*Journal of Chromatography*, 474 (1989) 197–207
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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# LIQUID CHROMATOGRAPHY–THERMOSPRAY MASS SPECTROMETRY OF DNA ADDUCTS FORMED WITH MITOMYCIN C, PORFIROMYCIN AND THIOTEPA

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## SUMMARY

High-performance liquid chromatography (HPLC) and thermospray mass spectrometry were combined for the analysis of DNA adducts formed from the interaction of the anticancer drugs mitomycin C, porfiromycin and thiotepa with calf thymus DNA. The adducts formed from reaction of mitomycin C and porfiromycin with DNA were separated from unmodified nucleosides by HPLC on a $C_{18}$ column and identified by thermospray mass spectrometry. Thiotepa DNA adducts readily depurinated from DNA and were chromatographed and identified by thermospray liquid chromatography–mass spectrometry as the modified bases without the ribose moiety attached. The utility of thermospray mass spectrometry for the identification of microgram quantities of nucleoside adducts and depurinated base adducts of these anticancer drugs was demonstrated.

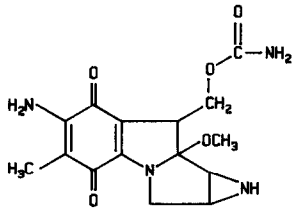
## INTRODUCTION

The ability to link the effluent from a high-performance liquid chromatograph to a mass spectrometer via a thermospray ionization source has proved to be very useful for the analysis of thermally labile, non-volatile compounds such as nucleotides and nucleosides[1–5]. Since the process does not require derivatization[6,7] or extensive sample workup[8,9] as required for other mass spectrometric techniques, thermospray mass spectrometry (MS) serves as an excellent method for rapid identification of nucleoside adducts observed in liquid chromatograms.

Many anticancer drugs are thought to exert their tumor cell killing potency by their ability to covalently bind to DNA forming mono and dialkylated adducts[10–12]. The identification of these adducts is important for understanding their therapeutic

0021-9673/89/$03.50 

importance and designing new drugs. Since thermospray liquid chromatography (LC)–MS is well suited for nucleoside analysis it could prove to be a useful tool in determining the structures of adducts created by the alkylation of DNA with anticancer drugs.



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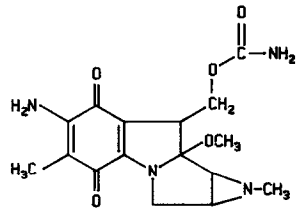

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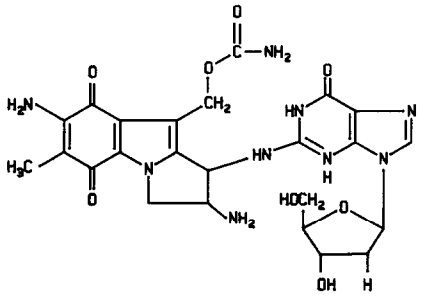
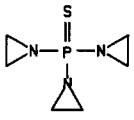


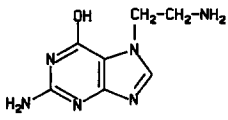
III

Mitomycin C (I) and its N-methyl analogue porfiromycin (II) are clinically effective anticancer drugs[13] that are known to alkylate DNA. The structure of the monoalkylation product formed between DNA and mitomycin C has been elucidated by Tomasz *et al.*[14] using NMR and Fourier-transform infrared (FT-IR) analysis. The alkylation product is a stable nucleoside adduct which occurs on the exocyclic N-2 position of deoxyguanosine residues (III). Porfiromycin has been shown to alkylate DNA by the same mechanism as mitomycin C, resulting in the formation of an analogous adduct[15].

Thiotepa (IV) is an anticancer drug which has been used clinically for over 30 years and has been used more aggressively in recent years in combination with autologous bone marrow transplantation[16–18]. Unlike mitomycin C, there has been little work done on the mechanism of alkylation of DNA by thiotepa. Several studies



IV



V

carried out on the alkylation of isolated nucleosides by thiotepa have suggested that alkylation occurs on the N-7 position of deoxyguanosine residues[19,20], leading to the formation of 7-(2-aminoethyl)guanine (V). Based on the instability of N-7 alkylated deoxyguanosine adducts formed by alkylation with ethylenimine or phosphoramide mustard [21,22], the adducts formed from reaction of thiotepa with DNA would also be expected to be unstable, leading to the formation of depurinated or imidazole ring opened products[21,22].

The purpose of this study was to demonstrate the utility of thermospray LC–MS for the identification of two types of drug–DNA adducts of potential clinical importance. The adducts of interest are stable nucleoside–drug adducts and adducts which cause depurination of the DNA. Since the adducts have been well defined by other methods, they should make good model compounds for comparative studies by thermospray LC–MS.

## EXPERIMENTAL

### *Reagents and materials*

Mitomycin C was provided by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute and by Dr. W. Bradner, Bristol Labs. (Syracuse, NY, U.S.A.). Porfiromycin was provided by Upjohn (Kalamazoo, MI, U.S.A.). Thiotepa was provided by Lederle Labs. (Pearl River, NY, U.S.A.). Unmodified nucleosides, calf thymus DNA, pancreatic DNAase (E.C. 3.1.4.5), snake venom phosphodiesterase (E.C. 3.1.4.1), and alkaline phosphatase (E.C. 3.1.3.1) were purchased from Sigma (St. Louis, MO, U.S.A.). Ethylenimine was prepared from the sulfate ester of ethanolamine according to the procedure of Wenker[23] and used immediately after preparation.

The water used for buffers was purified through a Milli-Q system, Millipore (Bedford, MA, U.S.A.) to give a resistivity of 18 MΩ/cm. Gold-label ammonium formate was obtained from Aldrich (St. Louis, MO, U.S.A.). HPLC grade methanol was used for all chromatography.

### *Preparation of mitomycin C and porfiromycin DNA adducts*

Adducts were prepared by a modification of the procedure of Tomasz *et al.*[24]. In a typical experiment calf thymus DNA (1 mg) was dissolved in 1 ml of 20 m*M* Tris buffer, pH 7.4. To this solution was added approximately 300 μg of mitomycin C or porfiromycin in buffer. Nitrogen was bubbled through the solution for 1 h, after which time 100 μg of $PtO_2$ was added. Hydrogen gas was bubbled through the solution for 10 min, followed by nitrogen gas for 20 min, air was then bubbled through the solution for 20 min. The platinum was removed by centrifugation.

The DNA was precipitated and digested according to the procedure of Pan *et al.*[25]. The DNA was precipitated by adjusting the NaCl concentration to 0.1 m*M* and adding two volumes of cold 95% ethanol (0°C) to the DNA–mitomycin C solution. The solution was centrifuged for 5 min at 5000 *g*. The supernatant was discarded and the DNA pellet was washed with cold ethanol to remove non-covalently bound mitomycin C or porfiromycin. The DNA was resuspended in 50 m*M* Tris buffer, pH 7.4. DNAase at 200 kallikrein units per mg DNA was added to the solution and allowed to digest the DNA for 4 h at 37°C. The pH was raised to 8.5 and $MgCl_2$ was added

such that the final concentration was 5 m*M*. The DNA was digested to nucleotides with 0.05 units of phosphodiesterase per mg DNA in 4 h at 37°C. Finally, the nucleotides were digested with 3.0 units of alkaline phosphatase per mg DNA for 12 h at 37°C to yield a mixture of alkylated and non-alkylated nucleosides. This mixture was then analyzed by thermospray LC–MS. An injection of 100 μl of the digested DNA was made onto the high-performance liquid chromatographic (HPLC) column amounting to approximately 1–3 μg of adduct being directed into the thermospray interface.

*Preparation of thiotepa–DNA and ethylenimine–DNA adducts*

To a solution of 1 mg/ml of calf thymus DNA in 25 m*M* phosphate buffer, pH 7.4, were added aqueous buffer solutions of thiotepa (100 μg) or ethylenimine (500 μg) along with 10 μg of toluene to prevent bacterial growth. The solution was stirred at 37°C for three days in a capped test tube. At the end of the reaction the DNA was precipitated and digested as described above in the mitomycin C experiments except the supernatant from the reaction mixture was lyophilized and stored at 0°C until analysis by thermospray LC–MS. The lyophylized residue was reconstituted in 1 ml of mobile phase and 250 μl of this solution was injected onto the column, representing 20–30 μg of the guanine adduct.

*HPLC instrumentation*

The HPLC system consisted of two Waters Assoc. (Milford, MA, U.S.A.) Model 501 pumps, a Model 660 solvent programmer, and a Model 440 UV absorbance detector. UV data were collected and processed through a Hewlett-Packard (Palo Alto, CA, U.S.A) Model 9000 series 216 computer using a Nelson Analytical (Palo Alto, CA, U.S.A.) series A/D converter with the chromatography software package XTRA CHROM. Brownlee (Santa Clara, CA, U.S.A.) RP-18 guard columns (30 × 4.6 mm) and RP-18 analytical cartridges (5 μm diameter particles; 220 × 4.6 mm) were used for all assays.

The mobile phase used for analysis of the enzymatically hydrolyzed DNA was 0.1 *M* ammonium acetate in 5% methanol changed linearly over 30 min to 0.1 *M* ammonium acetate in 50% methanol with a flow-rate of 1.2 ml/min. The mobile phase used for analysis of the hydrolyzed supernatant from the thiotepa–DNA reaction mixture was 0.1 *M* ammonium formate adjusted to pH 4.0 with formic acid that was changed linearly over 35 min to 0.05 *M* ammonium formate, pH 4.0, in 50% methanol, with a flow-rate of 1.0 ml/min.

*Thermospray mass spectrometry instrumentation*

A Vestec (Houston, TX, U.S.A) thermospray interface[1,2] connected to either a Finnigan MAT (San Jose, CA, U.S.A.) Model 4500 mass spectrometer, a Hewlett-Packard mass selective detector, or a quadrupole instrument constructed in our laboratory was used. In all cases the scan range of these instruments was set between 120 and 800 a.m.u. The thermospray interface temperatures were optimized for the molecular ion of deoxyguanosine such that the ratio of molecular ion (*m/z* 268) to base peak (*m/z* 152) was greater than 10%. The optimum temperatures for the vaporizer and source block varied slightly between instruments with the optimum vaporizer temperature being 210–220°C with the block temperature set at 295°C for the Hew-

lett-Packard mass selective detector, the optimum temperature for the vaporizer was 205–215°C with source block set at 310°C on the Finnigan ion source and the optimum vaporizor temperature on the laboratory-made instrument was 220–230°C with the source block set at 300 °C. The discharge electrode was operated at 1000 V, and the filament current at 300 mA.

## RESULTS AND DISCUSSION

The UV chromatogram of DNA that had been treated with mitomycin C and then enzymatically hydrolyzed (Fig. 1) showed a peak (retention time 20.1 min) corresponding to a monofunctional N-2 alkylated deoxyguanosine[14]. The UV chromatogram of the DNA treated with porfiromycin and then hydrolyzed (Fig. 1) showed an analogous peak (retention time 21.5 min) corresponding to a porfiromycin DNA adduct. The thermospray mass spectra (Fig. 2) of the adduct peaks detected in the UV chromatograms showed that the porfiromycin adduct corresponds closely to the mi-

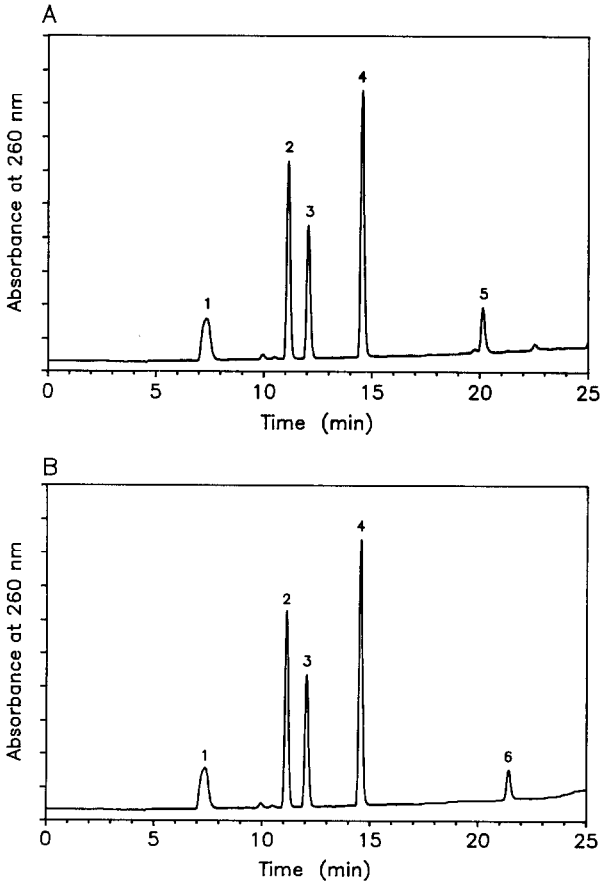



Fig. 1. UV chromatograms of DNA treated with (A) mitomycin C or (B) porfiromycin and then enzymatically digested. Peaks: 1 = deoxycytidine; 2 = deoxyguanosine; 3 = thymidine; 4 = deoxyadenosine; 5 = mitomycin C–deoxyguanosine adduct; 6 = porfiromycin–deoxyguanosine adduct.

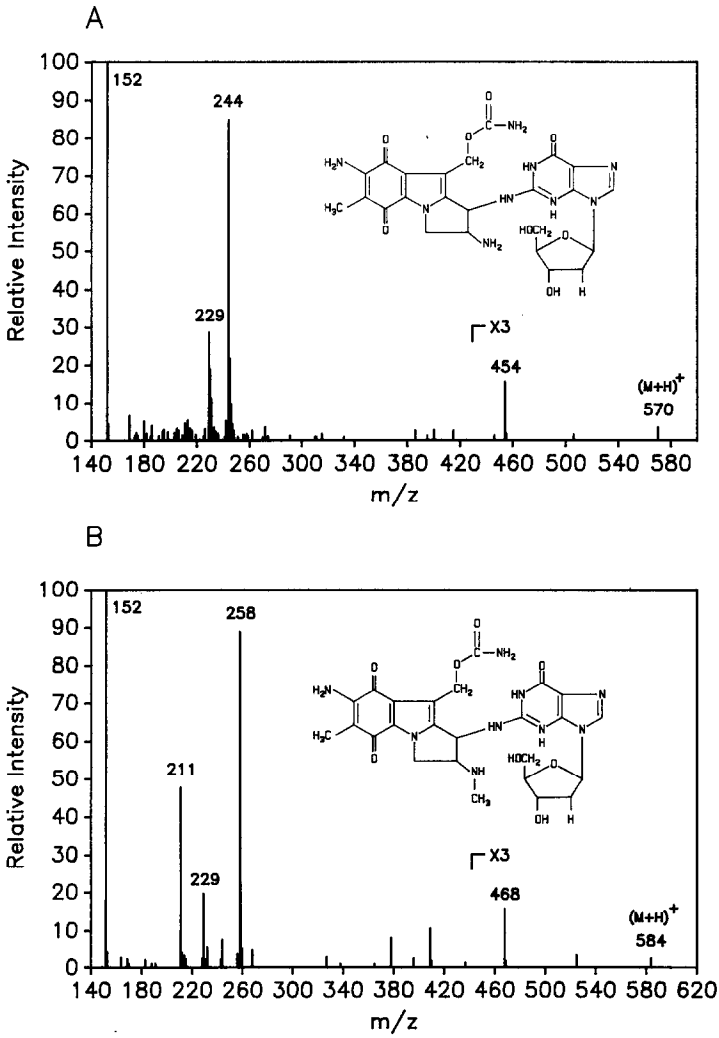
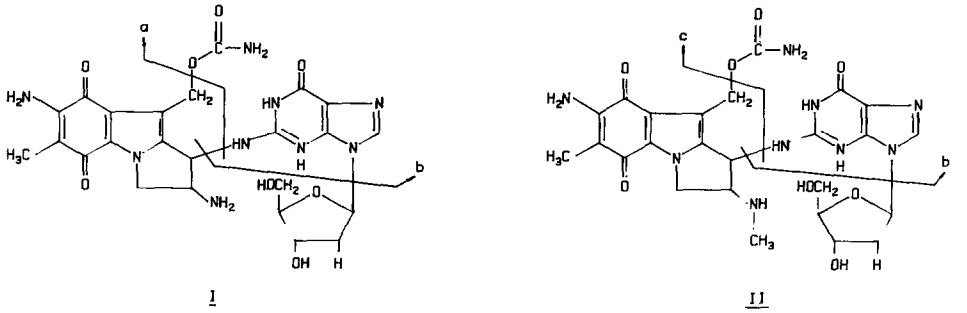



Fig. 2. Thermospray mass spectra of (A) mitomycin C–deoxyguanosine adduct and (B) porfiromycin–deoxyguanosine adduct.

tomycin C adduct except for a higher molecular weight. The difference in mass of 14 a.m.u. in the porfiromycin adduct in comparison with the mitomycin C adduct was accounted for by the presence of an N-methyl group in porfiromycin which is not present in mitomycin C.

The principal ions in the thermospray mass spectrum of the mitomycin C–deoxyguanosine adduct are the $[M+H]^+$ molecular ion at $m/z$ 570, and the ion at $m/z$ 244 which is proposed to be a protonated mitomycin C portion of the adduct as shown in Fig. 3.

The ion at $m/z$ 152 was assigned as the guanine base $[B+2H]^+$, accounted for by thermal cleavage of the ribose (Fig. 3), a common fragment observed in the ther-



I II

Fig. 3. Thermospray fragmentation of (I) mitomycin C adduct and (II) porfiromycin adduct. a, Protonated mitomycin C segment of the adduct, *m/z* 244; b, guanine base $[B+2H]^+$, *m/z* 152, and c, protonated porfiromycin segment of the adduct, *m/z* 258.

mospray spectrum of deoxyguanosine. The spectrum of the porfiromycin–DNA adduct is analogous to the mitomycin C adduct spectrum with an $[M+H]^+$ molecular ion at *m/z* 584, an ion at *m/z* 258 corresponding to the protonated porfiromycin segment of the adduct (Fig. 3) and the ion at 152 corresponding to the guanine base $[B+2H]^+$.

The UV chromatogram of the DNA which had been treated with thiotepa then enzymatically hydrolyzed (Fig. 4A) closely resembles that of untreated DNA (Fig. 4B), with no detectable nucleoside adduct present. However, close examination of the two chromatograms indicated that the deoxyguanosine peak was relatively smaller in the thiotepa treated DNA hydrolysate. On comparison of the peak area ratios of dG:dT and dA:dT (Table I) there is approximately a 14% reduction in the amount of deoxyguanosine and approximately 6% less deoxyadenosine found in the thiotepa treated DNA hydrolysate when compared with the untreated control.

Examination of the supernatant from the thiotepa–DNA reaction mixture showed two major peaks in both the UV chromatogram (Fig. 5A) and thermospray LC–MS total ion chromatogram (Fig. 5B). The identities of these two peaks were assigned as 7-(2-aminoethyl)guanine and 7-(2-aminoethyl)adenine since they coeluted with the adducts formed between ethylenimine and DNA (data not shown). In addition previous experiments with ethylenimine[21] and thiotepa[19] showed that the N-7 position is the preferred site of adduct formation. The thermospray mass spectra (Fig. 6A and B) consist mainly of a molecular ion at *m/z* 179 for the 7-(2-aminoethyl) adenine adduct and a molecular ion at *m/z* 195 for the 7-(2-aminoethyl)guanine adduct. Ions at *m/z* 136 for the adenine adduct and *m/z* 152 for the guanine adduct could be attributed to loss of the aminoethyl portion of the adduct. Use of the filament or discharge electrode to produce fragmentation provided no additional information.

While the lower limits of detection for the adducts were not determined, 200–300 ng of the mitomycin C or porfiromycin adducts and 50–100 ng of the thiotepa adducts produced full scan spectra. This sensitivity is comparable to that obtained by Moser and Wood[26] for non-alkylated nucleosides and nucleotides using fast atom-bombardment (FAB)–MS, although we were unsuccessful in obtaining a FAB mass spectrum of the mitomycin C adduct. Application of tandem MS with desorption chemical ionization (DCI)[27] has been shown to achieve detection of methylated nu-

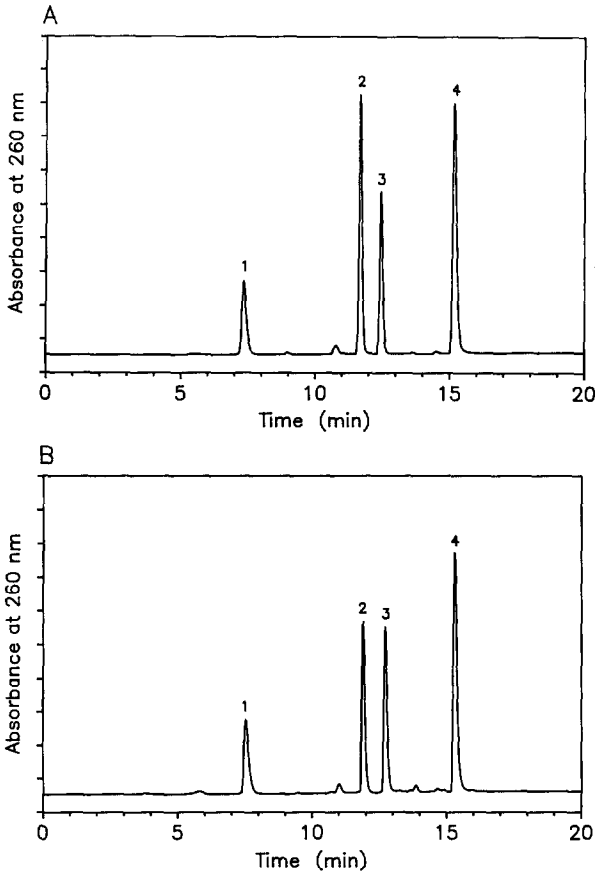



Fig. 4. UV chromatograms of (A) untreated DNA and (B) DNA which had been treated with thiotepa and then enzymatically hydrolyzed. Peaks: 1 = Deoxycytidine; 2 = deoxyguanosine; 3 = thymidine; 3 = deoxyadenosine.

TABLE I

EFFECT OF TREATMENT WITH THIOTEPA ON NUCLEOSIDE CONTENT OF DNA

dC = Deoxycytidine, T = thymidine, dG = deoxyguanosine and dA = deoxyadenosine.

| | *Area ratios of nucleosides* | | |
|---|---|---|---|
| | *dC:T* | *dG:T* | *dA:T* |
| Control DNA | 0.69 ± 0.006[a] | 1.61 ± 0.02 | 1.97 ± 0.01 |
| Thiotepa treated DNA | 0.68 ± 0.006 | 1.38 ± 0.01 | 1.88 ± 0.02 |

[a] Values represent the mean and the standard deviation of three runs.

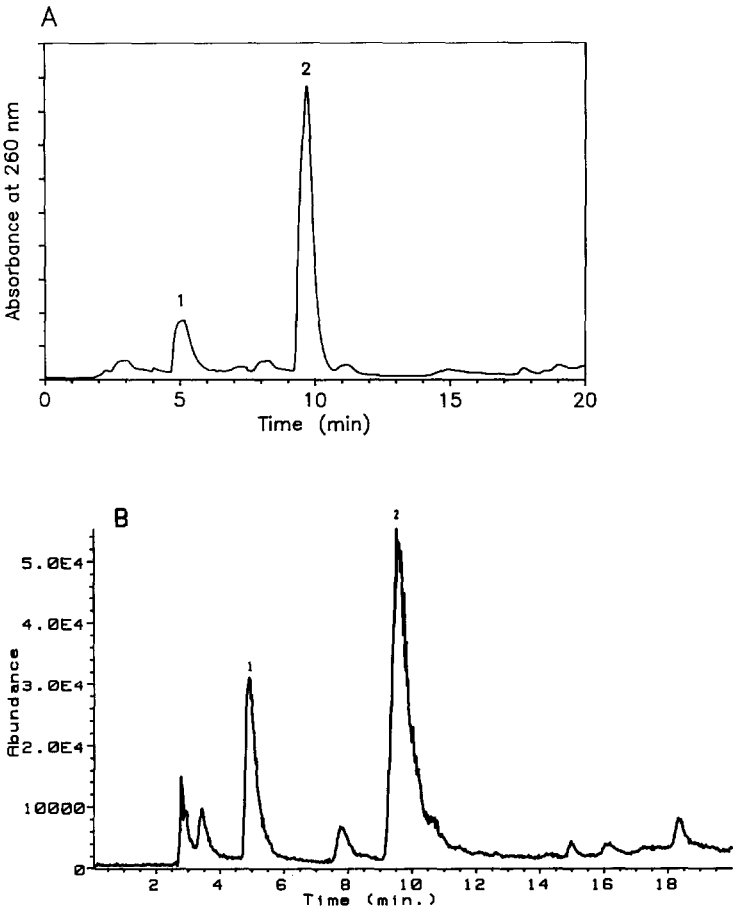



Fig. 5. (A) UV chromatogram and (B) thermospray total ion chromatogram of supernatant from DNA that had been treated with thiotepa. Peaks: 1 = 7-(2-aminoethyl)adenine, 2 = 7-(2-aminoethyl)guanine.

cleosides at a level of 1 ng, however molecular ions were not observed. Although FAB and DCI have been shown to be effective for the MS analysis of DNA adducts, thermospray provides an alternate method which is as sensitive and often produces spectra with molecular ion information not obtainable by other methods.

These results demonstrate that thermospray LC–MS is a powerful method for obtaining structural information on DNA adducts. Although thermospray yields mass spectra where molecular ions are almost always observed for nucleosides, it often fails to produce structurally important fragmentation. When fragmentation is observed, as demonstrated with the mitomycin C and porfiromycin adducts, the actual fragmentation mechanism is difficult to determine since fragments can be formed by several processes such as chemical ionization or as a result of protonating a pyrolysis product formed by the thermospray ionization process itself. Even when the fragmentation mechanism is not known this method provides useful molecular weight

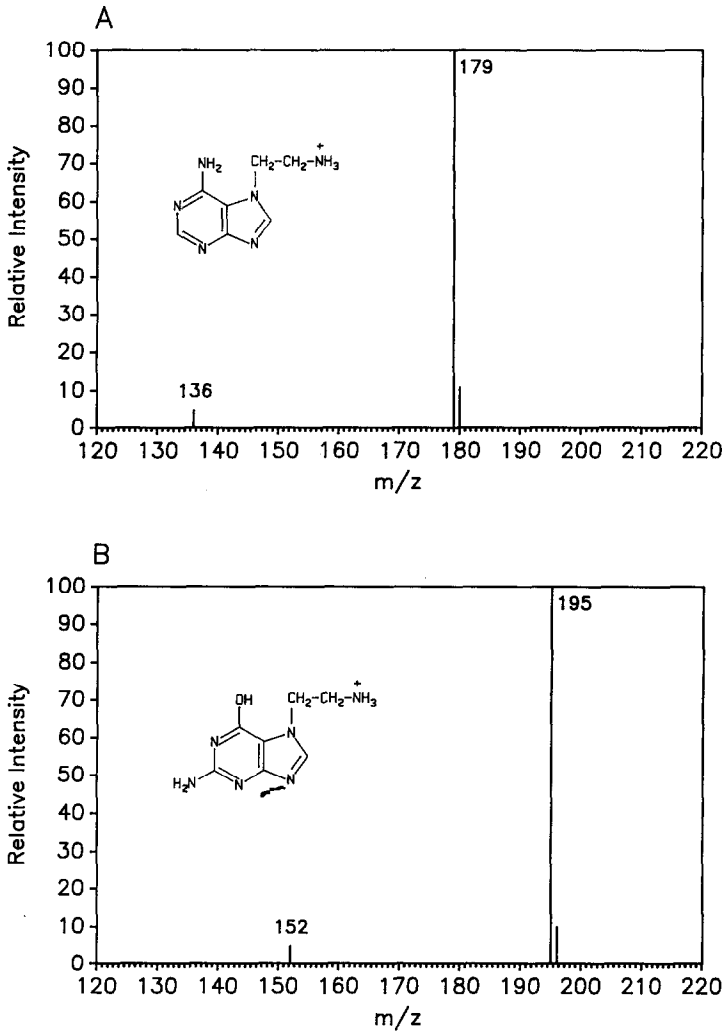



Fig. 6. Thermospray mass spectra of thiotepa DNA adducts found in the supernatant of the thiotepa–DNA reaction mixture. (A) 7-(2-aminoethyl)adenine; (B) 7-(2-aminoethyl)guanine.

information for the identification of nucleoside adducts as well as depurinated adducts resulting from the alkylation of DNA with the anticancer drugs mitomycin C, porfiromycin and thiotepa.

ACKNOWLEDGEMENTS

Supported by Hoffman-La Roche, Inc., Department of Drug Metabolism, University of Maryland Designated Research Initiative Funds, ACS grant No. IN-174D Maryland Cancer Program and ACS grant No. CH-412.

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