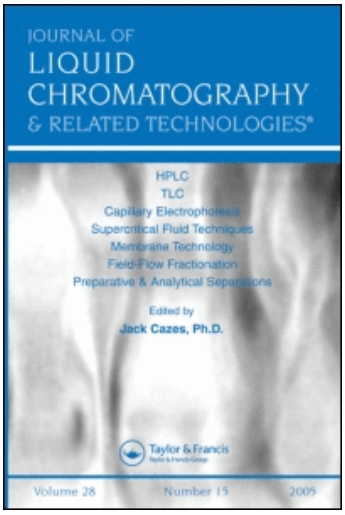

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Derivatization of Aminophosphonic Acids for HPLC Analysis

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DERIVATIZATION OF AMINOPHOSPHONIC ACIDS FOR HPLC ANALYSIS

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ABSTRACT

A general method for the derivatization of alpha-aminophosphonic acids for HPLC analysis is described. The method involves the conversion of the amino functionality to the corresponding 9-fluorenylmethoxycarbonyl (FMOC) urethane followed by esterification of the phosphonic acid moiety utilizing triethyl orthoformate. The sensitivity is compared to that achievable with a previously described N-trifluoroacetyl diethyl ester procedure on the basis of UV detection. Derivative separation using fluorescence detection is also shown.

INTRODUCTION

Since the original discovery of ciliatine (2-aminoethylphosphonic acid) by Kandatsu and Horiguchi[1] which represented the first report of a naturally occurring carbon-phosphorus bond, many relatives have been discovered and much activity has occurred in the preparation of similar compounds. Currently, there are several aminophosphonic acid derivatives either being used commercially or in the process of being approved for use. Just a couple of these are the antibacterial alafosfalin

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[(S,R)-alanyl-1-aminoethylphosphonic acid][2,3] and glyphosate (N-phosphonomethylglycine)[4], the active ingredient in the herbicide formulation Roundup®. In derivatizations of these types of compounds, especially when one focuses on the acidic groups, some differences are immediately seen between the reactivity of the phosphonic acid versus the carboxylic acid moiety. These differences present a further challenge to the chemist as far as derivatization is concerned.

Some of the more classical methods in the past of derivatizing the phosphonic acid function have involved the intermediacy of an acid chloride followed by alcoholysis of the resulting phosphonyl chloride giving the esters[5]. Other more recent approaches to the derivatization of the acidic portion involve trimethylsilylation,[6,7] reaction with orthoformates[8], and reaction with triazenes[9]. It is usually found that there is more effort involved in derivatizing the aminophosphonic acids than the related more naturally occurring aminocarboxylic acids. To further enhance this difficulty, there are some compounds, of which glyphosate is an example, in which both the carboxylic acid as well as phosphonic acid function reside in the same molecule. This represents an even more unique problem.

In the past it has been shown that the orthoformate-phosphonic acid reaction[8] to give phosphonate esters works well and appears to be a facile reaction to exploit with respect to derivatization of the phosphonic acid portion. This gives some chemical selectivity differences of the phosphonic acid group as opposed to the carboxylic acid group. Previous derivatizations with triethyl orthoformate of the N-trifluoroacetyl amides (N-TFA-Et_2) for GC/MS analysis have been reported.[10] We have found these same derivatives to be suitable for analysis by liquid chromatography but at low wavelength UV detection (220 nm). Since increasing the sensitivity would be especially desirable, other avenues of derivatization were explored. The possibility of using an aromatic orthoformate, in particular tribenzyl orthoformate, to give the benzyl esters for increased sensitivity was tried. The results were fairly complicated and many products apparently were formed and the reaction did not go as well as expected. No

chromatographic peaks were found which would correspond to derivative formation. One possible reason for lack of success which may be considered has already been suggested by Daughton[9], that the benzyl esters decompose under reversed phase chromatographic conditions. In further attempts to generate the benzyl esters by a different derivatization procedure for verification purposes, the method of Daughton[9] using 3-benzyl-1-p-tolyltriazene (BTT) was tried. When this was applied in our system, we found that it was also unsatisfactory based on the limited amount of work that was attempted.

Realizing that the amino group could also serve as a derivatization focal point, some possibilities were explored on this basis. Simple Schiff base formation using p-anisaldehyde followed by esterification was tried since the reactivity of the amino group is sufficiently great that it represents the basis of a synthetic procedure for aminophosphonic acids.[11-13] The results on this were not very encouraging either.

It has been reported that phenylisothiocyanate (PITC) can be used for derivatization of aminocarboxylic acids via the phenylthiohydantoin (PTH)[14] or phenylthiocarbamoyl (PTC)[15] derivatives. However, in dealing with a phosphonic acid moiety the same type of chemistry does not apply and prohibits formation of a cyclic derivative analagous to the PTH derivative. However, simple derivatization of the amino group as the PTC derivative followed by orthoformate esterification is possible, but our results by this route again were not encouraging.

At this point, some of the chloroformate based amino blocking groups used in peptide synthesis were considered followed by esterification with a lower alkyl orthoformate. This should convert the amino group into the corresponding urethane and the phosphonic acid group into the diester. The carbobenzoxy[16] (CBZ) group has been widely used and it would possibly serve our purposes due to its aromatic nature. In fact, the corresponding derivative prepared by this route has been reported.[3] However, the

CBZ group is acid labile and would possibly decompose upon being chromatographed in acidic mobile phases frequently employed. More recently the 9-fluorenylmethoxycarbonyl (FMOC)[17] group which has been widely used as a blocking group for peptide synthesis has been reported as a pre-column derivatization reagent for the aminoglycoside drug pirlimycin[18] and amino acids[19]. An advantage of the FMOC group is its acid stability as well as its UV and fluorescent properties[18]. This appeared to have the utility that was desired for our particular needs. Therefore, our attempts were then directed toward the possibility of derivatizing the amino group as the N-FMOC derivative followed by esterifying the phosphonic acid group with triethyl orthoformate. The use of an acid chloride as an amino derivatization reagent also allows for the determination of primary as well as secondary amines which would not be possible with the use of aldehydes as derivatization reagents. We wish to report herein some of our results pertaining to this derivatization procedure.

EXPERIMENTAL

Materials and Reagents

The aminophosphonic acids (APAs) used in this study were synthetic and prepared by a variety of methods as follows: 1-aminoethylphosphonic acid (1-AEPA) from the Curtius rearrangement[20] of triethyl 2-phosphonopropionate[21]; 1-aminopropylphosphonic acid[22] (1-APPA), 1-amino-2-methylpropylphosphonic acid[22] (1-AIBPA), and benzylaminophosphonic acid[23] (BzAPA) were prepared by the ureidophosphonate procedure. The APAs were of differing degrees of purity because of their method of preparation and purification. Because of this some impurities may be manifesting themselves as peaks in the derivative chromatograms. Triethyl orthoformate (TEO) and 9-fluorenylmethyl chloroformate (FMOC-Cl) were used as received from Aldrich Chemical (Milwaukee, WI). The FMOC-Cl was kept refrigerated. Sodium carbonate and concentrated hydrochloric acid were reagent grade. All solvents were B&J Brand high purity solvents (Burdick & Jackson, Muskegon, MI).

Instrumentation

LC analyses were performed on a Model 5060 liquid chromatograph (Varian, Walnut Creek, CA) equipped with a UV100 (Varian) variable wavelength detector. Sample injections were made manually through an electrically actuated valve (Valco EC6W, Houston, TX) which was attached through a precolumn filter (Upchurch A-315, Oak Harbor, WA) into an 9-port, 8-position electrically actuated column selector valve (Valco ECSD8U) with the analytical column in position 8. An OD5 (Burdick & Jackson, Muskegon, MI) C18 column (15 x 0.46 cm) was used for all analyses. The column effluentpassed through an 8-port manifold (Valco Z8M1) en route to the detector. All tubing was 0.010" or less. A precolumn (Upchurch C-130B) filled with C18 silica was placed between the pump and the injector to minimize analytical column damage due to the acidic mobile phases sometimes used. Chromatograms were recorded on a Model 4270 recording integrator (Spectra-Physics, Santa Clara, CA). Fluorescence data was determined on a Model 2070 dual monochromator fluorescence detector (Varian): Excitation 265 nm, Emission 340 nm. Reactions were carried out in a vortex evaporator equipped with a heating block (Buchler, Fort Lee, NJ) at atmospheric pressure.

Derivatization Procedure

A solution of FMOC-Cl was prepared by dissolving 200 mg in 2-5 mL of dioxane, making sure that an appropriate molar ratio was maintained for the experiment involved. A sample of aminophosphonic acid (APA) ranging from 10 µg to 10 mg was dissolved in 1-2 mL of 10% cold sodium carbonate solution in a 10-15 mL centrifuge tube. To this was added 1 mL of cold (12°C) FMOC-Cl solution. The mixture was vortexed at room temperature for 45 minutes to 1 hour. (More recent results indicate that approximately 5 minutes is sufficient reaction time.) This was extracted with several mL of ethyl ether in 2 or 3 portions. An additional mL of water must sometimes be added before the ether extraction. The aqueous phase was acidified with 100 - 200 µl of concentrated HCl and extracted with several mL of ethyl acetate in 2 or 3 portions.

To isolate the N-FMOC-APA residue, the ethyl acetate was removed by evaporation under a stream of nitrogen. To the N-FMOC-APA residue was added several mL of TEO. The mixture was heated and vortexed at 60 to 70 degrees for at least 3 hours. Excess TEO was evaporated under a stream of nitrogen at approximately 50°C. The residue was dissolved in an appropriate amount of acetonitrile-UV for LC analysis. In some cases where a precipitate formed upon addition of the acetonitrile, possibly due to inorganic salts carried through the procedure, the solution was filtered through an Acro LC13 filter, 0.45 micron (Gelman, Ann Arbor, MI) before being chromatographed.

Separation of Aminophosphonic Acids Derivatives

Various APAs were derivatized and their separation accomplished by reversed phase. The chromatographic assay of the mixture was achieved under linear gradient conditions from 20% to 100% acetonitrile in 15 to 20 min. as shown (Fig. 1). With the N-FMOC-Et_2 derivative of BzAPA, the conditions had to be slightly modified to get separation (Fig.2).

Sensitivity of N-FMOC-Et_2 Derivatives Compared to N-TFA-Et_2 Derivatives

A 10 µg sample of 1-AEPA was derivatized as the N-FMOC-Et_2 derivative and chromatographed under the above conditions (See Fig. 3). The response generated was approximately 7% of full scale at 0.160 AUFS for the derivative. In comparison, 10 µg of the 1-AEPA was derivatized to the N-TFA-Et_2 derivative and chromatographed at 254 nm as well as 220 nm. The N-TFA-Et_2 APA derivative was not detected with a reasonable S/N ratio in either case. (Although more sensitivity was potentially possible, the derivatives were still not detectable.) These results as well as those with a 10 mg sample are seen in Fig. 4.

RESULTS & DISCUSSION

Although the technique was not pushed to the absolute detection limits, it is apparent that much greater sensitivity is achieved over the underivatized phosphonic acids or

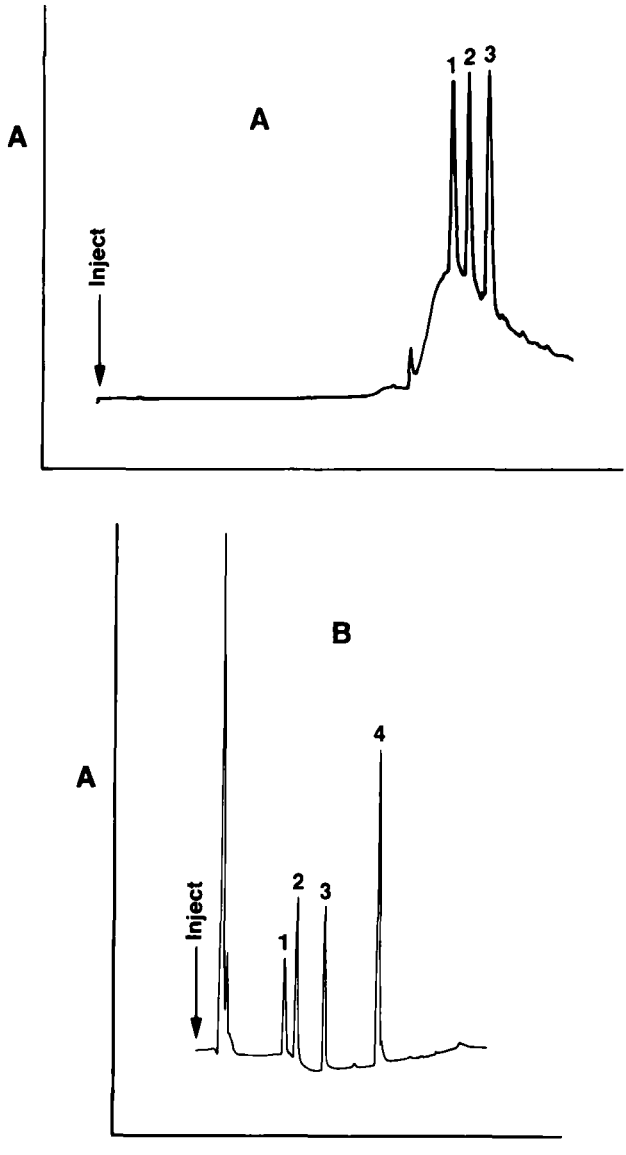


FIGURE 1

A) N-FMOC-Et_2 Derivative Separation - (1) 1-AEPA, (2) 1-APPA, (3) 1-AIBPA; UV-264 nm/0.16 AUFS.

B) N-TFA-Et_2 Derivative Separation - (1) 1-AEPA, (2) 1-APPA, (3) 1-AIBPA, (4) BzAPA; UV-220 nm/0.2 AUFS.

Chromatographic conditions (A&B): Column = OD5, C18, 5 µm, 15 x 0.46 cm; injection volume = 10 µl; solvents: A = water, B = acetonitrile-UV; flow rate = 1 mL/min; gradient conditions - 20-100% B/15 min; initial sample size - 10 mg each component. Abbreviations - see experimental section.

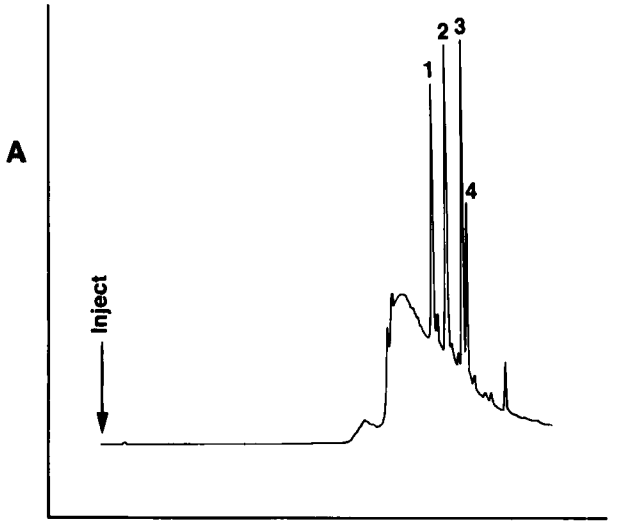


FIGURE 2 - N-FMOC-Et_2 Derivatives - Separation including BzAPA; (1) 1-AEPA, (2) 1-APPA, (3) 1-AIBPA, (4) BzAPA; UV-264 nm/0.08 AUFS.
Chromatographic Conditions:
20% B for 5 minutes, 20-100% B in 20 minutes; other chromatographic conditions as in Figure 1.

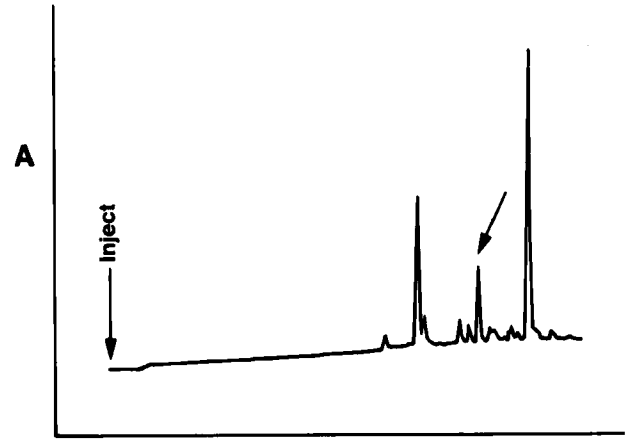
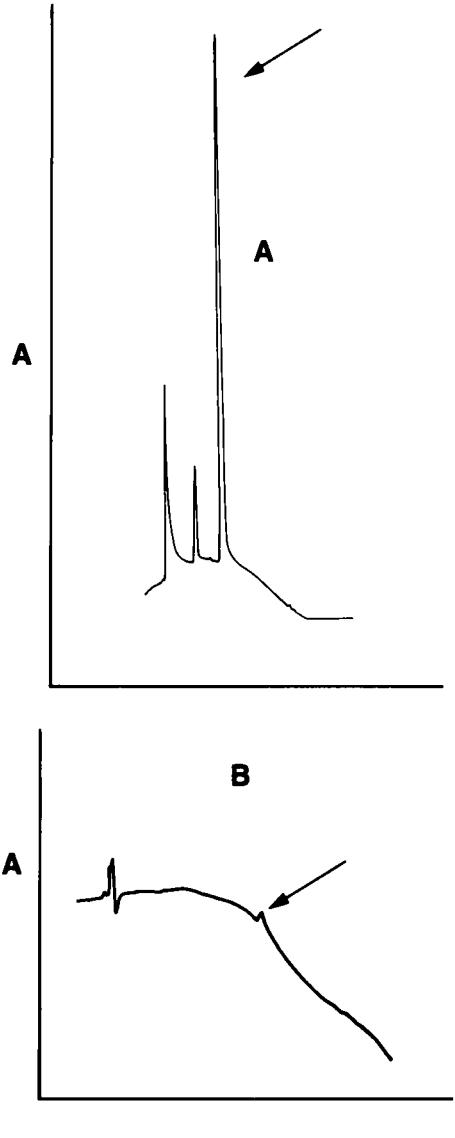


FIGURE 3 - 1-AEPA, N-FMOC-Et_2 Derivative Sensitivity -10 µg sample, pk - 10 ng (800 pmol)
Chromatographic conditions as in Figure 1.



(continued)

FIGURE 4 - 1-AEPA, N-TFA-Et_2 Derivative sensitivity.
Sample sizes: A - 10 mg, B - 10 µg, C - 10 mg; UV-220 nm/0.02 AUFS
Chromatographic conditions as in Figure 1.

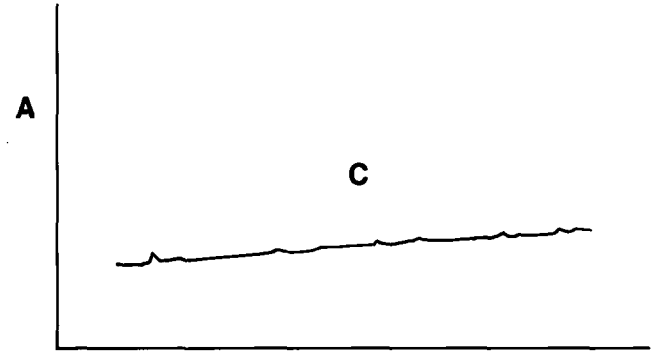


FIGURE 4C

the N-TFA-Et_2 derivatives (see Fig. 4). Because the standard procedure was not optimized with respect to different sample sizes, some variation was seen in the derivatization efficiency. This gave some non-linearity of response for some sample sizes. This can be alleviated by tailoring the method to anticipated sample concentrations. In some instances a broad peak is seen in the chromatograms of derivatized product. At this time, the cause of this is unknown but nevertheless it does not interfere with derivative identification. The possibility that it is due to a buffer component similar to that previously described[19] is possible. The dibenzofulvene polymer that sometimes accompanies FMOC derivatizations could also be responsible[17]. Also sometimes accompanying the derivative peaks are several other peaks the identity of which is unknown, but which again do not interfere with APA derivative identification with the compounds studied. Work is underway to identify these spurious peaks and possibly eliminate them if they turn out to be a consequence of the derivatization process and not due to sample impurities. From some of our work, it appears possible to eliminate most of these interferences by optimizing the overall derivatization technique. Some of our more recent data indicates that a 10:1 molar ratio of FMOC-Cl to APA is a good compromise giving good sensitivity and minimal interfering side peaks. However, in cases where maximum sensitivity is desirable, ratios up to 25:1 may be

necessary. We have also seen from more recent work that substitution of pentane for ethyl ether in the initial extraction step leads to a cleaner derivative chromatogram free from most of the spurious peaks.

When the UV detection is compared with fluorescence detection of the FMOC-Et_2 derivatives, it is seen that the magnitude of increase is approximately the same as has been described by others for aminocarboxylic acids[19]. Separation with fluorescence detection of APA derivatives is seen in Fig. 5. The concentrations of each derivative is approximately 1 µg.

Worthy of note is the fact that our results on "terminal" APAs, of the ciliatine type (e.g.,3-aminopropylphosphonic acid, etc.), were very unexpected. No discernible peaks attributable to derivative formation were detected under the standard procedure and chromatographic conditions used. This was not the case with the corresponding N-TFA-Et_2 derivatives. One possibility is that the lack of any alpha-steric bulk in these compounds resulted in a more linear derivative molecule with increased hydrophobic character which would require greater solvent strength than was used in the standard chromatographic procedure to elute the other alpha derivatives. We are still investigating these species in order to understand the nature of this unexpected behavior.

In conclusion, the N-FMOC-Et_2 derivatization procedure described is very adequate for determination of alpha aminophosphonic acids with good sensitivity. Although the derivatization procedure is more lengthy than the corresponding procedure for aminocarboxylic acids[19], it does include the additional esterification step. This second step is usually necessary for reversed phase separation because the low pKa (approx. 2) of the phosphonic acids many times does not allow for chromatographic retention even in strongly acidic mobile phases. Therefore, they usually elute around the column void volume even under acidic conditions unless esterified. We found this to be the case in the present work when attempts were made to chromatograph the N-FMOC-APA

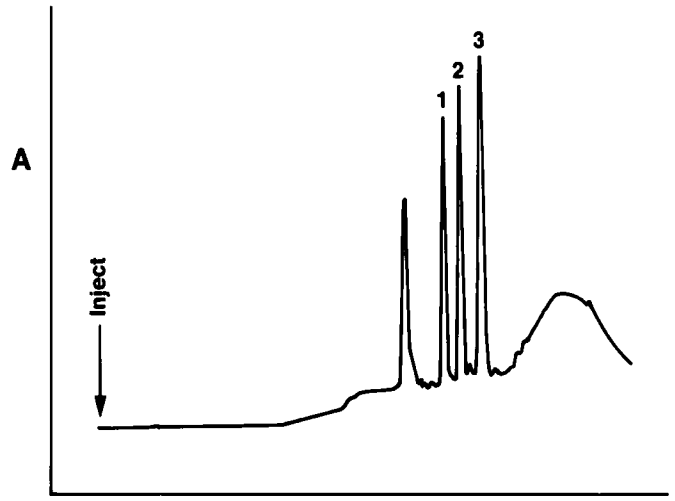


FIGURE 5 - N-FMOC-Et_2 Derivatives - Fluorescence Detection (excitation 265/emission 340)
Chromatographic conditions as in Figure 1.

intermediates. In contrast, the aminocarboxylic acid derivatives can be adequately chromatographed as the free acids[19] using acidic mobile phases. When optimized with regard to expected sample sizes even better results are obtainable with the present method.

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