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Determination of total and lactone form of a new camptothecin derivative gimatecan (ST1481) and its metabolite ST1698 in human plasma by high-performance liquid chromatography with fluorimetric detection

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ABSTRACT

A new sensitive high-performance liquid chromatographic (HPLC) method for the determination of gimatecan (ST1481), a new camptothecin derivative, and its metabolite (ST1698) in plasma sample has been developed. The method consisted of on-line column solid phase extraction of analytes from human plasma, chromatographic separation by isocratic elution, then fluorimetric detection. The limits of quantitation were 0.25 ng/mL for both the analytes. The recovery of the extraction procedure was in the range of 62.8–71.1% for all the compounds. Good linearity ($R^2 > 0.999$) was observed within the calibration ranges studied: 0.25–25 ng/mL for both ST1481 and ST1698. Precision was in the range 1.2–4.3%, and accuracy was always lower than 4.7%. Surprisingly, after administration of ST1481 to humans, plasma concentrations found were higher than expected, while metabolite plasma concentrations were negligible. For this reason, a second calibration curve range was validated to quantify ST1481 in human plasma, ranging from 5 to 200 ng/mL. A good accuracy and precision were obtained, confirming the usefulness of the procedure. By using neutral analytical condition the intact lactone form was estimated in plasma samples from a patient. The lactone form amounted to 80–100% of the total ST1481.

1. Introduction

7-[(E)-*tert*-butyloxyiminomethyl]-camptothecin (gimatecan, ST1481) is a new camptothecin derivative, active as inhibitor of Topoisomerase I. As a consequence, ST1481 is potentially a powerful anticancer agent; as a matter of fact, pre-clinical studies demonstrated that ST1481 is characterised by a significant *in vitro* and *in vivo* activity against a broad range of tumors [1,2].

ST1481 is characterised by an E/Z isomerism, due to the presence of an oxime group on the molecule. ST1481 is synthesised practically pure as E form; however, the inter-conversion occurs if the substance is solubilised and exposed to the light, [sigma-tau unpublished data]. For this reason, preparation of solutions and processing of samples for ST1481 analysis should avoid direct light as much as possible. After administration of ST1481 to mice, rats and dogs, a polar metabolite of the parent compound was found in plasma. It was identified by HPLC-MS/MS as the oxidation product of the t-butyl group of ST1481 and coded as ST1698. ST1698 shares with ST1481 the same E/Z isomerism; and in plasma just ST1698 E isomer was found after administration of ST1481 E isomer to animals. As a consequence, ST1698 had to be handled in the same way described for ST1481.

As all the other camptothecins, both ST1481 and ST1698 contain a lactone group, which hydrolyses under basic and neutral conditions. The method presented in this paper is able to determine concentration of total (lactone plus hydroxyl-carboxylic products) and lactone ST1481 and ST1698 in human plasma. Substantially, the method consisted of on-line column solid phase extraction of analytes from human plasma, chromatographic separation by isocratic elution, then fluorimetric detection. As internal standard (IS) 20-O-Butyryl-camptothecin was used.

A Phase I study was performed to assess the Maximum Tolerated Dose of ST1481 [3]. Patients received ST1481 by oral route. The pharmacokinetic profiles of ST1481 and of its expected metabolite ST1698 were a secondary objective of this Phase I study, so far the present method was developed. Surprisingly, after administration of ST1481 to humans, the plasma concentrations were higher than expected, while metabolite plasma concentrations were negligible. For this reason, a second calibration curve range was validated to quantify ST1481 in human plasma.

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The present paper reports results from both the validation studies which were differentiated as "low calibration curve range" and "high calibration curve range" method.

2. Materials and methods

2.1. Chemicals

7-[(E)-*tert*-butyloxyiminomethyl]-camptothecin (ST1481) and 7-(2 hydroxy-1,1 dimethyloxyiminomethyl) camptothecin (ST1698) were synthesised by Antibioticos S.p.A. (Milan, Italy), and 20-O-Butyryl-camptothecin (IS) was synthesised by sigma-tau Industrie Farmaceutiche Riunite (Pomezia, Rome, Italy). The chemical structures of the above compounds are shown in Fig. 1.

Acetonitrile (HiperSolv for HPLC) was purchased from BDH Laboratory Supply (Poole, UK), 2-propanol (Gradient grade); 37% hydrochloric acid and 98–100% formic acid were purchased from Merck (Milan, Italy). Bi-distilled water was obtained from a Milli-Q system (Millipore, Milford, MA, USA).

2.2. Equipment and chromatographic conditions

Chromatographic equipment was constituted of a model PU-980 solvent delivery pump and one PU-1580 solvent delivery pump; a model AS-1555-10 autosampler set at +4 °C (Jasco, Hachioji, Tokyo, Japan), a Six-Port LabPro Rheodyne valve with electric actuator (Rheodyne, Rohnert Parck, CA, USA) and a model FP-1520 spectrofluorimeter photometer (Jasco, Hachioji, Tokyo, Japan). A Jasco Borwin Ver. 1.5 (Jasco, Hachioji, Tokyo, Japan) chromatographic data collection station was used.

The method consisted of an on-line purification of plasma samples, followed by isocratic elution chromatography. The sample purification was obtained with a BioTrap 500 C_{18} 20 mm × 4.0 mm column (ChromTech L.t.d., Congleton, UK). The HPLC mobile phase for purification (A) was prepared by mixing 90 mL of 1 M HCOOH with 810 mL of water and 100 mL of acetonitrile, then 10 mL of 2-propanol were added. The solution was then filtered through 0.45 μm HV Millipore filters and degassed in an ultrasonic bath at room temperature for 5 min. The chromatographic separation was obtained with a Luna 5 μm Phenyl-Hexyl 150 mm × 4.60 mm column equipped with a Luna Phenyl-Propyl guard column 4 mm × 3 mm (Phenomenex, Torrance, CA, USA). The HPLC mobile phase for chromatographic separation (B) was prepared by mixing 100 mL of 1 M HCOOH with 500 mL of water and 400 mL of acetonitrile, then 10 mL of 2-propanol were added. The solution was then filtered through 0.45 μm HV Millipore filters and degassed in an ultrasonic bath at room temperature for 5 min.

A Six-Port Rheodyne valve with electric actuator was used (see Fig. 2) in the position 1 it allowed the flux of mobile phase A to pass through the purification column (operative flow rate: 3.2 mL/min), during this phase macromolecules of plasma are eluted from column and discharged to waste; whether analytes are retained on the head of the column; in the mean time, the valve allowed the chromatographic mobile phase B to pass through the chromatographic column at the flow rate of 1 mL/min. In the position 2, the valve allowed chromatographic mobile phase to pass back flow through purification column to the chromatographic column (flow rate 1 mL/min) and then to the detector. During this phase, the mobile phase A passed just through the valve towards waste (flow rate 0.2 mL/min).

Pump 1 flow rate program was set as follows: from 0 to 3.1 min: 3.2 mL/min; from 3.2 to 25 min: 0.2 mL/min; from 25.1 to end run: 3.2 mL/min. Pump 2 was set for an isocratic elution at 1 mL/min.

The switching column time program was set as follows: extraction for 3 min (position 1), elution for 22 min (back flow, position 2), then equilibration time for 5 min (position 1).

For the low calibration curve range, the excitation and emission wavelenghts of the spectro-fluorimeter were 370 and 510 nm, respectively; the detector gain was set at 1000 (band width 40 nm). For the high calibration curve range, the excitation and emission wavelenghts of the spectro-fluorimeter were 370 and 510 nm, respectively; the detector gain was set at 100 (band width 18 nm).

2.3. Standard solution preparation

2.3.1. Standard solution for calibration curve

2.3.1.1. Low calibration curve range. ST1481 stock solution was prepared dissolving 10 mg of the compound in 100 mL of acetonitrile; this solution was diluted 1:10 with acetonitrile for two times. The concentration of the final solution was 1 μg/mL.

ST1698 stock solution was prepared dissolving 10 mg of the compound in 100 mL of acetonitrile, this solution was diluted 1:10 with acetonitrile for two times. The concentration of the final solution was 1 μg/mL.

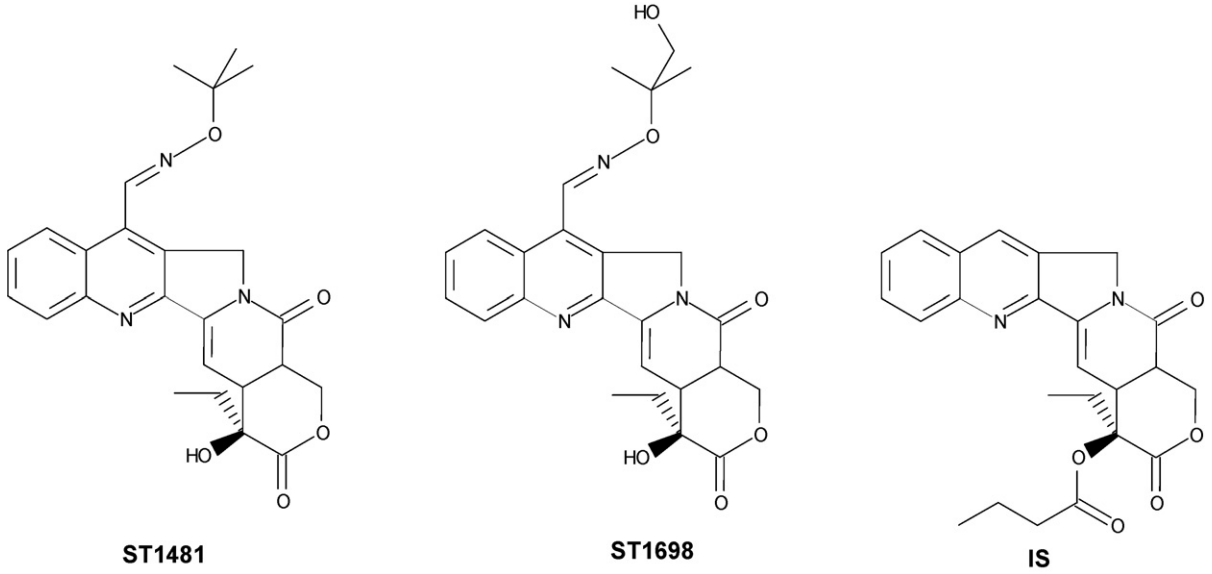

Fig. 1. Chemical structures.

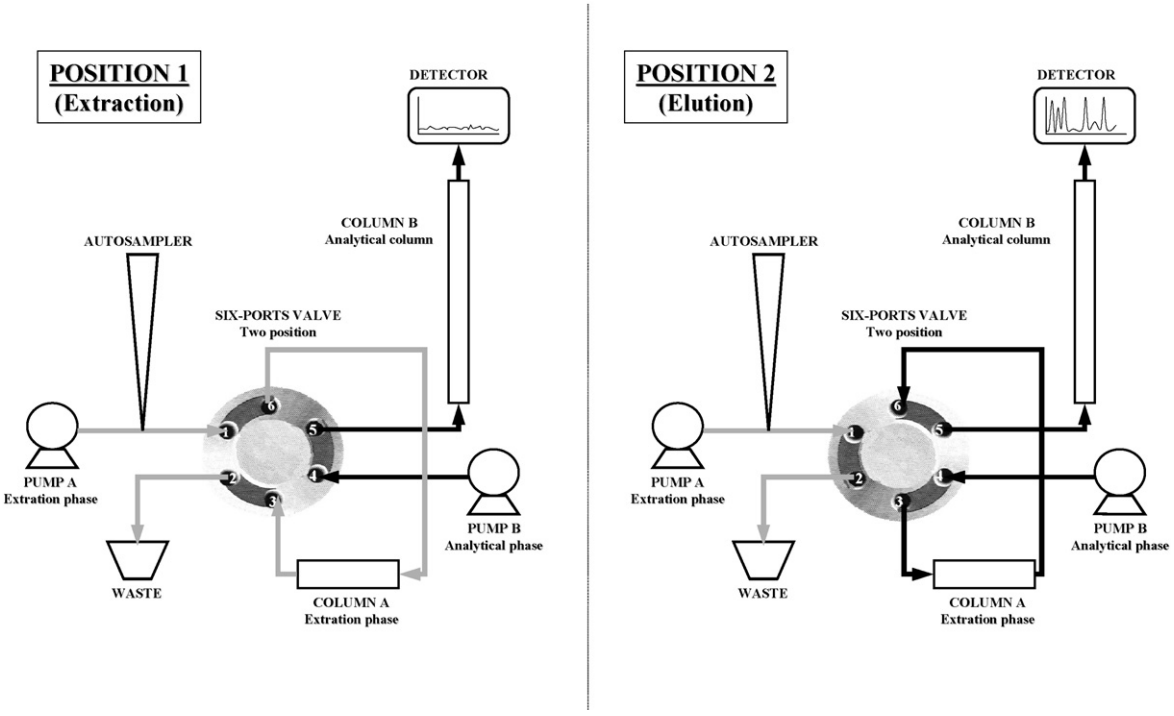

Fig. 2. Use of the Six-Port Rheodyne valve with electric actuator: in position 1 it allows the flux of mobile phase A to pass through the purification column, during this phase macromolecules of plasma are eluted from purification column and discharged to waste; whether analytes are retained on the column head; in position 2 it allows chromatographic mobile phase to pass back flow through purification and chromatographic columns, during this phase analytes are eluted from the column towards detector.

From 1 μg/mL ST1481 and 1 μg/mL ST1698 solutions 2.5 mL were diluted to 10 mL with acetonitrile. The solution obtained (250 ng/mL for both the analytes) was progressively diluted with acetonitrile to obtain solutions at the following concentrations: 100, 50, 10, 5 and 2.5 ng/mL of both the analytes.

2.3.1.2. High calibration curve range. ST1481 stock solution was prepared dissolving 10 mg of the compound in 100 mL of acetonitrile; this solution was diluted 1:10 with acetonitrile. The solution obtained (1 μg/mL) was progressively diluted with acetonitrile to obtain solutions at the following concentrations: 2000, 1000, 500, 250, 100 and 50 ng/mL of ST1481.

2.3.2. Standard solution for quality control (QC) samples

2.3.2.1. Low calibration curve range. ST1481 QC stock solution was prepared dissolving 4.8 mg of ST1481 in 100 mL of acetonitrile, the solution obtained was diluted 1:10, and then 1:2 with acetonitrile. The concentration of ST1481 QC final solution was 2.4 μg/mL.

ST1698 QC stock solution was prepared dissolving 4.8 mg of ST1481 in 100 mL of acetonitrile, the solution obtained was diluted once 1:10 then 1:2 with acetonitrile. The concentration of ST1698 QC final solution was 2.4 μg/mL.

From 2.4 μg/mL ST1481 and 2.4 μg/mL ST1698 solutions, 1 mL was diluted to 10 mL with acetonitrile. The solution obtained (240 ng/mL for both the analytes) was progressively diluted with acetonitrile to obtain solutions at the following concentrations: 60 and 6 ng/mL, for both the analytes.

2.3.2.2. High calibration curve range. ST1481 QC stock solution was prepared dissolving 4.8 mg of ST1481 in 100 mL of acetonitrile, the solution obtained was diluted 1.5:10 with acetonitrile. The solution obtained (7.2 μg/mL) was progressively diluted with acetonitrile to obtain solutions at the following concentrations: 1800, 720 and 144 ng/mL of ST1481.

2.3.3. Standard solution for IS

2.3.3.1. Low calibration curve range. Stock solution for IS was prepared dissolving 2.1 mg of IS with 50 mL of acetonitrile; this solution was diluted four times (1:20, 1:10 and twice 1:2) with acetonitrile to obtain a final concentration of 52.5 ng/mL.

2.3.3.2. High calibration curve range. Stock solution for IS was prepared dissolving 2.1 mg of IS with 50 mL of acetonitrile; this solution was diluted two times (1:20, 1:10) with acetonitrile to obtain a final concentration of 210 ng/mL.

2.4. Sample preparation

2.4.1. Calibration curve samples

A six-point calibration curve was prepared by adding 10 μL of each solution described above to 90 μL of human plasma. For the low calibration curve range the concentrations of 0.25, 0.5, 1, 5, 10 and 25 ng/mL, for ST1481 and ST1698 were obtained. For the high calibration curve range the ST1481 concentrations were the following: 5, 10, 25, 50, 100 and 200 ng/mL. Each calibration sample was processed as described below.

2.4.2. QC samples

QC samples at three different concentration levels were prepared by adding 10 μL of each solution described above to 90 μL of human plasma. For the low calibration curve range, the concentrations of 0.6, 6, and 24 ng/mL, for both ST1481 and ST1698 were obtained. For the high calibration curve range the ST1481 concentrations were the following: 14.4, 72, and 180 ng/mL. Each QC sample was processed as described below.

2.5. Processing of plasma samples

To samples of 100 μL of human plasma, 100 μL of 0.2 M HCl and 10 μL of the IS solution were added. After mixing, each sample was

transferred into an autosampler vial and injected (injection volume 100 μL for the low calibration curve range, 50 μL for the high calibration curve range).

2.6. Determination of the intact lactone form of ST1481

As mentioned in the introduction, ST1481 similarly to other camptothecins undergoes a reversible, pH dependent hydrolysis in which the closed lactone form is converted to the open carboxylate form under basic and neutral conditions.

The determination of the lactone form is particularly important as this form is responsible for the pharmacological activity. The procedure of analysis of the intact lactone form plasma samples consisted of the addition to 100 μL of plasma, of 100 μL of bi-distilled water and 10 μL of internal standard solution. The chromatographic separation was achieved under neutral pH conditions with a mobile phase of 50% acetonitrile in bi-distilled water. Samples were quantified by using the same standard calibration curve and QCs samples prepared as reported in Section 2.4.2 with procedure of extraction under acid conditions but using neutral pH chromatographic separation.

3. Results and discussion

The validation of both the analytical procedures was carried out calculating selectivity, extraction recovery, linearity, intra-assay and inter-assay precision and accuracy. Stability tests were carried out just for low calibration curve range method.

3.1. Chromatography and selectivity

Chromatograms of blank human plasma (A), blank human plasma spiked with ST1481 (6 ng/mL), ST1698 (6 ng/mL) and IS (B) and blank human plasma spiked with ST1481 (72 ng/mL) and IS (C) are shown in Fig. 3. These chromatograms indicate that no endogenous compound interfered with the detection of analytes at their retention times which are approximately ~8.5 min for ST1698; ~20.0 min for IS and ~24.9 min for ST1481. In the same figure also a chromatogram of a plasma sample from a patient treated orally for 5 consecutive days is shown (D). The sample was collected on day 5 of administration, 2 h after dosing with 0.9 mg/m^2/day of ST1481; this chromatogram is overlapped to the chromatographic profile of the pre-dose plasma sample collected before drug administration from the same patient. ST1481 and ST1698 found concentrations were 61 ng/mL and 8 ng/mL, respectively. At the retention time of 11.2 min the presence of another peak (M1) not detected in the pre-dose sample was observed. Preliminary mass-spectrometric studies indicate that M1 has camptothecin related structure and can be considered an unknown metabolite of ST1481.

3.2. Recoveries

The recoveries of ST1481, ST1698 and IS from human plasma were estimated by comparing the peak areas obtained from injections of standard solutions with those obtained from the injections of human plasma samples spiked with known concentrations of each analyte.

Low calibration curve range: for ST1481 the recovery ranged between 62.8% and 66.3%, for ST1698 it ranged between 63.6% and 71.1% and for IS, it was 61.4% (Table 1).

High calibration curve range: for ST1481 the recovery ranged between 63.3% and 65.3% and for IS it was 57.3% (Table 1).

Table 1
Recoveries of ST1481, ST1698 and IS from human plasma ($n = 3$).

	Concentration (ng/mL)	Recovery (%), mean ± SD
ST1481	0.6	66.3 ± 3.3
	6	64.8 ± 0.7
	24	62.8 ± 0.9
ST1698	0.6	63.6 ± 2.0
	6	71.1 ± 0.8
	24	67.9 ± 0.8
IS	5.25	61.4 ± 2.5
ST1481	14.4	64.6 ± 0.3
	72	63.3 ± 1.6
	180	65.3 ± 0.9
IS	10.5	57.3 ± 0.7

3.3. Linearity range

Calibration curves were constructed by plotting the peak area ratio of each analyte to IS against the concentration of the analyte. Data were fitted to least squares linear regression with a weighting factor of $1/y$.

Low calibration curve range: calibration curves ($n = 5$) for ST1481 and ST1698 were linear over the concentrations examined (0.25–25 ng/mL for both the analytes) and reproducible with a mean ± SD of $y = (0.1556 \pm 0.0070)x + (-0.0008 \pm 0.0078)$ for ST1481 and $y = (0.1442 \pm 0.0024)x + (-0.0015 \pm 0.0052)$ for ST1698. Regression coefficients (R^2) were greater than 0.9990 for both the molecules.

The suitable range for both ST1481 and ST1698 analyses was 0.25–25 ng/mL as also demonstrated by the obtained values of precision and accuracy from the back calculated concentrations (Table 2).

For ST1481 the precision ranged between 1.3% (25 ng/mL) and 6.2% (0.25 ng/mL); the accuracy ranged between 0.7% (25 ng/mL) and 3.8% (5 ng/mL), respectively.

For ST1698 the precision ranged between 1.1% (25 ng/mL) and 4.6% (0.5 ng/mL); the accuracy ranged between 0.8% (0.5 ng/mL) and 3.6% (10 ng/mL), respectively.

High calibration curve range: calibration curves ($n = 4$) for ST1481 were linear over the concentrations examined (5–200 ng/mL) and reproducible with a mean ± SD of $y = (0.0477 \pm 0.0004)x + (0.0041 \pm 0.0143)$ for ST1481. Regression coefficient (R^2) was greater than 0.9990.

The suitable range for ST1481 was 5–200 ng/mL as also demonstrated by the obtained values of precision and accuracy from the back calculated concentrations (Table 2). The precision ranged between 0.8% (200 ng/mL) and 2.1% (50 ng/mL); the accuracy ranged between 1.0% (100 ng/mL) and 3.0% (5 ng/mL), respectively.

3.4. Precision and accuracy

The intra-day precision and accuracy were evaluated by analysing the plasma spiked with ST1481 and ST1698 at concentrations of 0.6, 6 and 24 ng/mL in six replicates for low calibration curve range, and by analysing the plasma spiked with ST1481 at concentrations of 14.4, 72 and 180 ng/mL in six replicates for high calibration curve range (Table 3). Accuracy was based on the calculation of the found concentration compared to the actual concentration.

Low calibration curve range: the intra-day precision of this method for ST1481 and ST1698 were <1.7% and <1.6%, respectively. The accuracy ranged between 1.4% and 4.1% for both the analytes.

High calibration curve range: the intra-day precision and accuracy of this method for ST1481 were <2.0% and <3.9%, respectively.

Table 2
Precision and accuracy of the method for the determination of ST1481 and ST1698 according to calibration curve samples ($n = 5$ for low range calibration curve and $n = 4$ for high calibration curve range).

	ST1481						ST1698						ST1481					
Actual concentration (ng/mL)	0.25	0.5	1	5	10	25	0.25	0.5	1	5	10	25	5	10	25	50	100	200
Found concentration (ng/mL)	0.25	0.51	1.03	5.19	9.66	25.17	0.26	0.50	0.99	5.17	9.64	25.24	5.15	9.87	25.35	48.59	98.99	202.20
Precision (%)	6.2	3.4	3.4	2.1	3.4	1.3	4.2	4.6	1.5	2.7	3.1	1.1	1.5	1.1	1.5	2.1	1.5	0.8
Accuracy (%)	1.6	1.2	3.0	3.8	3.4	0.7	3.2	0.8	1.4	3.3	3.6	1.0	3.0	1.3	1.4	2.8	1.0	1.1

Table 3
Intra-day validation of quality control standards for the determination of ST1481 and ST1698 in human plasma ($n = 6$).

Actual concentration (ng/mL)	ST1481			ST1698		
	Found concentration (ng/mL)	Precision (%)	Accuracy (%)	Found concentration (ng/mL)	Precision (%)	Accuracy (%)
0.6	0.61	1.3	2.2	0.61	1.2	1.9
6	5.88	1.6	2.1	5.93	1.5	1.4
24	23.02	1.7	4.1	23.32	1.6	2.9

Actual concentration (ng/mL)	ST1481		
	Found concentration (ng/mL)	Precision (%)	Accuracy (%)
14.4	14.11	1.3	2.0
72	69.20	2.0	3.9
180	174.56	1.6	3.0

The inter-day precision and accuracy were evaluated at the same concentration levels (three replicates per concentration level per calibration curve range) over 4 different working days (Table 4).

Low calibration curve range: the inter-day precision of this method for ST1481 and ST1698 were <3.8% and <4.3%, respectively. The accuracy ranged between 1.4% and 5.1% for both the analytes.

High calibration curve range: the inter-day precision and accuracy of this method for ST1481 were <2.3% and <4.1%, respectively.

3.5. Quantitation limits

Low calibration curve range: the quantitation limits for the determination of ST1481 and ST1698 in human plasma were assessed as the lowest amount of each analyte which can be determined with precision and accuracy lower than 20% (six replicates per sample). They resulted to be 0.25 ng/mL for both the analytes, with a precision of 4.8% and 4.3% for ST1481 and ST1698, respectively; and an accuracy of 4.7% and 8.7% for ST1481 and ST1698, respectively.

High calibration curve range: The quantitation limits for the determination of ST1481 in human plasma were assessed as the lowest amount which can be determined with precision and accuracy lower than 20% (six replicates per sample). It resulted to be 5 ng/mL for ST1481, with a precision of 5.0% and an accuracy of 4.6%.

3.6. Stability

3.6.1. Freeze–thaw stability

The stability of ST1481 and ST1698 was over two freeze–thaw cycles. Both the molecules exhibited acceptable stability over two freeze–thaw cycles as can be seen from Table 5.

3.6.2. Plasma sample stability

The autosampler (+4 °C) stability was assessed analysing fresh samples, and analysing the same sample after 24 and 48 h after the first injection. Both the molecules exhibited acceptable stability as can be seen from Table 6.

The stability of ST1481 and ST1698 in human plasma samples stored at −20 °C was tested over 2 and 4 months. ST1481 and ST1698 showed a marked degradation at 4 months; therefore, they have to be quantified in human plasma within 2 months from the collection of samples.

3.7. Determination of the intact lactone form of ST1481

The use of neutral pH conditions was unequivocally able to determine the lactone form as illustrated in Fig. 4 that reports the plot of the equilibrium existing between the two forms of ST1481 as function of sample pH when a concentration of 50 ng/mL of ST1481 were incubated in aqueous media at different pH. The results of the

Table 4
Inter-day validation of quality control standards for the determination of ST1481 and ST1698 in human plasma ($n = 4$).

Actual concentration (ng/mL)	ST1481			ST1698		
	Found concentration (ng/mL)	Precision (%)	Accuracy (%)	Found concentration (ng/mL)	Accuracy (%)	Precision (%)
0.6	0.59	3.8	1.4	0.57	4.3	4.7
6	5.70	1.5	5.1	5.73	1.7	4.6
24	22.80	2.7	4.7	23.16	2.4	3.5

Actual concentration (ng/mL)	ST1481		
	Found concentration (ng/mL)	Precision (%)	Accuracy (%)
14.4	13.86	2.3	3.8
72	69.62	0.5	3.3
180	172.61	0.6	4.1

Table 5
Freeze–thaw stability of ST1481 and ST1698 in human plasma ($n = 3$).

	ST1481								
	Fresh			1st cycle			2nd cycle		
Actual concentration (ng/mL)	0.6	6	24	0.6	6	24	0.6	6	24
Found concentration (ng/mL)	0.61	5.60	22.20	0.56	5.65	22.68	0.60	5.80	23.65
Precision (%)	0.9	4.0	3.7	1.8	2.6	2.2	1.7	2.4	2.3
Accuracy (%)	2.2	6.6	7.5	6.7	5.8	5.5	0.0	3.3	1.5
	ST1698								
	Fresh			1st cycle			2nd cycle		
Actual concentration (ng/mL)	0.6	6	24	0.6	6	24	0.6	6	24
Found concentration (ng/mL)	0.56	5.68	22.59	0.55	5.67	23.00	0.60	5.87	23.9
Precision (%)	1.8	3.6	3.9	2.1	2.8	2.1	3.5	2.4	2.1
Accuracy (%)	6.7	5.4	5.9	8.9	5.5	4.2	0.6	2.2	0.4

analysis of the lactone form were independent from the use of the biotrap pre-column.

3.8. Application to clinical samples

The suitability of the developed method for clinical use was demonstrated by determining gimatecan and ST1698 in plasma obtained from patients participating to a Phase I study (3) in with the drug was administered per os, daily, for 5 consecutive days a week for 3 weeks.

Fig. 5 shows the plasma concentration versus time profiles of ST1481 and its main metabolite ST1698, measured with the present method in a patient who entered the study. The patient received gimatecan at a daily dose of 0.43 mg/m^2. Blood samples were collected at several times on day 1 (panel A) up to 24 h and on day 19 (panel B) up to 72 h after the administration. The drug appears well absorbed and it reaches a maximum of concentration 1.5 h after drug administration. The maximal concentrations for ST1481 were 23 and 36 ng/mL on days 1 and 19, respectively, indicating accumulation in plasma. Both ST1481 and ST1698 showed a long half-life of about 2 days with measurable concentrations at 72 h of

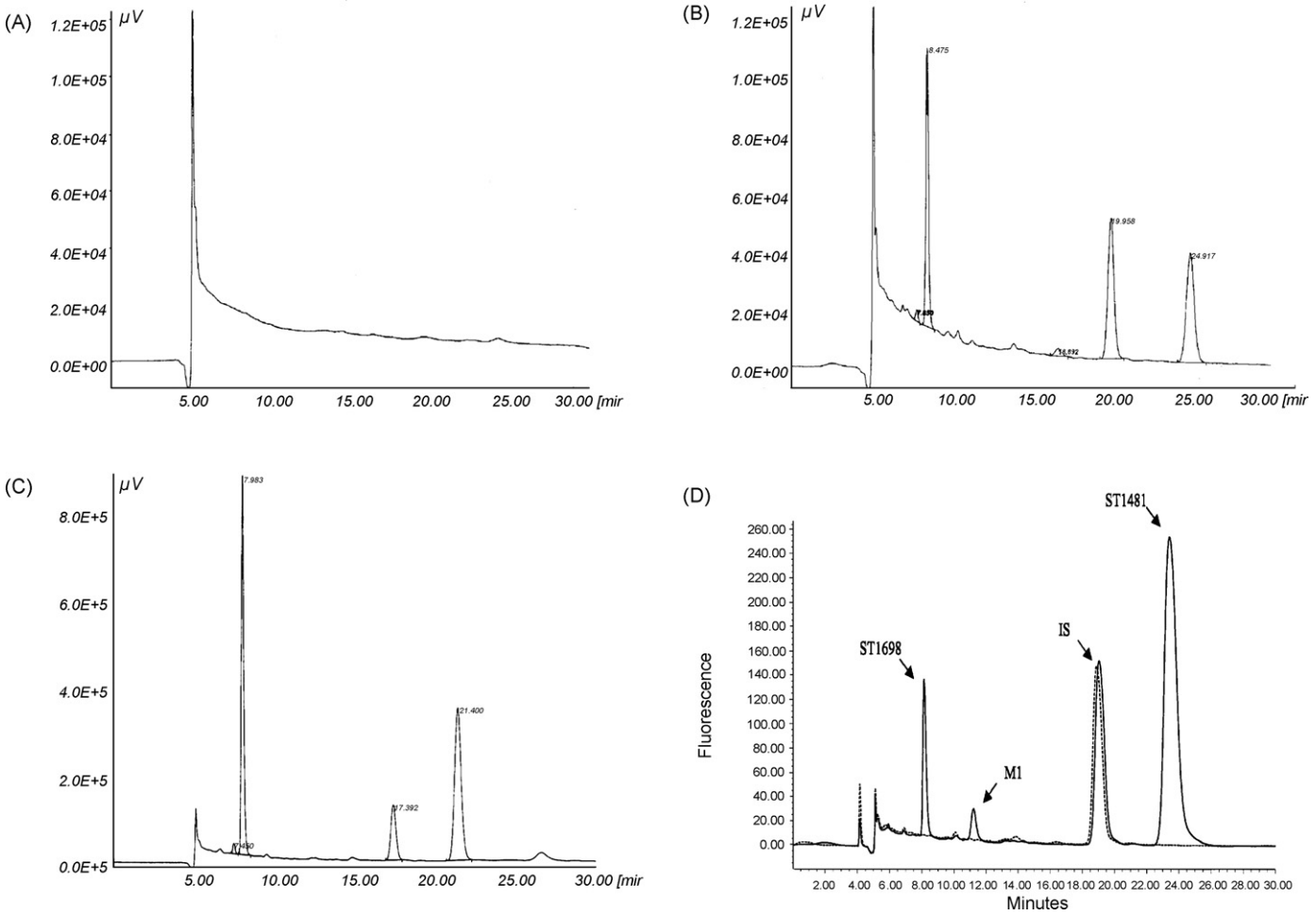

Fig. 3. Typical chromatograms of blank human plasma sample (A), human plasma sample containing 6 ng/mL of ST1481 and ST1698, and IS (B), human plasma sample containing 72 ng/mL of ST1481 and IS (C) and a human plasma sample from a patient treated with ST1481 overlapped to his/her basal plasma chromatogram (D).

Table 6
Autosampler stability (+4 °C) of ST1481 and ST1698 in human plasma ($n = 3$).

	ST1481								
	Fresh			24 h			48 h		
Actual concentration (ng/mL)	0.6	6	24	0.6	6	24	0.6	6	24
Found concentration (ng/mL)	0.56	5.82	23.48	0.62	5.96	23.72	0.61	5.92	23.57
Precision (%)	1.0	2.0	0.7	4.0	1.8	0.5	4.1	1.5	0.9
Accuracy (%)	7.2	3.0	2.2	3.9	0.7	1.2	2.2	1.3	1.8
	ST1698								
	Fresh			24 h			48 h		
Actual concentration (ng/mL)	0.6	6	24	0.6	6	24	0.6	6	24
Found concentration (ng/mL)	0.56	5.85	23.80	0.55	5.86	23.77	0.56	5.85	23.56
Precision (%)	2.7	1.7	0.7	0.0	1.8	0.5	1.8	1.7	1.2
Accuracy (%)	7.2	2.6	0.8	8.3	2.3	1.0	6.7	2.6	1.8

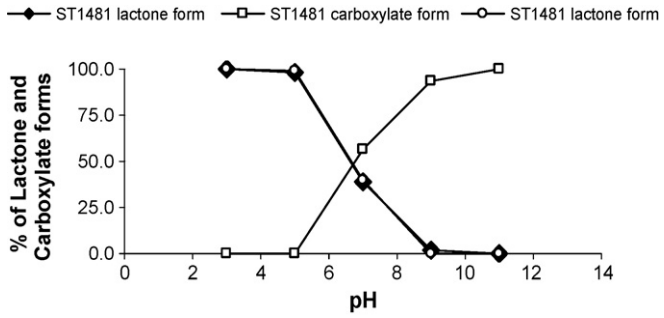

Fig. 4. Hydrolysis of ST1481 in aqueous media as function of pH. Symbols (♦) and (○) indicate the plot of the percentage of lactone form determined under neutral pH chromatographic condition with or without the use biotrap pre-column.

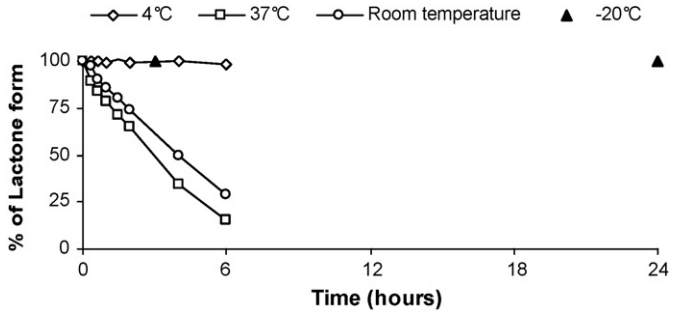

Fig. 6. Stability of ST1481 in fresh human plasma at different temperatures determined under.

7.0 and 0.92 ng/mL, respectively after 15 days of dosing. The intact lactone form was assayed in the same patient without acidification of plasma samples and performing the HPLC analysis under neutral pH conditions. The levels of the intact lactone form were found to be about 90–100% of the total drug. The unexpected observation of the very high percentage of the lactone form prompted us to conduct a study to verify the stability of the lactone form in plasma in an ex vivo incubation experiments. As showed in Fig. 6, the lactone form exhibited great stability in human plasma at 4 °C and in frozen plasma samples (−20 °C), when exposed to multi freeze–thaw cycles with undetectable change of the lactone group concentration over 24 h. The hydrolyses became evident at room temperature (20 °C) and at 37 °C, being after 6 h, the intact lactone form of 20% and 10% of the total ST1481 determined at time 0. These data indicated that the high stability observed in patients is due to *in vivo* mechanisms

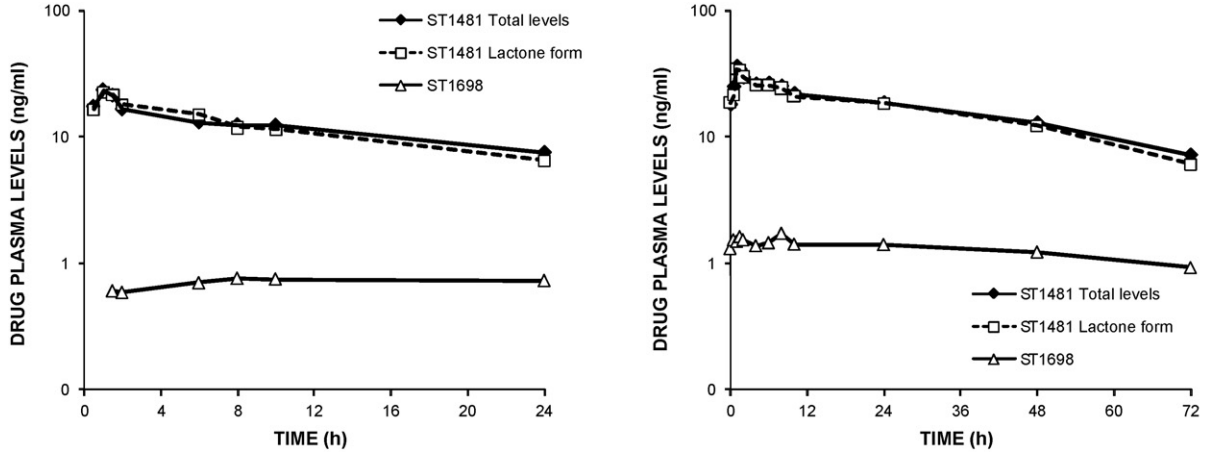

Fig. 5. Plasma profiles of ST1481 (total and lactone form) and its metabolite ST1698 in a patient entered the phase I study of this new camptothecin. The patient received oral ST1481 at a daily dose of 0.43 mg/m^2 for 5 consecutive days a week for 3 weeks. Blood samples were collected at several times on days 1 (panel A) and 19 (panel B) and up to 72 h after the last administration.

4. Conclusions

This paper describes a new and sensitive HPLC method for the determination of ST1481 and its metabolite ST1698 as total moieties, based on an on line-column sample purification, followed by chromatographic separation and fluorescent detection.

The method validation demonstrated that the assay showed very good accuracy and precision within a wide range of concentrations for the analytes. The linearity, reproducibility extraction recovery, selectivity and accuracy make this method suitable for pharmacokinetic investigations in humans.

Moreover, by using neutral chromatographic conditions, this method allows the determination in human plasma of the intact lactone which is considered the pharmacologically active form of camptothecins.

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