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Trace Derivatization of Cytosine with Pentafluorobenzoyl Chloride and Dimethyl Sulfate

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Methods are being developed for derivatizing trace amounts of DNA adducts for ultimate determination by gas chromatographic techniques. High-pressure liquid chromatography is used to optimize appropriate derivatization reactions for the determination of cytosine. A single vial reaction scheme involves acylation with electrophoric pentafluorobenzoylchloride followed by alkylation with dimethyl sulfate. Currently, the overall yield for this reaction starting with 50 nmole of cytosine is 59 ± 4.6%.

Introduction

Gas chromatography (GC) has become an advanced technique now applicable to the ultratrace analysis of DNA adducts. The important advances include sensitive and powerful detection methods such as electron capture detection (ECD) and negative ion chemical ionization mass spectrometry (NICI-MS); on-column injection and fused silica capillary columns; and new sample cleanup and derivatization procedures. Thus, derivatized cytosine, as a model for some adducts, was detected at the low femtogram (10^{-17} mole) level both with GC-ECD (*1*) and GC-NICI-MS (*2*).

In this prior work, cytosine, 5-methylcytosine, uracil, thymine, and some analogs were derivatized in millimolar amounts, and then standards were analyzed by GC-ECD (*1*). Progress in this research, as reported here, has involved the actual derivatization of trace amounts of the model analyte cytosine, including a procedure that confines the derivatization to a single vial.

Material and Methods

Apparatus

A Perkin-Elmer Series 4 liquid chromatograph equipped with a Rheodyne model 7125-075 at injector and a Kratos Spectroflow 773 Absorbance Detector (set st 254 nm) was used for the analyses by high-pressure liquid chromatography (HPLC). A reverse-phase precolumn immediately preceded a 15-cm, 4.6 mm ID LC-8 reverse-phase analytical column (5 μm particle size, Supelco). A 50-μL sample loop was used for most of the analyses. Liquid chromatograms were recorded with a Spectra-Physics SP4270 integrator.

Derivatization reactions were performed in Reactivials (Pierce Chemical) using block-module heaters (Pierce Chemical, Lab-Line, or American Scientific Products). Evaporative concentration at reduced pressure was performed with a Speed Vac Concentrator (Savant Instruments). Vapor-phase silanization was performed in a vacuum oven (VWR Scientific). The vortex mixer was obtained from American Scientific Products; Mininert valves were purchased from Pierce Chemical.

Reagents and Standard Compounds

Chemical reagents used for derivatizing cytosine (> 99%, Sigma Chemical) included *N*-methylmorpholine (NMM) and diisopropylethylamine (DIEA) (sequanal grade, Pierce Chemical); pentafluorobenzoyl chloride (PFBC) (98%, Columbia Organic Chemical); and dimethyl sulfate (Gold Label, Aldrich Chemical). Thymine (> 99%) was purchased from Sigma Chemical. Acetonitrile used as solvent for the derivatizations (Gold Label, Aldrich Chemical or UV-grade distilled-in-glass, Burdick & Jackson) was stored over 4A molecular sieve (Fisher Scientific). Methanol used for preparing derivatization standards was either HPLC-grade (Fisher Scientific) or glass distilled (OmniSolv, EM Science); water was HPLC grade (Baker Chemical). N^4-Penta-

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fluorobenzoylcytosine and N^4-pentafluorobenzoyl-1,3-dimethylcytosine were synthesized as described (*1*).

HMDS (1,1,1,3,3,3-hexamethyldisilazane) used for silanizing glassware was purchased from Aldrich Chemical (98%); Aquasorb (phosphoric anhydride mixture, analytical reagent) was obtained from Mallinckrodt. The pH 5.5 acetate buffer was prepared by dissolving 1.36 g of $CH_3COONa\text{-}3H_2O$ (Baker HPLC grade) in 1 L of Baker HPLC water and adjusting the pH to 5.5 with a solution of 1 N glacial acetic acid (Reagent grade) in water (Baker HPLC).

Silanization and Control of Blanks

All vials and other glassware (borosilicate) contacting the samples were cleaned and silanized. For the derivatization experiments analyzed by HPLC, glassware was cleaned by soaking it in dilute detergent (Liquinox, Alconox Inc.) followed by rinsing it with tap water, distilled water, and methanol. It was then air-dried and vapor-phase silanized.

Acylation of Cytosine

A 1-mL portion of a 1 μmole/mL standard of cytosine in methanol was added to a set of 5-mL clear Reacti-vials. (As an analytical blank, 1 mL of methanol was added to an extra vial.) All vials were transferred to a Speed Vac Concentrator, and the solvent was evaporated for 30 min at 44°C. A 1-mL portion of a solution containing 20 μmole/mL of pentafluorobenzoyl chloride and 40 μmole/mL of *N*-methylmorpholine in acetonitrile was then added to each vial. The vials were capped, and the reaction mixtures were vortexed. The vials were heated in a block-module heater at 35°C, and then 1 hr later the mixtures were again vortexed. At selected times, a set of three vials was removed and cooled to room temperature. The reaction solutions were diluted with either acetonitrile or a buffer solution which contained internal standard thymine and analyzed by HPLC.

Alkylation of N^4-Pentafluorobenzoylcytosine

A 1-mL aliquot of a 0.48 μmole/mL standard of N^4-pentafluorobenzoylcytosine in methanol was added to a set of 5-mL amber Reacti-vials. The vials were transferred to a Speed Vac Concentrator, and the solvent was evaporated for 30 min at 44°C. Another 1 mL of standard was added to the vials, and the solvent was again evaporated. (As an analytical blank, methanol without analyte was similarly added to another vial and evaporated.) Then 1 mL of a solution containing 250 μmole/mL of dimethyl sulfate and 500 μmole/mL of diisopropylethyamine in acetonitrile was added to each vial. The vials were capped, and the reaction mixtures were vortexed. The vials were heated in block-module heaters either for 24 hr at different temperatures or at 35°C for select periods of time. Replicate sets of vials were selectively removed and cooled to room temperature. The reaction mixtures were diluted with either acetonitrile or a buffer solution which contained internal standard thymine and analyzed by HPLC.

Single-Vial Derivatization of Cytosine

For the first derivatization experiments starting with 1 μmole of cytosine, the acylation procedure was as described above except that the reactions were performed at 35°C for either 48 hr or 6 hr. The vials were transferred to a Speed Vac Concentrator, and the solvent was evaporated for 30 to 45 min at 44°C. The alkylating reagents were added as described above, and the mixtures were heated at 35°C for either 24 or 12 hr. As before, the samples were diluted with acetonitrile and analyzed by HPLC.

For the derivatization of 1 μmole of cytosine with an intermediate hydrolysis, the acylation procedure was basically the same as that described above except for the following modifications: 3-mL or 5-mL amber Reacti-vials were used; the first evaporation of methanol was conducted in a Speed Vac Concentrator for 45 to 60 min at 44°C; the acylation reactions were performed for 6 hr at 35°C. After the 6-hr acylation, a 50-μL portion of water was added to each vial, and the solutions were vortexed and heated for 30 min at 35°C. The solvent was evaporated in a Speed Vac Concentrator for 45 to 60 min at 44°C. The alkylating agents were added as described above, and the solutions were heated for 24 hr at 35°C. As before, the samples were diluted with acetonitrile and analyzed by HPLC.

For the derivatization of 50 nmole of cytosine with an intermediate hydrolysis, special precautions were taken to minimize moisture in the sample vials: the amber 1-mL Reacti-vials were capped with Mininert valves and stored overnight in a dessicator containing Aquasorb; syringes were used to add reagents through the valves; after each evaporative concentration using the Speed Vac Concentrator, the vials were immediately capped. With these precautions, the same basic procedure described immediately above for the 1-μmole derivatization with an intermediate hydrolysis was followed, with the following modifications: 50 μL of a 1 nmole/μL standard of cytosine in methanol was used; 50 μL of a solution containing 20 nmole/μL of pentafluorobenzoyl chloride and 40 nmole/μL of *N*-methylmorpholine in acetonitrile were used for the acylations, and the acylation reaction mixtures were vortexed every hour during the 35°C, 6-hr reaction period; 2.5 μL of water were used for the hydrolysis; 50 μL of a solution containing 250 nmole/μL of dimethyl sulfate and 500 nmole/μL of diisopropylethylamine in acetonitrile were used for the alkylations, and the alkylation reaction mixtures were heated for 15 hr at 35°C. After all reactions were completed, the solvent was evaporated in a Speed Vac Concentrator for 45 to 60 min at 44°C. A 500-μL portion of acetonitrile was added, the solutions were vortexed, and the samples were analyzed by HPLC.

Liquid Chromatographic Conditions

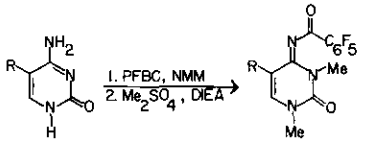
To monitor the acylation reactions, the following HPLC gradient program was used. The column was equilibrated for 10 min with 100% of pH 5.5 acetate buffer at a flow of 1 mL/min. Upon injection, the solvent composition was changed to 5:95 CH_3CN/buffer over a period of 3 min using a linear gradient. Over the next 17 min, the solvent composition was again changed to 76.5:23.5 CH_3CN/buffer using a linear gradient, and, at the same time, the flow was linearly increased to 2 mL/min. To decrease analysis time, the final product alone was monitored with the following gradient program: the column was equilibrated for 10 min with 5:95 CH_3CN/buffer at a flow of 1 mL/min; upon injection, the solvent composition was changed to 70:30 CH_3CN/buffer using a linear gradient over a period of 15 min, and, at the same time, the flow was linearly increased to 2 mL/min.

The alkylation and single stage derivatization reactions were monitored using the first program described above. To decrease analysis time, the final product, N^4-pentafluorobenzoyl-1,3-dimethylcytosine was monitored with the following gradient program: the column was equilibrated for 5 min with 30:70 CH_3CN/H_2O at a flow of 1 mL/min; upon injection, the solvent composition was changed to 60:40 CH_3CN/H_2O using a linear gradient over a period of 15 min, and, at the same time, the flow was linearly increased to 2 mL/min.

Results and Discussion

Although the ultimate goal in this project is to quantitate DNA adducts by gas chromatography with electron capture detection (GC-ECD), it is useful to use high-performance liquid chromatography (HPLC) initially to monitor the optimization of the derivatization reactions. By monitoring the reactions by HPLC, polar starting materials and side products either insufficiently volatile or thermally unstable for analysis by GC may be observed. HPLC may also be useful as a sample cleanup step prior to the final analysis by GC-ECD.

The reaction scheme for converting cytosine into its corresponding N^4-pentafluorobenzoyl-1,3-dimethyl derivative is shown in Eq. (1). In this procedure, the introduction of the electrophoric pentafluorobenzoyl (PFB) group imparts high sensitivity to this product for



(1)

detection both by GC-ECD and GC-NICI-MS (*1*,*2*). The second derivatization reaction, where remaining active hydrogens are replaced by methyl groups, serves to enhance the thermal stability and volatility of the derivative for analysis by GC.

Initially, each of these reactions was individually optimized and monitored by HPLC. Gradient elution conditions were developed so that starting materials and side products also could be observed, as shown by the analysis of a standard in Figure 1A. Thymine, peak 2 in Figure 1, was used as an internal standard to monitor the sample dilution steps and the HPLC quantitation.

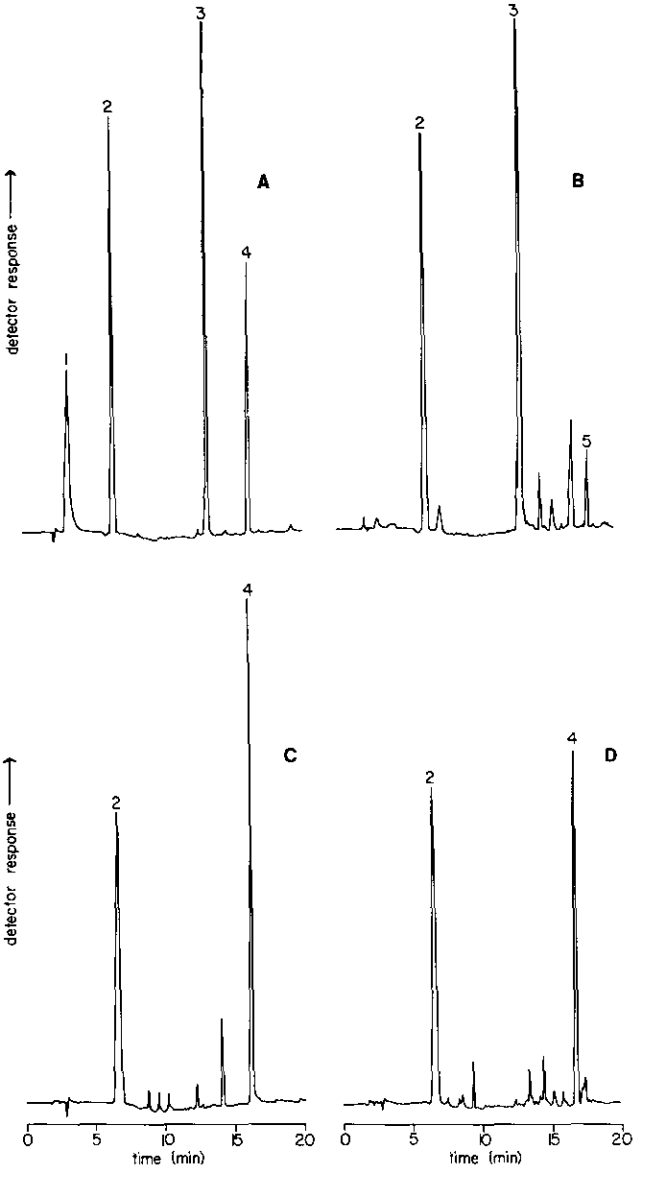


FIGURE 1. HPLC chromatograms for the derivatization of 1 μmole of cytosine: (*A*) standard mixture of cytosine (1), internal standard thymine (2), N^4-pentafluorobenzoylcytosine (3), and N^4-pentafluorobenzoyl-1,3-dimethylcytosine (4); (*B*) acylation of cytosine; (*C*) alkylation of N^4-pentafluorobenzoylcytosine; (*D*) single-stage derivatization of cytosine. The HPLC gradient program is the one described in Materials and Methods.

For the acylation reaction (Reaction I in Table 1), varying the reaction time from 4 hr to 32 hr had little effect on the yield: it increased only from 65 to 73% over this period of time. However, these results showed that the acylation product, N^4-pentafluorobenzoylcytosine, was stable under prolonged reaction times. A typical

Table 1. Optimization of derivatization conditions for the determination of cytosine (C) by GC-ECD.

	Reaction[a]	Amount of starting material	Time, hr	Temp, °C	Product yield ± SD, %[b]
I	C→PFB-C	1 μmole	4	35	65 ± 3.6
			8	35	71 ± 3.0
			12	35	73 ± 2.0[c]
			32	35	73 ± 1.9
II	PFB-C→$(Me)_2$PFB-C	1 μmole	24	25	79 ± 1.9
			4	35	81 ± 3.1[d]
			24	35	87 ± 3.1[d]
			48	35	86 ± 2.4[d]
			24	60	70 ± 1.4
			24	80	39 ± 2.0[c]
III	C→$(Me)_2$PFB-C	1 μmole	48/24[e]	35	65 ± 5.0
			6/12	35	17 ± 2.8
			6/24	35	64 ± 0.42[f]
		50 nmole	6/15	35	59 ± 4.6[f]

[a] PFB-C = N^4-pentafluorobenzoylcytosine; $(Me)_2$PFB-C = N^4-pentafluorobenzoyl-1,3-dimethylcytosine.
[b] Results of three replicates, unless otherwise noted.
[c] Results of two replicates.
[d] Results of four replicates.
[e] Represents periods of time for acylation and alkylation, respectively.
[f] Results of derivatization with an intermediate hydrolysis.

chromatogram of the acylation reaction is shown in Figure 1*B*. Here, starting material has disappeared, as the desired product, peak 3 has formed, whereas peak 5 is an unknown side product. The other peaks in this chromatogram are also present in the analytical blank.

For the alkylation reaction (reaction II in Table 1), temperature as well as time was varied. The result was that a reaction time of 24 hr at 35°C was optimum, giving a yield of 87% of the final product. As with the acylation reaction, a prolonged reaction time (48 hr) had no significant effect on the yield. A representative chromatogram for the alkylation reaction is shown in Figure 1*C*. The (intermediate) starting material, N^4-pentafluorobenzoylcytosine (peak *3*) has disappeared as the desired product (peak *4*) has formed. All of the extraneous (unlabeled) peaks in Figure 1C represent side products since none of them were present in the analytical blank.

After optimizing the reactions in two individual steps, they were then combined into a single vial reaction. This was accomplished by performing the acylation reaction, evaporating the solvent and reagents, adding the alkylating reagents, and then performing the alkylation reaction. As shown in Table 1 for reaction III, this revised protocol gave an overall yield of 65% based on results such as those shown by the chromatogram in Figure 1*D*. This was in agreement with the prior results for the two reactions conducted independently. However, when this single stage reaction was conducted for shorter periods of time (6 hr acylation and 12 hr alkylation instead of 48 hr acylation and 24 hr alkylation), an overall yield of only 17% was achieved. This significantly lowered yield clearly did not agree with the results from the two reactions conducted independently.

It appears that the cause of this reduced yield was the presence of a cytosine intermediate acylated with more than one PFB group at the end of the shortened reaction time. Such a side product was observed previously (*1*) and was found readily to undergo aqueous hydrolysis to form N^4-pentafluorobenzoylcytosine, the desired monoacylated product. Here, by examining the acylation mixture by thin layer chromatography after 6 hr, a spot corresponding to multi-acylated cytosine was seen to be present. However, by adding water to the acylation reaction after 6 hr prior to evaporation and alkylation, the yield of the single stage reaction conducted for shorter periods of time was increased to 64%. As shown in Table 1, a similar high yield of 59% was also achieved, starting with 50 nmole of cytosine and by using these shorter reaction times and an intermediate hydrolysis. A typical HPLC chromatogram showing the results of this 50-nmole reaction is shown in Figure 2.

Future work will extend the derivatization reaction to lower analyte levels based on further use of HPLC.

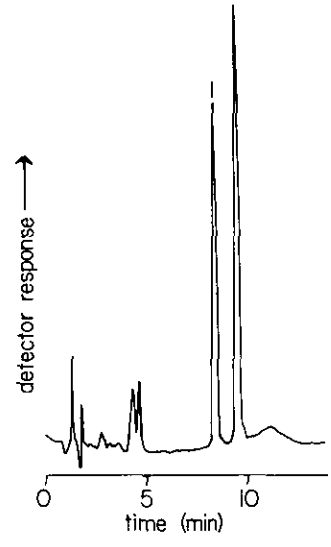


FIGURE 2. HPLC chromatogram for the derivatization of 50 nmole of cytosine. Peak 1 is N^4-pentafluorobenzoyl-1,3-dimethylcytosine. The HPLC gradient program is the one described for N^4-pentafluorobenzoyl-1,3-dimethylcytosine in Materials and Methods. The later-eluting peak is unidentified and present in the analytical blank containing no cytosine.

It is anticipated that the reaction can be monitored down to 0.10 nmole of cytosine or 5-methylcytosine by this technique, since the linear standard curve by HPLC for N^4-pentafluorobenzoyl-1,3-dimethylcytosine reaches this level with a precision of ± 4% (data not shown). GC-ECD will then be used to extend the reaction to still lower analyte levels, initially relying on the current HPLC conditions to give adequate post-derivatization cleanup of the desired product. The same, final procedure also should be applicable to the determination of 5-methylcytosine.

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