Analysis of Monoethanolamine by Derivatization with Marfey's Reagent and HPLC

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Abstract

A sensitive and selective method for determining the residual monoethanolamine in a developmental drug substance is developed and validated. Marfey's reagent, which is commonly used for the chiral analysis of amino acids, is reacted with the primary amine group of monoethanolamine and then analyzed by high-performance liquid chromatography–UV at 340 nm. Quantitation is performed by a standard addition method by preparing drug substance samples with added monoethanolamine ranging from 0.25–1.0 µg/mL (equivalent to 12.5–50 ppm with respect to the drug substance). The method performance is evaluated for linearity, specificity, detection and quantitation limits, accuracy, precision, and sample stability. The method is linear from 0.25–1.0 µg/mL with a coefficient of determination (r^2) > 0.95. The accuracy and precision obtained is 105.5 ± 4.8% (n = 3). The limits of detection and quantitation are 0.03 and 0.10 µg/mL, respectively. Instrument precision (% relative standard deviation of six injections of a derivatized 0.5 µg/mL monoethanolamine solution on two separate days) is ≥ 2.0%. This method is suitable for the determination of monoethanolamine at the 25 ppm level in drug substance.

Introduction

The development and validation of a quantitative method for the residual solvent monoethanolamine (MEA) in a developmental drug substance was completed. The drug substance has a molecular weight of less than 400 amu, and the structure contains both secondary and tertiary amine moieties. Although MEA is generally regarded to pose no significant toxicological hazard and is unspecified in International Conference on Harmonization guidelines for residual solvent limits, hypersensitivity reactions have been reported (1), and this work was driven by a European Union request to determine its levels in the drug substance. However, the analysis of MEA presents several unique challenges due to its low molecular weight (MW 61.0833), high polarity, relatively low volatility (0.404 mm Hg, 25°C), and lack of a sufficient UV absorbing chromophore. Although amino alcohols were previously determined by gas chromatography (GC) with flame ionization detection (FID) both directly (2) and as acyl derivatives (3,4), this technique was not feasible because of insufficient sensitivity at the 25 ppm level required, limited solubility of MEA in volatile solvents, and to numerous chromatographic interferences attributed to the thermal instability of the drug substance. Similarly, the high boiling point of MEA (171°C) precluded its analysis by headspace GC.

Therefore, derivatization of MEA with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide or FDAA) followed by analysis by high-performance liquid chromatography (HPLC) with variable wavelength UV detection (HPLC–UV) was pursued. The HPLC–UV analysis of Marfey's derivatives is typically employed for resolving amino acid enantiomers (5–7), and the advantages of applying this technique to MEA determination include improved UV sensitivity and selectivity over traditional GC techniques used for amino alcohols. Derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) dramatically improves the UV response of MEA and adds a lipophilic constituent to the molecule, which results in greater reversed-phase HPLC retention. The reaction between MEA and Marfey's reagent is shown in Figure 1. Perhaps the most important consideration in using Marfey's reagent in this study is its preferential derivatization with primary amines (8), which is a major consideration with respect to the amine structures present in the drug substance and the 7000-fold molar excess of drug substance com-

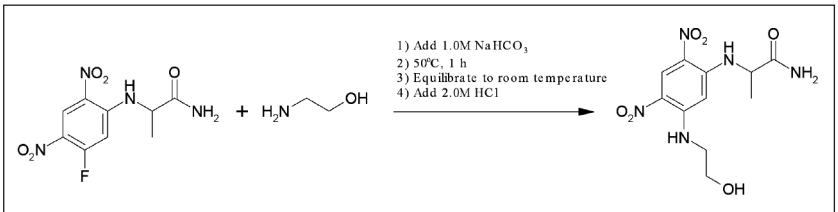

Figure 1. Reaction of monoethanolamine (MEA) with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent).

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pared with MEA (determined at 25 ppm).

The method was based on generic procedures obtained from Pierce Biotechnology (8) for the derivatization of primary amines and subsequent analysis by HPLC. The conditions were modified to optimize the HPLC separation and the method sensitivity. The final method was validated for accuracy and precision, sample stability, linearity, specificity, and detection and quantitation limits.

Experimental

Chemicals

Marfey's reagent was purchased from Pierce Biotechnology (Rockford, IL). Standard MEA (100.0%), sodium bicarbonate, and triethylamine were purchased from J.T. Baker (Phillipsburg, NJ). Acetonitrile, acetone, and methanol solvents (spectrophotometric grade) were purchased from Burdick & Jackson (Muskegon, MI). HPLC-grade water was produced with a Barnstead NANOpure II system (Dubuque, IA). Hydrochloric acid (conc.) and phosphoric acid (85%) were purchased from Mallinckrodt (St. Louis, MO).

Instrumentation

Sample injections (10 µL) were chromatographed on an Agilent 1100 Series HPLC–UV–vis system (Wilmington, DE) fitted with an Agilent Zorbax ODS 5 µm, 4.6 × 250 mm column. Sample absorbance was determined at 340 nm with a variable wavelength detector. Separation was achieved by gradient elution with a 1.5 mL/min flow of triethylamine–phosphate buffer (0.05M, pH 3.0) and acetonitrile (Table I).

Derivatization

The MEA derivative was prepared by combining 400 µL of 1% Marfey's reagent in acetone, 500 µL of the drug substance sample prepared in methanol, and 80 µL of 1.0M sodium bicarbonate. The mixture was placed in tightly capped 2-mL sample vials and was heated in a water bath at 50°C for 1 h. The vials were then equilibrated to room temperature, and 50 µL of HCl (2.0M) was added to each. Each preparation was then diluted by combining a 500-µL aliquot with 1000 µL of the triethylamine–phosphate (0.05M) mobile phase component.

System suitability solution

A solution of 0.5 µg/mL MEA in methanol was prepared, derivatized, and analyzed by HPLC–UV–vis. For six injections of the system suitability sample (0.5 µg/mL), the percent relative standard deviation (%RSD) of the MEA peak area should be less than or equal to 20% to verify adequate system performance.

Solutions for accuracy and precision

The accuracy and precision components of validation testing consisted of preparing two sets of standard addition samples in methanol. The preparation of each involved transferring 2.0-mL aliquots of MEA spiking solutions (0, 1.25, 2.5, 5.0 µg/mL) into 10-mL volumetric flasks. In order to determine the endogenous level of MEA in drug substance, one set was diluted to volume with a 25 mg/mL drug substance solution to produce 0, 0.25, 0.5, and 1.0 µg/mL MEA solutions in the presence of 20 mg/mL of drug substance. Similarly, a second set (n = 3 replicates) was diluted to volume instead with a mixture of 0.625 µg/mL of MEA in addition to 25 mg/mL of drug substance to produce the same standard addition levels in the presence of 20 mg/mL of drug substance as well as 0.5 mg/mL of MEA (equivalent to 25 ppm). This second set was prepared to represent drug substance containing 25 ppm of MEA.

Each standard addition sample was derivatized and analyzed by HPLC–UV. The added MEA concentration (µg/mL) was plotted against the observed peak area abundance, a linear regression was performed, and the concentration of MEA in the drug substance (ppm) was determined for each sample set as follows:

$$\text{MEA (ppm)} = (10 \times B / M) / (W / 25) \qquad \text{Eq. 1}$$

Where: 10 is volume of each standard addition sample preparation (mL); B is the y-intercept of the linear regression equation; M is the slope of the linear regression equation; W is the quantity of drug substance weighed for the 25 mg/mL drug substance stock solution [g]; and 25 is the dilution factor

The accuracy and precision of this method at the 25 ppm MEA level was determined upon subtracting the concentration of endogenous MEA.

Solutions for sample stability

Aliquots of one replicate of the 25 ppm fortified MEA set (0, 0.25, 0.5, and 1.0 µg/mL MEA with 0.5 µg/mL of MEA and 20 mg/mL of drug substance) were maintained at ambient room temperature (25°C) for approximately 36 h, followed by analysis by HPLC–UV. The concentration of MEA (ppm) was determined by the standard addition method and compared with the level determined in the originating 25 ppm fortified MEA set replicate.

Solutions for linearity

Linearity samples were prepared in duplicate at 0.25, 0.5, 0.75, 1.0, and 1.25 µg/mL of MEA in the presence of 20 mg/mL of drug substance (equivalent to 12.5, 25, 37.5, 50, and 62.5 ppm MEA in drug substance, respectively). The preparations were then derivatized and analyzed by HPLC–UV. The MEA concentration (µg/mL) was plotted against the observed peak area abundance, and linear regression was performed.

Solutions for specificity

The 0 and 0.25 µg/mL standard addition samples (each in the

Table I. Gradient Program for the HPLC Analysis of the Marfey's Derivative of MEA

Time (min)	Mobile Phase A Triethylamine-Phosphate (0.05M, pH 3.0)	Mobile Phase B Acetonitrile
0.00	85	15
5.00	85	15
45.00	60	40
55.00	10	90
65.00	10	90
68.00	85	15
75.00	85	15

presence of 20 mg/mL of drug substance), a 1.25 µg/mL MEA spiking solution, and a blank methanol sample were derivatized and analyzed by HPLC–UV. All samples were examined for the presence of the derivatized MEA peak.

Solutions for quantitation and detection limits determination

A 0.25 µg/mL MEA solution was prepared in methanol (equivalent to 12.5 ppm MEA with respect to the drug substance), followed by derivatization and analysis by HPLC–UV. Ten injections were performed on the system and the average signal-to-noise ratio (S/N) and precision (% relative standard deviation) were determined.

Results and Discussion

Method development

Because of the generic nature of the Pierce Biotechnology procedure (8), method development efforts were required and intended to tailor the analysis specifically to MEA.

Because the extent to which the drug substance is solubilized dictates the quantity of MEA available for analysis, maximizing the drug substance concentration was pursued as a means of lowering the MEA quantitation limit. Though approximately 200 mg/mL of drug substance could be dissolved in methanol with modest heating (~100°C), chromatographic interferences to the MEA derivative peak were severe and likely attributed to thermal degradants. Consequently, the maximum feasible drug substance concentration was established as 25 mg/mL, as this could be achieved with only brief sonication. Note that although the Pierce procedure specifies derivatization of a solid sample (8), the preliminary dissolution of the drug substance was necessary to ensure its solubility during the derivatization procedure and, thereby, eliminate any occlusion of MEA in the drug substance.

Reaction parameters (i.e., time, temperature, and reagent concentration) were never fully optimized to maximize the derivatization efficiency, but the relative proportions of reagents were altered to obtain adequate sensitivity. First, the 1% Marfey's reagent aliquot was increased from 200 µL to 400 µL, followed by a corresponding two-fold increase in the 1.0M sodium bicarbonate aliquot volume to 80 µL. These increases were meant to compensate for the apparent depletion of Marfey's reagent by the drug substance itself (i.e., its secondary amine group), which was proposed on the basis of diminished abundance of presumed Marfey's reagent peaks (as observed in derivatized blank methanol) upon inclusion of drug substance. Similarly, the aliquot volume of 2M HCl was increased from 25 to 50 µL to better solubilize the drug substance.

Chromatographic improvements were also pursued in this study. The HPLC gradient reflects modifications providing increased separation of the MEA derivative peak from other sample matrix components. However, baseline resolution of the MEA derivative peak was not obtained, requiring the use of the standard addition technique for quantitation. Furthermore, a three-fold dilution of the derivatized samples with triethylamine–phosphate (0.05M) was adopted to decrease the organic content of the sample to a level more consistent with the initial mobile phase composition (15% acetonitrile) and, thereby, minimize the asymmetry and broadening of the MEA derivative peak.

System suitability

The precision for replicate injections (n = 6) of the system suitability solution (derivatized 0.5 µg/mL MEA in methanol) was excellent. The precision was 2.0% and 1.5%, respectively, for two determinations.

Table II. Accuracy and Precision Summary for 25 ppm MEA Spiked Standard Addition Sets

Set	Linear regression		Conc. (ppm)	Conc. (less endogenous) (ppm)	Mean Conc. (ppm)	RSD (%)	Recovery (%)
Unspiked	y-Intercept	3.35740	6.4	N/A*	N/A	N/A	N/A
	Slope	26.1844					
	r^2	1.00					
Spike 1 (25 ppm)	y-Intercept	16.7576	31.7	25.3			
	Slope	26.2755					
	r^2	1.00					
Spike 2 (25 ppm)	y-Intercept	17.0576	32.8	26.4	26.5	4.8	105.7
	Slope	25.8260					
	r^2	1.00					
Spike 3 (25 ppm)	y-Intercept	17.3680	34.2	27.8			
	Slope	25.2178					
	r^2	1.00					

* N/A = not applicable.

Accuracy and precision

The accuracy and precision of quantitating MEA by standard addition was established in triplicate standard addition sample sets spiked with 25 ppm MEA, as compared with a single unspiked standard addition set used to determine the endogenous MEA in the drug substance. Table II shows a complete summary of these results. The results for each set were highly linear, as shown by a coefficient of determination (r^2) of 1.00 obtained for each set. The mean MEA content for the drug substance samples sets spiked with 25 ppm MEA was 26.5 ppm after normalizing the results for the endogenous MEA contained in the drug substance. The accuracy and precision of the method were excellent, as evidenced by an average recovery (n = 3) of 105.7% with a precision of 4.8% RSD.

Sample stability

Derivatized standard addition samples were stable for 35 h at ambient room temperature

(25°C) storage. The linearity of the stored standard addition sample set (see Accuracy and precision section) exhibited an r^2 of 0.99. The MEA content of the sample was 32.9 ppm initially, and decreased to 32.0 ppm after 35 h at room temperature or 97.4% of the initial concentration.

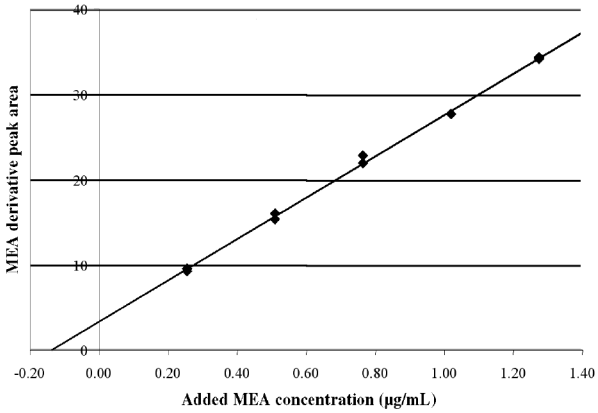

Figure 2. Linearity plot for 0.25–1.25 µg/mL of added MEA in the presence of 25 mg/mL of drug substance (equivalent to 12.5–62.5 ppm in the drug substance). MEA derivative peak area = 24.213 (added MEA conc.) + 3.3607 r^2 = 0.9981.

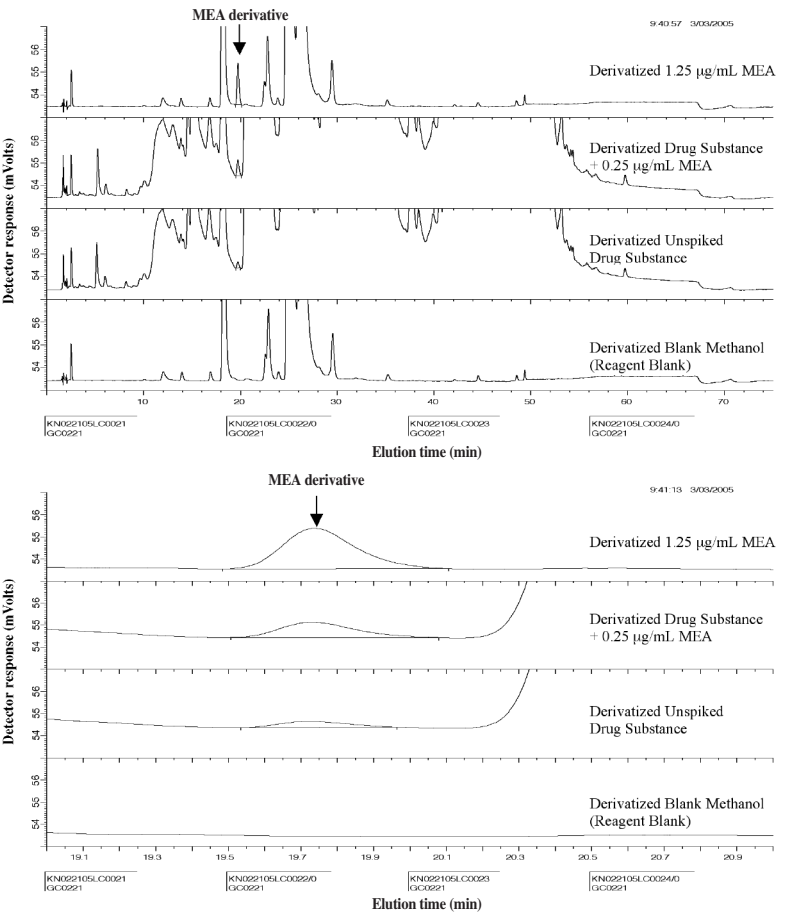

Figure 3. Stacked specificity chromatograms, including derivatized solution of 1.25 µg/mL MEA in methanol (no drug substance) (A), derivatized sample of drug substance containing 0.25 µg/mL of added MEA (B), derivatized solution of drug substance (C), and derivatized blank methanol (D).

Linearity

The linearity of 0.25–1.25 µg/mL of added MEA in the presence of drug substance (20 mg/mL) was determined for two derivatized standard sets. The linearity of response was excellent, and the linearity plot and regression equation are shown in Figure 2. The coefficient of determination for the combined sets was 1.00, verifying that this method is appropriate for determining MEA from 12.5–62.5 ppm in the drug substance.

Specificity

Stacked chromatograms of specificity injections reveal that the MEA derivative peak elutes at a retention time of approximately 19.7 min. Figure 3 shows chromatograms pertaining to the following: (*i*) derivatized solution of 1.25 µg/mL MEA in methanol; (*ii*) solution of derivatized drug substance containing an added 0.25 ug/mL of MEA; (*iii*) solution of derivatized drug substance containing endogenous MEA; and (*iv*) derivatized methanol (reagent blank). The reagent blank exhibited no potential interferences to the accurate quantitation of MEA in drug substance samples. In addition, the abundance of the MEA derivative peak increased between the unspiked and 0.25 µg/mL spiked MEA preparations in approximate proportion to the quantities of MEA added. Figure 3 shows that the most likely source of chromatographic interference is not due to reagent blanks, but from the drug substance itself or from degradation products of the drug substance presumably formed during the derivatization procedure. The development of robust chromatographic conditions that were able to sufficiently separate the derivatized MEA peak from any other interfering chromatographic peaks was instrumental to the success of this method.

Quantitation and detection limits

The limit of quantitation was determined by calculating the concentration of MEA that would result in a mean S/N ≥ 10 and a peak area RSD ≤ 35.0% for 10 replicate sample injections. A derivatized solution of 0.25 µg/mL MEA resulted in a mean S/N = 25.6 and a peak area RSD = 4.1%. Therefore, the LOD and LOQ for MEA were calculated to be 0.03 µg/mL (1.5 ppm in drug substance) and 0.10 µg/mL (5 ppm), respectively.

Conclusion

A sensitive, selective procedure for quantitatively determining MEA down to 25 ppm in a developmental drug substance has been developed and validated. Quantitation of MEA was accomplished by derivatization with Marfey's reagent, followed by chromatographic separation and quantitation by a standard addition method. The method was sufficiently robust for the accurate quantitation of MEA in a thermally labile, reactive drug substance.

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References

1. *Handbook of Pharmaceutical Excipients.* A. Wade and P.J. Weller, Eds. American Pharmaceutical Association, Washington, D.C., 1994. pp. 319–20.
2. W. Xu. Determination of industrial triethanolamine by gas chromatography. *Guangxi Huagong* **29:** 39–41,54 (2000).
3. I.N. Stan'kov, A.A. Sergeeva, and S.N. Tarasov. Gas-chromatographic determination of trace amino alcohols in water, air, and bitumen-salt masses forming in the detoxification of chemical warfare agents. *J. Anal. Chem.* **55:** 150–54 (2000).
4. I.N. Stan'kov, V.A. Yarova, A.A. Sergeeva, I.V. Potashova, S.N. Tarasov, and N.N. Samofalova. Gas-chromatographic determination of monoethanolamine and its salts of inorganic and organophosphorus acids present in combination. *J. Anal. Chem.* **55:** 155–59 (2000).
5. P. Marfey and M. Ottesen. Determination of D-amino acids. I. Hydrolysis of dinitrophenyl-L-amino acid methyl esters with carboxypeptidase-Y. *Carlsberg Res. Commun.* **49:** 585–90 (1984).
6. P. Marfey. Determination of D-amino acids. II. Use of a bifunctional reagent 1,5-difluoro-2,4-dinitrobenzene. *Carlsberg Res. Commun.* **49:** 591–96 (1984).
7. C. B'Hymer, M. Montes-Bayon, and J.A. Caruso. Marfey's reagent: Past, present, and future uses of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide. *J. Sep. Sci.* **26:** 7–19 (2003).
8. Pierce Biotechnology. Instructions for FDAA, Marfey's Reagent, 48895. Pierce Biotechnology, Rockford, IL, 2005.

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