Fluorescence Detection of Alkylphosphonic Acids Using \( p \)-(9-Anthroyloxy)phenacyl Bromide

Sir: Organophosphonates have found wide use as herbicides, insecticides, and antibiotics (1). Given their biocidal potency, sensitive means for monitoring trace amounts of organophosphonates in the environment are needed. Laser-based detection methods have provided excellent sensitivities in the determination of a variety of compounds (2-7). This report elaborates an analysis technique for methylphosphonic acid (1a), a residue resulting from complete hydrolysis of sarin.

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\begin{align*}
\text{H}_3\text{C} & \quad \text{P} \quad \text{F} \\
\text{O} & \quad \text{R} \quad \text{P} \quad \text{OH}
\end{align*}
\]

\( \text{sarin} \)

1a \( \text{R} = \text{CH}_3 \)
2a \( \text{R} = \text{CH}_3\text{CH}_2 \)
3a \( \text{R} = (\text{CH}_3)\text{CH}_2 \)
4a \( \text{R} = \text{CH}_3(\text{CH}_2)_5 \)

The analysis methodology is applicable to other alkylphosphonic acids such as 2a, 3a, and 4a. Laser-induced fluorescence is used in tandem with microcolumn high-pressure liquid chromatography. Central to the methodology is derivatization with a fluorescent labeling agent, \( p \)-(9-anthroyloxy)phenacyl bromide (5). Laser-induced fluorescence analysis allows detection of these derivatives in the femtomole range.

To date, organophosphonates have been detected via a variety of techniques. Conversion of organophosphonates to

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inorganic phosphate (8) facilitates quantitation by colorimetric assay. Similar colorimetric analysis is used for inorganic phosphate derived from organophosphonates separated by ion exchange chromatography (9, 10). Volatile organophosphorous compounds have been preferentially ionized and analyzed by using molecular secondary ion mass spectrometry (11). Determination of organophosphonates has been achieved by using a dual-flame photometric phosphorus-sensitive detector subsequent to ion-pair reverse phase, high-pressure liquid chromatographic separation (12). Sensitivities in the nanogram range are obtained by derivationization of the monooesters of alkylphosphonic acids with p-bromophenacyl bromide and determination of the adducts formed with absorbance detection (13). Derivatization of the phosphonic acid moiety is also useful in gas chromatographic analysis of organophosphonates. Derivatizing reagents include 3-benzyl-1-p-tolyltriiazene (14), diazoalkanes (15–17), O-methyl-N,N-di-cyclohexylisourea (18), BCl3/2-chloroethanol (19), N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (18), and trifluoroethanol/trifluoroacetic anhydride (20). Gas chromatographic analysis can facilitate detection in the 10–100 ppb range (20).

EXPERIMENTAL SECTION

Apparatus. The HPLC system consisted of an HPLC Micropump (Brownlee Labs., Santa Clara, CA), a Model 7410 Rheodyne injector fitted with a 1-μL loop and a 250 x 1 mm i.d. Adsorbosphere C18 column (Alltech Associates, Waukegan, IL). The 326-nm output of a Model 4060B Liconix He-Cd laser (Sunnyvale, CA) is utilized as the excitation source. The laser radiation (ca. 10 mW) was focused into a 75-μm fused silica capillary connected to the end of the microcolum. Fluorescence collected at right angles to the excitation beam was isolated with a saturated solution of sodium nitrite (to remove Rayleigh and Raman scatter) and a broad-band interference filter (no. 57530, ORiel Corp., Stratford, CT) and focused on a Centronic Model Q 4249B photomultiplier tube (Bailey Instruments Co., Saddle Brook, NJ). The FMT signal was conditioned with a current-to-voltage converter and a low-pass filter before being output to a strip-chart recorder.

1H NMR spectra were recorded on a Varian XL-400 spectrometer and chemical shifts reported in parts per million relative to tetramethylsilane ((CH3)2Si, δ 0.0) with CDCl3 as solvent. 13C NMR spectra were also recorded on a Varian XL-400 spectrometer. Chemical shifts were reported in parts per million relative to tetramethylsilane ((CH3)2Si, δ 0.0).

Chemicals. Phosphonic acids were purchased from Alfa Products (Danvers, MA) and water was removed as its azetrop with toluene immediately before use. Tetra-n-butylammonium hydroxide was purchased from Aldrich and distilled from calcium hydride under nitrogen. N,N-Dimethylformamide was distilled from activated Linde 4A Molecular sieves in the 10–100 ppb range (20).

RESULTS

Derivatization of alkylphosphonic acids such as methylphosphonic acid (1a) serves two purposes. First, the product of derivatization facilitates separation of the alkylphosphonic acid by C18 microcolumn high-pressure liquid chromatography. This is essential to observing the alkylphosphonic acid among a diverse array of other solutes in biological and environmental systems. Second, the derivatized alkylphosphonic acid is
detectable by laser-induced fluorescence which provides sensitivity far exceeding that available for this class of compounds by previous HPLC methods.

A straightforward, inexpensive synthesis can be used to prepare derivatizing agent 5 from readily available starting materials (5 is also available from Molecular Probes, Eugene, OR). Reaction of 5 and 1a affords bis[p-(9-anthroyloxy)phenacyl] methylphosphonate 1b. Formation of the diesterified adducts of alkylphosphonic acids appears to be general in view of derivatization of 2a, 3a, and 4a with 5 to form diesters 2b, 3b, and 4b. The alkylphosphonate must be neutralized for derivatization to occur although excessive amounts of base lead to hydrolysis of derivatizing reagent 5. Therefore, when the number of equivalents of alkylphosphonate are known, Method B is the most convenient protocol. Method A is designed for aqueous solutions of alkylphosphonic acids where the phosphonic acid concentrations are unknown. The tetra-n-butylammonium hydroxide functions as a titrant and, after removal of the water, as a phase transfer reagent which enhances the solubility of alkylphosphonates in dimethylformamide. Both methods afford approximately a 90% yield of derivatized methylphosphonic acid.

The excitation and emission spectra (uncorrected) of diester 1b is shown in Figure 1. A mixture of methyl-, ethyl-, isopropyl- and n-hexylphosphonic acids was derivatized by method B at the 10 mM level and injected after suitable dilution. Figure 2 shows the separation of 10 pmol each of the derivatized mixture of alkylphosphonic acids. The sensitivity of the method was determined by derivatizing methylphosphonic acid, diluting by appropriate volumes, and determining the signal-to-noise ratio down to the detection limit (S/N = 2). Using 8 mW of 325-nm radiation of a He–Cd laser, the detection limit was found to be 20 fmol.

Functionalization of 1a is unique to derivatizing agent 5. Attempted derivatization with a range of other reagents, including 4-bromomethyl-7-methoxycoumarin (6), under a variety of conditions failed to provide any derivatized alkylphosphonic acids. This is particularly notable in view of 4-bromomethyl-7-methoxycoumarin’s effective derivatization of monoalkyl esters of methylphosphonic acid (23). The precedent ability of phenacyl halides to esterify mono- (24), di-, and tribasic carboxylic acids (23) implies that phenacyl halide 5 is more reactive than coumarin 6. p-(9-Anthroyloxy)phenacyl bromide is thus a useful hybrid. As a phenacyl halide, 5 facilitates derivatization of alkylphosphonic acids, while its anthracyl moiety functions as the necessary chromophore for laser-induced fluorescence.

The results obtained show that alkylphosphonic acids can be readily derivatized by use of p-(9-anthroyloxy)phenacyl bromide. The reaction is rapid and the yield is quantitative, making this derivatizing agent an excellent choice for trace analysis. Disadvantages of the method are the low solubility of the resulting derivative in most HPLC compatible solvents, the need to remove water from the system during derivatization to minimize degradation of the reagent, and the fact that unreacted p-(9-anthroyloxy)phenacyl bromide is also fluorescent and shows up in the chromatogram requiring efficient separation of derivatives to avoid interference in quantitative analysis. The bulky derivatizing agent may tend to overwhelm the small structural variations of closely related alkylphosphonic acids making separation difficult. However, the use of a simple gradient can provide excellent separation between the derivatizing reagent and the alkylphosphonic acid
derivatives as shown in Figure 2. Derivatization with p-(9-
anthroyloxy)phenacyl bromide combined with laser-induced
fluorescence is an alternative to the aforementioned methods
currently exploited in organophosphonate detection (8–20).
The ease of derivatization and excellent sensitivity are particular-
ly appealing as a substitute for the use of radiolabels (26)
in organophosphonate analyses.

LITERATURE CITED
(1) The Role of Phosphonates in Living Systems; Hiderbrand, R. L., Ed.;
(7) Smith, L. M.; Sanders, J. Z.; Kaiser, R. J.; Hughes, P.; Dodd, C.; Con-
(13) Bossie, P. C.; Martin, J. J.; Sarver, E. W.; Sommer, H. Z. J. Chroma-
togr. 1983, 267, 209.
(15) Monsanto Chemical Co., Pesticide Analytical Manual; Food and Drug

Optical Activity and Ultraviolet Absorbance Detection of Dansyl
L-Amino Acids Separated by Gradient Liquid Chromatography

Sir: Dansyl [1-(dimethylaminonaphthalene-5-sulfonyl)]
chloride derivatization of amino acids was first used in peptide
sequencing (1) and determination of proteins by fluorescence
polarization (2). Recently dansylation has become a popular
precolumn derivatization method for fluorescence or UV ab-
sorbance detection of amino acids. Reversed-phase or ion pair
reversed-phase HPLC is employed in the separation of the
product mixture. Much work has been done with dansyl
derivatives including determination of reaction byproducts
and optimum conditions for the reaction (3,4). De Jong (5)
determined that quantitative data results if stringent reaction
and chromatography conditions are used. Other workers (6)
have found precolumn dansyl derivatization to be highly
reliable and reproducible, producing variations of less than 5%.
The separation of D and L isomers of dansyl amino acids has
also been accomplished by using chiral β-cycloextrin in the
mobile phase (7) and by mixed-chelate complexation (8).

Many scientific investigations (e.g., geochronology, pharma-
cuticals) have the need to determine enantiomeric ratios of
amino acids and other compounds. It has been reported that
OA/UV or OA/RI (refractive index) are ideal methods
for the determination of enantiomeric ratios without the need
for chiral columns, chiral eluents, or diastereomer preparation
(9). Unfortunately, only three amino acids are naturally UV
absorbing (254 nm), and RI sensitivity for amino acids is low.
Derivatization by several methods (o-phthalaldehyde, dansyl,
phenylisothiocyanate, fluorescamine, 2,4-dinitrofluorobenzene,
and phenylthiohydantoin) renders all amino acids UV ab-
sorbing and makes UV or fluorescence viable techniques for
amino acid determinations. A previously neglected aspect of
derivatization is the effect on optical activity. These highly
polar groups influence the chiral center of amino acids
drastically (electronic and steric effects). The shifting of
the absorption band to the proximity of the wavelength used for
OA measurements further enhances the importance of the
substituent. We report here the determination of 17 dansyl
amino acids in a mixture by UV absorbance and optical ac-
tivity. This involves gradient elution. Previously, the optical
activity detector (OAD) has been used only with isocratic
HPLC. Unfortunately, the OAD is not perfectly selective and
responds to large refractive index changes, very similar to
observations in UV detectors. This is most evident at the void
volume when eluent and sample solvent are not identical. The
change in refractive index displaces and disturbs the collu-
mation of the laser beam, which is interpreted by the detector
as a change in rotation. Thus, gradient HPLC could not be
coupled to the OAD without careful choice of eluents. By
using a previously reported mixture of 0.13 M ammonium
acetate and acetonitrile as eluent (10) in conjunction with a
small change in solvent composition per unit of time, it was
found that gradient separation of dansyl amino acids with the
OAD was possible.

EXPERIMENTAL SECTION
Reagents. The dansyl l-amino acids were obtained from Sigma
(St. Louis, MO). Dansyl-L-aspartic acid (Asp), N-dansyl-L-
asparginic acid (Asn), dansylglycine (Gly), dansyl-L-proline (Pro),
dansyl-L-phenylalanine (Phe), N,N'-dansyl-L-cystine (Cys),
dansyl-L-glutamine (Gln), dansyl-L-cysteic acid (Cya), and N-
dansyl-L-lysine (Lys) were obtained as the free acid; N-dansyl-
L-arginine (Arg), dansyl-L-alanine (Ala), dansyl-L-valine (Val),
dansyl-L-methionine (Met), dansyl-L-isoleucine (Ile), and dans-
yl-L-α-amino-n-butyric acid (Abu) as the cyclohexylamine salt;
N-dansyl-trans-4-hydroxy-L-proline (Hyp), dansyl-L-threonine
(Thr), and N-dansyl-L-tryptophan (Trp) as the cyclohexyl-

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