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High-performance liquid chromatographic analysis of imidazolium and pyridinium oximes in plasma and urine

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SUMMARY

Imidazolium oximes are useful in the treatment of organophosphorus agent poisoning. However, the extant methods for analyzing oximes in plasma and urine samples are not adequate. A unique high-performance liquid chromatographic assay method was developed for quantitating either imidazolium or pyridinium oximes. Plasma or urine samples can be injected directly onto the column after a centrifugation filtration step. This method demonstrates a different approach in the method development for quaternary ammonium compounds using non-end-capped reversed-phase columns and a mobile phase containing competing cations. In addition, a preliminary pharmacokinetic study of the imidazolium oxime in rabbits was carried out using this method.

INTRODUCTION

Imidazolium oximes have gained recognition as candidates for universal antidote for organophosphorus compound poisoning [1,2]. There is, however, no reported method to analyze this class of compounds in biological matrices. In the past, assay methods used for quantitating pyridinium oximes in biological samples included spectrophotometric and automated analyzer techniques. These methods are based on the characteristic shift of absorption wavelength of the oxime functional group in alkaline media to a longer-wavelength region and the assumption that the interferences from its metabolites or other impurities are minimal. We have found that imidazolium oximes do not shift as much as mono- and bispyridinium oximes in the alkaline solutions. Therefore,

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spectrophotometric methods are not applicable for quantitating imidazolium oxime class compounds in biological samples. High-performance liquid chromatography (HPLC) has been used successfully in analyzing pyridinium oximes in biological matrices. We used the end-capped μBondapak and compressed-type reversed-phase columns reported in the literature [3,4] and found the imidazolium oximes cannot be resolved from impurity peaks of the sample matrices.

This study reports a non-end-capped column system which can be used for either imidazolium or pyridinium oximes in both plasm and urine matrices. Only a simple centrifugation filtration step is needed in sample preparation. This method is used in a preliminary study of the pharmacokinetics of imidazolium oximes in rabbits.

EXPERIMENTAL

Materials

Imidazolium oximes, 1-(1,3-dimethyl-3-nitrobutyloxymethyl)-2-hydroxyiminomethyl-3-methylimidazolium chloride (I) and 1-[1-(3-butynyloxymethyl)]-2-hydroxyiminomethyl-3-methylimidazolium chloride (II), and 2-hydroxyiminomethylpyridiniummethyl-(4′-carbamoylpyridiniummethyl) ether dichloride monohydrate (HI-6) were synthesized by SRI International (Menlo Park, CA, U.S.A.) with Lot Nos. BL49877, BL49082 and BK75032 respectively. 1,1′-methylenebis{4-[(hydroxyimino)methyl]pyridinium} dichloride (MMB-4), Lot No. BL50736, was synthesized by Ash Stevens (Detroit, MI, U.S.A.). The structures of these compounds are depicted in Fig. 1. Organic solvents and PIC B8 reagent (1-octanesulfonic acid) were HPLC grade and purchased from Waters Assoc. (Milford, MA, U.S.A.). The Centricon-10 microconcentrator was obtained from Amicon Division, W.R. Grace (Danvers, MA, U.S.A.). The distilled water used was deionized by a Milli-Q water purification system. All other chemicals were of analytical-reagent grade. Male New Zealand rabbits were obtained from Hazelton Research Product (Denver, PA, U.S.A.)

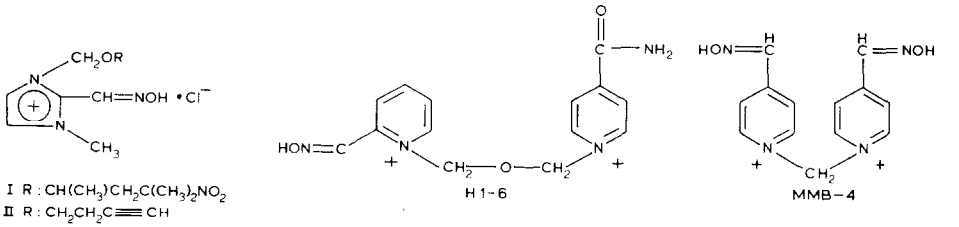

Fig. 1. Structures of imidazolium and pyridinium oximes.

Chromatography

Chromatography was carried out using equipment and Resolve C_{18} columns supplied by Waters Assoc. The equipment used consisted of an M45 pump, a 730 data module, a 720 system controller, a 710A WISP autoinjector and a 481 variable-wavelength UV detector. The separations were carried out using a stainless-steel Resolve C_{18} column (15 cm × 3.9 mm, 5-μm spherical particles, non-end-capped) and a Resolve C_{18} Guard-Pak precolumn. For comparison a μBondapak C_{18} column (end-capped) with the same length and diameter was used to study the effect of competing cation in the mobile phase on the capacity factor of these analytes. The mobile phase consisted of 25% acetonitrile, 5 m*M* PIC B8 reagent and tetramethylammonium chloride (TMA) and was delivered isocratically at a flow-rate of 1 ml/min. The TMA concentration used varied with the analyte as well as with the sample matrices as listed in Table I. Absorbance of the column effluent was monitored by setting the detector at maximum absorbance wavelength for each analyte.

Sample preparation

The plasma and urine samples were first diluted with the mobile phase without acetonitrile at a ratio of 1:1 and 1:10, respectively. The diluted samples were loaded in the Centricon-10 microconcentrator and centrifuged for 30 min in a Sorvall RC-5B refrigerated centrifuge at 10 000 *g*. Macromolecules with molecular mass larger than 10 000 will be retained by the membrane in the concentrator. After this ultrafiltration procedure 20 μl of the filtrate were injected. The validation studies were done using spiked rabbit plasma or urine. In the pharmacokinetic study 80-μl plasma samples were injected if the concentration was less than 0.5 μg/ml. The guard column was changed after every fifty (urine) or hundred (plasma) injections.

TABLE I

TMA CONCENTRATION, RETENTION TIMES (t_R) AND DETECTOR WAVELENGTH FOR EACH OXIME

Oxime	Plasma		Urine		Detector wavelength (nm)
	TMA (m*M*)	t_R (min)	TMA (m*M*)	t_R (min)	
II	1	8.7	0.2	14.4	270
I	6	9.3	3	12.0	270
HI-6	5	7.1	2	15.1	302
MMB-4	7.5	6.6	2.5	10.9	300

Preliminary pharmacokinetic study of imidazolium oxime (I) in rabbits

White New Zealand rabbits, weighing between 2.6 and 4.3 kg, were injected intramuscularly with I at a dose of 0.05 mmol/kg. Prior to the experiment the animals were fasted for 24 h but had access to water ad libitum. Blood samples were collected through the ear vein at 15 min and 1, 2 and 3 h. Pooled urine was collected through a catheter for a period of 6 h.

RESULTS

Chromatography

The retention times of the analytes were affected by the concentration of the competing tetraammonium cation. The retention times of these quaternary oximes and a tertiary amine compound decreased when the competing cation concentration increased. These effects are compared between end-capped and non-end-capped columns as illustrated in Table II. For all the compounds studied the effect is more pronounced on a non-end-capped column as indicated by the Diff value in the table. However, the impurity peaks in the chromatograms of control plasma or urine do not shift much as the TMA concentration changes when the non-end-capped column is used, as shown in Figs. 2 and 3. Therefore, the retention time of analyte can be optimized and the oxime can be eluted in the region free from interference of the impurity peaks by simply adjusting the TMA concentration in the mobile phase. Representative

TABLE II

COMPARISON OF THE EFFECT OF TMA ON THE RETENTION TIMES OF ANALYTES ON TWO TYPES OF REVERSED-PHASE COLUMN

(A) Non-end-capped column; (B) end-capped column.

TMA concentration (m*M*)	Retention time (min)									
	II		I		MMB-4		HI-6		Atropine	
	A	B	A	B	A	B	A	B	A	B
1	20.6	9.8	9.1	5.4	19.9	7.5	20.5	6.4	15.4	8.2
2	15.5	9.1	-	5.0	12.6	6.5	12.1	5.5	11.9	7.7
4	11.0	7.3	5.4	4.2	6.8	4.8	7.2	4.1	8.9	6.3
6	9.7	6.6	4.7	3.8	6.0	4.1	5.4	3.5	7.9	6.0
8	8.3	6.6	4.2	3.8	5.2	3.8	4.2	3.3	6.7	5.9
10	7.3	6.6	3.8	3.8	3.9	3.8	3.5	3.3	6.1	5.9
Diff[a]	13.3	3.2	5.3	1.6	16.0	3.7	17.0	3.1	9.3	2.3

[a]Difference in retention time between 1 and 10 m*M*.

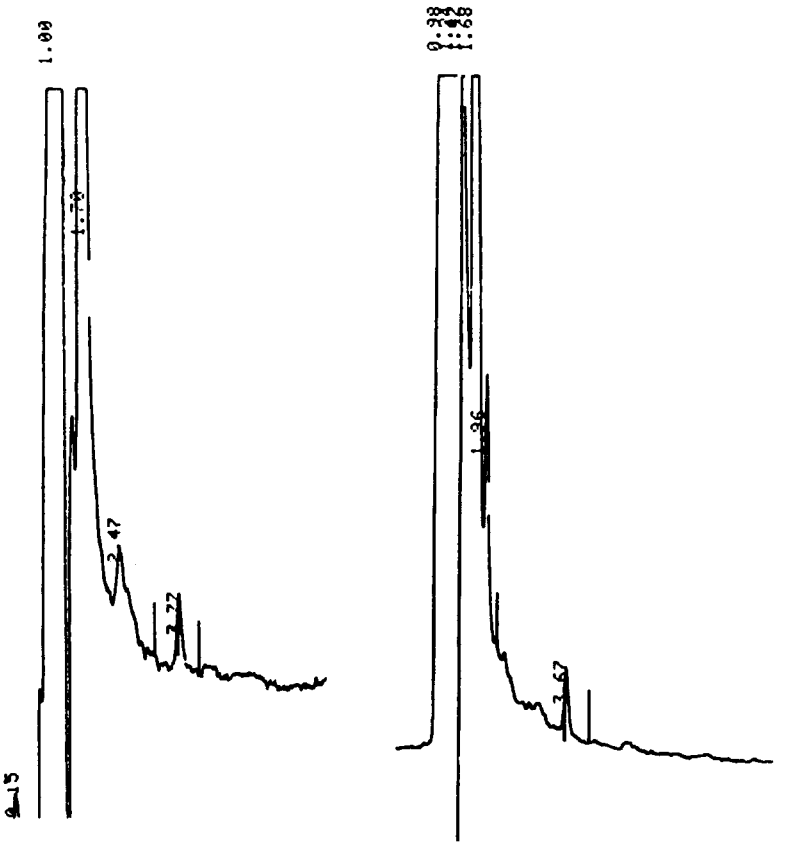

Fig. 2. Chromatograms of blank rabbit plasma. Column, Resolve C_{18}; mobile phase, 25% acetonitrile, 5 m*M* PIC B8 reagent and 1 m*M* TMA (left) or 10 m*M* TMA (right).

chromatograms of spiked rabbit plasma and urine samples are shown in Fig. 4.

Quantitation

The linearity for each oxime was determined at a concentration range of 5–100 μg/ml for urine and 0.05–15 μg/ml for plasma. The correlation coefficients of the straight line from the peak area count versus concentration plot for these oximes were at least 0.9993 or greater. The intra- and inter-day reproducibility data were collected at two concentration levels for the urine sample and at three concentrations for the plasma sample on three different days. The reproducibility data are expressed as percentage recovery and summarized in Tables III–V for I, HI-6 and MMB-4, respectively. The percentage recovery as calculated by comparing the peak areas of the sample filtrate and the aqueous standard spiked at the same concentration. For II, the intra-day reproducibil-

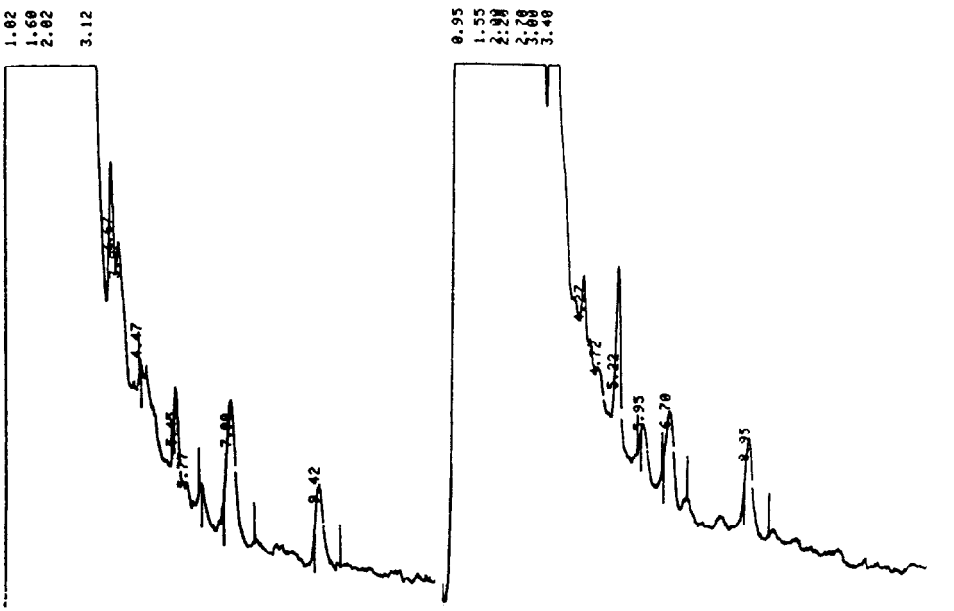

Fig. 3. Chromatograms of blank rabbit urine. Column, Resolve C_{18}; mobile phase, 25% acetonitrile, 5 m*M* PIC B8 reagent and 1 m*M* TMA (left) or 10 m*M* TMA (right).

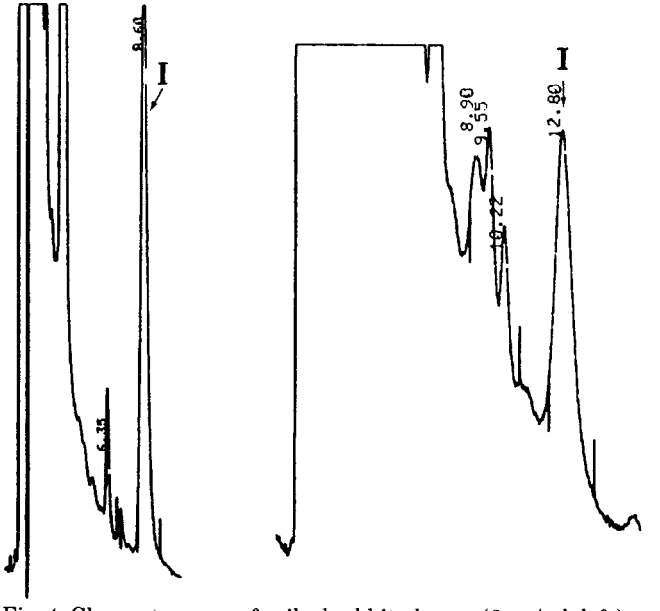

Fig. 4. Chromatograms of spiked rabbit plasma (2 μg/ml, left) and urine (10 μg/ml, right) with imidazolium oxime I. Chromatographic conditions and sample preparations as under Experimental. Sensitivity: 0.002 a.u.f.s.

TABLE III

INTRA- AND INTER-DAY RECOVERY REPRODUCIBILITY OF I FROM SPIKED PLASMA AND URINE SAMPLES

Values in parentheses are coefficients of variation (%).

Concentration (μg/ml)	Mean recovery (%)			
	Intra-day ($n=6$)			Inter-day
	Day 1	Day 2	Day 3	
Urine				
10	97.6 (5.5)	95.3 (2.9)	92.4 (2.9)	95.1 (2.7)
100	99.5 (0.6)	93.0 (0.4)	95.9 (1.3)	96.1 (3.3)
Plasma				
5	95.4 (1.2)	92.8 (1.0)	96.9 (1.7)	95.0 (2.1)
10	97.3 (0.5)	95.4 (2.8)	97.4 (0.7)	96.7 (1.1)
15	97.8 (0.2)	95.4 (1.1)	97.5 (1.0)	96.9 (1.3)

TABLE IV

INTRA- AND INTER-DAY RECOVERY REPRODUCIBILITY OF HI-6 FROM SPIKED PLASMA AND URINE SAMPLES

Values in parentheses are coefficients of variation (%).

Concentration (μg/ml)	Mean recovery (%)			
	Intra-day ($n=6$)			Inter-day
	Day 1	Day 2	Day 3	
Urine				
10	84.6 (6.2)	86.9 (6.7)	91.7 (2.9)	87.7 (4.1)
100	91.7 (1.0)	99.8 (1.0)	96.9 (2.1)	96.1 (4.2)
Plasma				
5	96.7 (0.6)	94.4 (0.6)	96.9 (0.8)	96.0 (1.4)
10	97.5 (0.8)	96.7 (1.5)	96.3 (1.1)	96.9 (0.6)
15	100.0 (0.9)	100.1 (0.6)	96.7 (1.0)	98.9 (1.9)

ity values are in the same range as the other oximes and the multi-day variation study was not carried out. The slopes of the calibration line for II in plasma and urine samples were greater than those for I over the same concentration ranges. The limit of quantitation was measured at a signal-to-noise ratio of 10:1 and found to be 0.05 μg/ml of plasma (injection volume of 100 μl of the diluted filtrate) with a coefficient of variation less than 10% for these oximes.

TABLE V

INTRA- AND INTER-DAY RECOVERY REPRODUCIBILITY OF MMB-4 FROM SPIKED PLASMA AND URINE SAMPLES

The values in parentheses are coefficients of variation (%).

Concentration (μg/ml)	Mean recovery (%)			
	Intra-day ($n=6$)			Inter-day
	Day 1	Day 2	Day 3	
Urine				
10	99.8 (1.7)	98.2 (2.7)	97.6 (5.5)	98.5 (1.1)
100	101.6 (0.7)	99.9 (1.0)	99.5 (0.6)	100.0 (1.1)
Plasma				
5	99.8 (0.4)	98.4 (2.2)	97.6 (0.8)	98.6 (1.1)
10	97.9 (0.3)	99.7 (0.5)	97.9 (0.5)	98.5 (1.0)
15	99.9 (0.4)	100.0 (0.8)	100.2 (0.2)	100.0 (1.5)

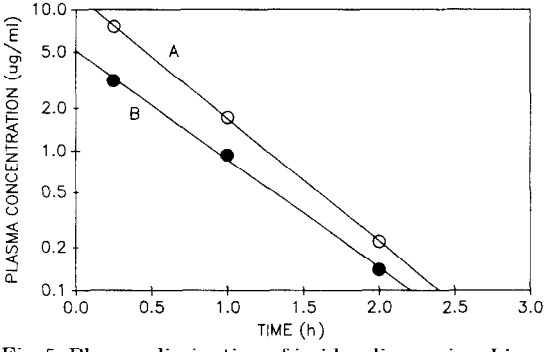

Fig. 5. Plasma elimination of imidazolium oxime I in rabbits. The total dose in the animal is (A) 66 mg and (B) 40 mg.

Preliminary pharmacokinetic study in rabbits

The plasma data for two rabbits dosed with I are plotted in Fig. 5. The compound was rapidly absorbed intramuscularly and eliminated at an average rate constant of 1.90 h^{-1}. A half-life of about 22 min was estimated. The amount excreted unchanged in the urine during a 6-h period was about 26% of the administered dose for I. Some metabolites were seen in the chromatograms but were not characterized structurely.

DISCUSSION

In chromatography the residual silanol groups on the silica-based columns are often undesirable and cause peak broadening and tailing of basic com-

pounds. Manufacturers use exhaustic end-capping procedures or heavy carbon-loading techniques in the production of bonded-phase HPLC columns to minimize the presence of residual silanol groups. Benschop et al. [3] indicated that the separation of HI-6 with an ion-pair reversed-phase system is not by partition but rather an adsorption mechanism. In the solid-phase extraction of the quaternary ammonium compound pyridostigmine, we also observed that the retention of this structure compound on a bonded-phase column depended on adsorption mechanism and the interaction with the residual silanol groups was implicated [5]. Stahlberg and Furangen [6] demonstrated that in ion-pair chromatography the capacity factor of charged analytes depended on the concentration of tetraalkylammonium modifier and can be predicted using electrostatic theory. This substantiated our observation in this study that a much greater effect could be expected when the column was not end-capped or not heavily carbon-loaded. This method further illustrates that the residual silanol groups on non-end-capped or less carbon-loaded reversed-phase columns can be beneficial. In the presence of abundant silanol sites the tetraalkylammonium modifier concentration can be adjusted to optimize the resolution of the cationic analytes in biological material.

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