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Journal of Chromatography B, 652 (1994) 187–194

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Assay of the antiangiogenic compound TNP-470, and one of its metabolites, AGM-1883, by reversed-phase high-performance liquid chromatography in plasma

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(First received August 9th, 1993; revised manuscript received November 1st, 1993)

Abstract

This paper describes a reversed-phase, high-performance liquid chromatographic (HPLC) method for the isolation, detection, and quantification of TNP-470 (I) and one of its active metabolites, AGM-1883 (II), from plasma. These compounds are initially extracted from plasma with an organic solvent and then separated from one another on a C_{18} column. Those fractions eluting from the C_{18} column and containing either I or II are then derivatized through their epoxide moieties with sodium 8-quinolinethiolate (SQT). This derivatization produces fluorescent species that are isolated and quantified by a second reversed-phase HPLC analysis. The assay yields a lower limit of reliable quantification of 2.5 ng/ml and is linear to a concentration at least as high as 160 ng/ml. The inter-assay percent coefficient of variation is less than 18%.

1. Introduction

The proliferation of new blood vessels is essential to the continued growth of solid tumors, and inhibition of angiogenesis has been proposed as a potential means for selectively impairing tumor growth. Inhibition of angiogenesis should be selective for deterring the growth of tumors inasmuch as the maintenance of most normal adult tissues does not require new blood vessel formation. Compounds that inhibit angiogenesis include protamine [1], interferon [2], specific steroids in combination with heparin [3], a sulfated polysaccharide–peptidoglycan complex [4], pentosan, platelet factor-4 [5], and D-penicillamine [6]. A new class of compounds, now called angioinhibins, was dis-

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SSDI 0378-4347(93)E0388-7

covered by Ingber *et al.* when it was noted that a fungal contaminant of a capillary endothelial cell culture produced a zone of endothelial cell rounding, a phenomenon characteristic of other antiangiogenic compounds [7]. The substance responsible for this phenomenon was found to be fumagillin, an antibiotic naturally secreted by the fungus *Aspergillus fumigatus* fresenius [7]. Although fumagillin was found to have antiangiogenic activity both in endothelial cell cultures and in an *in vivo* model of tumor growth, it was found to cause severe weight loss in mice with prolonged administration, and a search was conducted to identify less toxic analogues. TNP-470, *o*-chloroacetylcarbamoyl fumagillol (I), a semisynthetic derivative of fumagillin, was found to be 50 times more potent than fumagillin in inhibiting capillary growth and to be less toxic than the parent compound. I is now undergoing phase I testing in patients with solid tumors.

Compound I inhibits the proliferation of endothelial cells in the presence of basic fibroblast growth factor (bFGF) [7]. More recently, Saville *et al.* have found that I inhibits the growth of a spindle cell line derived from a patient with Kaposi s sarcoma (KS) at a concentration of 10 pg/ml, whereas the proliferation of peripheral blood mononuclear cells is not affected until a concentration of approximately 1 ng/ml is reached [8]. Angiogenesis is postulated to be important in the pathogenesis of Kaposi's sarcoma and two of the initial clinical trials of I are being conducted in patients with acquired immunodeficiency syndrome (AIDS) and Kaposi's sarcoma.

To support the pharmacokinetic and pharmacodynamic analysis of the National Cancer Institute's clinical trial of I, we have developed an HPLC assay for both I and one of its active metabolites, AGM-1883 (II). Neither compound possesses a useful chromophore, and the plasma concentrations anticipated to be achieved *in vivo* are in the low ng/ml range. Hence we took advantage of the presence of reactive epoxide groups in both compounds to subject them to nucleophilic substitution with sodium 8-quinoinethiolate (SQT), a thiolate anion which yields a fluorescent product [9]. Because the reactions of I and II with SQT yield identical products in an alkaline aqueous solution, it is necessary to separate these compounds prior to derivatization.

2. Experimental

2.1. Materials and reagents

Compounds I and II (see Fig. 1) were supplied as alkaline freeze-dried powders by the Research and Development Division of Takeda Chemical Industries (Osaka, Japan) through the Cancer Treatment Evaluation Program of the National Cancer Institute, National Institutes of Health. Both I and II were stored at − 80°C. 8-Mercap-

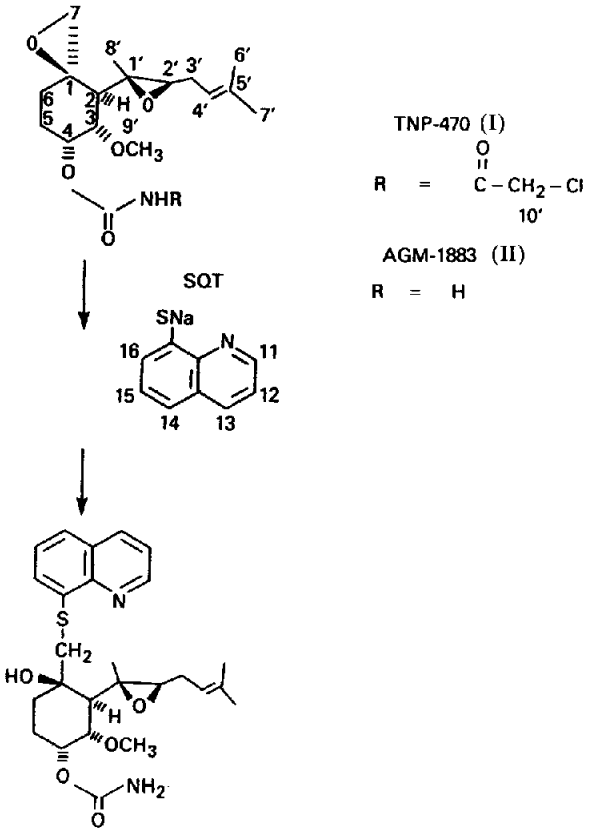

Fig. 1. Chemical structures of I, II, sodium 8-quinolinethiolate (SQT), and the derivatization product (bottom structure).

toquinoline hydrochloride (MW 197.7) was obtained from Sigma (St. Louis, MO, USA) and stored in a desiccator at +4°C. Twenty-five percent (wt.%) sodium methoxide was purchased from Aldrich (Milwaukee, WI, USA). HPLC grade acetonitrile from J.T. Baker (Phillipsburg, NJ, USA) was used both in sample extraction and in HPLC analysis. HPLC grade water was obtained with an in-house reverse osmosis system whose product was further processed through a Milli-Q UV Plus polishing unit (Millipore Co., Marlborough, MA, USA). All other chemicals were reagent grade or better.

2.2. Preparation of SQT from 8-mercaptoquinoline hydrochloride

The SQT powder was prepared by making a suspension of 8-mercaptoquinoline hydrochloride (1.0 g, 5.1 m*M*) in methanol (6.0 ml). Sodium methoxide (25 %) in methanol (2.2 ml, 10.2 m*M*) was added to the above suspension while being stirred and cooled on ice. After stirring for 10 min, the mixture was transferred to borosilicate glass tubes (VWR Scientific, San Francisco, CA, USA) and evaporated with moisture free air in a heated water bath (40°C) to produce a light yellow powder. The powder was washed three times with 10 ml of ether (99.9%, HPLC grade, Aldrich) and centrifuged (1202 *g* for 10 min, Sorvall RT 6000D, DuPont, Wilmington, DE, USA). The ether layer was decanted and the powder (sodium 8-quinolinethiolate and sodium chloride) was dried under the above conditions. This powder was stored in a light resistant vial at −80°C and was used repeatedly for 4 months (see Fig. 1).

2.3. Preparation of standards

Powder of both I and II was weighed on a Mettler AE 240 analytical balance (Mettler Instrument Co., Highstown, NJ, USA) and dissolved in acetonitrile–water (25:75, v/v) to yield a final concentration of 1 mg/ml for each compound. This solution was aliquoted into 2.0-ml cryotubes (Nunc Co., Denmark) (300 μl/tube) and stored in a −80°C Bio-Freezer (Forma Scientific, Marietta, OH, USA). A standard curve was prepared by thawing a 1 mg/ml aliquot for each of the compounds and diluting 160 μl of each in a 50-ml volumetric flask with acetonitrile–water (25:75, v/v) (concentration = 3.2 μg/ml). Serial dilutions (1:2) with acetonitrile–water (25:75, v/v) were conducted to further derive the appropriate concentrations. A 50-μl volume of each of the dilutions (both I and II) was added to 900 μl of plasma to generate a standard curve (final plasma concentrations 160, 80, 40, 20, 10, 5, 2.5, and 1.25 ng/ml). This spiked plasma then underwent the same procedures as the patient samples described below.

2.4. Sample preparation and extraction

Blood for the determination of circulating levels of I and II was drawn by venipuncture into green top (heparinized) Vacutainer collection tubes which were immediately placed on ice and then centrifuged at 1202 *g* at 4°C for 5 min in a Sorvall RT 6000D centrifuge. To 1 ml of plasma, 100 μl of 2% (wt.%) H_2SO_4 (Mallinckrodt, Paris, KY, USA) was added. The addition of sulfuric acid to the samples had the effect of acidifying the plasma to a pH of 4 to 5, a pH range in which I is most stable (see Table 1) [10]. Acidification of the plasma also served to partially denature plasma proteins. Plasma samples so prepared were then stored at −80°C until assayed.

Plasma to be assayed for I and II was thawed, and then 500 μl was placed into a 1.7-ml microcentrifuge tube (PGC Scientifics, Gaithersburg, MD, USA). To the 500 μl of plasma sample was added 1 ml of acetonitrile containing 0.01 *M* acetic acid (Mallinckrodt). Acetic acid was added to the acetonitrile to insure that the pH of the samples remained acidic during the extraction process. After addition of the acetonitrile–acetic acid solution the samples were vortex-mixed for 20 to 30 s and then centrifuged at 5654 *g* for 15 min at 4°C (horizontal rotor, 7.0 cm radius, IEC Centra MP4R, International Equipment Co., Needham Height, MA, USA). A 1-ml aliquot of supernatant was removed, placed into a borosilicate glass tube, and dried under a

Table 1
Stability of I

Time	Percentage				
	pH 1	pH 2	pH 4	pH 5	pH 7
25 μg/ml in water stored at 23°C[a]					
0hr	100	100	100	100	100
4 hr	0	88.3	98.6	97.2	81.6
24 hr	0	34.7	84.6	81.8	37.6
160 ng/ml in plasma stored at −80°C					
7 days	–	–	110.6	–	–
30 days	–	–	97.6	–	–

[a]The 25 μg/ml data is from ref. 10.

stream of nitrogen or moisture-free air while being maintained at 40°C (TurboVap LV, Zymark Co., Hopkinton, MA, USA).

The dried residue was redissolved in 100 μl of acetonitrile and vortex-mixed for 10 s and then 200 μl of water was added to yield a sample with a 33% (v/v) concentration of acetonitrile. This solution was then filtered with an Ultrafree MC centrifugal microfiltration device (0.46 μm pore size, Millipore Co., Boston, MA, USA) subjected to 3705 *g* for 15 min using a 45° fixed-angle rotor (10.5 cm radius, Centrifuge 5402, Eppendorf Inc.). The filtrate was placed into 300-μl borosilicate autosampler vials (Hewlett-Packard, Palo Alto, CA, USA) and maintained at 4°C pending injection onto the liquid chromatographic system described below.

2.5. Separation of I and II by HPLC

The chromatographic apparatus consisted of a Hewlett-Packard 1090 series II liquid chromatograph (Hewlett-Packard) (LC) equipped with a refrigerated autosampler compartment and a diode-array ultraviolet absorbance (UV) detector. The column used was a Nova-Pak C_{18} (Waters, Milford, MA, USA), 150 × 3.9 mm I.D., maintained at 40°C and protected by a Nova-Pak C_{18} Guard-Pak (Waters). The LC was coupled to and used to control a Pharmacia Frac 200 fraction collector (Pharmacia, Uppsala, Sweden), allowing the recovery of those column effluent fractions containing I and II, respectively. Eluates were maintained at 4°C via perfusion of the fraction collection tray with a circulating water bath. Injecting 200 μl of the 300-μl sample, I and II were separated from one another using a gradient of acetonitrile (mobile phase B) and water (mobile phase A) at a flow-rate of 1 ml/min. The mobile phase gradient for acetonitrile increased from 30% to 95% over 18 min. Fractions were collected between 6.4 and 8.8 min (for II) and between 9.0 and 12.5 min (for I). The delay volume between the UV detector and the fraction collector was 300 μl.

2.6. Derivatization of I and II with SQT

The fractions containing I or II were dried under nitrogen or moisture-free air at a temperature of 40°C. A 100-μl volume of acetonitrile was added to the dried fractions to redissolve the material, and then 200 μl of a 1 mg/ml solution of SQT in bicarbonate buffer [90% 0.1 *M* Na_2CO_3, 10% 0.1 *M* $NaHCO_3$, pH 10.8; sodium bicarbonate (Mallinckrodt) and sodium carbonate (Sigma)] was added. The SQT solution was always prepared immediately prior to derivatization. The fractions were vortex-mixed for 15 s and then placed into a 60°C water bath (Precision Water Bath 184, Precision Scientific, Chicago, IL, USA) for 15 min. The tubes were capped during derivatization to avoid sample

evaporation. Immediately upon completion of the 15 min of heating the samples were transferred to 300-μl borosilicate glass autosampler vials and maintained at 4°C until chromatographic injection.

2.7. Detection and quantification of I and II after derivation

The chromatographic apparatus for detection and quantification of derivatized I and derivatized II was identical to that used for the initial separation of the two compounds, with the exception that an HP 1046A fluorescence detector (Hewlett-Packard) was placed in line immediately after the UV detector. A 160-μl volume of the 300-μl sample was injected onto the 150 × 3.9 I.D. mm Nova-Pak C_{18} column (40°C) and was eluted with a gradient of acetonitrile (mobile phase B) and water (mobile phase A) at a flow-rate of 1 ml/min. The mobile phase gradient for acetonitrile increased from 30% to 95% over 15 min. The elution was monitored with an excitation wavelength of 250 nm and an emission wavelength of 426 nm, using an emission cut-off filter of 389 nm and a xenon lamp flash frequency of 220 Hz.

2.8. Characterization of the derivatives of I and II by nuclear magnetic resonance and mass spectroscopy

High concentrations (1 mg/ml) of chromatographically purified derivatives of I and II were evaporated to dryness with moisture-free air while being maintained at 40°C (TurboVap LV) and then subjected to mass measurement and NMR studies. Chemical ionization mass spectrometry (CI-MS) measurements were performed with a Finnegan Model 4600 mass spectrometer (Finnegan, San Jose, CA, USA) using NH_3 or ND_3 reagent gases, the latter permitting the determination of exchangeable hydrogens. ^{1}H-NMR spectra in $CDCl_3$ were measured with a Varian VXR-500S spectrometer (Varian Instrument Group, Columbia, MD, USA).

3. Results

3.1. Recovery of I and II following sample extraction

Recovery of I and II from plasma following extraction with acetonitrile, drying, redissolution in 33% (v/v) acetonitrile–water, and microfiltration was 85 ± 0.9% (mean ± S.D.) for II and 88 ± 0.6% (mean ± S.D.) for I. Insofar as the subsequent steps of the assay involved derivatization of these two compounds, it was impossible to characterize recovery beyond this point.

3.2. Chromatography of underivatized I and II

Injection of microgram quantities of I and II standards enabled peak visualization by UV absorbance at 210 nm (see Fig. 2). The LC was then programmed to activate fraction collection at the appropriate time points.

3.3. Chromatography of I and II following derivatization with SQT

Fig. 3a and 3b show the chromatograms of both I and II after derivatization with SQT. Inspection of these chromatograms, representing plasma samples initially containing 80 ng/ml (0.199 nM) of I and 40 ng/ml (0.123 nM) of II, demonstrates that the two compounds yield chromatographically indistinguishable products.

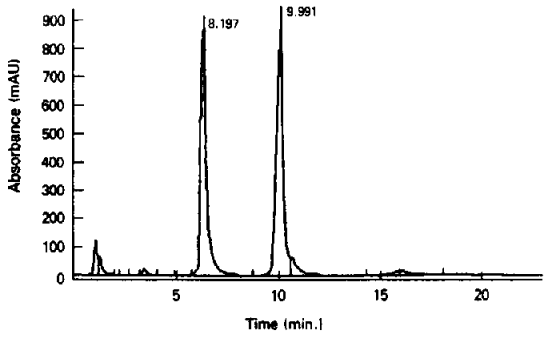

Fig. 2. Chromatogram of underivatized I (100 μg/ml) (peak at 9.991 min) and II (100 μg/ml) (peak at 8.197 min).

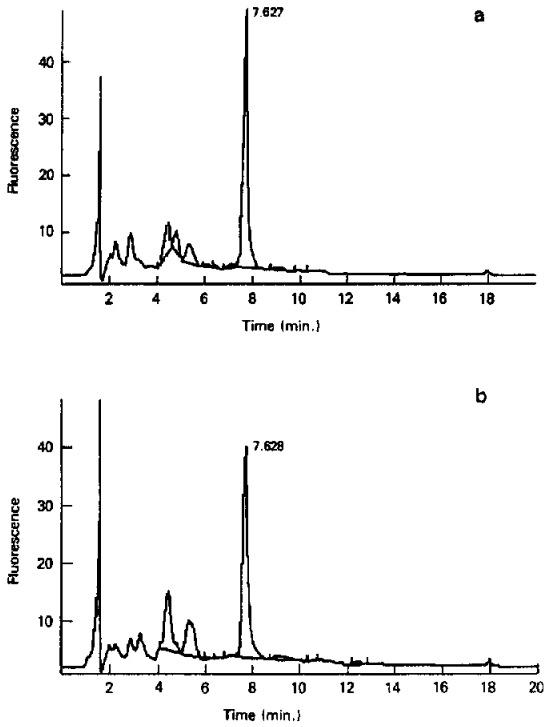

Fig. 3. (a) Fluorescence detection of derivatized I (80 ng/ml). (b) Fluorescence detection of derivatized II (40 ng/ml).

3.4. Structural characterization of the SQT derivative

CI-MS spectra revealed identical $[M+H]^+$ molecular ions, m/z 487 for the derivatives of I and II. Both spectra also shared all fragment ions in common, although some of their relative intensities differed slightly. The close similarity of their mass spectra suggested that derivatization of I and II with SQT under the current conditions afforded the same end product. The exact mass of the m/z 486 ion is consistent with a $C_{26}H_{34}N_2O_5S$ molecular formula.

The identity of the SQT derivatives of I and II is further supported by their indistinguishable 1D and 2D ^{1}H-NMR spectra. Analysis of the 500 MHz ^{1}H-NMR spectrum and the HH–COSY spectrum of the SQT derivative of I in $CDCl_3$ (at 55°C) [1] allowed the assignment of their structure as shown in Table 2, column 2. The downfield signals (δ 7.4–9.0, 6H) in the derivatized product indicated the presence of an SQT moiety. It is noted that the two signals (δ 2.33 and 2.97, J 4.4 Hz) assigned to the geminal protons in the exomethylene epoxide of the parent compounds were not observed in the product. Instead, two new signals at δ 3.56 and 3.40 with a larger coupling of 12.0 Hz were detected for the SQT-derivatized product. The disappearance of the characteristic coupling (4.4 Hz) for the geminal protons of the exo-methylene epoxide ring indicates that a ring opening occurred at this site as a result of an SN_2 nucleophilic attack on I and II by the SQT anion. We also noted that the methylene protons (δ 4.4, AB quartet, J 15.2 Hz) of the chloroacetyl moiety in the parent I compound were not present in the spectrum of the product, which suggested loss of this moiety during the preparation. This explains why reaction of I and II with SQT affords the same product. The remaining protons show NMR signals similar to those of the parent compounds. The signal at δ 5.4 is assigned to H-4 of the six-membered ring which shows spin connectivity to the resonances at δ 3.41 (H-2) and δ 1.86–2.0 (H-5s) which also show scalar coupling to H-2 (δ 2.43) and H-6s (δ 2.01 and 1.68), respectively. The resonances at δ 5.16 and 2.99 are assigned to H-4′ and H-2′, respectively, and both showed spin connectivity to the H-3′s at δ 2.38 and 2.17. The results indicated that the six-membered ring and the trisubstituted epoxide side-chain remain intact in the derivatization (see Fig. 1). Table 2 lists the NMR spectral data of these compounds.

3.5. Assay performance

The lower limit of quantitation for this assay was 2.5 ng/ml for both I and II, based upon a signal-to-noise ratio of 5:1. The assay was linear between concentrations of 2.5 and 160 ng/ml ($r = 0.998$ for I and $r = 0.987$ for II). The inter-assay coefficients of variation for both compounds were less than 18% and the intra-assay coefficients of variation for both compounds were less than 13% (see Table 3). The coefficients of variation remained constant throughout the concentration range. Fig. 4 shows a representative plasma concentration *versus* time course of I and II from a single patient who

Table 2
^{1}H-NMR (500 MHz) data for I[a] and its SQT derivative[b]

Proton(s)	δ (ppm), (J, Hz)	
	I	Derivatized I
2	1.92 ($J_{2,3}$ = 11.0)	2.43 ($J_{2,3}$ = 9.2)
3	3.68 ($J_{3,4}$ = 2.9)	3.43 ($J_{3,4}$ = 2.9)
4	5.5 ($J_{4,5a}$ = 2.9, $J_{4,5b}$ = 5.8)	5.37 ($J_{4,5}$ = 2.9)
5	1.92, 2.02 (m)	1.84-1.92 (m)
6	1.15, 2.07 (m)	1.68, 2.01 (m)
7	2.55, 2.97 ($J_{7a,7b}$ = 4.4)	3.40, 3.56 ($J_{7a,7b}$ = 12.0)
2′	2.58 ($J_{2',3'}$ = 6.4)	2.99 ($J_{2',3'}$ = 6.2)
3′	2.18, 2.34 ($J_{3'a,3'b}$ = 15.2, $J_{3',4'}$ = 7.0)	2.17, 2.38 ($J_{3'a,3'b}$ = 15.4 $J_{3',4'}$ = 7.0)
4′	5.21	5.16
6′	1.66(s)	1.62(s)
7′	1.75(s)	1.67(s)
8′	1.21(s)	1.49(s)
9′	3.45(s)	3.36(s)
10′	4.38, 4.42 ($J_{10a,10b}$ = 15.2)	–

[a] The ^{1}H-NMR and ^{1}H-COSY spectra were obtained with a Varian VXR-500S spectrometer in $CDCL_3$ at 55°C; δ (ppm) values were measured relative to $CHCl_3$ signal at 7.26 ppm.
[b] The proton signals for the SQT moiety are assigned as follows: δ 8.55 (H_{11}, $J_{11,12}$ = 4.0, $J_{11,13}$ = 1.8), 8.14 (H_{13}, $J_{12,13}$ = 8.0), 7.44 (H_{12}), 7.66 (H_{14}, $J_{14,15}$ = 8.0), 7.61 (H_{16}, $J_{15,16}$ = 7.3), 7.48(H_{15}).

received a 1-h infusion of I at a dose of 15.4 mg/m^2.

4. Discussion

The development of an analytical assay for both I and II was influenced by the fact that both compounds lack a chromophore and by the anticipation that, based upon preclinical data, phase I trials of I would generate plasma drug concentrations in the low ng/ml range. The epoxide groups of both compounds offered potential sites for nucleophilic substitution, and the thiolate anion of SQT provided a strong nucleophile which, after substitution, would yield a fluorescent product. The derivatization was carried out in an aqueous solution at pH 10.8 in order to insure that the thiolate anion of SQT (pK_a = 8.36) remained unprotonated and reactive.

Table 3
Precision of the assay

Concentration (ng/ml)	Intra-assay C.V.		Inter-assay C.V.	
	I	II	I	II
10 ng/ml	7	4	16	17
40 ng/ml	8	10	5	13
160 ng/ml	12	11	14	2

Although we were initially concerned that the two epoxides of I and II would yield two derivatization products for each compound, the chromatographic, mass spectroscopic, and NMR data indicate that nucleophilic substitution occurs only with the epoxide bearing the less substituted carbon atom (exo-methylene epoxide), which is characteristic of reactions of epoxides with basic reagents in general. The fact that I and II yield the same product after reaction with SQT requires that the two compounds be separated from one another prior to derivatization. While the approach we took involved separating the two compounds on an HPLC column and collecting appropriate fractions with

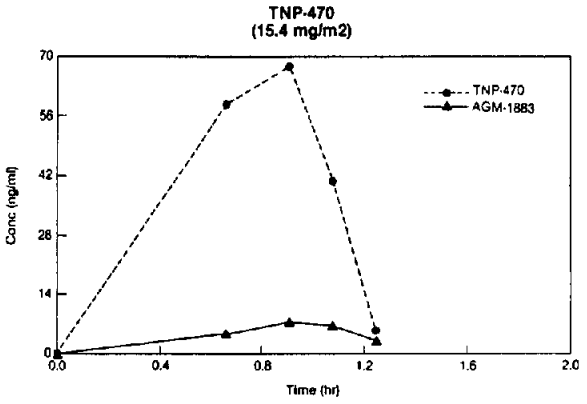

Fig. 4. Concentration–time curves of I and II for one patient following the i.v. administration (30-min infusion) of I (15.4 mg/m^2).

a fraction collector, it is conceivable that a more rapid separation of the two compounds could be effected by differential elution from a solid-phase extraction column; this approach is currently under investigation in our laboratory.

5. Acknowledgements

We would like to thank Angelo Russo, M.D., Ph.D., Department of Radiation Oncology, NCI, NIH for his assistance with optimizing the derivatization chemistry. We would also like to acknowledge the initial analytical work of Yoshinobu Yoshimura, Ph.D., Keji Yannshita, Ph.D, and Michio Motokashi, Ph.D. of Takeda Chemical Industries, Ltd. The laboratory assistance of Natalie McCall, B.S. and Tina Sogocio, B.S. is very much appreciated.

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