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Sensitive determination of irinotecan (CPT-11) and its active metabolite SN-38 in human serum using liquid chromatography–electrospray mass spectrometry

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Abstract

A couple of sensitive and accurate liquid chromatography–electrospray mass spectrometry (LC–ES-MS) methods for the determination of the total forms of irinotecan and its active metabolite SN-38 in human serum, using the same chromatographic and detection conditions, is presented. Both used camptothecin as internal standard (I.S.). The sample pretreatment for irinotecan involved a simple protein precipitation with acetonitrile, whereas a liquid–liquid extraction was necessary for SN-38. A Symmetry C_{18}, 3.5 μm (150×1 mm I.D.) reversed-phase column was used for the chromatographic separation, together with a gradient elution of acetonitrile in 5 m*M* ammonium formate buffer (pH 3) as mobile phase. After ionisation in the pneumatically-assisted electrospray source and in-source collision induced dissociation, acquisition was performed in the selected ion monitoring mode. Recoveries were 69 and 47% on average, detection limits 2.5 and 0.25 ng/ml and quantitation limits 10 and 0.5 ng/ml for irinotecan and SN-38, respectively. Reproducibility was good and the method was linear from limits of quantitation up to 10 000 ng/ml for irinotecan, and up to 100 ng/ml for SN-38. This sensitive and highly specific method is suitable both for pharmacokinetic studies and routine therapeutic drug monitoring.
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Keywords: Irinotecan; SN-38

1. Introduction

Irinotecan, also known as CPT-11 {7-ethyl-10-[4-(1-piperidino)-1 piperidino] carbonyl oxy-camptothecin} is a semi-synthetic and water-soluble derivative of 20(*S*)-camptothecin (CPT), an antineoplastic alkaloid [1]. CPT-11 has demonstrated pronounced antitumour activity against a large variety of malignancies, including colorectal cancer, refractory cervical cancer and other gynecological cancers [2,3]. The mechanism of action of CPT-11 is presumed to be mediated through topoisomerase I inhibition [4–6]. CPT-11 is converted in vivo into 7-ethyl-10-hydroxy camptothecin (SN-38) [7,8], whose in vitro cytotoxic activity is 100- to 1000-times greater than that of the parent compound [9]. Previous studies demonstrated

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that SN-38 serum levels were about 100-times lower than the corresponding CPT-11 levels [10] and that the pharmacokinetics of both compounds presented a high interpatient variability [11].

Moreover, like all other camptothecin analogues, CPT-11 and SN-38 contain an extremely labile α-hydroxy ring (Fig. 1). Therefore, they can undergo a reversible, pH-dependent hydrolysis during which the closed lactone form is converted to an open carboxylate form [12], while it has been shown that the intact lactone ring is necessary for biological activity [13,14]. CPT is in the lactone form at pH<5 and converts predominantly to the ionised carboxylate form at pH>8 [15]; the conversion of lactone to carboxylate forms of CPT-11 and SN-38 was reported to be slow at pH 5.5 [16].

To date, several high-performance liquid chromatographic methods have been developed for the determination of camptothecin derivatives, using mainly fluorometric detection after a liquid–liquid or solid-phase extraction. Some of these techniques concerned the qualitative identification of the lactone or total form only [17], or of the lactone and carboxylate forms simultaneously [18,19], and some reported the quantitation of these same forms [15,16,20–25]. Recently, high-performance liquid chromatography–atmospheric pressure chemical ionization-tandem mass spectrometry (HPLC–APCI-MS–MS) was used, after a solid-phase extraction, to assess the presence and structure of several metabolites of CPT-11 in biological fluids [26].

The aim of the present study was to design a couple of sensitive, specific and fully validated methods for the determination of the total concentrations of CPT-11 and SN-38 in human serum, applicable to pharmacokinetic studies as well as to routine therapeutic drug monitoring (TDM), using liquid chromatography–electrospray mass spectrometry (LC–ES-MS).

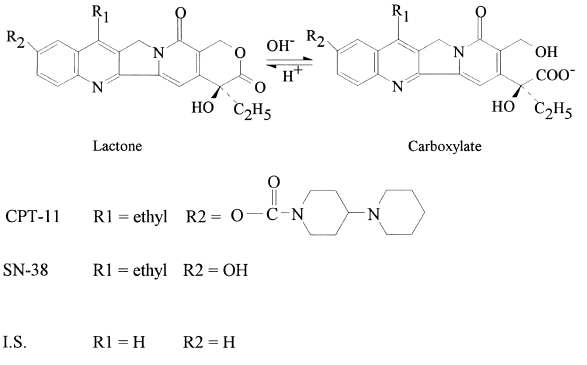

Fig. 1. Chemical structures of camptothecin (I.S.), irinotecan (CPT-11) and its active metabolite SN-38.

2. Experimental

2.1. Chemicals and reagents

CPT-11 and SN-38 were gifts from Rhône Poulenc Rorer (Vitry-sur-Seine, France) and the internal standard (I.S.) camptothecin was obtained from Aldrich (Saint-Quentin Fallavier, France). Acetonitrile, diethyl ether, dichloromethane and sodium hydroxide were purchased from Prolabo (Fontenay-sous-Bois, France), citric acid was obtained from Merck (Darmstadt, Germany), formic acid and ammonium formate from Sigma (St. Louis, MO, USA). All reagents were of chromatographic purity. Deionised water was prepared with a Milli-Q laboratory plant (Millipore, Bedford, MA).

2.2. Standards and solutions

Stock solutions were prepared at 1 g/l in methanol for CPT-11 and, due to a poor solubility in methanol, were prepared in methanol–dimethylsulfoxide (DMSO) (65:35, v/v) for SN-38 and in methanol–hexafluoro-2-propanol (HFIP) (40:60, v/v) for I.S. When kept at 4°C in the dark, they proved to be stable for several months. CPT-11 working solutions at 0.01, 0.1, 1 and 10 mg/l were freshly prepared by appropriate dilutions with acetonitrile and SN-38 working solutions at 0.01, 0.1 and 1 mg/l were prepared with deionised water. The internal standard working solution was prepared by dilution with acetonitrile.

2.3. Sample preparation

Fresh blood samples were centrifuged for 15 min at 2500 *g* and serum was frozen at −20°C until analysis. For the determination of CPT-11, a simple protein precipitation was performed: to 200 μl of thawed serum were added 50 μl of I.S. (4 mg/l) and

200 μl of acetonitrile in a polypropylene microtube (Eppendorf, CML, Nemours, France). The mixture was vortex-mixed for 30 s and centrifuged at 900 *g* for 10 min. A 100-μl aliquot of the supernatant was acidified with 20 μl of sodium citrate buffer (2.5 *M*, pH 2) and 2 μl of the mixture was directly injected into the HPLC system. The calibrating standards were prepared by spiking 200 μl serum with 50 μl I.S. and 50 to 200 μl of the appropriate working solution of CPT-11, eventually completed to 200 μl with pure acetonitrile. They were thereafter treated as detailed above. SN-38 was extracted by a rapid liquid–liquid procedure: to 1 ml of serum, 50 μl of internal standard (1 mg/l), 250 μl of a 2.5 *M* sodium citrate solution (pH 2) and 8 ml of diethyl ether–dichloromethane (4:1, v/v) were added in a 15-ml round-bottomed glass tube. To prevent emulsion, extraction was performed gently for 20 min on a test-tube rotator, keeping the rotating plate in an almost horizontal position. After centrifugation, the organic phase was transferred to a clean 15-ml glass tube and a spatula of sodium sulphate added to remove water traces. The tubes were briefly vortex-mixed (10 s), and centrifuged for 10 min. Then, the organic solvent was transferred to a 10-ml conical glass tube and evaporated to dryness under a gentle stream of nitrogen at 37°C. The dry extracts were redissolved in 50 μl of a mixture of acetonitrile–5 m*M* ammonium formate (pH 3) (70:30, v/v), of which 2 μl was injected into the chromatographic system.

2.4. HPLC conditions

The HPLC system included two Series 200 micro-LC chromatographic pumps and a Series 200 auto-sampler (Perkin-Elmer, Toronto, Canada). Chromatographic separation was performed on a Symmetry C_{18}, 3.5 μm (150×1 mm I.D.) reversed-phase column (Waters, Milford, MA, USA), together with a gradient of acetonitrile (from 15% to 50% in 6.5 min, from 50% to 70% in 2.5 min and finally decreased to 15% in 1 min) in 5 m*M* ammonium formate (pH 3) as mobile phase, which was delivered at a flow-rate of 50 μl/min. The column temperature was maintained at 35°C using a HPLC column oven (Waters).

2.5. Mass spectrometry

A PE-Sciex (Toronto, Canada) API-100 mass spectrometer was used, equipped with a pneumatically-assisted electrospray ionisation device. High-purity nitrogen was used as nebulisation and curtain gas. Calibration of the mass analyser was performed by infusion (5 μl/min) of a commercial mixture of polypropylene glycols (PE, Applied Biosystems, Courtaboeuf, France) using a Harvard Model 11 Syringe pump (Harvard Scientific, South Natick, MA, USA) and monitoring eight mass-to-charge ratios in the 55 to 2300 a.m.u. mass range. For optimising CPT-11, SN-38 and camptothecin ionisation and ion transmission conditions, 10 mg/l solutions of the pure drugs in the mobile phase (adjusted at pH 3 to obtain the lactone form only) were infused at 50 μl/min into the ion source. During this experiment, a mass range from m/z 65 to 550 was scanned with a resolution of 0.1 a.m.u. For each analyte, several ions were selected: the protonated molecule $[M+H]^+$ as quantifying ion and one or two main fragments as confirmation ions (Table 1). The main parameter settings of the MS system were as follows: nebulisation gas flow 1.55 l/min; curtain gas flow 1.36 l/min; ionspray voltage 5000 V; fragmentation energy (''orifice voltage'') optimised for each selected ion, as reported in Table 1.

2.6. Validation procedures

Extraction recoveries were determined in triplicate at concentrations of 50, 1000 and 5000 ng/ml for CPT-11 and 5, 25 and 75 ng/ml for SN-38. For a given concentration of SN-38, three serum samples were spiked with I.S. and the appropriate amount of SN-38, while three others were added the I.S. only; after extraction and evaporation, the first three samples were reconstituted with 50 μl mobile phase and the last three with 50 μl of mobile phase containing the same amount of SN-38, and representing 100% recovery. The same principle was used for CPT-11: for a given concentration of CPT-11, three serum samples were spiked with I.S. and the appropriate amount of CPT-11, three others with I.S. only; after protein precipitation, the first three supernatants were diluted with 100 μl of acetonitrile and the last three with 100 μl of acetonitrile containing the same

Table 1
Retention times, selected ions and related orifice voltages, and limits of detection of CPT-11, SN-38 and EI

Compound	Retention time (min)	Selected ions[a] (a.m.u)	Orifice voltage (V)	Relative intensity (%)	LOD (ng/ml)
CPT-11 (lactone)	3.9	**587.3**	60	100	2.5
		502.5	40	5	
		331.5	120	10	
CPT-11 (carboxylate)	3.5	**605.3**	40	50	N.D.[b]
		587.3	60	100	
SN-38 (lactone)	5.1	**393**	75	100	0.25
		349.6	70	50	
SN-38 (carboxylate)	5.5	**411.2**	80	50	N.D.
		393	75	100	
I.S.	5.4	**349.2**	20	100	N.D.
		305.1	80	60	

[a] Quantifying ions are in bold.
[b] N.D.: Not determined.

amount of CPT-11. Recoveries were calculated by comparison of the analyte/I.S. peak area ratios of the extracted samples with those of the unextracted standards, representing 100% recovery.

For the rest of analytical validation, the guiding principles of Shah et al. [27] were followed. These recommendations are briefly summarised as follows: the within-day precision should be studied at least at three concentrations; the intermediate (day-to-day) precision should be assessed using at least five determinations of five to eight concentrations (excluding blank values); within-day and day-to-day experiments should yield a precision relative standard deviation (RSD) of less than 15% and a deviation (mean relative error) of less than 15% from the nominal value at every concentration studied, except for the limit of quantitation (LOQ) where 20% is acceptable for both parameters.

Accordingly, within-day precision was assessed at concentration levels of 1, 25 and 75 ng/ml for SN-38 and of 50, 1000 and 5000 ng/ml for CPT-11, by extraction and analysis on the same day of six drug-free serum samples fortified with both compounds, for each level. For the determination of intermediate precision as well as for studying the linearity range of the method, drug-free serum samples spiked at 0, 0.5, 1, 5, 10, 25, 50, 75 and 100 ng/ml SN-38 and 0, 10, 50, 100, 1000, 5000 and 10 000 ng/ml CPT-11 were prepared in advance in 10-ml volumetric flasks and 1-ml aliquots stored at −18°C until analysis. A set of these calibrating samples was analysed each day for six days and a calibration graph of the drug-to-I.S. peak area ratio versus theoretical drug concentration was constructed, using a $1/x$ weighted least-squared linear regression.

3. Results

HPLC and MS conditions were optimised with pure standards of CPT, SN-38 and CPT-11 under their respective lactone forms. In order to be able to verify the absence of the carboxylate forms in the samples analysed, solutions of the pure drugs, diluted in mobile phase adjusted at pH 9, were injected. The mean retention times were: 5.4 min for I.S., 5.1 min for SN-38 and 3.9 min for CPT-11, lactone forms; 5.5 min and 3.5 min for the metabolite and drug carboxylate forms, respectively (Table 1). It is worth noting that the retention time of SN-38 carboxylate was higher than that of the lactone form, whereas the opposite was true for CPT-11. Fig. 2 shows typical chromatograms of a blank sample, of a serum sample spiked at LOQ (10 ng/ml for CPT-11, 0.5 ng/ml for SN-38) and of a serum sample spiked at a high concentration (5000 ng/ml for CPT-11, 75 ng/ml for SN-38) to demonstrate the absence of carboxylate

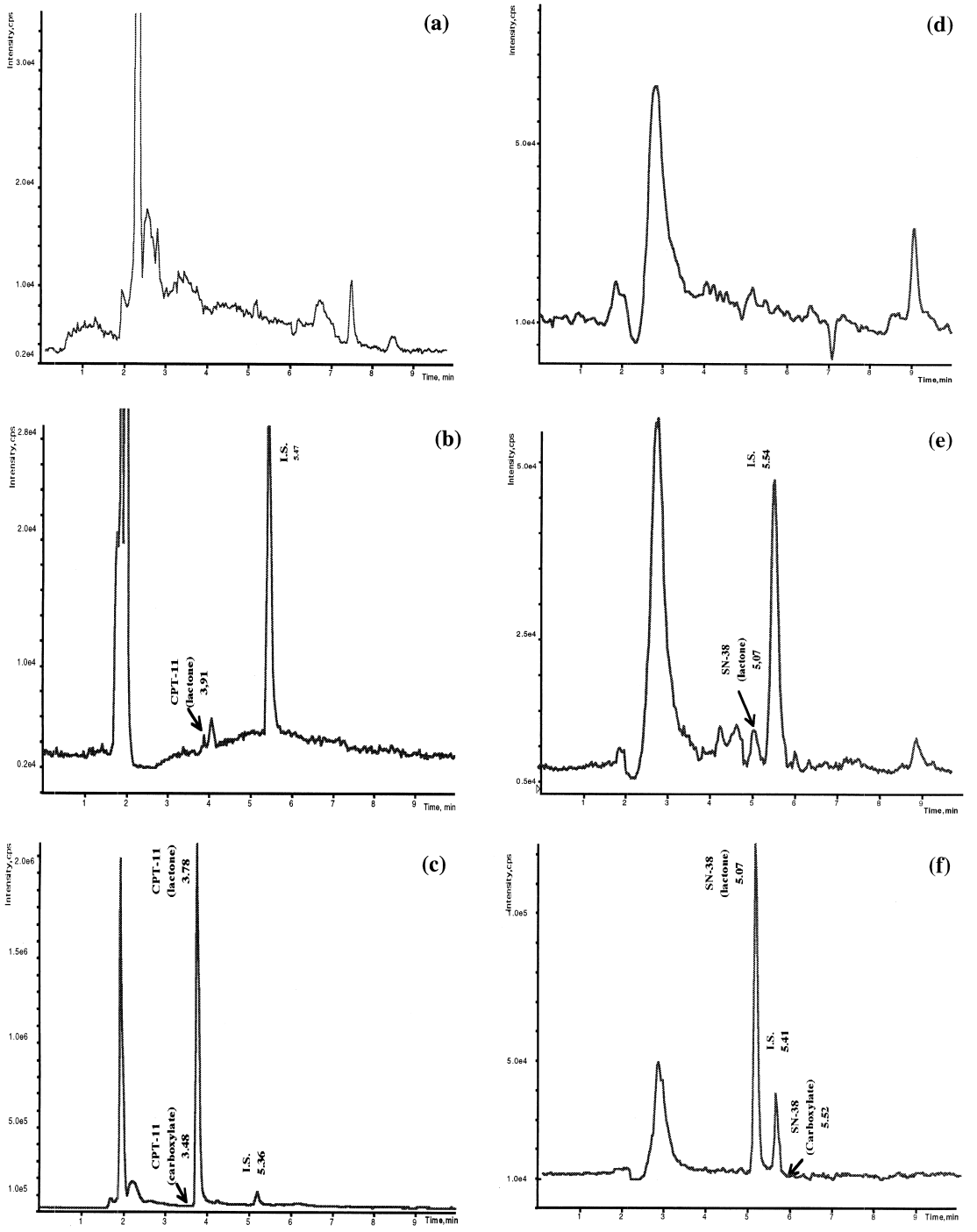

Fig. 2. Extracted ion chromatograms of extracts from (a) a blank serum of CPT-11, (b) a serum spiked at 10 ng/ml of CPT-11, (c) a serum spiked at 5000 ng/ml of CPT-11 (to demonstrate the absence of the carboxylate form), (d) a blank serum of SN-38, (e) a serum spiked at 0.5 ng/ml of SN-38 and (f) a serum spiked at 75 ng/ml of SN-38 (to demonstrate the absence of the carboxylate form).

forms. No unknown compounds resulting from the extraction procedure interfered with CPT-11 or its metabolite and no chromatographic peak corresponding to their carboxylate forms could be detected. Generally, no interferences were noted in the drug-free serum pools used for the setting and validation of these procedures, nor in the clinical samples analysed so far. With the instrument used in this study, fragmentation can be induced by means of the orifice voltage (OR), which controls the acceleration of the desolvated ion species in the intermediate pressure region, between atmospheric and high vacuum. As this parameter significantly modifies the absolute and relative abundances of the pseudomolecular and fragment ions, optimum settings for each ion were used. The m/z ratio of the pseudomolecular and fragment ions selected for each compound (including the carboxylate forms) as well as the corresponding optimised orifice voltages are reported in Table 1. Lower OR (40 V) were found to yield lower signal-to-noise ratios. Furthermore, as can be seen in Fig. 3, a higher OR (70 V) resulted in fragments characteristic of the loss of CO_2 (-44), although this was more pronounced for SN-38 and I.S. than for CPT-11. This last one yielded a fragment (m/z 502) consistent with the loss of the distal piperidine moiety.

Extraction recoveries were, respectively, 68.0±7.3%, 70.5±10.0% and 68.9±5.3% at 50, 1000 and 5000 ng/ml for CPT-11 and 48.6±3.2%, 43.8±1.7% and 49.6±3.7% at 5, 25 and 75 ng/ml for SN-38. For the internal standard, the extraction recovery was 86.6±6.7% with the CPT-11 deproteination step and 73.5±10% with the SN-38 extraction procedure. Detection limits were determined as 2.5 ng/ml for CPT-11 and 0.25 ng/ml for SN-38.

Intra-assay precision and accuracy were very satisfactory, as shown in Table 2: RSD values reached a maximum of 8.1% and 3.2% for CPT-11 and SN-38, respectively and mean relative error was less than 10% at all concentrations for both compounds. The limits of quantitation could then be assessed as 10 and 0.5 ng/ml for CPT-11 and SN-38, respectively. The intermediate precision RSD values were all satisfactory, i.e., between 2.5% and 11%, except for the LOQ where 12.1 and 17.4% were found for CPT-11 and SN-38, respectively. The intermediate mean relative error was less than 10%

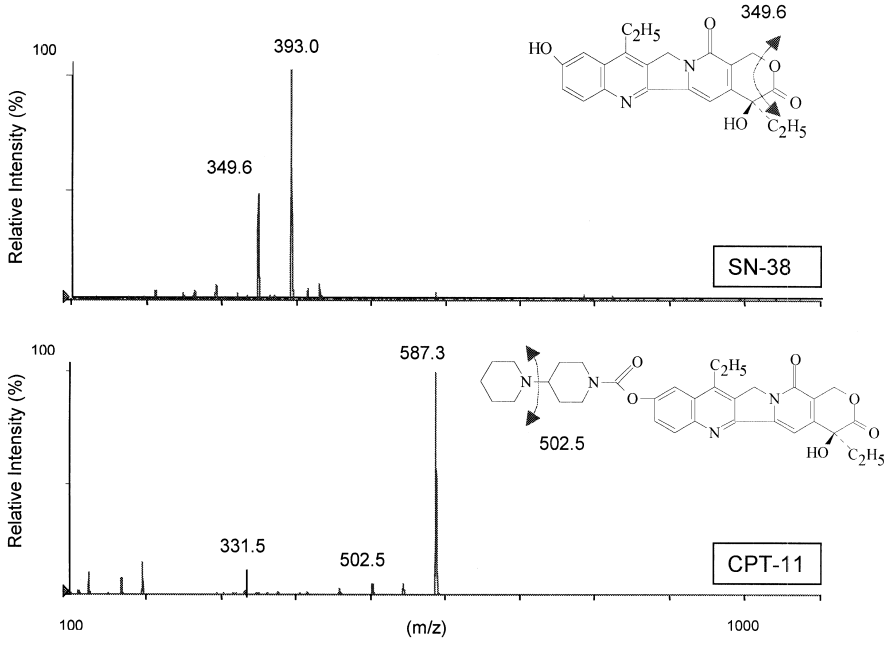

Fig. 3. Mass spectra and proposed fragmentation of CPT-11 and SN-38.

Table 2
Intra-assay precision and accuracy of the determination of total CPT-11 and SN-38 (under lactone forms) in human serum

Added concentration (ng/ml)	Mean found concentration ($n=5$) (ng/ml)	Precision (RSD, %)	Mean relative error[a] (%)
CPT-11			
50	50.0	8.1	0.0
1000	1079.2	3.7	7.9
5000	4949.2	3.0	1.0
SN-38			
1	1.1	3.2	5.5
25	22.6	2.2	9.8
75	69.6	2.6	7.2

[a] Mean relative error = |mean found *v*alue − true *v*alue| × 100/true *v*alue.

at all concentrations (Table 3). The correlation coefficients, calculated using a $1/x$ weighed regression analysis, were higher than 0.999 for both compounds.

The suitability of this method for clinical samples was demonstrated by the determination of CPT-11 and its metabolite in a blood sample obtained from a 31-year-old male patient with advanced cancer, treated with CPT-11 at a dose of 350 mg/m^2. The concentrations found in this sample, collected 14 h after the end of infusion, were 593.7 and 1.9 ng/ml for total CPT-11 and SN-38 (under lactone forms), respectively (Fig. 4). No residual carboxylate forms of either compound could be detected.

4. Discussion

The present procedure is among the most sensitive methods of determination of irinotecan and its metabolite in serum and doubtlessly one of the most specific, owing to MS. The quantitation limit of CPT-11 was similar to those obtained with conventional HPLC techniques with UV detection [25] and

Table 3
Intermediate precision and accuracy of the determination of total CPT-11 and SN-38 (under lactone forms) in human serum

Added concentration (ng/ml)	Mean found concentration (ng/ml)	Precision (RSD, %)	Mean relative error[a] (%)
CPT-11 ($n=6$)			
10	10.75	12.1	7.5
50	46.76	5.5	6.5
100	90.40	9.0	9.6
1000	991.73	5.7	0.8
5000	4902.0	5.0	2.0
10 000	10 137.0	2.5	1.4
SN-38 ($n=6$)			
0.5	0.49	17.4	2.1
1	1.01	11.0	0.7
5	5.00	10.6	0.0
10	9.99	8.7	0.1
25	25.42	7.4	1.7
50	50.74	5.8	1.5
75	74.26	3.8	1.0
100	99.46	4.9	0.5

[a] Mean relative error = |mean found *v*alue − true *v*alue| × 100/true *v*alue.

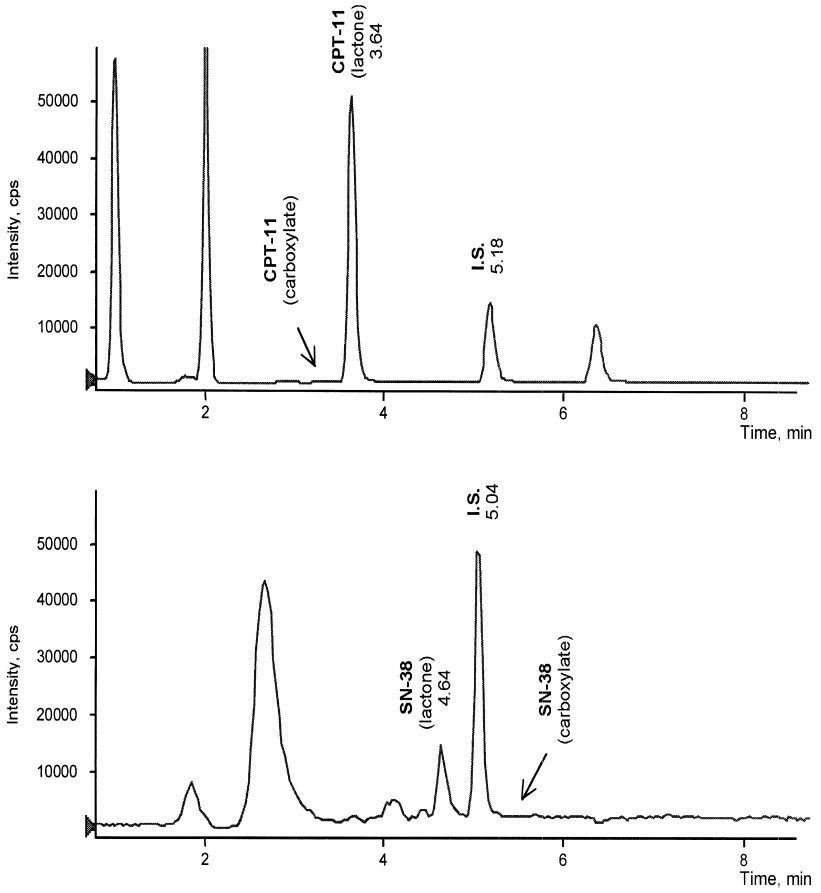

Fig. 4. HPLC chromatograms of a patient serum sample taken 14 h after i.v. infusion of 350 mg/m^2 CPT-11.

slightly higher than those reported with fluorescence detection (i.e., 2 or 5 ng/ml) [20,23]. However, it was low enough for pharmacokinetic studies, a reason why no concentration step was necessary in the extraction procedure. The SN-38 LOQ was considerably lower than those generally reported for HPLC with UV and fluorescence detection (i.e., 2 or 5 ng/ml) [20,23,25]; only Rivory et al. reported a fluorescence technique which was more sensitive than ours, with an LOQ at the low pg/ml level [24]. Moreover, UV or fluorescence detection may sometimes suffer from interfering compounds when samples from patients administered multiple drugs are analysed.

Several analytical methods for the simultaneous determination of the lactone and carboxylate forms of camptothecins have been described [13,18,19,22]. These methods were generally very complicated and an ion-pairing agent had to be employed to mask the charge of the carboxylate species. However, non-volatile counter-ions are incompatible with electrospray ionisation sources. Moreover, in these methods extraction and chromatography were generally performed at the pH of equilibrium (pH 5.5) between the two forms. As this pH is different from the physiological pH of serum, the calculated concentrations might not reflect the real serum concentrations. Furthermore, the albumin-bound carboxylate form might not be totally cleaved and extracted at this pH. Indeed, since the human serum pH is 7.4, a non-negligible fraction of CPT-11 is present in the inactive carboxylate form in serum and, under this form, preferentially binds to serum albumin by electrostatic bonds, displacing the equilibrium to-

wards carboxylate owing to the stabilisation of the bound form [28]. In other studies, the carboxylate form concentration was deduced from the difference between total and lactone form concentrations [16], which should be regarded as an approximation. Besides, determination of the lactone form requires immediate analysis because it has been shown that a significant variability may be introduced by the continuing hydrolysis of the lactone forms in the samples [29]: data from stability studies indicated that the lactone forms of CPT-11 and SN-38 were unstable during storage at room temperature or at 37°C [20,30]. This instability requires rapid freezing of clinical samples after blood collection to prevent significant degradation of lactone into carboxylate forms. The determination of total CPT-11 and SN-38 concentrations overrides this requirement. Chabot [10] reported that the area under the curve (AUC) ratio of lactone to total forms appeared to be constant between patients, and that monitoring the total form was probably a simpler and less biased alternative, as it was more convenient to assay in a clinical setting. Anyway, any attempt to study the ratio between lactone and carboxylate forms or between the lactone form and the total amount, based on any modification of the serum sample is flawed, as the equilibrium between the two chemical forms is set by experimental conditions. Therefore, in the present method, the extraction procedure and the mobile phase were optimised in order to respectively cleave the proteic bonds, transform the carboxylic into lactone form, and maintain the total amount of CPT-11 and SN-38 under this latter form through the chromatographic separation. This was verified by the absence of any chromatographic peak corresponding to the carboxylate form. Additionally, deproteinisation and extraction, though yielding mediocre recoveries, helped obtain a satisfying purity. Initially, we attempted to develop a common method to prepare and to analyse both compounds, but because of 100 times higher CPT-11 levels, as compared to SN-38 levels, we could not obtain both a high sensitivity for SN-38 and a large enough linearity range for CPT-11 and had to assay them separately. However, since extraction and chromatographic separation were very fast, this was not found a real limitation.

As illustrated by the increasing number of studies using LC–MS [31], the introduction of this reliable technology has doubtlessly improved drug bioanalysis in the field of pharmacokinetics, TDM or clinical toxicology. The present method for the quantitative determination of the total forms of CPT-11 and SN-38 in serum after conversion of carboxylate to lactone forms is rapid, sensitive, specific, precise and reliable. Its wide linearity range will be useful for drug monitoring and pharmacokinetic purposes.

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