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Determination of the heat shock protein 90 inhibitor 17-dimethylaminoethylamino-17-demethoxygeldanamycin in plasma by liquid chromatography–electrospray mass spectrometry

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Abstract

A rapid method was developed for the quantitative determination of the novel heat shock protein 90 inhibitor, 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG; NSC707545), in human plasma. Calibration curves were constructed, and were analyzed using a weight factor proportional to the nominal concentration. Sample pretreatment involved a one-step extraction with ethyl acetate of 0.5-ml samples. The analysis was performed in the range of 1–100 ng/ml on a column (75 mm × 2.1 mm internal diameter with 3.5 μm C18 particle size), using 55% methanol in water containing formic acid as the mobile phase. The column effluent was monitored by mass spectrometry with positive electrospray ionization. The values for precision and accuracy were always <8% and <10% relative error, respectively. The method was successfully applied to examine the pharmacokinetics of 17-DMAG in a cancer patient.
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Keywords: 17-DMAG; LC/MS; Plasma; Pharmacokinetics; Cancer

1. Introduction

The heat shock protein 90 (Hsp90) molecular chaperone has emerged as one of the most exciting targets for cancer drug development in recent years [1]. Hsp90 is overexpressed in many malignancies, possibly as a result of the stress that is induced both by the hostile cancer microenvironment and also by the mutation and aberrant expression of oncoproteins. A particularly attractive feature of Hsp90 as a cancer drug target is that it is required for the conformational stability and function of a wide range of oncogenic 'client' proteins, including c-Raf-1, Cdk4, ErbB2, mutant p53, c-Met, Polo-1 and telomerase hTERT, which processes are accompanied by dose-dependent inhibition of tumor growth [1]. Inhibition of Hsp90 should therefore block multiple critical oncogenic pathways in the cancer cell, leading to inhibition of all the hallmark traits of malignancy and induce a broad spectrum of antitumor activity across multiple cancer types. In the mid-1990s, it was discovered that certain natural products, including geldanamycin, exert their antitumor activity by inhibiting the essential ATPase activity associated with the N-terminal domain of the Hsp90 protein [2].

Although geldanamycin was not developed for clinical use because of excessive toxicity, the analogue 17-allylamino-17-demethoxygeldanamycin (17-AAG) is presently under evaluation as an anticancer drug in clinical trials [3–6]. In an attempt to improve the physicochemical properties and biological characteristics of 17-AAG, a library of more than 60 structurally related agents was developed [7], of which 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG; NSC707545; Fig. 1) was considered the most promising. Both 17-AAG and 17-DMAG have similar patterns of activity in the National Cancer Institute (NCI) 60 cell line screen, suggesting that the two compounds have similar mechanisms

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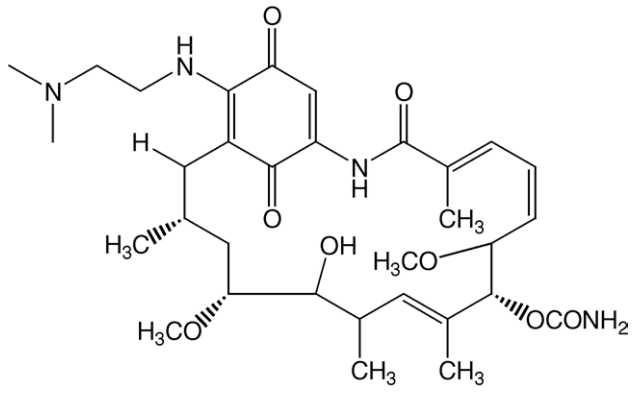

Fig. 1. Chemical structure of 17-DMAG.

of action (COMPARE analysis; http://www.dtp.nci.nih.gov/docs/dtp_search.html). 17-DMAG has shown antiproliferative activity in various in vitro human tumor models, including gynecologic cancer cell lines [8]. Furthermore, impressive in vivo antitumor activity in several human orthotopic tumor xenograft models with a remarkable lack of toxicity has been observed following 17-DMAG administration [9,10]. Compared to 17-AAG, 17-DMAG offers a potential advantage because its aqueous solubility eliminates the need for complicated formulations that are currently used for administration of 17-AAG. In addition, 17-DMAG undergoes only limited metabolism compared to 17-AAG in preclinical models [11,12], which may reduce drug clearance and interindividual pharmacokinetic variability in humans. Based on these promising data, we have initiated a Phase I clinical trial of 17-DMAG in patients with refractory solid tumors. Here, we report the development and validation of an analytical method for the quantitation of 17-DMAG in human plasma in support of a project to understand the clinical pharmacology of this agent.

2. Experimental

2.1. Chemicals and materials

17-DMAG was supplied as a crystalline white powder by the Pharmaceutical Management Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment and Diagnosis, NCI (Bethesda, MD, USA). HPLC-grade methanol was obtained from J.T. Baker (Phillipsburg, NJ, USA). Formic acid was purchased from Sigma (St. Louis, MO, USA). Deionized water was generated with a Hydro-Reverse Osmosis system (Durham, NC, USA) connected to a Milli-Q UV Plus purifying system (Malbourough, MA, USA). Drug-free heparinized human plasma was obtained from the National Institutes of Health Clinical Center Blood Bank (Bethesda, MD, USA).

2.2. Equipment

Chromatography was performed on an HP1100 system (Agilent Technology, Palo Alto, CA, USA), which included a binary pump, a vacuum degassing unit, a refrigerated autosampler, a temperature-controlled column compartment, a photodiode-array detector and a HP1100 single-quadrupole mass-spectrometric (MS) detector equipped with an electrospray source. The autosampler seat and needle sets consisted of a polyether–ether–ketone-based needle seat and assembly, and a Tefzel seal (Agilent Technology) was used in the injector valve to avoid carry-over. Data were acquired and integrated by the ChemStation software run on a HP Vectra 150/PC with a Windows NT operating system. A commercially available column composed of a C18 stationary phase packed in a stainless steel column (75 mm × 2.1 mm internal diameter [I.D.] with 3.5 μm particle size; Agilent Technology) attached to a column-inlet filter (3 mm × 0.5 μm; Varian, Walnut Creek, CA, USA). PEEK tubing of 0.127 mm I.D. (Upchurch Scientific, Oak Harbor, WA, USA) was used to connect the column to the pump and the MS detector with minimal tubing length to avoid an extensive post-column volume.

2.3. Chromatographic and MS conditions

Isocratic chromatographic separations were achieved using a mobile phase composed of 55% methanol in water with 0.2% formic acid with a flow rate set at 0.3 ml/min. The analytical column was kept at 40 °C. The column effluent was connected to an electrospray ionization MS interface without splitting. Nitrogen was used as the nebulizer gas at a pressure of 45 psi and as drying gas at a flow rate of 11 l/min and a temperature of 300 °C. The capillary voltage was set at 2000 V and fragmentor setting at 80 V. The MS detector was operated in the positive ion mode, with single ion monitoring set at *m/z* 617 for the protonated molecular ion of 17-DMAG. Monitoring was performed using a dwell time of 578 ms and was monitored in the high-resolution mode. After data acquisition, the selected-ion monitoring chromatograms were integrated using the HP ChemStation software and used for quantitation. No internal standard was used.

2.4. Preparations of standards

Stock solutions were prepared in triplicate by accurately weighting an appropriate amount of 17-DMAG and dissolving in DMSO, resulting in primary stock solutions containing 1 mg/ml, which were stored at −20 °C. The amount of compounds in the triplicate solutions was measured by injection of aliquots of 2500-fold diluted stock solutions in the mobile phase (all in triplicate) and injection into the liquid chromatographic system. The mean value of the individual stock solutions was within 2.0–4.6% of each other, and one solution was used in subsequent experiments.

Working standard solutions were prepared over a range of 0.02–20 μg/ml by serial dilution of the stock solution with methanol, and then were also stored at −20 °C. Plasma calibration standards of 1, 2, 5, 10, 20, 50 and 100 ng/ml were prepared by mixing the working standard solution with blank human plasma, such that the total amount of methanol added was identical in each sample. Quality control (QC) samples in plasma were prepared from an independent stock solution at concentrations of 3, 40 and 80 ng/ml by dilution of the working stock solution with blank human plasma. These QC samples

were subdivided into 0.5-ml aliquots, and stored at −20 °C until analysis.

2.5. Sample preparation

Standards, QCs samples and samples from patients treated with 17-DMAG were allowed to thaw at room temperature. Aliquots of 0.5 ml of sample were placed into 15 ml polypropylene tube (Greiner Bio-One, Frickenhausen, Germany), to which 4 ml ethylacetate were added. The mixture was vortex-mixed for 5 min, and then centrifuged for 10 min at 3000 rpm. The clear supernatant was transferred to a glass tube and evaporated to dryness under desiccated air in a water bath at 40 °C in a Zymark TurboVap LV (Hopkinton, MA, USA). The residue was reconstituted in 200 μl of 50% methanol in water containing 0.2% formic acid, followed by vortex-mixing and centrifugation for 5 min at 13,000 rpm. The supernatant was transferred to injection vials and 50 μl were injected into the chromatographic system.

2.6. Validation procedure

To evaluate the specificity of the analytical procedure, blank human plasma samples obtained from six different individuals were extracted and analyzed for the presence of interfering endogenous substances.

The absolute instrument response for standards at low (3 ng/ml), mid (40 ng/ml) and high concentrations (80 ng/ml) of 17-DMAG prepared in five different lots of blank human plasma from different donors was evaluated in quintuplicate. In addition, the absolute matrix effect was assessed on the ionization of the 17-DMAG. This was done by comparing the absolute responses of blank matrix extracted and spiked with 17-DMAG (at concentrations of 3, 40 and 80 ng/ml) post-extraction with the absolute response of neat analyte diluted in the mobile phase injected directly.

Calibration curves were constructed by plotting the peak area of the analyte *versus* the nominal concentration (x) of the calibration standards. The regression parameters of slope, intercept and correlation coefficient were calculated by least-squares linear-regression analysis using a weight factor of $1/x^2$. The linearity was evaluated by comparing the correlation coefficient (r^2), residuals and errors between theoretical and back calculated concentrations of calibration standard samples.

The accuracy and precision were assessed by analyzing QC samples prepared at three different concentrations equally distributed over the tested range (i.e., spiked at 3, 40 and 80 ng/ml) in six replicates on three different days. The accuracy of the assay was evaluated by the percentage deviation (DEV) from the theoretical concentration (TC) using the formula:

$$\text{DEV} = 100\% \times \frac{(\text{mean back calculated concentration} - \text{TC})}{\text{TC}}$$

Values for within- and between-assay precision were obtained by one-way analysis of variance (ANOVA) testing, and reported as relative standard deviation for each QC concentration. Estimates of the between-run precision were obtained by ANOVA using the run day as the classification variable. The between-groups mean square (MS_{bet}), the within-groups mean square (MS_{wit}) and the grand mean (GM) of the observed concentrations across runs were calculated using the software package NCSS 2001 (J. Hintze, Number Cruncher Statistical Systems, Kaysville, UT, USA). The between-run precision (BRP), expressed as a percentage relative standard deviation, was defined as:

$$\text{BRP} = 100 \times \left(\sqrt{\frac{((\text{MS}_{\text{bet}} - \text{MS}_{\text{wit}})/n)}{\text{GM}}} \right)$$

where n represents the number of replicate observations within each run. For each concentration, the estimate of the within-run precision (WRP) was calculated as:

$$\text{WRP} = 100 \times \left(\sqrt{\frac{(\text{MS}_{\text{wit}})}{\text{GM}}} \right)$$

The procedure used to assess extraction recovery was based on a comparison of the absolute response of samples of blank matrix spiked to contain 17-DMAG at concentrations of 3, 40 and 80 ng/ml in quituplicate after extraction with those of extracted spiked samples containing the same concentration.

The stability of 17-DMAG in human plasma was assessed at room temperature for up to 24 h and during three freeze-thaw cycles. Four aliquots of QC samples spiked to contain three different concentrations of 17-DMAG (3, 40 and 80 ng/ml) were thawed at room temperature, and kept at this temperature for 0, 12 and 24 h. After the indicated time period, the samples were immediately analyzed. For the freeze-thaw stability study, QC samples were prepared in quadruplicate at the same three concentrations, and then stored at −80 °C for 24 h. Next, the samples were thawed at room temperature, and were refrozen for 12 h under the same conditions. This freeze-thaw cycle was repeated two more times, and then all samples were analyzed after the final (third) cycle.

2.7. Clinical experiment

To demonstrate the applicability of the final analytical procedure, samples were obtained from a cancer patient, who participated in an ongoing multi-dose Phase I clinical trial with 17-DMAG in patients with solid tumors and lymphomas. The dose of 17-DMAG was 1.0 mg/m^2 (absolute dose, 2.4 mg in 15 ml of normal saline) and was given as a 1-h intravenous infusion. The current experiment was approved by the local Institutional Review Board, and the patient signed informed consent before study entry for the blood sampling procedure. Blood samples were be collected prior to the first drug infusion, then at approximately 5 min before the end of infusion, and 5, 10, 30 and 60 min after the end of the first infusion, and at 2, 4, 8, 16, 24 and 48 h after the end of the first infusion. All samples were collected in heparin-containing tubes and stored on ice, and were centrifuged at 3000 × g for 5 min to obtain the plasma fraction. Pertinent pharmacokinetic parameters, including peak plasma concentration, area under the plasma concentration–time curve (AUC), elimination half-life and total body clearance, were determined by a standard two-compartment open model

using the WinNonlin v4.0 software package (Pharsight, Mountain View, CA, USA), using equal weighting.

3. Results and discussion

3.1. Chromatography

The mass spectrum of 17-DMAG showed a protonated molecular ion ([MH^+]) at *m/z* 617, in accordance with the NTP chemical repository database, and a sodium adduct at *m/z* 639 ($MH^+ + Na$) (Fig. 2). Sample pretreatment was performed by a single solvent extraction using ethyl acetate, based on previous experience with extraction of the drug from biological matrices [11]. In spite of 17-DMAG's relatively high solubility in water (1.4 mg/ml) [7], which is more than 10-fold higher than that of 17-AAG, the molecule is presumably sufficiently hydrophobic for this procedure to result in acceptable extraction recovery (see below). Hence, no additional attempts were made evaluating alternative procedures, including solid-phase extraction. In the final procedure, only a small fraction of the sample after extraction was injected (i.e., 50 μl of 200 μl used for reconstitution) on the column to maintain high efficiency and resolution, and assay sensitivity was thus compromised. Although increased injection volumes could achieve higher response factors, overloading of the small column resulted in asymmetric sample bands. The absence of formic acid in the reconstitution mixture was found to induce a distorted separation artefact, which resulted in unstable response factors over time following repeat injections of extracted patient samples (not shown). In the final procedure, therefore, reconstitution of samples was performed with a mixture of methanol and water containing formic acid.

Out of various chemicals that were tested, geldanamycin was initially selected for use as internal standard. However, we were unable to use this compound due to incidences of high variability in extraction when plasma from different sources was used. Furthermore, the use of 17-AAG as a potential internal standard was unsuccessful because the agent produced very weak MS detector signals in the positive ion mode in contrast to the negative mode; the reasons for the discrepant behavior of 17-AAG and 17-DMAG are not entirely clear. At the time of development of this procedure, it was also unclear if 17-AAG would be a potential human metabolite of 17-DMAG [11]. Eventually, therefore, the current method was developed without an internal standard.

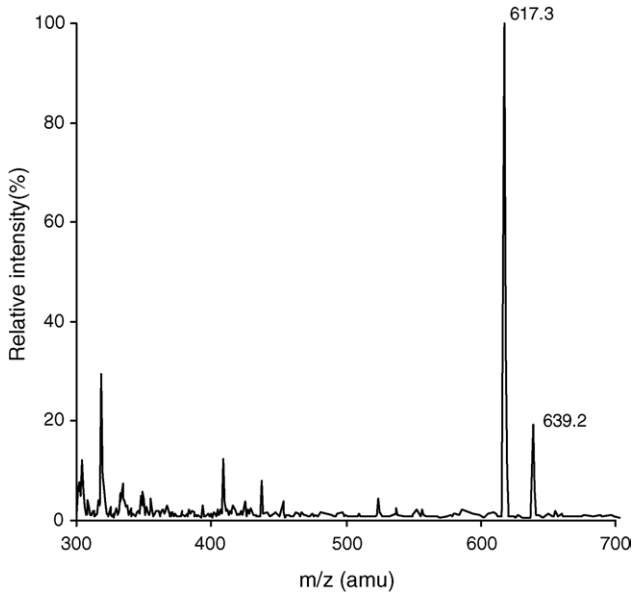

Fig. 2. Liquid chromatographic–electrospray mass spectrum of 17-DMAG, showing a protonated molecular ion ([MH^+]) at *m/z* 617, and a sodium adduct at *m/z* 639 ($MH^+ + Na$).

Variation of absolute response in an analytical assay employing MS detection that does not employ an internal standard could be of concern and would most likely render the assay unsuitable for use on clinical samples. Hence, the instrument response was recorded for standards at low (3 ng/ml), mid (40 ng/ml) and high concentrations (80 ng/ml) of 17-DMAG prepared in five lots of plasma from different donors. The results from this experiment indicate very limited variation of absolute response in the present assay (data not shown). In a second experiment, we assessed the absolute matrix effect on the ionization of the analyte. This data was generated by comparing, in quintuplicate, the absolute responses of blank matrix extracted and spiked at three different concentrations with analyte post-extraction with the absolute response of neat analyte injected directly. The mean overall difference between the two samples was 7.3%, indicating that this absolute matrix effect is not a cause for concern.

3.2. Validation characteristics

Fig. 3 displays chromatograms of an extract of a blank human plasma sample (A), and an extract of a plasma sample spiked with 17-DMAG at a concentration of 1.0 ng/ml (B). The mean retention time for 17-DMAG during the method validation was approximately 2.3 min, and the overall chromatographic run time was established at 6 min.

The assay for 17-DMAG analysis in plasma was found to be linear over the range of 1.0–100 ng/ml, applying the peak area in combination with a weighting factor of $1/x^2$, as indicated by the mean linear-regression correlation coefficient of >0.99 ($n = 3$). In blank human plasma spiked with 17-DMAG at 1.0 ng/ml, the mean percentage deviation from the nominal concentration and the within-run variability were both less than 20%. Based on these results, the lower limit of quantitation for 17-DMAG in human plasma was determined to be 1.0 ng/ml, using 0.5-ml sample volumes.

Validation data of the analytical method in terms of accuracy (percent deviation) and precision are shown in Table 1. At the upper limit of quantitation (i.e., 100 ng/ml), the mean percentage deviation and the within-run variability were less than 15%. The method was shown to be accurate, with an average accuracy at the three tested concentrations within ±10% of nominal values, and precise with a within-run and between-run variability of less than 8%. The mean overall extraction recovery, determined at three different concentrations, was 85.3%. A non-parametric Kruskal–Wallis one-way ANOVA indicated a lack of concentration-dependence ($P > 0.05$) (Table 2). In view of the relative consistency in the generated data, and the rapidity

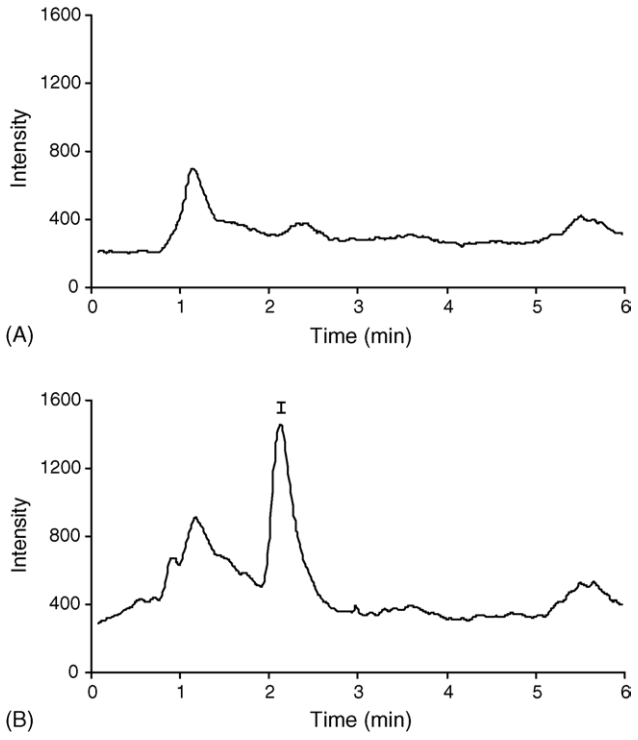

Fig. 3. Reversed-phase liquid chromatographic analysis of a blank human plasma sample (A), and a human plasma sample spiked with 17-DMAG at a concentration of 1 ng/ml, which analyte peak corresponds to the lowest calibration point (B). The labeled chromatographic peak indicates 17-DMAG (I).

Table 1
Assessment of accuracy and precision from quality control sample

Nominal (ng/ml)	Mean (ng/ml)	S.D. (%)	Accuracy (%)	BRP (%)	WRP (%)	*n*
3	2.72	0.16	−9.42	2.70	5.50	18
40	39.68	0.69	−0.81	[a]	1.75	18
80	78.17	5.20	−2.29	7.57	1.95	18

Abbreviations: S.D., standard deviation; WRP, within-run precision; BRP, between-run precision; *n*, total number of replicate observations during the validation runs.

[a] No additional variation was observed as a result of performing the assay on different days.

and ease of use, all experiments were performed using this one-step solvent extraction. Repeated freeze-thawing cycles had no influence on the stability, independent of the spiked concentration ($P > 0.05$, one-way ANOVA). In addition, plasma samples spiked with 17-DMAG and stored for variable time periods at ambient temperature were also stable (Table 3). On the basis of the generated validation parameters, the method was considered acceptable for the analysis of plasma samples in support of clinical pharmacokinetic studies [13].

Table 2
Recovery of 17-DMAG in human plasma[a]

Nominal (ng/ml)	Mean observed recovery (%)	Mean ± S.D.
3	84.1	85.3 ± 6.50
40	91.9	
80	89.7	

[a] The number of replicate sample was 4.

Table 3
Short-term temperature stability of 17-DMAG in plasma[a]

Time (h)[b]	Nominal (ng/ml)	Measured (ng/ml)[c]	Recovery (%)
0	3	2.4 ± 0.11	80.4
0	40	36.6 ± 0.19	91.6
0	80	73.2 ± 1.08	91.5
12	3	2.4 ± 0.07	80.9
12	40	36.9 ± 0.91	92.4
12	80	74.1 ± 2.18	92.6
24	3	2.3 ± 0.05	77.7
24	40	33.5 ± 0.88	83.8
24	80	67.4 ± 1.73	84.2

[a] The number of replicate sample was 4.
[b] Indicates the time lag between sample thawing and preparation.
[c] Data expressed as mean ± standard deviation.

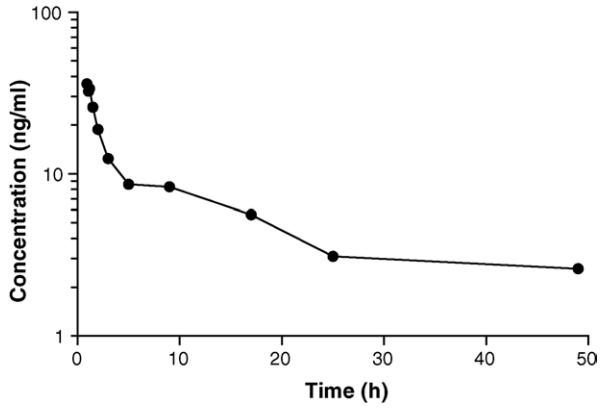

Fig. 4. Plasma concentration–time profile of 17-DMAG in a patient with cancer after a single intravenous administration of the drug at a dose of 1.0 mg/m^2.

3.3. Preliminary pharmacokinetics

The described analytical method was applied to a pharmacokinetic pilot study of 17-DMAG given intravenously to a single cancer patient. The observed concentration–time profile of 17-DMAG is shown in Fig. 4. The peak concentration of 17-DMAG was 36 ng/ml, and the area under the concentration–time curve amounted to 323 ng h/ml, with an apparent total body clearance value of 7.4 l/h and a terminal half-life of approximately 19 h. Although preliminary, this suggests that 17-DMAG is cleared much slower than the related compound 17-AAG, which has a mean overall clearance value of about 36 l/h, and a half-life of about 4 h [3]. UV detection was also carried out on all samples, but no additional peaks that might represent metabolites of 17-DMAG were detected, which is in line with preclinical data indicating the circulating concentrations of biotransformation products are insignificant [11].

4. Conclusion

In conclusion, the method presented for the determination of 17-DMAG in human plasma is specific, accurate and precise,

and is selective and sensitive enough to be used in clinical trials. The method permits the analysis of patient samples with low concentrations of 17-DMAG, and is currently being used in various Phase I clinical trials in patients with malignancies to further investigate the clinical pharmacologic profile of this agent.

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