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High-performance liquid chromatography *versus* solid-phase extraction for post-derivatization cleanup prior to gas chromatography–electron-capture negative-ion mass spectrometry of N1,N3-bis-(pentafluorobenzyl)-N7-(2-[pentafluorobenzyloxy]ethyl)xanthine, a product derived from an ethylene oxide DNA adduct

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ABSTRACT

N7-(2-Hydroxyethyl)xanthine (N7-HEX), as a standard, has been measured at the low picomole level by the following sequence of steps: (1) derivatization with pentafluorobenzyl bromide; (2) post-derivatization sample cleanup by reversed-phase high-performance liquid chromatography (HPLC) or silica solid-phase extraction; and (3) separation/detection by gas chromatography electron-capture negative-ion mass spectrometry (GC–ECNI-MS). The average yield of product from the two sample cleanup procedures applied to 95 pg (0.48 pmol) of N7-HEX was comparable: 60% for HPLC; 56% for solid-phase extraction. The reaction blanks (0 pg N7-HEX) showed an interfering GC–ECNI-MS peak after HPLC cleanup. This problem was not encountered with solid-phase extraction, which, along with its greater convenience, made it the preferred technique for post-derivatization sample cleanup.

INTRODUCTION

We are pursuing the detection of DNA adducts in physiological samples as biomarkers for human exposure to toxic chemicals. A DNA adduct is a site on the DNA where a toxic chemical has caused covalent damage. Many carcinogens and mutagens appear to act by producing DNA adducts [1]. High sensitivity is required to measure DNA adducts in physiological samples since the samples not only contain little DNA, but small numbers of such adducts may be toxic. The techniques which are being employed or developed for this purpose have been reviewed [2].

Previously we reported our initial progress on the detection of N7-(2-hydroxyethyl)guanine, an ethylene oxide DNA adduct, by gas chromatography–electron-capture negative-ion mass spectrometry (GC–ECNI-MS) [3]. In order to make this adduct sensitive for detection by GC–ECNI-MS, we converted it, as a standard at the milligram level, to N7-(2-hydroxyethyl)xanthine (N7-HEX) with nitrous acid, followed by derivatization with pentafluorobenzyl bromide to afford N1, N3-bis-(pentafluorobenzyl)-N7-(2-[pentafluorobenzyloxy]ethyl)xanthine, $(PFBz)_3$-N7-HEX. As little as 1.3 attomoles [signal-to-noise ratio (S/N) = 10] of the latter, as a diluted standard, can be detected by GC–ECNI-MS [4].

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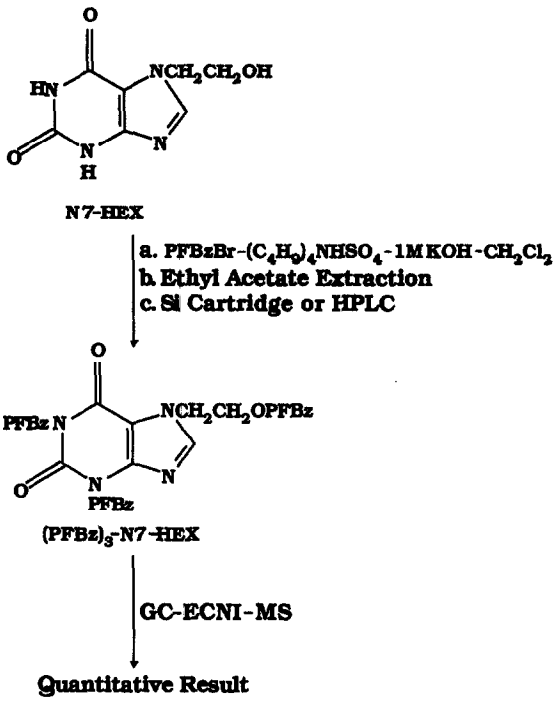

Fig. 1. Scheme for the detection of N7-HEX by GC–ECNI-MS.

Here we report additional progress towards the goal of detecting a trace amount of N7-(2-hydroxyethyl)guanine in a physiological sample. We have been working on the pentafluorobenzylation reaction (Fig. 1), and now are able to conveniently detect as little as 95 picograms (0.48 picomole) of standard N7-HEX by electrophore derivatization–post-derivatization sample cleanup–GC–ECNI-MS, as reported here. This progress has been made largely by focussing on the post-derivatization sample cleanup step. The derivatization reaction, even when performed in the absence of analyte, yields many interfering products that need to be removed prior to injection of the sample into the GC–ECNI-MS system.

EXPERIMENTAL

Reagents

N7-(2-Hydroxyethyl)xanthine was prepared as described [3]. Pentafluorobenzyl bromide, potassium hydroxide and tetrabutyl ammonium hydrogensulphate were purchased from Aldrich (Milwaukee, WI, USA). HPLC grade organic solvents were purchased from Doe and Ingalls (Medford, MA, USA).

High-performance liquid chromatography (HPLC)

A Microsorb silica reversed-phase column, 150 × 4.6 mm I.D., 10 μm (Rainin, Woburn, MA, USA) was used. The mobile phase was acetonitrile (ACN)–water (65:35, v/v) at 1 ml/min. Detection at 270 nm was done with a Spectra Monitor 3000 (LDC-Milton Roy, Riviera Beach, FL, USA) and an SP4270 integrator (Spectra-Physics, San Jose, CA, USA).

GC–ECNI-MS

The GC (5890 Series II), MS (5900A) and data system (59970 MS Chemstation) were from Hewlett-Packard (Palo Alto, CA, USA). Methane (2 Torr) and helium (20 p.s.i.) were used as reagent and carrier gases, respectively. Other conditions were as described before [4]. Specially cleaned and silanized glassware, pipettes and syringes were used to minimize adsorption losses and cross contamination [5].

Solid-phase extraction

The solid-phase extraction columns were prepared by dry-packing 200 mg of silica gel (60-Å pore, 40-μm irregular particles, J. T. Baker, Phillipsburg, NJ, USA) into a 5.25-in. borosilicate Pasteur pipette containing silanized glass wool (J. T. Baker).

Derivatization of N7-HEX

N7-HEX (95 pg, 0.48 pmol) in 10 μl of 1 *M* HCl was evaporated in a vial under nitrogen. From a stock solution of tetrabutyl ammonium hydrogensulphate (5 mg in 5 ml of 1 *M* KOH) was added 50 μl (0.15 μmol) to each vial with subsequent addition of 150 μl of CH_2Cl_2 and 10 μl (0.065 μmol) of pentafluorobenzyl bromide. The reaction mixture was stirred at room temperature for 20 h and the residual CH_2Cl_2 was slowly evaporated under nitrogen. Water (50 μl) and ethyl acetate (150 μl) were added, and the organic layer after vortexing and centrifugation was collected. Three more 150-μl ethyl acetate extractions were done, and the combined organic layer was evaporated under nitrogen, redissolved in 50 μl of acetonitrile prior to HPLC,

or in 50 μl of hexane–ethyl acetate (1:1, v/v) prior to solid-phase extraction, for post-derivatization sample cleanup as presented in Table I, followed by GC–ECNI-MS as also described in this table.

RESULTS AND DISCUSSION

For post-derivatization sample cleanup of $(PFBz)_3$-N7-HEX prior to detection by GC–ECNI-MS, we decided to explore both HPLC and solid-phase extraction. HPLC is attractive for this purpose because of its speed, high resolution, availability of automated equipment, the high quality of modern HPLC packings, and the use of stainless-steel hardware. These features collectively could be important in overcoming the classic, two general problems in trace organic analysis: losses and interferences. However, since all samples traverse the same HPLC system, it can be necessary to thoroughly clean the system between injections to minimize sample-to-sample effects such as carryover (ghosting) of the analyte. Below a certain level of analyte, on-line UV detection is no longer possible, so any shifts in retention of the trace analyte might be a second difficulty.

These two concerns about sample cleanup by HPLC prior to detection by GC–ECNI-MS should be easily avoided by solid-phase extraction. A new cartridge for each sample eliminates the difficulty of sample ghosting, and the use of optimized washing and elution conditions can assure the location of the analyte for a given batch of packing as long as the sample matrix is relatively constant. On the other hand, it is not clear that packings for solid-phase extraction are manufactured with as much attention to quality and reproducibility as those for HPLC. Also, solid-phase extraction is a lower-resolution technique. Thus we wanted to explore both HPLC and solid-phase extraction.

For HPLC, we selected a C_{18}-silica packing because of the well-deserved popularity of this type of column, particularly its resistance to contamination by conventional, small-molecule samples, and the speed and ease of achieving re-equilibration of the column after solvent changes. The latter property is important for the thorough washing of the HPLC system that we anticipated to be important for our trace samples.

The HPLC procedure that we developed for sample cleanup of $(PFBz)_3$-N7-HEX derived from subjecting 95 pg (0.48 pmol) of N7-HEX to our derivatization reaction is summarized in Table I. This procedure was the outcome of preliminary experiments (data not shown) that led to the conditions selected. Obviously the procedure is very tedious, particularly the extensive washing of the column

TABLE I

TWO PROCEDURES USED FOR POST-DERIVATIZATION SAMPLE CLEANUP

Double HPLC (C_{18}-silica)

(1) Inject 29 ng of standard $(PFBz)_3$-N7-HEX into the HPLC system to define the retention time *e.g.* 9.0 min for this compound (UV detection).
(2) Clean the HPLC system.[a]
(3) Inject 50 μl of acetonitrile (ACN blank A), collect 2 ml, and hold for steps 11, 12. Repeat HPLC cleaning and ACN injection twice (ACN blanks B, C).
(4) Inject reaction blank A, collect 2 ml, and hold for steps 11 and 12.
(5) Clean the HPLC system.[a]
(6) Repeat (steps 4 and 5) for reaction blank B, and for reaction blank C.
(7) Inject reaction sample A, collect 2 ml and hold for steps 11 and 12.
(8) Clean the HPLC system.[a]
(9) Repeat for reaction sample B, and for reaction sample C.
(10) Clean the HPLC system.[a]
(11) Evaporate ACN blank A, redissolve in 50 μl ACN, inject into the HPLC system, collect 2.0 ml, evaporate, redissolve in 50 μl of toluene, and inject 1 μl into the GC–ECNI-MS system.
(12) Wash the HPLC injector with 3 ml of hot ACN (as in step 2).
(13) Repeat steps 11 and 12 for each of the remaining blanks and samples.

Solid-phase extraction

(1) Wash the silica cartridge with 1 ml of ethyl acetate and 1 ml of hexane.
(2) Load the reaction blank (A, B) or reaction sample (A, B, C, D) in 50 μl of hexane–ethyl acetate (50:50, v/v).
(3) Wash with 4 ml of hexane and 8 ml hexane–ethyl acetate (90:10, v/v).
(4) Elute with 2 ml of ethyl acetate.
(5) Evaporate and continue as in above step 10.

[a] The HPLC column was cleaned by twice conducting a gradient from ACN–water (65:35, v/v) to 100% ACN over a 10-min period, holding for 4 min, and returning over a 10-min period to the initial condition. Before each gradient wash, the injector was washed with 0.5 ml of warm ACN in the inject position and the same in the load position.

and injector between injections, and the separation of each sample twice by HPLC. But our earlier experiments gave irreproducible results by GC–ECNI-MS in terms of both losses and interferences when less rigorous inter-sample washing of the HPLC system was done, or when the samples were subjected to a single HPLC separation.

The results of this procedure (Table I) are shown in Table II. Remarkably, once 29 ng of $(PFBz)_3$-N7-HEX are injected into the HPLC system in order to establish the retention time (with UV detection), extensive washing of the HPLC system is necessary before the compound is completely removed from this system. As seen, the first two ACN blanks (A and B; pure acetonitrile is injected) become contaminated [2.9 and 3.9 pg of apparent $(PFBz)_3$-N7-HEX, respectively, at the end of the overall procedure]. Finally the third ACN blank (C), collected after the column and injector have been subjected to a 3-h cleaning procedure (defined in Table I), shows no analyte, as seen in Table II.

For the reaction blanks A–C, it is not clear whether the observed, interfering peak by GC–ECNI-MS, as cited in Table II, is contaminating analyte or some other interfering compound derived from the derivatization reaction. Conceivably the HPLC system is still contaminated by analyte from the earlier, 29 ng injection, and the injection of a reaction matrix as opposed to injection of pure-solvent (the ACN blanks) displaces additional analyte from the system. At least the reaction itself is working at this level: the reaction sample A–C show an amount of product which is significantly higher than that present in the reaction blanks. If we subtract the average value for the reaction blanks from the average value for the reaction samples, we obtain a yield for the reaction of 60%. A representative GC–ECNI-MS chromatogram for a reaction blank is shown in Fig. 2a, and for a reaction sample in Fig. 2b. Note that the abundance scales are different, so the amount of the interfering peak (Fig. 2a) relative to the product peak (Fig. 2b) is different than the visual appearance. While we could have explored the origin of the contamination in more detail, we were disuaded from this by the much better performance provided by solid-phase extraction for sample cleanup, that we investigated in parallel, as presented below.

TABLE II

DETERMINATION OF N7-HEX BY PENTAFLUOROBENZYLATION–GC–ECNI-MS USING THE TWO POST-DERIVATIZATION SAMPLE CLEANUP PROCEDURES PRESENTED IN TABLE I

Sample cleanup procedure	Amount of $(PFBz)_3$-N7-HEX or interference[a] (pg) starting from 0 pg (ACN blanks and reaction blanks) or 95 pg (reaction samples) of N7-HEX. A 100% yield of product would be 356 pg.
Double HPLC (C_{18}-silica)	
ACN blanks (A,B,C)	2.9, 3.9, 0.0
Reaction blanks (A,B,C)	4.1, 10.5, 29.0
Reaction samples (A,B,C)	205, 305, 129
Solid-phase extraction (silica)	
Reaction blanks	0,0
Reaction samples (A,B,C,D)	260, 180, 124, 234

[a] The interference is quantified, although it is an unknown, by assuming that it is $(PFBz)_3$-N7-HEX.

For post-derivatization sample cleanup by solid-phase extraction, we selected a silica packing. While C_{18}-silica packings are also popular for solid-phase extraction, we wanted to avoid the potential for variability in the C_{18} bonding to silica by manufacturers. Also, we had previously found a silica cartridge to be useful for post-derivatization sample cleanup of an electrophoric derivative of 5-methylcytosine prior to detection by GC with electron-capture detection [6].

The convenient procedure that we developed for post-derivatization sample cleanup of $(PFBz)_3$-N7-HEX by solid-phase extraction on a silica cartridge is summarized in Table I. After the samples are loaded onto the cartridge in hexane–ethyl acetate (50:50) washing is done with hexane–ethyl acetate (90:10), and then product is eluted with ethyl acetate. The results from subsequent measurement of the reaction blanks and reaction samples by GC–ECNI-MS are shown in Table II. As seen, no interfering peaks are encountered in the reaction blanks, unlike what was observed after sample cleanup by HPLC. The average yield of product from the four reaction samples tested (A–E) is 56%. [In prior development work, spiking 57 pg in duplicate of $(PFBz)_3$-N7-HEX into a reaction blank followed by solid-phase extraction–GC–ECNI-MS gave a recovery of 84 and 85%.] Obviously this technique

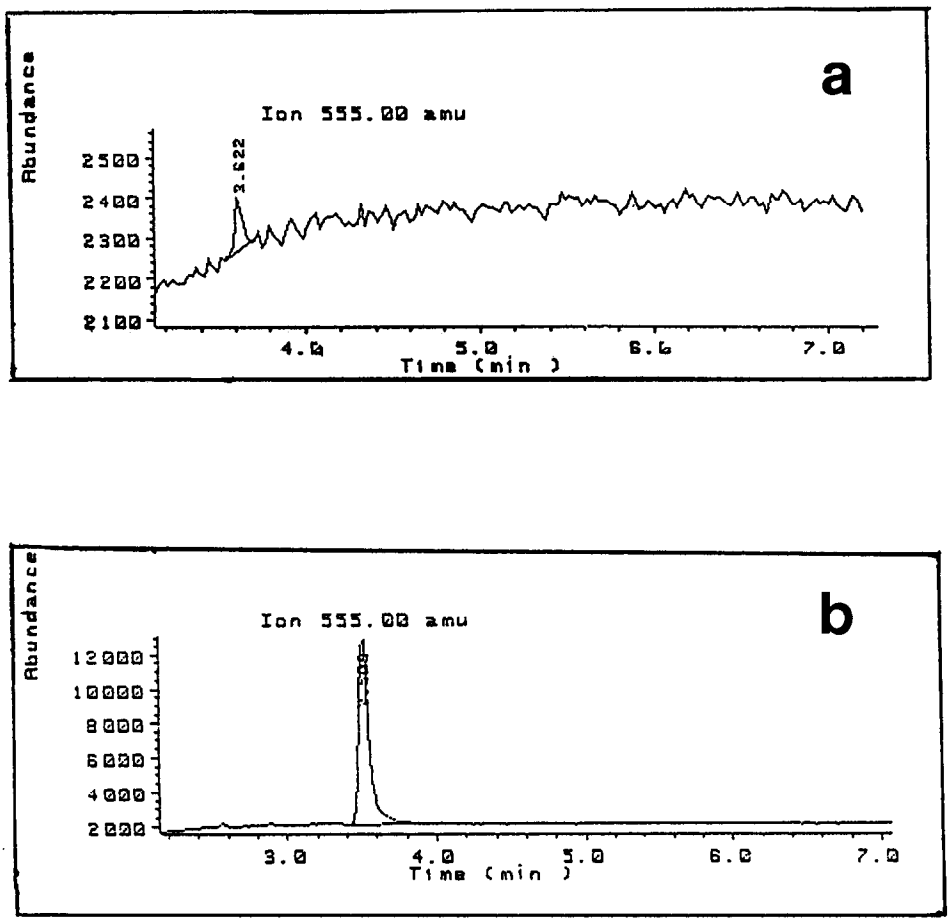

Fig. 2. Detection of $(PFBz)_3$-N7-HEX by GC–ECNI-MS obtained via the scheme shown in Fig. 1 and using double HPLC for post-derivatization sample cleanup as detailed in Table I. (a) Reaction blank A (see Table II); 0 pg of N7-HEX was derivatized. (b) Reaction sample B (see Table II); 95 pg of N7-HEX was derivatized. For both chromatograms, 1/50 of the final sample in 50 μl of toluene was injected. Each peak shown co-elutes with authentic $(PFBz)_3$-N7-HEX. GC column length: 12 m.

wins over the tedious, contaminant-prone double HPLC technique. A representative GC–ECNI-MS chromatogram for a reaction blank is shown in Fig. 3a, and for a reaction sample in Fig. 3b, after silica solid-phase extraction.

CONCLUSION AND FUTURE WORK

A convenient procedure utilizing silica solid-phase extraction for post-derivatization sample cleanup has been developed for the detection of standard N7-(2-hydroxyethyl)xanthine at the low picomole level by GC–ECNI-MS. Since the yield and signal-to-noise ratio are high, there is no interfering peak in the reaction blank, and only 1/50 of the final sample is injected into the GC–ECNI-MS, potentially the technique can be taken to a lower analyte level. We will be pursuing this goal in our future work, and also attempting to extend the method to the detection of a trace amount of the parent adduct, N7-(2-hydroxyethyl)guanine, in biological samples. It is desirable to reach at least the femtomole level in order to minimize the amount of such samples required.

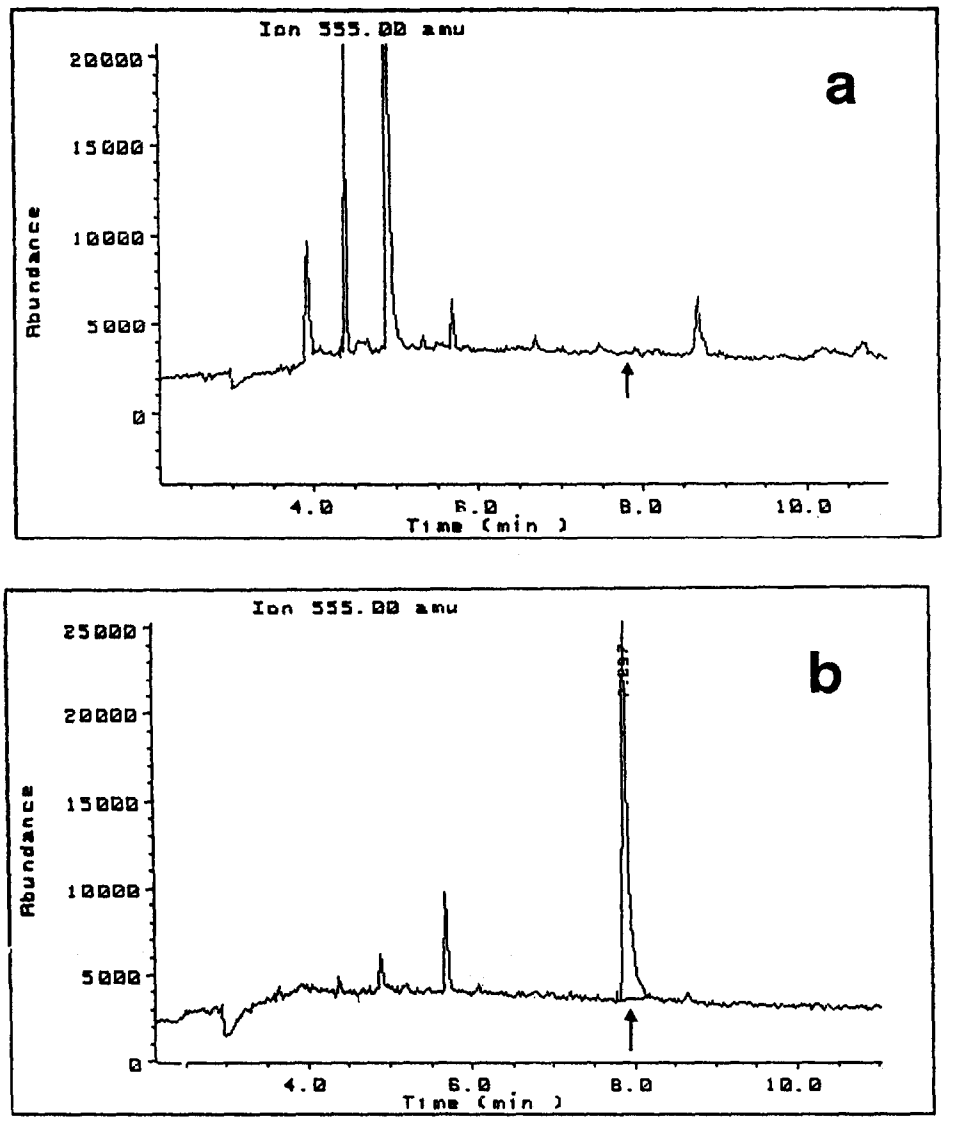

Fig. 3. Same as Fig. 2 except silica solid-phase extraction was used for post-derivatization sample cleanup (see Table II). (a) Reaction blank. (b) Reaction sample A (see Table II). The peak or retention position (based on injecting authentic product) for $(PFBz)_3$-N7-HEX is shown with an arrow. GC column length: 20 m.

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REFERENCES

1 B. Singer and D. Grunberger, *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York, 1983.
2 R. C. Garner, P. B. Farmer, G. T. Steel and A. S. Wright (Editors), *Human Carcinogen Exposure*, IRL Press at Oxford University, Press, Oxford, 1991.
3 K. Allam, M. Saha and R. W. Giese, *J. Chromatogr.*, 499 (1990) 571.
4 S. Abdel-Baky and R. W. Giese, *Anal. Chem.*, 63 (1991) 2986.
5 J. Adams, M. David and R. W. Giese, *Anal. Chem.*, 58 (1986) 345.
6 D. H. Fisher and R. W. Giese, *J. Chromatogr.*, 452 (1988) 51.