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ELECTROPHORE-LABELING AND ALKYLATION OF STANDARDS OF NUCLEIC ACID PYRIMIDINE BASES FOR ANALYSIS BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

The pyrimidine bases cytosine, uracil and thymine, along with some analogues, are electrophore-labeled either with pentafluorobenzoyl chloride (PFBC), pentafluorophenylsulfonyl chloride (PPSC), or heptafluorobutyric anhydride. Subsequent alkylation is most successful for PFB-cytosine, PPS-uracil, and PPS-thymine. These same alkylated compounds also have the highest aqueous stability and respond most strongly by gas chromatography–electron-capture detection. One of these derivatives, determined to be N^4-PFB-1,3-dimethylcytosine by authentic synthesis, and its 5-methyl analogue, can be detected with good precision down to the 100-fg level. Poor reproducibility is encountered at the 10-fg level.

INTRODUCTION

The analysis of altered forms of DNA bases is significant both for studies of normal as well as abnormal DNA function. For example, methylation of DNA bases influences vertebrate gene expression[1], and methylation patterns are tissue specific[2]. The ease of formation of Z-DNA can also depend on methylation[3]. Examples of abnormal DNA function arising from alterations of its bases are provided by carcinogen[4–6]– and mutagen[5,6]–DNA adducts. For all of these analyses, high sensitivity is useful to not only conserve samples, but these latter abnormal base alterations may be physiologically significant at a low level[6].

Gas chromatography with electron-capture detection (GC–ECD), complemented in its use by GC with negative ion chemical ionization mass spectrometry (GC–NICI-MS), is a sensitive technique that is potentially applicable to the ultratrace analysis of normal and lesion DNA bases. Aside from sample clean-up, derivatization of the bases is required prior to this analysis. The recent work of Gelijkens *et al.*[7]

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demonstrates this potential for GC. By attaching a trifluoroacetyl electrophore to two of the DNA bases, cytosine and guanine, followed by peralkylation, these workers obtained good solutes for GC analysis. In addition to standards, their overall methodology was applied to DNA samples as well. Although detection methods other than ECD and NICI-MS were employed, the potential for using these latter detectors to optimize the sensitivity was pointed out.

Two reports have appeared in which GC–ECD has been applied to the analysis of DNA bases or analogous substances. In the first, thymine was quantitated after derivatization with 1,3-bis(chloromethyl)tetramethyldisilazane for the microdetermination of DNA in biological samples[8]. Trifluoroacetylated derivatives of cytokinins were analyzed in the second case[9]. For both, detection limits were cited at the low picogram level.

In this paper we continue the work of quantitating DNA pyrimidine bases by GC–ECD. Employing pentafluorobenzoyl chloride (PFBC) for cytosine, and pentafluorophenylsulfonyl chloride (PPSC) for thymine, followed by alkylation, yields hydrolytically-stable, intensely electrophoric derivatives of these two nucleobases. Derivatized standards are detected at the femtogram level, and related bases are derivatized and analyzed by GC–ECD as well.

EXPERIMENTAL

Chemicals and reagents

All of the nucleobases were obtained from Sigma (St. Louis, MO, U.S.A.); PPSC was purchased from PCR Research Chemicals (Gainesville, FL, U.S.A.); PFBC from Columbia Organic (Camden, SC, U.S.A.); N-methylmorpholine, heptafluorobutyric anhydride and silylation grade acetonitrile were acquired from Pierce (Rockford, IL, U.S.A.); methyl iodide and ethyl iodide were obtained from Aldrich (Milwaukee, WI, U.S.A.); new fuchsin (Basic Violet 2) was from Matheson Coleman & Bell Manufacturing Chemists (Norwood, OH, U.S.A.); ethyl acetate, ethanol, methanol, chloroform, hexanes and cyclohexane were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.); toluene and isooctane were from Burdick & Jackson Labs (Muskegon, MI, U.S.A.); HPLC grade acetonitrile was purchased from Fisher Scientific (Medford, MA, U.S.A.); and silica gel 60, 230–400 mesh, was obtained from VWR Scientific (Boston, MA, U.S.A.).

High-performance liquid chromatography (HPLC)

This analysis of the derivatized bases was performed on a 15 cm × 4.6 mm I.D. Supelcosil LC-8 column (Supelco, Bellefonte, PA, U.S.A.) using isocratic acetonitrile–phosphate buffer, pH 5.0 (55:45, v/v). The effluent was monitored at 214 nm.

Preparative liquid chromatography

The injection valve was fitted with a 1-ml sampling loop and the separation was accomplished on a 25 cm × 10 mm I.D. column packed with Davisil C_8 (bonded and packed in-house) using either an acetonitrile (30 to 90%) gradient in water or isocratic acetonitrile–water (60:40) at a flow-rate of 4 ml/min. The effluent was monitored at 214 nm.

Flash column chromatography

Silica gel 60, 230–400 mesh was used with chloroform–ethyl acetate (1:1), hexanes–ethyl acetate (7:3), ethyl acetate–chloroform (6:4) and hexanes–ethyl acetate (3:1) as solvents.

Thin-layer chromatography (TLC)

GHLF silica gel Uniplates with fluorescence indicator (Analtech, Newark, DE, U.S.A.) were developed with either chloroform–methanol (3:1), chloroform–ethanol (19:1) or hexane–ethyl acetate (3:1) with detection by fluorescence quenching, new fuchsin and iodine.

Gas chromatography

A Varian Model 3700 gas chromatograph was used. It was fitted with a pressure regulator and a quartz direct vapor-injection insert (0.9-mm capillary bore, 11-cm length, cleaned and silanized as described[10]), unless indicated otherwise. The column was a fused-silica capillary column, 15 m × 0.25 mm I.D., DB-1701 (J&W Scientific, Rancho Cordova, CA, U.S.A.) and the detector was a conventional Varian constant-current variable frequency ^{63}Ni electron-capture detector unless indicated otherwise. The chromatograms were recorded with a SP4270 integrator (Specta-Physics, Santa Clara, CA, U.S.A.). The injector, column and detector temperatures were 300, 200 and 320°C, respectively, unless noted otherwise. The column carrier and detector make-up gases were ultra high purity helium and nitrogen (Matheson, Gloucester, MA, U.S.A.), respectively, filtered with 13 × molecular sieves, activated charcoal and an Oxyclear disposable purifier (Labclear, Oakland, CA, U.S.A.), with flow-rates of 5 $cm^3\ min^{-1}$ (helium) and 25 $cm^3\ min^{-1}$ (nitrogen), measured at room temperature and uncorrected. The septum-purge flow-rate with direct injection was in the range of 2 $cm^3\ min^{-1}$ per 5 p.s.i. column-head pressure. High temperature white septa (Alltech Assoc., Deerfield, IL, U.S.A.) were used. Direct injections into the gas chromatograph were made with 10-μl syringes, type 1701N (Hamilton, Reno, NV, U.S.A.) fitted with a type 26S needle and silanized as described[11]. Toluene was the injection solvent.

A Varian model 1095 on-column injector and a J&W 10-m fused-silica capillary column, DB-1701, 15 m × 0.25 mm I.D., were used to determine the standard curve and detection limit for derivatized cytosine and 5-methylcytosine. The flow-rates of helium and nitrogen in the column and detector were 8.3 $cm^3\ min^{-1}$ and 25 $cm^3\ min^{-1}$, respectively, measured as above. The injector temperature was 30°C during injection, and then immediately programmed at a setting of 180°C/min up to 250°C, whereas the column was held at 130°C for 1 min after injection and then was increased at a setting of 80°C/min up to 250°C. The on-column injections were made with 5-μl syringes (Varian Assoc., Sunnyvale, CA, U.S.A.) fitted with a fused-silica needle. The detector temperature was kept at 340°C.

All quantitative dilutions were made with silanized 701N syringes (Hamilton, Reno, NV, U.S.A.). Isooctane was the injection solvent.

Electropohore-labeling

The nucleobase (1 mmol) was added to acetonitrile (5 ml), followed by 4 mmol of N-methylmorpholine and 2 mmol of the electrophore reactant (PFBC, PPSC or

heptafluorobutyric anhydride). After 2 h stirring at room temperature, the reaction mixture was poured into a separatory funnel containing 20 ml of ethyl acetate and washed twice each with 0.01 *M* hydrochloric acid, 5% sodium bicarbonate, and water. After drying over anhydrous sodium sulfate, the ethyl acetate solution was evaporated at 50°C under reduced pressure on a rotary evaporator. Except for the different workups cited below, the product was dried *in vacuo* over phosphorous pentoxide and then it was purified either by recrystallization (acetonitrile or toluene), flash column chromatography or preparative HPLC. All structures were confirmed by MS. The melting points for the solids were: N^4-PFB-cytosine (267–271°C, d), PPs-cytosine (212–215°C, d), HFB-cytosine (221°C, d), PPS-uracil (215°C, d), PPS-thymine (261°C, d), N^4-PFB-3-methylcytosine (241–248°C,d), N^4-PFB-1-methylcytosine (171–176°C), PFB-3-methyluracil (161–163°C), and PFB-1-methyltymine (131–133°C).

The initial reaction mixture from the reaction of cytosine and5-methylcytosine with PPSC and PFBC was worked up differently. In the case of sulfonylation, after rotary evaporation the residue was dissolved in ethyl acetate–diethyl ether (2:1). The solution was washed twice with phosphate buffer (pH 6.0). After separation, the organic layer was acidified with a few drops of concentrated hydrochloric acid, and the resulting cloudy solution was refrigerated overnight. The yellowish white precipitate (the product as a salt) was filtered and washed three times with cold diethyl ether. It was dried under a stream of nitrogen and then under high vacuum over phosphorous pentoxide at room temperature. The salt was then converted to is free base form by combining it with ethyl acetate and sodium bicarbonate (0.1 *M*) in a separatory funnel. After shaking, the aqueous layer was discarded, the solvent was evaporated on a rotary evaporator under reduced pressure at 50°C, and the free base residue was dried *in vacuo* over phosphorous pentoxide. The product was pure based on HPLC.

In the case of pentafluorobenzoylation of cytosine and 5-methylcytosine, after rotary evaporation the solid residue was dissolved in ethyl acetate, and the product was extracted into 0.1 *N* sodium hydroxide. Acidification of the aqueous layer with concentrated acetic acid gave a pink precipitate, which was filtered, washed with phosphate buffer (pH 6.0) and then dried at 100°C followed by high vacuum overnight over phosphorus pentoxide. The product gave a single spot by TLC.

Alkylation

The nucleobase or electrophore-labeled nucleobase (1 mmol) was added to 5 ml of dimethylformamide, followed by the addition of 2 mmol of sodium carbonate and 5 mmol of methyl iodide or ethyl iodide for each mmol of replaceable hydrogen. After stirring for 2 h at room temperature, the reaction mixture was combined with 30 ml of ethyl acetate and washed six times with water. The organic portion was dried over anhydrous sodium sulfate, concentrated on a rotary evaporator under reduced pressure at 50°C, dried under vacuum over phosphorous pentoxide at room temperature, and purified either by recrystallization (ethyl acetate or ethyl acetate–hexane), flash column chromatography, or preparative HPLC. The molecular weights of all structures were confirmed by MS. The melting points for the solids were: 1,3-dimethyl-N^4-PFB-cytosine (185–186°C), 1,3-dimethyl-N^4-PFB-5-methylcytosine (152–153°C), dimethyl-HFB-cytosine (123–124°C), dimethyl-HFB-5-methylcytosine

(88–90°C), 1-ethyl-3-methyl-N⁴-PFB-cytosine (117–119°C), 1-methyl-3-ethyl-N⁴-PFB-cytosine (133–135°C), methyl-PPS-uracil (180–181°C), methyl-PPS-thymine (175–177°C), 1,3-dimethyl-PFB-5-fluorocytosine (142–144°C), 1,3-dimethyl-5-fluorouracil (128–131°C), 1,3-dimethyl-5-bromouracil (174–177°C), and 1,3-dimethyl-5-iodouracil (227–229°C). The reaction time for ethylation of N⁴-PFB-1-methyl-cytosine was 16 h.

RESULTS AND DISCUSSION

Fig. 1 shows the structures of cytosine (1) uracil (2) and thymine (3), the major pyrimidine bases in nucleic acids. The strategy of analyzing these bases and their altered forms by GC after prior electrophore-labelling and ethyl alkylation is demonstrated by the recent work of Gelijkens *et al.*[7]. However, electrophore attachment was not achieved onto uracil or thymine, perhaps because of the lability of the trifluoroacetyl electrophore that was used. For example, trifluoroacetamido groups are readily hydrolyzed[12,13].

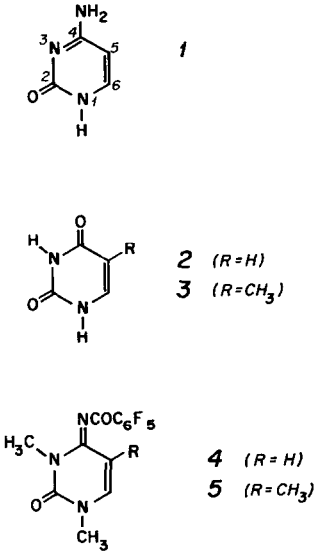

Fig. 1. Structures of cytosine (1); uracil (2); thymine (3); N⁴-PFB-1,3-dimethylcytosine (4); N⁴-PFB-1,3-dimethyl-5-methylcytosine (5).

In this work, we extend the work of Gelijkens *et al.* by forming hydrolytically-stable, strongly electrophoric, alkylated pyrimidine bases suitable for aqueous clean-up and analysis by GC–ECD. For this we use PFBC and PPSC. These two electrophoric labeling reagents have provided such derivatives for amino acids and peptides[14,15] and also tend to capture electrons more strongly than does trifluoroacetyl[15,16].

Labeling, alkylation and product stability

Both PFBC and PPSC readily label the pyrimidine bases 1, 2 and 3 in the

presence of N-methylmorpholine as an organic base. Two products are seen when excess PFBC reacts with cytosine and TLC analysis is carried out prior to sample workup. The higher R_F spot probably is N^4,N^4-di-(PFB)-cytosine that is hydrolyzed to N^4-PFB-cytosine during the aqueous work-up. This conclusion is based on results that we[17] and others (18) have obtained with related compounds. We also form a heptafluorobutyl (HFB) derivative of cytosine for comparison purposes.

Permethylated products of most of these compounds are then obtained and both these products and the corresponding precursor compounds are then evaluated for their dissolved aqueous stability giving the results shown in Table I.

TABLE I

STABILITY OF ELECTROPHORE-LABELED NUCLEIC ACID PYRIMIDINE BASES

The buffers were: acetate (0.1 *M* sodium acetate, pH 4); ACN–Phos. [acetonitrile–0.001 *M* sodium phosphate, pH 5 (55:45, v/v)] and Tris [0.2 *M* tris(hydroxymethyl)aminomethane].

Compound	*Recovery after 7 h (%)*		
	Acetate, pH 4	*ACN–Phos., pH 5 (55:45)*	*Tris, pH 8*
Cytosine			
N^4-HFB-1,3-dimethyl-	86	100	0
N^4-PFB-1,3-dimethyl-	100	100	100
N-PPS-*	61	84	59
Thymine			
PFB-*	100	100	7
PPS-methyl	100	100	64
Uracil			
PFB-*	75	94	19
PPS-methyl-	100	100	36

* Methylated derivatives of PPS-cytosine, PFB-thymine, and PFB-uracil are not reported due to the instability of these starting materials to our alkylation conditions.

The main point in this table is that the availability of PFBC and PPSC allows relatively stable electrophoric derivatives of each pyrimidine nucleobase to be obtained, at least under these conditions, with PFB preferred for this purpose on cytosine, and PPS better for thymine and uracil. It is particularly encouraging that highest stability is seen when these compounds are dissolved in an organic-aqueous solution typically used as a mobile phase for reversed-phase HPLC cleanup. Also, 7 h aqueous incubation in this experiment provides longer aqueous exposure than would ordinarily be encountered during an analysis. Thus, any of these derivatives might be used successfully under at least the acidic or organic–aqueous conditions that are cited. The lowest stability occurs when these compounds are dissolved in nucleophilic (Tris), alkaline buffer.

Storage conditions for dilute organic solutions were tested for two of the compounds, N^4-PFB-1,3-dimethylcytosine and its 5-methyl analogue. We found that a

toluene solution of these compounds, even at the 200-pg/μl level, is not stable to overnight storage even under dark conditions in a refrigerator. Earlier eluting, multiple peaks develop by GC–ECD analysis at the expense of the original, later-eluting single peaks for these compounds. Exposure of this sample to light accelerates its decomposition. Nevertheless, an isooctane solution of these compoounds, even at the 1-pg/μl level, is stable for at least one week under dark conditions in a refrigerator.

Structure proof for N^4-PFB-1,3-dimethylcytosine

We arbitrarily selected PFB-dimethylcytosine for structural elucidation. Due to the potential for different labeling–derivatization sites, including tautomeric forms, several structures were possible. The combination of several types of spectral data reduced the options, but the true structure was evident only after labeling–derivatization of authentic 1-methylcytosine[19] and 3-methylcytosine[20] gave the same final product, based on chromatographic analysis, spectral data and mixed melting point as that derived from cytosine. This structure is shown in Fig. 1 as compound 4, N^4-PFB-1,3-dimethylcytosine. The known tendency of the N^4 exocyclic amino group of cytosine to acylate[21] is consistent with this result.

GC–ECD characteristics

The labeled, methylated products shown in Table I, plus some additional methylated nucleobases, are evaluated by GC–ECD as shown in Table II. Chromatographic asymmetry values are not cited since these values are less than or equal to 1.1 in all cases, utilizing a DB-1701 column. (Initial work on a DB-5 column gave asymmetry values of 1.6 to 1.8 for the PFB-dimethyl derivatives of cytosine and 5-methylcytosine.) All products are reasonably volatile as demonstrated by the range of retention times from 0.6 to 15.4 min. In contrast, none of the corresponding non-methylated, electrophore-labeled nucleobases are detected by GC–ECD.

A high response, in the range of that for lindane, a strong electrophore, is observed for every pyrimidine in Table II labeled with a PFB or PPS electrophore. Subtle differences in response, *e.g.* by a factor of 2, for similar solutes are difficult to evaluate given the potential for variations either in recovery or sensitivity as discussed previously for another class of polar, strong electrophores[15]. The two-fold greater response for either N^4-PFB-1,3-dimethyl- or N^4-PFB-1,3-diethylcytosine relative to that given by either of the corresponding 1-methyl-3-ethyl or 1-ethyl-3-methyl derivatives apparently is due to surface effects in the GC–ECD system since the responses for these four compounds are all quite similar (0.7–0.9) on another GC–ECD system involving an on-column injector, DB-5 column, and experimental 350-μl electron-capture detector, as indicated in this table.

The high responses for the mono-Cl, -Br, and -I derivatives of uracil are noteworthy. Normally, organic molecules must be substituted with multiple halogen atoms, or corresponding electrophoretic groups, to reach the response range of lindane. Nevertheless, significant responses can arise in GC–ECD from a variety of heteroatom-containing, conjugated systems that lack conventional, electrophoric groupos[22–24]. The relatively high response for the HFB derivative of cytosine is consistent with the high response given by N-HFB-aniline[25].

TABLE II

STRUCTURAL AND GC–ECD CHARACTERISTICS OF ELECTROPHORE-LABELED, NUCLEIC ACID PYRIMIDINE BASES AND RELATED COMPOUNDS

Compound		*Retention time (min)*	*Response (relative to 1.0 for lindane)*
Core structure	*Degree and sites of heteroatom methyl or ethyl alkylation*		
N^4-PFB-Cytosine	1,3-Dimethyl	9.5	1.7 (0.8)[j]
	1,3-Diethyl	8.4	2.0 (0.9)[j]
	1-Methyl-3-ethyl[a]	8.8	0.8 (0.7)[j]
	1-Ethyl-3-methyl[b]	9.8	0.8 (0.9)[j]
5-Methyl-	1,3-Dimethyl[c]	15.4	1.8
5-F-	1,3-Dimethyl[c]	12.2	1.7
N^4-HFB-Cytosine	1,3-Dimethyl[c]	1.7	0.6
5-Methyl-	1,3-Dimethyl[c]	2.4	0.6
PFB-Thymine[d]	–	–	–
	1-Methyl[a]	4.7	1.3
PPS-Thymine	3-Methyl[e]	2.7	1.9
PFB-Uracil[d]	–	–	–
	3-Methyl[b]	2.6	1.5
PPS-Uracil	3-Methyl[f]	2.5	1.7
5-F-Uracil	1,3-Dimethyl[g]	1.6[h]	0.0003
6-Cl-Uracil	1,3-Dimethyl[g]	0.6	0.4
5-Br-Uracil	1,3-Dimethyl[g]	1.3	0.3
5-I-Uracil	1,3-Dimethyl[g]	1.9	0.6
Uracil	1,3-Dimethyl[i]	1.4[h]	0.00007

[a] The starting material for the labeling–methylation procedure was 1-methyl substituted.

[b] The starting material similarly was 3-methyl substituted.

[c] The sites of methylation were assigned based on the behavior of N^4-PFB-cytosine.

[d] Methylation of PFB-uracil and PFB-thymine gave, in each case, a major product with mass spectral characteristics that are not consistent with the expected structure. We are investigating the structure of these products.

[e] This assignment of methyl substitution was based on MS, NMR and IR characteristics of this compound and analogues, and on the known tendency of thymine to undergo N-methylation[28]. Although authentic 1-methylthymine reacted with PFBC, no reaction could be obtained with PPSC.

[f] The same final product was obtained when either uracil or authentic 3-methyluracil was the starting material.

[g] 5-F-Uracil and 5-Cl-uracil have been shown to undergo 1,3-N,N-dimethylation[29]; 5-Br- and 5-I-uracil are assumed to react similarly; 1,3-dimethyl-6-Cl-cytosine was obtained commercially.

[h] A lower column temperature of 160°C was used.

[i] Our product was identical with an authentic sample obtained commercially.

[j] Response with on-column injection, a DB-5 column, and an experimental, 350-μl electron-capture detector.

Calibration curve and detection limit

A calibration curve for compounds 4 and 5, based on absolute injected amounts involving on-column injection, is linear from 0.1 to 250 pg (data not shown). Representative chromatograms at the 100-, 1-, and 0.1-pg level are shown in Fig. 2 as A, B and C, respectively. The ratio of the peaks for compounds 4 and 5 remain constant within ± 5% throughout this range. These solutes can be routinely determined at the 0.1-pg level. However, at the 0.01-pg level several intermittent problems

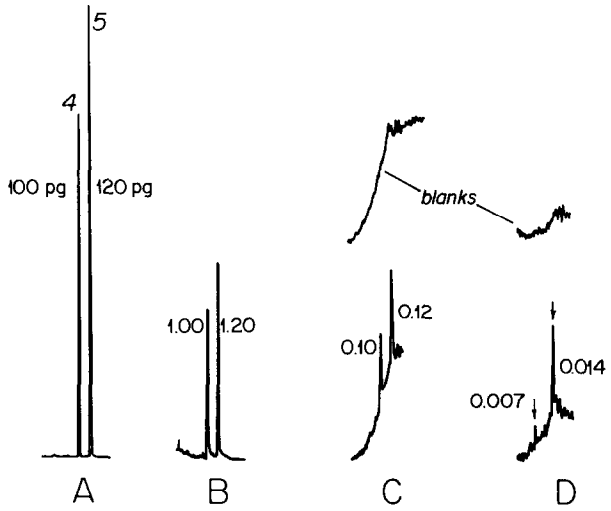

Fig. 2. GC chromatograms from on-column injection of a 1-μl solution of derivatized-cytosine (4, retention time, 3.4 min) and -5-methylcytosine (5, retention time, 3.8 min) in isooctane. The amount of 4 and 5 varied from 100 to 0.007 pg, and 120 to 0.014 pg, respectively, as shown, with attenuation settings of 128 × 10 (A), 32 × 1 (B), 8 × 1 (C) and 4 × 1 (D). An experimental electron-capture detector was used[30].

such as interferences and memory effects arise. For example, pre-injection of higher amounts, as little as 0.5 pg, of these analytes results in persistent peaks for them at the low-fg level when subsequent solvent blank injections are made. Also, this lower level is perturbed by changes in relative response, sudden disappearance of analyte peaks, and poor precision. Thus, the sample chromatogram shown in Fig. 2D, arising from the injection of a mixture of 0.007 and 0.014 pg of compounds 4 and 5, respectively, along with one of its accompanying blank injections, could not be obtained consistently. There also is a reproducible but unexplained tendency for the peak of compound 4 to decrease relative to that of compound 5 at these lower fg levels. This is seen by comparing chromatogram D with A, B and C in Fig. 2. These types of problems have been reported by others when polar solutes are analyzed at the femtogram level by GC[26], and are under investigation.

Future work

Potentially the PFB and PPS or related electrophores may react to form stable derivatives of purine nucleobases as well, and this work is underway. We are also pursuing a similar electrophore labeling-alkylation derivatization strategy for the analysis of the corresponding nucleosides. Finally, preliminary results encouragingly show that at least compounds 4 and 5 give intense molecular ions by GC coupled to NICI-MS, allowing a low-fg detection limit by this technique as well[27].

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