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Quantitation of TNP-470 and its metabolites in human plasma: sample handling, assay performance and stability

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Abstract

A selective and sensitive assay for the determination of TNP-470 and two of its metabolites, AGM-1883 and M-II, in human plasma was developed. The assay involved liquid–liquid extraction followed by analysis using high-performance liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry. Because TNP-470 is most stable in a pH of 4–5, an acidification procedure was utilized to prevent degradation of TNP-470 during sample collection which involved acidifying the whole blood sample collected with 5 mg of citric acid per ml of blood. Liquid–liquid extraction using an organic solvent mixture was chosen over solid-phase extraction to minimize the degradation of TNP-470 during solvent evaporation.

Keywords: TNP-470

1. Introduction

TNP-470 [*O*-(chloroacetylcarbamoyl)fumagillol], structure shown in Fig. 1, is a synthetic derivative of fumagillin, a natural product secreted by the fungus *Aspergillus fumigatus fresnius* [1]. TNP-470 has demonstrated angioinhibitory activity; i.e. blocking the response of endothelial cells to growth factors, which results in the inhibition of neovascularization [1]. The observed antiangiogenic effect of TNP-470 on different cell types has made it a potential drug in the treatment of cancer through the inhibition of tumor cell growth and/or metastasis [2–9]. Because plasma concentrations of TNP-470 are expected to be in the sub-ng ml^{-1} range [10], a sensitive and selective assay for the determination of TNP-470 concentrations in human plasma was needed to support pharmacokinetic analysis during clinical trials of TNP-470.

Cretton-Scott et al. recently reported that TNP-470 undergoes rapid and extensive metabolism [11]. Although a number of metabolites have been detected, there are two metabolites which have been characterized. Metabolite AGM-1883 is formed following cleavage of the chloroacetyl moiety of TNP-470, which is then further metabolized by hydrolysis of the epoxide ring to metabolite M-II. The proposed metabolic pathway is shown in Fig. 1. It was important to monitor for the concentrations of AGM-1883 and M-II in plasma because they were the major metabolites of TNP-470 and would provide

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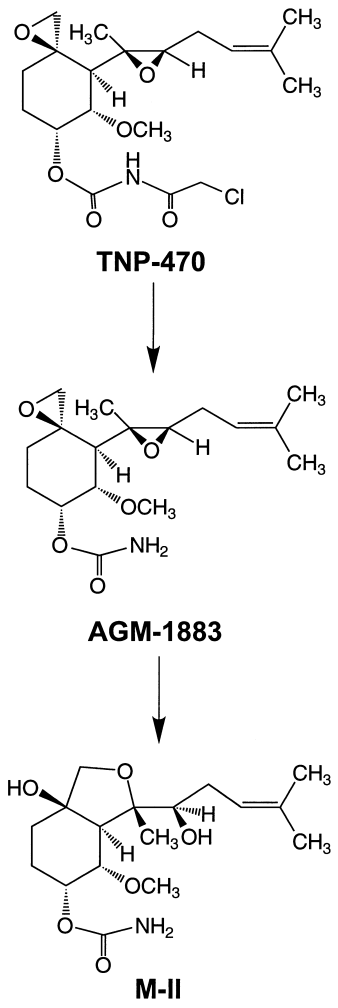

Fig. 1. Structures and proposed metabolic pathway.

further pharmacokinetic information in the absence of measurable levels of the parent drug.

Figg et al. [12] measured TNP-470 and AGM-1883 levels in human plasma using a method which involved acetonitrile precipitation, an initial high-performance liquid chromatography (HPLC) separation of the analytes, collection of HPLC fractions, followed by derivatization to incorporate a fluorescent chromophore to facilitate detection of the analytes by a second HPLC analysis. Only derivatization made the detection of low levels of the analytes possible because TNP-470 lacked a useful chromophore for analysis by HPLC with an ultraviolet detector. However, this procedure was labor-intensive and did not allow the measurement of metabolite M-II. Furthermore, the lower limit of quantitation of the method achieved was 2.5 ng ml^{-1} for TNP-470 and AGM-1883. This was not adequate because in vivo plasma concentrations were expected to be in the sub-ng ml^{-1} range [10]. Recently, Moore and Sommadossi reported a method for the determination of TNP-470, AGM-1883 and M-II which involved solid-phase extraction followed by HPLC with mass spectrometric (MS) detection [10]. In the study, the authors found that the solid-phase extraction procedure when used with HPLC–MS gave lower limits of quantitation for TNP-470 and AGM-1883 (0.62 ng ml^{-1}) which were better than those obtained from the acetonitrile precipitation procedure. This method was also used for the extraction and quantitation of M-II, with a lower limit of quantitation (LOQ) of 2.5 ng ml^{-1}.

In the studies discussed above, plasma harvested from collected whole blood samples was acidified with sulfuric acid to prevent the degradation of TNP-470. However, this acidification was carried out after the plasma had been harvested from whole blood. Our data indicated that significant degradation of TNP-470 occurred during this process. To obtain a more accurate measurement of circulating plasma levels of TNP-470, an alternative procedure for acidification was developed prior to the harvesting of plasma. In the present study, a liquid–liquid extraction procedure was used to minimize the degradation of TNP-470 during sample extraction. We also investigated the use of HPLC with tandem mass spectrometric (MS–MS) detection to improve the lower limits of quantitation, to shorten the analysis time and enable higher throughput in the measurement of TNP-470 and the two metabolites, AGM-1883 and M-II, in human plasma.

2. Experimental

2.1. Reagents and chemicals

All solvents used in this study were HPLC grade (EM Science, Gibbstown, NJ, USA). Ammonium acetate was obtained from J.T. Baker (Phillipsburg, NJ, USA). Mobile phase solvent (acetonitrile–2 m*M* ammonium acetate solution, 60:40, v/v) was filtered

through a 0.2 μm pore size nylon membrane (Alltech, Deerfield, IL, USA) prior to use. TNP-470, AGM-1883, and M-II were obtained from Takeda Chemical Industries (Osaka, Japan). The internal standard for the assay (a deuterated analog (d_3) of TNP-470) was also provided by Takeda Chemical Industries. For day-to-day analysis, a working internal standard solution (approximately 170 ng ml^{-1}) in water with 4% methanol was used.

2.2. Preparation of standard and quality control samples

Reference solutions of TNP-470, AGM-1883, and M-II were prepared as 1.0 mg ml^{-1} solutions in the HPLC mobile phase and stored at −70°C. Two reference solutions of each analyte were prepared from independent weighings; the first solution was used for preparing 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 10.0 μg ml^{-1}. This working reference solution was stored at −20°C and thawed at room temperature before use.

Preparation of standards: pooled normal human plasma was first acidified either with 10% of a 2% sulfuric acid solution (v/v) or with 5 mg of citric acid per ml of plasma. A stock solution in plasma (100 ng ml^{-1}) containing each analyte was then prepared by spiking the acidified plasma with the 10.0 μg ml^{-1} working reference solution. This stock solution of plasma was then serially diluted with acidified human plasma to obtain ten standards of plasma ranging in concentrations from 0.25–100 ng ml^{-1}.

Preparation of QC samples: QC samples were prepared similarly to the standard samples. A stock solution in plasma (68 ng ml^{-1}) containing each analyte was prepared by spiking acidified plasma with the working reference solution described above. This stock plasma solution was then serially diluted with acidified plasma to obtain QC samples at three concentration levels corresponding to 1, 11, and 68 ng ml^{-1} (designated as QC Low, QC Mid, QC High, respectively).

2.3. Sample extraction

A 1 ml aliquot of human plasma was mixed with 100 μl of internal standard, d_3-TNP-470. Five ml of extraction solvent (ethyl acetate–hexane, 1:1, v/v) were added to the tube containing plasma and internal standard. The tube was capped tightly and gently mixed on an Eberbach (Ann Arbor, MI, USA) reciprocating shaker for 5 min. The sample was centrifuged at 3080 *g* for 10 min at 4°C. The organic (top) layer was transferred into a 12×75 mm borosilicate glass tube and evaporated to dryness using a Hetovac vacuum evaporator (Heto Lab Equipment, Denmark). The dried extract was reconstituted with 100 μl of HPLC mobile phase solvent. Typically, 50 μl was injected into the HPLC–MS–MS system. If not analyzed immediately, the reconstituted extracts were stored at −20°C.

2.4. Apparatus

Mobile phase was delivered by an Isco 500D syringe pump (Lincoln, NE, USA) at a flow-rate of 0.60 ml min^{-1}. The analytes were separated using a reversed-phase analytical column (YMC ODS-AQ, 5 μm, 15 cm×2.6 mm, Wilmington, NC) at ambient temperature. Sample extracts were injected by a CMA/200 autosampler (CMA/Microdialysis, Stockholm, Sweden) equipped with a 50 μl injection loop.

A Sciex API III+ triple quadrupole mass spectrometer (PE-Sciex, Thornhill, Toronto, Canada) was used for analyte detection. Eluate from the HPLC column was ionized by atmospheric pressure chemical ionization via the heated nebulizer interface held at a temperature of 480°C. Nitrogen was used as the nebulizer gas at a pressure of 70 psi while the nitrogen auxiliary gas flow was held at a rate of 2750 ml min^{-1}. The curtain gas was ultra-high-purity nitrogen (99.999% pure) held at a flow-rate of 1.8 l min^{-1}. 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 (Argon) thickness was approximately 260×1013 molecules cm^{-2}. The following reaction channels were selected: m/z 326→215 for M-II, m/z 343→215 for AGM-1883, m/z 419→233 for TNP-470, and m/z 422→236 for the internal standard,

d_3-TNP-470. For data acquisition, the dwell time was set at 150 ms per channel with a pause time of 50 ms between channels. The standard software provided by Perkin–Elmer Sciex for data acquisition and quantitation (Tune 2.4, RAD 2.4, and MacQuan 1.3) was used in all experiments.

3. Results and discussion

3.1. Mass spectrometry

Full-scan positive ion mass spectrum of TNP-470 (molecular mass=401 g mol^{-1}), AGM-1883 (molecular mass=325 g mol^{-1}), and M-II (molecular mass=343 g mol^{-1}) was obtained by scanning from m/z 200–500 of a direct continuous infusion (150 $\mu l\ min^{-1}$) of a 1.0 $\mu g\ ml^{-1}$ solution of each analyte separately. Full-scan mass spectrum of each of the three analytes is given at the top of Figs. 2–4. The mass spectrum for each analyte was used to confirm the chromatographic elution order (on the C_{18} analytical HPLC column) of an injected reference mixture containing 100 ng of each analyte. In general, one of the more abundant ions (typically molecular adduct ion) in the mass spectrum of each analyte was selected and subjected to collision-activated dissociation to produce characteristic product ions. However, in the case of M-II, the base peak in the mass spectrum of M-II under liquid chromatographic conditions was found to be the ion with m/z 326 which was attributed to the $[M+H-H_2O]^+$ ion. The quantitation of TNP-470 (Fig. 2) was based on the selected reaction of the m/z 419 precursor ion $[M+NH_4^+]$ fragmenting to form the m/z 233 product ion. The product ion with m/z 233 was characterized in a separate experiment using high-resolution mass spectrometry. This product ion was determined to have the molecular formula of $C_{15}H_{21}O_2$ and the proposed fragment ion was $[M+H-CH_2C_1CONHCOOH-CH_3OH]^+$. Other reactions monitored were: m/z 343 to m/z 215 (for AGM-1883; see Fig. 3) and m/z 326 to m/z 215 (for M-II; see Fig. 4). The product ion (m/z 215) for these two reactions was believed to be structurally similar and was postulated to be an additional loss of H_2O from the m/z 233 ion. Because the internal standard used for quantitation in this study was a deuterated analog

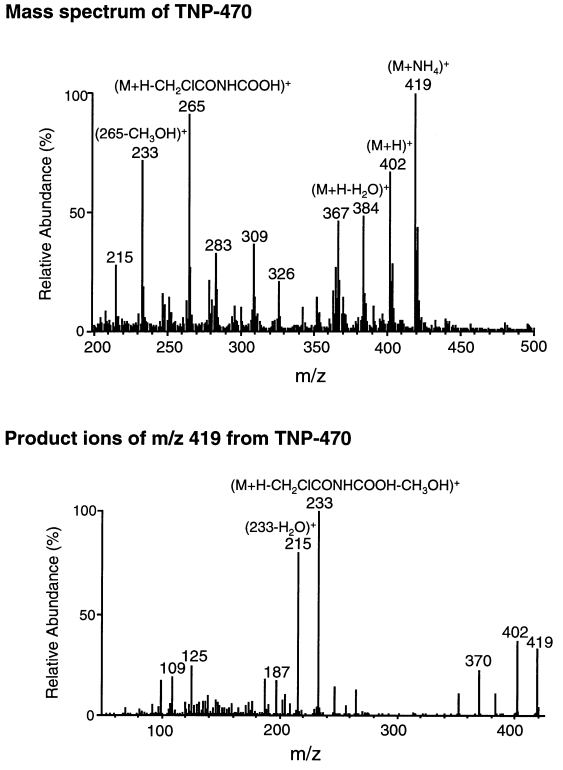

Fig. 2. Mass spectrum of TNP-470 from m/z 200 to 500 (top) and product ion mass spectrum of precursor ion with m/z 419 (bottom).

(d_3) of TNP-470, the selected reaction channel monitored for the internal standard was m/z 422 to m/z 236, three mass units higher than the precursor and product ions of TNP-470, respectively. Fig. 5 shows the selected reaction chromatograms corresponding to each analyte.

3.2. Sample collection and handling

Figg et al. suggested that TNP-470 would be most stable in a slightly acidic medium (pH 4 to 5) and developed plasma acidification by using a 2% sulfuric acid solution as part of the sample collection procedure [12]. However, this acidification procedure can only be carried out after the blood sample had been centrifuged and the plasma had been isolated. Thus, there is a risk of TNP-470 degrading in the matrix of whole blood prior to acidification. Therefore, we investigated an alternative procedure which

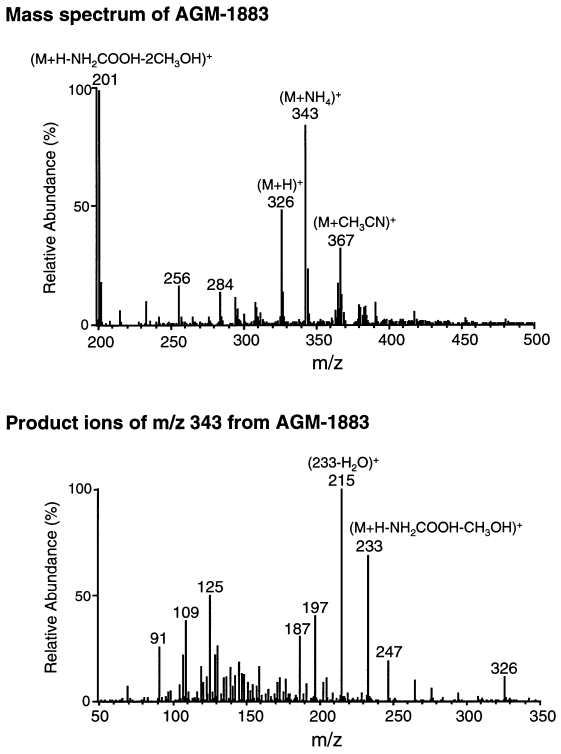

Fig. 3. Mass spectrum of AGM-1883 from m/z 200 to 500 (top) and product ion mass spectrum of precursor ion with m/z 343 (bottom).

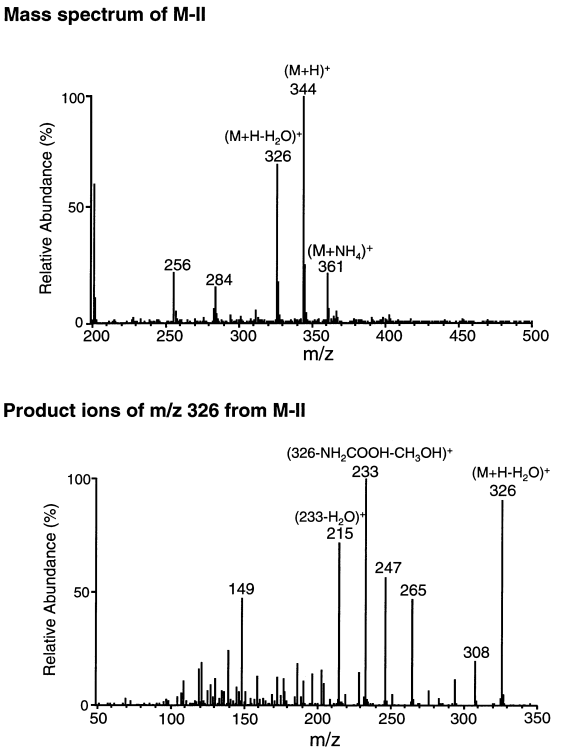

Fig. 4. Mass spectrum of M-II from m/z 200 to 500 (top) and product ion mass spectrum of precursor ion with m/z 326 (bottom).

would prevent or minimize degradation of TNP-470 in whole blood. In the course of our experiments, we found that the metabolites, AGM-1883 and M-II, are relatively more stable than TNP-470 and do not degrade as readily.

An experiment was first carried out to determine the extent of degradation of TNP-470 in whole blood prior to acidification. Whole blood was collected from six subjects into vacuum-filled collection tubes (Vacutainers®) containing sodium heparin and spiked with TNP-470. Each reservoir of blood was immediately placed in an ice bath and smaller aliquots of blood were then removed at different times to harvest the plasma. The volume of plasma collected was measured and the sample was acidified by the appropriate volume of 2% sulfuric acid solution. The acidified samples were then stored at −70°C until analysis. Table 1 shows the measured concentrations of TNP-470 (expressed as percent of the concentration at 0 min) for the six subjects after varying the time of exposure of untreated whole blood. The results indicated that there was a loss of TNP-470 in whole blood prior to plasma harvesting and addition of sulfuric acid. The rate of decline in the concentrations of TNP-470 varied from subject to subject. Although the whole blood samples were kept in an ice bath, after two hours there were significant decreases in the concentration of TNP-470 ranging from 17% (Subject 6 in Table 1) to 99% (Subject 4 in Table 1). These data indicate that an alternative acidification procedure was needed to minimize the degradation of TNP-470.

Citric acid was examined as the acidifying agent and as the anticoagulant for whole blood. By adding citric acid directly to the collection tubes, the blood samples can be immediately acidified. It was found that the 2% sulfuric acid solution (used in the preceding acidification procedure) causes severe hemolysis when it was added to whole blood samples, making it difficult for plasma harvesting.

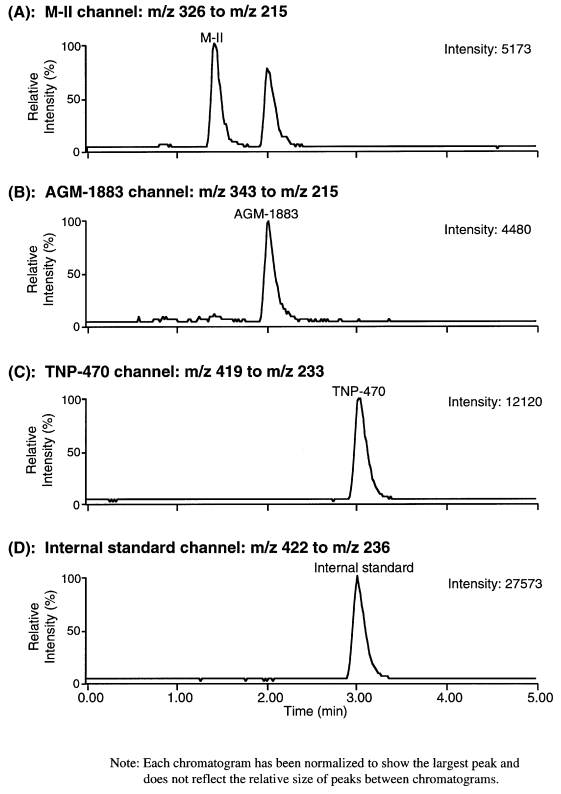

Fig. 5. Chromatograms of a human plasma extract spiked with approximately 6.5 ng ml^{-1} of (A) M-II, (B) AGM-1883, and (C) TNP-470. The concentration of the internal standard, d_3-TNP-470 (D), was approximately 17 ng ml^{-1}. Intensity counts (arbitrary units) are shown at the upper right of each chromatogram.

Initially, the solid form of citric acid was investigated because it can be added to the collected whole blood samples with negligible volume contribution. However, addition of a concentrated solution of citric acid was found to be more convenient. The harvested plasma would have a similarly acidic pH and would not require further processing until analysis. The amount of citric acid required to bring plasma pH to about 5 was determined experimentally to be 5 mg of citric acid per ml of plasma. The addition of 5 mg of citric acid per ml of whole blood also produced similar pH values in the harvested plasma without causing severe hemolysis.

To further simplify sample handling during clinical trials, acidification by citric acid was carried out in custom-made vacutainers containing a solution of citric acid in saline (250 mg ml^{-1}). The volume contribution using this solution was only 20 μl for every ml of blood collected, which represented a 2% increase in volume. The custom-made vacutainers (5 ml) containing citric acid in saline (100 μl of 250 mg ml^{-1} solution) were prepared by Haematologic Technologies Inc. (Essex Junction, VT, USA) for use during clinical trials. These vacutainers were used in an experiment to determine if the degradation of TNP-470 was minimized when the acidification was carried out in whole blood samples. Whole blood samples were collected from four of the six subjects used in the previous experiment into the custom-made vacutainers, spiked with TNP-470, and kept in an ice bath. Smaller aliquots of blood were drawn from the vacutainer at different times to harvest the plasma which were then analyzed for TNP-470 levels. The results are shown in Table 2 with concentrations expressed as percent of the initial concentration (concentration at 0 min). In Table 2, it can be seen that two hours after the collection of samples, Subjects 3 and 4 showed negligible losses, and Subjects 1 and 2 were still at 89% and 84% of initial TNP-470 concentrations, respectively. This suggests that acidification of whole blood samples by

Table 1
Stability of TNP-470 collected by the sulfuric acid procedure

Time in ice (min)	TNP-470 conc. expressed as percent relative to initial conc. (%)					
	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6
0	100	100	100	100	100	100
20	46	51	56	19	80	94
40	35	39	47	11	74	91
60	26	32	39	6	66	86
120	14	18	23	1	59	83

Table 2
Stability of TNP-470 collected in citric acid vacutainers

Time in ice (min)	TNP-470 conc. expressed as percent relative to initial conc. (%)			
	Subject 1	Subject 2	Subject 3	Subject 4
0	100	100	100	100
30	97	94	100	104
60	93	90	102	103
120	89	84	99	105

citric acid significantly minimized the degradation of TNP-470 when the samples were kept in an ice bath prior to plasma harvesting.

3.3. Sample extraction

Initially, a method involving solid-phase extraction (SPE) was used to isolate the analytes from human plasma as part of the sample preparation procedure. The SPE procedure was the same as the one reported by Moore and Sommadossi [10] which involved extraction with a C_{18} extraction cartridge. However, we found that solid-phase extraction produced inconsistent results even though a number of different extraction cartridges and elution procedures were tried. High variability in measured concentrations of replicate samples (particularly for the metabolites) was observed with relative standard deviation (R.S.D.) values that were more than 15%. Closer examination of the chromatographic data showed that peak areas of the internal standard (deuterated TNP-470) were highly variable within the analytical run although the same amount was added to each sample. TNP-470 peak areas were also variable among replicate samples although the peak area ratios (TNP-470/internal standard) were consistent. The peak areas for the metabolites, however, were similar among replicate samples. Since the measured concentrations of the metabolites were also based on peak area ratios (metabolite/internal standard), the high variability of the calculated results for the metabolites was due to variable recovery of the internal standard.

As mentioned earlier, Figg et al. had observed that TNP-470 was most stable in the pH range of about 4–5 [12]. We suspected that residual water eluted from an SPE cartridge caused non-uniform degradation in internal standard during the eluate evaporation process which, in turn, was seen as variability in recovery. This hypothesis was substantiated by an experiment in which a solution of the three analytes in acetonitrile with a small amount of water was evaporated to dryness. Fig. 6 shows (A) the total ion chromatogram (TIC) of a reference solution con-

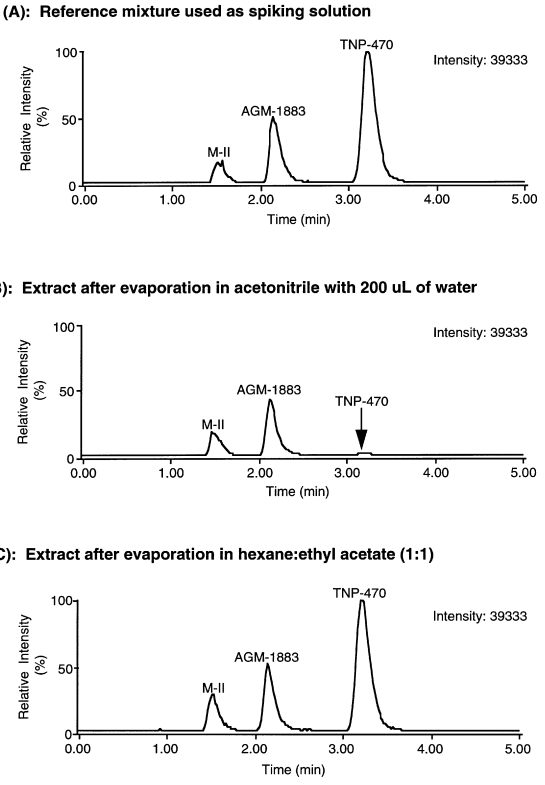

Fig. 6. Total ion chromatogram of (A) reference standard, (B) acetonitrile–water extract, and (C) hexane–ethyl acetate (1:1) extract. Intensity counts (arbitrary units) are shown at the upper right of each chromatogram.

taining the three analytes and (B) the TIC of the residue (reconstituted) after evaporation of a small volume of the reference solution in (A) pre-mixed with 3 ml of acetonitrile with 200 μl of water (similar to an extract obtained from the acetonitrile elution of an SPE cartridge). The TIC in Fig. 6B shows the extreme case in which greater than 90% of TNP-470 concentration had degraded. The data also showed that the metabolites are more stable than the parent drug and did not exhibit any notable degradation after evaporation.

We developed an alternative procedure involving liquid–liquid extraction because the amount of residual water could be minimized. A mixture of hexane and ethyl acetate (1:1) provided high extraction efficiency for all three analytes and the amount of water present in the organic extract was negligible. The complications associated with SPE were eliminated when this organic solvent mixture was used for extraction, and consistent recoveries of all analytes were achieved (R.S.D. $<10\%$). Fig. 6C shows the TIC of an extract obtained after reconstitution from evaporation of the hexane–ethyl acetate solvent.

3.4. Analytical method performance

Typically, pooled human plasma was used in the preparation of standard and QC samples. The pooled plasma samples (un-spiked) were extracted and analyzed for potential interferents but none were found, suggesting that each selected reaction was specific for the corresponding analyte. Because the peaks for the three analytes were well-resolved chromatographically (in under 4 min; see Fig. 5), there was no concern of 'channel cross-talk' (one analyte producing signals in two or more of the selected reaction channels) between the three analytes.

To assess the linear concentration range of each analyte, calibration curves consisting of human plasma standards at a range of concentrations (mean of three replicates at each standard level) were prepared and analyzed. The calibration curves consisted of peak area ratio (analyte/internal standard) versus concentration of analyte and were linear over the concentration range of 0.25–100 ng ml^{-1} for TNP-470 and 0.40–100 ng ml^{-1} for the metabolites with correlation coefficients better than 0.997 ($1/x$ weighted linear regression analysis).

The concentration of the lowest standard analyzed for TNP-470 was 0.25 ng ml^{-1}. This is a reliable lower limit of quantitation (LOQ) with a day-to-day R.S.D. of 3% and accuracy of 88%. The term accuracy is defined as percentage of theoretical concentration (mean calculated concentration/theoretical concentration$\times$100). The LOQ for both AGM-1883 and M-II was 0.40 ng ml^{-1}. For AGM-1883, the day-to-day R.S.D. at this concentration was 3% at an accuracy of 90%. The day-to-day R.S.D. for M-II was 5% at an accuracy of 94%. Fig. 7

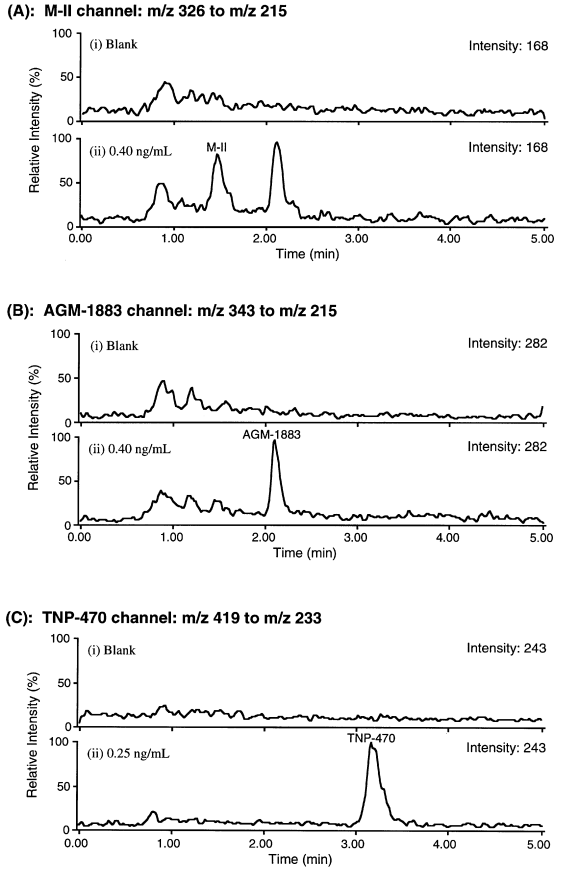

Fig. 7. Chromatograms of the extract of (i) human plasma blank vs. (ii) human plasma spiked with M-II (A), AGM-1883 (B), and TNP-470 (C) at their corresponding LOQ. Intensity counts (arbitrary units) are shown at the upper right of each chromatogram.

shows the selected reaction chromatograms obtained for each analyte at its corresponding LOQ.

Within-run and day-to-day accuracy and precision were evaluated by replicate analysis ($n=6$) of quality control (QC) samples on three different days. Results of the assay are presented in Table 3. Excellent accuracy and precision was obtained in the quantitation of all three analytes with mean values that were within 10% of the theoretical concentration and R.S.D. values of less than 10%.

The extraction recovery of the analytes from human plasma was estimated at three different concentrations (1.6, 13.2, and 53.0 ng ml^{-1}) constituting the low, middle, and high concentration region of the calibration curve. Recoveries were calculated as the relative peak areas of the sample extracts and the reference standard solution used for preparation of the spiked plasma standards. Mean recoveries (four replicates at each level) of TNP-470 from plasma were 72%, 75%, and 81% for the low, middle, and high concentrations, respectively. For AGM-1883, mean recoveries were 80% (low), 81% (middle), and 89% (high). For M-II, mean recoveries were 80% (low), 69% (middle), and 72% (high). These results suggest that, for all three analytes, there was no significant bias in the extraction recoveries at different concentrations.

3.5. Stability

The stability of TNP-470, AGM-1883, and M-II were evaluated in acidified human plasma because all samples were acidified at the time of collection and stored frozen until analysis. Acidified plasma was spiked with the analytes at three concentration levels corresponding to the low, middle, and high regions of the calibration curve and kept frozen at −70°C until analysis. These samples were thawed and analyzed at various intervals to determine the concentration of each analyte for comparison with the concentration measured on the first day of the stability study. All three analytes were found to be stable for at least one year when stored frozen at −70°C. Because samples may be analyzed more than once, the stability of the acidified plasma samples under freeze/thaw conditions was also investigated. Samples were found to remain stable for up to three freeze/thaw cycles prior to analysis.

In addition, spiked plasma samples were evaluated for in-process stability. The amount of time required to thaw a sample which had been stored frozen at −70°C was typically about 1 h. To assess in-process stability, frozen samples were thawed and maintained at room temperature for 4 h and 24 h prior to sample preparation and analysis and the measured concentration of each analyte was compared to the values obtained from samples that were prepared and analyzed immediately upon thawing. Results from the analysis suggest that the acidified plasma samples will remain stable for up to 24 h at room temperature.

The stability of the analytes as reconstituted sample extracts in HPLC mobile phase was evaluated on each day of analysis. During the course of a run, QC samples are strategically placed at the beginning and at the end of a run to monitor the integrity of the samples in the autosampler. Based on our experiences with numerous batch processing analyses, we have found that reconstituted sample extracts are stable in the autosampler for at least 8 h at room temperature.

Table 3
Within-run and day-to-day accuracy and precision

QC conc (ng ml^{-1})	Within-run accuracy and precision[a]			Day-to-day accuracy and precision[a]		
	TNP-470	AGM-1883	M-II	TNP-470	AGM-1883	M-II
67.5	95 (2)	92 (3)	94 (2)	94 (1)	92 (3)	93 (5)
11.3	100 (2)	97 (4)	93 (8)	99 (1)	96 (3)	95 (7)
1.2	104 (3)	99 (5)	100 (9)	101 (4)	99 (7)	98 (9)

[a] Accuracy defined as % of theoretical concentration; precision given as %R.S.D. in parentheses ($n=6$ for within-run and $n=18$ for day-to-day).

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