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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.