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Determination of 9-nitrocamptothecin and its metabolite 9-aminocamptothecin in human plasma using high-performance liquid chromatography with ultraviolet and fluorescence detection

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Abstract

A high-performance liquid chromatography assay is described for the determination of the investigational anti-cancer drug 9-nitrocamptothecin (9-NC) and its metabolite 9-aminocamptothecin (9-AC) as the total of their lactone and carboxylate forms. The sample pre-treatment consisted of a deproteinisation step and a quantitative acid-catalyzed conversion of all 9-NC and 9-AC into their lactone forms and a subsequent solid-phase extraction. Redissolved extracts were analyzed on a Prodigy analytical column, using a mixture of methanol–phosphate buffer (pH 2.5). Detection was concomitantly performed with UV for 9-NC and fluorimetrically for 9-AC. The lower limit of quantifications were 10 ng/ml and 2.5 ng/ml for the determination of 9-NC and 9-AC, respectively, using 500 μl of plasma. The presented method was successfully applied to a clinical pharmacokinetic study.

Keywords: 9-Nitrocamptothecin; 9-Aminocamptothecin

1. Introduction

9-Nitrocamptothecin (9-NC, RFS2000, Rubitecan, Fig. 1) is a newly developed orally administered poorly soluble derivative of the natural plant alkaloid camptothecin [1]. Camptothecin and analogues are an important class of anti-cancer drugs that exert their anti-tumor activity by specifically inhibiting the human topoisomerase I enzyme [2]. In vivo it was found that 9-NC is partly metabolized to 9-amino-camptothecin (9-AC, Fig. 1) [3,4]. Both 9-NC and 9-AC have shown significant activity against several tumor-types in vivo and in vitro [5–7]. In phase I/II studies with 9-NC clinical responses were observed in patients with various malignancies [8–10]. An important chemical feature for 9-NC, 9-AC and other camptothecin derivatives, is a chemically unstable E-ring (lactone function) which is essential for anti-

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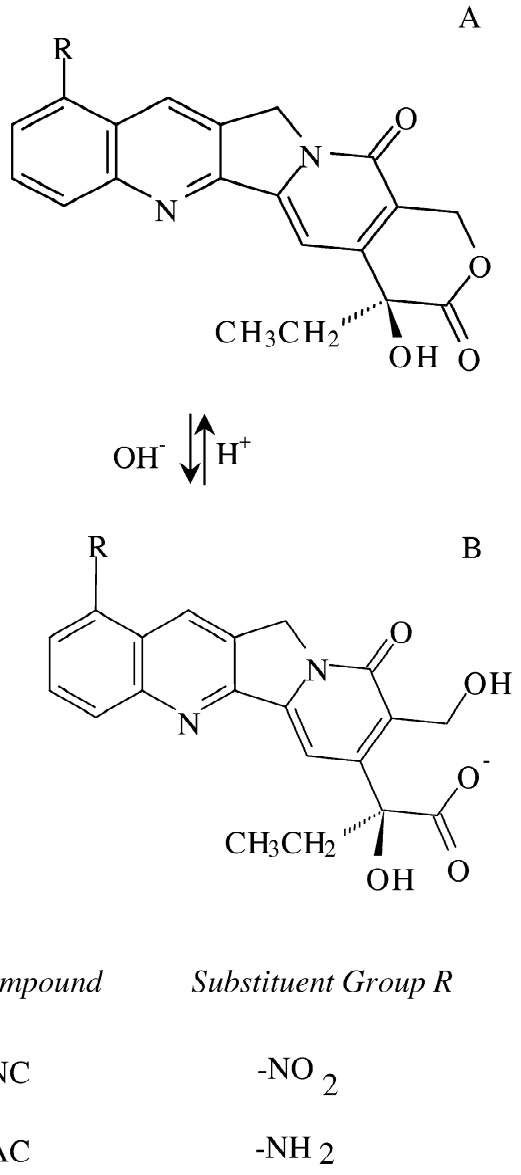

Compound	*Substituent Group R*
9-NC	$-NO_2$
9-AC	$-NH_2$

Fig. 1. The chemical structures of 9-NC and 9-AC in the lactone (A) and carboxylate form (B).

tumor activity. In plasma the pH favors formation of the inactive carboxylate form (Fig. 1) [2]. It was shown for camptothecin derivatives however, that monitoring the sum of the lactone and carboxylate forms is as effective, as a measure for pharmacodynamic outcome, as monitoring the lactone form separately [11,12]. To study the pharmacokinetics of 9-NC, both 9-NC and 9-AC should be considered. In the past, several methods to measure 9-AC levels in human plasma have been described, however thus far no method to measure both 9-AC and 9-NC in human plasma has been reported [13–15]. A selective high-performance liquid chromatography (HPLC) method with concomitant ultraviolet (UV) and fluorescence detection for the determination of the total concentrations (carboxylate and lactone forms) of 9-NC and 9-AC in human plasma is described, which is useful for clinical pharmacokinetic research. The stability of the analytes has been investigated under various relevant conditions.

2. Experimental

2.1. Materials

9-NC (potency 97.9%) and 9-AC (potency 99.13%) were obtained from Supergen (Pleasanton, CA, USA). Methanol (HPLC grade, ChromAR) was purchased from Promochem (Wesel, Germany). Acetic acid (analytical grade), dimethyl sulfoxide (DMSO, analytical grade), potassium dihydrogenphosphate (analytical grade), phosphoric acid (85%, analytical grade), and hydrochloric acid (analytical grade) were obtained from Merck (Darmstadt, Germany). Double distilled water was provided by the Slotervaart Hospital (Amsterdam, The Netherlands). Drug-free heparinized human plasma was obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands).

2.2. Equipment

Bondelut C_{18} phase extraction columns (100 mg, 1 ml; Varian, Palo Alto, CA, USA) were used for sample pre-treatment. The chromatographic system consisted of a solvent delivery system type P1000 and an automatic sample injection device Model AS3000 (Thermo Quest, Fremont, CA, USA). Separation of the compounds was performed on a Prodigy SB-C_8 column (5 μm, 150×4.6 mm) (Phenomenex, Torrance, CA, USA) and the column temperature was controlled with a column heater (Jones Chromatography, Lakewood, CO, USA). To protect the analytical column, a guard column (10×3 mm,

Chrompack, Middelburg, The Netherlands) packed with reversed-phase material was used. Detection was concomitantly performed by UV and fluorimetrically using first a UV1000 UV detector (Thermo Quest) and secondly a FP-920 Intelligent Fluorescence detector (Jasco International, Tokyo, Japan) for the detection of 9-NC and 9-AC, respectively. Retention times and peak areas were analyzed with Chromquest version 2.51 (Thermo Quest).

2.3. Preparation of stock and working solutions

To prepare a stock solutions of 9-NC and 9-AC of 100 μg/ml, 1 mg of 9-NC or 9-AC was accurately weighed in a 10.0-ml volumetric flask. The compound was dissolved in 8 ml DMSO, acidified with 10 μl of acetic acid and DMSO was added to a final volume of 10.0 ml. The stock solutions of 9-NC and 9-AC were diluted with DMSO to give working solutions of 1, 2.5, 5, 10, 25, 50, and 100 μg/ml. All stock and working solutions were stored at 2–8 °C.

2.4. Preparation of calibration standards, quality control (QC) samples and recovery samples

Independently prepared stock and working solutions were used for the preparation of the calibration standards and QC samples. Calibration standards and QC samples of 9-NC and 9-AC were prepared in 10-ml volumetric flasks by spiking 100 μl of the working solutions of both 9-NC and 9-AC to drug-free plasma. Calibration standards were obtained with concentrations between 10 and 500 ng/ml 9-NC and 2.5 and 50 ng/ml 9-AC. QC samples were obtained with concentrations between 10 and 2500 ng/ml 9-NC and 2.5 and 250 ng/ml 9-AC. Aliquots of 500 μl of the calibration standards and QC samples were stored at −70 °C. To calculate the recovery over the validated range, recovery samples of 9-NC and 9-AC were spiked with working solutions to obtain 9-NC and 9-AC concentrations in the eluent corresponding with the concentrations in the final plasma extracts.

2.5. Sample pretreatment

Total levels of 9-NC and 9-AC were determined after the acidic mediated proteinisation of the sample converting all 9-NC and 9-AC to their lactone form. Acidification was performed by adding 80 μl 1 *M* hydrochloric acid to 500 μl of plasma. Then 9-NC and 9-AC were extracted from plasma using solid-phase extraction (SPE) cartridges (Bondelut C_{18}, 1 ml, 100 mg). The columns were conditioned with methanol (3 ml) and distilled water (3 ml). After which they were loaded with the acidified samples. Washing was performed with distilled water (1 ml), methanol–water (25:75, v/v; 1 ml) and drying was performed for 5 min at maximal vacuum (10 mmHg; 1 mmHg=133.322 Pa). Elution took place with 750 μl of methanol and the eluate was evaporated to dryness under a stream of nitrogen at 40 °C. Plasma residues were redissolved in 100 μl of the eluent. Samples were centrifuged for 10 min at 10 000 *g* and 50 μl was injected into the HPLC column.

2.6. Chromatography

Chromatographic analysis was performed at 30 °C. The mobile phase consisted of a mixture of methanol–10 m*M* phosphate buffer, pH 2.5 (48:52, v/v). The flow-rate was 0.8 ml/min. Detection of 9-NC was performed with UV at 365 and the detection of 9-AC was performed fluorimetrically with the excitation wavelength at 380 nm and the emission wavelength at 440 nm. 9-NC eluted at a retention time of approximately 12.5 min and 9-AC after 6.0 min (Figs. 2 and 3).

2.7. Method validation

A three-run validation was completed, producing calibration lines ranging from 10 to 500 ng/ml for the determination of 9-NC and 2.5 to 50 ng/ml for the determination of 9-AC. The following parameters were determined: linearity, within run and between run precision, accuracy, specificity, selectivity and absolute recovery. To establish the best weighting factor, back-calculated calibration concentrations were calculated and the deviations from nominal concentrations were calculated. The model with the lowest total bias across the range was considered to be the best fit. The accuracy and the precision of the methods were determined by analyzing spiked quality control samples with analyte concentrations around the lower limit of quantitation (LLOQ), in

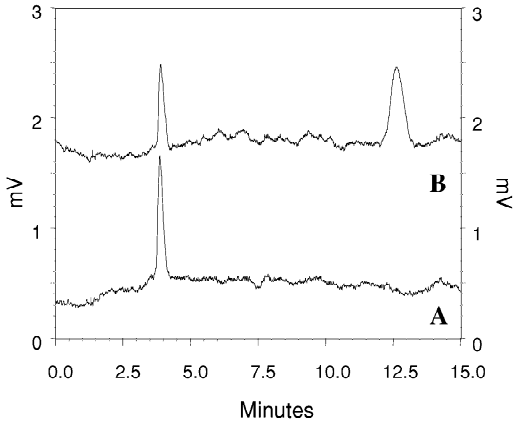

Fig. 2. Fluoresence chromatograms typical for the analysis of concentrations of 9-NC: before (A) and 6 h after (B) administration of 9-NC to a patient treated with 1.5 mg/m^2 orally administered 9-NC. 9-NC (39.55 ng/ml) is eluting after approximately 12.5 min.

the low, medium and high concentration range of the calibration curve and an additional quality control concentration above the upper limit of quantitation (ULOQ). The last-mentioned samples were measured after dilution with blank plasma (10-fold) to demonstrate parallelism. Each quality control sample was analyzed in a minimum of five replicates together with a calibration curve, in at least three analytical runs except for the quality controls above the ULOQ. These samples were assayed in one validation run using five replicates. The accuracy was determined in percent difference between the mean concentration and the nominal concentration. The relative standard deviation (RSD) was used to report the precision. Six batches control human plasma and four pre-dose patient samples were processed and analyzed to determine whether endogenous plasma constituents co-eluted with 9-NC and/or 9-AC. The overall 9-NC and 9-AC recovery was determined by comparing the slope of the processed human calibration curve (area versus concentration) to a standard curve prepared in the eluent.

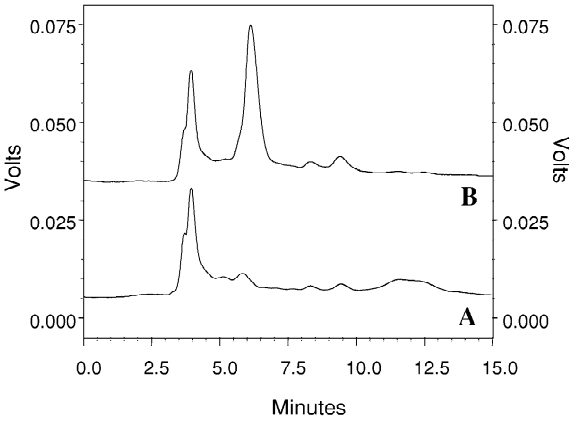

Fig. 3. UV chromatograms typical for the analysis of concentrations of 9-AC: before (A) and after 6 h after (B) administration of 9-NC to a patient treated with 1.5 mg/m^2 orally administered 9-NC. 9-AC (6.55 ng/ml) is eluting after approximately 6 min.

2.8. Stability

The stability of 9-NC and 9-AC in the biomatrix was investigated after three freeze–thaw cycles from −70 °C to ambient temperatures. The stability of 9-NC and 9-AC in the biomatrix during sample pretreatment was investigated at ambient temperatures. The 9-NC samples were assayed at $t=0$, 1, 2, 4, 6, and 24 h. The concentrations of 9-AC were determined at $t=0$ and 24 h. The stability of 9-NC and 9-AC in the final extract was investigated for 55 h at ambient temperatures. All samples were spiked at three concentration levels (in the low, mid and high concentration ranges of the calibration curve) and analyzed in triplicate. To investigate the stability of 9-AC and 9-NC in DMSO at 2–8 °C, stock and working solutions were re-assayed after 3.5 and 6 months. Solutions were diluted in the eluent and the recoveries were determined by comparing the slope of the calibration curve (area vs. concentration) of the stored solutions to a standard curve of freshly prepared solutions.

2.9. Application of the method

The presented method was used to support a phase II study of 9-NC. Patients were randomized during the first course to receive either 1.5 mg/m^2 oral 9-NC after an overnight fasting period or immediately after a high-calorie breakfast and crossed over to the alternative schedule after a 1 week washout period. Pharmacokinetic sampling was performed prior to dosing and 30 min, 1, 2, 3, 4, 6, 8, and 24 h after administration of 9-NC. Samples were immediately centrifuged and the plasma was directly stored

at −70 °C. Treatment was continued thereafter with 1.5 mg/m^2 9-NC given after an overnight fasting period daily for 5 days every week. Written informed consent was obtained from all patients and the study was approved by two independent ethics committees [16].

3. Results and discussion

3.1. Validation

A HPLC method with concomitant UV and fluorescence detection was validated for the determination of 9-NC and 9-AC in human plasma, respectively. Total levels of 9-NC and 9-AC were determined after converting all 9-NC and 9-AC to their lactone form. Sample clean-up was performed by SPE using Bondelut C_{18} material. Dry extracts were dissolved in eluent and injected into the HPLC system. For the determination of 9-NC and 9-AC the linear regression of the ratio of the area, versus the concentration was weighted by $1/x^2$ (the reciprocal of the CPT concentration). Correlation coefficients of 0.982 or better were obtained. The LLOQs were 10 and 2.5 ng/ml for the determination of 9-NC and 9-AC, respectively, using 500 μl of plasma. Concentration ranges were from 10 to 500 ng/ml for 9-NC and 2.5 to 50 ng/ml for 9-AC. For the calibration standards the back-calculated concentrations from the response were calculated (see Table 1). The deviation of the nominal concentration for all 9-NC concentrations were between −4.71 and 9.56% and for all 9-AC concentrations were between −14.04 and 17.53%. The RSD values ranged from 0.33 to 5.70% for 9-NC and from 0.65 to 6.76% for 9-AC. The assay performance data for the determination of 9-NC and 9-AC are shown in Table 2. The accuracy was for all tested concentrations of 9-NC within 20% and both the within- and between-run precisions were less than the required 20% [17]. Although the tested batches of control human plasma contained no co-eluting peaks >20% of the 9-NC and 9-AC areas at the LLOQ, interferences with 9-AC were detected when pre-dose patient samples were analyzed. For the measurement of clinical samples, the measurement of a blank sample is thus essential. The recovery for the determination of 9-NC was 82.7% and for 9-AC a recovery of 60.0% was found.

3.2. Stability

A number of stability experiments were performed and the results are summarized in Table 3. No significant changes in the 9-NC and 9-AC concentrations were measured after three freeze–thaw cycles. The stability of 9-NC was critical when plasma samples were kept at ambient temperatures. From data of the stability experiments it can be concluded that extraction should be performed within 2 h (Fig. 4). Since 9-NC is unstable in the biomatrix

Table 1
Back calculated concentrations of 9-NC and 9-AC

Analyte	Nominal plasma concentration (ng/ml)	Mean measured concentration (ng/ml)	RSD (%)	Deviation (%)
9-NC	10.42	10.10	1.9	−3.1
	26.04	28.53	5.7	9.6
	52.08	51.8	2.9	−1.9
	104.17	101.93	1.4	−2.2
	260.17	248.14	2.0	−4.7
	520.83	532.88	0.3	2.3
9-AC	2.66	2.76	1.5	3.8
	5.32	4.57	2.5	−14.0
	10.65	12.52	0.7	17.5
	26.62	26.79	6.8	0.7
	53.23	50.91	4.1	−4.4

Number of analytical runs was three. RSD, Relative standard deviation.

Table 2
Assay performance data for the determination methods of 9-NC and 9-AC

Analyte	Nominal plasma concentration (ng/ml)	Mean measured concentration (ng/ml)	Accuracy (%)	Within-run precision (%)	Between-run precision (%)	*n*
9-NC	11.44	11.48	0.4	5.4	8.6	15*
	28.61	29.82	4.3	4.3	4.6	15*
	286.11	286.39	0.1	2.3	3.9	15*
	429.11	420.64	−2.0	2.6	4.4	15*
	527.23	521.08	−1.2	1.2	4.5	15*
	2861.13	2633.60	−8.0	0.8	NA	5
9-AC	2.70	2.57	−4.7	7.8	11.5	15*
	5.41	5.85	8.1	4.8	7.3	15*
	27.04	30.69	13.5	3.9	4.1	15*
	40.56	44.02	8.5	5.4	6.3	15*
	54.08	58.09	7.4	4.6	4.5	15*
	270.38	308.89	14.2	1.6	NA	5

n, Number of replicates. NA, Not applicable. *Number of individual analytical runs was three.

at ambient temperatures, it is recommended to store the clinical samples at −70 °C until analysis. The analytes were stable in the final extract in the autosampler at ambient temperatures for 55 h. However, at the low concentrations of 9-AC a decrease of the concentration of 27% was observed. This is probably due to an interference in the concentration determination at time zero (measured concentration of 6.62 ng/ml, while the nominal concentration was 5.41 ng/ml). Stock and working solutions in DMSO of 9-NC and 9-AC were found to be stable up to 6 months when stored at 2–8 °C with a recovery above 90%.

3.3. Application of the method

The presented method was successfully applied to a phase II and pharmacokinetic study. In Fig. 5 the

Table 3
Stability data of 9-NC and 9-AC

Stability test	Conditions	Initial concentration (ng/ml)		Recovery (%)		RSD (%)	
		9-NC	9-AC	9-NC	9-AC	9-NC	9-AC
Stability after three freeze–thaw cycles	Storage at −70 °C, 12–24 h; thawed at ambient temperatures	25.00	5.00	104.7	104.5	6.0	1.8
		250.00	25.00	111.8	96.4	0.8	2.4
		375.00	37.50	110.4	97.1	0.5	8.5
Stability in human plasma	2 h at ambient temperatures	25.00	–	92.4	–	3.8	–
		250.00	–	87.1	–	0.9	–
		375.00	–	88.1	–	0.3	–
	24 h at ambient temperatures	25.00	5.00	64.5	100.2	2.1	4.5
		250.00	25.00	61.4	100.0	0.2	2.7
		375.00	37.50	63.5	100.7	1.5	8.9
Stability of the final extract	55 h at ambient temperatures	25.00	5.00	95.2	73.1	5.9	1.4
		250.00	25.00	99.2	92.6	0.8	3.2
		375.00	37.50	98.7	90.8	0.6	2.3

Number of replicates was three.

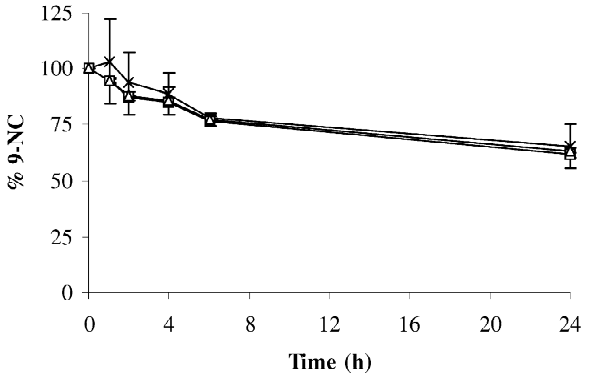

Fig. 4. Stability of 9-NC in human plasma at ambient temperatures. Samples were measured in triplicate at a low- (×; 25 ng/ml), mid- (□; 250 ng/ml), and high- (△; 375 ng/ml) concentration ranges after different times at ambient temperature. 9-NC was expressed as the recovered percentage of the amount of 9-NC measured at time 0 (error bars represent standard deviation).

plasma concentration–time curves of 9-NC and 9-AC are depicted of a patient treated with 1.5 mg/m^2 oral 9-NC after overnight fasting and after an high calorie breakfast. This demonstrates the applicability of the assay for complete pharmacokinetic evaluation of the drug in this study design.

4. Conclusions

We present a validated assay for the quantification of 9-NC and its metabolite 9-AC in human plasma.

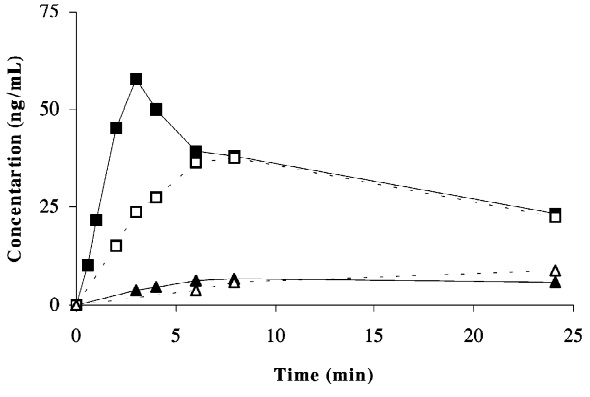

Fig. 5. Plasma concentration–time curves of 9-NC (■, □) and 9-AC (▲, △) of a patient treated with 1.5 mg/m^2 oral 9-NC after overnight fasting (solid symbols) and after an high calorie breakfast (open symbols).

Furthermore we demonstrated the applicability of this assay for pharmacokinetic evaluation of 9-NC in patients. Attention has been paid to the stability of the analytes, during storage and analysis. For the determination of 9-NC, it is of major importance that sample work up is performed within 2 h.

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