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General method for determining ethylene oxide and related N^7-guanine DNA adducts by gas chromatography–electron capture mass spectrometry

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Abstract

A 112-μg sample of DNA was spiked with 103 pg of N^7-(2'-hydroxyethyl)guanine and 100 pg of N^7-(2'-hydroxyethyl-d4)guanine, the internal standard. The sample was subjected to the following sequence of steps: heating at 100°C, precipitation of the DNA with HCl, reaction with nitrous acid to form the corresponding xanthines, reaction twice with pentafluorobenzyl bromide (first to derivatize NH, then OH), solid-phase extraction on silica and detection by gas chromatography–electron capture mass spectrometry. The absolute, overall yield of final product for both the analyte and internal standard was 9.7%. Conveniently, the three chemical reactions are conducted sequentially in the same vial and, aside from a washing step, are separated only by evaporations. Corresponding N^7-guanine methyl, phenyl and styrene oxide adducts were detected at about the 50-ng level by the procedure, to indicate the generality of the method.

1. Introduction

The N^7-position of guanine (G) is a prominent site of attack on DNA both in vitro and in vivo by a variety of alkylating agents, as has been reviewed [1]. Ethylene oxide, styrene, methylnitrosourea, vinyl chloride and aflatoxin (or their metabolites) are examples of such agents. For the "olefins" in this group, metabolic activation leads to a corresponding epoxide, which may then react with DNA. Certainly the exposure of the N^7-G site in the minor groove of DNA (N^7-G is not involved directly in Watson–Crick base pairing) is important for the propensity of this site to alkylate. It is therefore not surprising, perhaps, that at least some of these adducts are repaired enzymatically in vivo [1].

Humans are exposed occupationally, chemotherapeutically or otherwise (e.g., smokers) to alkylating agents [2]. For example, ca. $2.5 \cdot 10^6$ tons of ethylene oxide are produced annually in the USA, where 150 000 workers deal with it daily in various industrial processes [3] (1986 figures). Ethylene oxide is carcinogenic, mutagenic and teratogenic in animals, such as the rat, and is considered a probable carcinogen in humans [1]. The measurement of N^7-G adducts may be helpful as a biomonitor of such exposure,

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e.g., to facilitate epidemiological studies relating chemical exposure to health risks.

N^7-G adducts potentially are a favorable class of DNA adducts for measurement since they can be isolated conveniently from the DNA. Simply heating the DNA in water releases such adducts as free nucleobases [4]. Several procedures, even automated (e.g., from Applied Biosystems or Integrated Separation Systems) are available to purify DNA from biological samples.

The N^7-G adduct of ethylene oxide has been measured in biological samples by HPLC with fluorescence [5] and electrochemical detection [6] and GC–electron impact (EI) mass spectrometry [7]. Background adduct levels, per 10^6 nucleotides, of 2–6 (mice and rats), 0.15 (salmon sperm DNA) and 2 (rats), respectively, were reported. However, where the data were shown, the peak seen was at or near the detection limit of the method in a complex chromatogram, so the true background level remains to be confirmed. Walker et al. [5] pointed out the need for a more sensitive method for this adduct, since they were unable to measure the adduct in rats and mice exposed to 3 ppm of ethylene oxide.

We are developing a general method for the detection of "small" N^7-G DNA adducts by gas chromatography–electron capture mass spectrometry (GC–EC-MS). Our initial focus is the detection of N^7-(2′-hydroxyethyl)guanine, an ethylene oxide adduct, as a representative analyte of this type. Previously we reported the preparation of an electrophoric derivative of this compound at the milligram level [8]. We also compared its purification (as an electrophoric derivative) at the trace level by solid-phase extraction vs. HPLC [9], and determined that carryover of this compound in an HPLC system was largely due to the injector [10]. In additional work involving this analyte, a new electrophoric derivatizing agent was introduced [11].

Here we report further advances towards our long-term goal of measuring this class of adducts at trace levels in biological samples. We have succeeded in measuring 100 pg of N^7-(2′-hydroxyethyl)guanine spiked into about 100 μg of DNA, and demonstrate that the method also detects corresponding methyl, benzene and styrene DNA adducts (tested as standards at the nanogram level), which similarly are of toxicological interest [1,2,12].

2. Experimental

2.1. Materials

N^7-(2′-Hydroxyethyl)guanine was purchased from Chemsyn Science Laboratories (Lenaxa, KS, USA). *tert.*-Butyl nitrite (handle with caution [13]), pentafluorobenzyl bromide (PFBzBr), potassium carbonate, trifluoroacetic acid (TFA), potassium hydroxide and tetrabutylammonium hydrogensulphate were obtained from Aldrich (Milkwaukee, WI, USA). Organic solvents (HPLC grade) were purchased from Doe & Ingalls (Medford, MA, USA). Calf thymus DNA, N^7-methylguanine and guanosine were purchased from Sigma (St. Louis, MO, USA). Ethylene oxide-d4 gas was obtained from Cambridge Isotope Laboratories (Woburn, MA, USA). Racemic N^7-(2′-hydroxy-1′-phenylethyl)-guanine was prepared from the crude ribonucleoside preparation by heating 1.3 mg in 1.3 ml of 1 *M* HCl at 50°C for 53 h. The UV spectrum became constant after 18 h, matching that of 7-benzylguanine prepared similarly by others (see below). The starting ribonucleoside was prepared originally by Dipple and co-workers as described [14], and given to us by A. Dipple, who suggested the hydrolysis procedure [15]. He also gave us the UV spectrum of 7-benzylguanine. N^7-Phenylguanine was provided by K. Norpoth, who prepared it as described [16]. Shaking of reactions was done with a Mistral Multi-Mixer from Lab-Line Instruments (Melrose Park, Il, USA) at a setting of 0.3 full speed.

2.2. Internal standard

Racemic N^7-(2′-hydroxyethyl-d4)guanine was prepared by a procedure adopted from Brookes and Lawley [17]. Ethylene oxide-d4 gas was bubbled for 5 min through a mixture of 1.4 g of guanosine and 20 ml of acetic acid at 0°C (ice-

bath). After an additional 10 min, the mixture was heated for 1 h at 100°C with stirring under a reflux condenser. Evaporation (rotary evaporator) was followed by the addition of 10 ml of 1 *M* HCl and then similar heating for 1.5 h. The cooled (0°C) reaction mixture was neutralized with 1 *M* KOH and centrifuged at 3000 rpm for 10 min. The supernatant liquid was decanted twice, water was added to the residue and the centrifugation was repeated (until the supernatant liquid was colorless). Rotary evaporation of the residue gave a fluffy brown solid that was treated with 10 ml of 0.1% TFA–H_2O and centrifuged. The supernatant liquid was taken, and this process was repeated with 5 ml twice more. The pooled supernatant liquid was purified by HPLC (0.2-ml injections) using a Rainin Microsorb C_{18}-silica column (25 cm × 10 mm I.D.), 0.1% TFA–water as eluent at 5 ml/min and detection at 250 nm. The product eluted at 10.8 min. Some of this product was converted into a corresponding tris(pentafluorobenzyl) derivative, analogous to **3**, as described [8].

2.3. Methods

Solid-phase extraction (SPE) columns were prepared using disposable 5.25-in. borosilicate pasteur pipets firmly plugged with silanized glass-wool (J.T. Baker). Silica gel (60 Å pore, 40 μm irregular particles; J.T. Baker) was the packing material (200 mg per column). Each column was freshly washed with 1 ml of ethyl acetate and 1.5 ml of hexane prior to sample application. All washing and elution solvents were pushed to the upper bed surface with pressure from a rubber bulb.

All standard solutions were prepared in toluene for GC–EC-MS. Quantitative values were based on the masses of the free bases unless noted otherwise.

GC–EC-MS was carried out on a Hewlett-Packard Model 5988A mass spectrometer equipped with an HP5977 MS Chem-Station data system. Methane (2 Torr) and helium (20 p.s.i.) were used as the reagent and carrier gases, respectively. Injections were made in the on-column mode and the oven was programmed from 120 to 290°C at 70°C/min. A Hewlett-Packard Ultra-1 capillary column (25 m × 0.2 mm I.D., 0.11 μm film thickness) was used.

Step 1: neutral thermal hydrolysis

Based on mass, stock solutions of N^7-(2′-hydroxyethyl)guanine and N^7-(2′-hydroxyethyl-d4)guanine (the internal standard) were prepared in 0.1 *M* HCl, and a combined solution was prepared and diluted with this solvent to give a final solution containing 1.03 and 1.00 pg/μl, respectively. This latter solution was stored at 4°C in the dark and used for as long as 6 months. An aliquot (100 μl) of this latter solution was evaporated in a 2-ml conical vial in a Speed Vac, and then 100 μl of water containing 112 μg of dissolved DNA were added. The vial was heated at 100°C for 15 min and then placed in an ice-bath. Cold, 1 *M* HCl (150 μl) was added to precipitate the DNA. After 1 h, the cold solution was centrifuged (3100 rpm) at 0°C for 15 min, then the separated supernatant liquid was combined with a 100-μl wash (1 *M* cold HCl) of the pellet (with a second centrifugation) and evaporated in a Speed Vac.

Step 2: nitrous acid oxidation

The vial was placed in an ice-bath and 50 μl of 6 *M* HCl (degassed for 5 min with N_2) and 20 μl of *tert.*-butylnitrite were added. After shaking at 0°C for 4 h, the sample was evaporated under vacuum. The residue was subjected to liquid–liquid extraction using 150 μl of ethyl acetate and 50 μl of water. The separated aqueous layer was evaporated under vacuum.

Step 3: derivatization and detection

To the residue were added 5 mg of K_2CO_3 (stored as a powder at 60°C), followed by 100 μl of a freshly prepared solution of 10 μl of PFBzBr in 1 ml of acetonitrile. The sample was shaken for 20 h at room temperature and evaporated under nitrogen. The residue was treated with 50 μl of 1 *M* KOH containing 125 μg of Bu_4NHSO_4, 150 μl of CH_2Cl_2 and 5 μl of PFBzBr. After shaking for 20 h at room temperature, the CH_2Cl_2 was slowly evaporated under nitrogen and 3 × 200-μl ethyl acetate

extractions were combined and evaporated under nitrogen. The residue was dissolved in 25 μl of ethyl acetate and 25 μl of hexane were added. This solution was transferred to the silica SPE column, followed by washing (4.5 ml of hexane, then 6 ml of 10% ethyl acetate in hexane) and elution (2 ml of ethyl acetate). The ethyl acetate eluate was evaporated under nitrogen and the residue dissolved in 50 μl of toluene for injection of 1 μl into the GC–EC-MS system.

Detection of styrene oxide, methyl and phenyl N_7-guanine adducts

A vial was charged with 5 μl of 1 *M* HCl containing 50 ng of N^7-(2′-hydroxy-1′-phenylethyl)guanine. After evaporation in a Speed-Vac, 100 μl of water (not containing DNA) were added followed by heating, etc., as above. Similarly, 67 ng of N^7-methylguanine and 52 ng of N^7-phenylguanine were reacted, except that the latter was dissolved at the outset in 2 *M* HCl owing to its slow rate of dissolution in 1 *M* HCl. For the N^7-G methyl and phenyl adducts, step 5 was omitted: the evaporated residue after step 4 was dissolved in 50 μl of water and extracted with ethyl acetate as usual (step 6), etc. The final, evaporated samples (at the end of the procedure), were dissolved in 50 μl of toluene and 1 μl was injected into the GC–EC-MS.

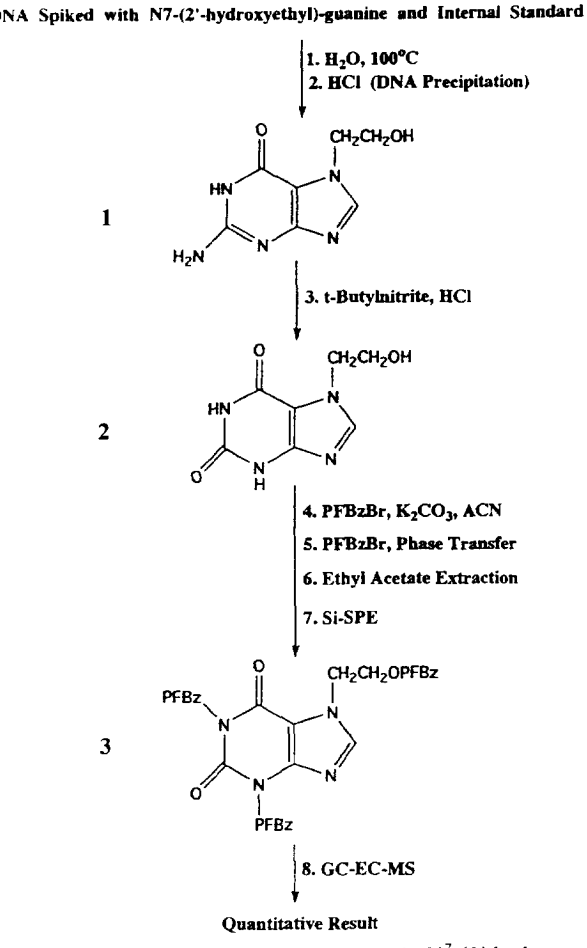


Fig. 1. Scheme for the detection of N^7-(2′-hydroxyethyl)guanine.

3. Results and discussion

The scheme that we have developed to detect N^7-(2′-hydroxyethyl)guanine spiked into DNA at the 100-pg level is summarized in Fig. 1. This scheme evolved from our earlier work in which we converted a standard of compound **1** to derivative **3** at the milligram level [8], and demonstrated that **3** as a standard could be detected at the low attomole level by GC–EC-MS [18]. In our earlier work, we also found that a derivatization precursor of **3**, in which the hydroxyethyl group was left underivatized, gave a good response by GC–EC-MS. However, peak tailing was more noticeable for the latter compound, suggesting that its performance would worsen more rapidly with column aging. We therefore elected to continue working only with **3**.

In step 1 of the analytical scheme, the spiked (analyte plus internal standard) DNA sample is heated in water at 100°C since adducts of DNA are well known to undergo depurination under such conditions, usually referred to as "neutral thermal hydrolysis" [4]. Although the step is unnecessary for a spiked DNA sample, it was incorporated here in anticipation of the future application of this method to biological samples containing DNA-bound N^7-G adducts. The simplicity and specificity of this step contribute significantly to the attraction of the overall

method. For example, the N^7-methyl-G adduct is reported to undergo release 30 000 times faster than normal guanine under typical such conditions [19].

Next (step 2), the DNA is precipitated with HCl. This step is carried out in the cold mainly to minimize additional depurination of the DNA. After evaporation of the supernatant liquid containing the analyte, nitrous acid is used to form the corresponding xanthine. Initially we formed the nitrous acid for this reaction from sodium nitrite and hydrochloric acid, and purified the intermediate product **2** by HPLC prior to conducting the two pentafluorobenzylation reactions (steps 4 and 5). Although this was successful, it was inconvenient. No final product **3** was detected, however, when xanthine **2** was not purified prior to steps 4–7. This was studied (data not shown) and found to arise from inhibition of the phase-transfer reaction (step 5) by sodium chloride derived from the nitrous acid reaction. Changing from sodium nitrite to *tert.*-butyl nitrite overcame this interference, allowing us to subject xanthine **2** without purification to the subsequent steps in the procedure.

Xanthine **2** can be converted into the final derivative **3** by omitting the first pentafluorobenzylation reaction (step 4, which utilizes potassium carbonate as a base). However, the overall yield is about 1.5-fold higher when step **4** is included. In this step the two NH sites on **2** are pentafluorobenzylated. We speculate that the product from step **4**, since it is less polar than **3**, more readily transfers into the organic phase of the phase-transfer reaction. As shown in Fig. 1, post-derivatization clean-up (steps 6 and 7) prior to detection by GC–EC-MS is accomplished by extraction with ethyl acetate (step 6) followed by solid-phase extraction on a silica packing (step 7).

The measurement of a sample in quadruplicate of 112 μg of DNA spiked with N^7-(2′-hydroxyethyl) guanine and internal standard (103 and 100 pg, respectively) gave absolute yields of $9.7 \pm 3.0\%$ and $9.7 \pm 3.3\%$ (mean $\pm$ S.D.), respectively, of final products. These yields were calculated by external calibration with standards of the final products (synthesized at the milligram level for both **1** and the internal standard). The ratio of analyte to internal standard (peak areas) in the four samples was 1.00, 0.96, 0.99 and 1.04. Representative GC–EC-MS traces for a sample and blank (only DNA) are shown in Fig. 2A and B, respectively. In Fig. 2C is presented the similar measurement of this analyte and internal standard (same yield) in the absence of DNA. As seen, the latter sample gives a cleaner chromatogram. The peaks for the analyte and internal standard in Fig. 2C elute later and are narrower than those in Fig. 2A and B because a longer, newer column was used, and because these peaks are plotted more compressed in Fig. 2A in order to reveal fully the other peaks in the chromatogram. (Compressing the display of a given peak makes it easier to see its asymmetry, as illustrated by the inset in Fig. 2C.)

The method was developed with the intent that it could be used, with little or no modification, to determine a variety of "simple" N^7-G adducts. To test this, we applied it to corresponding styrene oxide, methyl and phenyl adducts. When the procedure shown in Fig. 1 was applied to the styrene oxide adduct, step 5 (phase-transfer reaction) was omitted for the methyl and phenyl adducts, since it was not needed. Amounts near 50 ng of each were measured so that we could employ scanning conditions in the mass spectrometer to locate the desired products in the GC–EC-MS traces. The criterion selected initially for a peak to be the intended product was that the peak should be prominent, have a reasonable retention time and have a mass spectrum comprising a single ion at M − 181 (loss of pentafluorobenzyl from the parent anion radical), just like that of **3**. This was the case for the N^7-G methyl and phenyl adducts, which gave peaks at 4.1 min (m/z 345) and 5.1 min (m/z 407), respectively. Reconstructed selected ion mass chromatograms for these two products are shown in Fig. 3A and B, respectively. Interestingly, the apparent GC peak for the styrene oxide adduct gave an electron-capture mass spectrum in which two ions were prominent: m/z 603 and 631, in a ratio of about 2:1. The latter is the expected mass at

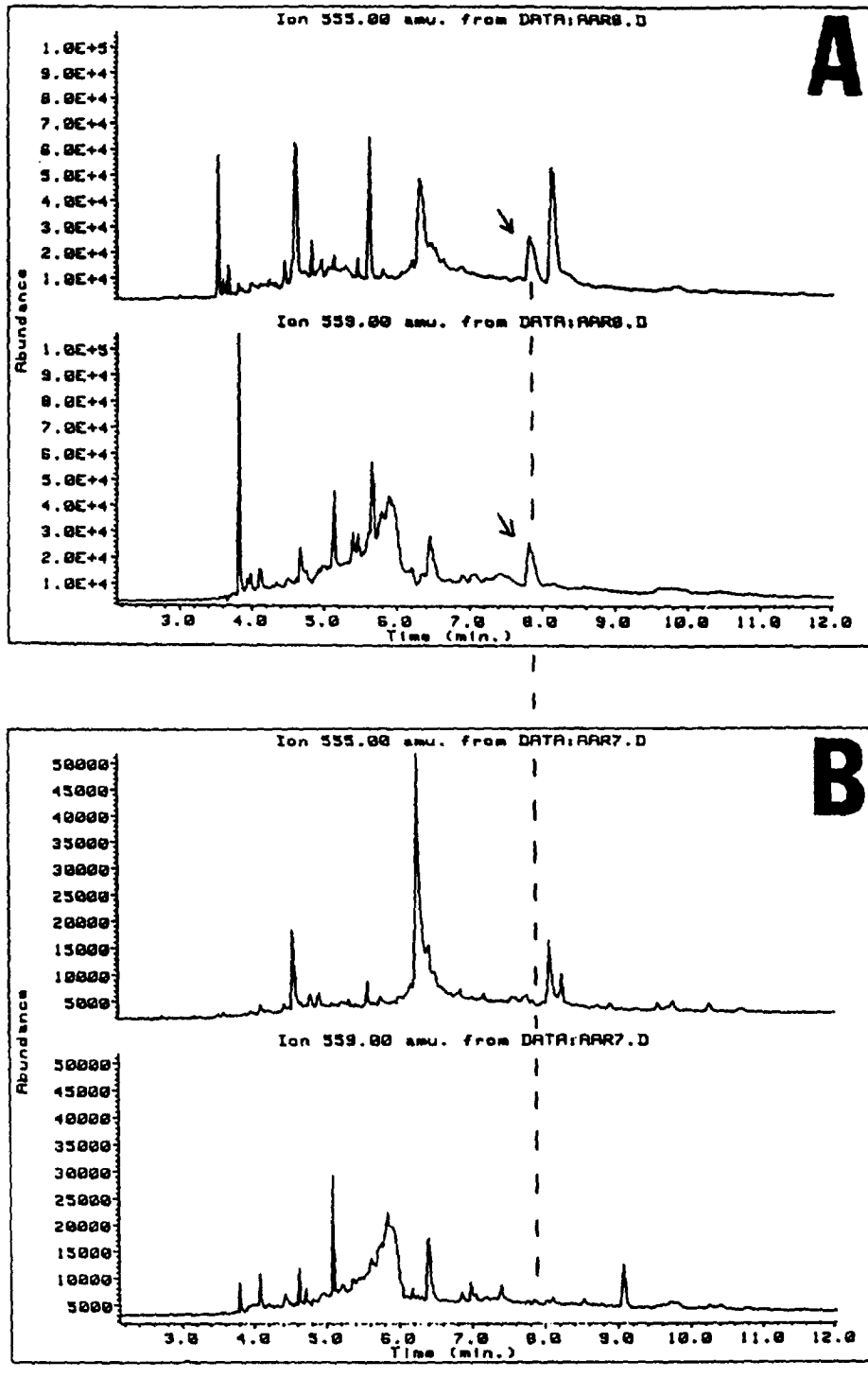


Fig. 2. GC–EC-MS traces obtained from the following samples using the scheme shown in Fig. 1: (A) 103 pg of **1** (m/z 555; upper chromatogram) and 100 pg of internal standard (m/z 559; lower chromatogram) spiked into 112 μg of DNA; (B) 112 μg of DNA; and (C) same as (A) except no DNA. In each case, 1 μl of a final sample volume (in toluene) of 50 μl was injected. Inset in C: same peak of a standard of **3** plotted at different heights.

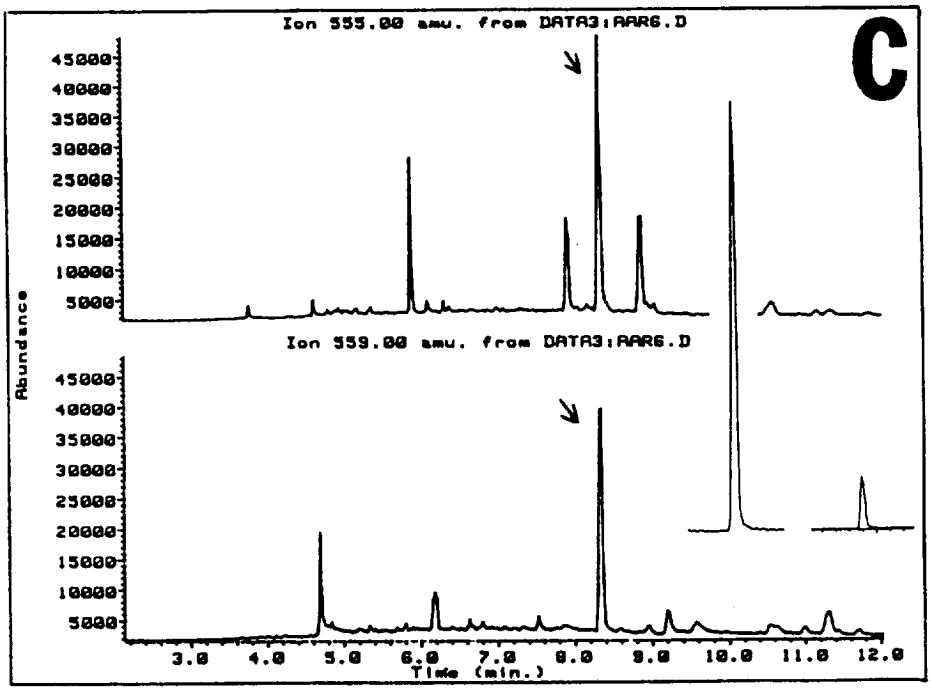
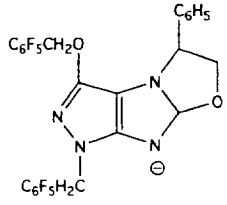


Fig. 2c. (*Continued*)

M − 181. A reconstructed selected ion chromatogram for this ion is shown in Fig. 3C. The ion at *m/z* 603 (data not shown) corresponds to an additional loss of 28 u. We speculate that the *m/z* 603 ion derives from *m/z* 631 starting with transfer of the PFBz group to O^6. This in turn changes the conjugation of the fused pyrimidine ring to which O^6 is attached, leading to loss of CO to form a final ion which might be the one shown here.



m/z=603

4. Conclusion and future

A procedure has been developed that detects an N^7-G ethylene oxide adduct spiked into about 100 μg of DNA at a level corresponding to one adduct in 10^6 normal nucleotides. Detection of corresponding N^7-G methyl, phenyl and styrene oxide adducts at the 50-ng level in the same way (except that one step was not needed for the first two compounds) implies the generality of the procedure. While the method involves multiple steps, this is the nature of definitive trace organic analysis. At least the steps are simple; for example, the three reactions are conducted sequentially in the same vial and, aside from a washing step, only evaporation is done between them.

Our original method, applied to 20 mg of a pure sample of the N^7-G ethylene oxide adduct, gave a 17% overall yield preparatively of the final product **3** [8]. Now, starting with about 10^8 times less analyte, and spiked into DNA, the absolute, analytical yield is 9.7%. We plan to focus next on finding out where the losses are taking place in the procedure, so that we might be able to raise the current yield. The current method has been transferred to another labora-

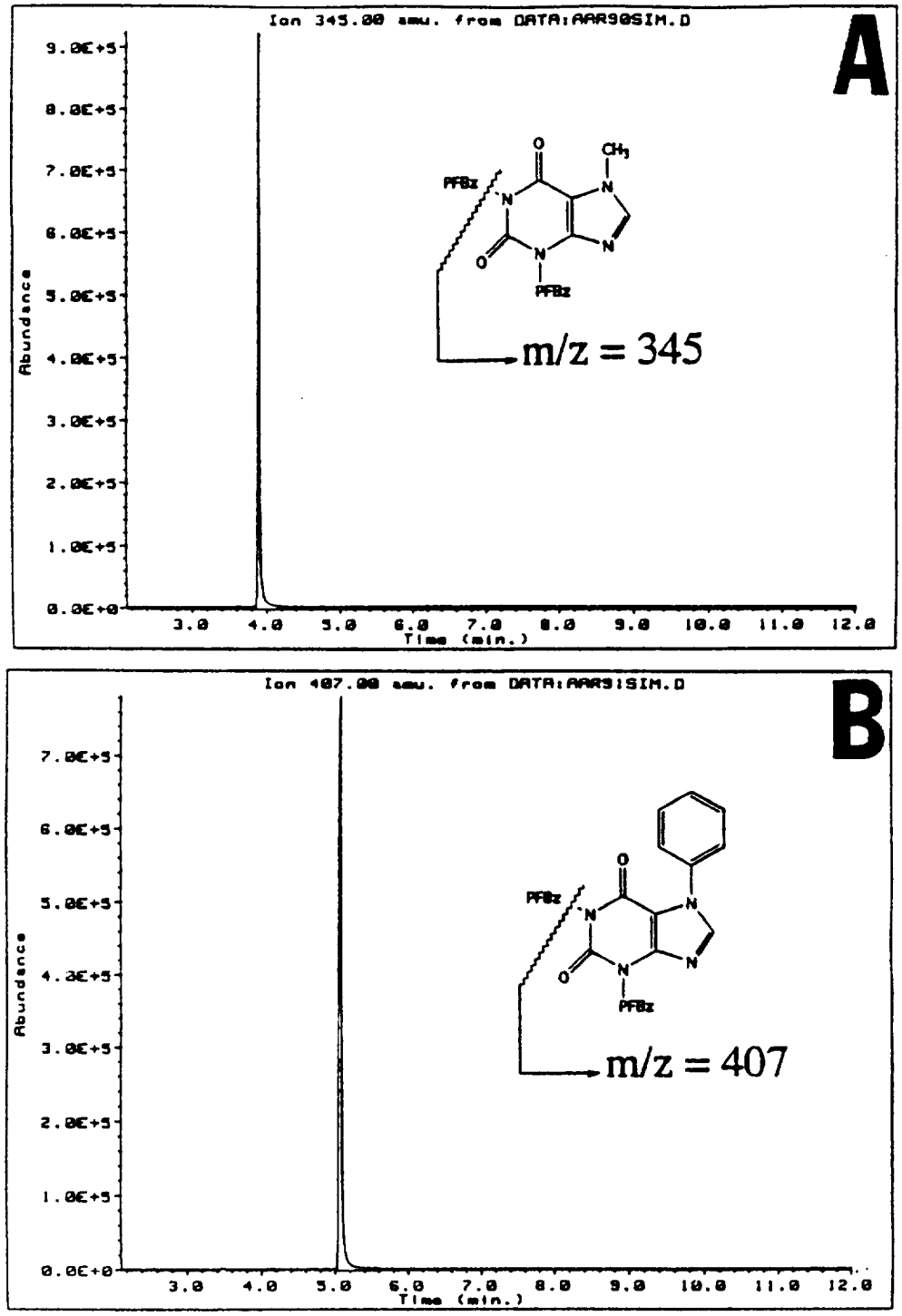


Fig. 3. Detection by GC–EC-MS (reconstructed selected-ion chromatograms) using the scheme shown in Fig. 1 (or this scheme without step 5; see text), of (A) 67 ng of N^7-methylguanine (m/z 345); (B) 52 ng of N^7-phenylguanine (m/z 407); and (C) 50 ng of N^7-(2′-hydroxy-1′-phenylethyl)guanine (m/z 631). In each case, 1 μl of a final sample volume (in toluene) of 50 μl was injected.

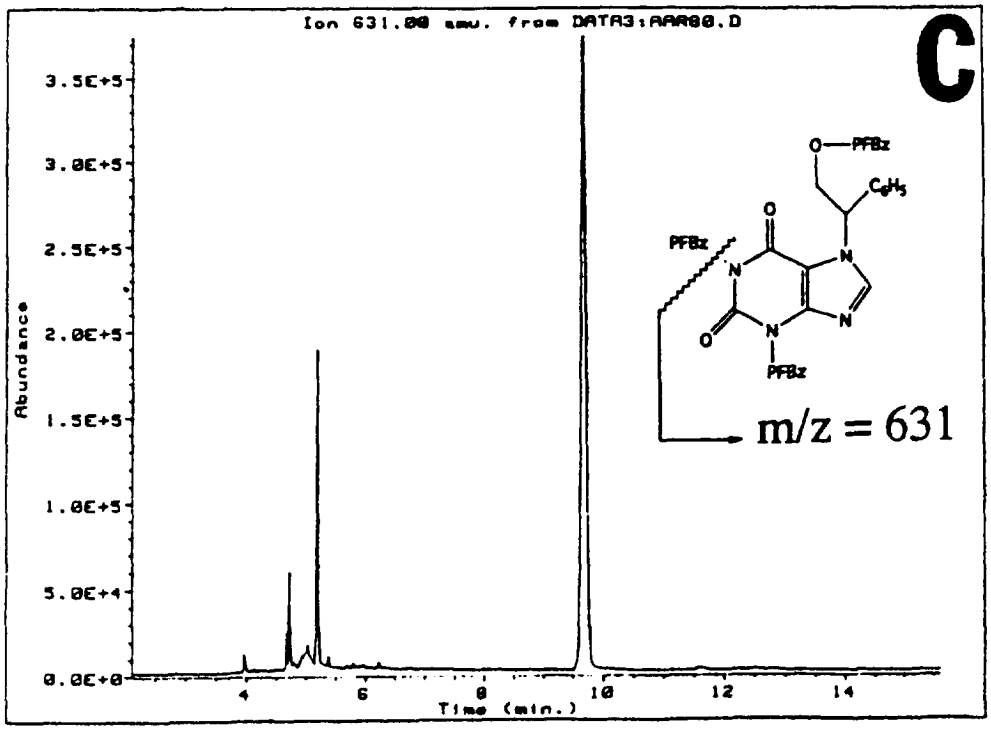


Fig. 3c. (*Continued*)

tory where it is being used successfully to measure this adduct in animal samples [20] and also in human liver samples [21].

Acknowledgements

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