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Determination of a benzamide histone deacetylase inhibitor, MS-275, in human plasma by liquid chromatography with mass-spectrometric detection

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Abstract

An analytical method was developed and validated for the quantitative determination of the histone deacetylase inhibitor MS-275 in human plasma. Calibration curves were linear in the concentration range of 1 to 250 ng/mL. Sample pretreatment involved a liquid-liquid extraction of 0.1 mL aliquots of plasma with methyl tert-butyl ether. MS-275 and the internal standard, benzanilide, were separated on a Zorbax SB-Phenyl column (4.6 × 75mm I.D., 3.5 μm), using a mobile phase composed of methanol and 10 mM ammonium formate (pH 2.9). The column eluent was monitored by mass spectrometry with electrospray ionization. Accuracy and precision of three concentrations of quality control samples ranged from 96.56 to 107.02% and 0.97 to 4.29%, respectively. This method represents an improvement over the previously published analytical assay for this agent, increasing the accuracy and precision (through addition of a suitable internal standard) and expanding the analytical range. The developed method was applied to study the pharmacokinetics of MS-275 in 724 clinical samples.

Keywords

Histone Deacetylase inhibitor; LC/MS; MS-275; Benzanilide

1. Introduction

MS-275 (pyridin-3-ylmethyl-N-({4-[(2-aminophenyl)carbamoyl]phenyl} methyl} carbamate; (Fig 1)) is a synthetic benzamide that acts as a histone deacetylase inhibitor. It has demonstrated potent antiproliferative activity in various *in vitro* human tumor models as well as several positive responses from patients on a phase I trial [1]. In addition to the ongoing single agent trials of MS-275 a combination Phase I clinical trial is currently underway, that includes MS-275 with the DNA methyltransferase inhibitor 5-azacytidine to promote optimal re-expression of cell regulatory genes, which are silenced through methylation [2].

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An analytical method was previously developed and validated in our laboratory for the quantitation of MS-275 in plasma, in support of a single agent dose-finding clinical trial [3]. However, this assay did not utilize an internal standard and was not validated for a combination study. Furthermore, the presence of co-eluting peaks (potentially a result of concomitant medications) required modification of the chromatography and re-validation. Although several potential compounds were tested to find the cause of the co-eluting peak nothing could definitively be determined, thus it is necessary to use our modified method. We report here an improved analytical method for quantitative determination of MS-275 in human plasma, based on liquid chromatography coupled with single-quadrupole mass-spectrometric detection.

2. Experimental

2.1. Chemicals

Schering-Plough Corporation (Kenilworth, NJ) supplied MS-275. The internal standard, benzanilide, was purchased from Sigma-Aldrich, (St. Louis, MO, USA). Methyl tert-butyl ether (MTBE) (Fisher Scientific, Fairlawn, NJ, USA) and methanol (J.T. Baker, Phillipsburg, NJ, USA) were of HPLC grade. De-ionized water was generated with a Hydro-Reverse Osmosis system (Durham, NC, USA) connected to a Milli-Q UV Plus purifying system (Millipore, Billerica, MA, USA). Drug-free heparinized human plasma was obtained from the National Institutes of Health Clinical Center Blood Bank (Bethesda, MD, USA). Prochlorperazine and valacyclovir were purchased from Toronto Research Chemicals Inc (North York, ON Canada).

2.2. Preparation of stock solutions and standards

A master stock solution of MS-275 was prepared by dissolving the drug in methanol at a concentration of 0.5 mg/mL and stored in a glass tube at -20 °C. The solution was made in triplicate to ensure accuracy; each solution was within 10% of the mean, as confirmed by LC-MS. From the master stock solution, a working solution containing 250 ng/mL of drug in methanol was prepared and stored at -20 °C between uses. Serial dilutions were prepared from this working solution for the preparation of calibration and quality control (QC) samples. A master stock of the internal standard, benzanilide, was prepared at a concentration of 1 mg/mL in methanol. From the master stock, a working solution of the internal standard was prepared by dilution to 1,250 ng/mL in methanol. Both the master and working internal standard solutions were stored at -20 °C.

With each analytical run, calibration standards in drug-free human heparinized plasma were freshly prepared in duplicate at MS-275 concentrations of 1, 5, 10, 25, 50, 125, and 250 ng/mL, such that the total amount of methanol added was identical in each sample (5%). QC samples were prepared in batch at concentrations of 3, 100, and 225 ng/mL, by adding plasma to the required amount of working solution in a volumetric flask. After vortexing to ensure complete mixing, these QC samples were subdivided into 0.6 mL aliquots in eppendorf tubes and stored at -20°C until the time of analysis.

2.3. Sample preparation

Calibration samples were prepared by spiking 95 µL of blank human plasma with 5 µL of the appropriate MS-275 working solution in 5 mL disposable glass centrifuge tubes (Kimble, Vineland, NJ, USA) for a total volume of 100 µL. Patient samples were allowed to thaw at room temperature, vortex-mixed for 20s to ensure uniformity, and a volume of 100 µL of each sample was transferred into a glass tube. QC samples were also thawed at room temperature, vortex-mixed and then 100 µL transferred into individual glass tubes. To each tube, 5 µL of working stock of internal standard and 1 mL of methyl tert-butyl ether were added. Tubes were then covered and vortex-mixed for 10 minutes, followed by refrigerated centrifugation for 10

min at $1200 \times g$. The clear supernatant was transferred to a clean glass drying 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 $100\ \mu\text{L}$ of a mixture of methanol/10 mM ammonium formate pH 2.9 (50:50, v:v), and vortex-mixed for 10 minutes. Finally, each sample was transferred to a glass vial and a volume of $20\ \mu\text{L}$ was injected into the system.

2.4 Equipment

Experiments were conducted on an Agilent 1100 system (Agilent Technology Palo Alto, CA, USA) which included a G1312 binary pump, a G1329 refrigerated autosampler, a mobile phase vacuum degassing unit, and a temperature-controlled column compartment, coupled with a single-quadrupole mass spectrometric (MS) detector (Agilent 1100 MSD) equipped with an electrospray ionization source, operating in positive mode. The autosampler was maintained at 4°C and the column was at 40°C . An Agilent ZORBAX SB-Phenyl column ($4.6 \times 75\ \text{mm}$) packed with $3.5\ \mu\text{m}$ packing material and a ZORBAX SB-Phenyl ($4.6 \times 12.5\ \text{mm}$, $5\ \mu\text{m}$) guard column were employed. Samples were eluted using a gradient at a flow rate of $1\ \text{mL/min}$, comprised of a 10 mM ammonium formate pH 2.9 (A) and methanol (B). After 9.5 minutes at 65% A and 35% B, the methanol was increased to 75% over the next 2.5 minutes. From 12 minutes until the end of the 14.5 minute run, a 65:35 A:B mixture was run to achieve re-equilibration. MS conditions were as follows: fragmentor, 85V, 80V; drying gas, $13\ \text{L/min}$; Vcap, 2000 V; Gas Temperature, 350°C ; Nebulizer Pressure, 60 PSI; and a Gain of 1. Selected-ion monitoring was accomplished at $m/z\ 377.1$ for MS-275, and $m/z\ 198.0$ for the internal standard. The chromatographic data were acquired and analyzed using the Chemstation software package (Agilent).

2.5. Validation procedures

Validation of the method with respect to accuracy and precision was carried out according to the FDA Guidance on Bioanalytical Method Validation [4]. Calibration standards and pools of QC samples were prepared as stated previously. QC samples at each concentration were analyzed daily in quintuplicate. Validation runs included blank (zero concentration) and internal standard only samples, along with calibration and QC samples. In addition to this at least 6 different lots of plasma were tested for endogenous interference and 5 different lots were used for validation of the LOQ response.

Calibration curves were calculated by least-squares linear regression analysis of the peak area ratio of MS-275 and the internal standard versus the nominal drug concentration. The regression parameters of slope, intercept and correlation were calculated using a weighting factor of $1/x^2$. The linearity was evaluated by comparing the correlation coefficient (r^2) and deviation between the nominal and back-calculated concentrations of calibration standard samples. The zero concentration (blank), and internal standard only samples were used to visually verify the purity of the reagents and the lack of other potentially interfering substances, but were not considered in the regression analysis. This calibration curve was then used to calculate measured QC concentrations, and that of unknown samples, by interpolation.

The lower limit of quantitation (LLOQ) of the assay was assessed by determining the concentration of MS-275 at which the deviation from nominal concentration was consistently less than 20%. At least five different lots of plasma were used, resulting in accurate and reproducible measurement at the concentration of the LLOQ, based on the deviation from nominal concentration.

The accuracy and precision of the assay were assessed by the mean relative percentage deviation (DEV) from the nominal concentrations and the within-run and between-run precision, calculated using previously published equations [5].

Estimates of the between-run and within-run precision were obtained by one-way analysis of variance (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 Microsoft Excel 2003 (Redmond, WA, USA).

The extraction efficiency of MS-275 and the matrix effect in human plasma, expressed as percentages, were determined at 5 and 225 ng/mL in five replicates, by comparing samples spiked into plasma prior to extraction, supernatant layer following extraction or mobile phase. Different lots of plasma were used to prepare QC samples and calibration curves that were analyzed on the same day, in order to assess relative matrix effect. Ion suppression or crossover of drug and IS was examined by comparing samples spiked with only IS, only MS-275 at the ULOD (250 ng/mL), or both.

Stability of the drug in the reconstitution solution was assessed by reinjection of calibrator and QC samples after remaining in the autosampler for 24 hours following initial injection. The stability of MS-275 in plasma was evaluated following three freeze-thaw cycles, using QC samples at concentrations of 3 and 225 ng/mL, in quadruplicate.

3. Results and Discussion

3.1. Specificity

Due to the previously reported difficulty in finding a suitable internal standard because of variability in extraction from different plasmas [3], a wider variety of compounds were tested, including 4,4'-diaminebenzanilide and N,N'-diphenylbenzidine. Finally, benzanilide (fig 1B) was chosen, using MTBE as the extraction solvent. Figure 2 displays typical 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 ng/mL and IS (B). The selectivity of the analysis is shown by the symmetrical resolution of the peaks, with no interference around the retention time of the analyte in drug-free plasma obtained from 5 different individuals. Overall chromatographic run time was established at 14.5 minutes with MS-275 eluting at t_R =8.567 minutes and the internal standard, benzanilide, at t_R =13.05 minutes.

In addition to the several different internal standards that were tested, several drugs were tested as possible co-eluting contaminants. MS-275 was given in concurrence with 5-azacytidine (MW 244.2). When spiked into mobile phase or blank plasma (with and without MS-275), no effect on MS-275 ionization or elution, nor any crossover was observed.

While processing patient samples using a previously validated assay, multiple patient samples had chromatograms with a peak that overlapped with MS-275 (fig 3). Upon review of patient charts, three concomitant medications were identified as being taken by all individuals whose samples exhibited poor chromatography: Compazine (prochlorperazine) (MW 374), Valtrex (valacyclovir) (MW 324), and Zofran (ondansetron) (MW 325). Prochlorperazine and valacyclovir were tested and shown not to be the co-eluting peak. Ondansetron was unobtainable and thus was not tested. Finally, a patient sample was used (fig 3) to test different gradient conditions, resulting in the method described here, which provides sufficient peak resolution (fig 2C).

3.2. Validation characteristics

The calculated detector response of the MS-275/IS ratio versus the nominal concentration displayed a linear relationship in the tested range of 1-250 ng/mL. However, variance increased proportionally with drug concentration. A weighting factor of $1/x^2$ was applied, x being the nominal MS-275 concentration. Using least-squares linear-regression, a mean ($\pm$ standard

deviation) correlation coefficient of 0.995 ± 0.0013 (range, 0.994-0.997) was obtained over 4 days.

In blank human plasma spiked with MS-275 at a concentration of 1 ng/mL, all of the 8 calibration samples run on four separate days were within $\pm 20\%$ deviation of the nominal value. The mean percentage deviation from nominal value for these 8 samples was -1.29% (Table 1). Based on these results, the lower limit of quantitation for MS-275 was established at 1 ng/mL.

Validation data for the analytical method in terms of accuracy and precision are summarized in Tables 1 and 2. Table 2 displays the data from the QC samples that were run in quintuplicate at each concentration, on each of four days. Values were back-calculated using the calibration curve from the same run. The assay was found to be accurate, within 7.02% at all four concentrations, and precise with within-run and between run precision error of less than 4.29%.

The mean overall extraction efficiency for MS-275, estimated by comparing the MS response of MS-275 spiked into plasma and extracted blank plasma, was approximately 73.44%, independent of the spiked concentration. The mean overall recovery of MS-275 was determined to be 75.86%, also independent of the spiked concentration. The matrix effect was determined by dividing the mean area count of five different lots of extracted blank plasma spiked with drug, by the mean area count of spiked mobile phase. This was estimated to be 3.13%, which was well within the range of experimental error, leading to the conclusion that no significant matrix effect is present. QC samples prepared using different lot of human plasma from the calibration curve did not show additional plasma-to-plasma variation. This result is also consistent with the slopes of calibration curves obtained from different lots of human plasma with a mean ($\pm$ S.D.) of $9.44 \times 10^{-4} \pm 4.17 \times 10^{-5}$, the y-intercept of $1.98 \times 10^{-4} \pm 8.54 \times 10^{-5}$.

Possible crossover interference was also evaluated by comparing the direct instrument responses of the following three sets of experiments in triplicate: samples spiked with IS only, 250 ng/mL MS-275 only, or both. No interference between MS-275 and the IS was found, as the response of each analyte did not vary with the addition of the other.

3.3. Stability

The reinjection measurements were consistent with the initial run, allowing samples to be reanalyzed on the following day when necessary (for example, in the case of machine failure). For the freeze-thaw stability test, back calculated values at both 3 and 225 ng/mL after each freeze-thaw cycle were within 10% of the nominal values, indicating no significant degradation.

4. Application

This validated method has been applied to study the pharmacokinetics of MS-275 in a combination study with the DNA methyltransferase inhibitor 5-azacitidine (5AC) for treatment of patients with acute myeloid leukemia, myelodysplastic syndromes and chronic myelomonocytic leukemia [2]. A typical chromatogram of a patient sample is presented in Fig. 2 (C) and a full PK profile is also provided in Fig. 4.

5. Conclusion

In conclusion, an improved chromatographic method with mass-spectrometric detection has been developed and validated for the quantitative determination of MS-275 in plasma. This method is specific, accurate and precise, and can be easily implemented into routine practice. Compared to our previously published LC/MS method [3], the addition of IS greatly reduced

assay variability, and allowed for the run to be adjusted to separated co-contaminates. Additionally, the range of the assay was increased to 250 ng/mL from a previously reported ULOQ of 100 ng/mL, as necessary for measurement of drug following administration of higher doses. This assay also provides comparable absolute sensitivity to other published methodology [6], without the need for tandem mass-spectrometric detection.

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Fig. 1.
Structure of Ms-275 (A), and internal standard, Benzanilide (B).

Fig. 2.

Chromatograms from reversed-phase HPLC of blank human plasma (A); a human plasma spiked with Benzanilide (IS) and 1 ng/mL (LLOQ) MS-275, (B); and a patient sample $\times$ hours after ingesting a dose of MS-275, spiked with IS (actual calculated concentration = X ng/mL) (C).

Fig. 3. Chromatograms from reversed-phase HPLC of patient sample, spiked with internal standard. Chromatogram A represents the original method that was developed Chromatogram B represents the final method. As is shown the MS-275 peak overlaps with another peak. The arrows denote the location of MS-275 in each chromatogram.

Patient Concentration Time Profile

Fig. 4.

A Concentration time profile for a patient on the Phase I combination study of MS-275 and 5-Azacytidine.

Table 1
Back-calculated concentrations from calibration curves run in duplicate on four occasions in human plasma

Nominal (ng/mL)	Gm (ng/ml)	S.D. (ng/mL)	Dev(%)	R.S.D.,D (%)	N
1	.99	0.10	-1.29	10.1	8
5	5.22	0.20	-4.44	3.83	8
10	10.4	0.50	4.19	4.81	8
25	25.23	0.90	0.9	3.56	8
50	49.59	2.10	-0.81	4.23	8
125	122.5	3.70	-1.97	3.02	8
250	236.36	13.4	-5.46	5.66	8

Abbreviations: GM, grand mean; S.D., standard deviation; DEV, percent deviation from nominal value; R.S.D., relative standard deviation; n, total number of replicate observations within the validation runs.

Table 2
Assessment of accuracy and precision from quality control samples^a in human plasma

Nominal (ng/mL)	GM (ng/mL)	S.D. (ng/mL)	DEV (%)	BRP (%)	WRP (%)	n
3	2.98	0.2	-0.62	3.98	4.27	20
100	96.56	4.8	-3.44	2.90	4.29	20
225	240.81	7.0	7.02	0.97	2.78	20

Abbreviations: GM, grand mean; S.D., standard deviation; DEV, percent deviation from nominal value; WRP, within-run precision; BRP, between-run precision; n, total number of replicate observations within the validation runs.

^aFive samples at each concentration were run on four different occasions.