plasma concentration–time curves of 9AC lactone and 9AC total forms of a patient treated orally with 2.7 mg of 9AC, are given in Fig. 4. The data indicate that the LLQs of 50 and 100 pg/ml for 9AC lactone and 9AC total, respectively, are sufficient for monitoring drug-plasma levels in samples obtained from patients treated at low oral doses.

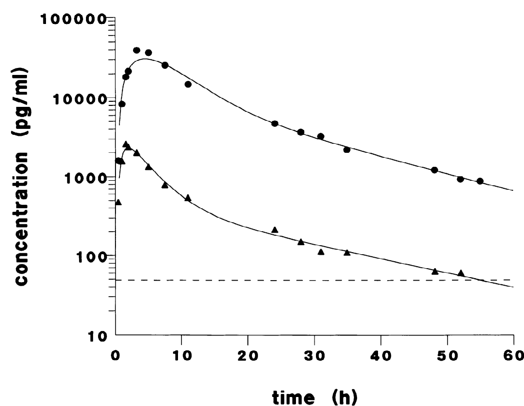

Fig. 4. Plasma concentration–time curves of 9AC lactone (▲) and 9AC total (●) in a patient after oral administration of 2.7 mg of 9AC. The dotted line indicates the lower limit of quantitation. Both curves were fitted to a two-compartment model using the MW\Pharm software package (Medi-Ware Groningen, Netherlands).

In conclusion, two sensitive, selective, accurate and reproducible isocratic reversed-phase HPLC methods have been developed for the analysis of 9AC lactone and 9AC total in human plasma. The sample pretreatment procedures are based on single solvent extractions, thereby eliminating the need of laborious solid-phase extraction techniques [12,13]. Compared to previously described assays for 9AC, our new methods provide equivalent to superior sensitivity with LLQs of 50 pg/ml for 9AC lactone and 100 pg/ml for 9AC total (lactone plus carboxylate). The methodologies described permit the analysis of patient samples, and will be implemented in future investigations on the clinical pharmacology of 9AC administered at low (oral) doses.

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