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Journal of Chromatography A, 797 (1998) 295–303

JOURNAL OF CHROMATOGRAPHY A

Determination of a new polymer-bound paclitaxel derivative (PNU 166945), free paclitaxel and 7-epipaclitaxel in dog plasma and urine by reversed-phase high-performance liquid chromatography with UV detection

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Abstract

A sensitive and selective high-performance liquid chromatographic method for the determination of PNU 166945, a new polymer-bound paclitaxel derivative, free paclitaxel and 7-epipaclitaxel in dog plasma and urine has been developed. The method involves a solid-phase extraction of free paclitaxel and its possible degradation product 7-epipaclitaxel from plasma and urine, previously buffered with an equal volume of 0.05 *M* or 1 *M* KH_2PO_4 respectively, on 1-ml cyanopropyl columns. Cartridges elution was performed with the mobile phase, 0.05 *M* (pH 4.6) monobasic potassium phosphate–acetonitrile mixture (45:55, v/v). The samples were chromatographed on a reversed-phase octyl 4-μm column with UV detection at 229 nm. The retention times of paclitaxel and 7-epipaclitaxel were about 14 and 22 min, respectively. Determination of total paclitaxel (free+polymer-bound) was performed after release of paclitaxel from the polymeric carrier by chemical hydrolysis at room temperature (22°C) for 20 h. After addition of 0.5 ml of methanol–0.1 *M* KH_2PO_4 mixture (50:50, v/v, pH=7.5) to 0.5 ml of plasma or urine, paclitaxel was analysed as described above. PNU 166945 concentration was then determined by subtraction of free from total paclitaxel. The linearity, precision, accuracy and recovery of the method were evaluated. The limit of quantitation of the method was 5 ng/ml for biological fluid for paclitaxel and 7-epipaclitaxel and 20 ng/ml for PNU 166945 (as paclitaxel equivalent). © 1998 Elsevier Science B.V.

Keywords: Taxanes; Paclitaxel; PNU 166945

1. Introduction

Paclitaxel (TX), the first taxane to become available for clinical use, exerts its antitumour action by promoting microtubule assembly and inhibiting tubulin depolymerization [1]. It has proven efficacious in several human cancers, such as ovarian, breast and nonsmall cell lung cancer, and has received marketing approval for metastatic ovarian and breast cancer. Encouraging activity has also been reported in oesophageal cancer, bladder cancer, germ cell tumors, Kaposi's sarcoma, endometrial cancer, lymphomas, and head and neck cancer [2]. The initial development of TX as an injectable agent was hindered by its low solubility in water. The drug delivery problem has been partly overcome by administering TX in a Cremophor EL–ethanol mixture, which is diluted to final volume with normal saline or 5% dextrose. This pharmaceutical form requires in-line filtration and avoidance of contact with plasticized polyvinyl chloride (PVC) equipment or devices. The high concentration of Cremophor EL

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0021-9673/98/$19.00
PII S0021-9673(97)01204-1

needed for TX formulation is considered a major cause of the hypersensitivity reactions occurring during the administration of the drug, and might also play a role in other TX-induced toxicities. In addition, recent data suggest that Cremophor EL might modify the pharmacokinetics of TX [3].

PNU 166945 (**I**) is a new highly water-soluble polymeric molecule (average M_r 25 000–35 000) containing the cytotoxic anticancer agent TX (about 5%, w/w). The compound consists of a hydroxypropyl-methacrylamide polymer bearing an amino acid chain (Gly-Phe-Leu-Gly) that is linked through an esteric bond at the 2′ position of TX (see Fig. 1) [4]. Cleavage of the esteric bond releases free TX into the blood stream.

To study the pharmacokinetics of **I**, two groups of compounds should be considered: drug which remains attached to the polymeric carrier, and free drug which has been cleaved from the polymer and may be subjected to subsequent metabolism. It was therefore necessary to select a method which would be suitable for the quantitation of both compounds within a sample (free and bound TX).

Determination of TX in plasma and urine by high-performance liquid chromatography (HPLC) has been previously described [5–12]. The hydrophilicity of **I** makes its extraction from biological matrix difficult. In addition, **I** is not a single chemical entity and its chromatography was not easy. For these reasons it was preferred to release TX from the polymeric moiety by chemical hydrolysis. The concentration of **I** was then estimated by subtraction of free from total TX determined after chemical hydrolysis.

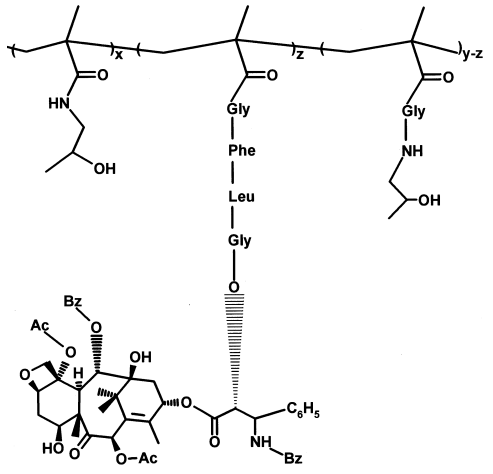

Fig. 1. Structure of compound **I**, a hydroxypropyl-methacrylamide polymer bearing an aminoacid chain (Gly-Phe-Leu-Gly) that is linked through an esteric bond at the 2′ position of TX.

The major metabolites of TX, 6α-hydroxyTX, 3′-*p*-hydroxyTX and 6α,3′-*p*-dihydroxyTX, were not available as pure reference standards and therefore were not considered in this study. On the other hand, since considerable amounts of 7-epiTX, a biologically active stereoisomer, were found during an in vitro study carried out to evaluate the kinetics of hydrolysis of **I** in dog plasma at 37°C, it was decided to determine this compound also. Since good results in terms of precision and accuracy were achieved, no internal standard was used in this study.

2. Experimental

2.1. Chemicals and solutions

Compound **I** and 7-epiTX were supplied by the Chemistry Department of Pharmacia & Upjohn (Milan, Italy). TX was supplied by Indena (Milan, Italy). All other chemicals and solvents were of analytical reagent grade from Carlo Erba Reagents (Milan, Italy). Stock solutions were prepared by dissolving accurately weighed amounts of TX and 7-epiTX in acetonitrile and of **I** in bidistilled water. Suitable working solutions were prepared by dilution with acetonitrile and bidistilled water, respectively.

After storage at 4°C, stock and working solutions of TX and 7-epiTX were found to be stable for at least four weeks. Stock and working solutions of **I** were found to be stable for one week.

2.2. Chromatographic equipment

The HPLC system used in this study consisted of an isocratic pump (Model Isochrom), an autosampler (Model AS3000) operating at room temperature and equipped with a Rheodyne injection valve (model 7010-090) with a 200-μl loop and a variable-wavelength UV-975 detector. A data acquisition system with WOW software was used for data processing. Real time chromatograms were obtained on an integrator (Model Chromjet). All these instruments

were from Thermo Quest (San Jose, CA, USA) except the detector which was purchased from Jasco (Hachioji, Japan).

2.3. Chromatographic conditions

The chromatographic separation was performed with a LiChroCART 250×4 mm I.D. Superspher 60 RP-8e reversed-phase column (particle size 4 μm, Merck, Darmstadt, Germany) equipped with a LiChroCART 4–4 mm I.D. LiChrospher 100 RP-8e guard column (particle size 4 μm, Merck, Darmstadt, Germany).

The mobile phase consisted of a 0.175 *M* (pH 4.6) monobasic potassium phosphate–acetonitrile mixture (45:55, v/v). The separation was performed at a flow-rate of 0.45 ml/min. The UV detector was set at 229 nm and wired to send a 1 V/AUFS signal to the data system.

2.4. Sample preparation

2.4.1. Free TX

The method involved a solid-phase extraction of free TX and its possible degradation product 7-epiTX from plasma and urine, stabilised with an equal volume of 0.5 *M* or 1 *M* KH_2PO_4 respectively, onto 1-ml cyanopropyl (CN) BakerBond cartridges. The cartridges were first conditioned with 1 ml of acetonitrile followed by 1 ml of the mobile phase and 1 ml of 0.05 *M* KH_2PO_4. The columns were not allowed to dry. The samples were loaded onto the columns and washed with 1 ml of water, 1 ml of acetonitrile–water (30:70) and then 1 ml of 0.05 *M* KH_2PO_4. The analytes were eluted using two 0.3-ml volumes of the mobile phase. The resulting solutions were transferred to autosampler polypropylene vials and an aliquot (0.2 ml) was injected into the HPLC column.

2.4.2. Total TX

Determination of total TX (free+polymer-bound) was performed after release of TX from the polymeric carrier by chemical hydrolysis at room temperature (22°C) for 20 h. After addition of 0.5 ml of methanol–0.1 *M* KH_2PO_4 mixture (0:50, v/v, pH= 7.5) to 0.5 ml of plasma or urine sample, released TX was extracted as described above. The concentration of **I** was then determined by subtraction of free from total TX.

2.5. Determination of TX, 7-epiTX and compound *I* concentration in quality control and calibration samples

Analyses of blank dog plasma and urine spiked with known amounts of TX, 7epiTX and **I** were carried out applying the above procedure. The linearity was evaluated from six calibration curves prepared and run on six different days in the concentration range, 5.13–512.5, 4.60–460.29, 21.4–214056 (as TX equivalent) ng/ml plasma and 5–10000, 5.80–9860 and 10.03–100280 (as TX equivalent) ng/ml urine for TX, 7epiTX and **I**, respectively. The precision and accuracy were evaluated by repeated analyses of the analytes at three concentrations (low, mid and high) in three replicate samples analysed on six different days. All chromatograms obtained were evaluated by peak area measurement. The concentration in quality control samples was calculated using the calibration curve generated on each day by linear regression (weighting factor $1/y$ except for urine total TX, $1/y^2$) of the analyte peak area against the concentration in plasma or urine.

To evaluate the extraction recovery, the peak area of extracted biological samples was compared to the peak area obtained with standards dissolved in the mobile phase and injected directly onto the chromatograph.

2.6. Kinetics of chemical hydrolysis

The study of the kinetics of chemical hydrolysis of **I** in plasma was carried out at room temperature (22°C) and at pH 7.5, since considerable amounts of degradation products were observed under more strongly acidic or alkaline conditions or at higher temperature. An aliquot of blank dog plasma (0.5 ml) was spiked with a weighed amount of **I** (1000 ng/ml as TX equivalents) and 0.5 ml of the hydrolysis mixture (methanol–0.1 *M* KH_2PO_4; 50:50, v/v, pH 7.5) was added. At predefined times (up to 24 h) the hydrolysis reaction was stopped by adding an equal volume of 0.5 *M* KH_2PO_4. The samples

were then processed as described for free TX. Control samples were prepared by spiking blank dog hydrolysed plasma with weighed amounts of TX.

To evaluate the hydrolysis yield, the peak areas of TX obtained from **I** hydrolysed samples were compared to the peak areas of TX control samples.

*2.7. Release of TX from **I** in plasma at 37°*

In order to evaluate the rate and extent of TX release in vivo from **I**, an in vitro study in plasma of different species (mice, rats, dogs and humans) at 37°C was carried out. Weighed amounts of **I** were added to three batches of blank fresh plasma, collected from three different animals or humans, and kept at 37°C. An aliquot of plasma was removed and processed as described above for free TX and 7-epiTX determinations. This procedure was repeated at 1, 2, 4, 6 and 24 h.

2.8. Stability

Since very high plasma levels of **I** (about 60–90 μg/ml at 5 min) were found in preliminary studies carried out in dogs given an i.v. dose of 9 mg/kg of **I**, the evaluation of its stability in whole blood and plasma during sample collection and analytical manipulations was essential in order to avoid analytical artifacts. Indeed, if only very small amounts of **I** (for example less than 1%) undergo in vitro spontaneous hydrolysis, results obtained for free TX (about 300 ng/ml) may be greatly overestimated. For these reasons the stability of **I** was evaluated both in dog whole blood and in plasma alone and after its stabilisation by addition of an equal volume of 0.5 *M* KH_2PO_4.

2.8.1. Whole blood

The short-term stability of **I** and TX in dog whole blood was investigated at 0°C, by adding weighed amounts of both compounds to three batches of blank blood, collected from three different animals, and kept on ice. An aliquot of blood was removed and centrifuged at 0–4°C at 1200*g* for 10 min; the plasma was separated and processed as described above for free TX determination. This procedure was repeated every 20 min up to 1 h. The stability of **I** in blood at room temperature was not tested since even TX was found not to be stable under these conditions.

2.8.2. Plasma

The stability of **I** in plasma alone and in stabilized plasma at room temperature (22°C) was also investigated. A stock solution of **I** was spiked into a blank pool of dog plasma, alone or stabilized, to give a concentration of 100 000 ng/ml. An aliquot of plasma was removed from the pool and processed as above. This procedure was repeated at 20, 60 and 180 min. The stability of **I** in stabilized plasma was also investigated at 0–4°C, up to 28 h.

In addition, the stability of **I** in dog plasma at 0–4°C, alone and in the presence of 5 mg/ml potassium fluoride, a most commonly used esterase inhibitor, was also investigated.

The stability of **I** in stabilized plasma was also checked under different conditions of freezing and thawing in order to find the most reliable method of handling compound **I** samples during preclinical and clinical trials.

Firstly, stabilized plasma spiked with **I** was frozen at −20°C or at −80°C, then thawed at 0–4°C after one day and analysed. Secondly, stabilized plasma spiked with **I** was flash frozen in a CO_2–ethanol bath, stored at −80°C for six days, rapidly thawed at room temperature and immediately analysed.

2.8.3. In vivo suitability of handling procedures

The suitability of the handling procedures for in vivo samples was assessed by the analyses of plasma samples obtained from a dog given 9 mg/kg **I** (as TX equivalents) as slow infusion (3 h). Samples ere analysed in duplicate: ‘on-line’, therefore without any need for storage, and adopting the second handling procedure described above.

2.8.4. Urine

In order to evaluate the stability of **I** during excretion in urine and while it remains in the bladder, the stability of **I** was checked in dog urine after incubation at 37°C up to 5 h. An aliquot of stock solution of **I** was spiked into a blank pool of dog urine to give a final concentration of about 100 000 ng/ml (as paclitaxel equivalent). An aliquot of urine was removed from the pool and processed as described above for free paclitaxel determination.

This procedure was repeated at 20, 40 min, 1, 2, 4 and 5 h.

2.9. Chromatographic system suitability test

The suitability of the chromatographic system for the analysis of TX, 7-epiTX and **I** was checked during the validation assay by calculating the column efficiency, the peak symmetry and the resolution factor of the peaks of the analytes. This evaluation was carried out according to USP [13] using the System Suitability Test software supplied by Thermo Quest. The column efficiency was expressed as the number of theoretical plates (N). This value must be higher than 4250 for both analytes. The tailing factor (T) must be less than 1.75 for both analytes. The resolution factor (R) between the peaks of the two analytes must be higher than 2.

3. Results and discussion

The chromatogram obtained for free and total TX assay from a spiked dog plasma and from a dog given a 9 mg/kg i.v. dose of **I** (30 h after administration) are shown in Fig. 2A, B, C and D. No interference from blank dog, rat, mouse and human plasma or urine was observed.

Sharp and symmetric peaks were obtained for TX and 7-epiTX under the chromatographic isocratic conditions chosen. Under these conditions, the analysis time was sufficiently short to allow about 48

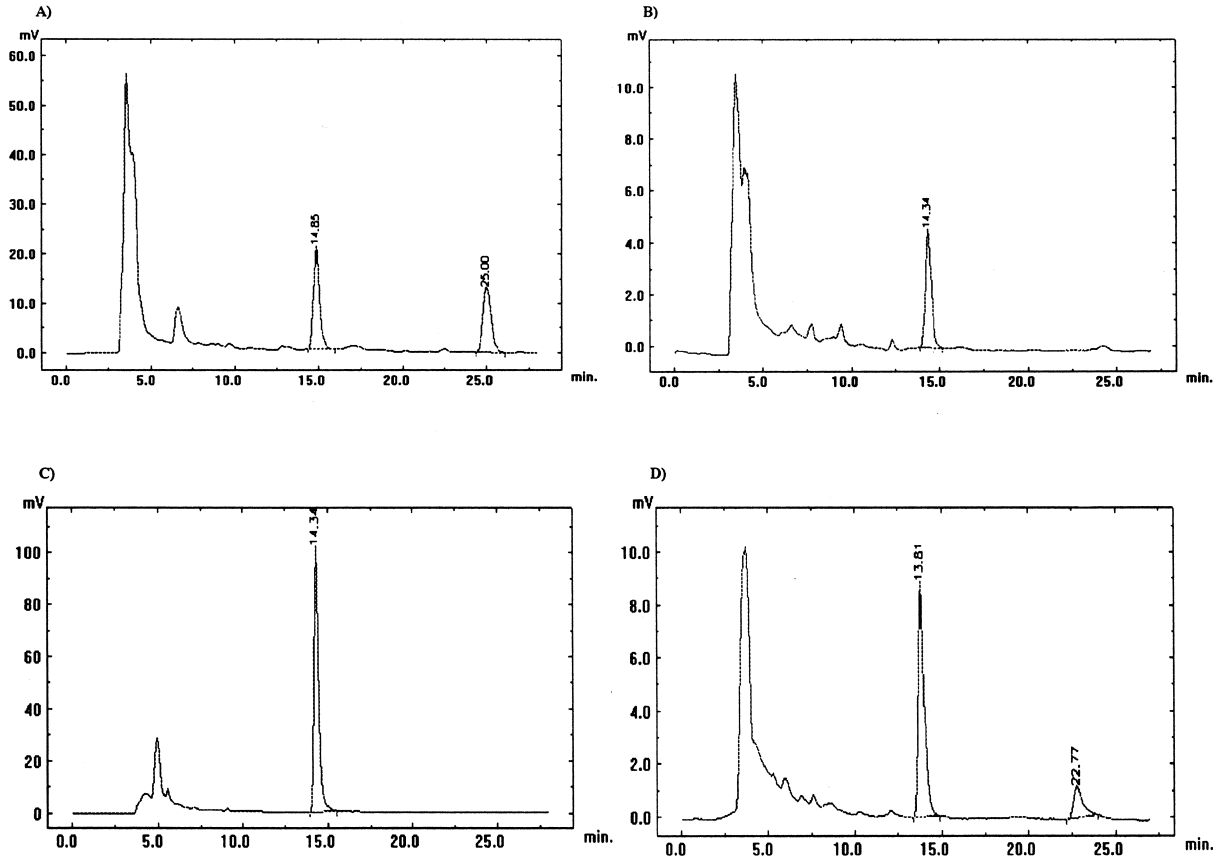

Fig. 2. Chromatograms of blank dog plasma spiked with (A) 333.13 ng/ml of TX (t_R 14.85 min) and 299.19 ng/ml of 7-epiTX (t_R 25 min) (quantitation of free TX) and with (B) 107.03 ng/0.5 ml of **I** (t_R 14.34 min) (quantitation of total TX) and of plasma from a dog given a 9 mg/kg i.v. dose of **I** (30 h after administration): (C) quantitation of free TX and (D) quantitation of total TX. No interfering peaks were observed in blank dog plasma and urine samples.

Table 1
Mean standard curve parameters ($n=6$) of TX, 7-epiTX and **I** obtained in dog plasma (free and total TX assay)

Compound	Linearity range (ng/ml)	Regression estimates		
		Slope	*y*-intercept	Slope R.S.D. (%)
TX	5.13–512.50	3404	6461	8.25
7-epiTX	4.60–460.29	3302	7885	8.93
I	21.40–214056[a]	1048	4265	3.34

[a] as TX equivalent.

samples per run/day to be assayed. The linearity of the method was evaluated from six calibration curves with five calibration points run on different days. The mean standard curves parameters of TX, 7-epi-TX and **I** obtained in plasma and urine are shown in Tables 1 and 2, respectively. The data were analysed by linear regression (weighting factor $1/y$ except for the urine total TX assay, $1/y^2$) of the peak-area versus the concentration. The limit of quantitation of the method was 5 ng/ml of biological fluid for TX and 7-epiTX and 20 ng/ml for **I** (as TX equivalent). Correlation coefficients (r) for the regression were always better than 0.99. Results for the intra-day and inter-day precision and accuracy are shown in Tables 3 and 4. For free TX assay, the mean ($n=3$) extraction recovery from plasma calculated at the concentrations of 512.50 ng/ml for TX and 460.29 ng/ml for 7-epiTX was 96.5% (R.S.D.=1.7%) and 97.7% (R.S.D.=3.7%), respectively. After hydrolysis, the mean ($n=3$) extraction recovery of **I** from plasma, calculated at the concentration of 500.0 ng/0.1 ml (as TX equivalent), was 98.7% (R.S.D.=0.4%)

The mean extraction recovery from urine calculated at the concentration of about 500 ng/ml was 80.6±9.5% for TX and 69.7±8.4% for 7-epiTX (mean+S.D., $n=6$). After hydrolysis of **I** at the concentration of about 5000 ng/ml (as TX equivalent) the mean extraction recovery was 73.7±8.9% (mean±S.D., $n=6$).

Table 2
Mean standard curve parameters ($n=6$) of TX, 7-epiTX and **I** obtained in dog urine (free and total TX assay)

Compound	Linearity range (ng/ml)	Regression estimates		
		Slope	*y*-intercept	Slope R.S.D. (%)
TX	5.0–10000.0	2554	4950	12.8
7-epiTX	5.80–9860.09	1916	−615	10.70
I	10.03–100280.0[a]	967	9110	12.1

[a] as TX equivalent.

Table 3
Accuracy and precision of the method (6 days, 3 concentrations in three replicates) for the determination of TX, 7-epiTX and **I** in dog plasma (free and total TX assay)

Compound	Concentration range (ng/ml)	Accuracy		Precision	
		% Recovery range (intra-day)	Pooled % recovery range (inter-day)	% R.S.D. (intra-day)	Pooled % R.S.D. range (inter-day)
TX	10.25–333.13	88.12–114.42	98.61–107.77	<18.74	7.10–10.62
7-epiTX	9.21–299.19	82.67–106.01	95.78–98.42	<19.18	9.20–12.01
I	107.04–107028[a]	95.13–118.98	101.01–108.79	<17.13	6.83–10.12

[a] as TX equivalent.

Table 4
Accuracy and precision of the method (6 days, 3 concentrations in three replicates) for the determination of TX, 7-epiTX and **I** in dog urine (free and total TX assay)

Compound	Concentration range (ng/ml)	Accuracy		Precision	
		% Recovery range (intra-day)	Pooled % recovery range (inter-day)	% R.S.D. (intra-day)	Pooled % R.S.D. range (inter-day)
TX	20.00–8000	92.73–115.35	102.71–106.86	<12.04	5.81–8.96
7–epiTX	23.20–7540	91.59–116.11	103.07–104.70	<17.91	8.61–10.91
I	50.14–501404.4[a]	92.03–117.16	98.16–107.15	<18.17	9.44–12.35

[a] as TX equivalent.

The results of the kinetics of chemical hydrolysis of **I** are shown in Fig. 3. The yield of hydrolysis (mean±S.D., $n=3$) was 6.3±1.4%, 10.7±0.9%, 22.2±0.7%, 39.7±0.6%, 57.7±0.3%, 70.9±5.2%, 86.7±5.7%, 86.8±3.1% and 88.4±6.1% after 1, 2, 4, 7, 15, 17, 20, 22, and 24 h, respectively. Therefore, the reaction time chosen was 20 h, since no statistically significant increase in released paclitaxel was observed after 22 or 24 h of hydrolysis.

The mean results ($n=3$) of in vitro release of TX from **I** (100 000 ng/ml, as TX equivalent) in dog, rat, mouse and human plasma at 37°C are shown in Fig. 4. The release was almost complete within 7 h in all species. The rate of release was similar in rats, dogs and humans (about 50% of the starting concentration is released within 2 h), while the process seems faster in mice (50% release reached at 1 h). However, considerable amounts of 7-epiTX were found after 24 h (about 30%). TX was found to be stable for at least 1 h, in dog whole-blood at 0°C, while **I** was stable only for up to 20 min. Therefore, immediate centrifugation (in every case within 20 min) of **I** blood samples is strictly necessary. After only 20 min of storage of **I** in dog plasma at room temperature (22°C), a dramatic increase in released TX (+889%) was observed. For this reason plasma was stabilized by adding an equal aliquot of 0.5 *M* KH_2PO_4. Under these conditions the increase in TX was much lower (+57.6%, after 20 min). No improvement in terms of **I** stability was observed when 5 mg/ml potassium fluoride were added to dog plasma at 22°C. In stabilized plasma at 0–4°C, **I** was found to be stable for at least 20 min.

When stabilized plasma spiked with **I** was normal-

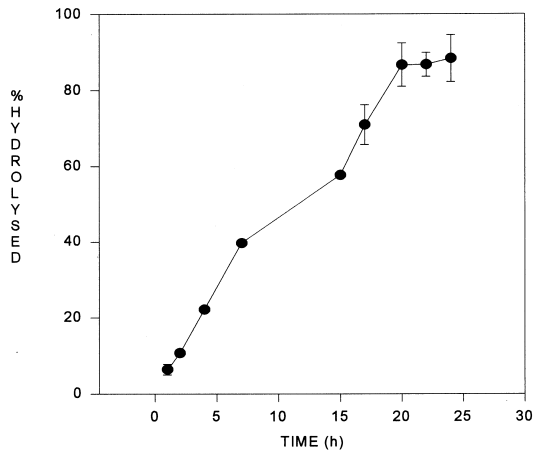

Fig. 3. Kinetics of chemical hydrolysis of **I** at room temperature (22°C) in dog plasma.

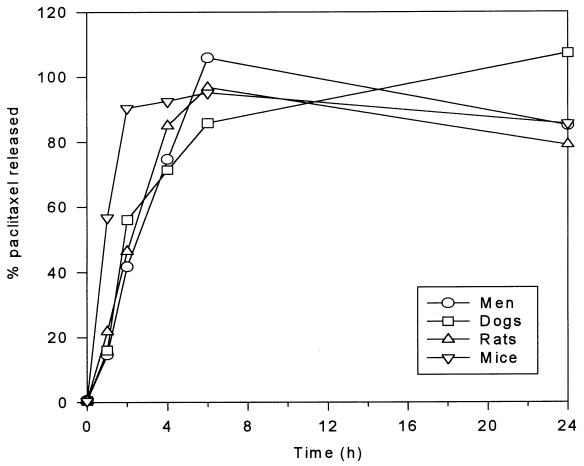

Fig. 4. In vitro release of TX from **I** in mouse, rat, dog and human plasma at 37°C. Each point is the mean of three replicates. **I** starting concentration: 102 μg/ml.

ly frozen at −20°C or at −80°C and then thawed at 0–4°C after one day and analysed, an increase in released TX of 43.2 and 31.8%, respectively, was observed. On the contrary, no increase in released TX was observed when stabilized plasma spiked with **I** was flash frozen in a CO_2–ethanol bath, stored at −80°C for six days, rapidly thawed at room temperature and immediately analysed.

In conclusion, collecting blood samples on ice, immediately centrifuging at 0–4°C (10 min at 1200 g), separating and stabilizing plasma by adding an equal aliquot of precooled 0.5 *M* KH_2PO_4, flash freezing in a CO–ethanol bath, storing at −80°C, thawing at room temperature and immediately analysing was the most reliable method of handling **I** samples, since no increase in TX content was observed when this procedure was followed scrupulously.

Seven in vivo samples obtained from a dog given 9 mg/kg of **I** as slow infusion (3 h) were assayed in duplicate 'on-line', therefore without any need for storage, and adopting the handling procedures described above. Comparison of plasma profiles obtained is shown in Fig. 5. Moreover, linear regression analysis was applied to the two sets of data (on-line vs. stored samples). A good correlation was found between the results of the two analyses (r= 0.9668). The correlation curve obtained was described by the equation y=0.9369+6.872. The slope was not significantly different from 1 and the intercept value was not significantly different from 0, as one would expect if the results obtained were in perfect agreement.

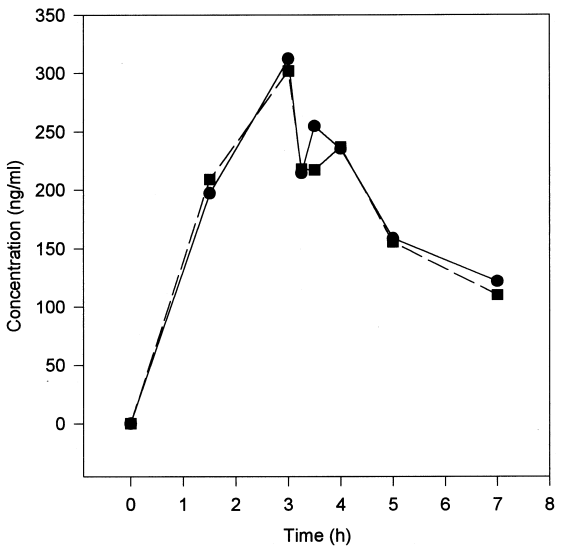

Fig. 5. Comparison of plasma profiles of TX obtained after on-line (●) and after stored (■) samples analyses in a dog given 9 mg/kg **I** as slow infusion (3 h).

The results obtained for the short-term stability of **I** in dog urine after incubation at 37°C, expressed as percentage increase, showed that after 20 min, an increase in free TX content higher than 50% was already observed, and after 5 h the increase was 211.5%. For this reason, urinary levels of free TX generated after **I** administration should be interpreted with great caution.

4. Conclusion

The proposed method is sensitive and selective for the determination of TX, 7-epiTX and **I** in dog plasma. It proved to be specific and sensitive enough to be applied to preclinical 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 and urine: samples obtained from dogs who had received a single i.v. dose of 3 and 9 mg/kg of **I** and TX.

The method was subsequently validated in mouse and rat plasma. Since blank human plasma and urine samples assayed according to the procedures described (free and total TX) show chromatograms free of interferences at the retention times of the compounds of interest the method may also be used for the determination of the same compounds in human plasma and urine.

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