ELSEVIER

Journal of Chromatography B, 716 (1998) 187–193

JOURNAL OF CHROMATOGRAPHY B

High-performance liquid chromatographic determination of N-[2- (hydroxyethyl)-N-(2-(7-guaninyl)ethyl)]methylamine, a reaction product between nitrogen mustard and DNA and its application to biological samples

Michelle L. Sperry[a], David Skanchy[b,*], Mark T. Marino[a]

[a]*Department of Pharmacology, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC 20307-5100, USA*

[b]*Department of Medicinal Chemistry, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC 20307-5100, USA*

Received 2 September 1997; received in revised form 2 March 1998; accepted 16 June 1998

Abstract

Nitrogen mustard (HN2) is a bifunctional alkylating agent which is thought to cause cytotoxicity by covalently binding to DNA. Most studies to date have looked at qualitatively determining the presence of DNA–HN2 adducts from reactions with native DNA. The adduct which is predominately formed in these reactions is N-[2-(hydroxyethyl)-N-(2-(7-guaninyl)ethyl]methylamine (N7G). A simple and sensitive reversed-phase high-performance liquid chromatographic (HPLC) method for the determination of N7G from DNA using ultraviolet detection is described. DNA samples having been exposed to HN2 treatment were hydrolyzed and preseparated from high-molecular-mass material by filtration using a molecular mass cut-off of 3000. The mobile phase consisted of methanol–26 m*M* ammonium formate, pH 6.5 (24:76, v/v). N7G, as well as the internal standard, methoxyphenol, were separated within 30 min. The recovery of N7G after hydrolysis of the DNA reaction product was quantitative and limits of detection and quantification of 10 and 20 ng/ml, respectively, were calculated. The method was validated in DNA–HN2 dose response experiments. The N7G reaction product appears to be the first reaction product formed at lower ratios of HN2/DNA but its production plateaus at higher ratios of HN2/DNA probably due to increased formation of hitherto unknown adducts. The method is simple and sensitive and for this reason, may be suited for the determination of DNA/HN2 reaction products.

Keywords: Nitrogen mustard; DNA; N-(2-hydroxyethyl)-N-[2-(7- guaninyl)ethyl]methylamine

1. Introduction

Nitrogen mustard (HN2) is one of the oldest anticancer agents in clinical use [1]. It is thought to exert its cytotoxic effect via reactions between HN2 and native DNA, principally guanine and adenine [2]. The monofunctional adduct N-[2-(hydroxyethyl)-N-(2-(7-guaninyl)ethyl)]methylamine (N7G) has been shown to be the predominant reaction product from reactions of HN2 with native DNA [3]. For this reason, the availability of a method to quantitate this adduct could provide a means to

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00296-5

assess the effect of nitrogen mustard on both normal and malignant tissue in vitro and in vivo experiments. It could also serve as a marker for the clinical use of HN2. Nitrogen mustard is also a potent vesicating agent in man [1].

N7G has been synthesized as an external standard for the quantitative determination of the N7G–DNA reaction product. The described method uses standard techniques for extraction of the N7G DNA–HN2 reaction product from intact DNA for the use in subsequent quantification experiments. Previously published papers reported isolation but no quantification of the adducts. In this paper, a two-step procedure involving hydrolytic cleavage of the HN2–DNA adducts and separation of the released N7G using high-performance liquid chromatography and ultraviolet detection is described.

2. Experimental

2.1. Chemicals

All solvents and reagents were of HPLC grade. Methyl-bis(ß- dichloroethyl)amine (nitrogen mustard, mechlorethamine·HCl, HN2) and calf thymus DNA were procured from Sigma (St. Louis, MO, USA). The synthetic guanine adduct, N7G, was provided by contract synthesis carried out by Starks Associates, (Buffalo, NY, USA) [4] (Fig. 1). Centricon-3 concentrator filters with a molecular mass cut-off of 3000 were purchased from Amicon (Beverly, MA, USA). Adduct solutions were prepared by dissolving weighed amounts of standard in deionized, distilled water. DNA solutions were prepared by dissolving weighed amounts of DNA in 0.1 *M* potassium phosphate buffer (pH 7.4) and homogenizing the DNA strands using a standard tissue grinder. HN2 solutions were prepared by dissolving weighed amounts of HN2 in ethanol.

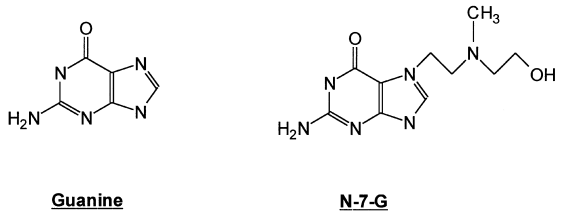

Fig. 1. Structures of guanine and N-[2-(hydroxyethyl)-N-(2-(7-guainyl)ethyl)]methylamine.

2.2. Apparatus

Thermospray mass spectrometry was performed on an HP Model 5989 operated in positive ion mode with the filament on. The MS source block temperature was set at 220°C and the MS quadrapole region was maintained at 100°C. The stem temperature was set at 114°C to maintain a tip temperature of 226°C.

The HPLC analysis system consisted of a Waters Model 600A pump, Model 712 upgrade autosampler and an Applied Biosystems Model 783A programmable absorbance detector set at 285 nm. The analytical process was controlled by Waters MAXIMA 820 program. Verification of peak identity was carried out using a Hewlett-Packard 1070 series HPLC system with a diode-array detector (284 nm) with peak spectral analysis capabilities. Thermospray MS identification was carried out on an HP 5989 mass spectrometer interfaced to the 1090 HPLC.

2.3. Liquid chromatographic procedures

The mobile phase consisted of 26 m*M* ammonium formate (pH 6.50)–methanol (76:26, v/v) at a flow-rate of 0.8 ml/min. Separation was achieved with a Keystone Scientific Betasil C_8 column, 250×4.6 mm. Run time for each 50-μl injected sample was 15 min.

2.4. Extraction

N7G was extracted from samples by heating for 30 min at 100°C to hydrolyze the modified bases. The heated sample was filtered using Centricon-3 concentrators. The filtrate was lyophilized and brought up to 100 μl with mobile phase.

2.5. Standard curve

For calibration curves, N7G serial dilutions of 10, 50, 100, 250, 500, 1000, 2500 and 10000 ng/ml were made in a DNA stock solution (1 mg/ml).

Samples were spiked with a constant amount of I.S. after thermal hydrolysis, prior to filtration. Samples were then processed under standard HPLC conditions. The peak-area ratios (N7G/I.S.) were calculated, graphed and used to calculate the concentrations of three controls made from the standard curve dilutions. Linear nonweighted regression was used to calculate the standard curves. Curves were fit to the equation $y=mx+b$, where y is the known concentration and x is the peak area ratio of N7G.

2.6. In vitro experiment

Formation of N7G from calf thymus DNA was accomplished by addition of weighted amounts of HN2 to 1 mg calf thymus DNA in solution (1 mg/ml). Experiments of increasing molar ratios of NM:DNA were performed to determine the percent formation of the N7G adduct and its dependence on the NM:DNA ratio. The reaction was incubated for 1 h at 37°C and extracted as previously described.

3. Results

3.1. Retention times

N7G standard and the corresponding sample peaks coeluted at 4.8 min. Retention time of the I.S. was 27.5 min (Fig. 2).

3.2. Extraction efficiency

Extraction efficiency with the Centricon-3 filters was 100% ($n=12$). No adduct was lost through the filtration process and interfering substances were cleared out of the sample.

3.3. Method validation

The standard curves ($n=4$) were analyzed using a least squares method of calculating a best fit line $y=mx+b$. Each standard curve was fitted with one equation for the lower ranges (10–500 ng/ml) and one for the higher ranges (500 ng/ml–10 μg/ml).

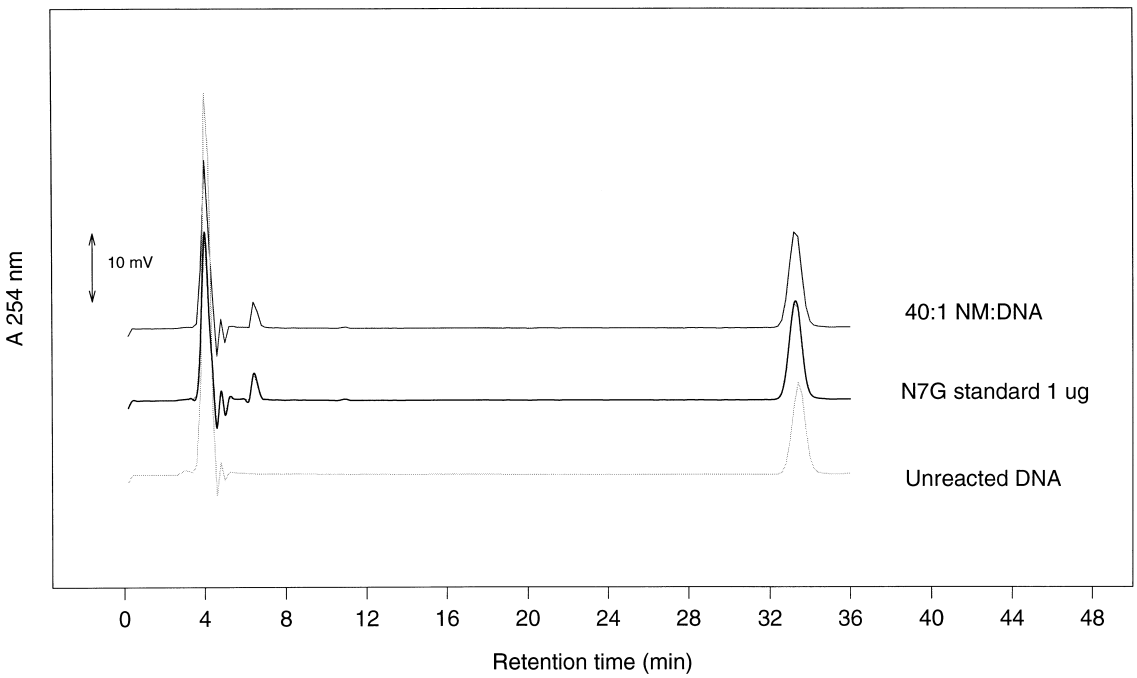

Fig. 2. Chromatograms of N7G standard (bottom) unexposed extracted from DNA reacted with HN2 in a 40:1 molar ratio and extracted as outlined (top).

The low limit of detection was 10 ng/ml and the low level of quantification was 20 ng/ml. The precision and accuracy of the method are outlined in Table 1.

3.4. In vitro experiment

Analysis of the reaction products of calf thymus DNA and HN2 showed coelution of the unknown sample peak with the N7G standard. The ultraviolet spectrum of the corresponding peaks were identical (Fig. 3a and c). This verification confirmed the identity of the in vitro sample product. Mass spectra results of the standard and DNA samples further confirmed the identity of the sample peaks (Fig. 3b and d). The production of the N7G adduct had minimal efficiency at low molar ratios of nitrogen mustard to DNA in that only 3% of the nitrogen mustard was used in the production of the N7G adduct. As the molar ratio of HN2 to DNA was increased, the efficiency of production of the N7G adduct increased to a maximum of 30%. The remaining nitrogen mustard appeared to produce other adducts. The N7G adduct appears to be the first adduct formed and as such would be the most sensitive marker of adduct formation from nitrogen mustard (Table 2).

4. Discussion

The formation of DNA adducts of nitrogen mustard has been known for some time but only recently has the identification of the major adducts been accomplished [3]. There have been no reports to date quantifying the amount of adduct formed. A method for quantifying adducts could be useful in in vitro dosimetry studies and in in vivo studies as a biomarker of alkylating agent exposure. Our methodology can detect down to 20 ng/ml or 79 ppm (1 ng or 4 ppm on column) using standard reversed-phase HPLC and UV detection. This would theoretically detect 1 adduct/10^7 bases from a 50 μg DNA

Table 1
Precision and accuracy of the assay of N7G[a]

	Concentration		S.D.	Error[b]	C.V. (%)
	Known	Predicted (ng/ml)			
Day 1	20 ng/ml	30.12	3.50	0.51	11.60
	577 ng/ml	604.81	32.93	0.05	5.45
	7.5 μg/ml	7508.04	278.44	0.00	3.71
Day 2	20 ng/ml	16.23	1.23	−0.19	7.56
	577 ng/ml	539.70	23.33	−0.06	4.32
	7.5 μg/ml	7507.85	484.49	0.00	6.45
Day 3	20 ng/ml	24.45	2.45	0.22	10.01
	577 ng/ml	530.92	21.58	−0.08	4.06
	7.5 μg/ml	7866.08	102.20	0.05	1.30
Day 4	20 ng/ml	24.50	3.78	0.23	15.43
	577 ng/ml	552.59	9.83	−0.04	1.78
	7.5 μg/ml	7256.27	654.06	−0.03	9.01
Day 5	20 ng/ml	19.79	2.72	−0.01	13.72
	577 ng/ml	538.94	25.25	−0.07	4.68
	7.5 μg/ml	7089.14	314.11	−0.05	4.43

Between-day C.V.[c]

Theoretical concentration (ng/ml)	C.V. (%)
20	23
577	5.4
7500	4.0

[a] Known and predicted concentrations with their S.D., error and C.V. values for days 1–5.
[b] Error is defined as (measured concentration−theoretical concentration)×100/theoretical concentration.
[c] Between-day C.V. values for each measured concentration over the 5-day period.
S.D.=standard deviation; C.V.=coefficient of variation.

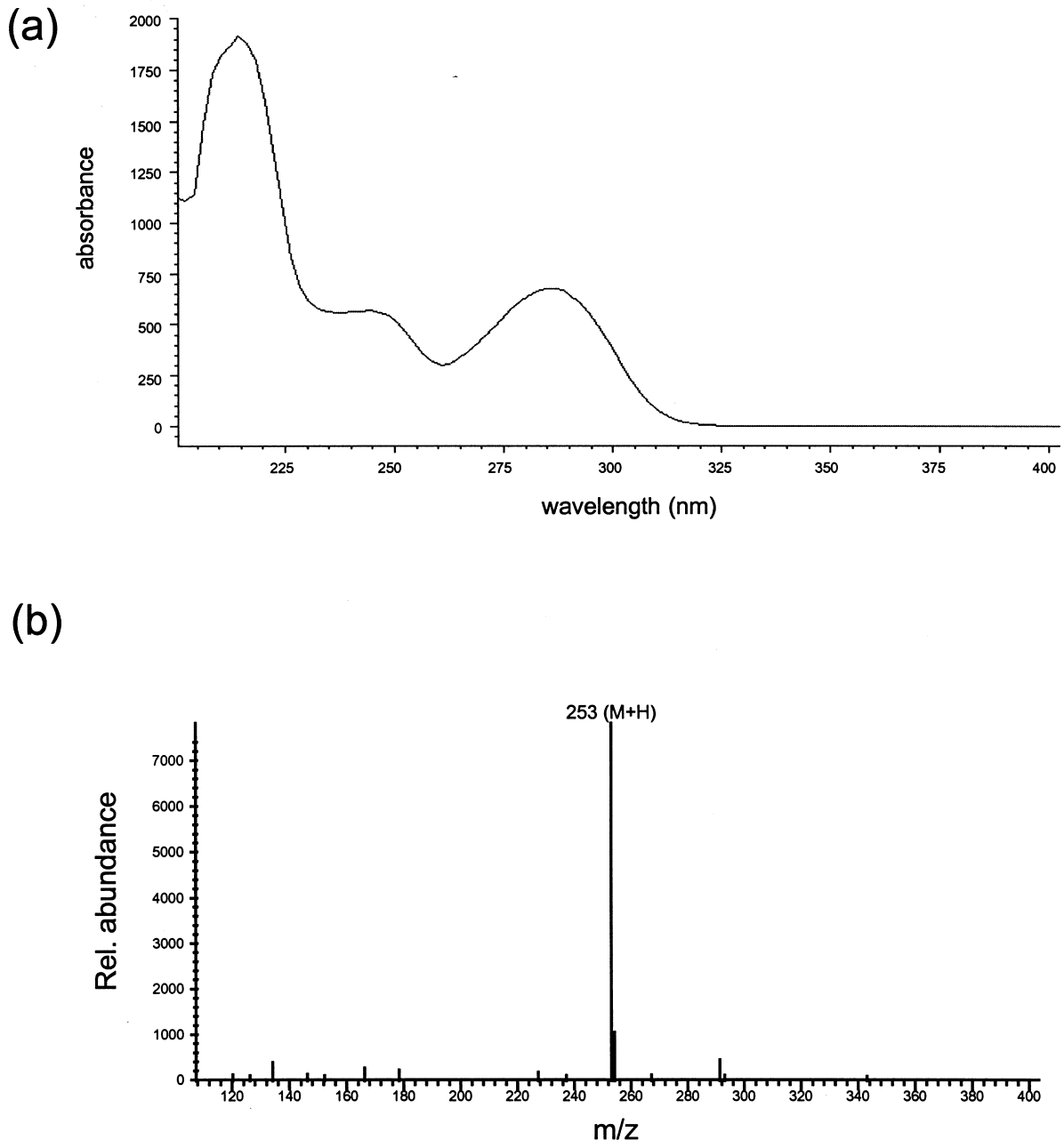

Fig. 3. (a) UV spectra of N7G (b) mass spectra of N7G (c) UV spectra of N7G extracted from DNA reacted with HN2 (d) mass spectra of N7G extracted from DNA reacted with HN2.

sample. While this may be sensitive enough to measure adducts from in vitro experiments, its usefulness in detecting adducts from in vivo experiments will need to be validated.

Other DNA adducts can be detected both in vitro and in vivo [5,6]. The measurement of adducts has been used to document exposure to carcinogens and has been proposed as a way to optimize chemo-

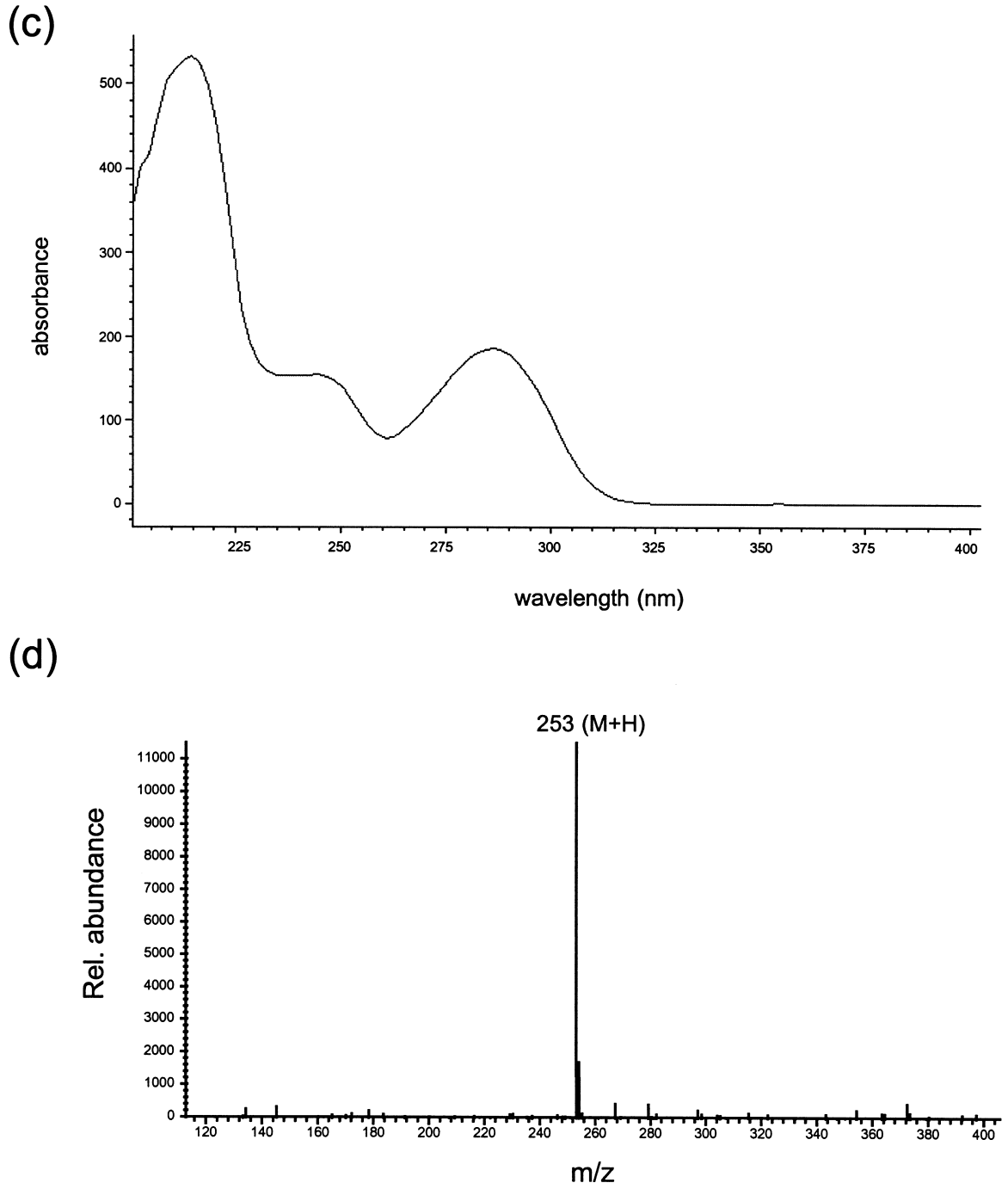

Fig. 3. (*continued*)

Table 2
N7G formation from HN2 exposed DNA

NM:DNA[a]	N7G conc.[b] (ng/ml)	Moles N7G formed[b]	HN2 used for N7G formation[c] (%)	*n*
1–100	48.26	$3.83 \cdot 10^{-10}$	1.21±.94	3
1–10	420.97	$3.34 \cdot 10^{-9}$	1.05±.05	3
1–2	1958.25	$1.55 \cdot 10^{-8}$	2.07±.30	2
1–1	4021.69	$3.19 \cdot 10^{-8}$	4.25±.52	3
2–1	6726.92	$5.33 \cdot 10^{-8}$	7.11±.81	3
10–1	16850.69	$1.34 \cdot 10^{-7}$	17.81±.19	3
20–1	21289.12	$1.69 \cdot 10^{-7}$	22.50 ±.66	2
40–1	27441.16	$2.18 \cdot 10^{-7}$	29.01±3.46	2

[a] Molar ratio of HN2 to DNA.
[b] Mean amounts measured.
[c] Mean±standard deviation.
n=Number of experiments.

therapy [7]. The routine use of these assays for both in vitro models and clinical purposes would enhance our understanding of their mechanism of action. This type of assay would also assist in dose response studies that look both at the production of adducts and cancer risk. While phenanthrine derivatives have been shown to form mainly DNA adducts, the alkylating agents may also form protein and RNA adducts. The relative contribution of these adducts needs to be studied both in vitro and in vivo.

Our assay has been shown to be sensitive and the N7G adduct of nitrogen mustard may be able to serve as a sensitive marker of nitrogen mustard exposure since it is the first adduct to be formed from the reaction of DNA and nitrogen mustard. Other agents which form DNA adducts have been detected both in vivo and in vitro [8]. The usefulness of adducts ranges from cancer epidemiology [9] to following the effect of antineoplastic agents [10].

The opinions and views in this paper are those of the authors and do not reflect the opinions of the Department of the Army or the Department of Defense.

References

[1] B.A. Chabner, C.J. Allegro, G.A. Curt, P. Calabresi, in: A.G. Gilman, T.W. Rall, A.S. Nies, P. Taylor (Editors), Goodman and Gilman's The Pharmacologic Basis of Therapeutics, Pergamon Press, New York, 1995, Ch. 51, p. 1233.
[2] J. Hansson, R. Lewensohn, U. Ringborg, B. Nilsson, Cancer Res. 47 (1987) 2631.
[3] M.R. Osborne, D.E.V. Wilman, P.D. Lawley, Chem. Res. Toxicol. 8 (1995) 316.
[4] Starks Associates, Buffalo, NY, US Army Medical Research and Material Command Contract No. DAND17-93-C-3003.
[5] R. Kumar, K. Hemminki, Carcinogenesis 17 (1996) 485.
[6] M. Suh, F. Ariese, G.J. Small, R. Jankowiak, A. Hewer, D.H. Phillips, Carcinogenesis 16 (1995) 2561.
[7] K. Hemminki, J. Occupational Environ. Med. 37 (1995) 44.
[8] L. Chen, P.D. Devanesan, S. Higginbotham, F. Ariese, R. Jankowiak, G.J. Small, E.G. Rogan, E.L. Cavalieri, Chem. Rsch. Tox. 9 (1996) 897.
[9] A.M. Simons, D.H. Phillips, D.V. Coleman, Br. Med. J. 306 (1993) 1444.
[10] P.A. Lawley, in: K. Hemminki, A. Dipple, D.E.G. Shuker, F.F. Kadlubar, D. Segerberck, H. Bartsch (Editors), DNA Adducts: Identification and Biological Significance, IARC, Lyon, 1994, No. 125, pp. 3–22.