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Short communication

# Development and validation of an HPLC–UV method for the analysis of methoxyamine using 4-(diethylamino)benzaldehyde as a derivatizing agent

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**Abstract**

Methoxyamine (MX) is a potential new anti-cancer drug. In this paper, a quantitative HPLC–UV method for MX using 4-(diethylamino)benzaldehyde (DEAB) as a derivatizing agent has been developed and validated. The studies showed that MX reacts with DEAB under acidic conditions to form protonated 4-(diethylamino)benzaldehyde *o*-methyloxime ($DBMOH^+$). The equilibrium between $DBMOH^+$ and its conjugate base 4-(diethylamino)benzaldehyde *o*-methyloxime (DBMO) is affected by both buffer concentration and organic solvent content in the solution. The method developed uses a reversed phase C18 column for the separation of MX derivatives, an internal standard benzil for method calibration, and a UV detector at a wavelength of 310 nm for analyte detection. The MX derivatives can be resolved in ca. 20 min. The method has a linear calibration range from 0.100 to 10.0 μM with a correlation coefficient of 0.999 for MX and a detection limit of 5 pmol with a 50 μl sample size. The intra-assay and inter-assay precision expressed in terms of percent relative standard deviation were ≤5 and 8%; and the intra-assay and inter-assay accuracy defined as the measured value divided by the accepted value multiplied by 100% were 94.2–100 and 92.6–111%, respectively. This method may be used for the analysis of MX in pharmaceutical preparations.


*Keywords:* Methoxyamine; 4-(Diethylamino)benzaldehyde; 4-(Diethylamino)benzaldehyde *o*-methyloxime; Liquid chromatography; Ultraviolet detection; DNA base excision repair

## 1. Introduction

Methoxyamine (MX, $CH_3ONH_2$, FW = 47.06) is a novel chemotherapeutic enhancer for alkylating agents [e.g., temozolomide and 1,3-bis(2-chloroethyl)-1-nitrosourea, etc.] that damage tumor cells by adding alkyl groups to DNA bases. MX can halt the DNA base excision repair (BER) pathway by chemically modifying DNA abasic sites generated by the BER enzymes and induces tumor cell death [1–3]. The combined use of MX with alkylating agents shows great promise in combating tumors that resist alkylating agents. MX has been supported by the RAID program of the National Cancer Institute for four consecutive cycles and is currently being prepared for an investigational new drug application.

A reversed phase liquid chromatographic assay for MX using *o*-phthalaldehyde (OPA) as a derivatizing agent was previously reported by Wang et al. [4]. In this assay, MX was first reacted with OPA to form MX derivatives; the reaction mixture was then subjected to chromatographic separation; and the quantitation of MX was done by measuring the UV absorbance of one of the derivatives, methoxyamine–OPA oxime, at 254 nm. Due to the uncontrollable side-reactions of OPA [4] this assay lacks specificity and is problematic when applied to biological samples. We recently developed a tandem mass spectrometric assay for the measurement of MX in plasma samples using 4-(diethylamino)benzaldehyde (DEAB) as a derivatizing agent. The MX derivative was

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recovered from plasma samples by on-line solid phase extraction and quantitated by positive-electrospray-ionization mass spectrometry (ESI-MS–MS) [5].

In this work, we have developed a cost effective HPLC–UV method for the quantitation of MX which exploits the resolving power of liquid chromatography, the chromogenic nature of the derivatizing agent DEAB, and the availability of ultraviolet detectors in a commercial HPLC system. Our studies showed that the equilibrium between the two MX derivatives [i.e., protonated 4-(diethylamino)benzaldehyde *o*-methyloxime ($DBMOH^+$) and 4-(diethylamino)benzaldehyde *o*-methyloxime (DBMO)] could be controlled by the pH of the solution and organic additives; and the conditions of chromatographic separation and UV detection for DBMO could be optimized.

## 2. Experimental

### 2.1. Chemicals and solutions

Glacial acetic acid (HPLC grade) was from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile (HPLC grade), benzil, DEAB, formic acid, methanol (HPLC grade) and potassium phosphate were from Aldrich (Milwaukee, WI, USA). MX hydrochloride (MX.HCl) was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical reagent grade. Deionized water was obtained from a NANOpure system (Barnstead, Dubuque, IA, USA) and was used to prepare aqueous solutions.

A stock solution of potassium phosphate (0.400 M) was prepared by mixing dibasic potassium phosphate and monobasic potassium phosphate to the desired pH 7. Stock solutions of MX, DEAB, and benzil were prepared at the concentrations of 100 mM (20.0 mg MX.HCl in 2.394 ml $H_2O$), 100 mM (46.3 mg DEAB in 2.610 ml of 66.7% acetic acid) and 2.5 mM (2.3 mg benzil in 4.290 ml acetonitrile), respectively.

The mobile phase was made up by 47.5% acetonitrile and 52.5% 200 mM potassium phosphate at pH 7.0 (v/v). Working solutions of MX (1.00–100 μM in $H_2O$), DEAB (1.00 mM in 33.3% formic acid) and benzil (100 μM in the mobile phase) were prepared by dilution of the stock solutions with the selected solvents. An aqueous solution of $NaBH_4$ (20 mg/ml) was prepared by dissolving appropriate amount of the solid compound in a known volume of $H_2O$. All these solutions were stored at 4 °C when not used.

### 2.2. Derivatization of methoxyamine

A 100.0 μl aliquot of MX standard solution (or sample) was mixed with an equal volume of DEAB (1.00 mM) in a 1.5 ml Eppendorf tube (Brinkmann Instruments, Westbury, NY, USA). The mixture was reacted in a dry bath incubator (Fisher Scientific, Pittsburgh, PA, USA) at 60 °C for 40 min. At the end of reaction, a 100.0 μl aliquot of internal standard benzil (100 μM) and a 700 μl aliquot of mobile phase were added to the mixture, and the resultant solution was subjected to the instrumental analysis.

### 2.3. Reduction of 4-(diethylamino)benzaldehyde *o*-methyloxime with $NaBH_4$

A 40.0 μl aliquot of MX standard solution (50.0 mM) was well mixed with an equal volume of DEAB solution (5.00 mM) in a 1.5 ml Eppendorf tube, and the derivatization reaction was allowed to proceed at room temperature for 60 min. At the end of the reaction, the solution was dried in a DNA120 SpeedVac® (ThermoSavant, Holbrook, NY, USA) at room temperature for 60 min. To chemically reduce the MX derivative DBMO, a 500 μl aliquot of $NaBH_4$ (20.0 mg/ml) was added to the Eppendorf tube containing the dried derivative. After mixing, the reaction was allowed to proceed at room temperature overnight. Prior to solid phase extraction, the resultant alkaline solution was first reacted with a 200.0 μl aliquot of HCl solution (1.00 M) and then diluted with $H_2O$ to a total volume of 1.50 ml (pH 5.5). Waters Oasis HLB extraction cartridge (3 ml) (Milford, MA, USA) was used for sample extraction. The extraction cartridge was first conditioned with 2.00 ml methanol, and then equilibrated with 2.00 ml water. After loading the sample solution the extraction cartridge was washed with 1.00 ml water and air-dried. The extracted compounds were eluted with 1.50 ml of methanol. The eluate was dried in the DNA120 SpeedVac® at room temperature for 60 min and reconstituted in 50% aqueous acetonitrile solution. The resultant solution was subjected to mass spectrometric analysis for the confirmation of the reduction product.

### 2.4. Instrumentation

An HP 8453 UV–vis spectrophotometer (Hewlett-Packard, Wilmington, DE, USA) and an HP personal computer with ChemStation software were used for taking the UV spectra of MX and DEAB, as well as their derivatives, in various test solutions.

A Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK) was used together with HPLC, or alone, for the confirmation of the MX derivative and its reduction product. The mass spectrometer was operated under the positive-electrospray-ionization mode ($ESI^+$). The ionization conditions were as follows: nitrogen sheath and desolvation gas at 10 and 350 l/h, capillary at 3.5 kV, HV lens at 0.5 kV, cone at 34 V, skimmer at 1.5 V, RF lens at 0.2 V, ion source temperature at 70 °C, ion energy at 1.2 V for quadrupole 1 and 2.0 V for quadrupole 2, low- and high-mass resolution at 15 for both quadrupole 1 and 3, multiplier at 650 V, dwell time of 0.5 s and inter-scan delay of 0.1 s. Full-scan spectra were acquired in the continuum mode at a rate 400 (*m*/*z*)/s. Daughter ion spectra were obtained by fragmenting the quasi-molecular ions of the analytes in the quadrupole 2 with argon collision gas at 0.54 μbar.

A Dionex (Sunnyvale, CA, USA) HPLC–UV system was used for the separation and detection of MX derivatives. It consisted of a GP40 gradient pump, a LC5 manual injector with a 50 μl sample loop, a stainless steel in-line filter (0.5 μm pore, 0.23 μl dead volume, Upchurch Scientific, Oak Harbar, WA, USA), an HyPURITY Elite C18 analytical column (150 mm × 2.1 mm, 5 μm, ThermalQuest, Runcorn, Cheshire, UK), and an AD20 absorbance detector. A Spectra Physics (San Jose, CA, USA) 4270 integrator was used for data recording.

### 2.5. Chromatographic conditions

The HPLC–UV system was operated at ambient temperature and the chromatographic separation was achieved by isocratic elution. Prior to the initial injection, the column was equilibrated with a mobile phase containing 47.5% acetonitrile + 52.5% 200 mM potassium phosphate buffer at pH 7.0 (v/v). The volume injection was 50 μl; the flow rate of mobile phase was 0.2 ml/min; and the wavelength detection was 310 nm.

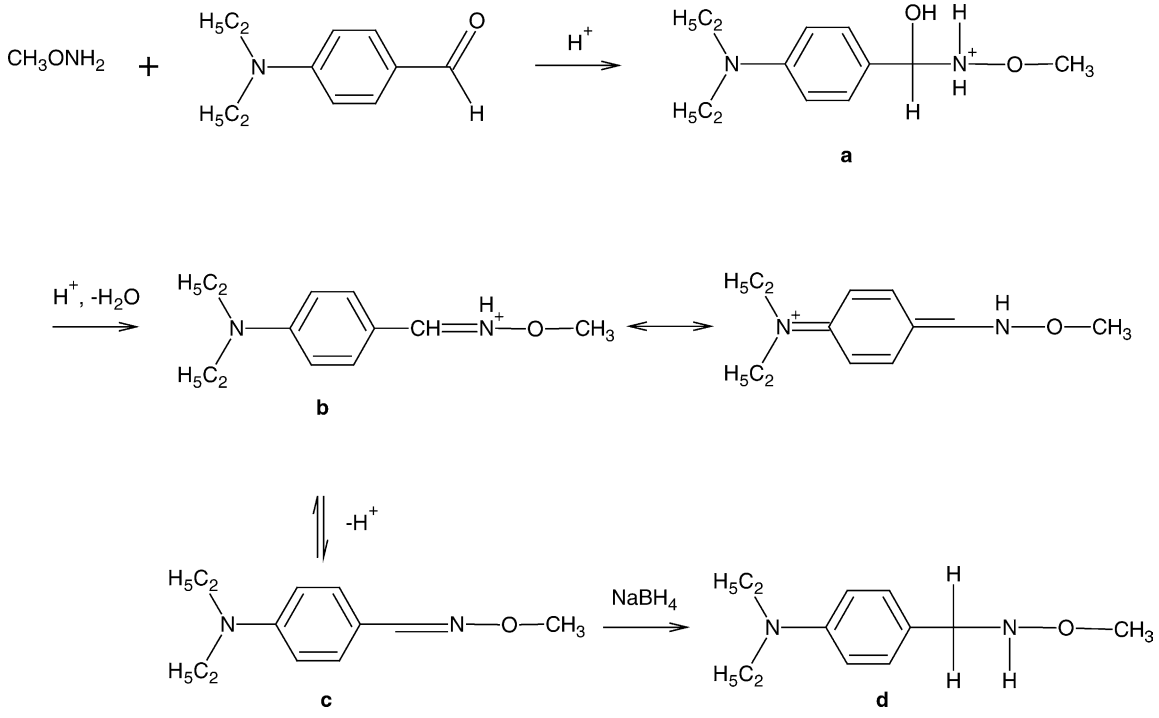
## 3. Results and discussion

### 3.1. Mechanism of derivatization reaction

The reaction mechanism of MX and DEAB has been postulated as in Scheme 1. The reaction is a Schiff reaction, where MX (a Schiff reagent) condenses with DEAB in an acidic solution through the bonding of the nitrogen atom of MX with the carbonyl carbon of DEAB. As shown in Scheme 1, the reaction initially forms an intermediate hemiaminal (compound **a**). Due to its instability, compound **a** loses a water molecule [6] and produces $DBMOH^+$ (compound **b**). When the pH of the solution and the percent content of organic solvent increase, compound **b** loses a proton ($H^+$) and forms a conjugate base, DBMO (compound **c**).

This mechanism was supported by the results from the chemical reduction, LC–MS, UV–vis spectrophotometric studies. From the UV–vis spectrophotometric studies, it was found that the compound **b** absorbed at 260 nm in the acidic aqueous solution (Fig. 1B). The increase of absorbance at 330 nm and the decrease of absorbance at 260 nm were observed when the concentration of phosphate buffer (pH 7.0) added to the reaction solution increased (Fig. 2). Since the compound **b** has a calculated p$K_a$ value of 6.26 and the derivatization reaction took place in the acidic conditions, the higher the concentration of phosphate buffer (pH 7.0) the greater the buffer capacity it had; therefore, more compound **b** was converted to compound **c** as the result of mass–action effect of the chemical equilibrium between these two compounds. The conversion of compound **b** to compound **c** was further facilitated by the addition of either methanol or acetonitrile (Fig. 3), which might be due to proton transfer from compound **b** to the organic solvents.

The presence of compounds **b** and **c** in the reaction mixture was confirmed by a reversed phase LC–MS experiment where




Scheme 1. The proposed reaction mechanism of MX and DEAB. Compound **a**, intermediate hemiaminal; compound **b**, protonated 4-(diethylamino)benzaldehyde *o*-methyloxime ($DBMOH^+$); compound **c**, 4-(diethylamino)benzaldehyde *o*-methyloxime (DBMO); and compound **d**, *N*,*N*-diethyl-*N*-{4-[(methoxyamino)methyl]phenyl}amine.

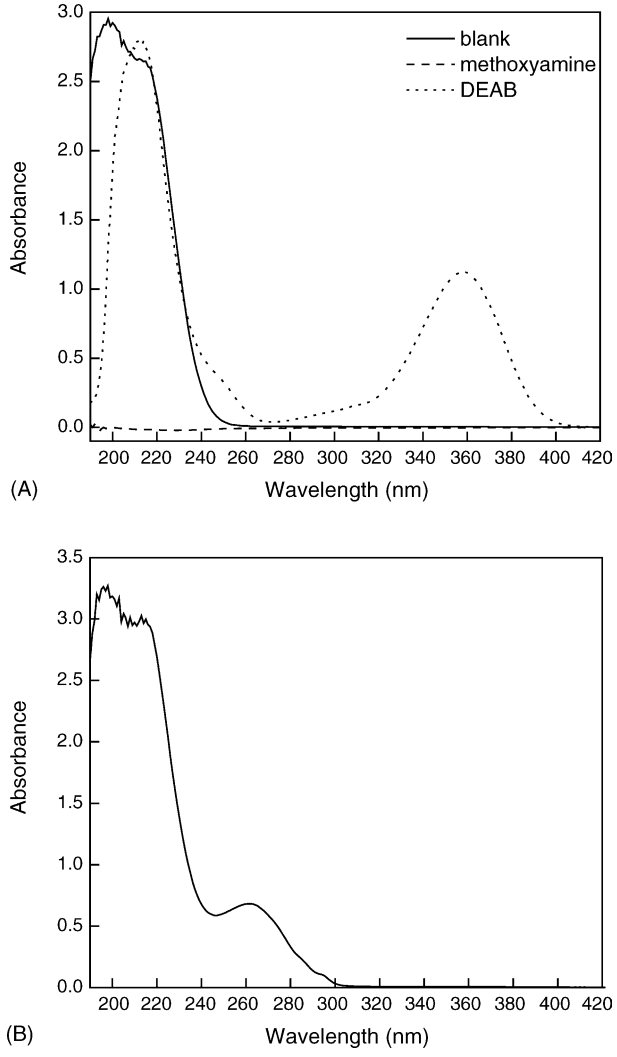



Fig. 1. The UV spectra of MX, DEAB, and $DBMOH^+$. (A) Blank solution, 40.0 μl of 33.3% formic acid was diluted to 4.0 ml with water; MX solution, 40.0 μl of 50.0 mM MX in water was diluted to 4.0 ml with water; DEAB solution, 40.0 μl of 5.00 mM DEAB in 33.3% formic acid was diluted to 4.0 ml with water. (B) A 40.0 μl of 50.0 mM MX in water reacted with 40.0 μl of 5.00 mM DEAB in 33.3% formic acid at room temperature, the reaction mixture was diluted to 4.0 ml with water.

the protonated compound **b** eluted earlier than the deprotonated compound **c** from a C-18 column, and both compounds **b** and **c** formed the identical quasi-molecular ion at *m*/*z* 207 in positive-electrospray-ionization mode ($ESI^+$-MS) (data not shown). Chemical reduction of compound **c** with $NaBH_4$ (Section 2.3) produced compound **d**, *N*,*N*-diethyl-*N*-{4-[(methoxyamino)methyl]phenyl}amine. Both compounds **c** and **d** could be detected by $ESI^+$-MS with scanning mode at *m*/*z* 207 and 209 from the solution of the reduction reaction (data not shown). Fragmentation of compounds **c** and **d** by $ESI^+$-MS–MS produced daughter ion spectra which revealed a high similarity in structures between both compounds (data not shown).

The proposed mechanism provided satisfactory explanations for the following experimental observations: (a) a single

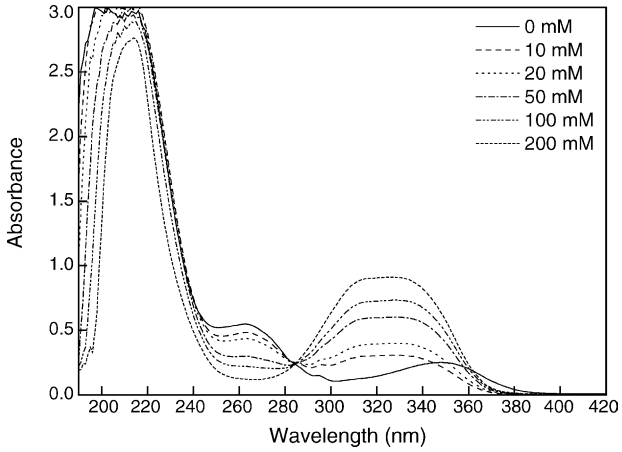



Fig. 2. The effect of phosphate buffer concentration on the UV absorbance of the MX derivatives. The derivatization reaction was the same as Fig. 1B. The reaction mixture was diluted in 50:50 (v/v) acetonitrile and phosphate buffer at pH 7.0 (0–200 mM).

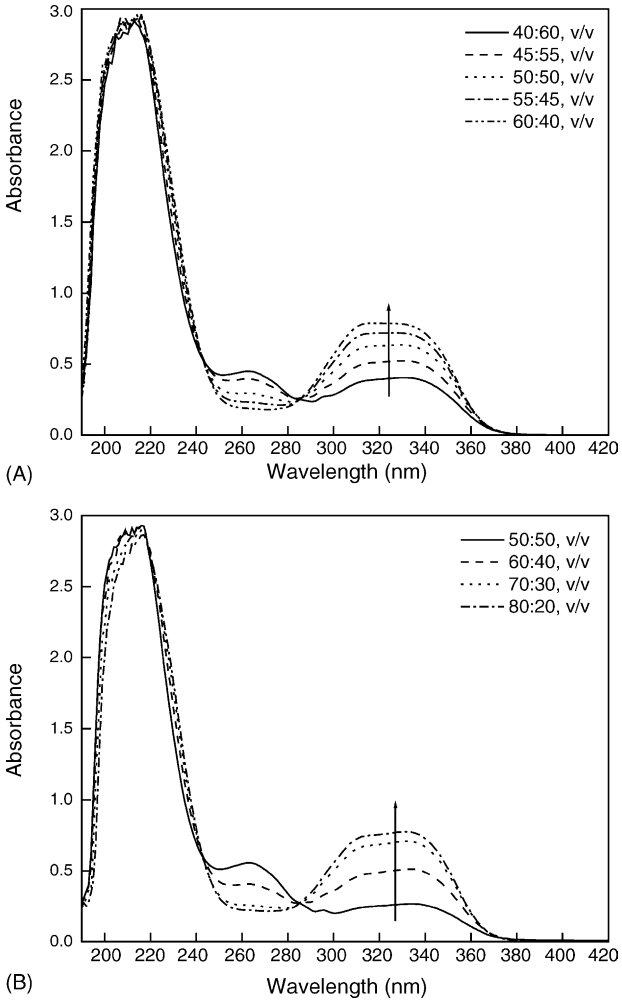



Fig. 3. The effect of organic solvent on the UV absorbance of the MX derivatives. The derivatization reaction was the same as Fig. 1B. (A) The reaction mixture was diluted in various ratios of acetonitrile and 50.0 mM phosphate buffer (pH 7.0); (B) the reaction mixture was diluted in various ratios of methanol and 50.0 mM phosphate buffer (pH 7.0).

product of the derivatized MX in our previously reported tandem mass spectrometric assay which employed the same derivatization reaction [5], and (b) two products of the derivatized MX in the HPLC–UV method (Section 3.2).

### 3.2. HPLC–UV method

A reversed phase HPLC–UV method was developed for the quantitation of the MX derivative DBMO (the compound **c** in Scheme 1). In this method, a HyPURITY Elite C18 column was used for analytical separation. Benzil was used as the internal standard for calibration. Since DBMO has an absorption plateau from 308 to 340 nm (Figs. 2 and 3) and benzil has an adequate absorption at 310 nm, the UV detector was set at 310 nm for quantitation. The optimal separation of DEAB, internal standard, and DBMO was achieved by isocratic elution with acetonitrile and 200 mM phosphate buffer at pH 7.0 (47.5:52.5, v/v) with a flow rate of 0.2 ml/min. Representative chromatograms are shown in Fig. 4, where the retention times of DEAB, internal standard, and DBMO were 7.6, 13.0, and 19.2 min, respectively. A trace amount of $DBMOH^+$ (compound **b** in Scheme 1) was observed at 14.9 min, which did not interfere with the quantitation of DBMO.

### 3.3. Effect of reaction time and temperature

In the spectrophotometric studies (Figs. 1–3), both reactants (i.e., MX and DEAB) were at millimolar concentrations, the derivatization reaction was completed at room tempera-

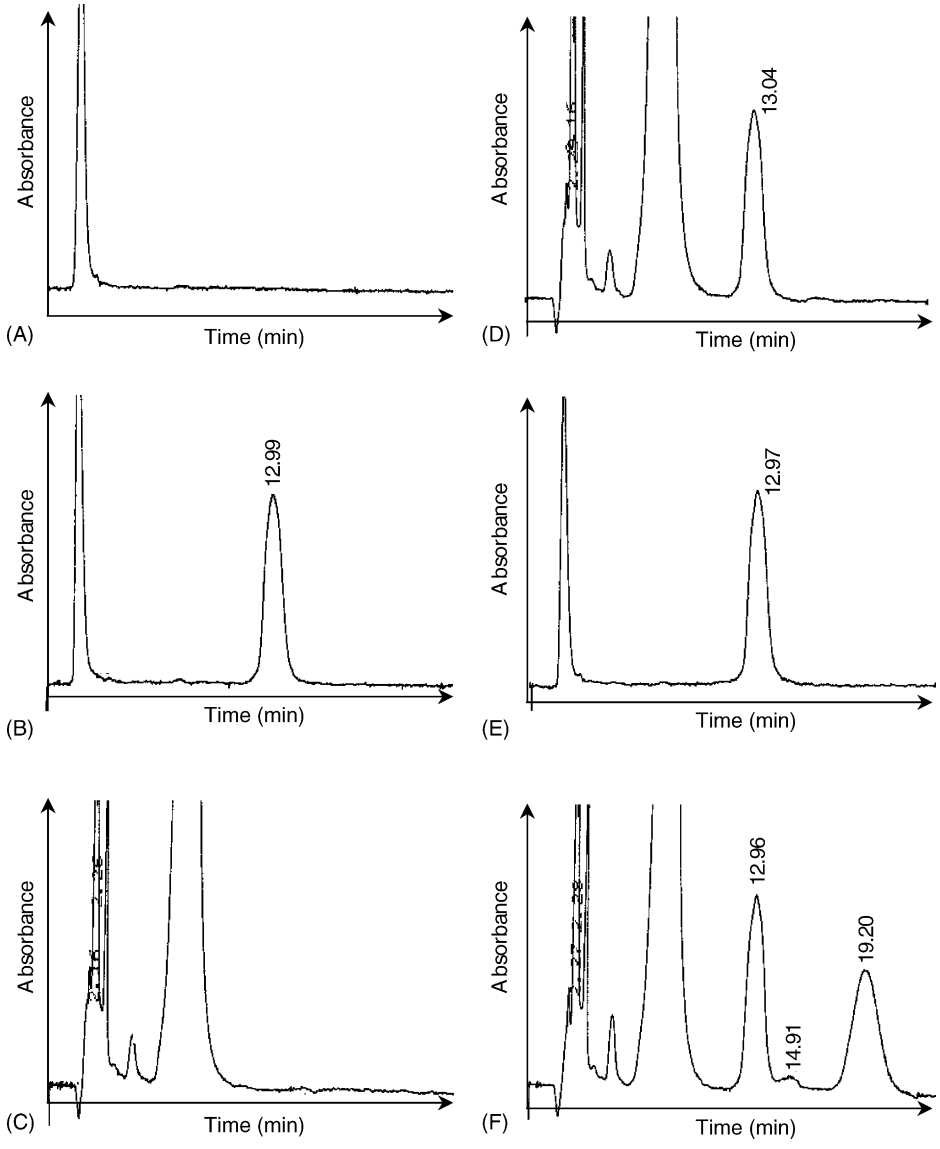



Fig. 4. Representative chromatograms of (A) MX, (B) benzil, (C) DEAB, (D) DEAB + benzil, (E) benzil + MX, and (F) MX + DEAB + benzil. Sample injection volume, 50 μl; mobile phase, 47.5:52.5 (v/v) of acetonitrile and 200 mM phosphate buffer (pH 7.0); flow rate, 0.2 ml/min; detection wavelength, 310 nm. The concentrations of DEAB, benzil, and DBMO were 100, 10.0, and 1.00 μM, respectively.

Table 1
The intra-assay and inter-assay precision and accuracy of DBMO

| MX added (μM) | Within-run ($n = 3$) | | | | Between-run ($n = 3$) | | | |
|---|---|---|---|---|---|---|---|---|
| | Peak area ratio[a] (mean ± S.D.) | R.S.D. (%) | MX measured (μM) | Accuracy (%) | Peak area ratio (mean ± S.D.) | R.S.D. (%) | MX measured (μM) | Accuracy (%) |
| 0.200 | 0.176 ± 0.009 | 5 | 0.201 | 100 | 0.202 ± 0.015 | 7 | 0.222 | 111 |
| 1.00 | 1.07 ± 0.03 | 3 | 0.942 | 94.2 | 1.14 ± 0.09 | 8 | 1.00 | 100 |
| 5.00 | 5.98 ± 0.05 | 0.8 | 5.00 | 100 | 5.53 ± 0.04 | 0.7 | 4.63 | 92.6 |

[a] The peak area ratio of DBMO to internal standard benzil (10.0 μM).

ture upon mixing. Since lower concentrations of MX were measured in the analytical procedures, the effects of reaction time and temperature on the formation of the MX derivatives were investigated. In this study, 100.0 μl of 10 μM MX in water was reacted with 100.0 μl of 1 mM DEAB in 33.3% formic acid. At the end of the reaction, the reaction mixture was subjected to HPLC analysis and the result was plotted as the peak area ratio of DBMO to internal standard versus reaction time at the chosen temperature. The data (not shown) revealed that the increase of reaction temperature favored the condensation of MX and DEAB in the acidic condition. At temperatures ≥40 °C, the peak area ratios reached a plateau at reaction times ≥20 min. Therefore, the derivatization reaction was carried out at 60 °C for 40 min in the subsequent analytical procedures.

### 3.4. Analytical performance

The intra-assay and inter-assay precision and accuracy of the method were assessed by measuring the peak area ratios of DBMO to internal standard for within-run ($n = 3$) and between-run ($n = 3$) studies at three concentration levels (0.200, 1.00, and 5.00 μM). As shown in Table 1, the intra-assay and inter-assay precision expressed in terms of percent relative standard deviation were ≤5 and 8%; and the intra-assay and inter-assay accuracy, defined as the measured value divided by the accepted value multiplied by 100%, were 94.2–100% and 92.6–111%, respectively. The linearity of the method was established over the concentration range of 0.100–10.0 μM. The mean calibration curve based on three separate calibration curves produced a mean calibration equation (by the method of least squares): $y = 1.21x - 0.0651$ with a correlation coefficient of 0.999, where $x$ is the concentration of MX and $y$ is the peak area ratio of the DBMO to the internal standard. The limit of quantitation of the method for MX was 0.100 μM, or 5 pmol with a 50 μl sample size, which was the lowest concentration on the standard calibration curve.

## 4. Conclusions

A quantitative HPLC–UV method has been developed and validated for the analysis of MX using DEAB as the derivatizing agent. The derivatization reaction between MX and DEAB is highly specific and its mechanism has been proposed. The conditions for formation and separation of the MX derivative DBMO have been investigated and optimized. The HPLC–UV method developed has good precision and accuracy. Its wide linear dynamic range and low limit of quantitation make this method applicable for pharmaceutical and formulation analyses of MX.

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