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Determination of MS-275, a novel histone deacetylase inhibitor, in human plasma by liquid chromatography–electrospray mass spectrometry

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Abstract

A rapid method was developed for the quantitative determination of the novel histone deacetylase inhibitor, MS-275, in human plasma. Calibration curves were constructed in the range of 1–100 ng/ml, and were analyzed using a weight factor proportional to the nominal concentration. Sample pretreatment involved a one-step protein precipitation with acetonitrile of 0.1 ml samples. The analysis was performed on a column (75 mm × 4.6 mm i.d.) packed with 3.5 μm Phenyl-SB material, using methanol—10 mM ammonium formate (55:45 (v/v)) as the mobile phase. The column effluent was monitored by mass spectrometry with positive electrospray ionization. The values for precision and accuracy were always ≤5.58 and <11.4% relative error, respectively. The method was successfully applied to examine the pharmacokinetics of MS-275 in a cancer patient.

Keywords: MS-275; Histone deacetylase inhibitor

1. Introduction

Acetylation and deacetylation of histones plays a major role in the regulation of gene transcription and in the modulation of chromatin structure [1]. The state of acetylation of these nucleosome core proteins is determined by two classes of enzymes with opposing activity, which are referred to as histone acetyltransferases and histone deacetylases (HDACs). During the last decade, various agents have been identified that inhibit HDAC activity and induce cell growth arrest, differentiation and/or apoptotic cell death [2]. These agents belong to diverse structural classes and include short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides, and synthetic benzamides. In the latter group, MS-27-275 (MS-275; 3-pyridylmethyl-*N*-{4-[(2-aminophenyl)-carbamoyl]-benzyl}-carbamate; Fig. 1) was found to be the most potent HDAC inhibitor [3]. In tumor cell lines, MS-275 induces the expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ and the developmental marker gelsolin, and changes the cell cycle distribution with a decrease of S-phase cells and an increase of G_1-phase cells [4]. In addition, the agent induces the transforming growth factor (TGF)-β type II receptor gene and, consequently, potentiates TGF signaling [5].

MS-275 has shown antiproliferative activity in various in vitro human tumor models, including breast, colon, lung, myeloma, ovarian, pro-myelocytic leukemia, and prostate carcinoma cell lines [4,5]. Furthermore, impressive in vivo antitumor activity in several adult and pediatric human orthotopic tumor xenograft models with a remarkable lack of toxicity has been observed following oral drug administration [4,6]. Based on these promising data, we have initiated a Phase I clinical trial of oral MS-275 in patients with refractory solid tumors. Here, we report the development and validation of an analytical method for the quantitation of MS-275 in human plasma in support of

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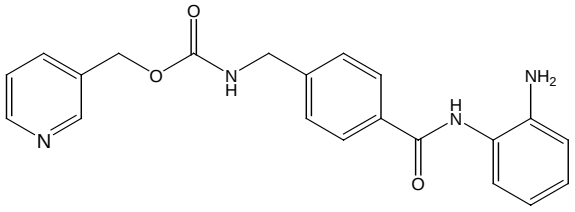

Fig. 1. Chemical structure of MS-275.

a project to understand the clinical pharmacology of this agent.

2. Experimental

2.1. Chemicals and materials

MS-275 (batch number: 81300002; HPLC purity, 99.82%) was supplied as a crystalline white powder by Schering AG (Berlin, Germany). HPLC-grade methanol and acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, USA). Ammonium formate and formic acid were 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

The experiments were carried out with a HP1100 system (Agilent Technology, Palo Alto, CA, USA). The system consisted of a G1312A binary pump, a mobile phase vacuum degassing unit, a G1329A autosampler, a temperature-controlled column compartment, 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. The stationary phase was composed of Phenyl-SB material (Agilent Technology) packed in a stainless steel column (75 mm × 4.6 mm i.d. with 3.5 μm particle size), and a Phenyl-SB guard column (12.5 mm × 4.6 mm i.d. with 5 μm particle size) 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

Chromatographic separations were achieved using a mobile phase consisting of methanol and 10 mM ammonium formate (pH 3, adjusted with formic acid) (55:45 (v/v)), with a flow rate set at 0.8 ml/min. The analytical column was kept at ambient temperature. The column effluent was connected to an electrospray ionization MS interface without splitting. The MS detector was operated in the positive ion mode, with single ion monitoring at a fragmentor setting of 65 V and a multiplier gain of 2. Nitrogen was used as the nebulizer gas at a pressure of 55 PSI and as drying gas at a flow rate of 13 l/min and a temperature of 350 °C. The capillary voltage was set at 2200 V, and selected-ion monitoring was accomplished at *m/z* 377 for the protonated molecular ion of MS-275. Monitoring was performed using a dwell time of 578 ms and was monitored in the high-resolution mode. Simultaneously, UV detection was done at 230 and 280 nm to detect possible metabolites in clinical samples. After data acquisition, the selected-ion monitoring chromatograms were integrated using the HP ChemStation software and used for quantitation.

2.4. Preparation of standards

Stock solutions were prepared in triplicate by accurately weighting, after correction for purity, an appropriate amount of MS-275 and dissolving in methanol. The final concentration of the stock solutions was 1 mg/ml, and these were stored at −20 °C. Working standard solutions were prepared over a range of 0.02–40 μg/ml by serial dilution of the stock solution with methanol, and then stored at −80 °C. Plasma calibration standards of 1, 5, 10, 20, 50, and 100 ng/ml were prepared by mixing 30 μl working standard solution with 570 μl blank human plasma. Quality control (QC) samples 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 0.1 ml aliquots, and stored at −80 °C.

2.5. Sample preparation

Standards, QCs samples, and patient samples were allowed to thaw at room temperature. A 0.1 ml aliquot of each was transferred to a 1.5 ml Eppendorf tube (Hamburg, Germany) and 500 μl of acetonitrile were added to precipitate plasma proteins. The mixture was vortex-mixed for 30 s, and then centrifuged for 5 min at 13,000 rpm. A volume of 500 μl of the clear supernatant was transferred to a glass tube and evaporated to dryness under desiccated air in a water bath at 45 °C in a Zymark TurboVap LV (Hopkinton, MA, USA). The residue was reconstituted in 200 μl of a mixture of methanol and water (50:50 (v/v)), followed by vortex-mixing. A 50 μl volume of the reconstituted sample was 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. In addition, plasma samples containing mixtures of several commonly used drugs were tested for potential chromatographic interference with MS-275.

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 a weighted ($1/x^2$) least-squares linear-regression analysis. 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:

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

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.

The extraction recovery for MS-275 in human plasma was determined at three concentration levels in triplicate using samples spiked to contain 3, 40, and 80 ng/ml, using comparison with samples prepared in 50% (v/v) methanol in water injected without extraction.

The stability of MS-275 in human plasma was assessed during three freeze–thaw cycles and at room temperature for up to 24 h. Four aliquots of QC samples of three different concentrations were thawed at room temperature, and kept at this temperature for 0, 12, and 24 h, and immediately analyzed. For the freeze–thaw stability study, QC samples at three different concentrations in quadruplicate, and 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. The freeze–thaw cycle was repeated two more times, and then analyzed on the 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 MS-275 tablets as single-agent therapy. The drug was administered orally with a meal at a dose of 10 mg/m^2. 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. A total of 11 blood samples (7 ml each) were obtained and collected in 10 ml glass tubes containing heparin as an anticoagulant. These samples were obtained before drug administration and at approximately 0.5, 1, 2, 6, 12, 24, 48, 60, 72, and 84 h after drug administration. Specimens were immediately centrifuged at 3000 × g for 5 min to separate the plasma supernatant, which was stored at −70 °C until the time of analysis. Plasma concentration–time data of MS-275 were analyzed by non-compartmental methods using the software package WinNonlin version 4.0 (Pharsight Corp., Mountain View, CA) using equal weighting.

3. Results and discussion

3.1. Chromatography

The mass spectrum of MS-275 showed a protonated molecular ion ([MH$^+$]) at m/z 377, in accordance with the NTP chemical repository database, a sodium adduct at m/z 399 (MH$^+$ + Na), and a prominent fragment ion peak at m/z 359 (MH$^+$ − H_2O) (Fig. 2). Sample pretreatment was initially performed by a solvent extraction (e.g., using ethyl acetate) or by solid phase-extraction (e.g., using C18 micro-extraction columns). However, these procedures resulted in poor extraction recovery, particularly at the upper limit of the expected concentration range (i.e., around 100 ng/ml). This is likely the result of the hydrophilic nature of MS-275, which is highly soluble in water (approximately 20 mg/ml at 20 °C in acidic buffers). Among various alternative procedures tested, MS-275 was eventually efficiently extracted with adequate elimination of endogenous

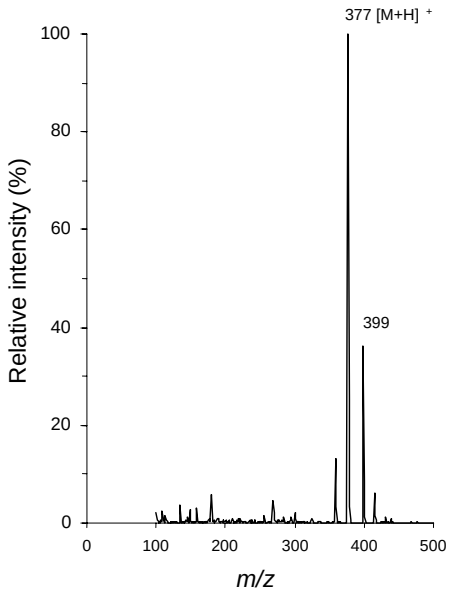

Fig. 2. Liquid chromatographic–electrospray mass spectrum of MS-275.

interfering compounds using a single protein precipitation step with acetonitrile. 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 presence of 10 mM ammonium formate (pH 3) in the reconstitution mixture was found to induce a distorted separation artifact, 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 (50:50 (v/v)). Out of various chemicals that were tested, 4,4′-diaminebenzanilide was initially selected for use as internal standard. But, we were unable to use 4,4′-diaminebenzanilide due to incidences of variability in extraction when plasma from different sources was used.

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 MS-275 at a concentration of 1.0 ng/ml (B). The mean retention time for MS-275 during the method validation was 4.3 min, and the overall chromatographic run time was established at 8 min. Several different drugs were tested for potential interference with MS-275 (Table 1), and none of these drugs was found to give an interfering peak during the analysis around the retention time of MS-275.

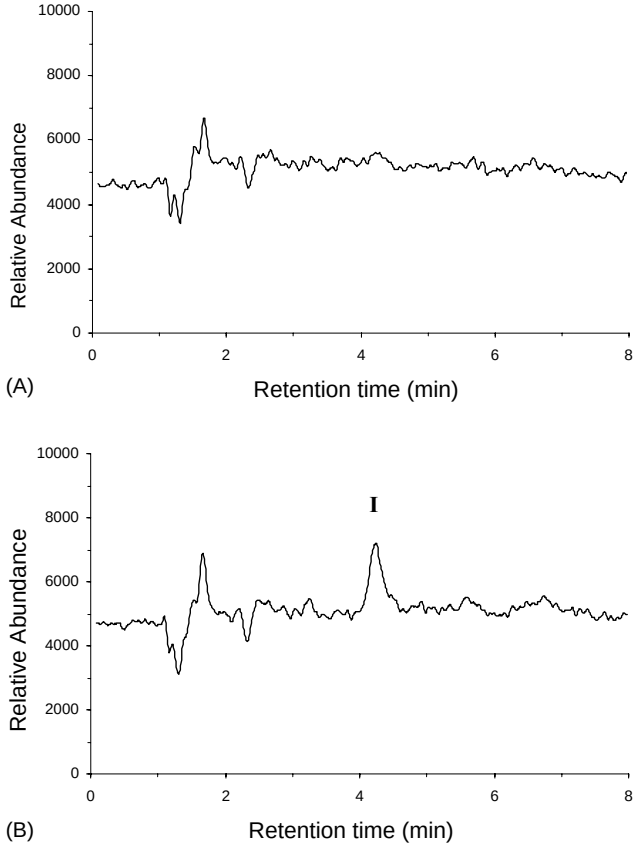

Fig. 3. Reversed-phase liquid chromatographic analysis of a blank human plasma sample (A), and a human plasma sample spiked with MS-275 at a concentration of 1 ng/ml (B). The labeled chromatographic peak indicates MS-275 (I).

Table 1
Interference analysis of various commonly administered drugs[a]

Amlodipine besylate	Loperamide
Atenolol	Metronidazole
Ciprofloxacin	Morphine sulfate
Clotrimazole	Omeprazole
Cyanocobalamine	Ondansetron
Dexamethasone	Oxycodone
Diazepam	Pamidronate disodium
Diphenhydramine	Phenytoin
Docusate sodium	Pseudoephedrine
Epoietin alpha	Pyridoxine hydrochloride
Fluticasone proprionate	Raloxifene
Folic acid	Ranitidin
Glucosamine sulfate	Rofecoxib
Hydromorphone	Sertraline hydrochloride
Hydroxyzine	Verapamil
Ketoconazole	Warfarin
Levofloxacin	Zolpidem tartrate
Levothyroxine	

[a] Plasma samples used for this analysis were taken from patients on these drugs.

The assay for MS-275 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 0.998 ($n = 3$). A comparative evaluation of accuracy between unweighted and $1/x^2$ weighted analysis is provided in Fig. 4. In blank human plasma spiked with MS-275 at 1.0 ng/ml, the mean percentage deviation from the nominal concentration and the within-run variability were both

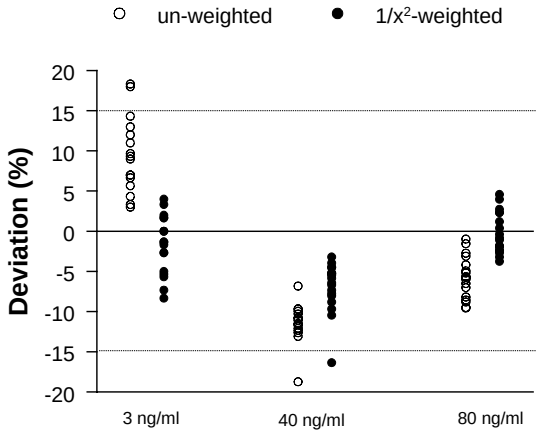

Fig. 4. Comparison of accuracy (percent deviation from nominal) for unweighted vs. $1/x^2$ weighted analysis of MS-275 in human plasma at three different concentrations. The horizontal dotted lines indicate the acceptable ±15% deviation range.

Table 2
Validation summary for the analysis of MS-275 in spiked human plasma samples

Parameter	Nominal concentration (ng/ml)		
	3	40	80
Accuracy			
Mean observed (ng/ml)	2.95	37.2	79.8
Deviation (%; $n = 18$)	−1.69	−6.92	−0.26
Precision			
Intraday (%; $n = 6$)[a]	4.58	1.13	1.56
Interday (%; $n = 18$)	3.75	3.31	2.56

[a] *Notation*: *n*, number of replicates analyzed.

less than 20% [7]. Based on these results, the lower limit of quantitation for MS-275 in human plasma was determined to be 1.0 ng/ml, using 0.1 ml sample volumes.

Validation data of the analytical method in terms of accuracy (percent deviation) and precision are shown in Table 2. The mean (±S.E.) equation was: $Y = 7388\,(\pm 335) \times X - 2091\,(\pm 571)$. 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 ±7% of nominal values, and precise with a within-run and between-run variability of less than 3.75%. The mean overall extraction recovery, determined at three different concentrations, was 37.9% (standard effect, 0.126%). A non-parametric Kruskal-Wallis one-way ANOVA indicated a minor concentration-dependence ($P = 0.027$), as determined by a Tukey–Kramer multiple comparison test for all pairwise differences between the means (Table 3). However, this effect is presumably due to normal analytical variability rather than reflecting a concentration-dependent extraction recovery. An improvement in recovery could be accomplished by using increased volumes of acetonitrile for primary isolation, followed by a repeat of the entire extraction procedure. However, in view of the relative consistency in the generated data, and the rapidity and ease of use, all experiments were performed using a one-step protein precipitation. Repeated freeze–thawing cycles had no influence on the stability. In addition, plasma samples spiked with MS-275 and stored for variable time periods at ambient temperature were also stable (Table 4). 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 [7].

Table 3
Recovery of MS-275 in human plasma

Nominal (ng/ml)	Plasma[a]	Methanol[a]	Relative recovery (%)
3	76984	181773	41.7
	76783	187090	
	77369	185003	
40	1152760	3269960	34.8
	1133914	3248894	
	1123663	3279752	
80	2419225	6517666	37.0
	2433135	6522078	
	2401394	6560018	

[a] Data expressed as chromatographic peak area of MS-275.

Table 4
Short-term temperature stability of MS-275 in plasma

Time (h)	Nominal (ng/ml)	Recovered (ng/ml)[a]	Deviation (%)[b]
0	3	3.00 ± 0.07	−0.08
	40	40.5 ± 0.73	1.29
	80	81.5 ± 0.86	1.83
12	3	2.55 ± 0.05	−15.0
	40	39.9 ± 0.53	−0.33
	80	78.0 ± 2.77	−2.50
24	3	2.71 ± 0.08	−9.75
	40	39.8 ± 1.89	−0.47
	80	78.9 ± 0.71	−1.37

[a] Data expressed as mean ± standard deviation.
[b] Percent deviation from the nominal value.

3.3. Preliminary pharmacokinetics

The described analytical method was applied to a pharmacokinetic pilot study of MS-275 given orally to a single cancer patient. The observed concentration–time profile of MS-275 is shown in Fig. 5. The time to peak concentration occurred before the first sampling time point, and hence the initial absorption phase of MS-275 was not observed in this patient. The peak concentration of MS-275 was 41.7 ng/ml, and the area under the concentration–time curve amounted to 400 ng h/ml, with an apparent oral clearance value of approximately 42 l/h per m^2). UV detection was also carried out on all samples, but no additional peaks that might represent metabolites of MS-275 were detected.

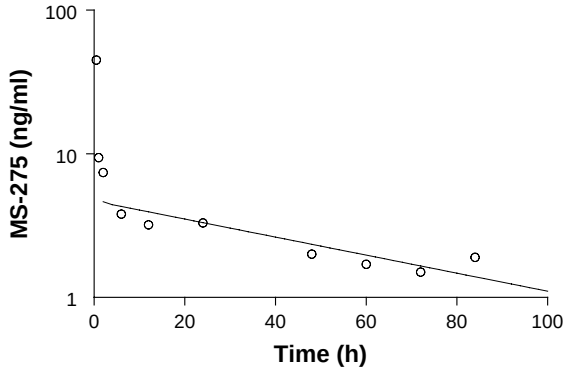

Fig. 5. Plasma concentration–time profile MS-275 in a patient with cancer after a single oral administration of the drug at a dose of 10 mg/m^2.

4. Conclusion

In conclusion, the method presented for the determination of MS-275 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 MS-275, and is currently being used in various Phase I clinical trials in patients with hematological malignancies or solid tumors to further investigate the clinical pharmacologic profile of this agent.

Acknowledgements

This paper is dedicated to the memory of our dear friend and colleague Eunhee W. Woo, who passed away in 2002.

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