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Simultaneous determination of the lactone and carboxylate forms of the camptothecin derivative CPT-11 and its metabolite SN-38 in plasma by high-performance liquid chromatography

Daniel F. Chollet[a,*], Laurent Goumaz[a], Alain Renard[b], Guy Montay[c], Laurent Vernillet[c], Valdo Arnera[a], David J. Mazzo[c]

[a]*CORNING SciCor SA, Bioanalytical Division, 7 Rue M. Marcinhes, CH-1217 Meyrin/Geneva, Switzerland*
[b]*Rhône-Poulenc Rorer Drug Metabolism and Pharmacokinetics Department, 13 Quai Jules Guesde, F-94403 Vitry-Alfortville, France*
[c]*Rhône-Poulenc Rorer Drug Metabolism and Pharmacokinetics Department, 20 Avenue Raymond Aron, F-92165 Antony, France*

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Abstract

CPT-11 (irinotecan) and mainly its metabolite SN-38 are potent antitumor derivatives of camptothecin. As the active lactone forms of both CPT-11 and SN-38 exist in pH-dependent equilibrium with their respective less potent open-ring hydroxy acid species, the simultaneous monitoring of both forms of both compounds is relevant. CPT-11 and SN-38 derivatives have quite different fluorescence responses. In order to avoid any compromise on the wavelength setting, we developed chromatographic conditions allowing simple automated wavelength setting changes which have been prevented using existing methods involving conventional C_{18} columns. This was achieved by means of a Symmetry C_{18} column combined to a gradient elution program using acetonitrile and 75 m*M* ammonium acetate plus 7.5 m*M* tetrabutylammonium bromide at pH 6.4. The developed conditions allowed an elution order suitable for a simple automated wavelength change in respect to reliable peak integration. CPT-11 and SN-38 derivatives were detected at λ_{ex}=362 nm/λ_{em}=425 nm and λ_{ex}=375 nm/λ_{em}=560 nm, respectively. The developed method allowed the detection of amounts less than 3 pg of each derivative injected on column. The method was successfully applied to pharmacokinetic and toxicokinetic studies in rat and dog.

Keywords: Irinotecan; CPT-11; SN-38; Camptothecin

1. Introduction

Camptothecin (CPT), an alkaloid isolated from the Chinese tree *Camptotheca acuminata*, and its semi-synthetic derivatives such as CPT-11 (irinotecan, **I**), are promising anticancer agents presently undergoing extensive clinical evaluation worldwide (reviews, see Refs. [1–4]). Each agent of this class of compounds contains a closed α-hydroxy-δ-lactone ring (lactone form) that undergoes reversible hydrolysis leading to the open-ring hydroxy acid (carboxylate form). The hydrolysis rate is dependent on several factors including pH [2], ionic strength [5] and protein concentration [6,7]. It has been demonstrated that the

*Corresponding author. Present address: COVANCE Central Laboratory Services SA, 7 Rue M. Marcinhes, CH-1217 Meyrin/Geneva, Switzerland.

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biological activities of camptothecins both in vitro and in vivo are significantly greater for the lactone than for the carboxylate species and that stabilization of the DNA–topoisomerase complex, which is considered to mediate the tumor inhibitory activity of camptothecins, requires the drug to be present in the lactone form [1].

Compound **I** undergoes deesterification to SN-38 (**II**) [8], which has 100–1000-fold more topoisomerase I-inhibitory activity than **I** in vitro [9]. Similarly to camptothecin derivatives, **II** exists in a equilibrium with less potent open-ring carboxylate form. Because all known active analogues of camptothecin are reversibly hydrolyzed to their inactive carboxylate forms and because the major activity of **I** may be attributed to its more active metabolite **II**, analytical methods suitable for the quantification of both forms of both **I** and **II** are required. In addition, due to stability issues e.g., lactone/carboxylate equilibrium, **I** photodegradation [10], proper sample handling throughout processing is of paramount importance to obtain reliable results.

Several high-performance liquid chromatographic (HPLC) methods have been developed for the determination of camptothecin derivatives in buffered solutions [11,12], in biological fluids [9,12–26] and in mice tumors [24]. Biological sample preparation has been performed either by fast deproteneization of chilled blood sample in cold methanol [9,13–16,21,25,26] or by regular solid-phase extraction on disposable extraction columns [17–20,24] or by liquid–liquid extraction [23,26]. Camptothecin derivatives were then separated using a C_{18} reversed-phase columns at temperature ranging from ambient temperature to 60°C in isocratic mode. They were detected by fluorometric detection. Only the method reported by Rivory and Robert [16] was able to determine simultaneously the lactone and carboxylate forms of both **I** and **II** in plasma in a single run. However, because the native fluorescence of **I** and **II** are quite different and because the plasma concentration of **I** are 10–20-fold higher than those of **II**, these authors [16] were constrained to use a compromise for the detection excitation and emission wavelengths. The elution order of the compounds of interest as well as the relative retention of those prevented a possible wavelength automated change.

In this paper, we described a HPLC method for the simultaneous determination of the lactone and carboxylate forms of **I** and its metabolite **II** in dog and rat plasma using a fluorometric detector set at the most favorable wavelength couples for each compound. This was achieved after chromatographic investigations leading to the modification of the elution order of the compounds of interest. The modified elution order was obtained by the combination of a suitable reversed-phase column and a gradient elution that allowed the necessary automated wavelength change.

2. Experimental

2.1. Chemicals

Compound **I** lactone form (CPT-11; irinotecan; RP 64174A; diethyl-4,11 hydroxy-4 (piperidino-4 piperidino-carbonyloxy)-9 1H-pyrano (3′,4′,6,7) indolizino (1,2-b) quinolein-(4H,12H) dione-3,14 hydrochloride trihydrate; M_r 677.20) and **II** lactone form (SN-38 monohydrate; M_r 410.43) were kindly supplied by Rhône-Poulenc Rorer (Vitry sur Seine, France). Compound **I** carboxylate form (free base, M_r 604.71) and **II** carboxylate form (M_r 410.43) were prepared from **I** and **II** lactone forms, respectively. Methanol, acetonitrile and tetrahydrofuran (THF) were of HPLC grade from Rathburn Chemicals (Walkerburn, UK). Water was purified by means of a Milli-Q Plus device from Millipore (Millipore, Le Mont, Switzerland). All other reagents were of analytical grade from either Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland).

2.2. Plasma samples

Male and female Sprague Dawley rats (Charles River, Saint Aubin les Elbeuf, France) as well as male and female beagle dogs (Marshall Farms or C.E.D.S.) were used.

As pre-requisite to this work, the plasma samples were collected according to the method developed by Rowinsky et al. [9]. Minor modifications were applied. Briefly, the heparinized dog blood samples chilled in ice were immediately centrifuged (1500 g/10 min/5°C). A 1-ml aliquot was then quickly placed into a polypropylene tube containing 6 ml of

cold methanol (−20°C). The tubes were carefully vortex-mixed, centrifuged (1500 *g*/10 min/−8°C) and frozen at −70°C. Rat samples were prepared as dog samples replacing 1-ml plasma aliquots with 0.5-ml aliquots and 6 ml of cold methanol with 3 ml, respectively. Both sample preparation procedures were shown to prevent lactone/carboxylate equilibrium displacement and hydrolysis of **I** to **II** [27].

2.3. Chromatographic equipment and conditions

LaChrom and a LiChrograph HPLC systems from Merck–Hitachi (Merck–ABS, Geneva, Switzerland) were used. The LaChrom system consisted of a Model L7100 pump, an autosampler Model L7250 equipped with a temperature-controlled rack and a Model L7480 fluorescence detector. The LiChrograph system consisted of a Model L6200A pump, an autosampler Model AS4000 equipped with a temperature-controlled rack and a Model F1080 fluorescence detector. The eluents of both systems were degassed by means of GasTorr Model GT-104 degassing units (Omnilab, Chavannes-des-Bois, Switzerland) and the temperature racks were maintained at 2±1.5°C. A Maxima 825 data station from Waters (Waters, Le Mont/Lausanne, Switzerland) was used for data acquisition from both systems. Cross-validation of the two HPLC systems showed equivalent results.

The HPLC separations were achieved using a Symmetry C_{18} column, 150×3.9 mm I.D., d_p=5 μm from Waters. In addition, the preliminary investigations were performed by means of a Supelcosil LC-ABZ column (150×4.6 mm I.D., d_p=5 μm) from Supelco (Bellafonte, PA, USA) and by means of either C_{18} or C_8 or CN Nucleosil columns (125×4.0 mm I.D., d_p=5 μm) from Macherey Nagel (Oensingen, Switzerland).

The final aqueous mobile phase (A) was a mixture 0.075 *M* ammonium acetate plus 7.5 m*M* tetrabutylammonium bromide adjusted to pH 6.40 with glacial acetic acid. The organic modifier (B) was acetonitrile. For dog plasma samples, the mobile phase was pumped in the isocratic mode with 18% B (v/v) for 3.50 min. An elution gradient increasing linearly the proportion of B from 18% to 29% was applied between 3.50 min and 10.50 min. The proportion of B was then returned to the initial conditions in 2.00 min. For rat plasma samples, the mobile phase was pumped in the isocratic mode with 16% B (v/v) for 7.00 min. An elution gradient increasing linearly the proportion of B from 18% to 29% was applied between 7.00 min and 14.00 min. The proportion of B was then returned to the initial conditions in 2.00 min. In both elution programs, the flow-rate of the mobile phase was 1.8 ml/min. The injected volume was set at 10 μl. The equilibration time was 4.50 min for both elution programs.

The detection program for dog plasma assay was t=0.00 min, autozero λ_{ex}=362 nm, λ_{em}=425 nm; t=7.20 min, autozero λ_{ex}=375 nm, λ_{em}=560 nm; t=17±1 min, autozero λ_{ex}=362 nm, λ_{em}=425 nm. For rat plasma assay, the detection program was t=0.00 min, autozero λ_{ex}=362 nm, λ_{em}=425 nm; t=11.8 min, autozero λ_{ex}=375 nm, λ_{em}=560 nm; t=17±1 min, autozero λ_{exc}=362 nm, λ_{em}=425 nm.

2.4. Sample preparation

The methanol extracts stored at −70°C were quickly transferred to a 1.5-ml autosampler amber vial stored at room temperature. They were then immediately capped and placed in the temperature-controlled rack. As a rule, a new sample was prepared as soon as the assay of the previous sample was finished.

2.5. Standard solutions

All solutions were prepared in amber volumetric flasks. Both stock solutions and working solutions of the lactone forms of **I** plus **II** were prepared in dimethyl sulfoxide (DMSO). The stock solutions of the carboxylate forms of **I** plus **II** were prepared by the dilution of the stock solution of the lactone forms of **I** plus **II** with acetonitrile–0.02 *M* borate buffer, pH 9.0 (50:50, v/v). The working solutions were prepared by successive dilution in acetonitrile–0.02 *M* borate buffer, pH 9.0 (50:50, v/v).

Standard samples were prepared by the dilution of the suitable either working or stock solutions in cold methanol (2.0±1.5°C) and immediately deep frozen in amber glass vials. The standard samples were stable at less than −70°C for six months.

2.6. Calibration curves

Typical calibration curves were constructed by means of standard samples (six levels) containing both lactone and carboxylate forms of both **I** and **II**. The calibration ranges were 0 to 1.604 nmol/ml, 0 to 16.041 nmol/ml, 0 to 1.274 nmol/ml and 0 to 12.737 nmol/ml for **I** carboxylate, **I** lactone, **II** carboxylate and **II** lactone, respectively. The calibration curves were obtained by plotting the peak area of the analytes versus their respective nominal concentrations expressed in nmol/ml. The equations were calculated using linear regression. Response weighting ($1/\text{conc.}$ or $1/\text{conc.}^2$) was applied.

2.7. Method validation

The validation of the methods was performed according to Ref. [28]. The extraction recoveries from both dog and rat plasma were calculated along the calibrated range (six calibration levels) by comparison of the peak area measured for extracted spiked plasma samples and that of the corresponding theoretical amount injected directly in the HPLC system (=100% recovery). The stability of the lactone and carboxylate forms of **I** and **II** in aqueous solutions was measured under darkness in 0.5 *M* phosphate buffer solutions (10 μl of either **I** lactone or **I** carboxylate or **II** lactone or **II** carboxylate stock solution in 1 ml of buffer) at pH 3.0, 4.0, 5.2, 6.4, 7.4 and 10.0. The solutions were placed in a temperature-controlled rack.

3. Results and discussion

3.1. Reference solutions

Because of the poor solubility of **II** and because the carboxylate form was not available as pure reference powder, difficulties were encountered for the preparation of concentrated **II** derivatives stock solutions. Some recommended media such as 0.1 *M* or 0.01 *M* sodium hydroxide [29,30], which are unsuitable for direct injection into the HPLC system, were avoided. Finally, the stock solutions of the lactone forms were prepared in DMSO. The stability of the lactone forms of both **I** and **II** was studied under darkness at 25°C for 20 h. The overall mean of remaining product within this time period was 98.8±1.1% (n=40) and 98.3±1.4% (n=40) for **I** lactone and **II** lactone, respectively. The stock solutions of carboxylate forms were prepared according to Rivory and Robert [16] by dilution of the lactone solutions in acetonitrile–borate buffer, pH 9.0. These solutions were found to be stable for up to 19 h under darkness at 2.0±1.5°C. The overall mean of remaining product within this time period was 97.9±2.0% (n=19) and 99.7±2.5% (n=19) for the carboxylate forms of **I** and **II**, respectively.

3.2. Stability data

3.2.1. Aqueous buffer solutions

The relative amounts of both **I** and **II** lactone/carboxylate at the equilibrium stage at different pH in aqueous buffer solutions at 10.0±0.2°C are shown in Fig. 1. The hydrolysis and the lactonization of both **I** and **II** in aqueous buffer solutions at 10.0±0.2°C are shown in Fig. 2. The apparent first-order rate constants (k) for the hydrolysis of **I** lactone were found to be $-0.344\ h^{-1}$ and $-1.533\ h^{-1}$ at pH 6.4 and 7.4, respectively. For the hydrolysis of **II** lactone these constants were found to be $-0.346\ h^{-1}$ and $-1.680\ h^{-1}$, respectively.

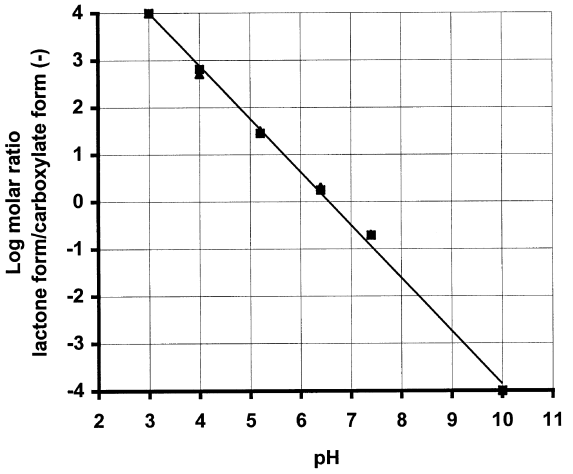

Fig. 1. The relative amounts of lactone/carboxylate forms at equilibrium in buffer solutions at different pH. Temperature 10±1°C, ■=**I**, ▲=**II**.

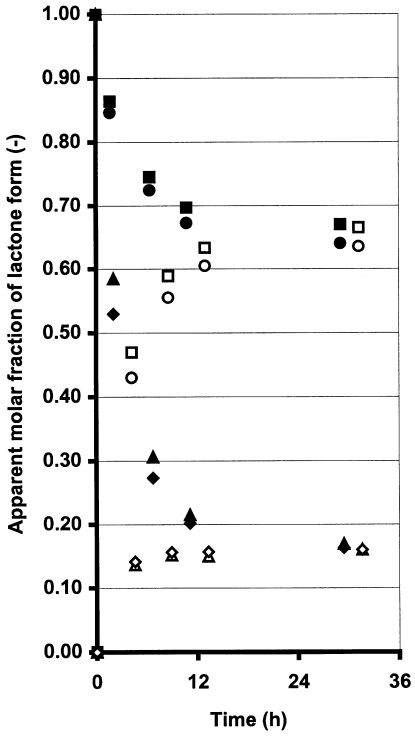

Fig. 2. Transformation of the lactone forms of both **I** and **II** (filled symbols) to the corresponding carboxylate forms and transformation of the carboxylate forms of both **I** and **II** (blank symbols) to the corresponding lactone forms at 10°C in buffered solutions at pH=6.4 and 7.4, respectively. ■=**I**, pH 6.4, ●=**II**, pH 6.4, ▲=**I**, pH 7.4, ♦=**II**, pH 7.4. Each point is the result of a single measure at the given time. The results obtained at pH 3.0, 4.0, 5.2 and 10.0 are not shown. The apparent molar fractions were measured under the recommended experimental conditions.

3.2.2. Aqueous buffer solutions containing organic modifiers

As reported by Rivory and Robert [16], equilibrium displacements were observed when organic modifiers were mixed to aqueous buffer solutions. Investigations performed showed that this displacement depended in a large extent of the buffer used and of the organic modifier content of the mixture. As an example, no equilibrium displacement was observed in the acetonitrile–borate buffer, pH 9.0 mixture used for the carboxylate reference solutions but 10% of both carboxylate and lactone forms of both **I** and **II** were converted to the other forms when borate buffer was replaced with phosphate buffer, pH 9.0. The stability of carboxylate and lactone forms of both **I** and **II** during the chromatographic run were studied at 3°C, 22°C and 37°C in the mobile phase A–B (82:18, v/v) for a time period greater than twice the retention time of the most retained analyte. Below 22°C, the rate of transformation of both **I** and **II** lactone forms were found to be less than 1.0%. Values below 1.8% were calculated for the transformation of their respective carboxylate forms into the lactones. At 37°C, these values were less than 5.4% (lactone forms to carboxylate forms) and less than 12.2% (carboxylate forms to lactone forms), respectively.

3.2.3. Methanol extracts

Methanol extracts have been found to be stable at −70°C for more than 1.5 year [9]. However, the transformation of either lactones to carboxylates or carboxylates to lactones from a form to another was observed at 2°C i.e., under the recommended experimental conditions. The transformation of a form to another versus time was approximately linear (r=0.96 to 0.99) for the four analytes within the first 15 h with a mean rate constant of 0.5% h^{-1}. Because of that transformation, the automated routine analysis runs were limited to 5 h. No noticeable variation of the transformation rate was observed for low and high concentrations of all analytes as well as for methanol extracts from either dog plasma or rat plasma or human plasma.

3.2.4. Dog and rat whole blood

Systematic investigations were performed with both dog and rat whole blood samples as well as with aqueous buffer pH 7.4 as reference medium to demonstrate that neither lactone/carboxylate equilibrium displacement nor hydrolysis of **I** to **II** occurred during collection of the samples. The results of the assay of samples, which were spiked with either **I** carboxylate (n=9) or **I** lactone (n=9) or **II** carboxylate (n=9) or **II** lactone (n=9) immediately after blood collection, demonstrated the reliability of the sampling technique combined to fast cold methanol extraction [27].

3.2.5. Plasma

In rat and dog plasma, the transformation of the lactone forms of both **I** and **II** to their corresponding carboxylates was found to prevent the reliable de-

termination of **I** and **II** derivatives from spiked rat and dog plasma samples. In addition, investigations performed in rat serum have shown that **I** was hydrolyzed to **II** with an initial burst phenomenon [8]. However, as the plasma samples were immediately extracted in cold methanol as pre-requisite to this work and as the standard samples were prepared in cold methanol, the plasma stability was not extensively investigated.

3.2.6. Sensitivity to light exposure

Photodegradation products of **I** lactone have been identified [10]. However, because the photodegradation study was performed in an acidic HPLC mobile phase containing an ion-pair reagent and acetonitrile as organic modifier, no data about **I** carboxylate forms have been reported. **II** derivatives stabilities under light exposure have not been reported. The investigations were performed using standard samples spiked with 8.02, 0.802, 6.37 and 0.637 nmol/ml of **I** lactone, **I** carboxylate, **II** lactone and **II** carboxylate, respectively. The time-course plots showed that both **I** and **II** derivatives were sensitive to sunlight. However, only the carboxylate forms of both **I** and **II** derivatives were found to be very sensitive to artificial light. The time courses of **I** and **II** derivatives after irradiation with regular laboratory neon lighting and in darkness at 28°C are given in Fig. 3. Under these conditions the half-life of **I** carboxylate and **II** carboxylate were about 3 h and 17 h, respectively. No additional chromatographic peaks were detected under the experimental conditions recommended for both dog and rat plasma samples assay.

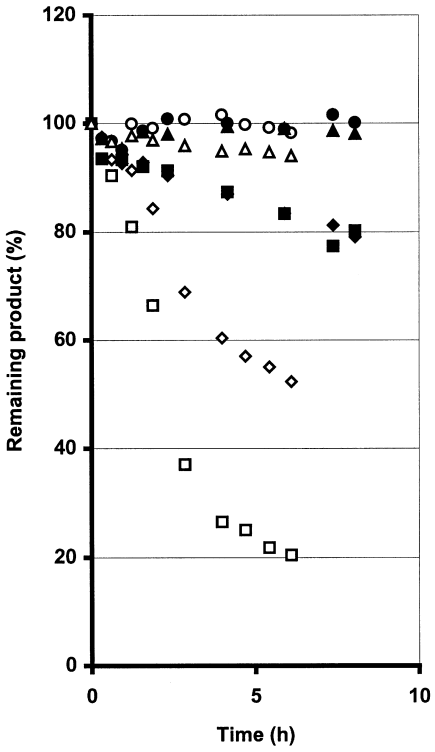

Fig. 3. Effect of artificial lighting on **I** derivatives in methanol extracts at 28°C (filled symbols=methanolic solution in darkness, blank symbols=methanolic solution under light exposure): ■=**I** carboxylate, ●=**I** lactone, ♦=**II** carboxylate, ▲=**II** lactone. The assays were performed under the recommended experimental conditions. The results obtained for the methanolic solutions under light exposure (blank symbols) were not corrected for the transformations occurring in methanol at 28°C (filled symbols).

3.3. Chromatography

Investigations were performed in order to obtain a chromatographic separation suitable for an automated wavelength change, acceptable peak shapes and suitable mobile phases in respect to lactone/carboxylate equilibrium. Several reversed-phase columns with either regular C_{18} or endcapped-like C_{18} or C_8 or CN packing were tested. The aqueous mobile phases with a pH of either 4.0 or 6.14 or 6.4 [9,11,13,14,16,17,20,21] were investigated as previously described. Additional investigations were performed at pH=7.4 with these mobile phases and with phosphate buffer solutions. Ion-pair reagents such as tetrabutylammonium bromide and heptanesulfonic acid sodium salt were also used. Binary mixtures of either methanol or acetonitrile or THF and the aqueous mobile phases were pumped in either isocratic or gradient mode. The most favorable detection wavelength couples were set for **I** forms and **II** forms, respectively. Those were obtained by on-line recording of the fluorescence spectra of each species in the final recommended mobile phase by means of the scanning facilities of the fluorescence detectors.

As illustrated in Fig. 4, the elution of **I** and **II** derivatives on the reversed-phase columns investigated by means of mobile phases containing methanol tended to favor peak asymmetries associated with

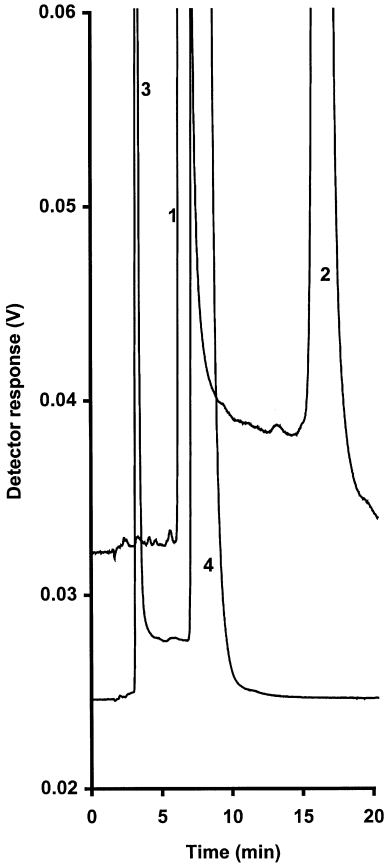

Fig. 4. Typical chromatograms of **I** derivatives and **II** derivatives showing no baseline separation despite suitable relative retention. The overlaid chromatograms were obtained with wavelengths optimized for either **I** or **II** derivatives. Upper trace: **I** derivatives, lower trace **II** derivatives. Experimental conditions: mobile phase: mixture methanol–0.1 *M* potassium phosphate buffer plus 3 m*M* heptanesulfonic acid sodium salt adjusted to pH 7.4 (50:50, v/v), column Supelcosil LC-ABZ, 1.0 ml/min, injection, 10 μl of a mixture of reference solutions containing either the two forms of **I** or the two forms of **II**. 1=**I** carboxylate, 2=**I** lactone, 3=**II** carboxylate, 4=**II** lactone.

no baseline separation despite adequate relative retention. This phenomenon suggested an equilibrium into the column that prevented any reliable peak integration. No additional investigations at either low or high temperature were performed to study this equilibrium. It can be also observed in the chromatograms of many published methods allowing the simultaneous separation of the lactone and carboxylate forms of either **I** or **II**. Acetonitrile and THF prevented in a large extent this phenomenon. However, as shown in Fig. 5, THF eluted the two **I** derivatives as multiple peaks. This artifact was already observed [16] in mobile phases containing acetonitrile when various solvents were used for protein precipitation or when plasma was substituted with phosphate buffer. **I** and **II** derivatives were not baseline resolved on the CN column tested. The elution order obtained onto the regular C_{18} and C_8

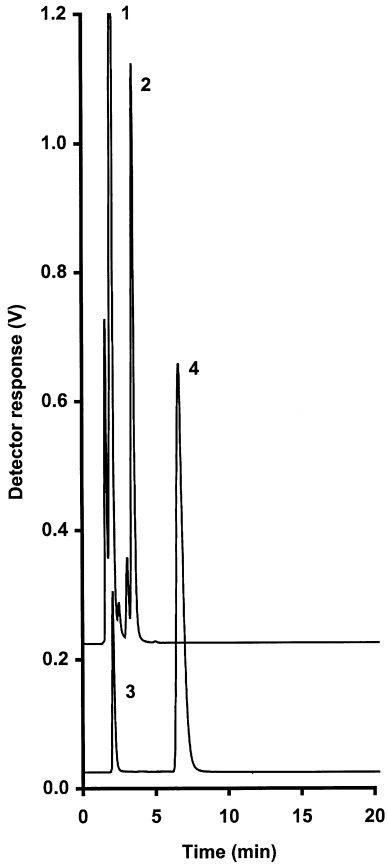

Fig. 5. Typical chromatograms of a **I** derivatives and **II** derivatives obtained with THF as organic modifier. The overlaid chromatograms were obtained with wavelengths optimized for either **I** or **II** derivatives. Upper trace: **I** derivatives, lower trace **II** derivatives. Experimental conditions: mobile phase: mixture THF–0.05 *M* potassium phosphate buffer plus 3 m*M* heptanesulfonic acid sodium salt 3 mM adjusted to pH 7.4 (25:75, v/v), column, Supelco LC-ABZ, 1.0 ml/min, injection, 10 μl of a mixture of reference solutions containing either the two forms of **I** or the two forms of **II**. 1=**I** carboxylate (probably eluted as twin peaks), 2=**I** lactone (probably eluted as twin peaks), 3=**II** carboxylate, 4=**II** lactone.

columns applying acetonitrile as organic modifier as well as those obtained onto NovaPack C_{18} [12] and Nova-Pack Radial-Pack C_{18} [16] columns were t_R **I** carboxylate$<t_R$ **II** carboxylate$<t_R$ **I** lactone$<t_R$ **II** lactone. The preliminary investigations performed showed that the Supelcosil LC-ABZ column gave very satisfactory results in term of peak symmetry and a modification of elution order (Fig. 6). However, the elution patterns obtained with all these columns prevented any relevant automated wavelength changes because of the relative retentions observed. Only the C_{18} Symmetry column was able to separate the two couple of compounds one after the other. Investigations done with camptothecin as possible internal standard showed that this compound interfered with **II** derivatives under the recommended experimental conditions. However, because the plasma samples were supplied as methanolic extracts as pre-requisite to this work and to avoid additional dilution step external standard quantification was applied.

For the assay in dog plasma samples, typical retention times were 4.1 min, 6.1 min, 8.9 min and 10.4 min for **I** carboxylate, **I** lactone, **II** carboxylate and **II** lactone, respectively. The final recommended aqueous mobile phase was a mixture of a buffer solution 0.075 *M* ammonium acetate adjusted to pH 6.4 plus 7.5 m*M* tetrabutyl-ammonium bromide. A typical chromatogram is given in Fig. 7. The elution was performed isocratically at the beginning of the run and then by a linear gradient both to reduce the run time and to improve the sensitivity of the **II** derivatives i.e., for the lowest expected concentrations to be measured. This was achieved by changing the wavelength setting after the elution of the two forms of **I** after about 8 min.

The chromatographic conditions developed for the determination of **I** derivatives in dog plasma were applied to the assay of rat plasma samples collected after **I** infusion. The investigations showed the presence of two partially resolved polar unknown xenobiotics eluted before **I** carboxylate. The spectroscopic properties of these compounds measured online suggested the presence of metabolites. No additional investigations were performed in order to show the possible presence of both the lactone and the carboxylate forms of **II** glucuronide already reported [16]. The gradient programme developed for the assay of dog plasma sample was slightly modified in order to improve the chromatographic separation. Under the recommended experimental conditions, the unknown metabolites and **I** carboxy-

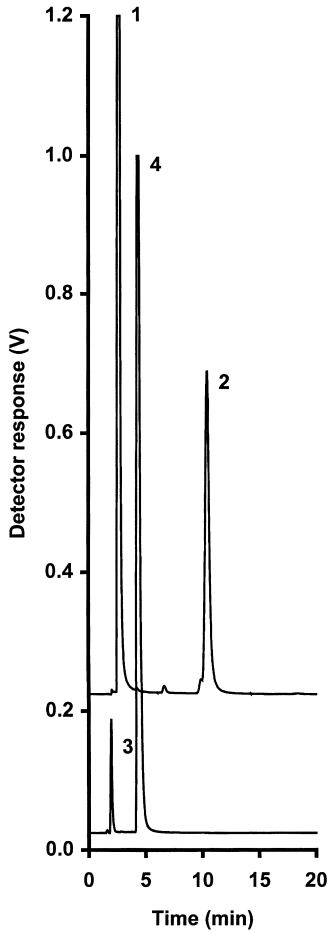

Fig. 6. Typical chromatograms of a **I** derivatives and **II** derivatives obtained using a Supelcosil LC-ABZ column in isocratic mode. The overlaid chromatograms were obtained with wavelengths optimized for **I** or **II** derivatives. Upper trace: **I** derivatives, lower trace **II** derivatives. Experimental conditions: mobile phase: mixture acetonitrile–0.1 *M* potassium phosphate buffer plus 3 m*M* heptanesulfonic acid sodium salt adjusted to pH 7.4 (25:75, v/v), 1.0 ml/min, injection, 10 μl of a mixture of reference solution containing either the two forms of **I** or the two forms of **II**. 1=**I** carboxylate, 2=**I** lactone, 3=**II** carboxylate, 4=**II** lactone.

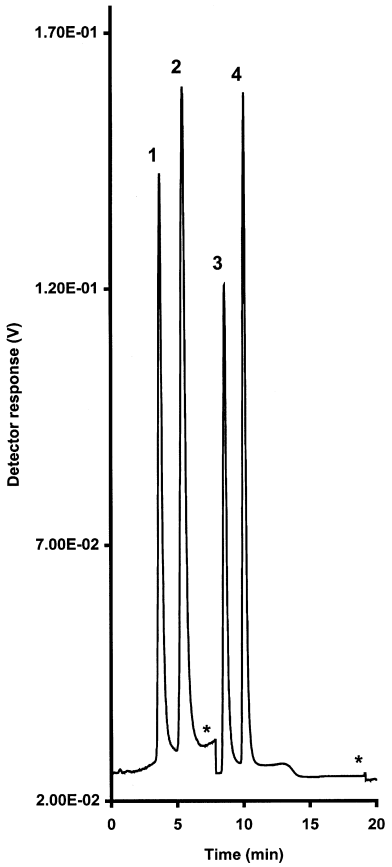

Fig. 7. Typical chromatogram of a mixture of working solutions of **I** derivatives under the recommended experimental conditions for dog plasma assay. Automated wavelength change at t=7.8 min and t=19 min combined with autozero. Experimental conditions, see Section 2.3. 1=**I** carboxylate, 2=**I** lactone, 3=**II** carboxylate, 4=**II** lactone.

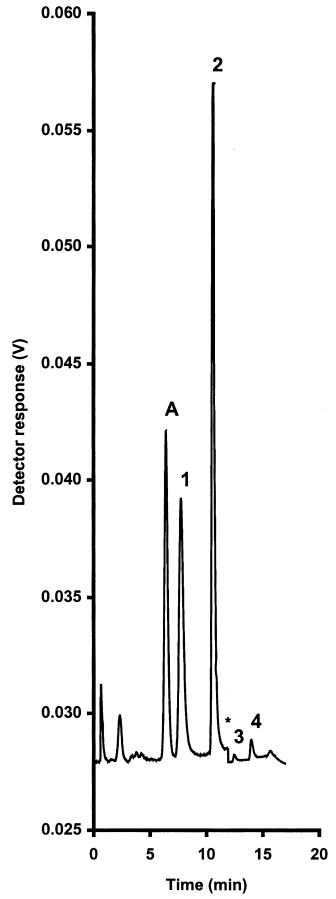

Fig. 8. Typical chromatogram of a rat plasma sample collected 7 h after i.v. infusion of **I** lactone. The sample was assayed under the recommended experimental conditions for rat plasma assay. Experimental conditions, see Section 2.3. *=Automated change of wavelengths, 1=**I** carboxylate, 2=**I** lactone, 3=**II** carboxylate, 4=**II** lactone, A=composite peak of unknown xenobiotics.

late were eluted in the isocratic part of the chromatogram while the **I** lactone and the **II** derivatives were eluted in the gradient mode. Typical retention times of 6.4 min, 7.7 min, 10.6 min, 12.5 and 14.0 min were measured for the composite peak of unknown metabolites, **I** carboxylate, **I** lactone, **II** carboxylate and **II** lactone, respectively. A typical chromatogram obtained applying the recommended experimental conditions to a rat plasma sample collected 7 h after **I** administration is given in Fig. 8.

No endogenous compound interfered with both assays. The retention times were found to be stable for hundreds of injections.

3.4. Dog plasma assay validation

The intra-day precision and accuracy as well as the inter-day precision and bias are summarized in Table 1. No reasonable explanation was found to explain the unexpected bias measured at low concentration for **II** carboxylate form. The detector

Table 1
Precision and accuracy of the simultaneous assay of the **I** derivatives in dog plasma

	Intra-day						Inter-day					
	CPT-11			SN-38			CPT-11			SN-38		
	Carboxylate	Lactone	Total	Carboxylate	Lactone	Total	Carboxylate	Lactone	Total	Carboxylate	Lactone	Total
Nominal concentration (nmol/ml)	0.0321	0.321	0.353	0.0255	0.255	0.280	0.0321	0.321	0.353	0.0255	0.255	0.280
Concentration found (nmol/ml)												
Mean	0.0319	0.308	0.340	0.0251	0.253	0.278	0.0291	0.300	0.329	0.0309	0.255	0.285
S.D.	0.0024	0.004	0.005	0.0009	0.001	0.001	0.0005	0.006	0.006	0.0016	0.003	0.004
n	6	6	6	6	6	6	6	6	6	6	6	6
R.S.D. (%)	7.6	1.3	1.5	3.4	0.4	0.5	1.6	2.1	1.9	5.3	1.0	1.4
Conf. interval (p=95%)	0.0025	0.004	0.005	0.0009	0.001	0.001	0.0005	0.007	0.007	0.0017	0.003	0.004
Deviation (%)	−0.6	−4.1	−3.8	−1.3	−0.7	−0.7	−9.4	−6.5	−6.8	21.2	0.0	1.9
Nominal concentration (nmol/ml)	0.802	8.02	8.82	0.637	6.37	7.00	0.802	8.02	8.82	0.637	6.37	7.00
Concentration found (nmol/ml)												
Mean	0.793	8.29	9.08	0.645	6.47	7.12	0.798	8.15	8.95	0.654	6.39	7.05
S.D.	0.019	0.07	0.08	0.006	0.14	0.14	0.032	0.31	0.34	0.031	0.27	0.30
n	6	6	6	6	6	6	6	6	6	6	6	6
R.S.D. (%)	2.4	0.9	0.9	1.0	2.2	2.0	4.1	3.8	3.8	4.7	4.2	4.2
Conf. interval (p=95%)	0.020	0.008	0.009	0.007	0.15	0.15	0.034	0.33	0.36	0.032	0.28	0.31
Deviation (%)	−1.2	3.3	2.9	1.3	1.6	1.6	−0.5	1.6	1.4	2.6	0.3	0.5

R.S.D.=Relative standard deviation, Conf. interval=confidence interval of the mean.

Table 2
Precision and accuracy of the simultaneous assay of the **I** derivatives in rat plasma

	Intra-day						Inter-day					
	CPT-11			SN-38			CPT-11			SN-38		
	Carboxylate	Lactone	Total	Carboxylate	Lactone	Total	Carboxylate	Lactone	Total	Carboxylate	Lactone	Total
Nominal concentration (nmol/ml)	0.0321	0.321	0.353	0.0255	0.255	0.280	0.0321	0.321	0.353	0.0255	0.255	0.280
Concentration found (nmol/ml)												
Mean	0.0327	0.305	0.338	0.0244	0.252	0.276	0.0300	0.303	0.333	0.0224	0.236	0.258
S.D.	0.0017	0.006	0.006	0.0013	0.007	0.008	0.0014	0.011	0.011	0.0011	0.003	0.003
n	7	7	7	7	7	7	6	6	6	6	6	6
R.S.D. (%)	5.2	1.9	1.7	5.2	2.9	3.0	4.7	3.6	3.4	4.9	1.2	1.2
Conf. interval (p=95%)	0.0018	0.006	0.006	0.0013	0.008	0.009	0.0015	0.012	0.012	0.0012	0.003	0.003
Deviation (%)	2.0	−4.8	−4.2	−4.2	−1.2	−1.4	−6.6	−5.5	−5.6	−11.9	−7.4	−7.8
Nominal concentration (nmol/ml)	0.802	8.02	8.82	0.637	6.37	7.00	0.802	8.02	8.82	0.637	6.37	7.00
Concentration found (nmol/ml)												
Mean	0.793	8.30	9.09	0.620	6.30	6.92	0.784	8.10	8.88	0.610	6.09	6.70
S.D.	0.008	0.11	0.11	0.007	0.09	0.09	0.004	0.22	0.22	0.014	0.06	0.08
n	7	7	7	7	7	7	6	6	6	6	6	6
R.S.D. (%)	1.0	1.4	1.3	1.2	1.4	1.3	0.5	2.7	2.4	2.3	1.1	1.2
Conf. interval (p=95%)	0.008	0.11	0.11	0.007	0.09	0.09	0.004	0.23	0.23	0.015	0.07	0.08
Deviation (%)	−1.1	3.5	3.0	−2.7	−1.1	−1.2	−2.2	0.9	0.7	−4.2	−4.4	−4.4

R.S.D.=Relative standard deviation, Conf. interval=confidence interval of the mean.

response was linear within the concentration ranges of 0.016 to 1.605 nmol/ml of plasma, 0.160 to 16.048 nmol/ml of plasma, 0.013 to 1.274 nmol/ml of plasma, 0.127 to 12.742 nmol/ml of plasma, 0.177 to 17.653 nmol/ml of plasma and 0.140 to 14.016 nmol/ml of plasma for **I** carboxylate, **I** lactone, **II** carboxylate, **II** lactone, **I** total (carboxylate+lactone) and **II** total (carboxylate+lactone), respectively. The coefficients of correlation calculated ranged from (n=7) 0.9997 to 1.0000, 0.9996 to 1.0000, 0.9996 to 1.0000 and 0.9988 to 1.0000 for **I** carboxylate, **I** lactone, **II** carboxylate and **II** lactone, respectively. The relative standard deviations calculated for the slopes (n=4) were found to be 2.6%, 1.9%, 1.5% and 2.2% for **I** carboxylate, **I** lactone, **II** carboxylate and **II** lactone, respectively. The intercept values were closed to the origin with calculated values of 0.001±0.001, 0.015±0.002, 0.002±0.002 and −0.001±0.004. With the injection of a 10-μl aliquot and a medium photomultiplier voltage setting, the detector cell was overloaded at a concentration of about 14 nmol/ml of plasma for both **I** and **II** lactone forms. This limitation in use, which was required by the necessity to avoid the dilution of the methanol extracts at −70°C, was overcome by means of a low voltage setting for the photomultiplier. The resulting signal was decreased by a factor 5 while the signal-to-noise ratio remained unchanged. The limits of quantification were found to be 0.008 nmol/ml (4.8 ng/ml), 0.010 nmol/ml (5.9 ng/ml), 0.004 nmol/ml (1.6 ng/ml) and 0.006 nmol/ml (2.4 ng/ml) for **I** carboxylate, **I** lactone, **II** carboxylate and **II** lactone, respectively. The limits of detection were estimated to 0.005 nmol/ml, 0.002 nmol/ml, 0.001 nmol/ml and 0.001 nmol/ml for **I** carboxylate, **I** lactone, **II** carboxylate and **II** lactone, respectively. These limits corresponded to an injected amount of less than 3 pg for all derivatives. As pre-requisite to this work, plasma samples were prepared according to Rowinsky et al. [9] i.e., by protein precipitation in large volumes of cold methanol (1 volume of plasma+6 volumes of methanol). The sensitivity of the assay can be noticeably improved applying the deproteinization step recommended by Rivory and Robert [16] and increasing the injected volume to 40 μl. The overall extraction recoveries calculated along the whole linear range were found to be 88.6±4.7% and 85.6±3.1% for **I** derivatives and **II** derivatives, respectively.

3.5. Rat plasma assay validation

The precision and accuracy data are summarized in Table 2. Both intra-day and inter-day precision and bias were found to be suitable for pharmacokinetic and toxicokinetic studies. The detector response was linear within the concentrations ranges investigated for dog plasma. The coefficients of correlation calculated (n=7) ranged from 0.9985 to 0.9999, 0.9982 to 0.9999, 0.9997 to 1.0000 and 0.9998 to 1.0000 for **I** carboxylate, **I** lactone, **II** carboxylate and **II** lactone, respectively. The intercept values of the calibration curves were closed to the origin with calculated values of 0.000±0.001,

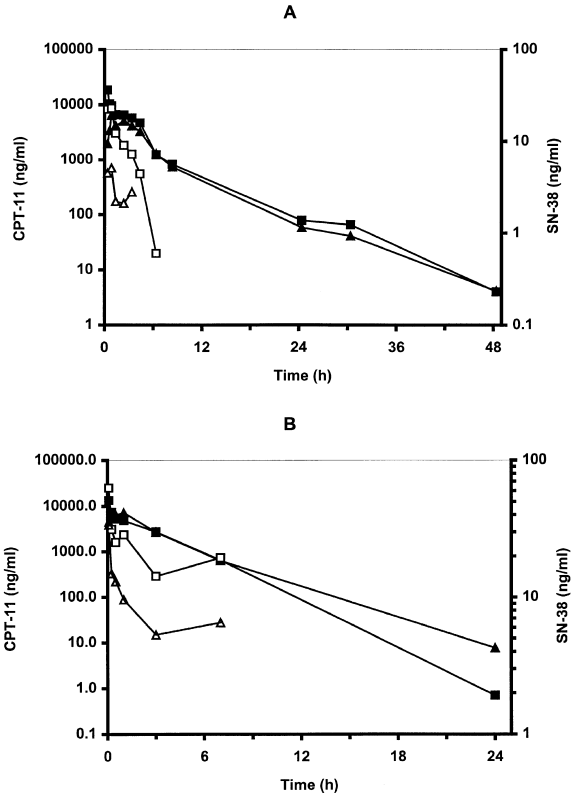

Fig. 9. Typical plasma concentration–time profiles for **I** carboxylate (▲), **I** lactone (■), **II** carboxylate (△) and **II** lactone (□) following i.v. infusion of **I** lactone in a male dog (A, dose 20 mg/kg, end of infusion at 0.37 h) and in a male rat (B, dose 80 mg/kg i.v. bolus).

0.007±0.005, −0.001±0.001 and −0.004±0.005, respectively. The relative standard deviations calculated for the slopes (n=7) were found to be less than 1.0%, 1.1%, 1.3% and 0.9% for all analytes, respectively. Low voltage setting of the photomultiplier was used as described for rat plasma. The limits of quantification and of detection were found to be similar to those obtained in rat plasma. The overall extraction recoveries calculated along the whole linear range were found to be 77.1±7.3% and 89.8±5.9% for **I** derivatives and **II** derivatives, respectively.

3.6. Method application

The developed methods were applied to hundreds of plasma samples from pharmacokinetic and toxicokinetic studies in rat and dog [31]. Fig. 9 shows representative plasma concentration–time profiles for **I** carboxylate, **I** lactone, **II** carboxylate and **II** lactone following i.v. infusion of **I** lactone in both rat and dog.

4. Conclusion

The HPLC method developed was suitable for the simultaneous, reliable and sensitive determination of the lactone and carboxylate forms of both **I** and **II** in dog and rat plasma in respect to stability issues. The combination of both a selected reversed-phase column and a gradient elution allowed the elution of the compounds of interest in such an order that automated changes of the most appropriate wavelength settings of the fluorescence detector were simple. As a consequence, the recommended experimental conditions avoided tedious wavelength settings compromises.

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