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Determination of bisnafide, a novel bis-naphthalimide anticancer agent, in human plasma by high-performance liquid chromatography with UV detection

Chii-Ming Lai, Dennis M. Garner, John E. Gray, Bernice L. Brogdon, Vanessa C. Peterman, Henry J. Pieniaszek Jr. *

Drug Metabolism and Pharmacokinetics Section, The DuPont Merck Pharmaceutical Company, Stine-Haskell Research Center, Building 112, 1090 Elkton Road, Newark, DE 19714, USA

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Abstract

A simple, specific, and sensitive high-performance liquid chromatographic (HPLC) assay utilizing ultraviolet (UV) detection for the determination of bisnafide in human plasma was developed, validated, and applied to plasma samples from patients undergoing cancer therapy. Plasma samples, containing an internal standard, XE842, were first deproteinized with 2.0 ml acetonitrile, and subsequently, 1.0 ml of pH 9 boric acid–potassium chloride–sodium hydroxide buffer (0.1 M) was added. To this mixture, 9.0 ml of ethyl ether was added then vortex mixed. Following centrifugation, the ether layer was back-extracted into 250 μl of 0.1 M phosphoric acid, then removed by vacuum aspiration. A portion of the remaining acid layer was directly injected onto the HPLC. Bisnafide was quantified using a Shiseido Capcell Pak C8 HPLC column and ultraviolet detection (274 nm). The lower limit of quantification was 10 ng ml^{-1} using 1.0 ml plasma. The intraday precision (RSD) ranged from 2.7 to 8.6% over a concentration range of 10–1000 ng ml^{-1}. The interday precision (RSD) ranged from 5.6 to 11.5%. Overall mean accuracy was $\pm 5.2\%$. The drug was stable in frozen heparinized human plasma stored at $-20°C$ for at least 1 year and stable throughout at least two freeze–thaw cycles. This method was successfully utilized for quantifying plasma concentrations needed to study the clinical pharmacokinetics of bisnafide in patients undergoing cancer therapy.

Keywords: Bisnafide; DMP 840; Bis-naphthalimide; Human plasma; Reversed-phase liquid chromatography; Quantitative determination; Anticancer drug

1. Introduction

Bisnafide (USAN; also known as DMP 840 in existing literature) is a novel bis-naphthalimide anticancer agent which binds with high affinity to DNA and has sequence specificity to multiple G

* Corresponding author. Tel.: +1 302 3665577; fax: +1 302 4510054; e-mail: henry.j.pieniaszek@dupontmerck.com

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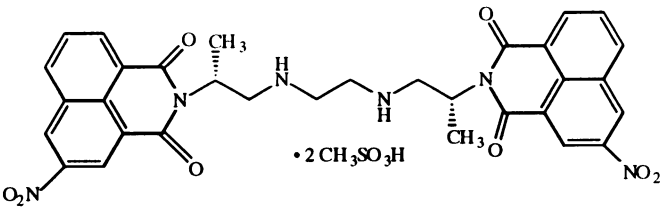
Bisnafide



XE842 (Internal Standard for Plasma Assay)

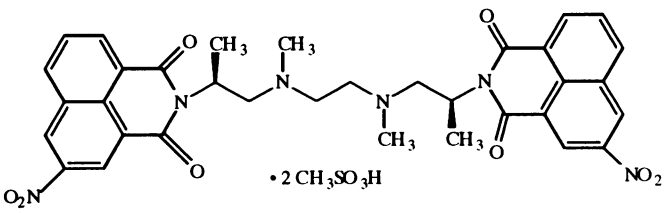


Fig. 1. Chemical structures of bisnafide and XE842 (internal standard for plasma assay).

and C bases causing single-strand DNA breaks [1,2]. In preclinical studies, bisnafide has shown remarkable in vitro activity against human tumor xenografts implanted in nude mice [3]. The drug caused regressions of tumor mass in DLD-2 colon adenocarcinoma, MX-1 mammary carcinoma, and LX-1 lung carcinoma xenografts lasting for 7–9 months. Bisnafide also has in vitro activity against a variety of human tumors, including a subgroup resistant to standard antineoplastic agents, in the human tumor cell clonogenic stem cell assay [4]. Bisnafide has been evaluated for treatment of human colorectal, lung, prostate, and breast cancers [5–8]. In an isolated case, the drug exhibited remarkable success in the treatment of retroperitoneal choriocarcinoma metastatic disease in the lungs [9]. To facilitate the determination of the clinical pharmacokinetics of bisnafide, a method of quantifying the compound in human plasma was developed and has been successfully applied to samples from cancer patients.

2. Experimental

2.1. Chemicals and reagents

Bisnafide, (*R*,*R*)-2,2′[1,2-ethanediylbis[imino(1-methyl-2,1-ethanediyl)]]-bis[5-nitro-1H-benz[de]-isoquinoline-1,3-(2H)-dione]dimethanesulfonate, and the internal standard XE842, (*S*,*S*)-2,2′[1,2-ethanediylbis[(methyl-imino)(1-methyl-2,1-ethanediyl)]]-bis[5-nitro-1H-benz[de]-isoquinoline-1,3-(2H)-dione]dimethanesulfonate, were obtained from The DuPont Merck Pharmaceutical Company (Wilmington, DE). The chemical structures of each are shown in Fig. 1. All chemicals and solvents were HPLC grade. Acetonitrile, glacial acetic acid, and ethyl ether were purchased from J.T. Baker (Phillipsburg, NJ). Ammonium acetate, pH 9 buffer solution (boric acid–potassium chloride–sodium hydroxide buffer 0.1 M), and *o*-phosphoric acid (85%) were purchased from Fisher Scientific (Fair Lawn, NJ). Tetrahydrofuran was from EM Science (Gibbstown, NJ).

Control (blank) heparinized human plasma was purchased from Biological Specialty Corporation (Colmar, PA).

Ammonium acetate buffer (pH 4.0; 0.05 M) was prepared by dissolving 3.854 g of ammonium acetate in deionized water in a 1000 ml volumetric flask. Next, 12.4 ml of glacial acetic acid was added to adjust the pH to 4.0. The mixture was then diluted to 1000 ml with deionized water. The phosphoric acid solution was prepared by diluting 6.8 ml of *o*-phosphoric acid (85%) to 1000 ml in a volumetric flask.

2.2. Standard solutions

Solutions of bisnafide used for constructing the standard curves were prepared by serial dilutions of a stock solution containing 10 μg ml^{-1} in acetonitrile. A series of standard solutions were prepared containing bisnafide in concentrations of 0.1, 0.15, 0.25, 0.5, 1, 2.5, 5 and 10 μg ml^{-1}. A nominal concentration of 3 μg ml^{-1} XE842 (internal standard, IS) was prepared in acetonitrile. All solutions were prepared in amber volumetric flasks and when stored at −20°C showed no deterioration after at least 113 days.

2.3. Instrument and chromatographic conditions

HPLC was performed with a Model 590 solvent delivery system, a Model 484 UV absorbance detector, a WISP Model 710A automatic injector (all from Waters Chromatography Division, Millipore, Milford, MA), and an HP Chemstation Data Acquisition System (Hewlett Packard, San Fernando, CA). A 100 μl aliquot of the extracted sample was injected onto a Shiseido Capcell Pak C8 column (5 μm, 150 × 4.6 mm i.d., DyChrom, Santa Clara, CA). These aliquots were protected from light while on the automatic injector. The mobile phase was ammonium acetate (pH 4.0; 0.05 M)–acetonitrile–tetrahydrofuran (57.7:37.5:4.8, v/v/v). Column temperature was maintained at 30°C by a Sys-Tec CH-1448 dual zone column temperature controller (Systec Inc., Minneapolis, MN). The flow rate was 1.0 ml min^{-1} and UV detection was carried out at 274 nm.

2.4. Sample preparation and extraction procedure

All frozen plasma samples were thawed at room temperature in the dark just prior to analysis. The extractions were carried out in amber 16 × 125 mm borosilicate screw cap culture tubes with Teflon®-lined screw caps. Standard curve samples were prepared each day by pipetting 100 μl of the standard solutions containing 0.1–10 μg of bisnafide into a tube containing 1.0 ml of blank plasma. Similarly, 100 μl of acetonitrile was added to the tubes containing 1.0 ml of plasma taken from human patients that had received bisnafide. A 100-μl aliquot of the working internal standard solution was added to each tube and vortex mixed for 1 min. Afterwards, 2.0 ml of acetonitrile was added to each tube. The tubes were vortex mixed for 5 min and 1.0 ml of pH 9 buffer solution was added. The tubes were again vortex mixed for 5 min. Ethyl ether (9.0 ml) was added to all samples tubes. After mechanically shaking (Eberbach Co., Ann Arbor, MI) for 20 min, the samples were centrifuged (Sorvall RT 6000D, DuPont Co., Wilmington, DE) for 5 min at approximately 2300 × *g*. The ether layer was transferred to culture tubes containing 250 μl of 0.1 M phosphoric acid. After shaking again for 20 min and centrifuging for 5 min, the ether layer was vacuum aspirated and discarded. A sample containing 100 μl of the remaining acid layer was directly injected onto the HPLC system.

2.5. Validation procedures

A calibration curve was constructed using the peak height ratio (y) of bisnafide to XE842 versus the actual spiked standard bisnafide concentrations (unweighted). Parameters 'a' and 'b' (where $a, b > 0$) and the correlation coefficient of the curve fitting for bisnafide were determined by power curve regression analysis. Once 'a' and 'b' were determined, the concentration of bisnafide in the unknown plasma sample was

Table 1
Intra- and interday bisnafide assay precision (% RSD) in human plasma during validation and interday bisnafide assay precision in human plasma over an 18 month period

Concentration (ng ml^{-1})	Intraday ($N=6$)	Interday ($N=3$)	Interday($N=26$)[a]
10.0	4.8	5.6	9.0[b]
50.0	6.3	8.5	7.8[c]
250.0	2.7	5.8	5.9[d]
1000	8.6	11.5	5.2[e]

[a] Interday precision for controls processed during 26 clinical sample batches assayed over an 18 month period; [b] actual concentration of control plasma sample was 12.5 ng ml^{-1}, $N=39$; [c] actual concentration of control plasma sample was 31.3 ng ml^{-1}, $N=38$; [d] actual concentration of control plasma sample was 438.4 ng ml^{-1}, $N=30$; [e] actual concentration of control plasma sample was 876.6 ng ml^{-1}, $N=44$.

back-calculated with reference to the calibration curve coefficients by using the following equation: concentration $=(y/a)^{1/b}$.

Plasma standards ranging from 10 to 1000 ng ml^{-1} of bisnafide were prepared and analyzed on the same day. The intraday precision of the assay was assessed by the analysis of replicate plasma quality control samples (at concentrations of 10.0, 50.0, 250.0 and 1000 ng ml^{-1}) and obtaining relative standard deviations (RSD) for each replicate set.

Similarly, plasma samples containing bisnafide were prepared and analyzed on three different days. The interday precision of the assay was again assessed as RSD. The specificity of the assay was checked by analyzing three different plasma lots, and pre-dose plasma samples from several different patients ensuring that no endogenous interferences were observed. The accuracy of the assay was assessed by single-blinded assays performed at bisnafide concentrations of 10.0, 50.0, 250.0 and 1000 ng ml^{-1}. An independent calibration curve was used to calculate the concentrations. The percent difference between assayed and actual spiked concentrations was determined as:

$$\%\ \text{Difference} = \frac{(\text{Measured} - \text{Actual Concentration})}{\text{Actual Concentration}} \times 100.$$

2.6. Application of the method to clinical samples

The assay method was utilized to determine bisnafide concentrations in plasma samples from patients administered bisnafide intravenously. Patients at the time of initial diagnosis with relapsed solid tumors at the Johns Hopkins Oncology Center (Baltimore, MD) were studied after each gave informed consent. The study protocol was approved by the institutional review committee. The plasma samples were obtained from a single patient who had received a 60 mg m^{-2} dose of bisnafide by intravenous infusion. In this patient, the infusion lasted 6.7 h. Blood samples were drawn through a cannula from a peripheral vein from the forearm opposite that used for infusion. Samples were collected just prior to administration and at approximately 1/4, 1/2 and 3/4 of the calculated infusion time, 5 min prior to the end of infusion, and at 5, 10, 20, 30, 40, 60 min, 1.5, 2.0, 4.0, 6.0, 8.0, 11, 24, 48, 72, 96 h post infusion. Blood was collected in silicon-coated glass heparinized tubes and plasma was separated by centrifugation of the blood samples within 1 h. Plasma samples were stored at -20°C for subsequent analysis.

3. Results

3.1. Extraction efficiency

The extraction efficiency of bisnafide from human plasma was obtained by comparing the peak heights of bisnafide extracted standards over the concentration range 10–1000 ng ml^{-1} to those of unextracted standards. The overall extraction effi-

ciency of bisnafide from human plasma using the described extraction procedures was $97 \pm 17\%$ (mean $\pm$ SD) and appeared to be concentration independent. The overall extraction efficiency of the 300 ng ml^{-1} (internal standard) XE842 from human plasma was $92 \pm 4.5\%$.

3.2. Chromatography, calibration curve characteristics and UV detection

Typical chromatograms of the extracts from blank plasma, plasma spiked with 1000 ng ml^{-1} of bisnafide and 300 ng ml^{-1} of XE842, and a 2 h plasma sample from a patient dosed with bisnafide, respectively, are shown in Fig. 2. The analyte and internal standard displayed good chromatographic separation and there was no chromatographic interference from the endogenous plasma components in the extract. The retention times for bisnafide and XE842 were approximately 4.8 and 7.5 min, respectively. The mean correlation coefficient of the calibration curves was 0.9989 (range: 0.9984–0.9996) during the validation runs. The molar absorption coefficient of bisnafide in methanol was determined to be 36.6 μM^{-1} cm^{-1}.

3.3. Precision

The intraday precision of the assay method was assessed by determining the relative standard deviations (RSD) of peak height ratios obtained from six replicate assays at concentrations of 10.0, 50.0, 250.0 and 1000 ng ml^{-1}. Interday precision was assessed by determining the RSD of measured concentrations of bisnafide from single plasma samples over the same concentration range on 3 separate days. The RSD (%) for intraday analysis ranged from 2.7 to 8.6% (Table 1). The RSD for interday analysis ranged from 5.6 to 11.5% (Table 1). In addition, for plasma, interday precision was assessed over an 18 month time period. Plasma samples were assayed at concentrations of 12.5, 31.3, 438.4 and 876.6 ng ml^{-1}. The RSD (%) ranged from 5.2 to 9.0%.

3.4. Accuracy

Twenty-four single-blinded samples at bisnafide concentrations of 10.0, 50.0, 250.0 and 1000 ng ml^{-1} were assayed as if they were patient samples. An independent calibration curve was used to calculate the concentration of the samples and accuracy was determined by comparing the measured concentration to the actual value. This assay could accurately determine bisnafide concentrations within $\pm 15\%$ difference in 23 of the 24 samples evaluated (Table 2). Overall mean accuracy was $\pm 5.2\%$. In addition, plasma samples (concentration range: 12.5, 31.3, 438.4 and 876.6 ng ml^{-1}) were assayed during 26 separate clinical sample batches. The overall mean accuracy was 5.5% supporting the utility of this method over an extended period of time.

Table 2
Accuracy in the analysis of bisnafide unknown plasma samples ($N = 24$)

Actual concentration (ng ml^{-1})	Measured concentration (ng ml^{-1})	Accuracy[a] (%)
10.0	10.7	7.0
10.0	10.2	2.0
10.0	10.7	7.0
10.0	11.1	11.0
10.0	9.9	−1.0
10.0	9.7	−3.0
50.0	57.1	14.2
50.0	49.5	−1.0
50.0	51.0	2.0
50.0	49.1	−1.8
50.0	48.1	−3.8
50.0	49.7	−0.6
250.0	268.9	7.6
250.0	275.0	10.0
250.0	265.0	6.0
250.0	256.3	2.5
250.0	273.7	9.5
250.0	261.7	4.7
1000	1190	19.0
1000	974.2	−2.6
1000	972.8	−2.7
1000	988.5	−1.2
1000	975.7	−2.4
1000	972.4	−2.8

[a] $\left(\frac{\text{Measured Concentration} - \text{Actual Concentration}}{\text{Actual Concentration}}\right) \times 100.$

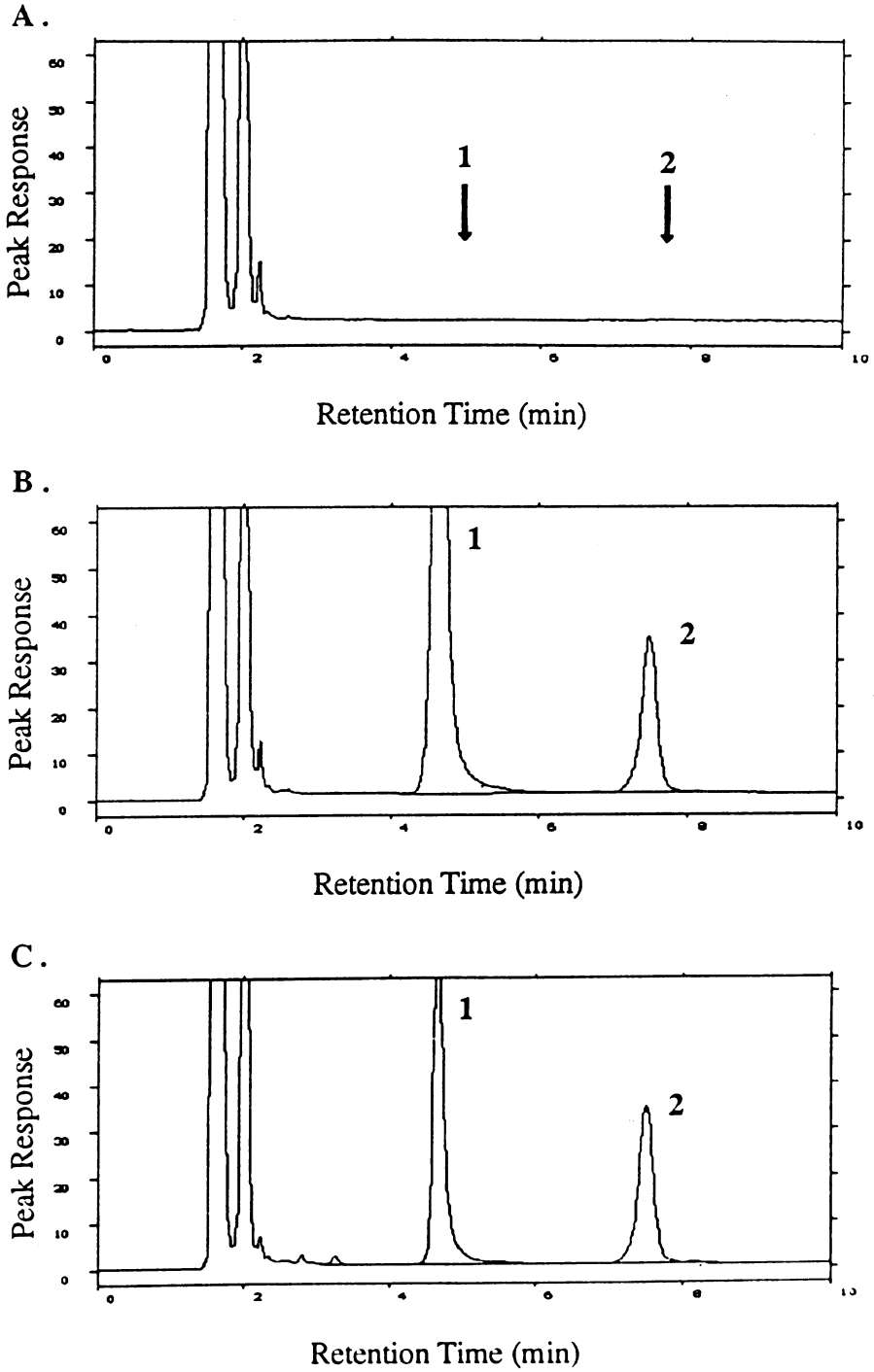


Fig. 2. Representative HPLC chromatograms of bisnafide and internal standard (XE842) in human plasma using UV (274 nm) detection: (A) control human plasma (drug free) extract; (B) control plasma extract spiked with 1000 ng ml^{-1} bisnafide and 300 ng ml^{-1} of XE842. Retention times: bisnafide = 4.8 min, XE842 = 7.5 min; (C) extract of a 2 h post-dose plasma sample (concentration: 80 ng ml^{-1}) from a cancer patient treated with bisnafide. Bisnafide and internal standard (XE842) are denoted as 1 and 2, respectively.

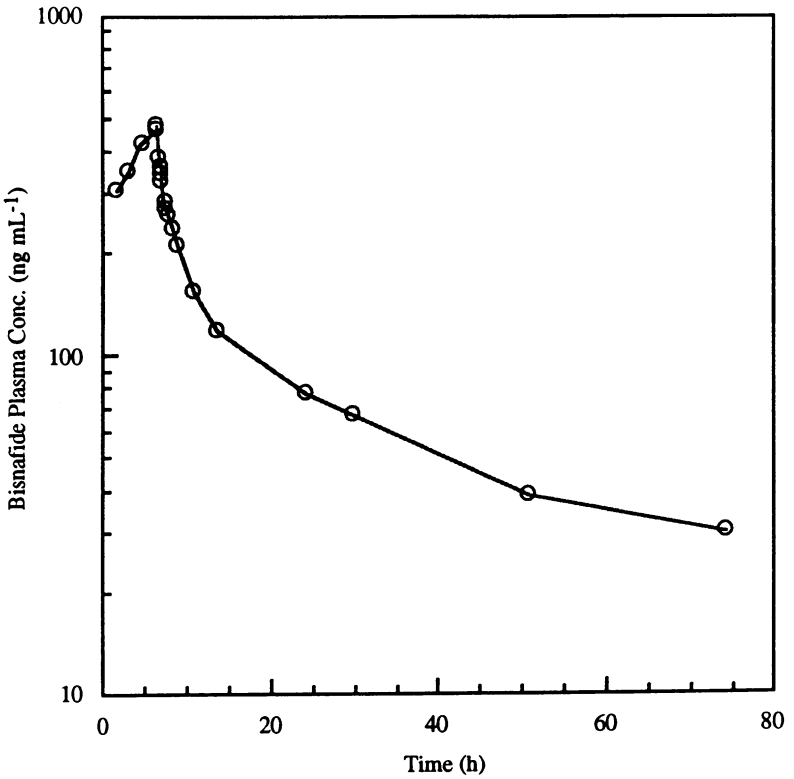


Fig. 3. Bisnafide plasma concentration vs time profile for a cancer patient receiving a 60 mg m^{-2} bisnafide dose infused over 6.7 h.

3.5. Stability

The stability of bisnafide was determined in acetonitrile stock solutions and the drug was found to be stable for at least 113 days. The stability of bisnafide was determined in spiked blank plasma at concentrations of 45 and 800 ng ml^{-1}. The plasma samples were assayed on the day they were prepared (day 0) and kept frozen (-20°C) for subsequent analyses up to 372 days. No degradation of bisnafide concentrations over time was observed. The average recoveries from plasma samples containing bisnafide after 1, 7, 21, 100 and 372 days were 95, 90, 93, 101 and 90%, respectively at the 45 ng ml^{-1} concentration and 101, 121, 108, 98 and 99%, respectively at the 800 ng ml^{-1} concentration. In addition, no decrease in bisnafide concentrations was observed after two freeze–thaw cycles. No degradation of bisnafide was observed in human plasma containing bisnafide following storage at room temperature for at least 5 h. Finally, the stability of bisnafide extracted from human plasma into 0.1 M phosphoric acid was demonstrated for up to 45 h, indicating that extracted samples could remain on the HPLC autosampler for this time period.

4. Discussion

The extraction of bisnafide from plasma is rapid and involves relatively few steps. In development of this assay, it became apparent that the bisnafide readily adhered to glassware in the absence of protein. Pre-treatment of the glassware with silicanizing agent did not prevent loss of drug to glass surfaces. Isolation of bisnafide from plasma never involves evaporation of organic solvent containing drug. Amber glassware is needed for all extraction steps to protect bisnafide from photodegradation.

Also, samples in the autosmpler must be protected from light. A simple, specific, and sensitive high-perfor- mance liquid chromatographic assay utilizing UV detection for the determination of bisnafide in human plasma is described. Although bisnafide contains two oxidizable tertiary amines and is amenable to electrochemical detection, the current method provided sufficient sensitivity to quantitate bisnafide in human plasma following clinical doses (8–80 mg m^{-2}) in cancer patients. A plasma concentration profile from a patient who received a 60 mg m^{-2} dose of bisnafide is shown in Fig. 3. In this patient, the infusion ended at 6.7 h and the maximum plasma concentration was 447 ng ml^{-1}. The plasma concentration-time plot indicates that the decrease in bisnafide concentration is polyphasic with a long terminal half life (ca. 30 h) for the drug. The described assay procedure has been extensively used in both Phase I and Phase II clinical trials of bisnafide.

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