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# Identification of potentially mutagenic contaminants in the aquatic environment by liquid chromatographic–thermospray mass spectrometric characterization of in vitro DNA adducts

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**Abstract**

Liquid chromatographic–thermospray mass spectrometric (LC–TSP-MS) characterization of chemical adducts of DNA formed during in vitro reactions is proposed as an analytical technique to detect and identify those contaminants in aqueous environmental samples which have the propensity to be genotoxic, i.e. to covalently bond to DNA. The approach for direct-acting chemicals includes the in vitro incubation of DNA with contaminated aqueous samples at 37°C, pH 7.0 for 0.5 to 6 h, followed by enzymatic hydrolysis of the DNA to deoxynucleosides and LC–TSP-MS analysis of the resultant solution. A series of allylic reagents was used as model reactive electrophiles in synthetic aqueous samples to demonstrate that adduct formation was linear with both contaminant concentration and electrophilic reactivity potential. The characterizations can also estimate the proportion of bonding to different sites on a base, for instance, the ratio of $O^6$- to 7-alkylguanine (oxygen vs. nitrogen bonding) products, which is an important parameter in assessing the genotoxicology of chemicals.

## 1. Introduction

Potentially mutagenic chemical agents are recognized as being contaminants in the aquatic environment, including municipal and industrial discharges, hazardous waste leachates and contaminated sediments. The development of protocols for the regulation of discharges and the remediation of contaminated areas is dependent upon the careful detection and identification of potential mutagens. Those identifications, however, can be very difficult because the potentially mutagenic chemicals are often (1) at very low concentrations, (2) in the presence of much higher concentrations of non-mutagenic contaminants and (3) poorly recovered during sample processing. Their identification has been based primarily upon extensive gas chromatographic–mass spectrometric (GC–MS) characterizations of all (or as many as possible) chemicals in an extract, followed by an assessment of the structures to determine which might possibly be mutagenic [1]. The use of MS–MS spectra [2] and the integration of GC–MS with mutagenic response bioassays [3] have been added to improve the chances of mutagen detection. An

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alternative approach for the selective detection and identification of mutagens was proposed by Cheh and Carlson [4]. They assessed the ability of low-molecular-mass nucleophilic reagents to selectively label electrophilic (mutagenic) contaminants in complex solutions after sample enrichment. The label provided an easily identifiable moiety with which to detect potential mutagens.

The use of selective labeling to identify potentially mutagenic contaminants in complex environmental samples is also being investigated in these laboratories. First, using a gas chromatograph interfaced to the collision cell of a tandem quadrupole mass spectrometer, it was demonstrated that potentially mutagenic environmental contaminants could be selectively detected after reaction with gas phase nucleophilic labeling reagents in the collision cell of the mass spectrometer [5–7], and second, we have been assessing aqueous in vitro reactions of electrophiles with their ultimate in vivo target, DNA, as the labeling reagent. In contrast to reactions with low-molecular-mass nucleophilic reagents, reactions with DNA are important because many environmental contaminants which are metabolically activated to reactive electrophiles, such as polynuclear aromatic hydrocarbons, require the DNA double helix structure for adduct formation to occur [8]. Further, DNA adducts formed in vitro have the analytical advantage over adducts formed in vivo of not being destroyed by cellular DNA repair mechanisms before they can be identified.

The second technique, using DNA as a labeling reagent, is the subject of this report. Our objective was to conduct a feasibility study to determine if liquid chromatography–thermospray mass spectrometry (LC–TSP-MS) can be used to identify potentially mutagenic chemicals isolated from aqueous environmental samples (effluents, leachates, etc.) by selectively bonding to DNA during in vitro reactions. Although the non-chemospecific measurement of DNA adducts by $^{32}$P-postlabelling has previously been proposed as a technique to assess the presence of mutagenic contaminants in environmental samples [9], to the best of our knowledge, LC–TSP-MS has not been applied to the characterization of potentially mutagenic chemical contaminants isolated in such a manner.

A series of allylic reagents has been selected to test the proposed protocol. The potential of these reagents to alkylate nucleophiles has previously been correlated to their mutagenic potential [10,11]. Some of the compounds selected, because of their highly reactive nature, may not necessarily be identified as an environmental contaminant. However, they can serve as a good set of demonstration chemicals because of their previous extensive characterization.

## 2. Experimental

### 2.1. Chemical reagents

Acetonitrile (spectrograde) was obtained from EM Scientific (Gibbstown, NJ, USA). Ammonium acetate (ACS reagent grade), allylchloride, allylbromide, allyliodide and allylisothiocyanate were obtained from Aldrich (Milwaukee, WI, USA) and used without further purification. Nucleosides, enzymes and calf thymus DNA were obtained from Sigma (St. Louis, MO, USA).

*Caution:* Allyl reagents should be used with care in a well ventilated area or chemical fume hood.

### 2.2. Instrumentation

A Finnigan-MAT (San Jose, CA, USA) Model TSQ-70 triple quadrupole mass spectrometer coupled through a Finnigan-MAT Model TSP-2 thermospray interface to a Beckman Model 340 liquid chromatograph was used to characterize DNA hydrolysates. The HPLC system was equipped with a Supelcosil LC-18s reversed-phase column (Supelco, Bellefonte, PA, USA). The mobile phases were programmed after 3 min from 3% to 15% B in 6 min and to 85% B in an additional 6 min. The system was then maintained at 85% B for an additional 10 min. Simultaneously, the flow-rate was increased after 3 min from 1.2 to 1.4 ml/min in 12 min,

and similarly maintained at that value for an additional 10 min. Mobile phases A and B were 1.5% and 30% acetonitrile in water, respectively. Both mobile phases were 0.05 *M* ammonium acetate, pH 6.5. The mass spectrometer was operated in both the positive ion and pulsed positive/negative ion scan modes. The positive ion scan was 105–405 u/s. The pulsed scan was 105–405 u/0.5 s for positive ions and 120–405 u/0.5 s for negative ions. Argon at approximately 1 Torr (1 Torr = 133.322 Pa) was used as the collision gas for MS–MS experiments. Collision energies varied from −0.5 to −20.0 V, and mass spectrometer–mass spectrometer voltage correction (MSMSC) was 50 V.

### 2.3. Allylation of deoxyguanosine

To 100 μmol of deoxyguanosine in 500 μl 50 m*M* Tris–HCl (pH 7.0) were added 100 μmol allylbromide in 100 μl dimethyl sulfoxide (DMSO). The reaction mixture was stirred at 37°C for 2 h. The reaction was stopped by placing it on ice, and analyzed directly by LC–TSP-MS.

### 2.4. In vitro DNA alkylation reactions

Calf thymus DNA (200 μg) was dissolved in 100 m*M* Tris–HCl pH 7.0 (500 μl). To this was added from 0.5 to 25 μmol allyl reagent in 10 to 100 μl DMSO. The reaction was maintained at 37°C for 0.5 to 6 h. The reactions were stopped by placing the reaction on ice for 10 min. The DNA was then precipitated from the reaction mixture by the addition of 50 μl of 3 *M* LiCl followed by the slow addition of 450 μl isopropanol, chilling the mixture to −20°C, and centrifuging for 30 min at 10 000 *g*. The solvent and excess reagent were removed by drying the DNA in a rotary vacuum evaporator.

### 2.5. DNA hydrolysis

Hydrolysis of DNA to deoxynucleosides was achieved by the consecutive application of nuclease $P_1$, phosphodiesterase I and alkaline phosphatase as previously described [12].

### 2.6. Quality assurance/quality control

Performance of the LC–TSP-MS for analyte elution, signal response and mass calibration was evaluated using calibration standards of deoxynucleosides [12]. Alkylation reactions were conducted and analyzed in replicate.

## 3. Results and discussion

Products formed during the in vitro reaction of unsubstituted allylic compounds with DNA have previously been identified by Eder et al. [13]. Product distribution was dominated by reactions with the purine bases, guanine and adenine. They observed that 7-allylguanine (7-A-Gua) and 3-allyladenine were released spontaneously during alkylation while $N^6$-allyladenosine and $O^6$-allylguanosine ($O^6$-A-Dguo) were released only during enzymatic hydrolysis of DNA to free deoxynucleosides. Alkylation of guanine at the 7 position (nitrogen) is expected to be the predominant product formed during reactions of double-stranded DNA with low-molecular-mass direct-acting electrophiles [14]. The proportion of bonding at $O^6$ relative to N-7, however, is an important parameter in the evaluation of genotoxic risk [15].

The experiments performed here were based upon the results of Eder et al. [13] and were designed to assess several factors necessary to determine if the proposed protocol was a feasible approach to developing a method for the chemospecific identification of environmental mutagens. It was necessary to demonstrate whether (1) alkylation product formation was linear with the concentration of a mutagenic contaminant in an aqueous sample, (2) alkylation product formation was proportional to the relative reactivity of various mutagenic contaminants in an aqueous sample and (3) it was possible to determine the relative proportion of guanine alkylation products which had been formed, i.e. N-7 vs $O^6$ allylguanine.

Identification of the products of alkylation of deoxyguanosine (dGuo) and DNA by allylbromide using LC–TSP-MS are presented in

Table 1
Identification of three alkylated guanines produced during in vitro reactions of allylbromide with deoxyguanosine and calf thymus DNA

| Analyte | M + H | RRT (dCyd = 0)[a] | Nucleophile | |
|---|---|---|---|---|
| | | | dGuo (%)[b] | DNA (%)[b] |
| dGuo | 268 | 1.00 | – | – |
| $N^2$-A-dGuo | 308 | 2.61 | 53 | 0.4 |
| 7-A-Gua | 192 | 2.89 | 35 | 99 |
| $O^6$-A-dGuo | 308 | 3.90 | 12 | 0.6 |

The table provides $m/z$ values of M + H ions and relative LC retention time of products. Data also show the difference in product distribution of modified dGuo relative to the total amount of modified dGuo obtained when dGuo is free in solution vs. held in the DNA double helix during the reaction. (Product distributions from the two reactions are estimated by calculating $[M + H]^+$ TSP peak area ratios, and are not quantitative.) Reaction conditions: 37°C, pH 7.0, 2 h, 5 μmol allylbromide, 100 μg DNA or 5 μmol dGuo.

[a] RRT = Relative retention time.

[b] Modification/total modification × 100.

Table 1. Identification of modified products was based upon increasing reversed-phase liquid chromatography elution order presented by Eder et al. [13] ($N^2$-allylguanosine before 7-A-Gua followed by $O^6$-A-dGuo), upon appropriate mass spectral $m/z$ values ($m/z$ 192, B + 2H allylguanosine and M + H allylguanine; and $m/z$ 308, M + H allylguanosine), and upon the detection of a predominant product resulting from N-7 alkylation of guanine in the DNA structure compared to dGuo free in solution. Further, the N-7 reaction product was confirmed to be a modified base by the inability to detect an ion at $m/z$ 308 during a MS–MS product ion experiment. The reaction product determined to be $O^6$-A-dGuo was further characterized by determining that the ion at $m/z$ 308 produced an ion at $m/z$ 192 during a MS–MS precursor-ion experiment.

DNA alkylation product formation, as a function of the concentration of the mutagenic contaminant in the sample, was assessed by determining the amount of 7-A-Gua and $O^6$-A-dGuo formed relative to the amount of dGuo present in the final reaction product hydrolysate. The results are presented in Table 2. The production of 7-A-Gua increased rapidly with the dose of allylbromide, exceeding the amount (peak area ratio, $m/z$ 192 M + H 7-A-Gua:$m/z$ 152 B + 2H dGuo) of dGuo at a dose of just over 5.0 μmole allylbromide. In spite of the large loss of dGuo, the formation of $O^6$-A-dGuo increased linearly from 0.5 to 25 μmol allylbromide ($r^2$ = 0.995).

DNA alkylation product formation, as a function of the reactivity of individual mutagenic components in an aqueous sample, was assessed by determining the relative amount of 7-A-Gua formed during the reaction of 5 μmol of each of four allylic reagents, allylisothiocyanate, allylchloride, allylbromide and allyliodide, with DNA. The amount of product formed relative to

Table 2
Product formation during in vitro reactions of allylbromide with DNA

| Dose (μmol) allylbromide | Modification/dGuo | |
|---|---|---|
| | 7-A-Gua | $O^6$-A-dGuo |
| 0.5 | 0.12 | <0.0001 |
| 5.0 | 0.93 | 0.015 |
| 7.5 | 2.11 | 0.025 |
| 12.5 | – | 0.05 |
| 25.0 | – | 0.11 |

Data show an increase in product formation relative to dGuo as the amount of allylbromide added to the reaction increases. Product formation calculated from $[M + H]^+$ TSP peak areas; not quantitative. Reaction conditions: 37°C, pH 7.0, 6 h, 100 μg DNA.

dGuo (peak area measured as before) was compared to alkylating activity [4-nitrobenzylpyridine (NBP)] and mutagenic activity (*Salmonella typhimurium* TA 100) previously presented by Eder et al. [11] (Table 3). NBP activity was found to correlate with mutagenic activity at $r^2 = 0.999$. The correlation between DNA alkylation product formation determined by LC–TSP-MS with NBP reactivity was $r^2 = 0.891$. Allyliodide was not found to be as reactive in the DNA alkylation experiment as was expected from the mutagenicity results. The reason for this is not known; however, it is possible that because of the high reactivity of this compound, some of it had hydrolyzed to the less reactive allyl alcohol.

The propensity of an environmental contaminant to be more or less carcinogenic relative to other contaminants can potentially be estimated by evaluating its initial N-7:$O^6$ alkylation ratio [15]. The data presented in Table 1 indicate that it was possible to estimate this ratio using LC–TSP-MS characterizations of in vitro DNA alkylation reactions. For instance, for the reaction conditions of 5 μmol allylbromide, 37°C and 6 h and 100 μg DNA, the estimated ratio of N-7:$O^6$ calculated using peak areas of M + H ions was found to be 62:1. Accurate ratio determinations, of course, would require analytical quantification standards.

These studies indicate that the proposed protocol for the chemospecific detection of potentially mutagenic environmental contaminants is feasible, and therefore further experiments should be conducted to extend the technique. Additional studies will include sample-enrichment procedures [4,16], integration of the protocol into toxicity identification evaluation (TIE) procedures [17] and complex mixture analysis. Our preliminary investigations into LC–TSP-MS characterization of contaminants metabolized by liver microsomal preparations to reactive species indicate that the protocol can be readily extended to contaminants such as polynuclear aromatic hydrocarbons (PAHs) [18], where the reactive form of the molecule, the diol epoxide metabolite, covalently bonds with $N^2$ of dGuo.

It must be remembered that the modifying moiety which has covalently bonded to DNA is not the intact environmental contaminant which one wishes to identify, but a only portion of the original molecule which remains after it has been modified to become the reactive species. Although systematic studies to optimize TSP analyses have shown that TSP can be reproducible and highly sensitive [19], TSP spectra of deoxynucleosides are generally quite simple and usually do not contain sufficient information for a complete characterization of the modifying moiety. However, numerous instrumental techniques are available to enhance fragmentation and thus provide more structural information. These techniques include variation of vaporizer and/or source temperature, selection of filament and/or

Table 3
Comparison of the formation of 7-A-Gua ($[M + H]^+$ TSP peak area ratio) produced during the in vitro alkylation of DNA by four different allyl reagents to the alkylating (NBP) and mutagenic activity obtained by Eder et al. (11)

| Allyl reagent | LC–TSP-MS, 7-A-Gua/dGuo | Alkylating activity, NBP ($\Delta E_{560}$)[a] | Mutagenic activity (revertants/μmol) |
|---|---|---|---|
| Thiocyanate | <0.0001 | 0.03 | 1 |
| Chloride | 0.002 | 0.3 | 9 |
| Bromide | 0.012 | 54 | 700 |
| Iodide | 0.018 | 164 | 2000 |

Formation of 7-A-Gua relative to dGuo was estimated from $[M + H]^+$ TSP peak area; not quantitative. Reaction conditions: 37°C, pH 7.0, 2 h, 5 μmol allylbromide, 100 μg DNA.
[a] Data from Ref. 11. $\Delta E_{560}$ = Molar extinction coefficient measured at 560 nm.

discharge ionization and collision-induced dissociation with the TSP repeller [20] and by MS–MS. A review of MS techniques for the characterization of the modifying moiety in DNA alkylations has been presented by McCloskey and Crain [21].

Sensitivity (full mass range scanning, positive ion) for the characterization of a modifying moiety using the protocol proposed here was assessed by analyzing a series of standards each containing Guo (500 pg/$\mu$l) and decreasing amounts of 7-methylGuo (to 1 pg/$\mu$l). It was found that at the greatest concentration ratio for Guo to 7-methylGuo (500:1), the ratio of the TSP peak area of M + H for each analyte was 228:1, and that 1 pg/$\mu$l (100 $\mu$l injected) of 7-methylGuo produced a signal-to-noise ratio ($S/N$) of 7:1. For an injection volume of 100 $\mu$l taken from 500 $\mu$l of solution produced by the hydrolysis of 100 $\mu$g DNA, this sensitivity represents the detection of approximately 1 modified guanine per 10 000 guanine residues.

Alternative analytical techniques to LC–TSP-MS, such as LC–continuous-flow fast atom bombardment MS (LC–FAB-MS) may also be feasible for these types of analyses. For instance, Kostiainen et al. [22] have recently used LC–FAB-MS to successfully characterize butadienemonoxide/deoxyadenosine reaction products, and Wolf and Vouros [23] have used it to characterize N-acetoxy-N-acetyl-2-aminofluorene/dGuo adducts with a sensitivity of one adduct per $10^6$ normal bases. Finally, we believe that low-molecular-mass nucleophilic reagents, such as 4-nitrothiophenol [4] and NBP [11], which have been used to assess the reactivity of electrophilic chemicals can be used to conduct a preliminary assessment of environmental samples for reactive contaminants. We have therefore also been pursuing the development of alternative nucleophilic reagents suitable for high-sensitivity characterization by GC–MS, using highly selective precursor and product ion scans in both positive and negative ionization modes [24]. No single analytical technique or biological assay can be expected to completely characterize all potentially mutagenic contaminants in an environmental sample, but the protocol described in this paper can serve as a complementary technique to other existing techniques.

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