ELSEVIER

Journal of Chromatography A, 721 (1996) 289–296

JOURNAL OF CHROMATOGRAPHY A

2-Phosphoglycolate and glycolate-electrophore detection, including detection of 87 zeptomoles of the latter by gas chromatography–electron-capture mass spectrometry

Poguang Wang[a], Veeravagu Murugaiah[a], Bernice Yeung[a,b], Paul Vouros[a,b], Roger W. Giese[a,b,*]

[a] *Department of Pharmaceutical Sciences, Bouvé College of Pharmacy and Health Professions, Barnett Institute, Northeastern University, Boston, MA 02115, USA*

[b] *Chemistry Department, Northeastern University, Boston, MA 02115, USA*

First received 17 May 1995; revised manuscript received 13 July 1995; accepted 17 July 1995

Abstract

As a first stage towards a goal of studying some aspects of oxidative damage to DNA and its subsequent repair, we set up three techniques for the detection of 2-phosphoglycolate (PG). This compound is released as a metabolite from the DNA in certain cases of this process. We explored three techniques because we wanted to learn which one(s) would be most sensitive, given the anticipated availability of small biological samples for analysis. By employing indirect detection with fluorescein as the fluorophore in capillary electrophoresis, we detected $5 \cdot 10^{-6}$ *M* PG (corresponding to 5 pmol/μl, projecting that a final sample volume of 1 μl could be handled). The specificity of this technique can be enhanced by converting the PG to glycolate enzymatically. Flow injection analysis (FIA; 1.0-μl injection volume) negative-ion electrospray mass spectrometry was similarly sensitive ($1.1 \cdot 10^{-6}$ *M*). Based on our prior experience, substituting capillary HPLC for the FIA in this technique is anticipated to lower the detection limit by 20- to 150-fold. Gas chromatography–electron-capture mass spectrometry (1.0-μl injection volume) was able to detect, as a standard, 87 zmol of O^2-pivalyl-3′,5′-bis(trifluoromethyl)benzylglycolate, a product that can be obtained from PG via hydrolysis followed by derivatization. We plan to continue working with all three techniques since each is very sensitive, and has certain advantages.

1. Introduction

The biomolecule 2-phosphoglycolate (PG) arises in biological systems in two ways, both of which begin with oxidative, apparently damaging events. In the first way, oxygen rather than the intended substrate, carbon dioxide, reacts with ribulose-1,5-biphosphate, a component of photosynthesis [1]. The enzyme involved, ribulose-1,5-diphosphate carboxylase/oxygenase, is the most abundant enzyme in the world [2], due to the magnitude of plant life. The resulting metabolic degradation of oxidized ribulose-1,5-biphosphate leads to PG. In the second case, damage to DNA by ionizing radiation [3] or bleomycin [4] can lead, via free radical abstraction of the C4′ hydrogen on deoxyribose and subsequent reaction with oxygen, to strand cleavage in which a 3′-phosphoglycolate terminus results (3′-position

* Corresponding author.

0021-9673/96/$32.00
SSDI 0021-9673(95)00791-1

on the DNA) which in turn undergoes enzymatic repair [5,6] to release PG.

Towards a long-term goal of contributing to a better understanding of oxidative damage to DNA and its associated repair, we have begun to explore sensitive methodology for measuring PG. To date, PG has been detected after a combination of acid and enzymatic hydrolysis of the 3′-DNA precursor to glycolic acid, which in turn has been subjected to thin layer chromatography and also trimethylsilylation GC–MS [7].

Two of the techniques available in our laboratory seemed to have a good potential to detect unmodified PG with high sensitivity: capillary electrophoresis with indirect laser fluorescence detection (CE), and electrospray tandem mass spectrometry. As reported here, each was successful, and comparable detection limits for PG were achieved (about 10^{-6} *M* in each case, although the potential of the latter technique remains to be fully evaluated). We also determined glycolate, a hydrolysis product of PG, via derivatization followed by GC–MS utilizing ionization by electron capture (GC–EC-MS).

2. Experimental

2.1. Materials

Fluorescein (sodium salt), 3,5-bis(trifluoromethyl)benzyl bromide, 4-dimethylaminopyridine, trimethylacetic anhydride (pivalic anhydride), and potassium carbonate were purchased from Aldrich (Milwaukee, WI, USA). Glycolic acid, phosphoglycolic acid as a tri(monocyclohexylammonium) salt, 2-[N-morpholino] ethanesulfonic acid (MES), and phosphatase, acid, were purchased from Sigma (St. Louis, MO, USA). Acetone, hexane, ethyl acetate, methylene chloride, propyl sulfonic acid silica and hydrochloric acid were from J.T. Baker (Phillipsburg, NJ, USA). HPLC grade acetonitrile was from Fisher (Pittsburgh, PA, USA). Thin-layer chromatography (TLC) plates (250-μm plates for monitoring reactions and 1000-μm plates for preparative TLC) with silica gel GF were from Analtech (Newark, DE, USA). Gases for GC–EC-MS were from Med-Tech (Medford, MA, USA). Milli-Q water was produced in-house using a Milli-Q Plus system (Millipore, Milford, MA, USA).

2.2. Electrospray mass spectrometry

This work was done using a Quattro triple quadrupole mass spectrometer (Fisons Instruments, Beverly, MA, USA) equipped with a Fisons electrospray ion source. Data were acquired in the negative-ion mode by flow injection. An Isco μLC-500 solvent pump (Lincoln, NE, USA) was used to deliver an infusion solution (ACN–H_2O, 50:50, v/v) at 5 μl/min through a fused-silica capillary (75 μm I.D. × 280 μm O.D.; Polymicro Technologies, Phoenix, AZ, USA) to the electrospray probe. Injections were made using a Valco injector fitted with a 1-μl internal sample loop, and the phosphoglycolate was dissolved in ACN–H_2O (50:50, v/v) for all injections. The electrospray ion source temperature was maintained at 80°C, and N_2 was used as both the coaxial and bath gas for desolvation of the droplets. The focus and skimmer lenses were optimized to 25 and 30 V, respectively. For collision-induced dissociation (CID) analysis, collision energy was set to 20 eV and high purity Ar was used as the collision gas with a pressure of $\sim 1.3 \cdot 10^{-3}$ mbar in the gas cell. For multiple-reaction monitoring (MRM) analysis, a pressure of $\sim 3.2 \cdot 10^{-3}$ mbar was maintained and a collision energy of 80 eV was used.

2.3. Electron-capture mass spectrometry

A Model 5988A mass spectrometer from Hewlett-Packard (Palo Alto, CA, USA) was used. It was fitted with an Everlast Model 1305 continuous dynode electron multiplier (Detector Technology, Sturbridge, MA, USA). The gas chromatograph, Hewlett-Packard 5890 Series II, was connected to the mass spectrometer with the capillary interface kept at 300°C. A Hewlett-Packard 59970 MS Chemstation data system was used to record the data. Methane and helium were used as reagent and carrier gases, respectively, and each was filtered through an Oxisorb cartridge (MG Scientific, MA, USA). Injections

were made in an on-column mode onto an HP Ultra 2 (5% phenylmethylsilicone gum phase) 25 m × 0.2 mm I.D., 0.33 μm film thickness capillary column (Hewlett-Packard). The oven was programmed from 50 to 140°C at 45°C/min, held for 1 min, then ramped at 20°C/min to 170°C, held for 2 min, then finally up to 290°C at 60°C/min. The instrument was autotuned and the conditions for GC–MS were: column head pressure (He) 20 p.s.i., ion source pressure (CH_4) 2 Torr, ion source temperature 250°C, ion source energy 240 eV, emission current 300 μA, dwell time 900 ms, and the cycle time 1.0 s. Detection mode was selected-ion monitoring at *m/z* 159.

2.4. Capillary electrophoresis

A home-built capillary electrophoresis (CE) apparatus with laser-induced fluorescence detection (Ar ion laser with excitation at 488 nm) was used [8]. The CE unit was interfaced to a Macintosh Centris 610 computer through DYNAMAX MacIntegrator I (Rainin Instrument Co., MA, USA). One of the contact input ports was used to trigger the data acquisition, and one of the contact outputs was used to interlock and trigger the regulated high-voltage d.c. power supply (Glassman High Voltage, NJ, USA). CE was performed in a 70-cm long fused-silica capillary (75 μm I.D.) with the detection window 45 cm from the injection end. Samples were injected hydrodynamically: anode end 5 cm higher for 20 s. This gives an injection volume of about 10 nl. The applied voltage across the capillary was 30 kV. CE buffer: $3.3 \cdot 10^{-4}$ *M* fluorescein at pH 8, or $3.3 \cdot 10^{-4}$ *M* fluorescein adjusted with 0.005 volume of 0.2 *M* MES, giving pH 6.0. In order to obtain a stable baseline, it was necessary to operate the electrophoresis continually for 5 h, or let the buffer incubate (0 V) in the capillary overnight.

2.5. Synthesis

3,5-Bis(trifluoromethyl)benzylglycolate

Glycolic acid (268 mg, 3.52 mmol) and powdered, dried potassium carbonate (40 mg, 0.29 mmol) were combined with 10 μl of acetone (dried over molecular sieve) and 200 μl of 3,5-bis(trifluoromethyl)benzyl bromide (335 mg, 1.09 mmol). The resulting solution was refluxed (60–70°C) with stirring for 80 h. The reaction mixture was centrifuged and the organic layer pipetted away from the potassium carbonate and evaporated, giving a residue which was dissolved in 0.5 ml ethyl acetate, and purified by preparative silica TLC with hexane–ethyl acetate (4:1, v/v). Extraction of desired product with ethyl acetate from the TLC plate gave the product as a viscous liquid on evaporation. ^{1}H NMR ($CDCl_3$): δ 7.87 (Ph-H, 1H, singlet), 7.82 (Ph-H, 2H, singlet), 5.53 (Ph-CH_2, 2H, singlet), 4.28 (HO-CH_2, 2H, doublet, $J = 5.7$ Hz) and 2.61 (-OH, 1H, triplet, $J = 5.7$ Hz).

O^2-pivalyl-3′,5′-bis(trifluoromethyl)-benzylglycolate

Based on our prior experience [9], the product from the above synthesis (71 mg, 0.23 mmol) and 4-dimethylaminopyridine (18 mg, 0.16 mmol) were combined with 2 ml of freshly distilled pyridine and 100 μl of pivalic anhydride (92 mg, 0.49 mmol) and stirred for 20 h. The reaction mixture was treated with 5 ml of saturated ammonium chloride and extracted twice with 5 ml of methylene chloride. The methylene chloride extract was washed twice with 0.07 *M* hydrochloric acid followed by 5 ml of water, dried over anhydrous sodium sulfate, and evaporated under vacuum. Redissolving in 0.5 ml of ethyl acetate and purification by silica preparative TLC gave the product. ^{1}H NMR ($CDCl_3$): δ 7.88 (Ph-C4-H, 1H, singlet), 7.82 (Ph-C2,4-H's, 2H, singlet), 5.28 (Ph-CH_2, 2H, singlet), 4.68 (CO-CH_2, 2H, singlet) and 1.22 (C-CH_3, 9H, singlet).

The latter compound was further purified by solid-phase extraction (SPE) for GC–EC-MS. A SPE column was prepared using a Fisher brand disposable 10-ml borosilicate pipette which was firmly plugged with a piece of Kimwipe and packed with 2 g of silica gel [60-Å pore size, 40 μm irregular particles (J.T. Baker)]. The column was conditioned with 5 ml of ethyl acetate and 5 ml of hexane prior to sample application in 1 ml of hexane. After washing the column with 10 ml hexane, elution with hexane–ethyl acetate (3:1,

v/v) followed by evaporation under nitrogen gave an oil that was dissolved in acetonitrile for detection by GC–EC-MS.

Enzymatic digestion of phosphoglycolate

Propyl sulfonic acid silica, 0.2 g, was packed in a Micro-Spin centrifuge filter (Nylon 66, 0.2 μm) unit with polypropylene housing (Alltech Associates, Deerfield, IL, USA) and washed with distilled water (3×1.0 ml). Phosphoglycolate ($2.7 \cdot 10^{-3}$ *M* in 200 μl of water) was centrifuged through to remove the monocyclohexyl ammonium ion. To 100 μl was added 1 μl of acid phosphatase (0.5 unit/μl), followed by incubation at 37°C for 40 min and then analysis by CE.

3. Results and discussion

3.1. Capillary electrophoresis

For determination of 2-phosphoglycolate (PG) by capillary electrophoresis (CE), we adopted the indirect detection technique of Xue and Yeung [10] based on fluorescein as the fluorophore and utilizing laser-induced fluorescence detection. (More recently, the technique of fluorescein indirect detection CE has been used to measure some metal cations [11].) Our initial pH of 8.0 for the running buffer was similar to that used by Xue and Yeung (pH 8.5) for measurement of lactate and pyruvate. This gave a peak for PG at 4 min as shown in Fig. 1A, along with three impurity peaks also derived from the commercial sample of PG. One of these peaks (migration time of 3 min) apparently was phosphate, since it co-migrated with added phosphate (Fig. 1B). No effort was made to identify the other impurity peaks.

PG carries a significant negative charge at pH 8, giving it a higher mobility than fluorescein, which in turn results in a broad peak for PG as seen in Fig. 1. To improve this peak shape, we lowered the charge on the PG by adjusting the pH to 6.0 with MES, giving a narrower peak for this compound as seen in Fig. 2. Since this change in pH also lowered the electroendosmosis, the migration time for PG shifted to 6

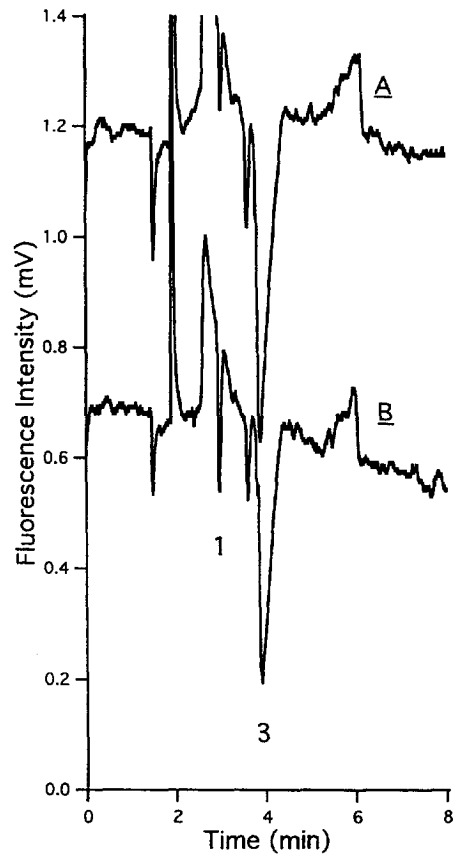

Fig. 1. Detection of 2-phosphoglycolate (PG, peak 3) by CE at pH 8.0 with indirect detection using fluorescein as the fluorophore. (A) Commercial sample of PG; (B) PG sample spiked with phosphate (peak 1).

min. Also included in this separation were added phosphate and acetate, to establish that they do not interfere. In another sample, sulfate and chloride were added and observed to co-migrate as a broad band near 9 min (data not shown), indicating that they also did not interfere. The inset in Fig. 2 shows the detection of $5 \cdot 10^{-6}$ *M* phosphoglycolate. If a 1-μl sample volume were handled for such detection (from which 10 nl was injected), the 1 μl would correspond to 5 pmol of analyte.

For two reasons we wanted to know whether PG could be enzymatically hydrolyzed to glycolate: (i) this could help to confirm a CE peak for PG, and (ii) it could open up an opportunity to detect PG by measuring the latter compound

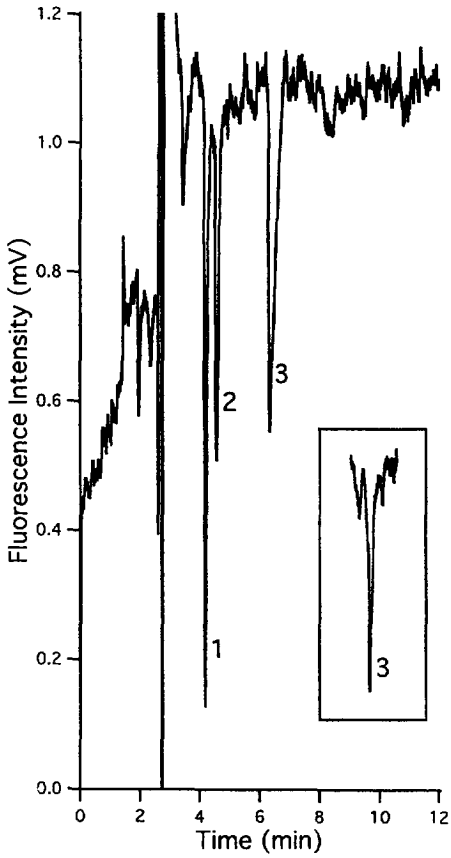

Fig. 2. Electropherogram as in Fig. 1 except that the pH for the separation was 6.0, a higher amount of phosphate was spiked (peak 1), and acetate (peak 2) was added as well. The inset shows the detection of $5 \cdot 10^{-6}$ *M* PG.

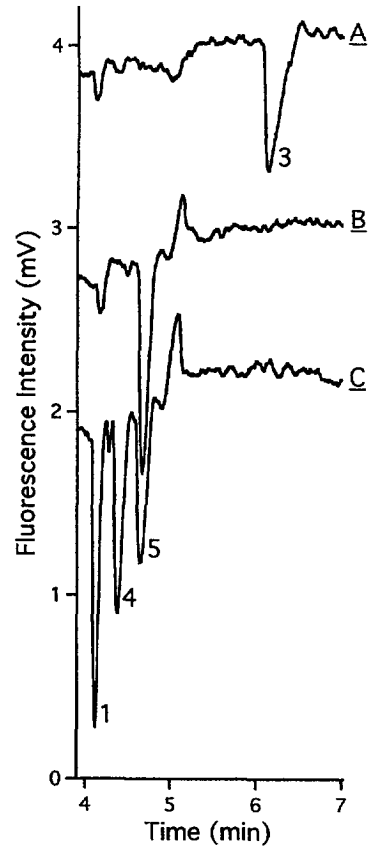

Fig. 3. Electropherograms utilizing Fig. 2 conditions. (A) Commercial sample of PG; (B) commercial sample of glycolate; (C) sample A after hydrolysis with acid phosphatase. Peaks: 1 = phosphate, 3 = PG, 4 = unknown (present in the enzyme sample), 5 = glycolate.

as a specific hydrolysis product (see below). Such hydrolysis was successful as demonstrated in Fig. 3. The peak for PG (Fig. 3A) disappears after incubating the sample with acid phosphatase forming, as expected, peaks for phosphate and glycolate (Fig. 3C). Fig 3B is from an authentic sample of glycolate.

3.2. Electrospray mass spectrometry

Initially, the phosphoglycolate tri(monocyclhexylammonium) salt was examined by electrospray mass spectrometry in the positive-ion mode. However, only the quaternary cyclohexylammonium ion was observed as an intense peak at *m/z* 100; no ion pertaining to the phosphoglycolate portion was present. More successful was negative-ion electrospray MS as shown in Fig. 4. After optimizing the conditions for the molecular ion $[M]^-$ at *m/z* 155, a full scan spectrum of 100 ng of PG salt (Fig. 4A) showed, aside from this ion, only a single fragment ion at *m/z* 79. The origin of this ion is explained in Fig. 5. When 1.0 μg of the PG salt was injected and CID conditions were applied to M^-, an additional fragment at *m/z* 97 showed up which also is explained in Fig 5. Detection in triplicate of 500 pg of the PG salt (1.0-μl injections from a $1.1 \cdot 10^{-6}$ *M* stock solution) utilizing multiple-reaction monitoring of the ion transition *m/z* 155→79 is shown in Fig. 4C.

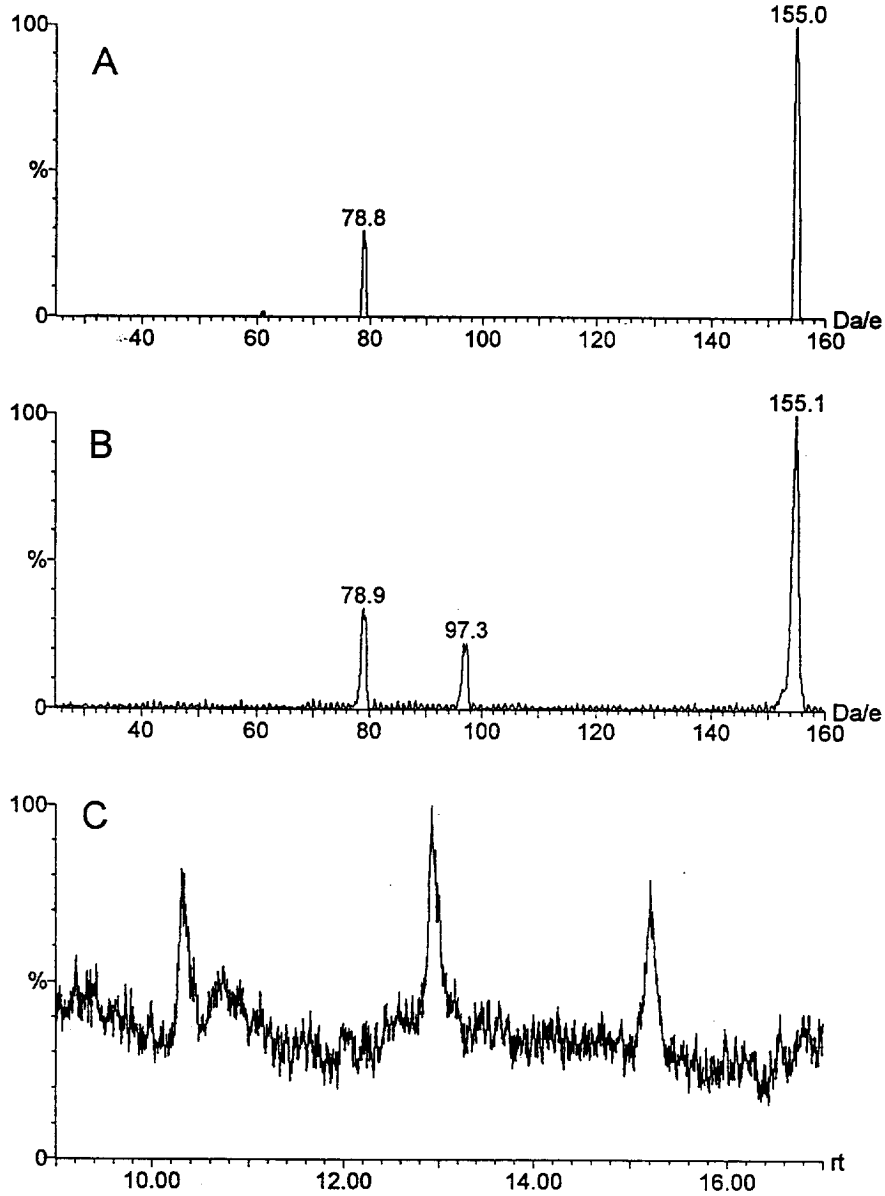

Fig. 4. Detection of PG, as a tri(monocylohexlyammonium) salt, by flow-injection analysis electrospray negative-ion mass spectrometry. (A) Full scan mass spectrum; (B) CID mass spectrum; (C) multiple-reaction monitoring mass spectrum of the ion transition m/z 155→79 of PG in triplicate. Amounts injected in 1.0 μl: (A) 100 ng; (B) 1.0 μg; and (C) 500 pg in triplicate.

Based on our prior experience [12,13], one can project that a 20- to 150-fold lower amount can be detected by introducing the 1-μl sample via capillary HPLC instead of by simple flow injection into the electrospray-MS system, in order to narrow the peak width.

3.3. GC–EC-MS

The detection of 33 ag (87 zmol) of a derivatized standard of glycolate, considered here as a hydrolysis product of PG, by gas chromatography–electron-capture mass spectrometry (GC–

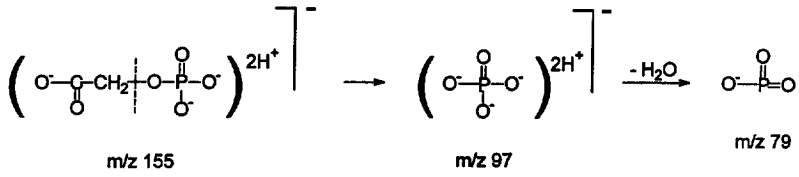

Fig. 5. Origin of the peaks shown in Fig. 4.

EC-MS) is shown in Fig. 6. The response was linear up to at least 72 fg (data not shown). Previously the smallest amount of an electrophore measured in this way was 200 zmol of a different compound, as reported before [14], while attomol amounts are more routine. This small improvement in detection limit can be attributed to the favorable detection properties of the current compound, since we concurrently confirmed that the same GC–EC-MS was operating at the previously obtained sensitivity level by re-determining a detection limit that we had obtained before, using the same type of electron multiplier, at the time of the 200 zmol result.

We chose to electrophore-derivatize the carboxyl of glycolate with 3,5-bis(trifluoromethyl)-benzyl bromide, rather than with the more conventional pentafluorobenzyl bromide [14], since others have reported higher responses with the former reagent [15,16]. A pivalyl ester was chosen as a derivative for the residual hydroxy group based on our earlier work in which several derivatives of such a group were compared [9].

4. Conclusion

A first stage, i.e. the detection of standards and determination of their detection limits, has been accomplished for the sensitive measurement of 2-phosphoglycolate. Losses and interferences certainly will compromise these detection limits when the methods are applied to biological samples. Only after the latter work is accom-

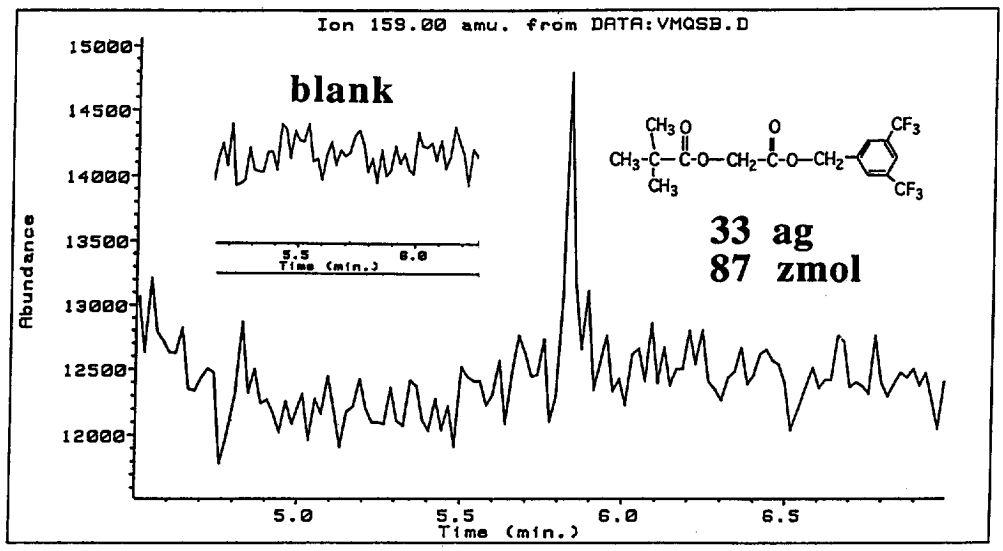

Fig. 6. Detection limit by GC–EC-MS of O^2-pivalyl-3′,5′-bis(trifluoromethyl)benzylglycolate.

plished can the relative sensitivity and usefulness of the three techniques be truly compared. Ultimately we plan to detect this analyte in human samples. Since it is always useful to minimize the size of such samples, it is important to lower the detection limit as much as possible.

Acknowledgements

This work was funded by the Office of Health and Environmental Research Division, Department of Energy, as Grant DE-FG02-90ER60964. We thank Bruce Demple for encouraging this study and for valuable discussions. Publication number 663 from the Barnett Institute.

References

[1] A. Lehninger, D.L. Nelson and M.M. Cox, Principles of Biochemistry, Worth, New York, 1993, p. 633.
[2] C.K. Mathews and K.E. van Holde, Biochemistry, Benjamin/Cummings, New York, 1990.
[3] C. von Sonntag, The Chemical Basis of Radiation Biology, Taylor and Francis, London, 1987, p. 248.
[4] J. Stubbe and J.W. Kozarich, Chem. Rev., 8 (1987) 1107.
[5] W.D. Henner, S.M. Grunberg and W.A. Haseltine, J. Biol. Chem., 258 (1983) 15198.
[6] B. Demple and L. Harrison, Ann. Rev. Biochem., 63 (1994) 915.
[7] L. Giloni, M. Takeshita, F. Johnson, C. Iden and A.P. Grollman, J. Biol. Chem., 256 (1981) 8608.
[8] E.S. Yeung, P. Wang, W. Li and R.W. Giese, J. Chromatogr., 608 (1992) 73.
[9] G.M. Kresbach, M. Itani, M. Saha, E.J. Rogers, P. Vouros and R.W. Giese, J. Chromatogr., 475 (1989) 423.
[10] Q. Xue and E.S. Yeung, J. Chromatogr. A, 661 (1994) 287.
[11] P.L. Desbène, C.J. Morin, A.M. Desbène Monvernay and R.S. Groult, J. Chromatogr. A, 689 (1995) 135.
[12] B. Yeung, P. Vouros and G.S. Reddy, J. Chromatogr., 645 (1993) 115.
[13] B. Yeung, P. Vouros and G.S. Reddy, American Laboratory, 26 (1994) 12.
[14] S. Abdel-Baky and R.W. Giese, Anal. Chem., 63 (1991) 2986.
[15] A. de Jong, personal communication.
[16] B.C. Blount and B. Ames, Anal. Biochem., 219 (1994) 195.