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Analytical procedure for the determination of the new antitumour drug N-benzoylstaurosporine and three potential metabolites in human plasma by reversed-phase high-performance liquid chromatography

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Abstract

The staurosporine derivative, N-benzoylstaurosporine (CGP 41 251; I), is a protein kinase C inhibitor that has been selected for phase I clinical evaluation in cancer patients. We have developed a selective and sensitive assay of the drug and three potential metabolites in human plasma. The method is based on reversed-phase high-performance liquid chromatography with fluorescence detection. The sample pretreatment involves liquid–liquid extraction with diisopropyl ether with recoveries over 88%. The limit of detection and limit of quantitation of the parent compound and two metabolites were 0.5 and 1.0 ng/ml, respectively. For the third metabolite the limit of detection and limit of quantitation were 1.0 and 2.0 ng/ml, respectively. Linear calibration lines were obtained over the range of 1–1000 ng/ml. The between-day and within-day precisions were $<7.1\%$ for all the analytes. In plasma the compounds were stable for at least one month if stored at $-30°C$ or below. The applicability of the method for in vivo studies has been demonstrated in a pharmacokinetic study in rat receiving 0.5 mg/kg of the drug as an intravenous bolus injection. Compound I and two metabolites were detected.

1. Introduction

N-Benzoylstaurosporine (CGP 41 251, I, Fig. 1) is a derivative of the microbial alkaloid staurosporine and an inhibitor of the protein kinase C (PKC) enzyme family. PKC comprises a group of at least ten isoenzymes with the capability to phosphorylate a variety of intracellular target proteins on serine and threonine residues. The enzymes are involved in the transduction of cell proliferation and differentiation signals from the cell membrane to cytoplasm and nucleus. Currently it is believed that by inhibition of abnormally expressed signalling pathways

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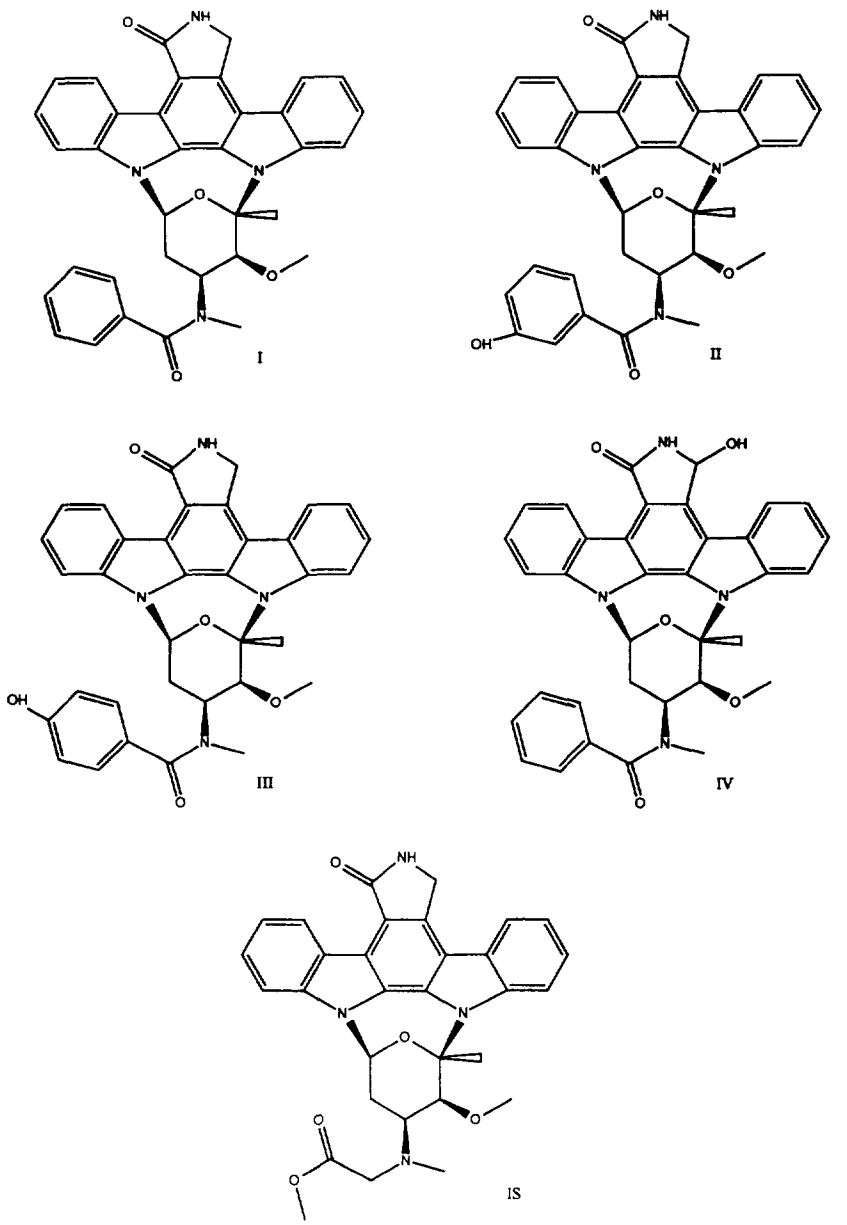

Fig. 1. Structures of N-benzoylstaurosporine (CGP 41 251; I), CGP 50 750 (II), CGP 50 723 (III), CGP 52 421 (IV) and CGP 41 126 (I.S.).

in malignant cells, cytostasis and cytotoxicity can be achieved. Compound I has been found to be a potent reversible inhibitor of PKC and platelet-derived growth factor (PDGF) mediated signal transduction. In comparison with the parent compound staurosporine, I displays a more selective inhibition of PKC [1]. In mice, I is better tolerated than staurosporine and shows an improved efficacy in tumour growth inhibition [1]. This might be explained by the different selectivity in inhibition of various PKC isoenzymes in comparison with staurosporine [2]. Activity of I has been demonstrated in vitro against a wide variety of malignant cell lines

including leukaemia, bladder carcinoma, colon carcinoma, melanoma, renal carcinoma and prostate carcinoma and in vivo in nude mice with melanoma, bladder carcinoma, sarcoma and lung carcinoma [1–3]. Both oral and intra-peritoneal administration gave activity against primary tumour and metastases in vivo. Based of these interesting properties I has been selected for further phase I testing. In the proposed phase I study the drug will be given orally during a 21-day schedule. The primary aims will be to evaluate the safety of drug administration, to establish the maximum tolerated dose (MTD) and to study the pharmacokinetics of the drug. On the basis of the chemical structure of the drug several metabolic products can be expected.

We have developed a selective and sensitive assay for the determination of I and three potential metabolites, CGP 50 750 (II); CGP 50 723 (III) and CGP 52 421 (IV) (Fig. 1), in human plasma. CGP 41 126 was used as an internal standard (I.S.). The reported assay was validated, and the chemical stability of the analytes in plasma and in dimethyl sulfoxide (DMSO) was investigated. This is the first report in the literature describing the analysis of I and metabolites in plasma.

2. Experimental

2.1. Equipment

The HPLC system consisted of a solvent delivery system type SP 8800 (Spectra-Physics, Santa Clara, CA, USA), an LS 40 fluorescence detector (Perkin-Elmer, Norwalk, CT, USA), a SP 8880 automated sample injection device and a SP 4600 integrator (Spectra-Physics). The analytical column (300 × 3.9 mm I.D.) was packed with μBondapak RP-18 material (particle size 10 μm) (Waters Assoc., Milford, MA, USA) and was protected by a Chromspher RP (10 × 3.0 mm I.D.; particle size 5 μm) (Chrompack) guard column. UV spectra of I, II, III, IV and I.S. were recorded on a GBC 918 UV–Vis spectrophotometer (GBC, Dandenong, Virginia, Australia). Fluorescence emission and excitation spectra of the analytes were recorded on-line with the HPLC LS-40 fluorescence detector.

2.2. Chemicals

The test compounds I, II, III, IV and the I.S. originated from Ciba Geigy (Basel, Switzerland) and were used as supplied. Acetonitrile (ChromAr) was obtained from Mallinckrodt (Paris, KY, USA), ammonium acetate (p.a.), sodium hydroxide (p.a.), dimethyl sulfoxide (p.a.) (DMSO), ethanol abs.(p.a.) and diisopropyl ether (Lichrosolv) were from Merck (Darmstadt, Germany). Home made distilled water was used throughout.

Drug-free heparinized human plasma was obtained from the Central Laboratory of the Netherlands Red Cross (Amsterdam, Netherlands).

2.3. Stock solutions

Stock solutions of I, II, III, IV and I.S. (1.00 mg/ml) were prepared by dissolving the appropriate amount of each drug, accurately weighed, in DMSO–ethanol (1:9, v/v). The stock solutions were stored at −30°C and were stable for at least 2 months under these conditions.

2.4. Standard solutions

The stock solutions were diluted with DMSO–ethanol (1:9, v/v) to give standard solutions of each component with concentrations of 100, 10 and 1 μg/ml.

2.5. Mixed standard samples

Aliquots (100 μl) of each standard solution of I, II, III and IV, were transferred to a polypropylene Eppendorf cup and the total volume of DMSO–ethanol (1:9, v/v) of each calibration standard was brought to 1000 μl to give mixed standard solutions with concentrations of 10, 1 and 0.1 μg/ml of each component.

2.6. Internal standard (I.S.) solution

The stock solution of the I.S. was diluted with DMSO–ethanol (1:9, v/v) to give an I.S. solution with a final concentration of 0.5 μg/ml.

2.7. Calibration samples

Calibration samples were prepared with each run by adding 0, 5, 10, 25, 40 and 50 μl of each mixed standard solution to 1000 μl of drug free plasma–water mixture (1:1, v/v) in a polypropylene tube. The total volume of each sample was supplemented to 1050 μl with DMSO–ethanol (1:9, v/v). Calibration samples were prepared in the range 1–1000 ng/ml in plasma.

2.8. Quality control samples

Quality control (QC) samples were prepared from freshly made stock and standard solutions. Volumes of 500 μl of plasma, containing 5, 50 or 500 ng/ml of I, II, III and IV, were prepared in polypropylene test tubes and were stored at −30°C until analysis.

2.9. Sample pretreatment

To a 500-μl sample in a 10-ml polypropylene tube, 500 μl of water and 50 μl of the DMSO–ethanol (1:9, v/v) solution were added.

2.10. Extraction procedure

To each sample (patient, calibration or QC) 20 μl of the internal standard solution was added and subsequently the samples were thoroughly mixed with 5.00 ml of diisopropyl ether on a vortex-mixer for 1 min. Next, the samples were centrifuged at 2500 *g* for 10 min at room temperature, the aqueous phase was frozen on dry ice–ethanol and the supernatant was decanted into a clean tube. The solvent was evaporated under a gentle stream of nitrogen at 40°C. The residue was redissolved in 200 μl of DMSO. An aliquot of 20 μl of this solution was subjected to chromatography.

2.11. Chromatography

Chromatographic analyses were performed at ambient temperature with a mobile phase of acetonitrile–ammonium acetate buffer (0.001 *M*, pH 4.0) (45:55, v/v). Prior to mixing the acetonitrile and the ammonium acetate buffer the solutions were filtered through a 0.22-μm filter. The fluorescence was monitored with excitation and emission wavelengths set at 286 nm and 386 nm, respectively. The flow-rate was maintained at 2.0 ml/min. Aliquots of 20 μl were injected onto the chromatographic system.

2.12. Validation

The absolute recovery of the extraction procedure was calculated by dividing the slope of the calibration line in the biological matrix by the slope of the calibration line in the mobile phase (untreated standards). The accuracy and precision of the method were determined by replicate analysis ($n = 5$) of the quality control samples in three individual analytical runs. Ratios of the peak areas of I, II, III or IV and the I.S. were used for quantitative computations. Calibration lines were calculated by weighted ($1/x^2$) least squares linear regression analysis [4] using a commercial software package (NCSS, Kaysville, UT, USA). The limit of detection was defined as the concentration of drug giving a signal-to-noise ratio of 3:1. The lower limit of quantitation was defined as the concentration of the lowest standard in the analytical run which was quantitated with a definite level of certainty (accuracy and precision $< 20\%$) [5].

2.13. Determination of stability

The chemical stabilities of I, II, III and IV were investigated by adding known amounts of the analytes to plasma. The spiked samples were stored in the dark at 4°C, −30°C, −70°C and at ambient temperature and the drug concentrations were determined at $t = 0$, $t = 1$ week and $t = 1$ month. The stability of the compounds dissolved in DMSO, exposed to normal fluorescent light, and kept at ambient temperature was

also studied. The stability of I, II, III, IV after two freeze (−30°C)–thaw cycles was tested in plasma spiked with 50 and 500 ng/ml.

2.14. Pharmacokinetics

Two male rats (WAG/Rij; weight approximately 200 g) received 0.5 mg/kg of I dissolved in 50 μl of DMSO–ethanol (1:1, v/v) formulation. The compound was given as an intravenous bolus injection in the tail vein. At serial time points blood samples of 300 μl were withdrawn from a cannula in the carotid artery, collected in Eppendorf cups containing 10 μl of heparin solution (equivalent to 50 I.U.) and centrifuged immediately (3 min at 2500 *g*) to obtain the plasma fraction. An aliquot of 100 μl was mixed with 400 μl of drug free human plasma and stored at −30°C prior to analysis.

3. Results and discussion

3.1. Chromatography and detection

Satisfactory separation of I, II, III, IV and the internal standard was accomplished using a μBondapak C_{18} column in combination with a mobile phase of acetonitrile–acetate buffer pH 4. Baseline separation between compounds II and III could, however, not be obtained (Fig. 2A). Other columns tested showed even worse selectivity for these compounds. Changing the pH value of the mobile phase only affected the retention of the I.S., while the chromatography of I, II, III and IV remained unaltered. Apparently II is not formed in rats after i.v. administration of I and this does not jeopardize the quantitation of III here (Fig. 2D). Compound I is virtually insoluble in water but can be readily dissolved in DMSO [3]. In a DMSO–ethanol mixture (9:1, v/v) the UV absorption spectrum of I showed a maximum around 294 nm (molar absorptivity: 68 500). However, at this wavelength there were many endogenous interferences in the chromatograms of plasma samples which will preclude UV absorption for sensitive detection. The staurosporine part of the compounds shows native fluorescence which can be utilized for the detection in HPLC. The selection of fluorescence wavelengths was based on scanning experiments with the drug in the mobile phase, and optimum excitation and emission wavelengths were found at 286 and 386 nm, respectively for all compounds I, II, III and IV. The optimum excitation wavelength of the internal standard was at 335 nm, but at 286 nm the compound still absorbed sufficient energy to obtain a useful excitation signal at 386 nm. Representative HPLC chromatograms of a blank human plasma and human plasma spiked with I, II, III, IV and I.S. are depicted in Fig. 2A,B, respectively.

3.2. Sample pretreatment

Liquid–liquid extraction of plasma samples with diisopropyl ether appeared to be a simple sample pretreatment procedure yielding high recoveries (Table 1). In combination with the HPLC system this procedure also provided high selectivity. The chromatograms of six different blank plasma samples obtained from healthy volunteers were free of any interfering substances (Fig. 2A).

3.3. Validation of the assay

The analytical methodology was validated in terms of recovery, detection limit, precision, accuracy and linearity for all compounds I, II, III and IV [4,5].

The overall mean recoveries ranged from 88.0% to 99.7% (Table 1). The calibration lines in plasma and DMSO were linear ($r > 0.995$) over the range of interest (1–1000 ng/ml) for all the investigated compounds using 500-μl samples. The limit of detection of I, II and III was 0.5 ng/ml using 500 μl of plasma and the limit of quantitation of these compounds was 1.0 ng/ml. Using the same amount of plasma the limit of detection and the limit of quantitation for compound IV were 1.0 and 2.0 ng/ml, respectively. The between-day and within-day precision were $< 7.1\%$ for all the analytes (Table 2).

Several drugs including acetylsalicylic acid,

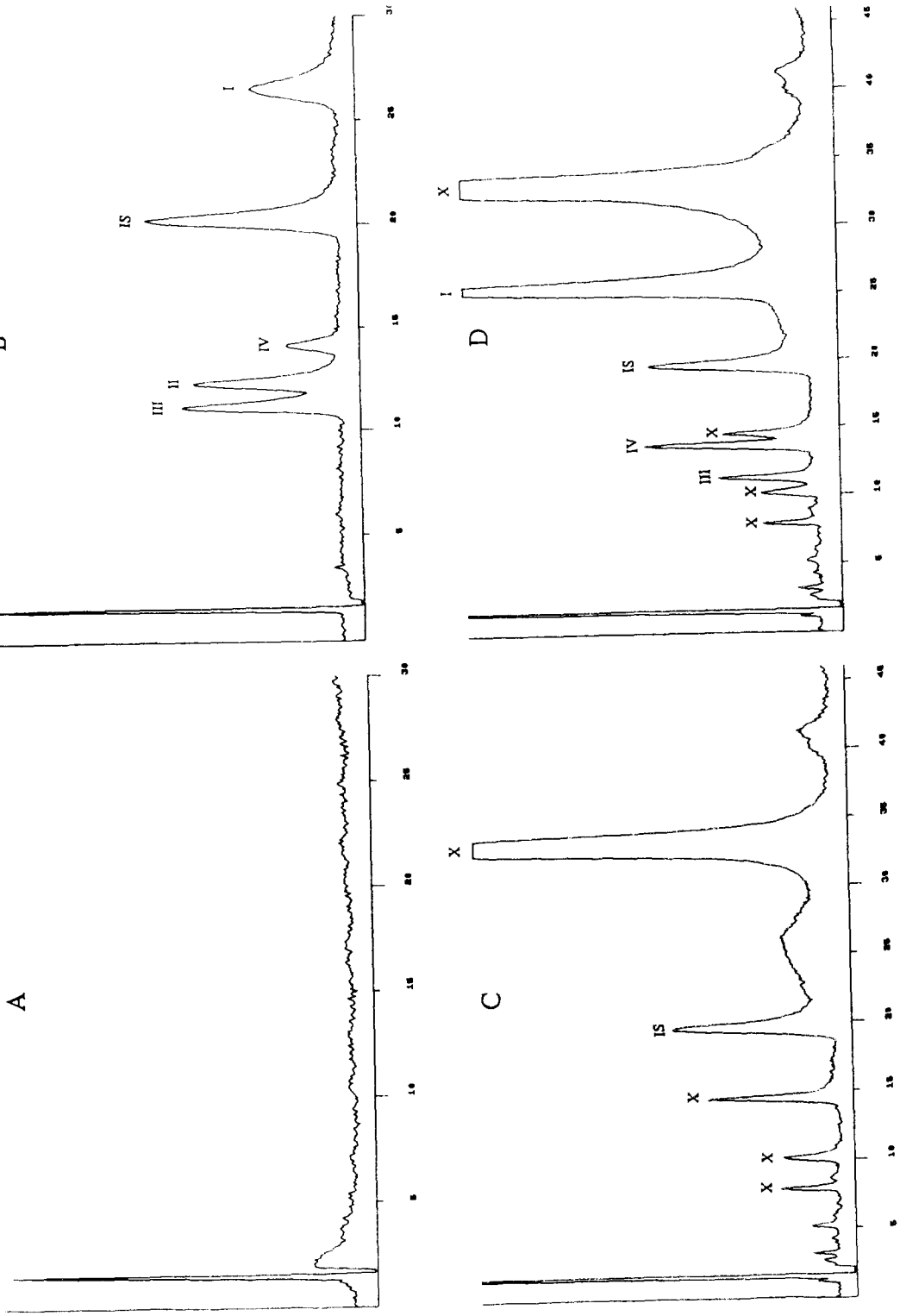

Fig. 2. HPLC chromatogram of a blank human plasma (A), a spiked human plasma (B) (concentration of I, II, III and IV: 2.0 ng/ml; concentration of I.S.: 400 ng/ml), a blank rat plasma (C) (endogenous substances are marked with X) and a rat plasma sample (D) after receiving 0.5 mg/kg of I as an intravenous bolus injection (concentrations: I = 11.2 ng/ml; III = 1.8 ng/ml; IV = 8.8 ng/ml; I.S. 400 ng/ml).

Table 1
Recoveries of I, II, III and IV calculated from the ratio of the slopes of processed and non-processed standard curves (concentration range 1–1000 ng/ml)

Component	Recovery (%)	R.S.D. (%)	n
I	99.7	7.1	3
II	88.0	7.0	3
III	90.0	10.5	3
IV	88.5	11.3	3

dexamethasone, acetaminophen, ibuprofen, megesterol acetate, morphine, procainamide and ranitidine, each in its own therapeutic concentration and dissolved in mobile phase, were injected onto the HPLC system. None of these compounds interfered with the compounds to be determined.

3.4. Stability

At concentrations of 50 and 500 ng/ml compound I was stable in plasma at 4 and 25°C for at least one week when protected against light. Compounds I, II, III and IV (concentrations of 50 and 500 ng/ml) were stable at −30 and −70°C when stored in the dark for at least one month.

All compounds were also stable (concentration range 1–1000 ng/ml) in DMSO at ambient temperature, when protected against light, for at least 24 h. This enables the use of an autosampler for overnight HPLC analyses.

When exposed to normal fluorescent day light

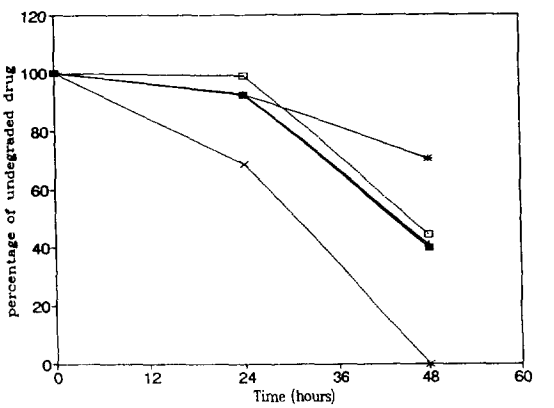

Fig. 3. Degradation of I (□), II (+), III (■), IV (☆) and I.S. (×) in DMSO at ambient temperature and exposed to normal fluorescent day light. The curves of II (+) and III (■) coincide.

Table 2
Accuracy and precision (C.V.) for the bio-analysis of I, II, III and IV in human plasma

	Theor. conc. (ng/ml)	Measured conc. (ng/ml)	Accuracy (%)	Precision[a] (%)	
				Between-day	Within-day
I	5.00	4.52	90.4	1.2	5.2
	50.0	47.3	96.6	2.1	4.9
	500	534	106.8	5.1	3.2
II	5.00	4.72	94.4	3.6	3.9
	50.0	50.3	100.7	5.9	4.3
	500	508	101.6	–[b]	3.1
III	5.00	4.82	96.4	3.4	3.0
	50.0	48.9	97.8	1.6	5.4
	500	502	100.7	1.7	2.6
IV	5.00	4.99	99.9	3.3	7.1
	50.0	51.9	103.7	5.1	6.9
	500	505.7	101.2	2.9	2.8

[a] Between day precision was calculated from three independent runs. Values are the mean of five determinations.
[b] No significant additional variation was observed as a result of performing the assay in different runs.

the drugs, dissolved in DMSO or DMSO–ethanol, rapidly degraded at ambient temperature (Fig. 3). No degradation of I, II, III and IV has been observed when the analytes (concentrations: 50 and 500 ng/ml) were subjected to 2 freeze–thaw cycles.

3.5. Applicability

Although the assay has been validated for the determination of the compounds in human plasma, the applicability of the method, so far, could only be demonstrated in an experiment with I given to rats. No human samples were available, because the clinical phase I study has not yet been started. Chromatograms of blank samples from rats contained substantially more peaks (Fig. 2C) although none of the interferences co-eluted with the reference compounds. Fig. 4 depicts the plasma concentration–time curves of I, III and IV obtained in a rat after an intravenous bolus injection of I. For the analysis of the rat plasma samples, the samples were diluted first (1:5) with blank drug free human plasma. Already in the first sample, obtained 2 min after drug administration, III and IV were present. Metabolite II was not detected in any of the samples obtained within the tested time interval. After 24 h, levels of III and IV became undetectable whereas the concentration of I was still detectable (1.1 ng/ml). We expect that the sensitivity of this assay, using a 500 -μl plasma sample, should be sufficient for the planned human pharmacokinetic studies.

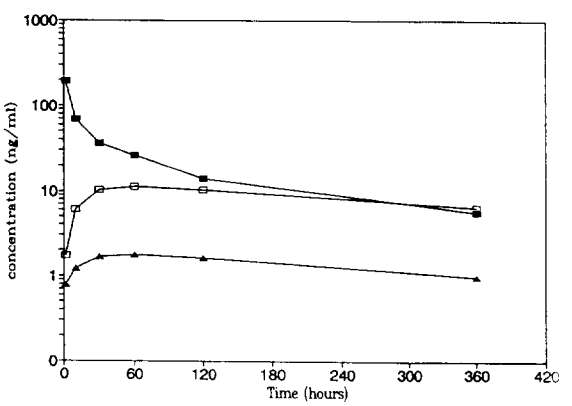

Fig. 4. Log concentration–time curves of I (■), III (▲) and IV (□) in plasma of a rat after receiving 0.5 mg/kg of I as an intravenous bolus injection.

4. Conclusions

A simple, sensitive, selective and validated HPLC method for the simultaneous analysis of the new investigational PKC inhibitor N-benzoyl-staurosporine and three potential metabolites has been presented.

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