Assay of TNP-470 and Its Two Major Metabolites in Human Plasma by High-Performance Liquid Chromatography–Mass Spectrometry

Carl T. Whalen[1], Glenn D. Hanson[1], Kathy J. Putzer[1], Michael D. Mayer[2], and **Darcy J. Mulford[2]**

[1]Covance Laboratories Inc., 3301 Kinsman Blvd., Madison, WI 53704 and [2]TAP Pharmaceutical Products, Inc., 675 N. Field Drive, Lake Forest, IL 60045

Abstract

In this study, a sensitive, specific assay for the determination of TNP-470 and its two major metabolites M-IV (also know as AGM-1883) and M-II in human plasma is reported. The assay involves liquid–liquid extraction of acidified plasma followed by reversed-phase high-performance liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry. A liquid–liquid extraction using an organic solvent mixture (methyl-*tert*-butyl-ether–hexane, 1:1, v/v) is used in place of solid-phase extraction because it provides consistent recoveries for all analytes, including the internal standard. Retention times for the analytes and internal standard are less than 7 min. Within- and between-day precision is ≤ 6.5% and ≤ 13.3% relative standard deviation, respectively, for the three analytes. The lower limits of quantitation are 0.25, 0.5, and 1.0 ng/mL for TNP-470, M-IV, and M-II, respectively.

Introduction

O-(Chloroacetylcarbamoyl) fumagillol, or AGM-1470 (later termed TNP-470), is a semisynthetic analog of fumagillin, which is an angiostatic antibiotic derived from *Aspergillus fumigatus fresenius*. This agent is a potent inhibitor of endothelial cell proliferation in vitro, being 50 times more potent than fumagillin ($IC_{50} \cong 10$ pg/mL). It inhibits angiogenesis in vivo in a variety of assays, including the chorioallantoic membrane, rat corneal micropocket, and murine sponge implantation assays (1).

TNP-470 has demonstrated activity against a wide variety of rodent and human tumor types, inhibiting tumorigenesis and growth of de novo tumors by inhibiting neovascularization. Direct cytotoxicity is not felt to play a role in the antitumor activity of this agent. Clinical trials are currently evaluating the role of this agent as monotherapy and as a component of combination chemotherapy of a variety of malignancies, including prostate, breast, lung, and cervical cancer (1).

Preliminary pharmacokinetic studies reveal that this agent is rapidly metabolized to a number of metabolites that exhibit very short terminal disposition half-lives (< 1 h). The major metabolic pathway involves ester hydrolysis to M-IV (an active metabolite, also known as AGM-1883) followed by metabolism by epoxide hydrolase to M-II (2,3). The structures of TNP-470 and its two major metabolites in humans are shown in Figure 1.

Other investigators have published analytical procedures for one or more of these metabolites. Figg et al. (4) have published a procedure for quantitating TNP-470 and AGM-1883 plasma concentrations using tandem high-performance liquid chromatography (HPLC)–HPLC technology; however, this method is laborious, does not allow for the quantitation of M-II, and has a lower limit of quantitation of only 2.5 ng/mL for both analytes. More recently, Moore and Sommadossi (5) published a method for quantitating TNP-470, AGM-1883, and M-II using HPLC–mass spectrometry (MS). Although this method was less labor-intensive, exhibited lower limits of quantitation than the method of Figg et al. (4), and was able to quantitate M-II concentrations, the solid-phase extraction procedure when used in this investigation proved unreliable (as will be described). Moreover, in the previously mentioned studies, plasma was acidified after harvesting from untreated whole blood, a process associated with the significant degradation of TNP-470 (as will be

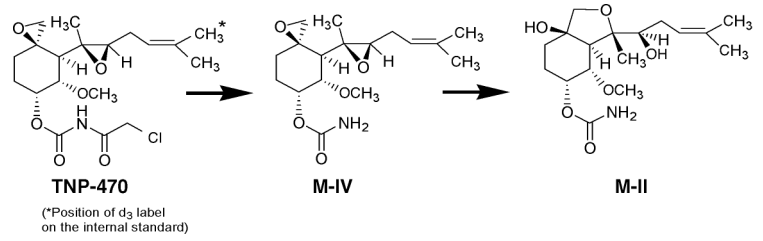

Figure 1. Chemical structures and the major metabolic pathway of TNP-470 in humans.

described). This study was an extension of the work of Ong et al. (6), examining liquid–liquid extraction as a means to reduce TNP-470 degradation followed by HPLC–tandem MS analysis.

Experimental

Reagents and chemicals

All solvents used in this study were HPLC grade and obtained from Fisher Scientific (Fair Lawn, NJ), EM Science (Gibbstown, NJ), and Burdick & Jackson (Muskegon, MI). Ammonium acetate was obtained from Fisher Scientific. A mobile phase solvent (0.02M pH 4.0 ammonium acetate–acetonitrile, 25:75, v/v) was mixed and degassed with helium prior to use. TNP-470, M-IV, and M-II were obtained from TAP Pharmaceutical Products, Inc. The internal standard for the assay, a deuterated (d_3) analog of TNP-470, was also provided by TAP Pharmaceutical Products, Inc. For day-to-day analysis, a working internal standard solution (1700 ng/mL) in water–acetonitrile (1:1, v/v) with 0.1% acetic acid was used.

Table I. QC Sample Within-Day and Between-Day Accuracy and Precision*

QC concentration (ng/mL)	Within-day†			Between-day‡		
	TNP-470	M-IV	M-II	TNP-470	M-IV	M-II
1.5	109(4)	104(7)	99(8)	106(4)	105(7)	96(11)
15	108(1)	113(5)	100(3)	104(2)	108(5)	95(8)
75	101(1)	104(6)	94(4)	101(2)	106(4)	92(7)
200 (5X dilution)	107(1)	113(9)	104(13)			

* Accuracy defined as the percent of theoretical concentration. %RSD in parentheses.
† $n = 6$.
‡ $n = 18$.

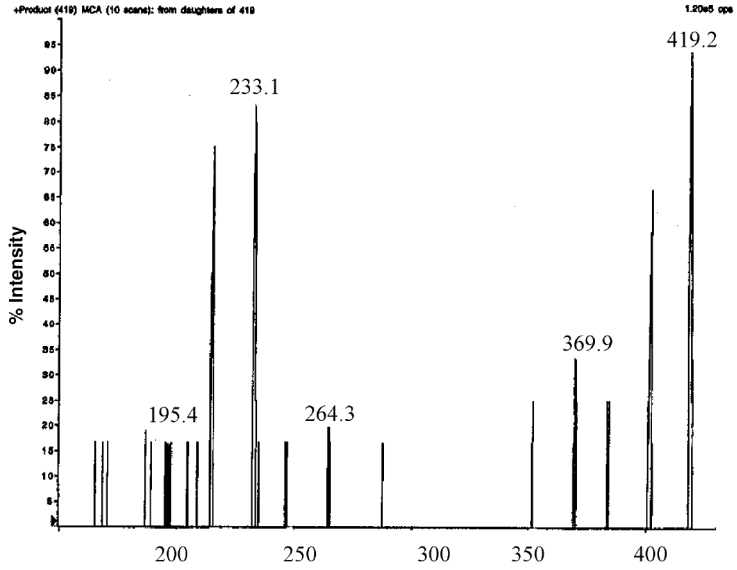

Figure 2. Mass spectrum of daughter ions of *m/z* 419 (TNP-470).

Preparation of standard and quality control samples

Reference solutions of TNP-470, M-IV, and M-II were prepared as 1.0-mg/mL solutions in water–acetonitrile (1:1, v/v) with 0.1% phosphoric acid and stored at –10°C to –30°C. Two reference solutions of each analyte were prepared from independent weighings; the first solution was used to prepare calibration standards in human plasma and the second was for quality control (QC) samples (also in human plasma). These reference solutions were further diluted in the same solvent to obtain a single working reference solution containing a mixture of each analyte at a concentration of 10,000 ng/mL. This working reference solution was stored at –60°C to –80°C and thawed at room temperature before use.

Preparation of standards

Pooled control human plasma was first acidified with 0.2 mL of a 250-mg/mL citric acid solution per 10 mL of whole blood or equivalent. Working calibration solutions containing 2.5, 5, 10, 25, 50, 100, 250, 500, and 1000 ng/mL of each analyte were prepared by diluting the working reference solution with water–acetonitrile (1:1, v/v) with 1% acetic acid. A 0.1-mL aliquot of each of these calibration solutions was added to 1 mL citrated human plasma to obtain nine plasma standards ranging in concentration from 0.25 to 100 ng/mL of each analyte.

Preparation of QC samples

QC samples were prepared in a manner similar to the calibration standards. Two stock spiking solutions in plasma (1000 and 10,000 ng/mL) containing each analyte were prepared by spiking acidified plasma with the reference solutions (1 mg/mL) described previously. These stock spiking solutions in plasma were then diluted with acidified plasma to obtain QC samples at four concentration levels corresponding with 1.5, 15, 75, and 200 ng/mL (designated as low-level, intermediate-level, high-level, and over-curve-level QC, respectively). Over-curve QC samples were analyzed using a 5X dilution of the aliquot volume diluted to full volume with blank plasma. The accuracy, defined as a percentage of the theoretical concentration (mean concentration / theoretical concentration × 100), and precision (percent relative standard deviation, RSD) of the method are presented in Table I.

Sample preparation

A 1-mL aliquot of human plasma was mixed with 0.05 mL of the internal standard (d_3-TNP-470) solution. To this mixture was added 1 mL of a TRIZMA buffer (Sigma, St. Louis, MO) followed by vortexing for 10 s. Five milliliters of the extraction solvent (methyl-*tert*-butyl-ether–hexane, 1:1, v/v) was then added. The sample was vortexed for 5 min, centrifuged at 3000 rpm for 10 min, and then placed in a dry ice/acetone bath in order to freeze the aqueous

layer. The upper organic layer was transferred into a clean tube and evaporated under nitrogen to dryness using a Turbo-Vap apparatus (Zymark, Hopkinton, MA) set at 40°C. The residue was reconstituted with 100 µL of water–acetonitrile (1:1, v/v) with 0.1% acetic acid. Typically, 25 µL was injected into the HPLC–MS–MS system.

Apparatus

Mobile phase was delivered by a Hewlett-Packard (Wilmington, DE) 1090 L system at a flow rate of 0.40 mL/min. Analytes were separated using a reversed-phase analytical column (ODS-AQ, 5 µm, 150 × 4.6 mm) (YMC, Wilmington, NC) at a column temperature of 50°C.

A Sciex API III triple quadrupole MS (PE-Sciex, Thornhill, Canada) was used for analyte detection. Analytes from the HPLC column were ionized by atmospheric pressure chemical ionization via a heated nebulizer interface at 475°C. Nitrogen was used as the nebulizer gas at a pressure of 80 psi, and the nitrogen auxiliary gas flow was 3600 mL/min. The curtain gas was nitrogen, and it was held at a flow rate of 1800 mL/min. Mass analysis and quantitation of the analytes were performed in the positive-ion mode under selected reaction monitoring conditions. The orifice potential was maintained at 40 V, and the collision gas (10% nitrogen BAL argon) thickness was approximately 260×10^{13} atoms/cm^3. A MS–MS spectrum of TNP-470, M-IV, and M-II are shown in Figures 2, 3, and 4, respectively. The following mass transitions were selected: *m/z* 344 → 326 for M-II, *m/z* 343 → 215 for M-IV, *m/z* 419 → 233 for TNP-470, and *m/z* 422 → 236 for the internal standard at a collision energy of –12 eV. For data acquisition, the dwell time was set at 150 ms per channel. Sciex API Version 2.7 software was used in all experiments. Figure 5 shows the selected reaction chromatograms corresponding with TNP-470, the internal standard, M-IV, and M-II.

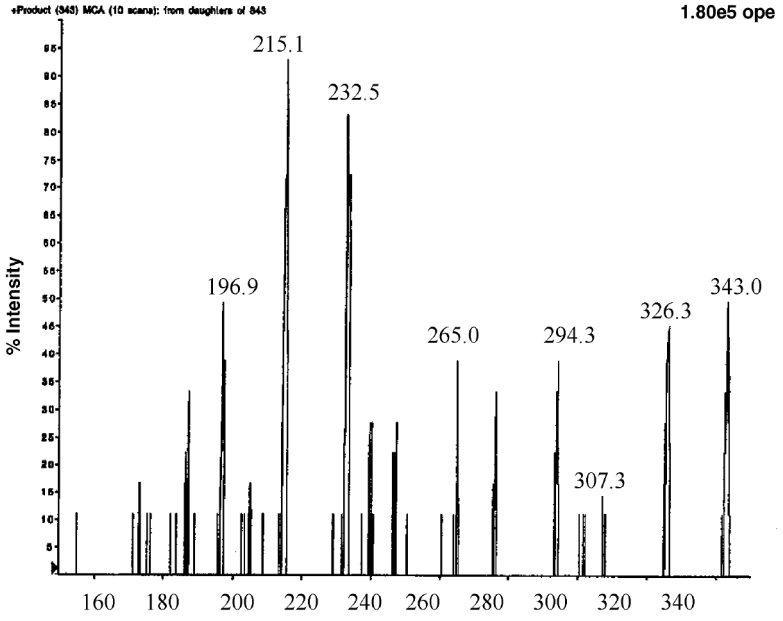

Figure 3. Mass spectrum of daughter ions of *m/z* 343 (M-IV).

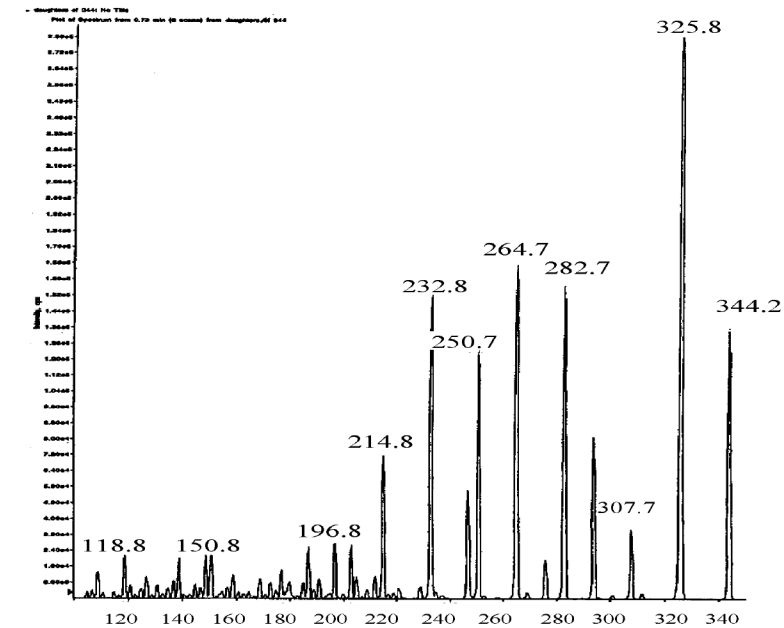

Figure 4. Mass spectrum of daughter ions of *m/z* 344 (M-II).

This methodology was also cross-validated using a Sciex API 3000 triple quadrupole MS (PE-Sciex). The heated nebulizer interface was held at a temperature of 450°C. Nitrogen was used as the nebulizer gas at a flow setting of 10 psi, and the air auxiliary gas flow was held at a rate of 8000 mL/min. The curtain gas was nitrogen at a flow setting of 10. The collision gas was 10% nitrogen BAL argon at a flow setting of 4. Dwell times were 100 ms (internal standard) or 200 ms (other analytes).

Results and Discussion

The mass chromatographic conditions for this study were selected based upon previous experience as described by Ong et al. (6). Full-scan positive-ion mass spectra for the three analytes were used to confirm the chromatographic elution order (on the C18 analytical HPLC column). Characteristic product ions were produced via collision-activated dissociation, and these data provided the selected reaction channels to be monitored for analyte quantitation.

Again, based in part upon the work of Ong et al. (6), it was decided to use acidified whole blood to provide plasma samples for analysis. This was because of the variable, but extensive, loss of TNP-470 prior to plasma harvesting (range of loss = 17% to 99%). Citric acid proved to be an ideal acidifier because it can act as an

anticoagulant at the same time.

Solid-phase extraction, using the method of Moore and Sommadossi (5), produced inconsistent results even when using a variety of extraction cartridges and elution procedures. High variability in replicate samples, with RSD values exceeding 15%, were observed, especially with the metabolites. It was felt that the high variability of results for the metabolites was caused by the variable recovery of the internal standard. Liquid–liquid extraction using an organic solvent mixture was used in order to reduce the residual water content, which has been previously proven to cause nonuniform degradation of the internal standard during the eluate evaporation process (6). Liquid–liquid extraction provided consistent recoveries for all analytes (RSD < 11%).

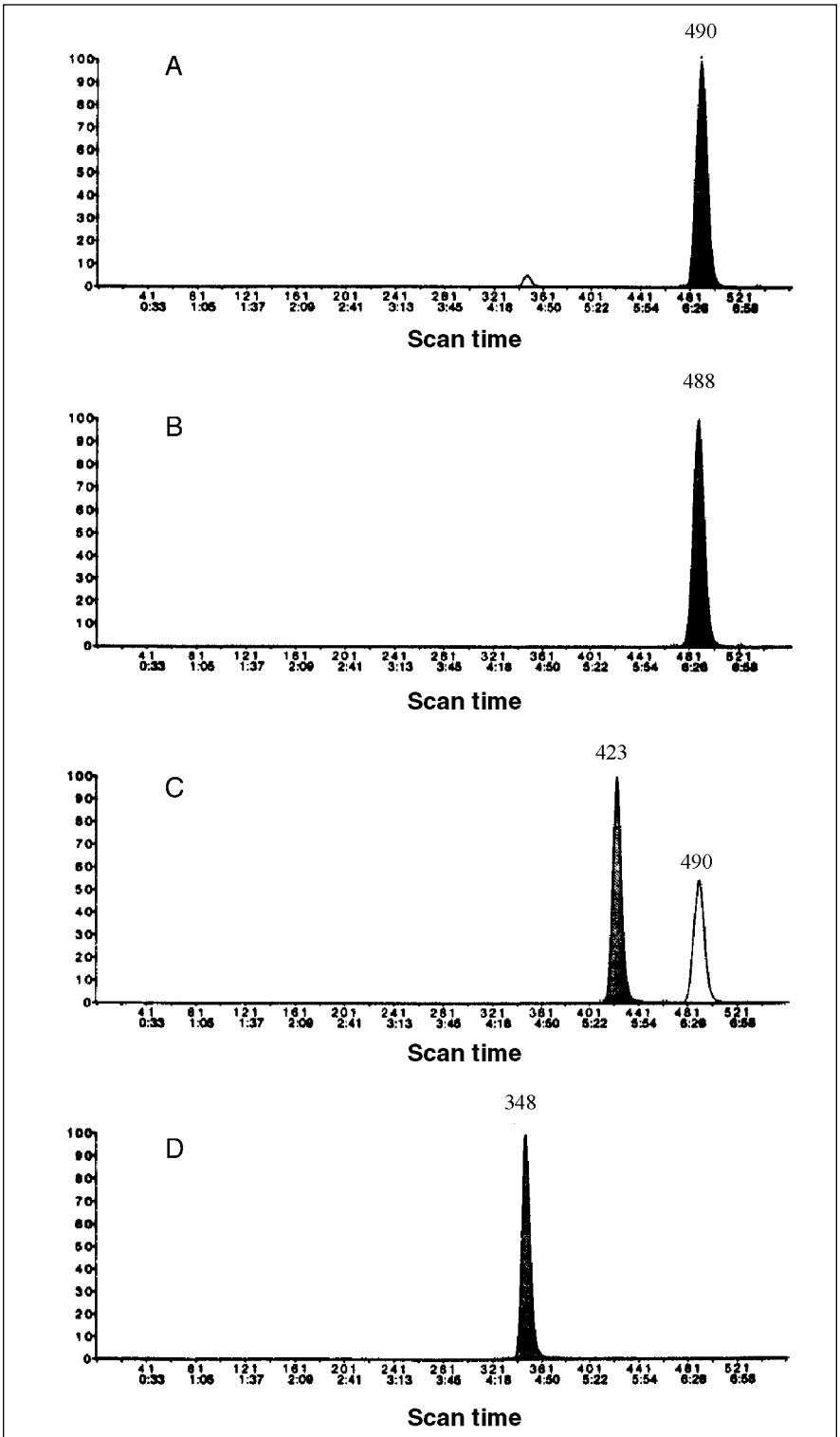

Figure 5. Chromatograms of a 100-ng/mL calibration standard containing (A) TNP-470, (B) the internal standard, (C) M-IV, and (D) M-II.

The analytes were well-separated with mean retention times of 6.56, 5.66, 4.65, and 6.55 min for TNP-470, M-IV, M-II, and the internal standard, respectively. No significant interfering peaks have been found in blank plasma lots tested to date.

Calibration curves were linear for all three analytes over concentration ranges of 0.25 to 100 ng/mL, 0.5 to 100 ng/mL, and 1 to 100 ng/mL for TNP-470, M-IV, and M-II, respectively, with correlation coefficients ≥ 0.9954 for all three analytes. Interday precision ranged from 0.4% to 3.8% RSD for TNP-470, 1.9% to 6.0% RSD for M-IV, and 1.7% to 6.5% RSD for M-II. Intraday precision ranged from 0% to 3.5% RSD for TNP-470, 3.1% to 7.8% RSD for M-IV, and 2.8% to 13.3% RSD for M-II at the three QC sample concentrations. Precision was maintained even when high-concentration (outside the linear range) QC samples were diluted five-fold with blank acidified plasma.

The lower limits of quantitation were 0.25, 0.5, and 1.0 ng/mL for TNP-470, M-IV, and M-II, respectively.

Absolute recoveries of the analytes were determined by comparing peak areas of the extracted QC samples with the peak areas of unextracted recovery standards at the same nominal concentrations. Overall mean absolute recoveries of TNP-470, M-IV, M-II, and the internal standard were 67.8%, 51.6%, 22.1%, and 69.2%, respectively.

Three freeze/thaw cycles and storage of plasma samples at room temperature for 4 h had no significant effect on assay precision or accuracy. The storage of extracts at room temperature for 24 h similarly had no significant effect on assay precision or accuracy.

Using the Sciex API 3000 MS, the mean retention times of TNP-470, M-IV, M-II, and the internal standard were 7.2, 5.9, 4.7, and 7.2 min, respectively. Calibration curves for the three analytes were linear over the concentration ranges of 0.25 to 100 ng/mL, 0.5 to 100 ng/mL, and 2.5 to

100 ng/mL for TNP-470, M-IV, and M-II, respectively. Precision of the QC samples ranged from 1.0% to 1.5% RSD, 3.0% to 6.6% RSD, and 1.3% to 3.3% RSD for TNP-470, M-IV, and M-II, respectively. Accuracy, determined by comparing the measured with theoretical concentrations of calibration standards, ranged from –7.6% to 15%, –15.0% to 12.0%, and –4.7% to 4.6% for TNP-470, M-IV, and M-II, respectively. Accuracy, determined as percentage deviation from theoretical concentrations for QC standards, ranged from –10.6% to –5.2%, 2.0% to 4.1%, and –10.2% to –4.0% for TNP-470, M-IV, and M-II, respectively. Thus, use of the Sciex API 3000 MS demonstrated acceptable precision and accuracy as compared with results with the API III apparatus.

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