ELSEVIER

Journal of Chromatography B, 661 (1994) 133–141

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Reversed-phase high-performance liquid chromatographic method for the simultaneous quantitation of the carboxylate and lactone forms of the camptothecin derivative irinotecan, CPT-11, and its metabolite SN-38 in plasma

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First received 10 May 1994; revised manuscript received 22 July 1994

Abstract

Irinotecan, also known as CPT-11 (I), is a potent semi-synthetic derivative of 20(*S*)-camptothecin (CPT). Like all known active derivatives of CPT, the lactone forms of I and its active metabolite SN-38 (II) are reversibly hydrolysed to inactive carboxylate forms. We describe a sensitive and selective HPLC method using the ion-pairing reagent tetrabutylammonium phosphate (TBAP) which allows the simultaneous determination of the carboxylate and lactone forms of I and II in human plasma samples following the precipitation of plasma proteins with an ice-cold mixture of acetonitrile and methanol. The mobile phase was 0.075 *M* ammonium acetate buffer (pH 6.4)–acetonitrile (78:22, v/v) containing 5 m*M* TBAP. Separation of the compounds was performed on a radially-compressed C_{18} column. The limits of quantitation in human plasma were 2 and 10 ng/ml for the two forms of II and I, respectively. In addition, we propose the use of CPT lactone both as an internal standard and as a "watchdog" for sample status. Under unsuitable storage conditions, CPT elutes increasingly in its carboxylate form thereby alerting the operator of possibly erroneous determinations of the concentrations of both forms of I and II. The retention times of the peaks of interest are well separated from the solvent front. This enables the detection of early eluting metabolites. The method was successfully used for monitoring of the two forms of I and II in patients treated with I.

1. Introduction

20(*S*)-Camptothecin (CPT, see Fig. 1), is a potent antitumour alkaloid isolated from the Chinese tree *Camptotheca acuminata* [1]. Its α-hydroxy-δ-lactone ring undergoes reversible hydrolysis (Fig. 1, scheme II) at a rate dependent on a multitude of factors including pH [2], ionic strength [2] and protein concentration [3]. The poor water solubility of the lactone form of CPT led to the use of the ring-opened sodium carboxylate salt in early clinical trials in which occasional promising anti-tumour activity was marred by unpredictable and often severe urinary toxicities [1]. It has since been demonstrated that stabilization of the DNA/topoisomerase I complex, which is considered to mediate the

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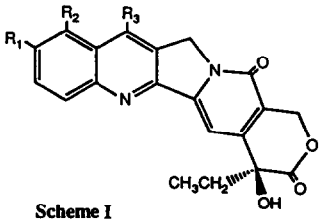
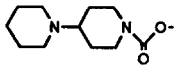
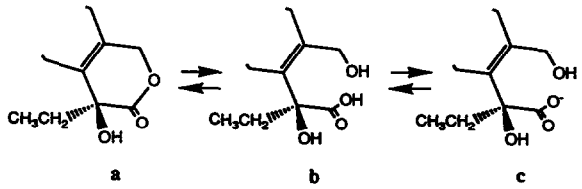
0378-4347/94/$07.00
SSDI 0378-4347(94)00340-8



Scheme I

Compound	Substituent Group R_1	R_2	R_3
CPT	H	H	H
CPT-11		H	$-CH_2CH_3$
SN-38	OH	H	$-CH_2CH_3$
Topotecan	OH	$-CH_2-N(CH_3)_2$	H



Scheme II

Fig. 1. Scheme I: structure of 20(*S*)-camptothecin (CPT) and several of its derivatives. Scheme II: schematic representation of the reversible hydrolysis of the lactone ring (ring E in scheme I). a = lactone, b = carboxylic intermediate and c = carboxylate ion.

tumour inhibitory activity of CPT, requires the drug to be present in the lactone form [1].

The search for active CPT analogues that are water-soluble in the lactone form has led to the discovery of several new derivatives. One of these, irinotecan (7-ethyl-10-{4-[1-piperidino]-1-piperidino}carbonyloxycamptothecin), also known as CPT-11 (I), has recently been synthesised at the Yakult Central Institute for Microbiological Research (Tokyo, Japan) [4]. Following the administration of I in vivo, the dipiperidino moiety is cleaved by serum and tissue esterases [5,6], yielding the metabolite SN-38 (7-ethyl-10-hydroxycamptothecin, II). This compound is 100-fold or more active in vitro than I itself [7] and I can be considered to be a prodrug of II [1].

Unfortunately, both I and II, like all known active analogues of CPT are reversibly hydrolysed to their inactive carboxylate forms and it is clear that further understanding of the pharmacology of these and other CPT analogues requires the development of a methodology enabling the quantitation of both forms.

Several methods have been described for the measurement of CPT analogues using HPLC separation and fluorescence detection. In general, however, these techniques are limited in that the carboxylate forms elute with the solvent front and only total concentrations can be estimated following the acidification of the sample to convert all the drug into its lactone form [5,8]. When samples are collected in the appropriate fashion (rapid cooling of the blood, centrifugation and protein precipitation, e.g. see Ref. [9]), the hydrolysis of lactone to its corresponding carboxylate is stopped [9,10]. Such methods can then be used to estimate selectively the concentration of the lactone form present in the plasma. The samples can then be acidified for the estimation of the total concentration and, subsequently, of the carboxylate form by subtraction. In the case of I and II, such techniques required the use of separate columns and HPLC conditions and no less than four injections of a single sample [11,12]. Furthermore, since hydrolysis of the lactones may continue with improper storage or handling of the samples [9,13], there is no means of verifying the quality of the data thus obtained. Recently, HPLC methods using the cationic ion-pairing reagent tetrabutylammonium phosphate (TBAP) have been developed [13,14]. This compound causes an increased retention of the carboxylate forms of CPT analogues such that they can be dissociated from the solvent front.

The aim of this study was to develop a sensitive and selective HPLC method using TBAP which would enable the simultaneous determination of both forms of I and II in plasma and allow an assessment of sample quality to be made.

2. Experimental

2.1. Chemicals and HPLC reagents

The lactone forms of I and II, prepared by Yakult Honshua (Tokyo, Japan), were obtained from Bellon (Groupe Rhône-Poulenc Rorer, Neuilly, France). Compound II was dissolved in DMSO at a concentration of 5 mg/ml and stored at −20°C. CPT, obtained from Sigma (St. Louis, MO, USA), was purified further by HPLC (as per below) to remove minor but potentially interfering peaks. The collected fractions corresponding to CPT lactone were extracted with chloroform, dried and redissolved in DMSO. TBAP was purchased from Waters (Millipore, St. Quentin-en-Yvelines, France) as a ready-to-use solution (PIC A). The water used was of Milli-Q grade and all solutions used in the HPLC analysis were filtered and degassed using a 0.5-μm filtration system (Millipore). The mobile phase was 0.075 *M* ammonium acetate buffer (pH 6.4)–acetonitrile (78:22, v/v) to which one vial of PIC A solution was added. The final concentration of TBAP was 5 m*M*.

2.2. Apparatus

Separation of the compounds of interest was carried out at ambient temperature on a Waters (Millipore) Nova-Pak Radial-Pak C_{18} reversed-phase column (100 × 5 mm I.D., 4 μm particle size) preceded by a matching C_{18} guard column (Guard-Pak, Nova-Pak, Waters). The mobile phase was delivered by a M-45 Waters HPLC pump at a flow-rate of 1.5 ml/min. Detection was carried out with a Hitachi F-1050 fluorimeter (Merck, Nogent-sur-Marne, France) and peak data were recorded by a Perkin-Elmer FCI-100 integrator (Perkin-Elmer, Toulouse, France). Samples were injected onto the chromatogram with a Hamilton syringe (Millipore) by means of a Waters U6K manual injector equipped with a 2-ml sample loop.

2.3. Method optimisation

Standards of I, II and CPT were prepared in acetonitrile–0.01 *M* citric acid (pH ~ 3) (50:50, v/v) for the lactones and acetonitrile–0.01 *M* sodium tetraborate (pH ~ 9) (50:50, v/v) for the carboxylates. Conversion of the lactones into carboxylates with the acetonitrile–sodium tetraborate solution was virtually complete (> 99%) as determined by HPLC. Addition of citric acid to the lactone standards ensured that conversion to the carboxylates did not occur. The standards were stored in sterile silicone treated glass tubes (Venoject) at 4°C and appeared to remain stable for several months, yielding identical standard curves over this period of time. Injections of 1 ng (10 μl) of each of the six compounds, prepared by further dilution of the appropriate standard into buffer (pH 3 or 9 as required), were made directly onto the column and peak area data were recorded at several different excitation (355–385 nm) and emission (450–550 nm) wavelengths.

2.4. Method validation

Purified CPT lactone stock solution was diluted in ice-cold acetone and 50-μl aliquots (containing 0.05 μg CPT) were added to each of a series of polypropylene Mini-Eppendorf tubes (Treff Lab, Degersheim, Switzerland). The solution was evaporated under a stream of nitrogen and the tubes were stored at −20°C. Plasma standards were prepared by the addition of 5 μl of the appropriate standard solution and 50 μl of fresh human plasma to one of these tubes. These were rapidly vortex-mixed and the plasma proteins were precipitated by the addition of 100 μl of a solution of ice-cold methanol and acetonitrile (50:50, v/v). The tubes were vortex-mixed for 5 s and centrifuged briefly (max 8000 *g*). Part of the supernatant (100 μl) was transferred to a fresh Mini-Eppendorf tube and 70 μl of the mobile phase buffer were added. This solution was briefly vortex-mixed and 5–20 μl injected onto the column.

The recovery of the carboxylate and lactone forms of I and II was investigated using similarly prepared standards. Three different concentrations of both forms of I and II were analysed, whereas the internal standard (CPT lactone) was investigated at the concentration used in sample

analysis. The measured peak areas were then compared to those recorded when the plasma was replaced by an equal volume of phosphate-buffered saline (PBS, pH 7.4).

Repeated injections ($n = 6$) of carboxylate and lactone standards of I prepared with plasma as above at five different concentrations (10 ng/ml–12.5 μg/ml) were performed on a single day to establish the within-day coefficient of variation (precision) and the mean accuracy. A slightly different range of concentrations (2 ng/ml–2.5 μg/ml) of the carboxylate and lactone standards of II were used in the same manner.

Carry-over between injections was minimal (typically $< 1\%$). However, prior to each sample run, the syringe was rinsed with 0.2 *M* NaOH, water and methanol and the injector loop was back-flushed for approximately one minute with mobile phase at a flow-rate of 1.5 ml/min. Standards and samples were assayed in order of increasing concentration.

2.5. Pharmacokinetics

The pharmacokinetics of I and II were investigated in several patients undergoing therapy with I. Blood samples were withdrawn at various times during and after the infusion. A blank sample was taken prior to treatment. The samples were collected in heparinised tubes and immersed briefly into a dry ice/acetone bath kept at the bedside taking care not to freeze the blood. The sample was rapidly aliquoted into cold Eppendorf tubes, centrifuged for 2 min (4°C, 8000 *g*) and the resulting plasma was then treated in a way identical to that described in section 2.4. The supernatants were transferred to fresh Eppendorf tubes, immediately stored at −20°C and later transferred to a −70°C freezer.

Prior to analysis, stored samples were transferred to a −20°C freezer for 1 h, vortex-mixed and centrifuged briefly (0°C, 8000 *g*). Aliquots of the supernatant (100 μl) were diluted with 70 μl of mobile phase buffer just prior to injection (5–20 μl). The samples were constantly kept on ice since this prevented significant degradation of the sample (increased presence of CPT carboxylate).

3. Results and discussion

3.1. Method optimisation

As expected, we found that the strength of the buffer (0.05–0.10 *M*) had a large influence on the retention of the carboxylate forms of I and II (increasing strength resulting in decreasing retention) and that the retention of I (both its lactone and carboxylate form) was sensitive to the buffer pH in the range of 5.0 to 6.5 (increasing pH giving increasing retention). By combining these two factors, we developed the conditions as described in the section above. This gave retention times of 4.1, 5.2 and 6.5 min for the carboxylates and 8.2, 9.3 and 10.4 min for the lactones, respectively (Fig. 2A).

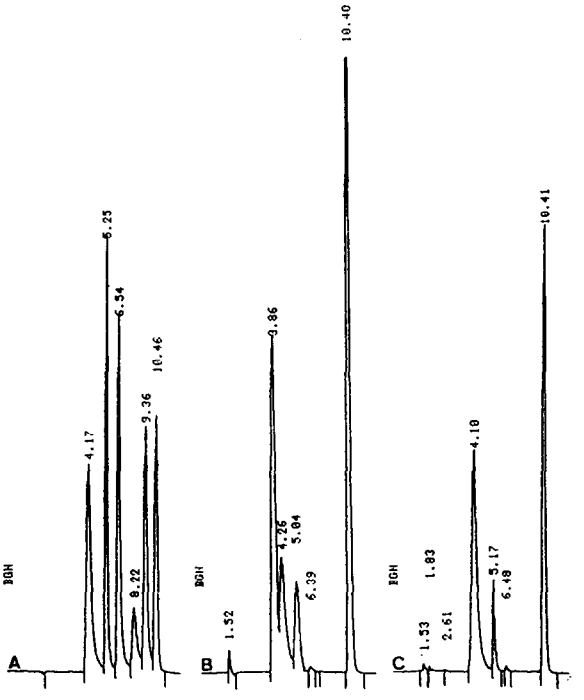


Fig. 2. Chromatograms of (A) mixture of the carboxylates of I, II and CPT (4.2, 5.2 and 6.5 min) and their corresponding lactones (8.2, 9.4, 10.5 min) in aqueous solution (~ 1 ng of each); (B) carboxylate standards of I and II in the presence of the internal standard CPT lactone (10.4 min); note that the carboxylate forms of I and II elute as twin peaks (I: 3.86 and 4.26 min) and an unresolved complex (II: 5.04 min), respectively; (C) the same sample as in (B) after dilution with mobile phase buffer.

Wavelength optimisation revealed that the fluorescence responses of I and CPT were very different from those of II under the assay conditions employed (Fig. 3) when the most favourable excitation wavelength was used (355 nm). Studies published on the pharmacokinetics of I showed that the plasma concentrations of I are often 10–20 times higher than those of II. Hence, we selected the emission wavelength (515 nm) so as to bias the detector response in favour of II. The resulting wavelength couple (ex = 355 nm, em = 515) seemed satisfactory for both the lactone and carboxylate forms of I, II and CPT (Fig. 3).

3.2. Method validation

The recoveries of the compounds of interest from fresh human plasma are shown in Table 1. The recovery of the internal standard, CPT lactone, measured at the concentration used was 101.1 ± 5.8% ($n = 20$). The limit of quantitation (LOQ) in plasma was approximately 2 ng/ml for both forms of II and 10 ng/ml for those of I. Peaks for each of the four compounds could be identified by the detector at concentrations as low as 0.5 ng/ml (II lactone) whereas higher concentrations were required for the detection of I (~2 ng/ml). All four compounds gave linear responses as a function of concentration. Because of the large range of concentrations studied (10 ng/ml–12.5 μg/ml and 2 ng/ml–2.5 μg/ml for I and II, respectively), we opted for log–log linear regression to prevent domination of the higher concentrations. The mean correlation coefficients (r) for the daily calibration curves were all >0.999 ($n = 6$). The validation data in terms of within-day precision and accuracy are represented in Table 2. Similar variability was observed for the between-day precision and accuracy (data not shown).

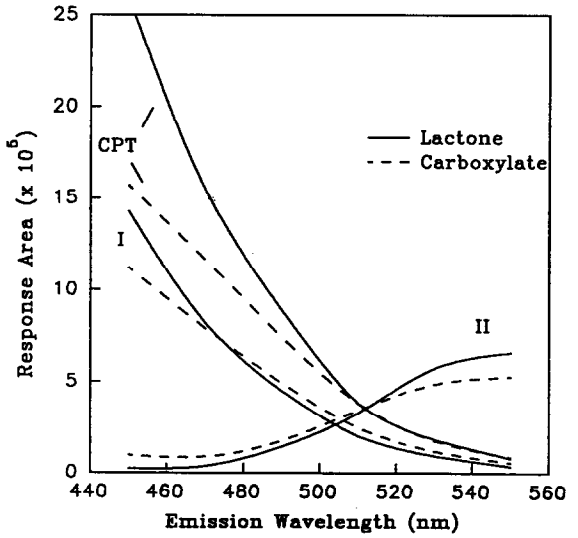


Fig. 3. Detector response during the assay of I, II and CPT (1 ng each) in their carboxylate and lactone forms as a function of the emission wavelength (ex = 355 nm).

Table 1
Recovery (%) of drug from spiked human plasma as compared to that from spiked phosphate-buffered saline (mean ± S.D.)

Concentration	n	Recovery (%)	
		Carboxylate	Lactone
Compound I			
10 ng/ml	4	110.9 ± 10.6	96.4 ± 22.0
0.5 μg/ml	3	93.8 ± 2.1	105.1 ± 3.4
12.5 μg/ml	3	97.1 ± 1.5	89.6 ± 17.3
Compound II			
2 ng/ml	4	132.2 ± 29.6	105.8 ± 15.1
0.1 μg/ml	3	94.6 ± 2.1	105.4 ± 4.1
2.5 μg/ml	3	97.6 ± 1.7	90.4 ± 5.8

3.3. Sample treatment and stability

We compared the protein-precipitating properties of several organic solvents (methanol, acetonitrile, ethanol, acetone and tetrahydrofuran) and their mixtures using as criteria: (1) the volume required for protein precipitation, (2) the resulting baseline distortion, (3) the tendency to freeze at −70°C, and (4) the homogeneity of the precipitate. Based on these criteria, we found that the 50:50 (v/v) mixture of methanol and acetonitrile was the most satisfactory. The addition of buffer to the protein-free supernatant was found to be an essential step in the assay. Omission resulted in the carboxylate forms of I and II eluting as twin peaks or as a

Table 2
Within-day precision (C.V.) and accuracy of the HPLC determination of the carboxylate and lactone forms of I and II in human plasma

Concentration (ng/ml)	Carboxylate		Lactone	
	C.V. (%)	Accuracy (%)	C.V. (%)	Accuracy (%)
Compound I				
10	15.4	98.9	20.2	110.0
50	3.8	101.3	5.7	85.8
500	2.8	100.7	2.8	104.4
2500	3.1	100.9	2.3	102.1
12 500	1.0	100.0	2.3	100.9
Compound II				
2	17.0	105.9	19.6	110.0
10	11.8	94.9	9.5	92.4
100	2.6	97.3	2.4	90.1
500	2.9	101.8	3.3	94.8
2500	0.8	104.1	3.7	111.8

$n = 6$.

complex of unresolved peaks (see Fig. 2B,C). This artefact was also seen when other solvents were used to precipitate the plasma proteins or when plasma was substituted with PBS. However, the appearance of the complexes varied largely, depending on the composition of the mixture. Addition of water, although equally good in correcting this problem, was not as satisfactory, since the hydrolysis of CPT lactone increased to ~5% of the total within 15–20 min at 4°C. In comparison, the same degree of hydrolysis was observed only after 2–3 h in samples treated with buffer under identical conditions. The buffer composition (pH 6.4) corresponds to the conditions in which lactone hydrolysis is slowest [2].

The fact that the artefact mentioned above could be removed by the addition of buffer suggests that it might be due to self-association of the carboxylate forms of the drugs. Indeed, it is well recognised that carboxylic acids are largely present as dimers in organic solvents due to the formation of hydrogen bonds between the carboxyl functions [15]. In water, these are almost totally replaced by hydrogen bonding with the surrounding water molecules. Dimerisation would explain the existence of multiple forms of the carboxylate and the dimers could elute faster if they have less interaction with TBAP.

Besides its use as the internal standard, CPT also served as a "watchdog" for sample freshness (Fig. 4B). Since A-ring substituted camptothecin analogues have similar ring-hydrolysis kinetics in protein-free media [2], the conversion of CPT to the carboxylate form should be representative of that of I and II, thereby serving as an indicator of continued hydrolysis during storage and handling. Protein-free supernatants of plasma samples kept at −70°C appeared to be stable for several months. However, samples degraded rapidly if they were inadvertently left at room temperature. We set an arbitrary limit of 5% as the maximum tolerable area ratio of CPT carboxylate to total CPT for accurate analysis.

3.4. Pharmacokinetics

The method appears to be sensitive and linear, and therefore, suitable for the performance of pharmacokinetic studies in patients. Before attempting patient studies, we first confirmed that I in the infusion was present only as the lactone (data not shown). Plasma collected prior to the

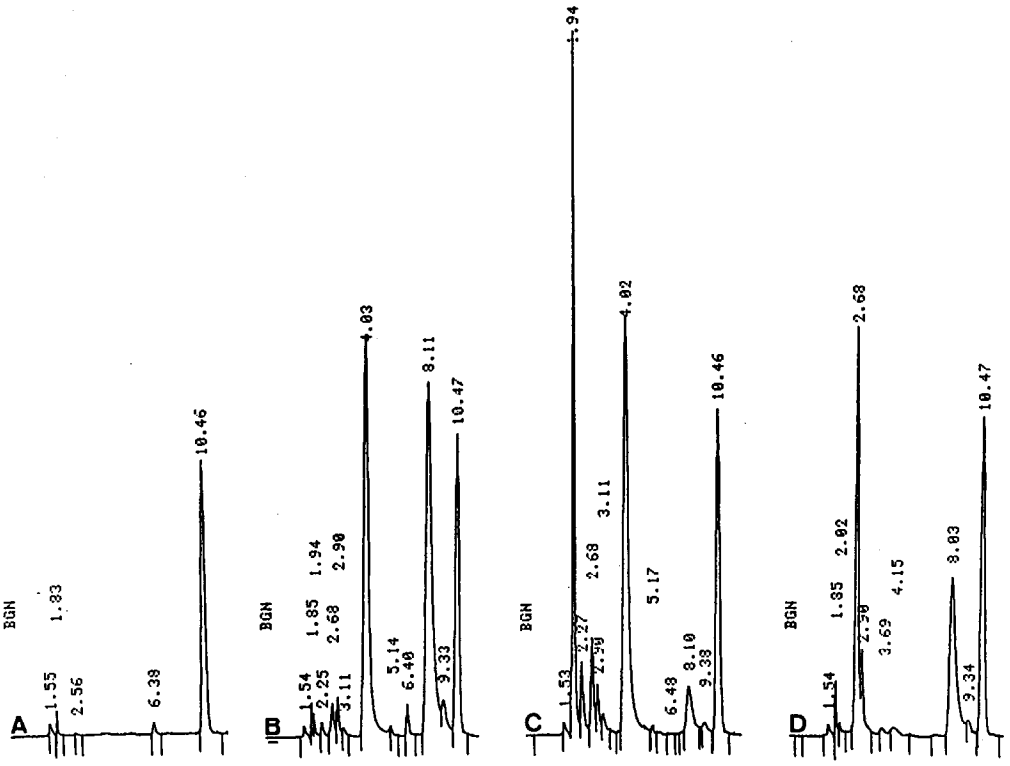


Fig. 4. Representative chromatograms of plasma extracts of a patient treated with I. (A) Pre-infusion sample; the internal standard (CPT lactone), which has been slightly degraded to its carboxylate (6.4 min) elutes at 10.46 min. (B) Sample taken during infusion; the hydrolysis of the internal standard to its carboxylate (> 5%) indicates that the assay of this sample may be erroneous due to continued hydrolysis during storage and handling. (C) Sample taken 6 h after the end of the infusion. (D) The same sample as in (C) after acidification.

infusion revealed one small endogenous peak (Fig. 4A). Concentrations of the carboxylate and lactone forms could be readily estimated from the protein-free extracts and acidification of such samples (addition of 5 μl of 0.1 *M* HCl) reconverted the carboxylates into their corresponding lactones (Fig. 4D). Under the current chromatographic conditions, four additional peaks eluted early on the chromatograph. These are likely to represent the carboxylate and lactone forms of two metabolites since they were reduced to two peaks following acidification of the sample. Indeed, two of these peaks (at 2.3 and 2.9 min, respectively, Fig. 4) have been identified as the carboxylate and lactone forms of a β-glucuronide of II [16].

In the patient plasma, I was present mostly as the lactone until the end of the infusion (Fig. 5). Continued transformation of the lactone form of I into the carboxylate form resulted in the concentrations of the carboxylate peaking later than those of the lactone. The proportion of I present as the carboxylate then reached a relatively stable value of approximately 75% some 2–3 h after the end of the infusion (Fig. 6). The observed post-infusion predominance of the carboxylate form is in agreement with previously reported studies [11,12,17]. The situation is very much different for II, with the lactone being the predominant form at all times in the plasma of the patient studied. This could be partly explained by the preferential binding of the carboxylate form of II to serum albumin [18], which greatly reduces both the rate and the extent of the hydrolysis of II in plasma compared with other camptothecin derivatives [19]. Further

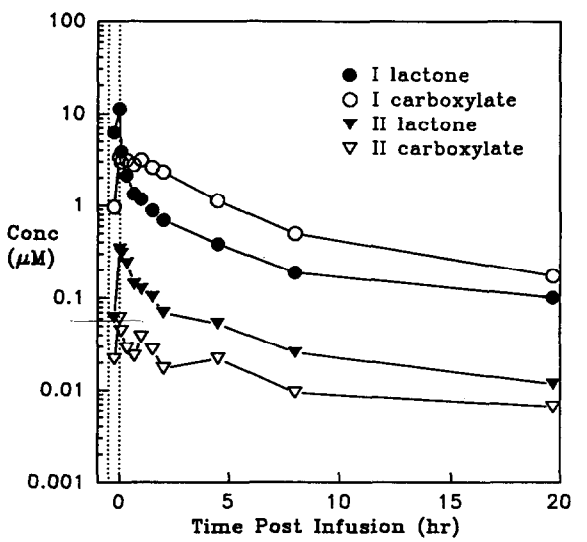


Fig. 5. Concentration–time profile of the carboxylate and lactone forms of I and II determined in the plasma of a patient. The vertical dotted lines delimit the period of infusion.

modification of the plasma lactone/carboxylate ratio of II in vivo could also arise from different metabolism/elimination of the two forms. In fact, large interindividual variability in the contribution of the lactone form to the total AUC of II has recently been reported, with values ranging from 7 to 75% [12]. The mechanisms underlying such variability clearly merit further investigation.

We have also used this HPLC method successfully in studies of the pharmacokinetics of I in rats. We also intend to modify this technique to enable the simultaneous quantitation of the other metabolites once they are identified. Such studies are vital to the continued elucidation of the pharmacology of I and II.

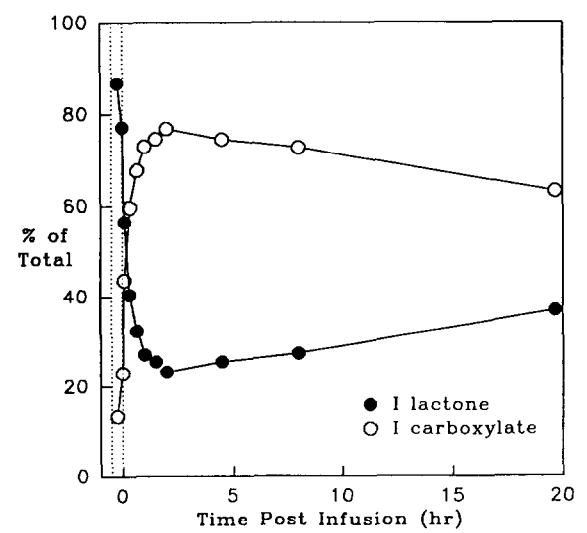


Fig. 6. The percentage of total I present as carboxylate and lactone in the plasma of the same patient as in Fig. 5.

Acknowledgements

The authors wish to thank Dr. Y. Bécouarn for his assistance with the patient study. This work was funded in part by the Fédération Nationale des Groupements des Entreprises Françaises dans la Lutte Contre le Cancer. L.P.R. is the recipient of a NHMRC/INSERM exchange fellowship.

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