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Chromatographic separation and NMR characterization of the isomers of MMB-4, a bis-(pyridiniumaldoxime)☆

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ABSTRACT

1,1′-Methylenebis{4-[(hydroxyimino)methyl]pyridinium) dichloride (MMB-4), a promising antidote for organophosphate poisoning, has been shown by chromatography and NMR to be a mixture of geometric isomers, predominantly the *E*/*E* form. The chromatographically separated isomers have been isolated, directly characterized by NMR to be *E*/*E* and *E*/*Z* isomers of high purity, and shown by HPLC and NMR to re-equilibrate in solution to the isomeric mixture found in bulk MMB-4. These findings clearly show that a minor component in MMB-4 is not an impurity, but a geometric isomer of the principal component and demonstrate the need to understand equilibrium processes for drug characterizations and isomer distributions of chemicals proposed for animal and human clinical trials. Evidence for the presence of the *Z*/*Z* isomer could not be found.

1. Introduction

Toxic effects of chemical warfare nerve agents as well as those of organophosphorus pesticides continue to challenge scientists in their quest for effective antidotes. Among the potential antidotes, the more promising candidates are derivatives of bis-(pyridiniumaldoximes) [1]. Although no single antidote is effective against all known nerve agents, MMB-4, Fig. 1, a potential mixture of three geometric isomers (*E*/*E*, *E*/*Z*, and *Z*/*Z*), has shown considerable promise and has been selected by the US Army as a clinical candidate. Because such a candidate will be dosed in humans, complete characterization of the material is needed prior to clinical investigations and eventual IND submission to the FDA for approval.

Isomerization of *E* and *Z* isomeric aldoximes/oximes has long been known. Mono-functioned derivatives, having only two isomeric forms, have been well characterized [2] and proton NMR has proved useful for elucidating geometric configurations. Detailed reports on this topic include those by Phillips [3], Karabatsos and Taller [4], and Poziomek et al. [5] separated the two geometric isomers of isonicotinaldehyde oxime by fractional crystallization, methylated each isomer to the corresponding pyridiniumaldoxime,

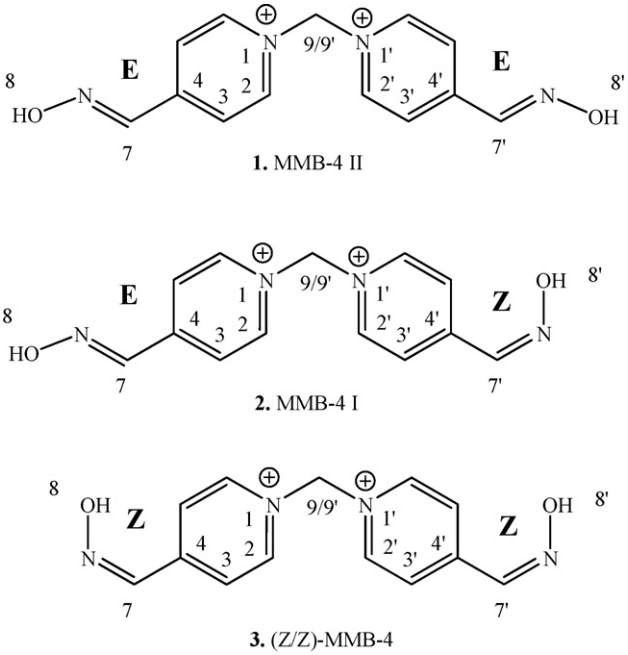

Fig. 1. Structures of the isomers of MMB-4, with the descriptor *E* is equivalent to the descriptors *syn* and *trans* used in earlier literature; and, analogously, the descriptor *Z* is equivalent to earlier descriptors anti and cis.

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and determined proton spectra on the four aldoximes. The authors reported that the aryl protons chemical shifts in the *E* isomers are upfield from their counterparts in the *Z* isomers, but that the aldehydic proton chemical shifts in the *E* isomers are downfield from those in the *Z* isomers. Lustig [6] recorded spectra of the *E* and *Z* isomers of p-chlorobenzaldoximes whose structures were known from X-ray studies and found the aldehydic proton in the *E* isomer lies at a lower field than that of the *Z* isomer by about 0.7 ppm. Bi-functioned derivatives, such as bis-aldoximes, have received less attention; however, Spohrer and Eyer [7] separated the *E*/*E* and E/*Z* isomers of obidoxime, determined their proton spectra, and found the NMR data consistent with those cited above for the mono-aldoximes.

Chemical shifts of the aldoxime–OH in the *E* and *Z* isomer also differ. Kleinspehn et al. [8] reported that most simple oximes at ≤5 mol% solutions in DMSO exhibit hydroxyl proton chemical shifts that are essentially concentration independent, and thus, are characteristic of the respective isomeric oxime. For simple aliphatic aldoximes, the OH protons in *E* isomers range from 10.25 to 10.31 ppm, while those for the corresponding *Z* isomers are from 10.60 to 10.68 ppm, indicating that the *E*-form hydroxyls are more shielded. Based on reported chemical shift data for aryl, aldehydic, and OH protons of mono- and bis-(pyridiumaldoximes), the geometric isomers of MMB-4 and related components can be readily assigned.

With support from the NMR data, confirmation of configurations can be achieved by chromatographic methods. By design, pyridiniumaldoximes are ionic, and the conjugate anions are, in general, hydrophilic. Such compounds do not lend themselves to the usual reverse phase HPLC; in general, separations have been based on ion-exchange or ion-pair chromatography [7]. Such separations have shown the bis-(pyridiumaldoximes) to be mixtures of geometric isomers [9–11]. In our laboratory, we have used C18 columns with heptanesulfonate as the pairing ion; however, we found using diphenyl columns and aqueous acidic mobile phases were more effective for analytical and preparative separations of the MMB-4 components and permit LC/MS determinations for NMR and LC/MS analyses. Moreover, by applying the water suppression technique (WET) [12], direct proton NMR determinations on isolated fractions can also be achieved. Thus, by coupling chromatographic and spectrometric techniques, we are able to unequivocally deduce structures for two MMB-4 geometric isomers.

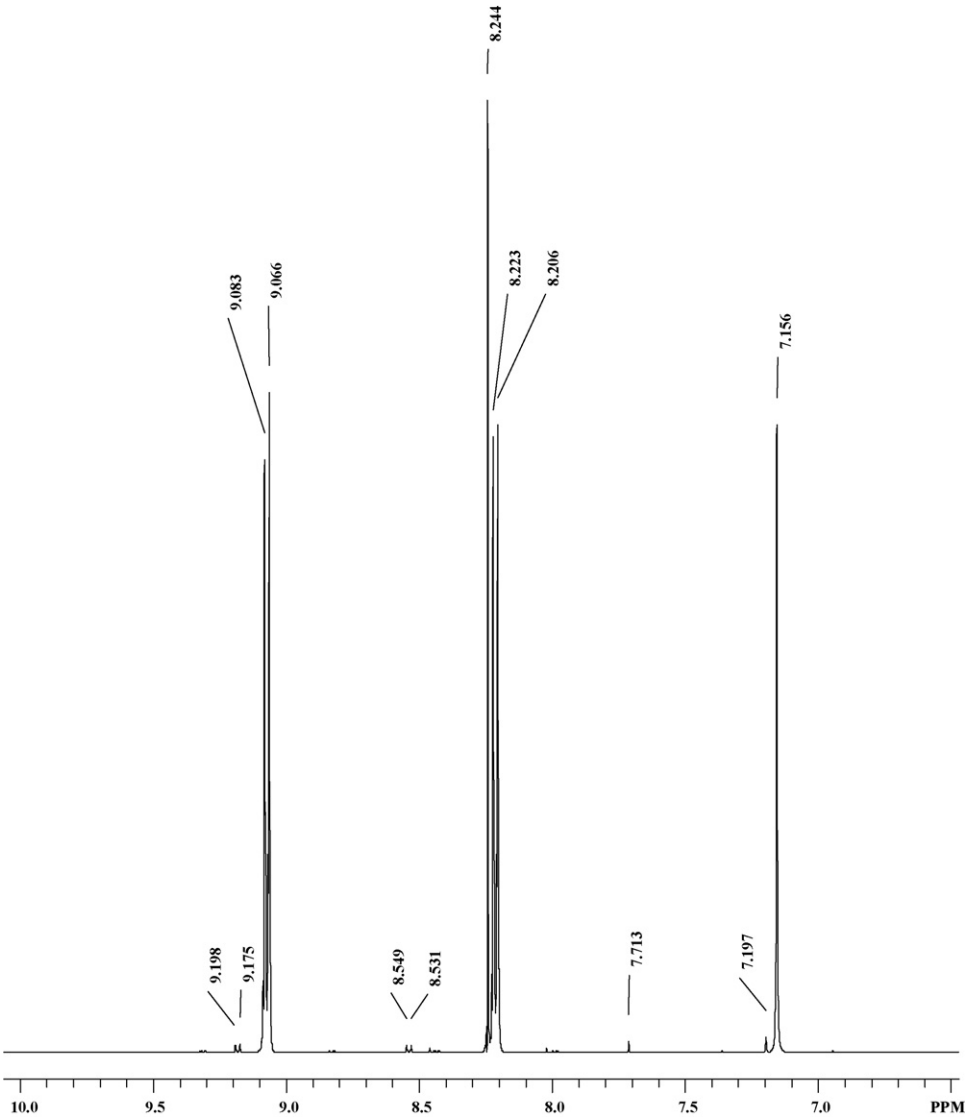

Fig. 2. ^{1}H NMR spectrum, of bulk MMB-4 at 200 mg/mL in water (with DMSO-d_6 added to provide signal lock), showing weak *Z* signals analogous to the predominant *E* signals (see text).

2. Experimental

2.1. Materials

MMB-4 dichloride (WR249943AK:BM0444, Lot SW-04-63), was obtained from the Walter Reed Army Institute of Research (WRAIR), Silver Spring, MD. 4-Pyridinealdoxime was purchased from Sigma–Aldrich Chemical Co. (Milwaukee, WI). Millipore (Bedford, MA) Elix/Milli-Q > 18 MΩ resistivity water was used throughout. Chemicals and solvents were of ACS reagent grade.

2.2. Instrumentation

Chromatography was performed using a Waters 600 pump and controller, a Waters 717-plus auto-sampler, and a Waters 996 photodiode array detector controlled by Waters Empower software (Build 1154). An analytical wavelength of 297 nm was used throughout.

Quantitative and preparative chromatographic analyses were performed using a Varian Pursuit XRs Diphenyl column (10.0 mm × 250 mm, 5 μm), a mobile phase of 25 mM ammonium formate/0.70% formic acid (v/v), solution pH 2.8, and a flow rate of 2.0 mL/min.

LC/MS analyses were performed using a Waters Acquity Ultra-Performance Liquid Chromatograph (UPLC) coupled to a Waters Acquity Triple Quad Detector operated in the ESI mode. The stationary phases were a Waters BEH C18 (2.1 mm × 50 mm, 1.7 μm) in tandem with a Waters BEH Phenyl column (2.1 mm × 50 mm, 1.7 μm), a mobile phase of 50 mm ammonium formate/0.5% formic acid (v/v), and a flow rate of 0.25 mL/min. The ion spray voltage was set at 3.0 kV with a source temperature of 120 °C and a desolvation temperature of 250 °C and operated in the positive ionization mode. The desolvation gas flow was set to 500 L/h. The MS1 mode was used to characterize the precursor ion (258 *m*/*z*) with a corona voltage of 1.5 kV and cone voltage of 20 V. The MS/MS daughter and parent modes were used to find the product (daughter) ions (123, 135 and 136 *m*/*z*), and confirm the precursor ion as the parent ion. Waters MassLynx 4.1 software was used for instrument control and data acquisition.

The isolation of separated components for NMR studies was performed by collecting 0.8 mL of separated fractions in the mobile phase to which was added one drop of deuterated DMSO for a deuterium lock. NMR spectra were determined with Varian NMR System spectrometers (300 and 400 MHz) equipped with VnmrJ software. NMR tubes, 3 and 5 mm, were purchased from Wilmad Glass, Buena, N.J. NMR solvents were purchased from Aldrich.

2.3. Preparation of samples for analyses

For preparative chromatography, 215 mg of MMB-4 dichloride was dissolved in 1.0 mL of the ammonium formate/formic acid mobile phase, the solution transferred to an auto-sampler, and 50 μL injections were made. After ensuring that each isolated fraction was >95% isometrically pure by re-chromatography, it was transferred to an NMR tube for spectral and equilibration studies.

For LC/MS studies, solutions of 14 mg MMB-4 dichloride/mL ammonium formate/formic acid mobile phase were prepared, from which appropriate-volumes were injected.

3. Results and discussion

3.1. NMR spectrum of bulk MMB-4

The presence of isomeric MMB-4 can be observed in a proton spectrum of the bulk chemical. Though the signals from the minor isomer are weak, they can be assigned with considerable certainty to a 4-pyridiumaldoxime moiety. From a solution of 200 mg MMB-4/mL water containing 3% DMSO-d_6, the recorded spectrum is dominated by signals of the major component which can be assigned to the (*E*/*E*)-MMB-4 isomer (Fig. 2) based on the literature references described earlier and by Nuclear Overhauser Effect (nOe) experiments that verified these assignments. In the 7.0–10.0 ppm region, where all the C-protons in MMB-4 resonate, a set of weak signals having an overall pattern analogous to that of the intense *E*/*E* signals (9.1 and 8.2 ppm), are found. These weak signals consist of two apparent doublets (9.2 and 8.5 ppm), assignable to H-2′,-6′ and H-3′,-5′, and two singlets, assignable to H-7′ (7.7 ppm) and H-9′ (7.2 ppm). These assignments are consistent with those reported for protons in 4-pyridinium-*Z*-aldoxime [7].

The chemical shifts for the *Z*- and *E*-aldoxime OH protons in MMB-4 also differ. A spectrum (not shown) of 3 mg MMB-4/mL DMSO-d_6 shows a weak 13.47 ppm and a strong 13.17 ppm signal, both exchangeable with the trace water in DMSO-d_6. The weak signal, 0.30 ppm downfield from the strong, is consistent with chemical shifts difference for *Z*- and *E*-aldoxime OH protons reported by Kleinspehn et al. [8]. Although nOe results correlated the strong OH signal with the *E* aldehydic proton, verifying an *E*-aldoxime geometry in the major component, no nOe correlation between the weak OH signal and the H-7′ signal could be found, possibly owing to the weakness of the signals.

The aforementioned 4-pyridinium-*Z*-aldoxime moiety in the MMB-4 minor component can be part of the (*E*/*Z*)-MMB-4 isomer or of the (*Z*/*Z*)-MMB-4 isomer. The former is expected to show *E* and Z aryl protons that possibly differ in chemical shifts; the latter is expected to show only identical *Z* aryl protons, analogous to the *E*-aryl protons in the *E*/*E* isomer. Spohrer and Eyer [7] in their NMR study on isolated *E*/*Z* and *E*/*E* isomers of obidoxime cited

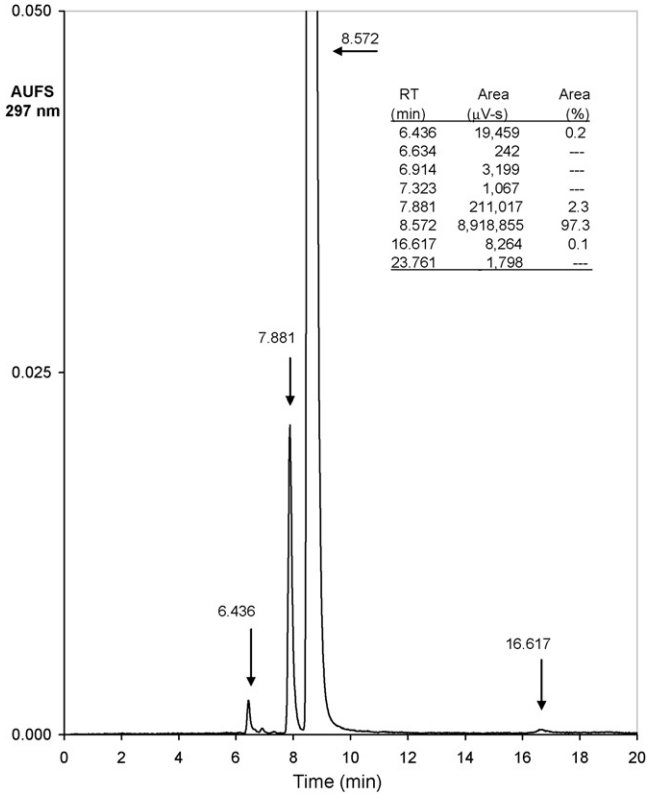

Fig. 3. Typical HPLC chromatogram of bulk MMB-4 showing an unknown at 6.4 min, MMB-4-I at 7.9 min, and MMB-4-II (the major component) at 8.6 min. The signal at 16.6 min co-elutes with 4-pyridinealdoxime, a known precursor to MMB-4.

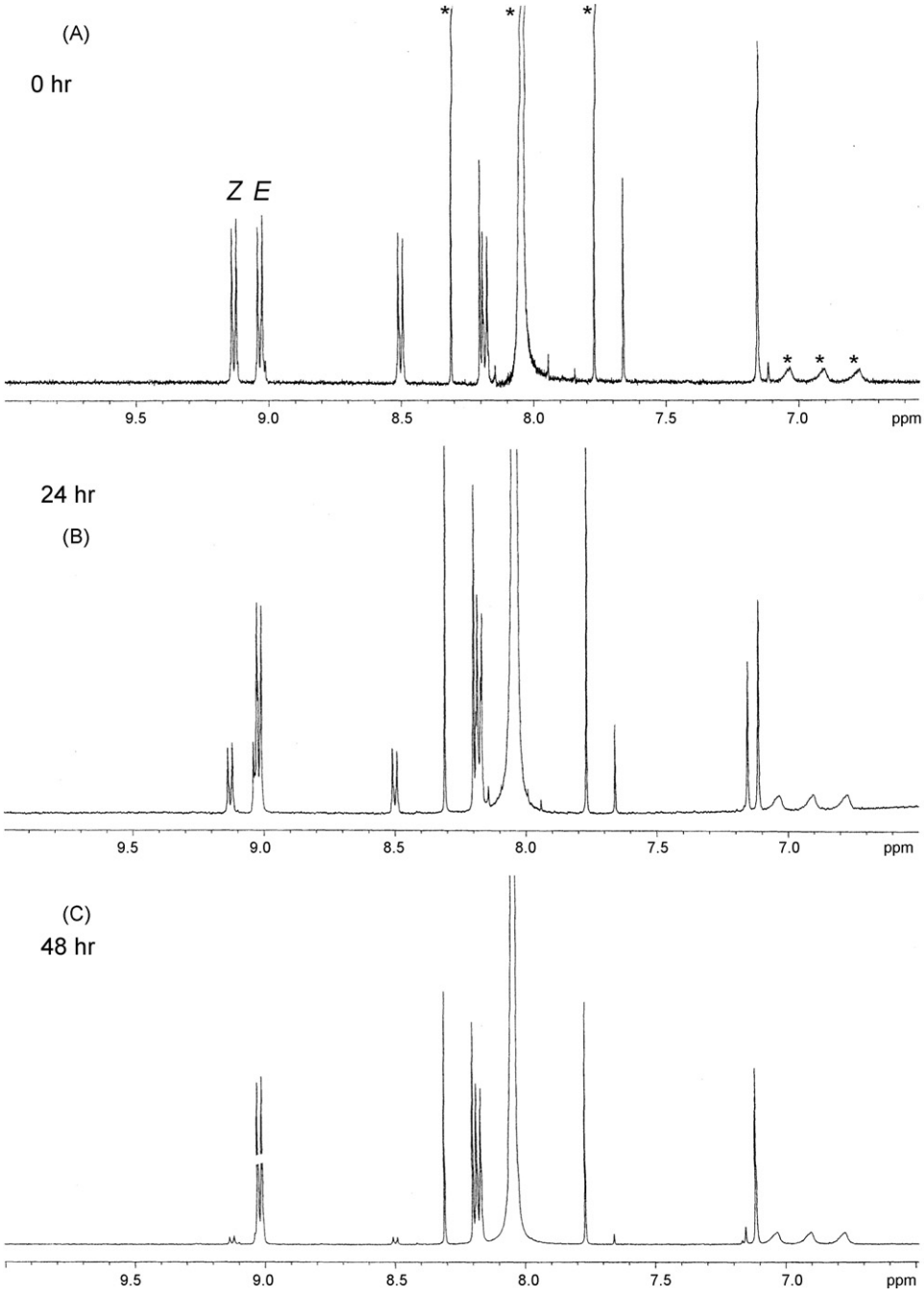

Fig. 4. ^{1}H NMR spectra of isolated component I in pH ~ 4.5 ammonium formate recorded at 0, 24, and 48 h, showing 96% (*E*/*Z*)-MMB-4 (4% (*E*/*E*)-MMB-4) equilibrating to 2% (*E*/*Z*)-MMB-4 (98% (*E*/*E*)-MMB-4) over 48 h. Asterisks identify solvent (ammonium formate) peaks.

small chemical shift differences between protons in the *E* portion of the *E*/*Z* isomer and those in the *E*/*E* isomer. If such differences between the *E* protons in the *E*/*Z* and protons in the *E*/*E* isomers can be found in spectra of bulk MMB-4, identity of the minor component as (*E*/*Z*)-MMB-4 would be achieved. Because a second set of *E*-aryl protons in spectra of bulk MMB-4 could not be found, isolation of the minor component and determination of its spectrum are needed to unambiguously identify the *E* and *Z* moieties in the MMB-4 minor component, MMB-4-I.

3.2. Chromatography and LC/MS of MMB-4

A typical chromatogram of MMB-4, showing its major (MMB-4-II), minor (MMB-4-I), and trace components appears in Fig. 3. Because the mobile phase is 100% aqueous, the separation mode is most likely selective π–π interactions common for diphenyl columns. This is evidenced by the longer retention of an MMB-4 synthetic starting material, 4-pyridium aldoxime, eluting at 16.6 min, and identified by retention and UV spectrum compared to an authentic standard. The major component (8.57 min, identified as MMB-4-II) was estimated to be 98.4% pure with a minor component (7.88 min, identified as MMB-4-I) representing 1.6% of total peak areas. A trace component, eluting at 6.43 min, representing 0.2% of total peak areas, has not been identified and is under investigation. Two other trace components, estimated at less than 0.1%, are also found but not being investigated. Decreasing the analytical wavelength to 210 nm showed no additional components other than those observed at 297 nm.

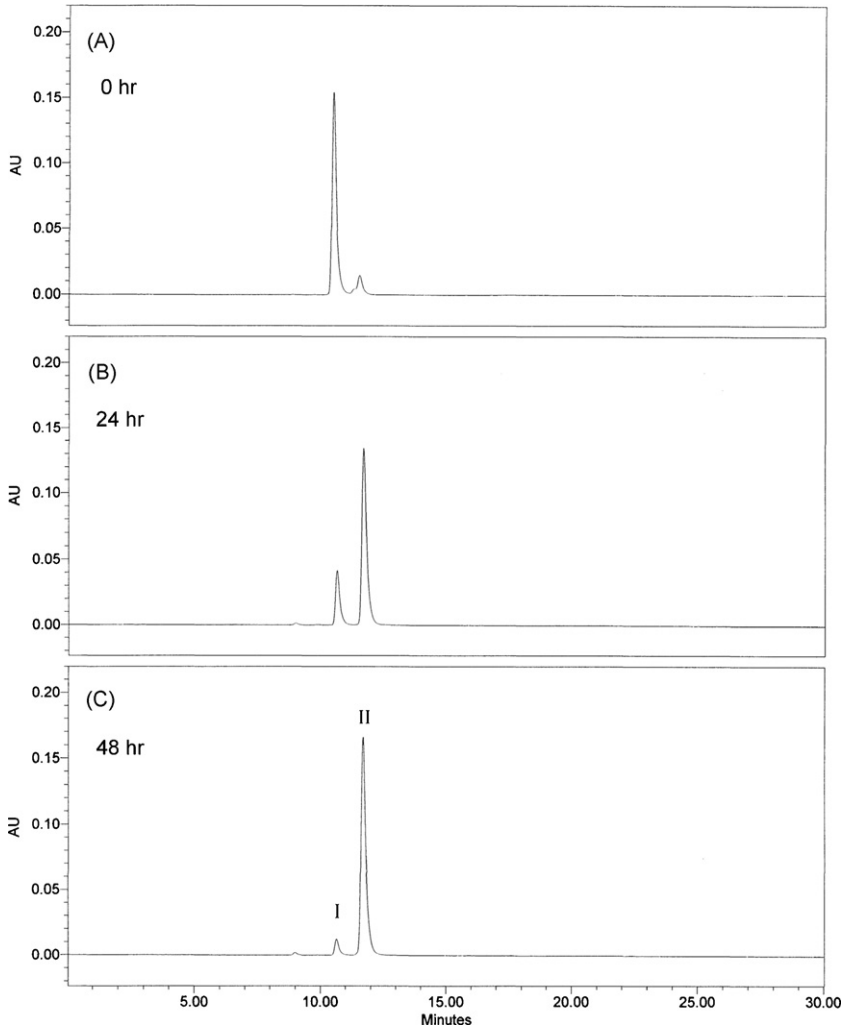

Fig. 5. Chromatograms of isolated component I in pH ~ 4.5 ammonium formate developed after 0, 24, and 48 h, showing 96% (*E*/*Z*)-MMB-4 (4% (*E*/*E*)-MMB-4) equilibrating to 2% (*E*/*Z*)-MMB-4 (98% (*E*/*E*)-MMB-4) over 48 h.

LC/MS results for the major component show an $M-H^+$ of *m*/*e* 257, corresponding to the loss of a proton from an aldoxime–OH. MS/MS data from this ion yields a *m*/*e* 135 base peak plus an intense *m*/*e* 123; these ions are consistent with cleavage of MMB-4 at the methylene bridge. Identical mass data were obtained from the minor component, indicating the two components are isomeric. LC/MS on the 6.43-min component yielded a $M-H^+$ of *m*/*e* 258, suggesting it is not an isomer of MMB-4.

3.3. NMR studies on MMB-4-I

After collecting the minor component, MMB-4-I, and showing it to be 96% MMB-4-I and 4% MMB-4-II by re-chromatography, it was analyzed by proton NMR using the WET technique significantly reduces the water resonance at 4.5 ppm, leaving only the major resonance from the mobile phase ammonium formate and its ^{13}C-sidebands and the sample resonances. This direct approach eliminates extraneous sample handling which becomes important when equilibriums are involved. The resulting spectrum, Fig. 4a, clearly indicates two sets of equal-intensity aryl protons, leaving no doubt that MMB-4-I is (*E*/Z)-MMB-4. The 9.04- and 9.13-ppm doublets in Fig. 4a can be assigned, respectively, H-2,6 and H-2′,6′ in the *E* and *Z* portions of (*E*/*Z*)-MMB-4. Moreover, a weak, partially resolved signal, slightly upfield from the 9.04-ppm doublet (*E* in *E*/*Z*), can be assigned to the H-2,6 in the *E*/*E* contaminant in the collected (*E*/*Z*)-MMB-4 fraction, adding support to the 9.04-ppm assignment for the H-2,6 in the *E* portion of MMB-4-I. Spectrum of the isolated MMB-4-I fraction, maintained in its capped NMR tube at ambient temperature for 24 h, was re-recorded. This spectrum, Fig. 4b, shows the *E*-aryl protons in Fig. 4a are much enhanced, with a corresponding loss of the Fig. 4a *Z*-aryl protons. After another 24 h, when its spectrum, Fig. 4c, was again recorded, the spectral profile has become similar to that of bulk MMB-4 shown in Fig. 2. From these spectra, isomerization of (*E*/*Z*)-MMB-4 to (*E*/*E*)-MMB-4 is evident, and after 2 days, the isomeric mixture has reverted nearly to that of bulk MMB-4, showing beyond doubt that the MMB-4-I and MMB-4-II are isomeric and are in equilibrium. These spectral data on MMB-4 isomerism and isomeric equilibration are corroborated by chromatographic results from a second isolate of MMB-4-I obtained on days 0, 1 and 2, for which the chromatograms are depicted in Fig. 5a–c. The respective chromatograms clearly corroborate the respective spectra in Fig. 4a–c, demonstrating beyond doubt that the minor component is (*E*/*Z*)-MMB-4. While the chromatographic data shows trace amounts of additional components, no spectral evidence could be obtained to deduce the presence of a *Z*/*Z* isomer as part of the MMB-4 mixture.

Chemical shift and coupling constant data for the protons in (*E*/*Z*)-MMB-4 and (*E*/*E*)-MMB-4 are tabulated in Table 1; nOe experiments, where applicable, have been performed to verify these assignments. Comparisons of the chemical shift data for the respec-

Table 1
Proton chemical shifts for (*E*/*Z*)-MMB-4 and (*E*/*E*)-MMB-4[a,b].

Position		(*E*/*Z*)-MMB-4			(*E*/*E*)-MMB-4	
		δ (ppm)	*J* (Hz)		δ (ppm)	*J* (Hz)
2,6	*E*	9.04	6.72	*E*	9.03	6.88
3,5		8.19	6.72		8.18	6.68
7		8.21			8.21	
8		13.17	–		13.17	–
9		7.16	–		7.12	–
2′,6′	*Z*	9.13	6.92	*Z*	9.03	6.88
3′,5′		8.51	6.96		8.18	6.68
7′		7.66	–		8.21	–
8′		13.47	–		13.17	–
9′		7.16	–		7.12	–

[a] Except for the 8,8′–N–OH chemical shifts, which were assigned from a spectrum obtained from a 3-mg MMB-4/mL DMSO-d_6 solution referenced to the DMSO peak at 2.54 ppm, all other chemical shifts were assigned from spectra of the isolated (*E*/*Z*)-MMB-4 and its isomerized (*E*/*E*)-MMB-4, referenced to the formate peak at 8.048 ppm.

[b] Coupling constants in Hz were determined from apparent doublets.

tive protons in the *E* portion of the *E*/*Z* isomer with those in the *E* portion of the *E*/*E* isomer show small to very small differences, however, they are consistent with those reported in other bis-(pyridiumaldoximes) [7].

The exact solid phase distribution of *E*/*E* and *E*/*Z* isomers in MMB-4 is unknown and likely unimportant. We suspect, however, that clinical trials will involve aqueous solution preparations in which isomer distributions like those reported here will be involved. Having established reliable analytical methods to determine MMB-4 isomer distributions will allow the clinician to determine the actual composition of drug being dosed.

4. Conclusions

The minor chromatographic component in samples of MMB-4 has been identified as (*E*/*Z*)-MMB-4 by proton NMR and its isomeric relationships to (*E*/*E*)-MMB-4 confirmed by chromatographic and mass spectral evidence. When isolated and kept in solution at room temperature, it isomerizes to a mixture of (*E*/*E*)-MMB-4 and (*E*/*Z*)-MMB-4 in 48 h. Evidence for a Z/Z component could not be found, indicating its contribution to MMB-4 isomeric equilibria is very small, if at all. Thus, while such isomerizations appear universal for many mono- and bis-aldoximes, assignment of equilibrium distributions can be studied by both chromatographic and spectrometric techniques.

Acknowledgements

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