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Trace Detection of Glycolic Acid by Electrophore Labeling Gas Chromatography−Electron Capture Mass Spectrometry

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As little as 10 pg of standard glycolic acid (glycolate) was detected in a method comprising the following sequence of steps: (1) add glycolate-2,2-d_2 as an internal standard and exchange the carboxylate oxygens in hot HCl/[^{18}O]-water; (2) form an amide derivative with a water-soluble carbodiimide and the electrophoric amine, AMACE1; (3) purify by bypass HPLC; (4) derivatize the residual hydroxy with butyric anhydride; (5) partition with acetonitrile/2 M NaCl; and (6) detect by GC−ECMS. At an intermediate stage in method development, 1 pg of glycolate-2,2,-d_2 could be detected by subjecting it to the above steps 2−6, forming product in an overall, absolute yield of 76%. Step 1 was added after an effort to fully overcome background contamination by glycolate was unsuccessful. For example, background contamination by glycolate could increase rather than decrease when the methanol reagent in the procedure was "carefully purified." The work extends the sensitivity for glycolate detection by ∼100-fold and provides high-performance conditions for the analytical steps employed.

We are interested in the trace detection of the polar metabolites that arise from oxidative damage to the sugars of DNA.[1] In part, the measurement of these metabolites is of interest to better understand how this type of damage is repaired enzymatically.[2] More broadly, oxidative damage to DNA is of interest because it may play a role in aging and some disease processes such as cancer and heart disease. We selected glycolic acid (glycolate) as an initial analyte to test. This compound can form as a secondary metabolite of phosphoglycolate, which in turn arises from oxidative damage at the 4′ position of the deoxyribose residues of DNA.[3] Due to its water solubility and ease of occurrence as a background contaminant, trace glycolate is a challenging analyte. Phosphoglycolate is a product of a reaction catalyzed by ribulose-1,5-biphosphate carboxylase-oxygenase (rubisco), the most abundant protein on earth.[4] Glycolate is an important chemical industrially, environmentally, cosmetically, and clinically as has been summarized recently.[5]

Prior assays for glycolate, whether as a standard, an industrial chemical, or a metabolite, have provided moderate sensitivity, when handling a detection limit[6] is considered. For example, a detection limit of 12 ng for standard glycolate was achieved recently using a plant tissue-based chemiluminescence flow biosensor.[7] An amperometric biosensor was used to detect as little as 90 ng (S/N = 6) of glycolate.[5] Yao and Porter reported the detection of spiked glycolate in serum down to 0.5 ng based on trimethylsilylation/GC-FID,[8] while showing a chromatogram from a sample containing a spike of 25 ng. Soga and co-workers, using capillary electrophoresis-electrospray ionization-mass spectrometry, showed an electropherogram obtained by making an injection from a 20-μL sample containing 152 ng of glycolate and reported a corresponding detection limit of 9 ng considering S/N =3.[9] In an HPLC procedure with UV detection, glycolate was measured in cosmetic products in the low-microgram range.[10] Although previously we reported the detection of a diluted standard of *O*^{2}-pivalyl-3′,5′-bis(trifluoromethyl)benzylglycolate by GC−ECMS at the zeptomole level, anhydrous conditions were used for the derivatization, and the derivatization was performed at the milligram level.[11]

Methods also have been reported for measuring analogues of glycolate. For example, γ-hydroxybutyric acid has been detected at the low-nanogram level by gas chromatography-positive chemical ionization mass spectrometry after conversion to the corresponding lactone,[12] and at the mid-nanogram level by HPLC with UV detection.[13] A low-nanogram level detection limit was reported for lactic acid when measured by trimethylsilylation-GC-FID.[14]

Previously we reported the synthesis of an electrophoric reagent, "AMACE1", which can be coupled onto carboxylic acids such as glycolate in an aqueous buffer, yielding, after further derivatization as necessary, products with excellent detection

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characteristics by GC−ECMS.[15] In part the favorable detection properties come from a built-in site for dissociative electron capture in AMACE1 that yields an analyte-characteristic anion upon electron capture. Here we report the use of this reagent to detect glycolate at the low-picogram level. This required us to overcome two general problems: (1) some of the conditions that worked well at microgram or nanogram levels of glycolate gave poor recoveries or interferences at lower levels, and (2) contamination by glycolate was persistent at the low-picogram level.

EXPERIMENTAL SECTION

Chemicals and Materials. Glycolic acid (99% pure), triethylamine, 1-[(3-dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), 2-(morpholino)ethanesulfonic acid (MES), 1-hydroxybenzotriazole hydrate (HOBT), 4-(dimethylamino)pyridine (DMAP), 1,1,1,3,3,3-hexamethyldisilazane, and butyric anhydride (all used as received) were purchased from Aldrich (Milwaukee, WI). Glycolic acid-2,2-d_2 (glycolate-d_2, 98% isotopic purity) was from Cambridge Isotope Laboratories, Inc. (Cambridge, MA). Acetamide, 2-amino-*N*-[[3,5-bis(trifluoromethyl)phenyl]methyl]-*N*-methyl monohydrochloride (AMACE1: Aminoacetamide Electrophore 1) was prepared as described.[15] Triethylamine phosphorus pentoxide and all organic solvents (including those used for cleaning) were Optima Grade from Fisher (Pittsburgh, PA). Glass inserts for derivatization and HPLC purification (250 and 350 μL, types 500−304 and 500−305, respectively) were from Sun International (Wilmington, NC). Reagent test tubes (14-961-26) for preparation and dilution of stock solutions were from Fisher and were capped as needed with aluminum foil followed by caps 540-001 from PGC Scientific (Frederick, MD). All solutions were v/v unless indicated otherwise. "Al-P capping" refers to capping a vial or tube capped firmly with aluminum foil and then Parafilm. Sonication was done in a model 9331 apparatus (Elma, Dubuque, IA). Vortexing was done on a Genie 2 (Fisher Scientific). Evaporations were done in a SpeedVac SC 100 (Savant Instruments, Inc., Farmingdale, NY) Automatic shaking at room temperature was done on a Mistral Multi-Mixer (Lab-Line Instruments, Melrose Park, Ill). Heating/stirring of the AMACE1 reaction at 40 °C was done in a Genie 2 placed in an oven. Double-distilled (dd) water was prepared by first passing distilled water through a D3700 series Nanopure II cartridge deionization system (Barnstead Co., Boston, MA), fitted with an ion-exchange and a carbon column, and then distilling it twice in an all-glass apparatus that was dedicated to this purpose, rinsing the apparatus between the distillations with the water that was just distilled.

Glassware Cleaning. Glassware was cleaned in one of three ways. Procedure **A** (distillation apparatus including flasks for synthesis or nanogram-level analysis): soak glassware overnight in KOH-saturated 2-propanol (solution was reused until it became colored; after ~1 month); place in a tray and flush with tap water for 4 h with flask emptying after each half hour; shake water out and put onto Kimwipes EX-L paper in a clean basket until almost dry; sonicate in methanol and then water for 15 min and cap loosely with aluminum foil; dry in 120 °C oven for 1 h; cool in desiccator and use within 1 day. Procedure B (disposable inserts; insert holder vials; reagent test tubes for picogram or lower level analysis): soak in methanol overnight; change and 15-min sonicate in methanol twice and in dd water once; rinse with dd water again and put up-side down and air-dry in a clean beaker, then cover with aluminum foil and dry in 120 °C oven for 1 h, cool to ambient T, and gas-phase silanize as described below. Procedure C (Pasteur pipets): sonicate twice in methanol and then gas-phase silanize the ones for use in the analytical procedure.

Gas-Phase Silanization. A model 1410 vacuum oven from VWR Scientific (Boston, MA) was used that was modified as follows: the line from the vent valve was connected to an injection septum followed by a valve (a) to N_2, and the vacuum valve was connected to an external three-way valve (b) that gave the options of venting to a hood, to an oven closure, or connection to a high vacuum. By relying on the vacuum gauge on the oven, glassware in the oven preset to 200 °C was subjected to the following conditions: vacuum (to 28 in. of Hg) and N_2 fill (to 20 in. of Hg and wait 10 min before next vacuum) 3×; vacuum (28 in. of Hg), N_2 (25 in. of Hg), inject 0.8 mL of 1,1,1,3,3,3-hexamethyldisilazane (Aldrich, 99.9%), and stand overnight (vacuum 20 in. of Hg observed next day); vacuum and N_2 purge as above except 30 min of vacuum and N_2 to 0 in. of Hg each time) 5×; turn off heat, open oven, and remove glassware when cool enough to handle.

Synthesis. *Dimethyl Amide, 2-Tritylamino-N-propionic Acid Diethylammonium Salt.* To a solution of 1.6 g (5 mmol) of triphenylmethyl bromide in 10 mL of $CHCl_3$/DMF (2:1), 0.18 g (2 mmol) of alanine was added, followed by vigorously stirring until a clear solution resulted. After adding 8.0 mmol of TEA dropwise in 2 mL of $CHCl_3$/DMF slowly and waiting 30 min, 10 mL of methanol was added followed by heating at 50 °C for 2 h. Evaporation under reduced pressure gave a residue that was dissolved in 20 mL of diethyl ether, and the solution was washed with 3 × 10 mL each of 10% citric acid and then water. After drying over anhydrous sodium sulfate, 2 mmol of dimethylamine in 2 mL of diethyl ether was added, followed by evaporation under reduced pressure and purification by flash column chromatography (acetone:hexane = 2.5:1), giving a white powder (0.69 g, 86%)): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.54−7.25 (m, 15H), 3.22−3.10 (dd, 1H), 3.10−2.95 (dd, 4H), 1.30−1.27 (t, 6H), 0.90−0.86 (d, 3H). *Propionamide,2-amino-N-[{(3,5-bis(trifluoromethyl)phenyl}-methyl]-N-methyl-, Monohydrochloride (AMACE II)*. By relying on the same reactions used to synthesize AMACE I,[15] 0.31 g of the prior acid was converted into a white powder (0.26 g, 92%): ^{1}H NMR (DMSO, 300 MHz) δ 8.23 (s, 2H), 8.06−7.95 (m, 3H), 4.80−4.65 (dd, 2H), 4.18 (s, 1H), 3.11 (s, 3H), 1.29−1.24 (d, 3H).

Reagent Purification. Methanol and acetonitrile were distilled twice in this order before each procedure (use within same day) in distillation apparatus 9317-03 (ACE Glass, Vineland, NJ) as follows: charge a 250-mL round-bottom flask with 200 mL of solvent (plus 2 g of $LiAlH_4$ for the methanol) and a magnetic stirring bar, distill at 20 drops/min and discard first 20 mL, collect 100 mL in 200-mL round-bottom flask, and repeat the distillation of the latter in the same way (but omitting $LiAlH_4$) to yield 30 mL in a 50-mL round-bottom flask that was glass-stoppered and kept in a glass box until use. Between each solvent purification, the distillation apparatus was air-dried and then oven-dried. Acetoni-

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trile was stirred with 5 g of P_2O_5 for 1 h at room temperature before subjecting this mixture to the first distillation and, similarly, 1 g of P_2O_5 for the second distillation. In a second, equivalent distillation apparatus, similarly distill triethylamine twice (recovering in a 50-mL flask), apply a glass stopper, and store in a refrigerator for use within 1 month. A separate stirring bar was dedicated to each of the eight distillations that had first been cleaned by sonication in its assigned solvent. The dd water was prepared on the day of use as described earlier.

Stock Solutions. *Glycolate.* A 20-mg sample of glycolic acid was dissolved in 1 mL of methanol, and a 4× dilution of 10 μL into 1 mL gave 1 pg/5 μL for testing. The stock solution was stored with AL-P capping.

MES, HOBT, and EDC Solutions. MES (213 mg) was dissolved in 10 mL of methanol/water (1:1) with stirring, diluted 100× to 1 mM, and the pH adjusted to 5.0 by 1 N NaOH (freshly prepared). HOBT (6.8 mg) was dissolved in 1 mL of methanol and diluted 100× with pH 5.0 MES buffer to give 2.5 nmol/5 μL. EDC was divided into 1-g amounts and placed in reagent test tubes followed by nitrogen filling, AL-P capping, and storage in a vacuum desiccator up to at least 6 months. Each tube was opened only once, and then 8.4 mg was dissolved and diluted 100× in methanol to give 2.5 nmol/5 μL. These solutions were used within 6 h of preparation.

AMACE1 and TEA Solutions. AMACE1 (7 mg) was dissolved in 0.5 mL of methanol and diluted 100× with pH 5.0 MES buffer to give 2 nmol/5 μL. TEA (8.4 μL) was dissolved in 1.0 mL of methanol and diluted to 3 nmol/5 μL for the AMACE1 reaction and to 10 nmol/5 μL for butyrylation. These solutions were used within 6 h of preparation.

Instrumentation. A SPD 111V SpeedVac with a UVS400 universal vacuum system (Thermo Savant, Holbrook, NY) was used for drying of glycolate and glycolate-d_2 solutions. For HPLC purification of AMACE1 derivatives of glycolate and glycolate-2,2-d_2 after step 2 (see Figure 1), a bypass HPLC method was used as described.[16] Two, nearly equivalent (6-month difference in prior use) Zorbax SB-C18 columns (4.6 × 150 mm, 5 μm) were used in a HP 1100 series HPLC system (Agilent Technologies, Wilmington, DE**)** in contact with a G1313A autosampler and a G1315A DAD detector. Acetonitrile/0.1% trifluroacetic acid (40:60) at a flow rate of 1.2 mL/min was used as the mobile phase with a detection at 265 nm. Gas chromatography electron capture mass spectrometry (GC−ECMS) was performed with a HP-5MS (HP−Ultra 2) cross-linked 5% phenyl methyl siloxane capillary column (25 m × 0.25 mm × 0.25 μm) installed in a GC 6890 series gas chromatograph connected to a 5973 Network mass-selective detector controlled by an HP5973 MS ChemStation data system (Agilent). An Agilent 7683 autosampler was used in the cold, on-column injection mode with the inlet pressure at 18.5 psi. The oven temperature was begun at 70 °C, ramped immediately after injection at 35 °C/min up to 290 °C, and held for 3 min. The source was zoned at 230 °C with the MS Quad at 106 °C. Single ions were monitored at 219, 217 and 215 *m*/*z* with a dwell time of 50 ms. Helium (40 psi) and methane (20 psi; UHP Grade, Medical-Technical Gases, Inc., Medford, MA) were used as carrier and reagent gases, respectively.

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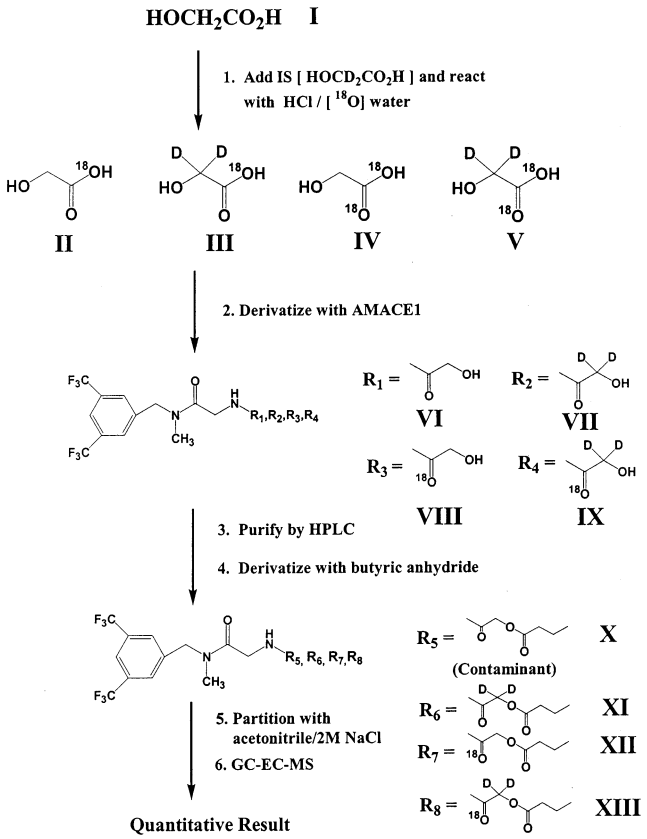

Figure 1. Analytical scheme for detection of glycolic acid.

Determination of 10−100 pg of Glycolate. *Step 1*. Capillary reaction tubes were prepared by flame-sealing one end of a 100-μL disposable glass pipet (Catalog No. 21-164-2H, Fisher, Pittsburgh, PA) with a Microflame Gas Torch (Microflame Inc., Plymouth, MA). Ten picograms of glycolic acid-2,2-d_2 in water was added to each tube followed in duplicate by 10, 20, 50, or 100 pg of glycolic acid in water. The tubes were dried at room temperature in a SpeedVac system and then charged with 10 μL of HCl-saturated [^{18}O]water. After each tube was flushed through a needle with argon for 10 s, the closed end was touched to solid CO_2, the other end was flame-sealed, and the tube was placed in a 90 °C oil bath for 3 h. The contents were transferred into a 250-μL glass insert tube and dried under nitrogen at room temperature.

Step 2. To the dried insert tube was added 2 nmol of AMACE1, 2.5 nmol of HOBT, and 2.2 nmol of EDC (using the above stock solutions), giving 12 μL in pH 5.0 methanolic MES. The tube was Al-P capped, placed in a vial, and vortexed at room temperature overnight.

Step 3. After evaporation under nitrogen, the residue was dissolved in 10 μL of acetonitrile/water, 9:1, vortexed for 20 s, and autoinjected into the purification HPLC column of a bypass HPLC system[16] (which indirectly monitors retention time for quality control), and the product in 0.2 mL was collected into a borosilicate glass tube (12 × 75 mm, Fisher Scientific) followed by evaporation in a SpeedVac system.

Step 4. The evaporated residue was transferred with 2 × 30 μL of acetonitrile into a new 250-μL insert tube and evaporated. A total of 120 nmol of TEA was added followed by shaking (5 s) and addition of 110 nmol of butyric anhydride and 110 nmol of DMAP from the above stock solutions, yielding a total volume of

15 μL in acetonitrile. The tube was Al-P capped, shaken at room temperature overnight, and evaporated.

Step 5. The residue was dissolved with 0.5 min of manual shaking in 30 μL of acetonitrile and transferred into a 350-μL insert tube followed by two 20 μL of similar transfers. A 30-μL aliquot of 2 M NaCl was added. After 2 min of vortexing, both phases were pulled into a silanized Pasteur pipet. A sharp boundary formed, and only the upper phase was transferred to a new 250-μL conical insert followed by evaporation.

Step 6. The residue of one tube at a time was dissolved in 10 μL of hexane and placed in the GC autosampler containing two washing solvents (methanol and ethyl acetate) in separate vials. After washing the injector to waste with 3 × 4 μL of methanol and 2 × 4 μL of ethyl acetate, 1 μL of the sample was injected.

RESULTS AND DISCUSSION

Our method for determining glycolate is summarized in Figure 1. In this method, the carboxyl oxygens of glycolate are exchanged with ^{18}O at the outset to distinguish analyte glycolate from background (method-derived) glycolate. The following discussion is organized according to the six steps of this procedure.

A. Analytical Procedure. *Step 1. Add Internal Standard ($HOCD_2CO_2H$) and React with HCl/[^{18}O]Water*. This step was introduced into our procedure after we were unsuccessful in overcoming glycolate contamination at the low-picogram level by purifying reagents, using clean conditions, and testing alternative reagents and conditions (see below). The conditions that we adopted (90 °C for 3 h in [^{18}O]water saturated with gaseous HCl) were selected after we found this exchange reaction to proceed much slower when conducted at room temperature or with 2 M HCl at 60 °C. The reaction was done in a small volume (10 μL) in a flame-sealed glass tube, since [^{18}O]water is expensive, and for the purpose of minimizing contamination. The carboxyl oxygens of amino acids have been exchanged in 1 N HCl in [^{18}O]water at 60 °C for 2−3 days,[17] and of valproic acid (9.8% single labeled and 90.2% double labeld) in 0.4 M HCl at 75 °C after 4 days.[18]

Step 2. Derivatization with AMACE1. In this step, ^{18}O-exchanged glycolate and glycolate-2,2-d_2 in MES buffer are derivatized with AMACE1 in the presence of HOBT and a water-soluble carbodiimide (EDC) to form products **VI**−**IX** (Figure 1). Conditions for this step were optimized by testing nanogram amounts of glycolate where the yield of product could be monitored by HPLC. At this level of analyte, the final conditions selected gave 90% conversion of glycolate to product. Initially *N*-hydroxysulfosuccinimide was tested instead of HOBT, but HOBT was easier to purify. As reported before, the low pK_a (8.2) of AMACE1, by design, enables it to react efficiently with active ester derivatives of carboxylic acids at pH 6.0 in MES buffer.[15] Here we found that the yield is slightly higher at pH 5.0, but it drops to 40−50% when the reaction is conducted outside the pH range of 4−7. Initially, 100 mM MES was employed when nanomole levels of glycolate were tested, but later the concentration of this buffer was lowered to 1 mM in order to yield a smaller residue of it after evaporation prior to the next step. While this step also can be performed at room temperature for 24 h, we observed that the yield similarly went to completion (tested at the nmol level) after 4 h at 40 °C. A 5-h reaction at 40 °C was selected. Before step 1 was added to the procedure, we tested alternate reagents in this derivatization reaction (dicydohexylcarbodiimide and diisopropylcarbodimide instead of EDC, and cacodylic acid buffer instead of MES). This was not successful in reducing glycolate contamination. Substituting AMACE2 for AMACE1 also failed to reduce this contamination. The latter experiment was motivated by the concern that the $COCH_2NH_2$ moiety of AMACE1 might degrade to form glycolate prior to or during the procedure.

Methanol is a cosolvent in step 2. When we began to focus on the problem of glycolate contamination, methanol was not one of the first reagents we worried about. This is because we already were doubly distilling HPLC-grade methanol on the day of use. Further, methanol is a single-carbon reagent, which might obviate its oxidative conversion to glycolate. But when the problem of glycolate contamination persisted after we purified or substituted the other reagents in the procedure, we turned our attention to the methanol.

To investigate methanol more carefully as a potential source of contamination by glycolate in step 2 of our method, and also the water in this reaction at the same time, we conducted the reaction in methanol-d_4 (99.8 atom % D, Aldrich) and water-d_2 (99.9 atom % D, Aldrich). (These solvents were used as received because of their cost.) However, the contamination level of glycolate increased 100-fold. Instead, more rigorous conditions were studied for purifying the methanol. We redistilled the methanol as separate batches in four different ways: from $NaBH_4$, metallic sodium spheres, $KMnO_4$/HCl, and $LiAlH_4$. Relative to our usual technique of purifying the methanol twice by distillation without additives, all of these techniques, except the use of $LiAlH_4$, increased rather than decreased the level of contamination by glycolate. Subsequently we purified the methanol by distilling it from $LiAlH_4$.

Apparently glycolate contamination is both ubiquitous and dynamic: it can be constantly and irreproducibility produced by oxidation of ubiquitous organic matter at the trace level in many reagents and under many conditions, even purification conditions. (We were reminded of our observation that a highly purified reagent—essentially a single peak by capillary electrophoresis with laser-induced fluorescence detection—was actually contaminated with many oxidative degradation products as seen by injecting an off-scale amount, observing many nearby contamination peaks, and then discovering that most of the peaks became intensified upon addition of H_2O_2.[19]) At this point in our work, we added step 1, in which glycolate analyte is converted to 1,1-[^{18}O]$_2$-glycolate as a way to minimize contamination by adventitious glycolate.

Step 3. Purify by HPLC. We studied several techniques for removal of residual AMACE1 from intermediate **VI** (see Figure 1) before we selected HPLC for this purpose. (At this stage in method development, only **VI** was formed.) These techniques fell into the two categories of extractions (both liquid and solid phases) and reactions (e.g., react residual AMACE1 with a liquid- or solid-phase reagent such as poly(aspartic acid) active ester, sulfosuccinic acid active ester, activated agaroses (aldehyde-,

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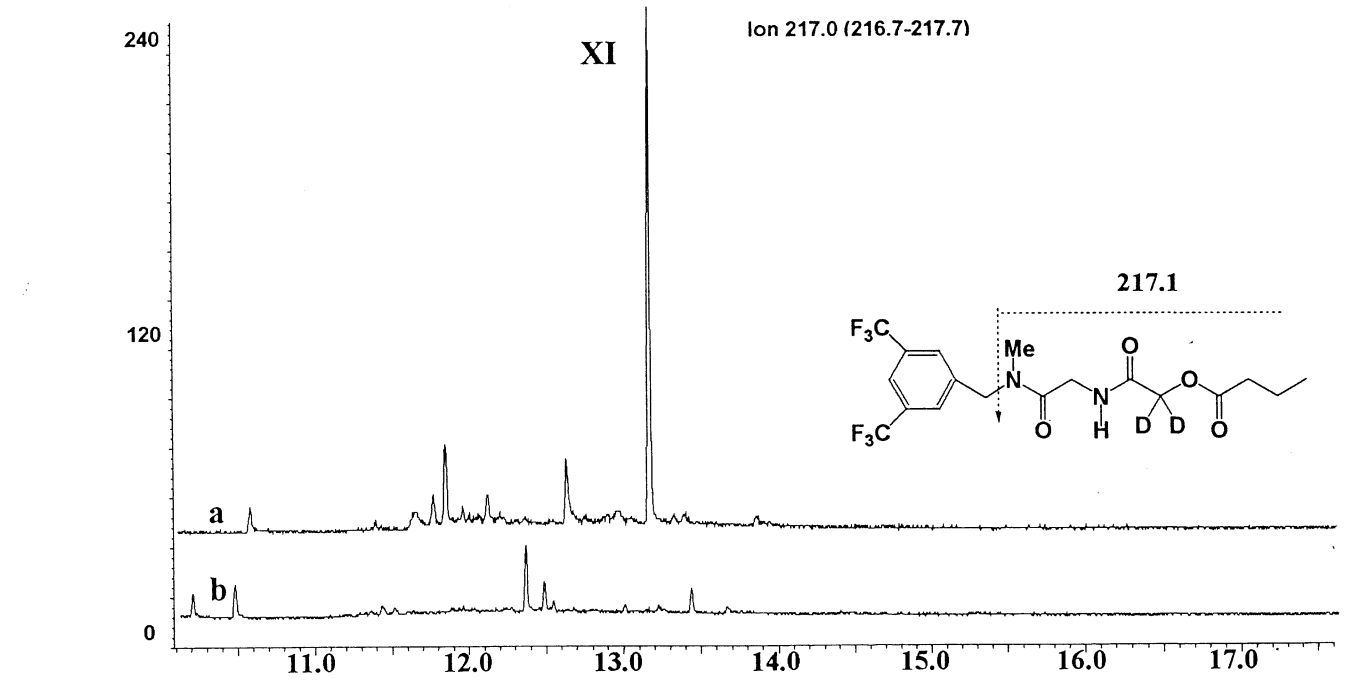

Figure 2. GC−ECMS chromatograms obtained by subjecting 1 pg of glycolate-2,2-d_2 (a) and a blank (b) to the steps 2−6 of the procedure shown in Figure 1. Of the final sample volume of 10 uL, 1 uL was injected.

N-hydroxysuccimidyl- and 1,1′-carbonyldiimidazole-activated agarose from Sigma, and epoxy-activated agarose from Pierce) and a 4-benzyloxybenzaldehyde polystyrene resin from NovaBiochem (La Jolla, CA). Use of these techniques was fairly successful at the nanomole level (e.g., removal of 70−90% AMACE1 with a 80−90% recovery of **VI** by using 4-benzyloxybenzaldehyde resin or aldehyde-agarose from Sigma), but 100% removal of AMACE1 was never observed, and higher amounts of these reagents lowered the recovery of **VI** or added interferences, especially when smaller amounts of **VI** were tested. HPLC vastly outperformed the other strategies that we tested (complete removal of AMACE1, no added interferences, quantitative recovery of **VI**), and so was adopted.

Step 4. Derivatization with Butyric Anhydride. The butyrylation reaction was initially performed in pyridine in anhydrous acetonitrile at 60 °C overnight. While the reaction proceeded quantitatively and cleanly for micromole amounts of glycolate, interferences showed up at lower levels of glycolate, and the yield dropped markedly, for example, to 83% and then 19% when 320 and 40 pmol, respectively, of glycolate were derivatized. High yield and absence of interferences were maintained at this level by using triethylamine (TEA) instead of pyridine and conducting the reaction at room temperature. Lowering the reaction temperature is a well-known technique for minimizing interferences in trace derivatization.

Step 5. Partition with Acetonitrile/2 M NaCl. Initially HPLC was used successfully for step 5, but later work showed that significant purification could be achieved by organic/aqueous partitioning. We chose a nonsaturating concentration (2 M) of NaCl in the aqueous phase to minimize NaCl fines in the acetonitrile phase. When 1 pg of standard **X** was extracted using these conditions, a quantitative recovery was observed.

At one stage in method development (prior to adding step 1), step 5 was done as follows: extract the reaction solution with 2 M NaCl and inject into HPLC. This led to GC−ECMS chromatograms in which the peak for **X** progressively tailed as samples were injected. We postulated that either residual triethylamine or NaCl in the HPLC fraction for **X** was responsible for this behavior. A subsequent experiment involving the presence and absence of these ingredients demonstrated that triethylamine was the origin of this problem, which, in part, led to the final conditions for step 5 (removal of triethylamine by evaporation) that avoid this problem.

Step 6. GC−ECMS. This is the first time that we have conducted trace GC−ECMS with a GC autoinjector. By redissolving our samples one at a time in hexane for injection in this way, we were able to handle redissolved volumes as small as 3 μL, where 1 μL was injected. A comparison of the splitless, pulsed splitless, and cold on-column injection modes with the autoinjector, conducted at the 10-fg level, showed that the latter technique gave about a 2-fold higher response than the others and therefore was selected. Loss in peak efficiency (after ∼150 injections) was overcome by removing the first 20 cm of the GC column.

B. Detection of 1 pg of Glycolate-2,2-d_2. We first encountered the contamination problem of background glycolate when we began to develop our method at the subnanogram level, after conditions had been set up that performed well at the microgram and nanogram levels. Prior to bringing step 1 (^{18}O exchange reaction) into the procedure, we tested glycolate-2,2-d_2 as an analyte in order to focus on recovery. For this analyte, contamination was not a problem. As shown in Figure 2, 1 pg of glycolate-2,2-d_2 can be detected by subjecting it to steps 2−6 of Figure 1. The absolute yield throughout the overall procedure is 76% (SD = 17%, n = 3). The precision for this measurement is low due to the absence of a stable isotope internal standard.

Detection of 10 pg of Glycolic Acid. As explained above, much work remained after 1 pg of glycolate-2,2-d_2 was detected

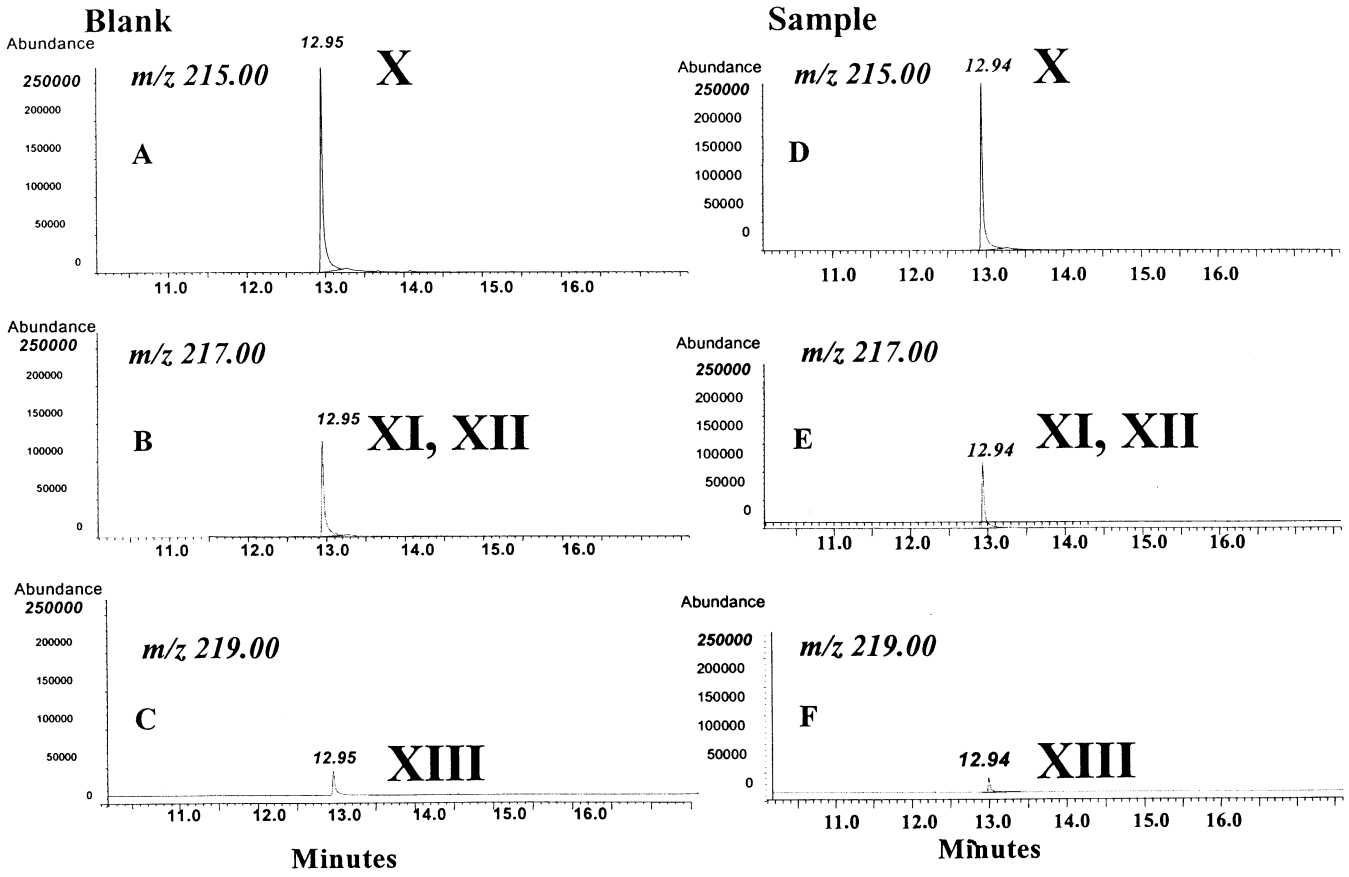

Figure 3. Representative GC−ECMS chromatograms for the detection of 10 pg of glycolate according to the scheme shown in Figure 1. Blank peak heights: X, 294,570; XI + XII, 119,048; XIII, 24,390. Sample peak heights: X, 271,818, XI + XII, 127,065: XIII, 31, 956.

before we were able to manage glycolate contamination (by introducing step 1), so that glycolate itself could be detected at a trace level (10 pg). Representative GC−ECMS chromatograms for the blank and 10-pg samples are shown in Figure 3. As seen in Figure 3A, even the blank sample gives a peak for glycolate, apparently because of glycolate contamination in step 1, step 2, or both, of the procedure. Since glycolate-d_2 is added to the blank, and the exact percent of [^{18}O] exchange at the trace-analyte level is unknown, then it is unclear how much the peak at m/z 217 is due to [^{18}O]glycolate versus residual glycolate-2,2-d_2. Only the percentage of the glycolate peak in Figure 3B (2.3%) due to the presence of natural isotopic forms of glycolate (1.1% of the glycolate peak in Figure 3A) is known. Because of this uncertainty, one can only set up an empirical calibration plot, based on the amounts of authentic glycolate present in a series of standard samples. Plotting the ratio of peak height at m/z 217 to that of m/z 219 against added amounts of glycolate (5 data points, each from duplicates) from 0 to 100 pg gave a linear curve ($y = 7.8329X + 0.1246$ with $r^2 = 0.9993$; data not shown). In the calculation for this plot, the peak height of m/z 217 first was corrected by removing the isotopic contribution (1.1%) from m/z 215 to 217.

CONCLUSION

A sensitive method is reported for detecting standard glycolate in water. Application of the method to biological and other real samples remains to be done. Highly optimized conditions for sample handling including derivatization explain the trace detection limits achieved. These high-performance conditions should be useful not only in applying the method to related analytes but also more generally in setting up procedures of this type. If the other fractions of the HPLC separations were collected from real samples and subjected to GC−ECMS, many analytes of this type no doubt would be detected.

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