Journal of Chromatography, 603 (1992) 145–150
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 147

Determination of hydroxylamine in aqueous solutions of pyridinium aldoximes by high-performance liquid chromatography with UV and fluorometric detection☆

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(First received February 11th, 1991; revised manuscript received March 3rd, 1992)

ABSTRACT

A high-performance liquid chromatographic assay using UV and fluorescence detection was developed that monitored hydroxylamine as the vanillin oxime derivative while simultaneously monitoring concentrations of a complex mixture of a pyridinium aldoxime and its degradation products. The technique should be useful for measuring hydroxylamine levels in other aqueous systems.

INTRODUCTION

During the investigation of the stability of dosage concentrations of various organic aldoximes that have potential pharmaceutical application as antidotes to organophosphorus anticholinesterase poisoning, there arose a need to monitor the concentration of hydroxylamine in the acidic aqueous solutions of degrading aldoximes. Although the equilibrium associated with the hydrolysis of aldoximes (eqn. 1) usually lies far to the left in weakly acidic solutions [1,2],

$$RCH{=}NOH + H_2O \overset{H^+}{\rightleftharpoons} RCHO + H_2NOH \quad (1)$$

participation of the components of the equilibrium in side reactions during long term storage can lead to a significant loss of pharmacologically active aldoxime [3–6]. For example, hydroxylamine was reported to be a reactant in the hydrolysis of the amide group during the degradation of several pyridinium aldoximes under pharmaceutical evaluation which contained both aldoxime and amide functional groups [6]. While studying these complex reactions of aldoximes, a convenient high-performance liquid chromatography (HPLC) method that allowed simultaneous determination of hydroxylamine, a pyridinium aldoxime, and the major degradation products was developed.

Numerous methods have been reported for measuring the concentration of hydroxylamine [7–12]; however, only Lombardi and Crolla [12] adressed the possibility of an HPLC assay. Although their paper primarily focused on a gas chromatographic (GC) procedure for hydroxylamine found in medicinal preparations containing hydroxamic acids, they did briefly mention that a similar method involving the pre-chromatographic formation of the 4-methoxybenzaldehyde oxime derivative of hydroxylamine had been applied to HPLC with some success.

This report will describe how the more water soluble aldehyde vanillin was used as the derivatizing agent to enhance the ultraviolet (UV) and fluores-

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☆ The opinions or assertions contained herein are the private views of the author and are not to be construed as official or as reflecting the views of the Army or the Department of Defense.

0021-9673/92/$05.00

cence detectability of hydroxylamine in solutions of three pyridinium aldoximes with different degradative pathways. The aldoximes selected for investigation were 2-[(hydroxyimino)methyl]-1-methylpyridinium chloride (2-PAM chloride), 1,1′-[oxybis-(methylene)]bis{4-[(hydroxyimino)methyl]-pyridinium} dichloride (toxogonin), and 1-({[3-(aminocarbonyl)pyridinio]methoxy}-methyl)-2-[(hydroxyimino)methyl]pyridinium dichloride (**I**, HS-6). Degradation pathways for these aldoximes have been extensively studied and the concentration of hydroxylamine present under several reaction conditions determined [4,5,13,14] by modifications of the Csaky method [7] so that comparisons with the procedure described herein could be made.

EXPERIMENTAL

Equipment

The HPLC system included a reversed-phase 10-μm μBondapak C_{18} column (30 cm × 3.9 mm I.D.), Model 6000A pump, a WISP 710B autoinjector, a Lambda-Max 481 spectrophotometer, and a Model 730 data module, all from Waters Assoc. (Milford, MA, USA). A McPherson FL-749 spectrofluorimeter (McPherson, Acton, MA, USA) equipped with a 200-W xenon–mercury lamp and a 12-μl flow cell was used for fluorescence detection with the HPLC system. Fluorescence spectra were obtained with a Perkin-Elmer MPF-44B fluorescence spectrofluorimeter (Perkin-Elmer, Norwalk, CT, USA), and UV spectra were obtained with a Carey Model 219 spectrophotometer (Varian, Palo Alto, CA, USA).

Materials

1-Heptanesulfonic acid, sodium salt, was purchased from Sigma (St. Louis, MO, USA), tetramethyl ammonium chloride (TMAC) from Mallinkrodt (Paris, KY, USA), and potassium dihydrogen phosphate (KH_2PO_4), 85% phosphoric acid, sodium acetate, and vanillin were purchased from Fisher Scientific (Pittsburgh, PA, USA) and used without purification. Vanillin has UV absorption maxima [molar absorptivity (l mol^{-1} cm^{-1}) in parentheses] in ethanol at 309 nm ($10.5 \cdot 10^3$), 279 nm ($10.2 \cdot 10^3$) and 232 nm ($14.4 \cdot 10^3$) [15]. The broad emission band was centered at 412 nm. Vanillin oxime was synthesized from vanillin and hydroxylamine hydrochloride according to the procedure of Vogel [16], and recrystallized from ethanol (m.p. 121–122°C, Lit. [17] 121–122°C). UV absorption maxima in water–ethanol (5:1, v/v) were at 298 nm ($9.6 \cdot 10^3$), 268 nm ($13.8 \cdot 10^3$) and 213 nm ($21.0 \cdot 10^3$). The broad emission band was centered at 367 nm. Hydroxylamine hydrochloride was obtained from Eastman (Rochester, NY, USA) and recrystallized from a mixture of ethanol and methanol (m.p. 151–152°C, Lit. [18] 151–152°C). 4-Cyanophenol was obtained from Aldrich (Milwaukee, WI, USA). Acetonitrile was obtained from Burdick & Jackson (Muskegon, MI, USA).

2-PAM chloride was obtained from Ayerst (New York, NY, USA) and toxogonin and **I** (HS-6) were obtained from the Walter Reed Army Institute of Research (Washington, DC, USA). HPLC analysis indicated that the three crystalline pyridinium aldoximes contained less than 1% of the starting material and were used without further purification. Outdated 2-PAM chloride autoinjectors manufactured by Survival Technology (Bethesda, MD, USA) were obtained from the Safety Officier (USAMRICD, Aberdeen Proving Ground, MD, USA).

Derivatization procedure

Vanillin [200 μl, 0.1 *M* in ethanol–water (1:1, v/v)], sodium acetate (100 μl, 0.2 *M*) and the aldoxime solution to be tested (10–100 μl) were mixed in a 1-ml reaction vial and allowed to stand at room temperature for 30 min. At hydroxylamine concentrations greater than $5 \cdot 10^{-5}$ *M*, the reaction took less than 10 min to obtain a constant concentration of the hydroxylamine derivative, vanillin oxime. The entire contents of the vial were then transferred to a 10-, 25- or 50-ml volumetric flask, depending on the expected concentration of hydroxylamine, and diluted with water to the desired volume for HPLC analysis.

Chromatography

Several different mobile phases were used during the study to optimize the separation of components in the mixtures of the different aldoximes and degradation products. The data in Table I were collected from separations using three different mobile phases varied only by the quantity of acetonitrile used in the solvent mixture. These mobile phases

were prepared by taking from 90 to 120 ml of acetonitrile (90 ml for **I** assays, 100 ml for toxogonin assays and 120 ml for 2-PAM assays), 5 ml of 0.1 *M* H_3PO_4, 0.2 ml of 1.0 *M* TMAC, 120 mg of 1-heptanesulfonic acid, sodium salt, and 70 mg of KH_2PO_4 and mixing them with water to make 1 l of solution. The flow-rate was 0.5 ml/min and the chart speed on the data module set at 0.2 cm/min. The UV detector was set at 255 nm and the fluorescence detector set at 270 nm (16 nm slitwidth) for excitation and 355 nm (8 nm slitwidth) for emission. The sample size was usually 10 μl, but it was increased to as much as 50 μl for samples with low concentrations of hydroxylamine.

The vanillin oxime standard solutions and the vanillin reagent were prepared fresh daily. 4-Cyanophenol was a satisfactory internal standard for UV detection with a retention time greater than that of vanillin; however, the fluorescence signal was very small at the excitation/emission wavelengths used for this study.

Degradation reactions

Solutions of pyridinium aldoximes (0.8 ml) at various concentrations (see Table I) were placed in one ml reaction vials and either heated in an oven at 60°C or left at room temperature for the desired period of time. The heated vials were cooled before testing. The rate of the reverse reaction, hydroxylamine with the pyridinium aldehyde (eqn. 1), has been shown to be slow at the low pH of the degradation reactions [1]; therefore, the concentration of hydroxylamine was expected to remain constant during the short cooling period.

TABLE I

HYDROXYLAMINE LEVEL IN PYRIDINIUM ALDOXIME SOLUTIONS RELATIVE TO THE INITIAL CONCENTRATION OF PYRIDINIUM OXIME[a] AND AS A FUNCTION OF TEMPERATURE, pH AND TIME

Oxime (concentration)	Temperature	pH	Hydroxylamine (%)				
			24 h	48 h	96 h	144 h	Other time
2-PAM (0.5 *M*)	60°C	1.1	2.38	2.62	2.66	2.63	2.38[b]
		2.0	0.75	0.81	0.66	0.65	0.52[b]
		3.1	0.15	0.13	0.15	0.17	0.05[b]
	Room temperature	1.1	1.68	2.05	2.13	2.26	
		2.0	0.18	0.41	0.61	0.62	
		3.1	0.01	0.03	0.10	0.14	
2-PAM (1.8 *M*)	AT[c]	2.5					0.17[c]
Toxogonin (0.25 *M*)	60°C	1.1	2.64	2.62	2.81	2.83	2.50[b]
		1.9	0.44	0.67	0.80	0.84	
		2.9	0.08	0.11	0.19	0.17	
	Room temperature	1.1	2.55	2.46	2.44	2.56	
		1.9	0.07	0.49	0.70	0.72	
		2.9	0.01	0.08	0.15	0.17	
I (0.5 *M*)	60°C	1.1	0.60	1.44	1.64	1.32	
		2.7	—[d]	0.01	1.06	0.70	
	Room temperature	1.1	0.84	1.86	1.84	1.80	
		2.7	—[d]	—[d]	—[d]	0.01	

[a] Calculated as per cent yield of hydroxylamine from the initial concentration of pyridinium oxime: (mol hydroxylamine/mol oxime) × 100. All values are averages of two or more determinations.

[b] Measured after heating 2 h.

[c] Average value of samples from two six-year-old autoinjectors maintained at ambient temperature (AT).

[d] Not detectable with this assay.

RESULTS AND DISCUSSION

This HPLC assay was designed to measure hydroxylamine in concentrations ranging from $5 \cdot 10^{-2}$ to $5 \cdot 10^{-5}$ *M* in aqueous solutions containing high concentrations of pyridinium aldoximes and their degradation products. By using an HPLC procedure instead of a GC procedure, the ionic aldoximes and both ionic and neutral decomposition products could be monitored simultaneously with the hydroxylamine.

Initial aldoxime concentrations, 0.25 *M* for toxogonin and 0.5 *M* for 2-PAM and **I** for the degradation reactions, were selected to reflect dosage preparations. The 1000-fold range of concentrations for hydroxylamine was expected based on previous studies concerning the degradation of pyridinium aldoximes [4,5,13,14].

Derivatization

Vanillin reacted readily, in less than 10 min at room temperature, with hydroxylamine in the presence of sodium acetate to form vanillin oxime over the entire expected concentration range. A large excess, at least 40-fold, of vanillin and an extended reaction time of 30 min was used to ensure reaction. Hydroxylamine was derivatized in yields between 92 and 95% when tested with either a standard solution of hydroxylamine hydrochloride or a standard solution containing hydroxylamine hydrochloride and the freshly dissolved pyridinium aldoxime. Fresh solutions of the three pyridinium aldoximes contained less than $1 \cdot 10^{-5}$ *M* hydroxylamine. A yield less than 100% was expected for the derivatization reaction because a small amount of material assumed to be the *anti*-stereoisomer of vanillin oxime was also formed.

With the 30-min derivatization time, the presence of the derivatization reagents did not lead to the formation of significant quantities of base promoted pyridinium aldoxime degradation products. The chromatograms of the aging pyridinium oxime solutions were consistent with previously reported HPLC analysis at low pH (<4) [19,20]. If the derivatization reaction was extended past 1 h or subjected to heat, additional products generated from the decomposition of the pyridinium oximes at high pH (>4), particularly for the relatively base-sensitive **I**, were observed in the chromatogram.

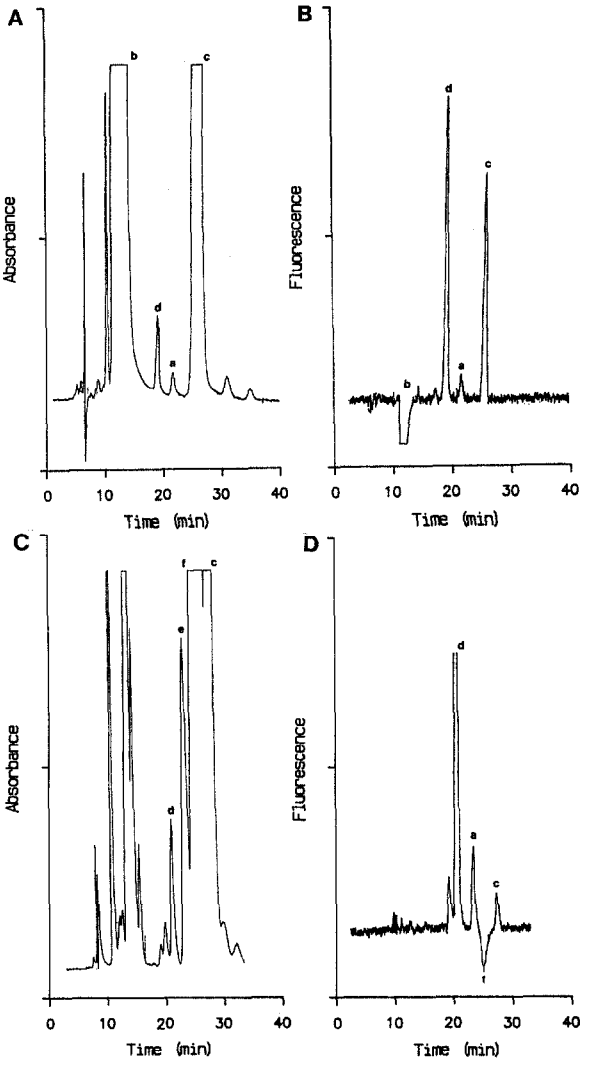

Fig. 1. (A) Chromatogram of a sample of a 2-PAM solution (pH 3.1) after 2 h at room temperature followed by derivatization with vanillin and analyzed with the UV detector (10 μl injected), (B) the same sample as in (A) but analyzed with the fluorometric detector, (C) a sample of a solution of **I** (pH 2.7) after 48 h at 60°C followed by derivatization with vanillin and analyzed with the UV detector (10 μl injected) and (D) the same sample as in (C) but analyzed with the fluorometric detector. Peaks: a = vanillin oxime; b = 2-PAM; c = vanillin; d = a by-product of vanillin presumed to be vanillin hydrate; e = a decomposition product of **I**; f = **I**. The mobile phases are described in the *Chromatography* section under Experimental.

Chromatography

Chromatograms A and B in Fig. 1 show the relative intensities of the components in the 0.5 *M* 2-

PAM (pH 3.1) solution when the concentration of hydroxylamine was near the detection limit of the assay. The vanillin oxime (peak a) represents hydroxylamine at the 0.01% level (a 1/10 000 mol ratio of hydroxylamine to 2-PAM, peak b). The large excess of vanillin (peak c), did not interfere with quantitation of vanillan oxime or 2-PAM with either UV or fluorescence detection.

Because the retention times for toxogonin and **I** were considerably greater than the retention time of 2-PAM and closer to that of vanillin oxime under many chromatographic conditions, there was a distinct advantage to using fluorescence detection for monitoring the vanillin oxime in the mixture of degrading bis(pyridinium)aldoximes, toxogonin and **I**. In Fig. 1C, the vanillin oxime signal was buried under a decomposition product of **I** (peak e), and a UV measurement was impossible even at high concentrations of vanillin oxime. With fluorescence detection, Fig. 1D, the vanillin oxime (peak a) is free from interference even when measured near the detection limit and in the presence of high concentrations of **I** (peak f).

Fluorescence detection also had the additional capability of observing fluorescent degradation products, such as the pyridones, which are formed in weakly acidic solutions of pyridinium oximes [3].

Quantitation

To minimize the effect of the large signal from the excess of vanillin on the vanillin oxime signal in both UV and fluorescence detection, the ratio of the two signals was optimized by the selection of detector wavelengths. The ratio of the vanillin oxime to vanillin signal was 3:1 with the UV detector set at 255 nm and the ratio was 250:1 with the fluorimetric detector set at 270 nm for excitation and 355 nm for emission. With these detector parameters, the limit of detection (a 2:1 signal-to-noise ratio) for vanillin oxime was 0.8 ng using the UV detector and 1.5 ng using the fluorimetric detector. Both detector responses were linear over the range of on-column quantities (20–2000 ng) monitored.

The precision of the assay was tested at several conditions with relative standard deviations ranging from 1 to 2% when the stable 2-PAM and toxogonin solutions at equilibrium were monitored to as high as 27% when the rapidly decomposing samples of **I** with low concentrations of hydroxylamine were monitored. Insight into the accuracy of the method was gained by the observation that 92 to 95% of the hydroxylamine added to solutions of pyridinium aldoximes was monitored after derivatization. In addition, hydroxylamine levels measured by this HPLC method in decomposing solutions of pyridinium aldoximes, described below, were compared with hydroxylamine levels reported [5,13] using Csaky's colorimetric method for hydroxylamine [7].

Application: pydridinium aldoxime decompositions

Table I summarizes some selected data for the hydroxylamine level found in aged oxime samples. The data collected over a six-day period indicated that equilibrium for the hydrolysis process (eqn. 1) was attained quite slowly with 2-PAM and toxogonin, about six days at room temperature and pH 3.1, and about 4 days at room temperature and pH 2.0. Both the 0.5 *M* 2-PAM and 0.25 *M* toxogonin solutions showed similar concentrations of hydroxylamine at corresponding pH values after reaching equilibrium and the hydroxylamine levels were clearly pH dependent as expected [1,2]. At pH 1.1 and 60°C the hydroxylamine level was 2.63% for 2-PAM and 2.83% for toxogonin, at pH 2 the levels were 0.65% and 0.84% respectively, and at pH 3 the levels were 0.17% for both 2-PAM and toxogonin solutions.

The anomalous lack of pH dependence reported by Christenson [5] for a 0.28 *M* toxogonin solution analyzed by Csaky's method was not observed in this study. However, the hydroxylamine level (2.83%) at pH 1.1 with 0.25 *M* toxogonin was reasonably consistent with a value of 3.9% for a 0.28 *M* solution at pH 1.7 calculated from Christenson's data [5]. The hydroxylamine level of 0.17% obtained for the 2-PAM solution at pH 3 was slightly higher than a value of 0.11% calculated from Barkman's [13] data for a buffered pH 3 2-PAM methanesulfonate solution (0.6 *M*) stored for two years and analyzed by Csaky's method. Two six-year-old samples of buffered (pH 2.5 when opened) 1.8 *M* 2-PAM chloride from out-dated autoinjectors also contained 0.17% hydroxylamine and only small quantities of by-products were detected which indicated that further chemical change occured very slowly once the hydrolysis equilibrium was attained.

A previous report of the degradation of **I** over the

pH range 0 to 3 indicated that both the degradation mechanism and hydroxylamine levels as measured by the Csaky method were similar for both toxogonin and **I** [14]. In this study, the assay of 0.5 *M* **I** at pH 2.7 led to unexpectedly low levels of hydroxylamine and a more complicated degradation pattern than at pH 1.1. At pH 1.1 and room temperature, **I** appeared to be relatively stable and the hydroxylamine level of 1.86% after 48 h was similar to the 2.46% level observed with toxogonin. At the higher pH (2.7) at room temperature only a trace of hydroxylamine was observed over the entire six day reaction period and a new major degradation product, the carboxylic acid derivative of **I** was identified [6]. The 60°C reaction at pH 2.7 also showed only trace quantities of hydroxylamine for the first 48 h and then an increase to 1.06% by 96 h. However, by 96 h approximately 50% of **I** had decomposed to a complex mix of by-products. The low levels of hydroxylamine in the reactions at pH 2.7 may be accounted for by the previous observations that hydroxylamine reacted with either formaldehyde formed from the cleavage of the aminal-acetal bridge which links the two pyridinium rings [4], or the amide group [6] found in **I**.

ACKNOWLEDGEMENTS

The author appreciates the generous support of the USAMRICD under the provisions of the Intergovernmental Personnel Act of 1970 and the cooperation and technical assistance of Dr. Ming L. Shih, Rick Smith and George Salem.

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