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# Determination of MAG-Camptothecin, a new polymer-bound Camptothecin derivative, and free Camptothecin in dog plasma by HPLC with fluorimetric detection

D. Fraier *, E. Frigerio, G. Brianceschi, M. Casati, A. Benecchi, C. James

*Pharmacia and Upjohn, Drug Metabolism Research, Pharmacokinetics and Metabolism Department, Viale Pasteur 10, 20014 Nerviano, Milan, Italy*

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**Abstract**

A high throughput, selective and sensitive high-performance liquid chromatographic (HPLC) method for the determination of a water-soluble polymer-bound Camptothecin conjugate (MAG-CPT) and Camptothecin (CPT) in dog plasma has been developed and validated. The method involved the analysis of free and total CPT (free + polymer-bound). Free CPT (intact lactone plus carboxylate) was extracted from acidified plasma using Oasis™ SPE material in 96-well plates. For the assay of the total CPT, plasma proteins were first precipitated with methanol in a 96-well plate containing a 10-μm melt blown polypropylene membrane. The methanolic supernatant was separated and collected into a second 96-well plate by simply applying vacuum to the plate. After hydrolysis at pH 9.8 for 18 h and re-acidification, samples were injected directly from the collection plate onto the HPLC system. MAG-CPT concentration was then calculated by subtraction of free from total CPT. The LLOQs of the method were 1.17 ng/ml for free CPT and 103.10 ng/ml (as CPT equivalent) for MAG-CPT using 0.1 and 0.05 ml of plasma, respectively. Linearity, precision, accuracy and recovery of the method were evaluated. The stability of MAG-CPT in plasma alone and after its stabilisation was carefully evaluated. No interference from blank dog, mouse and human plasma was observed. The suitability of the method for in vivo samples was assessed by the analysis of samples obtained from dogs that had received a single and 5-day repeated dose of MAG-CPT. 

*Keywords:* Polymer-bound Camptothecin derivative; Camptothecin; HPLC determination; Fluorimetric detection; Dog plasma

## 1. Introduction

Suppression of toxicity of existing drugs, without impairment of their therapeutic activity, is one of the major research tasks in chemotherapy.

* Corresponding author.

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Soluble polymer conjugates are a significant step in the evolution of a new approach for delivery of cytotoxic drugs that have the potential to improve anticancer activity and reduce toxicity [1–3]. The enhancement of the efficacy is mainly due to the alteration of the organ distribution of the drugs, with increase of tumour retention and accumulation. Different water-soluble polymeric molecules containing anticancer agents such as Doxorubicin (PK1) [4–6], Taxol (MAG-taxol) [7] and Camptothecin (MAG-CPT) have been investigated. PNU-166148 (MAG-CPT) (I) (Fig. 1), is the lead compound of this novel delivery system, where CPT (Fig. 2) is linked ( $\approx 10\%$, w/w), with an ester bond at the 20 position, to a soluble polymeric carrier based on *N*-(2-hydroxypropyl)-methacrylamide (HPMA) through a spacer consisting of glycyl-aminohexanoyl-glycyl. Cleavage of this linkage releases free CPT into the bloodstream, tumour and tissues.

Hence I is the result of the combination of three major moieties: the drug, the spacer and the carrier. CPT was selected as the drug because of its well-documented antineoplastic efficacy in experimental tumours. However it has an unfavourable toxicological profile [8], poor solubility and instability at physiological pH of the δ-lactone [9] whose stability is of paramount importance for activity [10,11]. Coupling of CPT to the polymer was performed in an attempt to maintain the efficacy whilst improving the biological profile, solubility and stability.

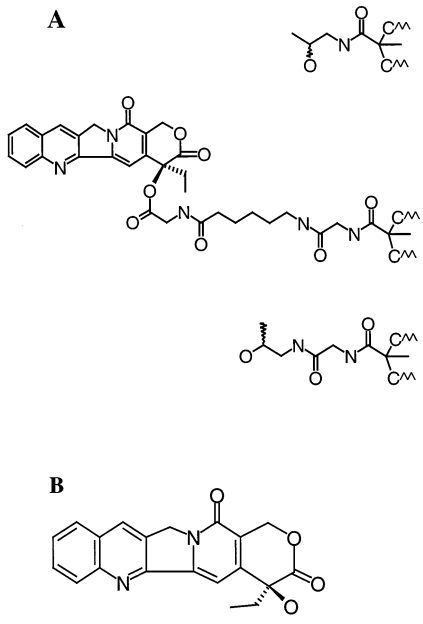


Fig. 1. (A) Structure of MAG-CPT, copolymer of (1-methacryloylamido-2-hydroxypropane) and [20-O-(methacryloyl-glycyl-6-aminohexanoyl-glycyl) CPT] and [1-(methacryloyl-glycyl) amido-2-hydroxypropane] and, (B) of CPT.

In order to study the pharmacokinetics of I, two groups of compounds should be considered: drug which remains linked to the polymeric carrier, and free drug which has been cleaved from the polymer. It was therefore necessary to select a method which would be suitable for the quantitation of both compounds within a sample (free and bound CPT). Determination of CPT in plasma by high-performance liquid chromatography (HPLC) has been already described [12–14]. Plasma levels of total CPT (free + polymer-bound) were determined after release of CPT from polymeric carrier by chemical hydrolysis. I concentration was then estimated by subtraction of the free CPT, previously determined, from the total CPT quantified after chemical hydrolysis.

## 2. Experimental

### 2.1. Chemicals and solutions

I, free CPT and Camptosar (CPT-11, IS) were supplied by the Pharmaceutical Development Department of Pharmacia and Upjohn S.p.A. (Milan, Italy). All other chemicals and solvents were of HPLC or analytical reagent grade from Carlo Erba Reagents (Milan, Italy). Stock solutions were prepared by dissolving accurately weighed amounts of I in a mixture of methanol and 25 mM phosphoric acid (50:50), and of CPT and the IS in methanol containing 10 mM phosphoric acid to prevent the lactone ring from opening. Suitable working solutions were prepared by dilution of I stock solution with 25 mM phosphate buffer (pH 4.0), while dilution of CPT and IS stock solutions was performed with water and methanol containing 10 mM phosphoric acid, respectively.

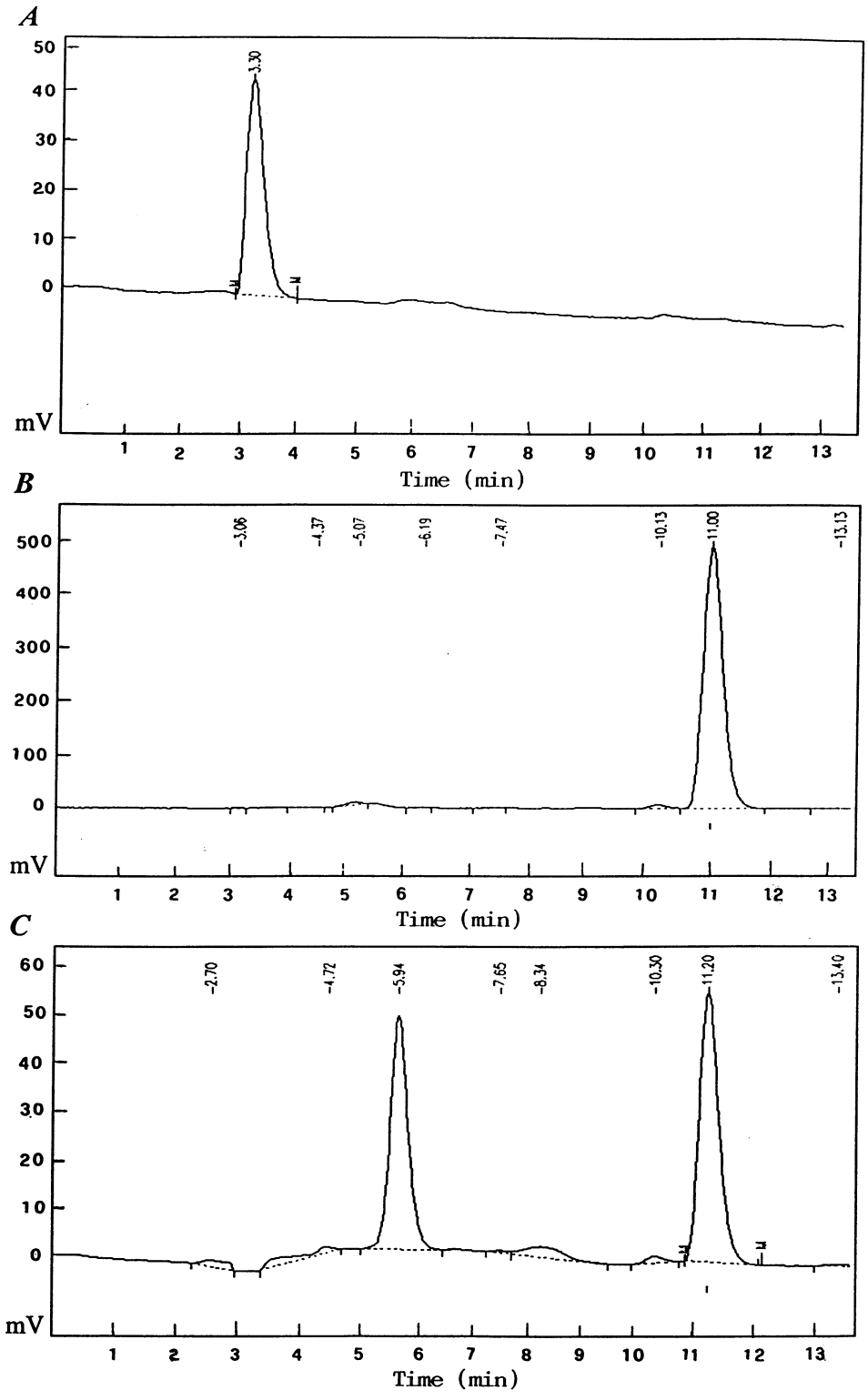


Fig. 2. Chromatograms (free CPT determination assay) of (A) blank dog plasma, (B) dog plasma spiked with 2332 ng/ml of CPT (Rt: 11.00 min) and, (C) of plasma from a dog given 12.5 mg/kg i.v. dose of I, 48 h after treatment (113 ng/ml, Rt: 11.20 min).

After storage at 4°C, stock solutions of I and CPT were found to be stable for at least 1 month.

### 2.2. Chromatographic equipment

The HPLC system used in this study consisted of a pump (Model P4000, Thermo Quest), an autosampler with 96-well injection plate (Model 233XL, Gilson) operating at room temperature and equipped with a Rheodyne injection valve (model 7010-090) with a 200-μl loop, a fluorescence detector (Model 474, Waters) and a column oven (Model CTO-10A VP, Shimadzu). Data acquisition and elaboration were performed using TurboChrom Professional System, Software Version 4.1 (Perkin Elmer).

### 2.3. Chromatographic conditions

The chromatographic separation was performed with a Zorbax SB C8 4.6 × 250 mm i.d., 5 μm particle size, reversed-phase column (Hewlet Packard) equipped with a RP-18 guard column (Applied Biosystem). Column and guard column were kept at the temperature of 30°C. The mobile phase consisted of a 0.025 M monobasic potassium phosphate (pH 4)–acetonitrile mixture (70:30, v/v). The separation was performed at a flow-rate of 1 ml/min. The fluorescence detector was set at 380 and 440 nm (excitation and emission, respectively) and wired to send a 1 V signal to the data system. The gain was set at × 10 (total CPT) and × 100 (free CPT). Typical back-pressure was ≈ 90 bar.

### 2.4. Sample preparation

#### 2.4.1. Free CPT

The method involved a solid-phase extraction of free CPT from plasma (0.1 ml), stabilised with 0.9 ml of 8.5% phosphoric acid, using Oasis™ HLB Extraction Plates (96-wells) with 30 mg of sorbent in each well. The plate sorbent was a copolymer designed to have a hydrophilic-lipophilic balance (HLB).

The plate was first conditioned by adding to each well 1 ml of acetonitrile followed by 1 ml of 8.5% phosphoric acid. The stabilised samples were passed through the wells and washed with 2 ml of water and 1 ml of acetonitrile:water mixture (15:85, v/v). The analyte was eluted using 0.5 ml of acetonitrile:0.025 M monobasic potassium phosphate mixture (60:40, v/v) into a poypropylene 96 deep well plate (1 ml; Stepbio, Bologna, Italy). After each solution was added, vacuum was applied to gently pull all solutions through the cartridges.

For the sample load and elution steps, a flow rate of ≈ 2 ml/min was used. In all other steps higher flow rates up to 10 ml/min were applied.

Prior to the analysis, 0.5 ml of 0.025 M (pH 4.0) monobasic potassium phosphate were added to the sample in order to make the solvent composition similar to the mobile phase.

The collecting plate with the resulting solutions was transferred to the autosampler and an aliquot (0.050 ml) was injected onto the HPLC system.

#### 2.4.2. Total CPT

Plasma concentrations of total CPT (free + polymer-bound) were determined after release of CPT from polymeric carrier by chemical hydrolysis. Plasma proteins were precipited by addition of 0.25 ml of methanol to 0.05 ml of plasma in a 96-well plate containing a 10-μm melt blown polypropylene membrane (MBPP; WHATMAN, Kent, England). After vortex mixing, the methanolic supernatant was separated and collected into a poypropylene 96 deep well plate (1 ml; Stepbio, Bologna, Italy) by applying vacuum to the filter plate. Then, 0.15 ml of 0.05 M borate buffer at pH 9.8 were added, the plate capped and the samples left to hydrolyse at 50°C for 18 h. Aliquots of 0.05 ml of acetonitrile containing 20 064 ng/ml of IS and of 0.45 ml of 8.5% phosphoric acid were added and, after 15 min of reaction, an aliquot of the samples (0.02 ml) was injected directly from the collection plate onto the HPLC. The internal standard was added after the hydrolysis because it could be hydrolysed itself. I concentration was then estimated by subtraction of free from total CPT determined after chemical hydrolysis.

### 2.5. Determination of CPT and I concentration in quality control and calibration samples

Analyses of blank dog plasma spiked with known amounts of CPT and I was carried out applying the above procedure. The linearity was evaluated from six calibration curves prepared and run on 3 different days in the concentration ranges 1.17–2332.0 and 103–430 000 (as CPT equivalent) ng/ml plasma for CPT and I, respectively. The precision and accuracy were evaluated by repeated analyses of the compounds at four concentrations (low, mid and high) in six replicate samples analysed on the 1st day, and three replicate samples analysed on the 2nd and 3rd day. All chromatograms obtained were evaluated by peak area measurement. Calibration curves were constructed by plotting the area of the compound (free CPT assay) or the ratio of the area of the compound divided by the area of IS (total CPT assay) against the concentration of the compound (free CPT assay) or the concentration ratio of the compound and the IS (total CPT assay).

A weighted linear regression function (weighting factor $1/y$) was used to fit the calibration line and hence to calculate I and CPT concentrations in quality control and unknown samples. The concentrations of I and CPT were expressed in ng/ml, in terms of CPT equivalent. In order to evaluate the extraction recovery, the Relative Weight Ratio [RWR, (CPT area/CPT amount) × IS amount/IS area)] of extracted biological samples was compared to the mean RWR obtained with unextracted samples dissolved in the same solvent mixture of the extracted samples and injected directly onto the chromatograph. The IS was added after the extraction, just before the sample injection.The hydrolysis yield of I after 18 h at 50°C was evaluated at three different concentrations comparing the RWR of the I plasma samples extracted after hydrolysis and the mean RWR of CPT extracted samples.

### 2.6. Stability

Since very high plasma levels of I and low concentrations of free CPT were found in preliminary studies, the release of CPT from I under different conditions during the analytical manipulations and after three freeze/thaw cycles was investigated in order to avoid analytical artefacts. Indeed, if only very small amounts of I (for example $< 1\%$) undergo in vitro spontaneous hydrolysis, results obtained for free CPT may be greatly overestimated. For these reasons the release of CPT from I was evaluted in dog plasma alone and after its stabilisation by addition of 0.9 ml of 8.5% phosphoric acid to 0.1 ml of plasma at room temperature (22°C) and at 0–4°C. A stock solution of I was spiked into a blank pool of dog plasma, alone and stabilized, to give four different concentrations (385, 1540, 123 224 and 308 060 ng/ml). An aliquot of plasma was removed from the pool at the time zero and processed with the method for the free CPT. This procedure was repeated after 0.5, 1, 2 and 3 h.

In addition the stability of I and CPT stock and working solutions, after storage in plasma at room temperature for 24 h, during storage of the extracts at room temperature for 48 h, in plasma after three freeze/thaw cycles, and after 1 and 3 months at $-80$ and $-30$°C was investigated.

### 2.7. Chromatographic system suitability test

The suitability of the chromatographic system for the analysis of CPT and I was checked during the validation assay by calculating the column efficiency and the peak symmetry. This evaluation was carried out according to USP [15] using the System Suitability Test of the TurboChrom Professional Software.

The column efficiency was expressed as the number of theoretical plates ($N$). This value must be higher than 5800 for CPT and 2740 for the IS. The tailing factor ($T$) must be $< 1.6$ for both analytes.

## 3. Results and discussion

The chromatograms obtained from blank and spiked dog plasma and from a dog given a 12.5 mg/kg i.v. dose of I for free and total CPT assay, respectively, are shown in Figs. 2 and 3. No interference from blank dog, mouse and human plasma was observed.

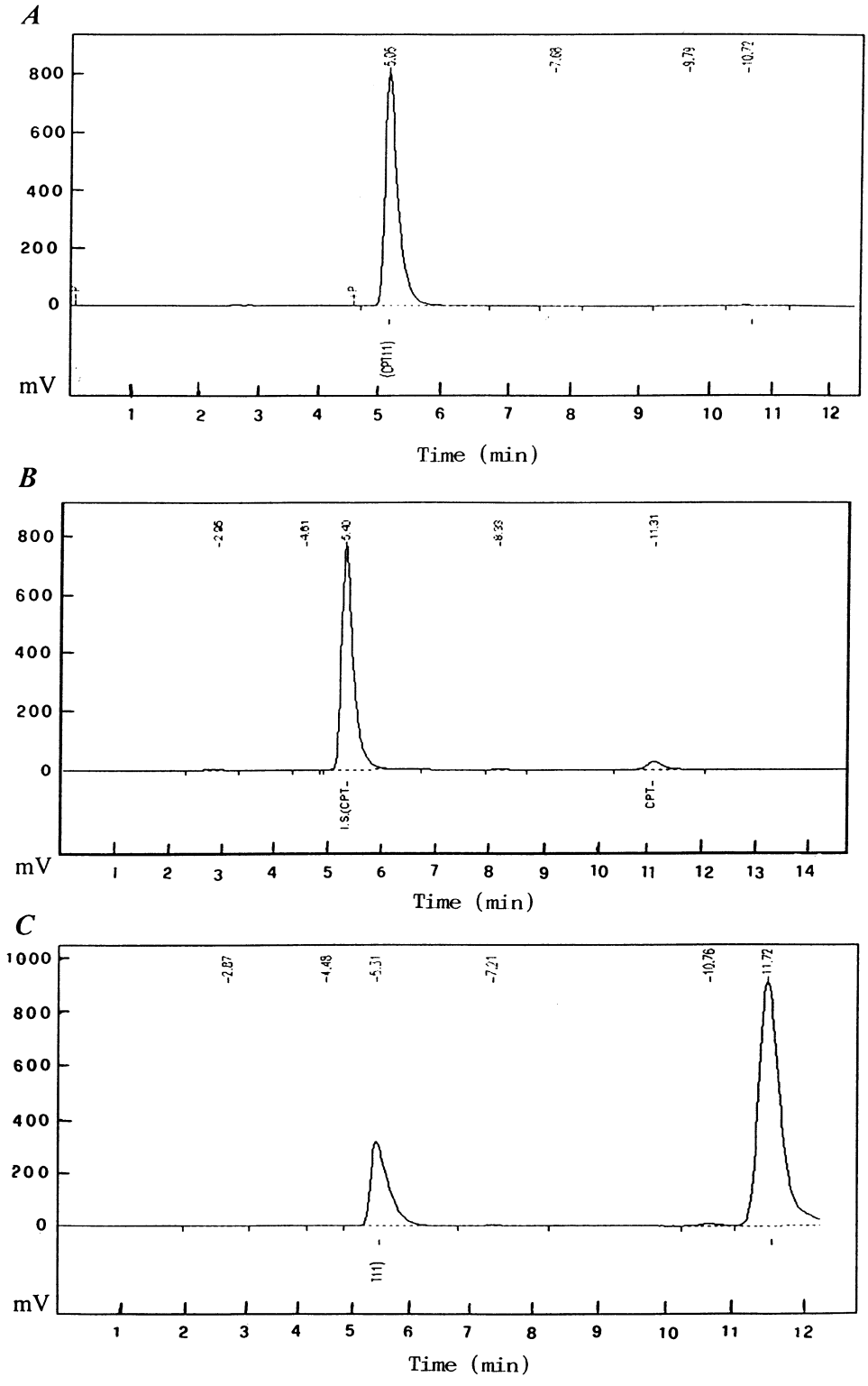


Fig. 3. (A) Chromatograms (total CPT determination assay) of blank dog plasma, (B) dog plasma spiked with 385 ng/ml of CPT (Rt: 11.31 min) and 20 064 ng/ml of IS (Rt: 5.04 min) and, (C) of plasma from a dog given 12.5 mg/kg i.v. dose of I, 72 h after treatment (75 197 ng/ml, Rt: 11.70 min).

Sharp and symmetric peaks were obtained for CPT under the chromatographic isocratic conditions chosen. Under these conditions the total analysis time was 15 min, sufficiently short to allow 96 samples per day to be assayed. The linearity of the method was evaluated from six calibration curves with six and eight calibration points for free and total determination, respectively, run on 3 different days. The method proved to be linear in the range of concentrations of 1.17–2332 and 103–430 000 ng/ml for CPT and I, respectively. The data was analysed by linear regression (weighting factor $1/y$) of the peak-area versus the concentration (free determination) or the ratio of peak-area of the compound and the IS versus the concentration ratio of the same (total determination). Due to the presence of a chromatographic interference at the retention time of the IS in some of the real study samples and since good results in terms of precision and accuracy were achieved, no internal standard was used in this study for the free CPT determination.

The limits of quantitation of the methods were 1.17 ng/ml of biological fluid for CPT and 103.10 ng/ml for I (as CPT equivalent). Correlation coefficients ($r^2$) for the regression were always better than 0.99. Results for the intra-day and inter-day precision and accuracy are shown in Tables 1 and 2. The bias for all the concentrations was within $\pm 15\%$ of the target except at the lowest concentration that showed a bias within $\pm 20\%$ for both methods.

The robustness and ruggedness of the method were checked. Equivalent results were obtained with different batches of analytical columns, solid phase extraction and filtration plates. Moreover small changes in the preparation of the mobile phase did not seem to affect the optimum performance of the method.

The mean ($n = 3$) extraction recovery of I from plasma evaluated at the concentrations of 582, 1166 and 3498 ng/ml ranged from $97.7 \pm 0.02$ to $101.1 \pm 0.05\%$ (mean $\pm$ S.D.).

The yield of the chemical hydrolysis of I in dog plasma after 18 h at 50°C evaluated at the same concentrations ranged from $98.4 \pm 0.034$ to $110.1 \pm 0.13\%$ (mean $\pm$ S.D., $n = 3$).

There was no evidence of degradation of I (in terms of total CPT determination) and CPT after storage in plasma at room temperature for 24 h, during storage of extracts at room temperature for 48 h, after three freeze/thaw cycles of the plasma sample, and after 1 and 3 months in plasma at $-80$°C.

Also, there was no evidence of degradation of I (in terms of total CPT determination) and CPT working solutions after 1 week at 4°C.

However, after 30 min of storage of I in dog plasma at room temperature (22°C), a high increase in released CPT was observed. For this reason the plasma was stabilized by adding 8.5% phosphoric acid and the stability of I was checked at room temperature and in a ice bath. No release of free CPT was noted in stabilized plasma kept in an ice bath not longer than 30 min. A slight increase of free CPT was observed after three freeze/thaw cycles of I in stabilized plasma samples whilst no release was observed after the first cycle.

From the results obtained, the recommended sample treatment involves the collection of the blood in pre-cooled tubes. Blood should be placed on ice and immediately centrifuged at 0–4°C (10 min at 1200g) to separate the plasma. Plasma should then be sub-divided into two tubes. An aliquot of 0.05 ml of plasma should be put in a tube for the total determination. For the free determination, 0.1 ml of plasma should be immediately put in a pre-cooled tube containing 0.9 ml of 8.5% phosphoric acid solution and flash frozen in a $CO_2$/ethanol bath. Both the tubes should be stored at $-80$°C until the analysis.

Keeping the stabilized plasma sample in ice bath and within 30 min extracting the sample, was the most reliable method of handling I plasma samples when determining for the free CPT.

## 4. Conclusions

A highly efficient method binding the 96-well plate technology to the use of the novel MBPP filtration plate for plasma precipitation, sensitive and selective for the determination of I and CPT in dog plasma has been developed and validated.

Table 1
Accuracy and precision data of the method for CPT determination in dog plasma

| Control sample (ng/ml) | | | Accuracy | | | Precision | | |
|---|---|---|---|---|---|---|---|---|
| | Day | *n* | Mean found (ng/ml) | Mean (%) recovery (intra-day) | Polled (%) recovery (inter-day) | RSD | %RSD (intra-day) | Pooled %RSD (inter-day) |
| 2.5 | 1 | 6 | 2.49 | 99.6 | | 0.35 | 14.1 | |
| | 2 | 3 | 2.75 | 110.0 | | 0.17 | 6.2 | |
| | 3 | 3 | 2.61 | 104.4 | 103.2 | 0.26 | 10.0 | 11.2 |
| 49.97 | 1 | 6 | 51.93 | 103.9 | | 3.70 | 7.1 | |
| | 2 | 3 | 52.03 | 104.1 | | 3.48 | 6.7 | |
| | 3 | 3 | 51.95 | 104.0 | 104.0 | 4.49 | 8.6 | 6.7 |
| 499.71 | 1 | 6 | 511.89 | 102.4 | | 26.19 | 5.1 | |
| | 2 | 3 | 552.53 | 110.6 | | 10.99 | 2.0 | |
| | 3 | 3 | 498.97 | 99.6 | 103.8 | 21.99 | 4.4 | 5.7 |
| 1415.86 | 1 | 6 | 1306.35 | 92.3 | | 61.52 | 4.7 | |
| | 2 | 3 | 1567.97 | 110.7 | | 27.21 | 1.7 | |
| | 3 | 3 | 1462.87 | 103.3 | 99.6 | 30.56 | 2.1 | 8.8 |

Table 2
Accuracy and precision data of method for I determination in dog plasma[a]

| Control sample (ng/ml) | | | Accuracy | | | Precision | | |
|---|---|---|---|---|---|---|---|---|
| | Day | *n* | Mean found (ng/ml) | Mean (%) recovery (intra-day) | Polled (%) recovery (inter-day) | RSD | %RSD (intra-day) | Pooled %RSD (inter-day) |
| 385 | 1 | 6 | 353 | 91.7 | | 36 | 10.2 | |
| | 2 | 3 | 327 | 85.0 | | 7 | 2.1 | |
| | 3 | 3 | 401 | 105.9 | 93.2 | 59 | 14.7 | 12.5 |
| 1540 | 1 | 6 | 1563 | 101.5 | | 35 | 2.2 | |
| | 2 | 3 | 1289 | 83.7 | | 11 | 0.9 | |
| | 3 | 3 | 1495 | 97.1 | 97.0 | 203 | 13.6 | 9.5 |
| 123 224 | 1 | 6 | 119 789 | 97.2 | | 4838 | 4.0 | |
| | 2 | 3 | 122 652 | 99.53 | | 13 731 | 11.2 | |
| | 3 | 3 | 126 154 | 102.4 | 99.1 | 18 732 | 14.8 | 8.8 |
| 308 060 | 1 | 6 | 307 437 | 99.8 | | 15 409 | 5.0 | |
| | 2 | 3 | 307 931 | 99.96 | | 13 774 | 4.5 | |
| | 3 | 3 | 301 098 | 97.7 | 99.3 | 41 347 | 13.7 | 7.0 |

[a] All the concentrations are expressed as CPT equivalent.

Subsequent experiments were performed with the dual membrane polypropilene filters (Whatman, Kent, England). These plates showed improved filtration properties over the plates previously used. The centrifugation step that sometimes occurred with the use of the monolayer filters (MBPP) was found to be unnecessary and filtration was performed simply applying vacuum. The proposed method proved to be specific and sensitive enough to be applied to pre-clinical studies carried out to evaluate the pharmacokinetics of I in dogs. The suitability of the method for in vivo samples was assessed by the analysis of plasma samples obtained from dogs that had received a single i.v. dose of 12.5 mg/kg of I.

The method was subsequently applied to the determination of I and CPT in mouse and human plasma.

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