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DETERMINATION OF 5-METHYLCYTOSINE IN DNA BY GAS CHROMATOGRAPHY–ELECTRON-CAPTURE DETECTION

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SUMMARY

The following sequence of analytical steps was used to determine the amount of 5-methylcytosine (mol-%) in calf thymus and human lymphocyte DNA: acid hydrolysis of the DNA, derivatization (pentafluorobenzyl bromide, solid phase extraction, pivalic anhydride), internal standard addition, solid phase extraction, high-performance liquid chromatography, and gas chromatography with electron-capture detection. The steps were carefully optimized, leading to a recovery of 30 ± 1.0% starting with a nucleobase standard containing 1.25 ng of 5-methylcytosine. A second analysis of this sample gave a 30 ± 0.3%, demonstrating a high precision for the method. In good agreement with earlier work by others, 1.2 ± 0.10 mol-% of 5-methylcytosine was then found in a 350 ng sample of calf thymus DNA, and values of 0.9 ± 0.07 and 0.8 ± 0.04 mol-% (two runs) were found in hyman lymphocyte DNA.

INTRODUCTION

There is increasing concern about the risks to human health, especially carcinogenesis and mutagenesis, from exposure to chemicals. Since DNA is an ultimate target for this exposure, the measurement of chemicals attached to DNA, or of "DNA adducts", is of considerable interest[1,2]. High sensitivity is required since a tiny amount of a chemical attached to DNA may pose a significant risk.

Our approach to quantify a DNA adduct with high sensitivity is to isolate it from a physiological sample as a modified nucleobase or nucleoside, derivatize the adduct with an electrophore, and quantify the derivatized adduct by gas chromatography (GC) or high-performance liquid chromatography (HPLC) utilizing electrophore detection. The latter refers to either the use of electron-capture detection (ECD) or electron-capture negative-ion mass spectrometry (ECNIMS).

Thus far we have formed sensitive electrophoric derivatives from standards of

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pyrimidine[3] and purine[4] nucleobases. We have utilized a mild, chemical oxidation reaction to release pyrimidine and purine nucleobases from their corresponding nucleosides[4]. We have detected $1.3 \cdot 10^{-15}$ mol of an electrophoric derivative of the DNA adduct, 5-hydroxy-methyluracil, as a standard by HPLC–ECNIMS using a belt interface[5].

Our continuing work on the quantitation of DNA adducts by chromatography with electrophore detection is proceeding along two lines. First, we are preparing suitable electrophoric derivatives of other DNA adducts. Second, the subject of this paper, we are developing sample cleanup procedures to bring this electrophore methodology to real samples.

As an intermediate stage towards the determination of a trace amount of DNA adduct obtained from a real sample, we chose to set up an electrophore-based method for quantifying 5-methylcytosine (5-MC) in calf thymus DNA and in DNA obtained from human lymphocytes. This analyte is a normal, minor base in DNA, typically reported to be about 1–2 mol-% of mammalian DNA. Thus its measurement presents an intermediate level of difficulty relative to much smaller amounts of DNA adducts that need to be measured[2]. 5-MC has been determined in these DNA samples by several techniques (see Table I). Recently, an HPLC–UV method using rechromatography was used to quantify 5-MC in trace amounts (1 in 100 000 nucleotides) in other types of DNA samples[15].

Here we present the determination of 5-MC in both calf thymus DNA and human blood lymphocyte DNA by electrophoric derivatization followed by GC–ECD. A small amount of DNA (350 ng) was analyzed in order to test the suitability of our sample handling steps for eventual application to trace amounts of DNA adducts. We also analysed larger amounts of the same calf thymus DNA by HPLC with UV detection in order to establish our own reference values for this sample.

TABLE I

DETERMINATION OF 5-METHYLCYTOSINE IN DNA SAMPLES

Sample	*Method*	*Reference*	*Amount 5-methylcytosine (mol-%)*
Calf thymus	HPLC–UV*	6,7	1.7
		8	1.4 ± 2.2
	GC–MS of an alkylsilyl derivative	7,10	1.28 ± 0.09
		11	1.39 ± 0.09
	^{32}P-Postlabeling–TLC–autoradiography	12	1.07 ± 0.06
	Fluoroimmunoassay	13	1.0
	GC–ECD	This work	1.2 ± 0.10
Human blood lymphocyte	HPLC–UV	14	0.96 ± 0.01
	GC–ECD	This work	0.9 ± 0.07**
			0.8 ± 0.04**

* Using such a method, DNA was hydrolyzed in aqueous hydrofluoric acid to prevent deamination of 5-methylcytosine.

** Different runs.

EXPERIMENTAL

Chemicals and reagents

The DNA bases and calf thymus DNA (Type I: sodium salt; highly polymerized), were obtained from Sigma (St. Louis, MO, U.S.A.). Pentafluorobenzyl bromide (>99%), trimethylacetic anhydride (99%), and 4-dimethylaminopyridine (99%) were from Aldrich (Milwaukee, WI, U.S.A.). Tetrafluorobenzyl bromide was from Alfa Products, (Danvers, MA, U.S.A.). Organic solvents, GC/HPLC or GC^2 grade, were from American Burdick and Jackson (American Scientific Products, Boston, MA, U.S.A.). Distilled-deionized water was purified to HPLC grade with an Organopure system (Barnstead, Boston, MA, U.S.A.). HPLC grade water was also purchased from J. T. Baker (Phillisburg, NJ, U.S.A.). Formic acid (88%) was from Baker. For the reactions, the acetonitrile was dried with type 4A molecular sieves.

All solution compositions were v/v except as noted. Values for precision were based on analysis of samples in triplicate unless indicated otherwise.

Apparatus

The glassware was soaked in hot liquid detergent, washed with water, kept in hot conc. HCl for >2 h, washed with water and methanol, thermally cleaned (250°C), silanized[16], and thermally cleaned again.

Solid phase extraction columns were prepared using 5.25-in. borosilicate Pasteur pipets. They were packed with 500 mg of either silica gel (60-Å pore size, 40-μm irregular particles) or end-capped cyanopropylsilica (60-Å pore size, 40-μm irregular particles), from Baker. The column bed was sandwiched between two plugs of silanized glass wool. Solvents and samples were eluted immediately after application with 1 p.s.i. of nitrogen. This pressure was removed when the level of solvent (or sample) was 2–3 mm above the column bed, and the next aliquot of solvent was applied before the level of liquid reached the bed.

The analytical HPLC separations were done at 1.0 ml/min on a LC-18-DB HPLC column (250 × 4.6 mm, 5-μm diameter particles; Supelco, Bellefonte, PA, U.S.A.) fitted with a LC-18-DB guard column (20 × 4.6 mm, 5-μm diameter particles; Supelco). The column temperature was kept at 30°C. Detection was at 254 or 260 nm for the nucleobases, 284 nm for N1-pentafluorobenzyl-5-methylcytosine (PFBz-5-MC), and 314 nm for the pivalyl-N1-pentafluorobenzyl-5-methylcytosine (Piv-PFBz-5-MC) and internal standard, pivalyl-N1-(2,3,5,6-tetrafluorobenzyl)-5-methylcytosine. A Perkin-Elmer Series 4 HPLC pump was used (Perkin-Elmer, Norwalk, CT, U.S.A.).

A Model 3740 gas chromatograph was fitted with a ^{63}Ni electron-capture detector and Model 1095 on-column capillary injector (Varian, Palo Alto, CA, U.S.A.). Compounds were separated on a HP-Ultra, 5% phenylmethylsilicone, fused-silica capillary column (32 m × 0.32 mm I.D., 0.52-μm film thickness; Hewlett-Packard, Palo Alto, CA, U.S.A.). The flow of the carrier gas, helium, was set to 4 ml/min at 250°C. The flow of make-up gas, nitrogen, was set to 26 ml/min at 250°C.

Peak areas for both HPLC and GC were obtained manually using the scanner mode of a Waters 840 data system (Millipore-Waters, Milford, MA, U.S.A.).

Synthesis

PFBz-5-MC. 5-MC hydrochloride (485 mg, 3 mmol), pentafluorobenzyl bromide (1.36 ml, 9 mmol) and potassium carbonate (2.07, 15 mmol) were refluxed at 50°C in 100 ml of dry acetonitrile with stirring until no starting material remained by silica TLC. Ethyl acetate (100 ml) was added and the mixture was transferred to a silica flash chromatography column (20 cm × 19 mm I.D. bed). This was repeated two more times and the column was washed with 500 ml of hexane. The compound was eluted with acetonitrile, and further purified after evaporation by preparative HPLC on a C_8 silica column (25 cm × 10 mm I.D.) using a gradient of acetonitrile in water. Evaporation gave a white solid that was a single peak by HPLC. IR, 2.86 and 3.03 cm^{-1} (NH_2), 5.9–6.1 (C=O); ^1H NMR, δ 1.9 (s, 3H, CH_3), 4.9 (s, 2H, CH_2), 7.5 (s, 1H, ring CH); MS(EI), *m/z* 305 (M, 100%), 263 (17%, [M − $NCNH_2$]).

Piv-PFBz-5-MC. N1-PFBz-5-MC (305 mg, 1 mmol), pivalic anhydride (203 ml, 10 mmol), N-methylmorpholine (1.1 ml, 10 mmol) were refluxed in 100 ml of dry acetonitrile for 20 h at 50°C with stirring. The product was purified by flash chromatography, as described above, followed by spotting onto two 1000 μm thick, 20 × 20 cm silica TLC plates (Analtech, Newark, DE, U.S.A.). After development with methanol–methylene chloride (1:9), the scraped band was boiled in ethyl acetate, filtered through sintered glass, and evaporated yielding a white solid that was a single peak by HPLC and GC–ECD. IR, new peak at 6.3 cm^{-1} (C=O, pivalyl); ^1H NMR, new peak at δ 1.2 (s, 9H, pivalyl); MS(ECNI), *m/z* 208 (100%, [M − $CH_2C_6F_5$]).

Pivalyl-N1-(2,3,5,6-tetrafluorobenzyl)5-methylcytosine. This compound was synthesized and purified using the procedures just described except 2,3,5,6-tetrafluorobenzyl bromide was substituted for pentafluorobenzyl bromide. MS(EI), *m/z* 372 (2%,[M + 1]), 314 (85%,[M − $C(CH_3)_3$]) 208 (100%,[M − $CH_2C_6F_4H$]).

Analytical procedure

DNA hydrolysis. An aliquot of an aqueous DNA sample (*ca.* 350 ng) or of an external nucleobase standard (1.25 ng or 10 pmol of 5-MC and 200 pmol each of the other four DNA bases) was added to a 1-ml crimp-top Wheaton Micro-V vial (Aldrich). The sample was dried by evaporation in a Speed-Vac Concentrator (Savant Instruments, Hicksville, NY, U.S.A.) at 45°C for 1 h. Formic acid (200 μl) was added to the vial, and the vial was sealed with a PTFE-faced silicone septum and aluminum seal (Aldrich). After heating for 3 h at 150°C in a Reacti-Therm (Pierce, Rockford, IL, U.S.A.) in which the vial holes were half-filled with sand, the sample was evaporated as before for 45 min. A crimp-top cap was used because the plastic caps were digested by the acid vapors. The elevation of the vial with sand minimized contact of the acid vapors with the vial seal.

Alkylation. Pentafluorobenzyl bromide (100 μl of a 100 nmol/μl solution in dry acetonitrile) was added followed by a spatula tip (1–2 mg) of potassium carbonate that had been dried at 250°C. The vial was capped with a PTFE-faced silicone septum and an open-top screw cap (Pierce) and kept at 60°C for 3 h with vortexing every 30 min.

Cyano solid phase extraction. The column was conditioned with 2 ml of methanol followed by 2 ml of ethyl acetate–dichloromethane–acetronitrile (3:1:1) (solvent A). The sample was treated with 0.5 ml of solvent A and transferred with a Pasteur pipet to the column. This was repeated two more times, and the column was washed with 2 ml of solvent A and 4 times 0.5 ml of methanol, the last two portions of which were

collected in a 2-ml Reacti-Vial (Pierce) since they contained the product. The sample was evaporated at 45°C for 45 min.

Acylation. The sample was treated with 100 μl of a solution of pivalic anhydride (100 nmol/μl) and 4-dimethylaminopyridine (20 nmol/μl) in dry acetonitrile. The vial was sealed and heated at 60°C for 1 h with vortexing every 15 min. The internal standard was added (0.65 pmol, 241 pg).

Silica solid phase extraction. The column was conditioned with 2 ml of acetonitrile–2-propanol (9:1) (solvent B) followed by 4 ml of hexane–dichloromethane (3:1) (solvent C). The reaction mixture was transferred to the column using 500 μl of solvent C and this step was repeated twice. The column was washed 2 times with 1 ml of solvent C and 4 times with 0.5 ml of solvent B. The last two fractions, which contained the product, were collected in a 2-ml Reacti-Vial and evaporated as before.

HPLC cleanup. The column was cleaned at 1 ml/min starting with acetonitrile–water (2:8), by progressive gradient changes, each 10 min in duration, to 100% acetonitrile and then isopropanol–methylene chloride (1:9). The latter mobile phase was maintained for 2 h, and the starting mobile phase was re-established by reversing the gradient program. The "analytical gradient" later to be used for the samples was then begun: a gradient from acetonitrile–water (2:8) to (7:3) in 10 min, to (8:2) in 10 min, and similarly back to (2:8).

A solution containing approximately 20 pmol each of the internal standard and derivatized analyte was injected and detected by UV to establish their retention times, typically 15 and 16 min, respectively. After washing the injector with 1 ml of hot acetonitrile, mobile phase was injected and the "analytical gradient" was done. This hot wash/injection/gradient sequence was repeated. It was then done twice more except the fraction eluting from 14.5 to 16.5 min was collected, evaporated, and analyzed by GC–ECD (see below) to assure that the HPLC system was clean. The injector was hot washed.

The sample was reconstituted in 150 μl of hot acetonitrile (50–60°C) with vortexing. After the solvent drained off the walls (10–20 min), essentially the entire volume was injected onto the HPLC column and the "analytical gradient" (see above) was performed. A fraction was collected from 14.5 to 16.5 min in a 3-ml Reacti-Vial and evaporated in a Speed-Vac Concentrator at 45°C for 1.5 h. The vial was washed with 100 μl of hot methanol (50–60°C) followed by thorough vortexing and evaporation at 60°C under a gentle stream of nitrogen.

The column was equilibrated for 10 min with the starting mobile phase and the injector was hot washed. The next and subsequent samples were treated the same, with a hot wash of the injector and 10 min column re-equilibration between each sample.

GC–ECD. The sample was reconstituted in 10 μl of toluene and 1 μl was injected into the gas chromatograph. The data were calculated relying on a linear calibration curve obtained by injecting standards containing known amounts of Piv-PFBz-5-MC and internal standard.

Mol-% values of nucleobases determined by HPLC

DNA (4–6 μg) was acid-hydrolyzed as above in 200 μl of formic acid, evaporated, dissolved in mobile phase, and the amount of the nucleobase (mol-%) was determined using HPLC. The peak areas for the bases were calibrated using standard solutions prepared from weighed, vacuum-dried bases. The bases, used as received,

were single peaks by HPLC. The base ratios ([C plus 5-MC]/G and T/A) for both types of DNA samples were 1.0 in all cases except A/T was 0.95 for human lymphocyte DNA.

RESULTS AND DISCUSSION

Our method for quantifying 5-MC in DNA by GC–ECD is summarized in Fig. 1. The DNA sample was either commercial calf thymus DNA, or human lymphocyte DNA that we isolated from blood by a conventional technique.

Hydrolysis

The DNA was hydrolyzed in formic acid for 3 h at 150°C (Fig. 1, step 1), conditions known to completely hydrolyze DNA to its bases[14,17,18]. The amount of DNA was 350 ng for each sample later subjected to quantitation by GC–ECD; whereas 4–6 μg was hydrolyzed for independent measurement of the mol-% values of all nucleobases in the DNA by HPLC.

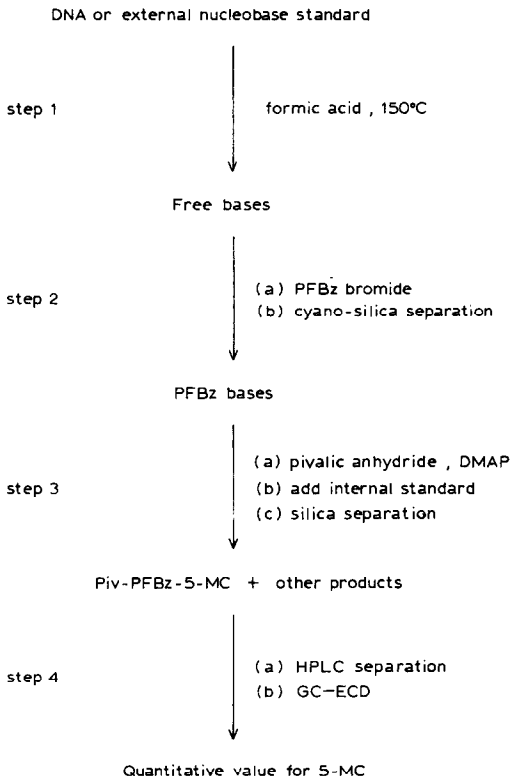

Fig. 1. Scheme for the quantitation of the amount of 5-MC (mol-%) in DNA by GC–ECD.

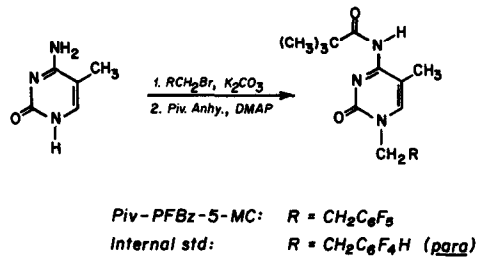

Fig. 2. Derivatization of 5-methylcytosine. Piv. Anhy. = pivalic anhydride; DMAP = 4-dimethylaminopyridine.

Derivatization

Previously we derivatized 5-MC with pentafluorobenzoyl chloride followed by methyl iodide[16]. Although the product had good electrophore detection characteristics, the yield varied, particularly when a few nanograms as opposed to milligrams of 5-MC were reacted. Apparently this problem was due to the formation of both mono-(desired) and di-substituted products in the acylation reaction.

Derivatization was done here in two steps, with a solid phase extraction after each. This is shown in Fig. 1 as steps 2 and 3. The overall derivatization reaction, including the likely structure of the final product, is presented in Fig. 2. In the first derivatization reaction, 5-MC is alkylated with pentafluorobenzyl bromide in the presence of solid potassium carbonate. Analysis of the product by electron impact mass spectrometry reveals a fragment corresponding to loss of $NCNH_2$, proving that the PFBz group is not attached to N^4 (exocyclic nitrogen) or to N3 (ring nitrogen closest to N^4) of 5-MC. Based on analysis by ^{13}C NMR, the PFBz group is attached to N (ref. 19). Thus the PFBz group is attached to the N1 position of 5-MC.

A reaction time of 3 h was selected for the conversion of 5-MC to PFBz-5-MC since the yield of this product (48.1 $\pm$ 6.3% by HPLC starting with 50 nmol of 5-MC) was maximum at this point. Analyte is lost in this step, especially with a longer reaction time, because of the formation of two, less polar side products in the first derivatization reaction. The major one is a dialkylated product, based on mass spectrometry, and the minor one, which was not investigated, is probably a trialkylated product.

The intermediate product, PFBz-5-MC, is next semi-purified by extraction on a cyanosilica cartride. Smaller or less polar contaminants including residual PFBz bromide are washed away initially with ethyl acetate–dichloromethane–acetonitrile (3:1:1) and then PFBz-5-MC is eluted with methanol. The cartridge, acting as a filter, also removes the solid potassium carbonate. To avoid contact of the sample with plastic, a source of interferences in work with GC–ECD[20], the cartridge is prepared in a silanized Pasteur pipet. The recovery of PFBz-5-MC is quantitative in this extraction step.

In the second derivatization reaction, PFBz-5-MC is acylated with pivalic anhydride in the presence of 4-dimethylaminopyridine (DMAP). The yield as determined by HPLC of this step (step 3a in Fig. 1) is 89.4 $\pm$ 0.7% starting with 50 nmol of PFBz-5-MC. This reaction is followed by semi-purification of the product with a quantitative recovery on a silica cartridge. The DMAP remains on the column, and the pivalic anhydride/acid is washed out prior to elution of the product.

The structure of the final product, Piv-PFBz-5-MC, is probably as shown in Fig. 2, with the pivalyl group attached to the N^4 site on PFBz-5-MC. This site is where acylation of cytosine and 5-MC has always been observed to take place[3,14].

This derivative, Piv-PFBz-5-MC, is a good choice for the determination of 5-MC by electrophore detection. As just cited, its yield is moderate and reproducible starting with a trace amount of 5-MC. The compound is sensitive by both ECD (peak area molar response is 0.16 relative to that of lindane) and ECNIMS. Essentially only a peak for [M − 181], due to loss of a PFBz group, is seen in the latter spectrum[21]. Although the compound possesses an active hydrogen, shielding of this hydrogen by the adjacent pivalyl group, a strategy that we have used before[22], allows the compound to be determined by GC. Interestingly, the corresponding methylated product (obtained by reacting Piv-PFBz-5-MC with methyl iodide) tails more on GC than Piv-PFBz-5-MC. Apparently methylation of Piv-PFBz-5-MC significantly changes its electronic structure, the orientation of its pivalyl group, or both.

Continuing with step 3 (Fig. 1) of the analytical procedure, an internal standard was added at the end of the derivatization reaction. This standard has the same structure as the final derivatized product except for the presence of a tetra- *vs.* pentafluorobenzyl group (see Fig. 2). While such an internal standard fails to monitor the earlier step in the procedure, these steps are checked by the external standards. This compound also is conveniently obtained and structurally similar to the derivatized analyte.

HPLC

Because electrophore detection by ECD is non-specific, we have resorted in general to the use of HPLC as a post-derivatization cleanup step in analytical procedures of this type[20]. Thus we next semi-purified the sample here by HPLC (step 4a in Fig. 1). The chromatogram (not shown) from the C_{18} silica column is featureless, aside from the early elution of non-retained components. A prior injection of a larger amount of product and internal standard, detectable by UV, established the collection window. Typical retention times for product and internal standard are 15 and 16 min, and the collection window is 30 s wider on each side. Apparantly derivatized 5-MC is largely resolved from earlier-eluting, derivatized cytosine in this step.

GC–ECD

The evaporated sample collected from the HPLC column is dissolved in hot methanol, thoroughly vortexed and re-evaporated. Now that the sample is focused low in the vial it is efficiently dissolved in 10 μl of toluene. Injection of 1 μl of this latter solution into the GC–ECD gives a chromatogram such as that shown in Fig. 3A. This chromatogram is obtained from a sample derived from human lymphocyte DNA; similar chromatograms (not shown) are obtained from calf thymus DNA. A corresponding chromatogram from a nucleobase standard solution is shown in Fig. 3B: a mixture of the DNA bases corresponding to the base composition of human lymphocyte DNA is prepared and subjected to the overall procedure including initial acid hydrolysis. Fig. 3C shows the chromatogram of a blank sample (all steps were done except no DNA was present at the outset of the procedure). Although there is a small peak in the latter chromatogram that has the same retention time as that of the

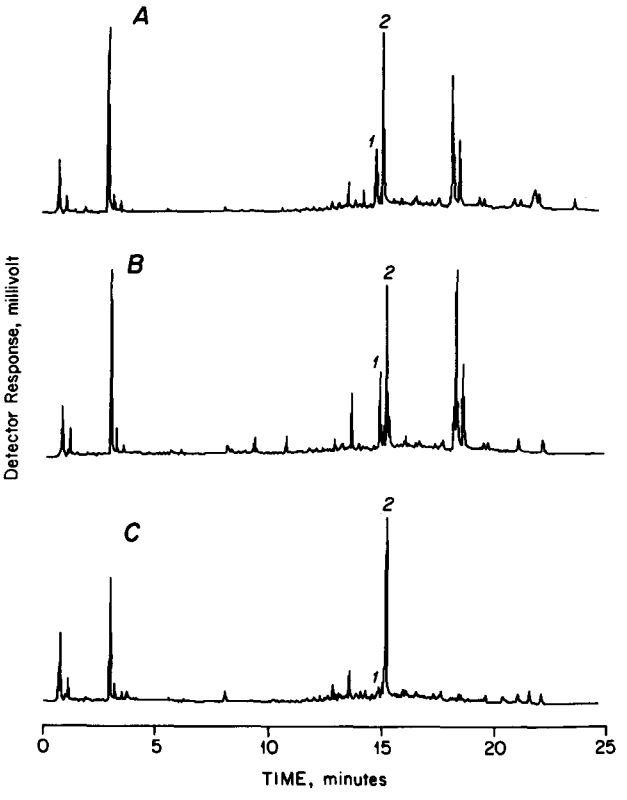

Fig. 3. Representative GC–ECD chromatogram from a human lymphocyte DNA sample (A), nucleobase standard (B), and reaction blank (C). 1 = Piv-PFBz-5-MC; 2 = internal standard. In B, the peaks represent 0.3 pmol or 117 pg of 1, and 0.65 pmol or 241 pg of 2.

product, this peak is resolved from the product on a new 50-m GC column of the same type (data not shown).

Two runs were made using the scheme of Fig. 1, one on human lymphocyte DNA sample plus an external standard nucleobase sample (defined above), and one on a calf thymus and human lymphocyte DNA sample plus an external standard nucleobase sample. The two yields for 5-MC from the external standard nucleobase samples from the two runs were 30 ± 1.0 and 30 ± 0.3%. This high precision is significantly better than that cited above for one of the individual steps due to refinements in our sample handling techniques, as detailed in the Experimental, which were developed during the course of the work. Thus a yield of 30% was used in the calculation for the amount of 5-MC in the DNA samples. Accordingly, the amount of 5-MC in the calf thymus DNA was 1.2 ± 0.10 mol-%, and in the human lymphocyte DNA was 0.9 ± 0.07 mol-% (one run) and 0.8 ± 0.04 mol-% (second run). These values compare favorably with literature values of 1.39 ± 0.09 mol-% of 5-MC in calf thymus DNA based on isotope dilution–MS[11], and 0.96 ± 0.01 mol-% of 5-MC in human lymphocyte DNA by HPLC[14].

CONCLUSION

A multi-step but sensitive and precise method relying on GC–ECD has been developed to quantify 5-MC in DNA. The experience of developing this procedure is intended to help us set up similar methodology for the determination of DNA adducts. The GC–ECD chromatograms shown in Fig. 3 are relatively clean when compared to other GC–ECD chromatograms in the literature in which low-molecular-weight analytes from biological samples are similarly derivatized and quantified. Nevertheless, refinements in the GC stage will be necessary to overcome the interferences which are present before such methodology can be successfully applied to DNA adducts, which tend to be present in much smaller amounts in DNA than 5-MC. Most attractive for this purpose will be the use of GC with detection by ECNIMS.

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