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Normal-phase and stability-indicating reversed-phase high-performance liquid chromatographic methods for the determination of the novel antitumor agent: 1-methylpropyl-2-imidazolyldisulfide

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Abstract

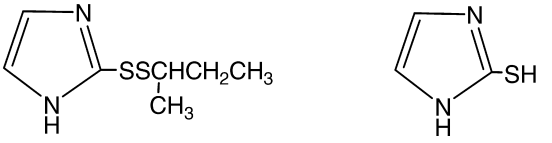
1-Methylpropyl-2-imidazolyl disulfide (MID) is a novel antitumor agent currently in Phase I clinical trials. The chromatographic behavior of MID and its potential impurity, degradation product, and metabolite 2-mercaptoimidazole (2MI) was studied under reversed-phase (RP) and normal-phase (NP) conditions. Both RP- and NP-HPLC separation methods were developed. RP-HPLC was validated as a stability-indicating assay for MID. NP-HPLC retained both MID and 2MI and pending further validation, could prove useful in the study of MID pharmacokinetics.

Keywords: 1-Methylpropyl-2-imidazolyldisulphide

1. Introduction

1-Methylpropyl-2-imidazolyl disulfide (MID) (Fig. 1) is a member of the novel antitumor series comprising unsymmetrical alkyl-2-imidazolyl disulfides [1]. The series was identified from its in vitro inhibitory activity against thioredoxin [2]. MID is cytotoxic to cancer cells and has in vivo antitumor activity in animals [3]. MID is under development as an antineoplastic agent and is currently in Phase I clinical trials.

Sulfur-containing compounds are susceptible to various redox levels (see reference [4] for more



MID 2MI

Fig. 1. Chemical structures of MID and 2MI.

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information). Depending on the severity of the applied conditions, various species of MID could be present as a result of oxidation–reduction reactions. However, a stability-indicating assay should be capable of resolving the analyte of interest from all potential impurities and degradation products. 2-Mercatoimidazole (Fig. 1) is a synthetic precursor for MID [5] and could be present as an impurity or form as a result of a degradation reaction. Furthermore, 2MI is a potential metabolite of MID. Using UV and mass spectral analysis [6], in vitro studies on the interaction between MID and its proposed target, thioredoxin, indicate the occurrence of a thiol–disulfide exchange between MID and thioredoxin with the release of 2MI. Thus, it is of interest to develop quantitative analytical methodologies for both MID and 2MI.

In this report, chromatographic separations of MID and 2MI were studied. RP-HPLC method was developed as a stability-indicating assay for MID. The method was validated for specificity, limit of detection, limit of quantitation, linearity, system precision and assay accuracy and precision. Elucidation of the identity of the degradation product(s) from the solution currently employed in Phase I studies (Phase I solution) was performed. MID and 2MI were retained in a NP-HPLC method. The potential utility of the NP-HPLC method is discussed.

Data presented in this study are useful in guiding the optimization of separation conditions for MID and 2MI to meet other specific chromatographic needs as well as in development of analytical methods for other analogs in the alkyl-2-imidazolyl disulfide series.

2. Experimental

2.1. Reagents and instruments

MID was provided by ProlX Pharmaceuticals (Pittsburgh, PA, USA) both as a reference standard material and as a production batch. MID certified as a reference standard material was characterized by DSC, TGA, IR, NMR, GC and elemental analysis. 2MI was purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile, methanol, isopropanol, hexane, methylene chloride, methyl terbutyl ether and ACS grade phosphoric acid, monobasic and dibasic potassium phosphate, 30% H_2O_2, 1.0 *M* HCl and 1.0 *M* NaOH were all purchased from Fisher (Pittsburgh, PA, USA).

Phosphate buffers were prepared by adding either 50 m*M* H_3PO_4 or 50 m*M* K_2HPO_4 to 50 m*M* KH_2PO_4 until the desired pH was obtained. Mobile phase pH in RP-HPLC corresponds to that of the aqueous buffer before the addition of acetonitrile as an organic modifier.

The HPLC–UV systems consisted of Waters 2690 Alliance® system; Waters 996 photodiode array detector; Waters Symmetry® column; Waters cyano column; YMC-Pack® ODS-AQ column (all from Waters, Milford, MA, USA).

2.2. Procedures

2.2.1. Degradation of MID

2.2.1.1. Forced degradation. Solutions of MID were prepared at a 1.5 m*M* concentration in 1.0 *M* HCl, water, 1.0 *M* NaOH and 3.0% H_2O_2 in 100-ml borosilicate glass bottles. The bottles were incubated in a water bath at 65 °C for 16 h. MID solutions were diluted 5-fold in 0.2 *M* phosphate buffer, pH 2.2, before analysis.

The intravenous solution of MID currently employed in Phase I studies (Phase I solution) was incubated in 4-ml amber borosilicate glass vials in a heating block at 65 °C for 5 days. Phase I solution contained MID at an initial concentration of 13.3 m*M* (2.5 mg per 1.0 ml of solvent, each 100 ml of solvent consisted of 11.7 ml of 0.1 *M* HCl solution, 7.0 ml PEG 400 and 81.3 ml water) and was diluted 10-fold in 50 m*M* phosphate buffer, pH 2.2, before analysis.

2.2.1.2. Degradation of MID in human plasma. From the MID Phase I solution, 52.5 μl were transferred to 947.5 μl of human plasma. These plasma samples spiked with MID, were then shaken in a shaking water bath at room temperature. A 1-ml volume of acetonitrile was then added to each 200 μl

of blank or MID-spiked plasma in 1.8-ml polypropylene (PP) tubes. Samples were vortexed for 3 min, centrifuged for 5 min at 17 500 relative centrifugal force in a Microfuge® followed by the transferral of 1 ml of the supernatant to separate 1.5-ml PP tubes and drying to completeness in a Centrivap®. Dried plasma samples were then reconstituted in 200 μl of methanol–isopropanol–hexane (22:10:68, v/v/v).

2.2.2. RP-HPLC–UV

Unless otherwise indicated, RP-HPLC utilized a C_{18} Symmetry® column (3.9×150 mm, 5 μm particle size) with a Symmetry guard column (3.9×20 mm, 5 μm particle size); column temperature, 30 °C; flow-rate, 1 ml/min; injection volume, 10 μl; samples were dissolved or diluted in aqueous phosphate buffer at pH 2.2; λ of 254 nm for quantitative analysis and a λ range of 200–400 nm for qualitative analysis. The column dead time (t_0) was determined from the time of negative peak from water injection.

2.2.3. NP-HPLC

Unless otherwise indicated, NP-HPLC utilized a cyano column (Nova-Pack® CN HP, 3.9×150 mm, 5 μm particle size) with a cyano guard column (3.9×20 mm, 5 μm particle size); column temperature, 30 °C; flow-rate, 1 ml/min; injection volume, 10 μl; samples dissolved in methanol–isopropanol–hexane (22:10:68, v/v/v); λ of 254 nm for quantitative analysis and a λ range of 200–400 nm for qualitative analysis. The column dead time (t_0) was determined from the time of the negative peak from hexane injection.

3. Results and discussion

3.1. Effect of mobile phase pH in RP-HPLC

The relationship between the mobile phase pH and analyte retention (k) was studied and the results are shown in Fig. 2. When the mobile phase pH was raised, the retention of MID on the ODS column increased, a typical chromagraphic behavior of a weak base. The pK_a of MID was 5.06 as determined by potentiometric titration. However, due to the presence of organic modifier (15% acetonitrile) in the mobile phase, MID acted as a weaker base with an apparent pK_a of about 4.5. On the other hand, 2MI retention showed no dependence on the mobile phase pH within the range tested. When 2MI was acid titrated, the titration curve was identical to that of acid titration of pure water, explaining the lack of dependence of 2MI on pH in the range tested.

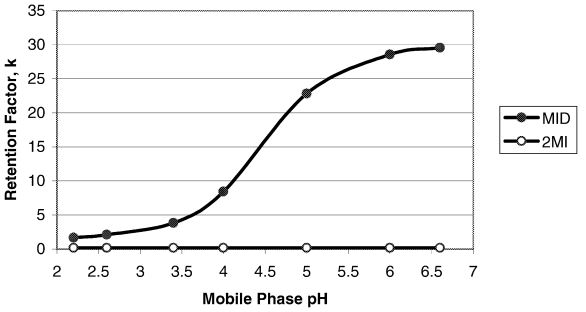

Fig. 2. Analyte retention as a function of pH of the mobile phase. The mobile phase consisted of 15% acetonitrile in 50 m*M* aqueous phosphate buffers. Analyte samples were dissolved in 15% acetonitrile in unbuffered water.

3.2. Effect of organic modifier in RP-HPLC

Analyte retention as a function of percentage organic modifier was studied in the range of 7–22% acetonitrile in aqueous phosphate buffer, pH 2.2. Fig. 3 shows the retention as a function of the percentage of acetonitrile. Relationships between retention and percentage organic modifier were well described by

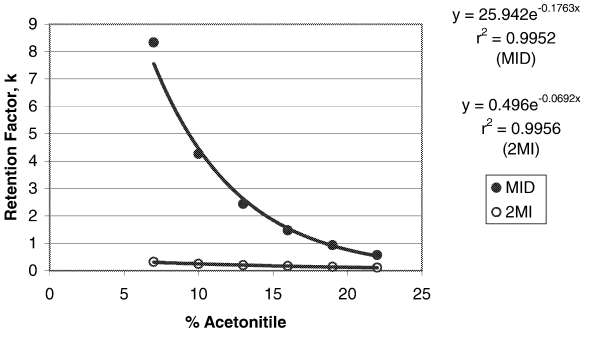

Fig. 3. Analyte retention as a function of percentage acetonitrile in the mobile phase. Mobile phase consisted of aqueous phosphate buffer, pH 2.2, and acetonitrile. Analyte samples were dissolved in 50 m*M* aqueous phosphate buffer, pH 2.2.

exponential functions. Within the range tested, MID retention varied by an order of magnitude, while that of 2MI, did not vary to any appreciable degree.

3.3. Separation and validation in RP-HPLC

Since 2MI was not retained in RP-HPLC to any practical degree, choice of separation conditions focused on yielding a rugged and rapid method for the quantitative analysis of MID. The pH value of 2.2 was chosen for the mobile phase to allow for retention that is minimally dependent on pH. Solutions of MID in phosphate buffer at pH 2.2 are stable for at least 72 h at room temperature. However, stability decreases as the pH of the phosphate buffer is increased. From Fig. 3, using 18% acetonitrile as the organic modifier, a retention factor of about 1 is obtained. Typical chromatograms for MID under these conditions are shown in Fig. 4.

For this separation procedure to be considered stability indicating, it should be capable of resolving MID from any potential impurities or degradation products. To test for this, MID in 1.0 *M* HCl, water, 1.0 *M* NaOH and 3% H_2O_2, respectively, was stressed at 65 °C for 16 h. Also, Phase I solution of MID was stressed for 5 days at 65 °C.

In all cases of forced-degradation, the degradation products eluted before MID and near the dead volume of the column. An example of that is shown in Fig. 4A for the degradation of the Phase I solution. Incubation of the Phase I solution for 5 days at 65 °C resulted in 16.7 % degradation of MID. Assuming that the degradation product was 2MI, 100±3% of the initial concentration of MID in the Phase I solution, could be accounted for by the sum of the MID and 2MI concentrations in the degraded solution. Fig. 4B is a chromatogram of a solution containing standard materials of MID and 2MI injected in 50 m*M* phosphate buffer, pH 2.2. UV spectra of the main peaks in Fig. 4A and B are shown in Fig. 5A and B, respectively. The UV spectrum of the degradation product is identical to that of the 2MI standard material, suggesting that the degradation product was 2MI. The identity of 2MI as a degradation product was confirmed by the use of LC–MS analysis where conditions were modified to suit LC–MS method of detection, i.e. by changing the column to a narrow-bore column (2×150 mm,

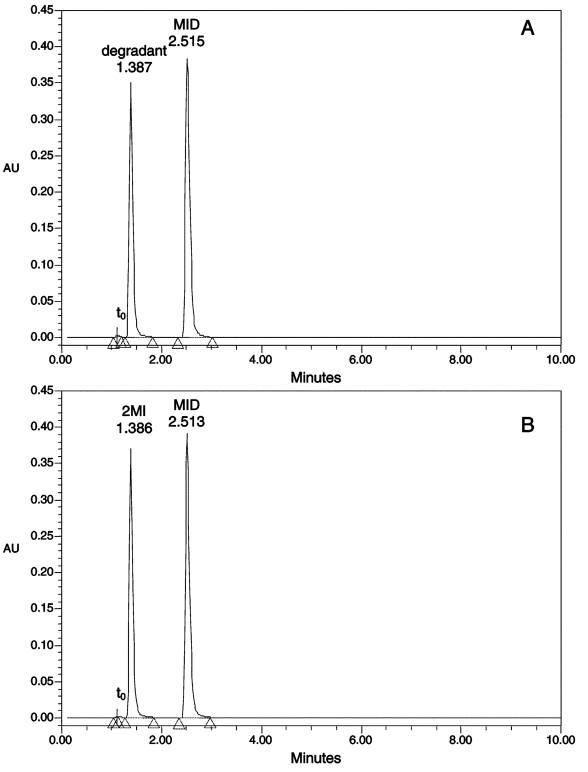

Fig. 4. Chromatograms under RP-HPLC conditions. (A) Chromatogram of Phase I solution of MID maintained at 65 °C for 5 days (the solution contained MID at a theoretical initial concentration of 13.300 m*M* and was diluted by a factor of 10 for analysis). (B) Chromatogram of MID (1.107 m*M*) and 2MI (0.223 m*M*) injected in 50 m*M* phosphate buffer solution, pH 2.2.

YMC-Pack ODS-AQ) and the phosphate buffer to 1% formic acid (data not shown). Unretained moieties in the cases of acid, water and base also yielded UV spectra identical to that of standard 2MI. From these results, it is concluded that the appearance of a new peak near the dead volume of the column would be indicative of MID degradation.

RP-HPLC for the quantitative analysis of MID was validated for the limit of detection (LOD), limit of quantitation (LOQ), linearity, system precision, assay precision and accuracy. Based on a signal-to-noise ratio of at least 3:1, the LOD was found to be 20 ng/ml. Based on a relative standard deviation of <20% for the response of three different solutions at the same concentration, the LOQ was found to be 170 ng/ml (6.1% RSD). The linearity was tested using ten different concentration levels equally

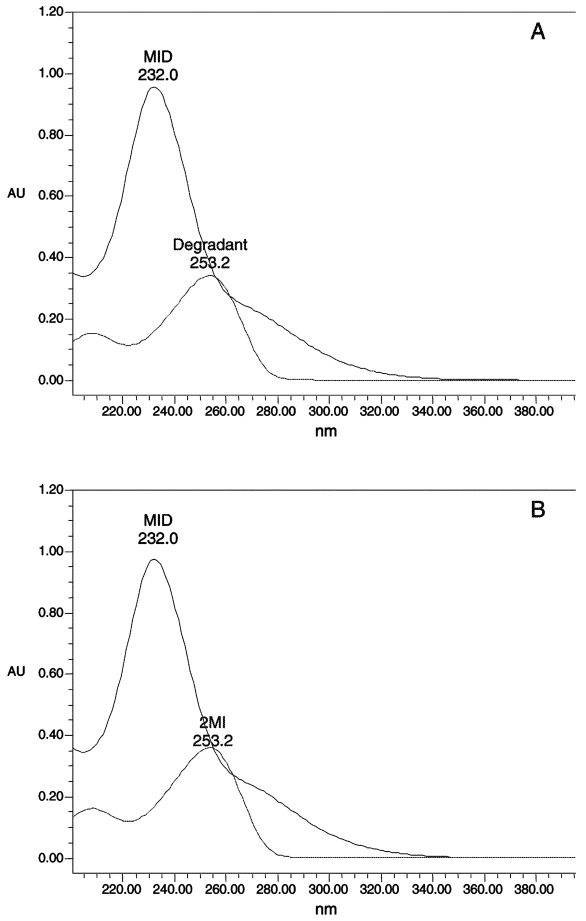

Fig. 5. UV spectra under RP-HPLC conditions. (A) UV spectra of the main analyte peaks shown in Fig. 4A. (B) UV spectra of the main analyte peaks shown in Fig. 4B.

spaced between the LOQ and 150% of the target concentration of the method, which is 0.25 mg/ml. Three different solutions were tested at each concentration level. The method was found linear ($r^2 >$ 0.9999). The slope was 11 200 000 with standard deviation of 13 530. The intercept was 6257 with a standard deviation of 2174. The coefficient of variation of the slopes from three different calibration plots was 0.34%. The system precision was 0.04% for retention time and 0.15% for response as determined from six injections from the same MID solution at 0.25 mg/ml. The intra-assay precision as assessed from analyzing six different solutions of similar concentration (0.25 mg/ml) was 0.9%. The assay intermediate precision from analyzing six different solutions at the target concentration level on 3 different days was 0.2%. Table 1 shows the accuracy of the assay based on the analysis of five different solutions at 50, 75, 100, 125 and 150% of the target level. The assay accuracy was well within 1%.

Table 1
Accuracy data for the validation of RP-HPLC assay for MID[a]

Solution	% of Target level	Concentration (mg/ml)		Error (%)
		Actual	Found	
1	50	0.1250	0.1242	−0.6335
2	75	0.1875	0.1871	−0.2063
3	100	0.2500	0.2499	−0.0319
4	125	0.3125	0.3120	−0.1732
5	150	0.3750	0.3748	−0.0474
		Average error	absolute	0.2185

[a] See text for HPLC conditions and the calibration plot data for MID.

3.4. Separation in NP-HPLC

From the preceding sections, it was seen that RP-HPLC conditions did not afford adequate control over 2MI retention. C_{18} columns other than Symmetry, such as YMC-Pack ODS-AQ and highly aqueous mobile phases were tried, and in all cases 2MI eluted near the dead volume of the column. Thus, NP-HPLC was attempted for the simultaneous retention of MID and 2MI.

In the development of an NP-HPLC method, a cyano column and mobile phases consisting of mixtures of hexane as solvent A and isopropanol (IP) as solvent B, were used. As shown in Table 2, 14% IP in hexane yielded reasonable retention factors for both MID and 2MI. IP in the mobile phase was then replaced in part or in full—depending on solvent miscibility—with solvents B of different selectivities, keeping the overall eluting strength, however, constant among the different mobile phases [7]. Examples of solvents B used were; non-localizing solvents such methylene chloride (MC); basic localizing solvents such as MTBE; nonbasic localizing solvents such as acetonitrile (ACN); and methanol (MeOH) that has similar selectivity to that of IP. Both IP and MeOH are

Table 2
Analyte retention as a function of solvent B selectivity in NP-HPLC

Mobile phase	Solvent B	*k* (MID)	*k* (2MI)
1	14% IP	1.00	1.64
2	32% MC	>40.00[a]	>40.00[a]
3	17% MTBE	>40.00[a]	>40.00[a]
4	12% ACN+13% MC	1.26	>40.00[a]
5	4% IP+8.5% MeOH	0.82	1.79

Mobile phases consisted of the indicated proportions of Solvent B in hexane (solvent A). Solvents B were isopropanol (IP), methylene chloride (MC), methyl ter-butyl ether (MTBE), acetonitrile (ACN), and methanol (MeOH).

[a] Analyte did not elute from the column in a 1-h run time at 1 ml/min.

known to have variable selectivity that is dependent on the analyte [7]. Table 2 shows the analyte retention factor as a function of solvent B selectivity.

For the most part, retention of both MID and 2MI in NP-HPLC was highly similar. Both analytes were eluted by IP. Neither analyte was eluted in the course of a 1-h run, at a flow-rate of 1 ml/min, irrespective of whether a non-localizing or basic localizing solvent, was used. Differences between MID and 2MI retention were evident only when the mobile phase was modified by the non-basic localizing solvent, ACN, as it selectively eluted MID. Partial replacement of IP with MeOH while keeping the mobile phase at the same strength, did not affect analyte retention. However, the resolution (R_s) between the two analytes increased by a factor of 5 from mobile phase 1 to mobile phase 5. This improvement in the R_s could be ascribed to the slightly more acidic behavior of MeOH when contrasted with IP, as the analytes seem to have interacted with the cyano column with acidic functionalities. Further gains in R_s by replacing IP with MeOH were not achieved as a result of the limited solubility of MeOH in the mobile phase. The use of MC as a cosolvent to solubilize more MeOH was undesirable due to its relatively high UV cut-off and low boiling point [8].

Keeping the ratio of MeOH to IP at 2.125, as in mobile phase 5, but varying the overall eluting strength of the mobile phase, did not result in any change in the selectivity (α) between the two analyte peaks. The selectivity between MID and 2MI remained at 2 whether 7.5, 10, 12.5, 15 or 17.5% B was used in the mobile phase, confirming the highly similar nature of interaction of the two analytes with the cyano column. Gains in α were achieved when ACN was added to the mobile phase as ACN selectively elutes MID rather than 2MI (Table 2). However, this approach resulted in a multicomponent solvent B containing ACN, MC, MeOH and IP. This approach could be reserved for situations were higher selectivity is needed than that afforded by MeOH and IP alone. Controlling α by mixtures of ACN and IP without MeOH resulted in broad and tailing peaks of 2MI.

Based on the retention factors for the analytes and the resolution between them, 12.5% solvent B (32% IP and 68% MeOH) in hexane was chosen as the mobile phase components. NP-HPLC when compared to RP-HPLC is known for slow column equilibration time that could result in variable retention [9]. This variability in retention time has been ascribed to absorption of atmospheric moisture by the mobile phase and subsequent adsorption by the column. Conditions employed here were meant to minimize the problem of variable retention time, as this problem is less pronounced when a bonded polar phase (like the cyano column) is used instead of a bare silica column. Also the presence of MeOH and IP in the mobile phase is supposed to make the presence of water molecules inconsequential [10]. For the method described here, the relative retention time for any of the analytes did not change by more than 2% RSD for any six injections chosen randomly from any given day of analysis. Examples of chromatograms obtained under these conditions are shown in Figs. 6 and 7.

The developed NP-HPLC was equivalent to the

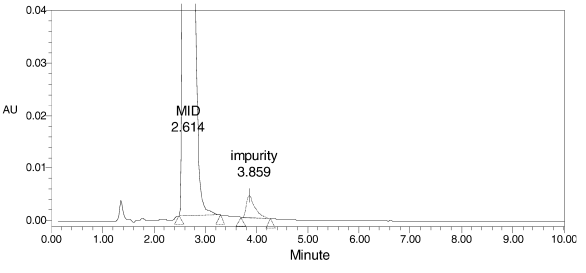

Fig. 6. NP-HPLC chromatogram of MID from lot no. CSS387 showing an impurity at retention time 3.8 min (see text for more information). MID solution was at 1330 μM (0.25 mg/ml).

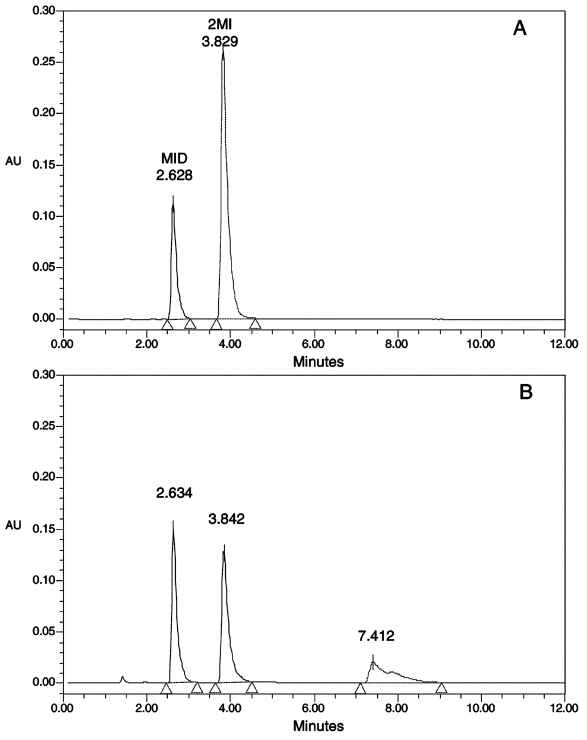

Fig. 7. Chromatograms under NP-HPLC conditions. (A) Chromatogram of a solution of standard materials of MID and 2MI, each present at 350 μ*M* concentration. (B) Chromatogram of MID incubated in a human plasma at a theoretical initial concentration of 700 μ*M*. The peak at 7.41 min was also present in blank plasma samples.

RP-HPLC method in assaying percentage purity of the drug substance. When MID from a production batch (lot no. CSS837) was analyzed against the reference standard by RP-HPLC and NP-HPLC, both methods assigned it a value of 95% purity. Unlike RP-HPLC however, NP-HPLC was capable of retaining an impurity in MID from lot no. CSS837. From relative retention times and UV spectra, the impurity was identified as 2MI (see Fig. 7A for relative retention time of 2MI). However, the detected levels of 2MI only amounted to 0.25% (w/w) of MID. Thus, other impurities besides 2MI must be present in lot no. CSS837. Symmetrical aliphatic disulfides could be present as synthetic byproducts. These materials are expected to have low UV molar absorptivity, and thus may not be apparent in an LC–UV chromatogram. This latter possibility highlights the need that new batches of the drug substance should always be analyzed against a well-characterized reference standard material if LC–UV is to be used to assess the purity of new batches.

The proposed NP-HPLC method may prove useful in the study of the pharmacokinetics of MID. Fig. 7B shows a chromatogram of a human plasma sample spiked with MID at a concentration of 700 μ*M* and incubated at room temperature for 3 h. The plasma sample was pretreated with acetonitrile for protein precipitation, dried, and reconstituted with the solvent for injection (see Procedures Section). Fig. 8A is the UV spectra of the standard materials of MID

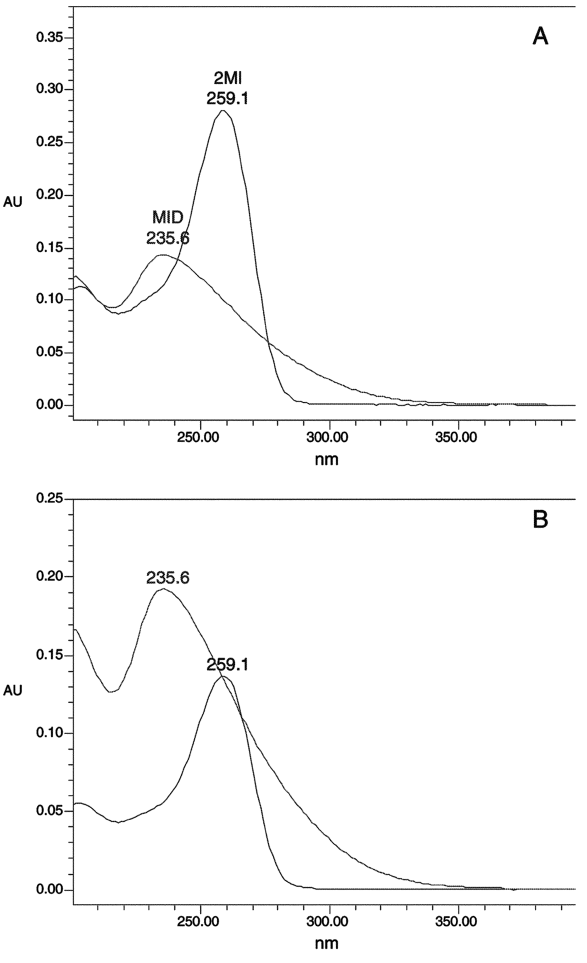

Fig. 8. UV spectra under NP-HPLC conditions. (A) UV spectra of standard MID and 2MI shown in Fig. 7A. (B) UV spectra of analytes shown in Fig. 7B; UV spectrum with λ_{max} 259.1 is that of the analyte with $t_R = 2.63$ min; UV spectrum with λ_{max} 235.6 is that of the analyte with $t_R = 3.84$ min.

and 2MI shown in chromatogram 7A. Fig. 8B is the UV spectra of the main peaks in the chromatogram in Fig. 7B. These figures show that MID has degraded or reacted with thiol-containing moieties in the plasma and released 2MI. The molar sum of the recovered quantities of MID and 2MI in Fig. 7B accounted for 100% of the theoretical initial concentration of MID in the plasma sample. Degradation of MID in the plasma to give 2MI is thought to have resulted from a disulfide exchange reaction of MID with plasma thiols. Boiling plasma for 5 min and allowing cooling to room temperature prior to incubation with MID yielded results similar to the ones reported here (data not shown), indicating that a chemical rather than enzymatic pathway was responsible for MID breakdown in the isolated plasma. 2MI was also found to be the main breakdown product/metabolite of MID from cell culture and animal studies [11]. Validation of this method for pharmacokinetic studies and pharmacokinetic data on MID will be published in a separate report.

In summary, a rapid and simple RP-HPLC assay has been developed for the determination of the novel antitumor agent: 1-methylpropyl 2-imidazolyl disulfide (MID). The assay is stability indicating; it resolves MID from its degradation products. The method is linear ($r^2 > 0.9999$), sensitive (LOD = 20 ng/ml), reproducible (RSD = 0.2%) and accurate (error $< 1\%$). MID and its potential metabolite: 2-mercaptoimidazole, were both retained in NP-HPLC that upon passing validation criteria could prove useful in the study of the pharmacokinetics of MID.

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