ELSEVIER

Journal of Chromatography B, 694 (1997) 65–70

JOURNAL OF CHROMATOGRAPHY B

Detection of N7-(2-hydroxyethyl)guanine adducts in DNA and 9L cells treated with 1-(2-chloroethyl)-1-nitrosourea

Qiuping Ye, William J. Bodell*

Brain Tumor Research Center, Department of Neurological Surgery, Box-0806, University of California, San Francisco, CA 94143-0806, USA

Received 7 November 1996; revised 30 January 1997; accepted 13 February 1997

Abstract

A sensitive analytical method, HPLC–ED, was developed for the measurement of N7-(2-hydroxyethyl)guanine (N7-HOEtG). A detection limit of 3.2 N7-HOEtG/10^8 nucleotides was obtained with this method. Linear dose response curves for the formation of N7-HOEtG were obtained following treatment of either calf thymus DNA or 9L cells with 1-(2-chloroethyl)-1-nitrosourea (CNU). Using HPLC–ED a significant increase in the level of N7-HOEtG could be detected in 9L cells following treatment with 5 μM CNU. Our study suggests that with this analytical method the formation of N7-HOEtG in the white blood cells of patients treated with chloroethylnitrosoureas may be determined.

Keywords: DNA; 1-(2-Chloroethyl)-1-nitrosourea; N7-(2-Hydroxyethyl)guanine adducts

1. Introduction

N-(2-Chloroethyl)-N-nitrosoureas are a group of clinically useful antineoplastic agents employed in the treatment of brain tumors [1,2]. As illustrated in Fig. 1, 1-(2-chloroethyl)-1-nitrosourea (CNU) undergoes hydrolysis to form both chloroethylating and hydroxyethylating species. The DNA adducts formed by these bifunctional nitrosoureas have been extensively characterized by Ludlum [3]. The products identified include N7 and O^6 alkylated guanines, intra- and interstrand cross-linked bases, ethano derivatives and phosphotriesters [3]. The principal base alkylation products formed by CNU treatment of DNA are N7-(2-hydroxyethyl)deoxyguanosine (N7-HOEtdG) and N7-(2-chloroethyl)deoxyguanosine (N7-ClEtdG) shown in Fig. 1.

Studies to date have suggested that the cytotoxic properties of CNU are related to the formation of DNA adducts by these agents [3–5]. A variety of procedures have been applied to measure the formation of alkylation products following treatment of either DNA or cells with chloroethylnitrosoureas. These methods include the use of alkaline elution to investigate DNA interstrand cross-link formation [6], radiolabelled CNU [7] and the application of HPLC with fluorescence detection [8].

Recent studies have suggested that the levels of DNA platination products in white blood cells of patients treated with *cis*-platinum is correlated with therapeutic response [9,10]. In order to determine if similar relationships are also observed in patients treated with chloroethylnitrosoureas, a non-radioactive based method for the detection of these DNA adducts is required. The purpose of this study was to develop a simple and sensitive method for the

*Corresponding author.

PII S0378-4347(97)00161-8

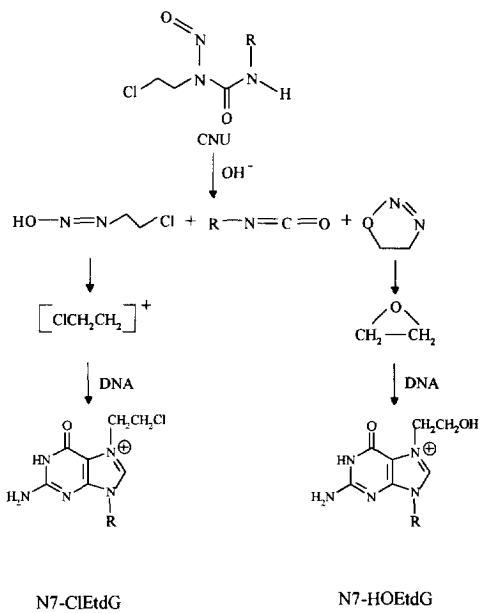

Fig. 1. Proposed decomposition pathway of CNU leading to the formation of the N7-HOEtdG and N7-ClEtdG.

quantitative determination of N7-HOEtG formed in DNA and 9L cells treated with CNU.

2. Experimental

2.1. Chemicals and instruments

The standards of N7-HOEtG and N7-ClEtG were synthesized and their structures were confirmed by UV spectroscopy and mass spectrometry [11,12]. CNU was obtained from the Drug Development Branch of the National Cancer Institute (Bethesda, MD, USA). All chemicals were purchased from Aldrich (Milwaukee, WI, USA) and Sigma (St. Louis, MO, USA). Deoxyribonuclease I (DNase I) (EC 3.1.21.1), snake venom phosphodiesterase (EC 3.1.4.1) and alkaline phosphatase (EC 3.1.3.1) were purchased from Worthington Biochemicals (Freehold, NJ, USA).

The HPLC-fluorescence system consisted of a model 250 Perkin–Elmer solvent delivery system with an ISS 200 LC sample processor coupled to a LC 235 UV detector and a LC 240 fluorescence detector. UV and fluorescence responses were digitized with single channel interfaces and recorded using a Omega data system (Perkin–Elmer, Cupertino, CA, USA). The HPLC–ED system consisted of a model 250 Perkin–Elmer solvent delivery system coupled to a Coulochem II electrochemical detector with a ESA 5010 analytical cell (Chelmsford, MA, USA). Electrochemical response was digitized with a Nelson interface and analyzed using TurboChrom 4 (Perkin–Elmer). An Alltech 5 μm C_{18} reversed-phase analytical column with column dimensions of 250×4.6 mm I.D. (Alltech Associates, Deerfield, IL, USA) was employed for the separation in both systems.

2.2. Reaction of calf thymus DNA with CNU

The CNU-modified DNA samples were prepared by reacting 1 mg purified calf thymus DNA in 10 m*M* sodium cacodylate buffer (pH 7.0) with varying amounts of CNU dissolved in ethanol. The total volume of the reaction mixture was 1 ml. The reaction mixture was incubated at 37°C for 6 h. The DNA was precipitated with ethanol and 0.4 *M* NaAc and redissolved in H_2O.

To some of the incubations 250 μCi of [^{3}H]CNU 7.14 Ci/mmol (Moravek Biochemicals, Brea, CA, USA) was added instead of the unlabelled CNU. The reactions were incubated as described above for 6 h. The samples were repeatedly precipitated with ethanol and 0.4 *M* NaAc until a constant specific activity (^{3}H cpm/mg DNA) was obtained.

2.3. Treatment of 9L cells with CNU

9L cells were grown in 175 cm^2 flasks containing 50 ml of Eagle's minimum essential medium supplemented with 10% newborn calf serum and gentamicin (50 μg/ml) [13]. The cells were maintained and treated at 37°C in a humidified 5% CO_2/95% air atmosphere. Various amounts of CNU dissolved in ethanol were added to the cells. After 6 h the cells were treated with a trypsin cocktail and collected by centrifugation. The cells were washed once with phosphate buffered saline and collected by centrifugation. The cells were quick frozen and stored at −70°C until the DNA was isolated. The DNA was isolated using a modified Marmur procedure as previously described [14]. The concentration of

DNA in the samples were determined by UV analysis.

2.4. DNA hydrolysis methods

2.4.1. (a) Enzymatic

Approximately 4.5 μg of [^{3}H]CNU–DNA was digested overnight at 37°C with a mixture of pancreatic Deoxyribonuclease I, snake venom phosphodiesterase and alkaline phosphatase as previously described [7].

2.4.2. (b) Acid

4.5 μg of [^{3}H]CNU–DNA was added to 300 μl of 0.1 *M* HCl and incubated at 100°C for 1 h. After cooling, the hydrolysate was adjusted to pH 6 with the addition of 4 *M* NaAc, pH 6.4.

2.4.3. (c) Thermal

4.5 μg of [^{3}H]CNU–DNA in 100 μl of H_2O was heated at 100°C for 30 min. The sample was subsequently plunged into an ice bath and 100 μl of cold 1 *M* HCl added. The sample was centrifuged at 1300 *g* at 0°C for 10 min to collect the DNA. The pellet was washed once with 100 μl of cold 1 *M* HCl and centrifuged as described above. Both supernatants were combined and adjusted to pH 6 with the addition of 4 *M* NaAc, pH 6.4.

Each of the hydrolysates were filtered with 0.2 μm centrifugal microfilters at 1300 *g*, 4°C for 5 min. Ten μl of each filtrate was mixed with 2 ml of liquid scintillation cocktail and their radioactivity determined by scintillation counting. The remaining filtrate was separated by reversed-phase HPLC–UV with a mobile phase of 10 m*M* $NH_4H_2PO_4$, pH 5.1, and varying amounts of methanol. UV absorption at 254 and 280 nm were monitored simultaneously. Thirty-second fractions were collected for 85 min. Ten pmol of N7-HOEtG was spiked into the sample to identify the position of elution of the radiolabelled N7-HOEtG peak. Comparison of the amount of radioactivity in the sample with the amount of radioactivity collected from the HPLC fractions was used to determine recovery of radioactivity for each of the methods.

2.5. Detection of N7-HOEtG with HPLC-fluorescence

In duplicate, various amounts (0.8, 1, 3, 5, 8 pmol) of N7-HOEtG were introduced to the HPLC with an isocratic program of 90% 0.05 *M* sodium acetate buffer, pH 5.4, and 10% MeOH at a flow-rate of 1 ml/min. The fluorescence intensity was measured with excitation at 295 nm and emission at 370 nm.

2.6. Voltagram analysis of N7-HOEtG and N7-ClEtG

One pmol N7-HOEtG and 50 pmol N7-ClEtG were chromatographed on a reversed-phase column using a isocratic program of 90% 0.05 *M* sodium acetate buffer, pH 5.4, and 10% MeOH at a flow-rate of 1 ml/min. The relative responses to each of these compounds were measured with electrode 1 set at +100 mV and electrode 2 varied from 0 to +800 mV.

2.7. Detection of N7-HOEtG and N7-ClEtG with HPLC–ED

Varying amounts of N7-HOEtG (0.003 to 0.35 pmol) and N7-ClEtG (3 to 10 pmol) were analyzed in duplicate by HPLC–ED. The isocratic system described above was used for chromatography. For these studies electrodes 1 and 2 were set at +400 and +750 mV, respectively.

2.8. Prepurification of N7-HOEtG for HPLC–ED detection

Fifty to 100 μg of DNA from individual samples was thermally hydrolyzed. N7-HOEtG was initially purified on HPLC–UV system with a gradient program as follows: 100% A to 94% A, 6% B in 20 min; 94% A, 6% B to 70% A, 30% B in 20 min and 70% A, 30% B for 5 min (solvent A: 0.05 *M* sodium acetate buffer, pH 5.4; solvent B: MeOH). Under these conditions N7-HOEtG had a retention time of 15.4 min. We collected the products eluting from 14–18 min. The sample volumes were reduced to about 400 ml by a speed vacuum and subsequently analyzed on HPLC–ED using the isocratic solvent described above. For quantitation of N7-HOEtG

electrodes 1 and 2 were set at +400 and +750 mV, respectively.

3. Results

The hydrodynamic voltammograms of N7-HOEtG and N7-ClEtG were investigated. For this, the electrochemical response of N7-HOEtG and N7-ClEtG were detected over a range of oxidation potentials. Neither N7-HOEtG or N7-ClEtG showed significant electrochemical response below 700 mV. Above 700 mV, the electrochemical response of N7-HOEtG increased significantly, while that of N7-ClEtG increased only slightly. Based on these results oxidation potentials of +400 and +750 mV were selected for electrodes 1 and 2, respectively. Under these conditions, a instrument detection limit of 5.0 fmol for N7-HOEtG and 5.0 pmol for N7-ClEtG was achieved. Attempts to increase the sensitivity detection of N7-ClEtG by derivatization with a more electrochemically active compound were not successful (results not shown). Fig. 2 demonstrates that the EC response of N7-HOEtG standard was linear between 0.05 and 0.35 pmol.

The detection of N7-HOEtG requires its release from DNA. Three methods, namely enzymatic digestion, acid hydrolysis and thermal depurination, were compared for their efficient release of radiolabelled N7-HOEtG from DNA reacted with [^{3}H]CNU. As a percentage of total radioactivity, the levels of N7-HOEtG in the hydrolysates from enzymatic, acid and thermal hydrolysis were 35.7±0.7, 34.8±0.3 and 34.2±0.8%, respectively. Therefore the release of N7-HOEtG from DNA by each of these procedures was similar.

Since neutral thermal hydrolysis was expected to be the most selective in the release of N7-HOEtG adducts from DNA, it was chosen for the remaining studies. However even with this procedure, other modified and unmodified bases which are electrochemically active at +750 mV may also be released from CNU modified DNA. In order to circumvent this problem, we developed a two step chromatography procedure for the electrochemical-detection of N7-HOEtG. After neutral thermal hydrolysis, the hydrolysate was separated on a reversed-phase column and fractions corresponding to the retention time of N7-HOEtG were collected. These samples were reduced in volume and subsequently analyzed by HPLC–ED.

One hundred μg of purified calf thymus DNA was spiked with N7-HOEtG standard over a range of 0.010, 0.030, 0.050, 0.10 and 0.20 pmol in duplicate. The recovery of N7-HOEtG from these DNA samples are shown in Table 1. The recovery of N7-HOEtG was not significantly dependent upon its concentration. Using this method of analysis a detection limit of 3.2 N7-HOEtG/10^8 nucleotides (0.01 pmol N7-HOEtG/100 μg DNA) was obtained.

Purified calf thymus DNA was reacted with varying amounts of CNU. HPLC–ED chromatograms of control DNA and calf thymus DNA reacted with 1 μ*M* CNU are shown in Fig. 3A and Fig. 3B.

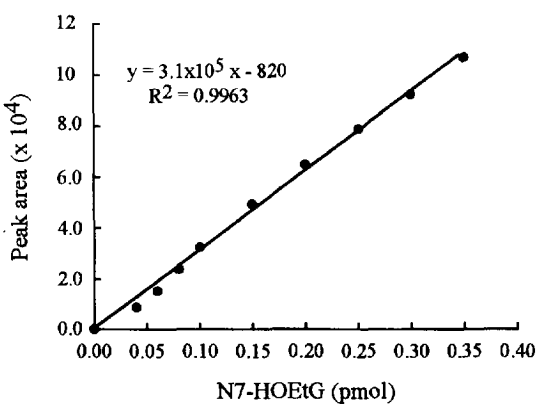

Fig. 2. Electrochemical response to varying amounts of N7-HOEtG.

Table 1
Recovery of N7-HOEtG from spiked DNA samples

pmol N7-HOEtG/100 μg DNA	Recovery±S.D. (%)[a,b]
0.010	81.5±5.7
0.030	82.2±6.3
0.050	87.0±3.8
0.10	92.7±6.6
0.20	96.2±5.1

[a] Recovery was based on the amount of N7-HOEtG added to the DNA. Each concentration was analyzed in duplicate three times.
[b] The relative electrochemical responses were measured with electrode 1 set at +400 mV and electrode 2 set at +750 mV. HPLC was performed using an isocratic program of 90% 0.05 *M* sodium acetate buffer, pH 5.4, and 10% MeOH at a flow-rate 1 ml/min.

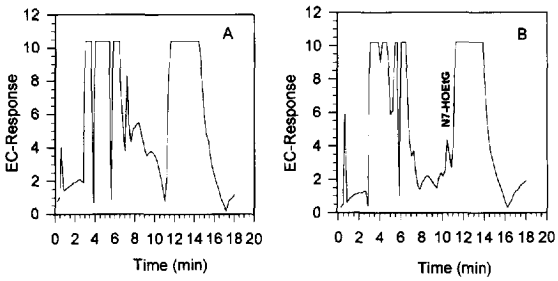

Fig. 3. HPLC–ED chromatograms of (A) control calf thymus DNA, (B) calf thymus DNA treated with 1 μ*M* CNU. Response of electrode 2 set at +750 mV is shown. For HPLC–ED conditions, see Table 1.

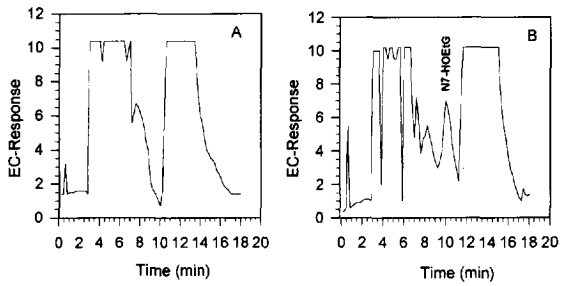

Fig. 5. HPLC–ED chromatograms of DNA from (A) control 9L cells, (B) 9L cells treated with 5 μM CNU. Response of electrode 2 set at +750 mV is shown. For HPLC–ED conditions, see Table 1.

The peaks were quantified by comparison with the electrochemical-response obtained with known amounts of N7-HOEtG. The amount of N7-HOEtG in control DNA was $4.2\pm0.5\times10^{-4}$ and $1.0\pm0.2\times10^{-3}$ pmol/μg DNA in calf thymus DNA treated with 1 μ*M* CNU. The levels of N7-HOEtG in DNA after treatment with 1 to 25 μ*M* of CNU were linearly related to treatment dose (Fig. 4).

In subsequent experiments, 9L cells were treated with varying amounts of CNU. HPLC–ED chromatograms of control 9L cells and 9L cells treated with 5 μ*M* CNU are shown in Fig. 5A and Fig. 5B. The amount of N7-HOEtG in untreated 9L cells and 9L cells treated with 5 μ*M* CNU was $8.7\pm7.0\times10^{-5}$ and $4.3\pm1.9\times10^{-4}$ pmol/μg DNA, respectively. A dose-dependent increase in the levels of N7-HOEtG in 9L cells treated with varying amounts of CNU was found (Fig. 6).

4. Discussion

We have developed a method for the detection of N7-HOEtG in DNA. This procedure employs neutral thermal hydrolysis for release of the modified base. Initial separation of the product by reversed-phase HPLC with UV detection followed by HPLC with electrochemical detection provided a simple but sensitive method for the quantitation of this DNA adduct.

Previous studies have reported that HPLC–ED can

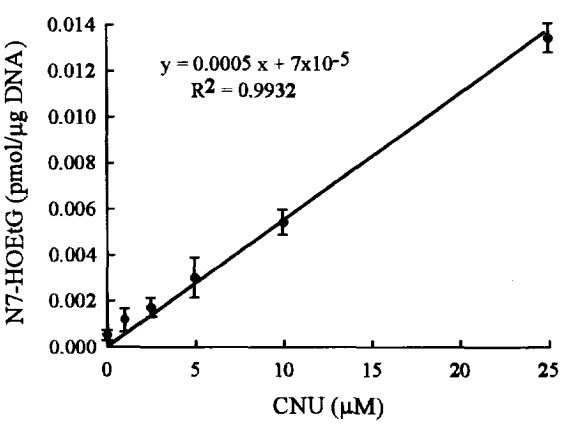

Fig. 4. Dose–response curve for the formation of N7-HOEtG in calf thymus DNA treated with varing concentrations of CNU. For HPLC–ED conditions, see Table 1.

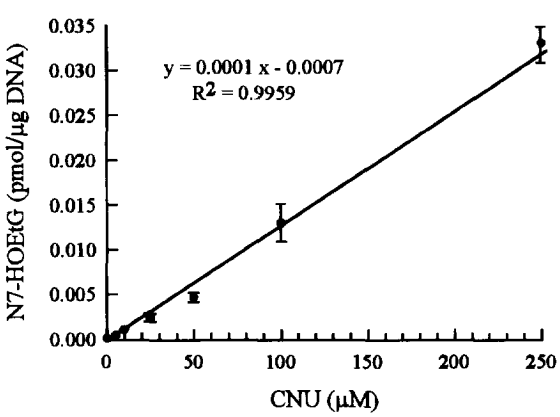

Fig. 6. Dose–response curve for the formation of N7-HOEtG in 9L cells treated with varying concentrations of CNU. For HPLC–ED conditions, see Table 1.

be used for the detection of N7-alkylguanines [15–17]. In our study, the sensitivity of detection of N7-HOEtG by HPLC–ED was increased, after N7-HOEtG was pre-purified by HPLC–UV (result not shown). Using this approach a sensitivity of 3.2 N7-HOEtG/10^8 nucleotides was obtained. In addition, the EC response was linear from 0.05 to 0.35 pmol of N7-HOEtG.

With the method developed, the formation of N7-HOEtG in either purified DNA or 9L cells treated with CNU was readily detectable. The amount of N7-HOEtG in calf thymus DNA treated with 1 μM CNU was 2.4-fold higher than that in control calf thymus DNA. The levels of N7-HOEtG formed in calf thymus DNA treated with CNU were linear with dose, with a slope of 5.3×10^{-4} pmol/μg DNA per μM CNU (0.17 μmol/mol DNA per μM CNU). The amount of N7-HOEtG in DNA of 9L cells treated with 5 μM CNU was 4.9-fold higher than that in DNA of control 9L cells. Treatment of 9L cells with CNU also gave a linear dose response with a slope of 9.1×10^{-5} pmol/μg DNA per μM CNU (0.029 μmol/mol DNA per μM CNU). The results with HPLC–ED detection of N7-HOEtG in 9L cells treated with CNU are similar to those we have previously reported for the formation of N7-HOEtG formed in 9L cells treated with [^{3}H]CNU [5].

When HPLC-fluorescence was used in this study, an instrument detection limit of 1 pmol N7-HOEtG standard was obtained (unpublished result Q. Ye). Using 1 mg of DNA, 2 pmol of N7-HOEtG/mg DNA (6.4 N7-HOEtG/10^7 nucleotides) have been detected with HPLC-fluorescence after ethylene oxide treatment of rats [18]. With immunoassays, adduct levels of 1 N7-HOEtG/10^7 nucleotides have been determined [19–21]. Using GC–electron capture mass spectrometry, 1.6 N7-HOEtG/10^6 nucleotides was measured in spiked DNA after N7-HOEtG was derivatized with pentafluorobenzyl bromide [22]. With HPLC–ED, 2 N7-HOEtG/10^7 nucleotides [15] and 3 N7-ethylguanines/10^7 nucleotides [16] have been detected. In our study we have obtained a sensitivity of 3.2 N7-HOEtG/10^8 nucleotides. Comparison of these results with those previously reported for the detection of N7-HOEtG indicate that this method is about one order of magnitude more sensitive than previously reported methods. Our study suggests that with this analytical method the formation of N7-HOEtG in the white blood cells of patients treated with chloroethylnitrosoureas may be determined.

Acknowledgments

These studies were supported by CA-13525 from the National Cancer Institute.

References

[1] V.A. Levin, C.B. Wilson, Cancer Treat. Rep. 60 (1976) 719.
[2] A.W. Prestayko, S.T. Crooke, L.H. Baker, S.K. Carter, P.S. Schein (Eds.), Nitrosoureas: Current Status and New Developments, Academic Press, New York, NY, 1981.
[3] D.B. Ludlum, Mutat. Res. 233 (1990) 117.
[4] W.J. Bodell, K. Tokuda, D.B. Ludlum, Cancer Res. 48 (1988) 4489.
[5] W.J. Bodell, Mutat. Res. 233 (1990) 203.
[6] R.A.G. Ewig, K.W. Kohn, Cancer Res. 38 (1978) 3197.
[7] W.J. Bodell, K. Pongracz, Chem. Res. Toxicol. 6 (1993) 434.
[8] W.P. Tong, K.W. Kohn, D.B. Ludlum, Cancer Res. 42 (1982) 4460.
[9] E. Reed, R.F. Ozols, R. Tarone, S.H. Yupsa, M.C. Poirier, Proc. Natl. Acad. Sci. USA 84 (1987) 5024.
[10] S. Gupta-Burt, H. Shamkhani, E. Reed, R.E. Tarone, C.J. Allegra, L.H. Pai, M.C. Poirier, Cancer Epidemiol., Biomarkers Prev. 2 (1993) 229.
[11] P. Brookes, P.D.J. Lawley, J. Chem. Soc. (1961) 3923.
[12] W.P. Tong, D.B. Ludlum, Cancer Res. 41 (1981) 380.
[13] W.J. Bodell, H.T.R. Rupniak, J. Rasmussen, W.F. Morgan, M.L. Rosenblum, Cancer Res. 44 (1984) 3763.
[14] W.J. Bodell, M.R. Banerjee, Nucleic Acids Res. 3 (1976) 1689.
[15] J.H.M. van Delft, E.J.M. van Weert, M.J.M. van Winden, R.A. Baan, Chem.-Biol. Interact. 80 (1991) 281.
[16] J.H.M. van Delft, M.J.M. van Winden, A.M.C. van den Ende, R.A. Baan, Environ. Health Perspect. 99 (1993) 25.
[17] J. Park, K.C. Cundy, B.N. Ames, Carcinogenesis 10 (1989) 827.
[18] V.E. Walker, T.R. Fennell, P.B. Upton, T.R. Skopek, V. Prevost, D.E.G. Shuker, J.A. Swenberg, Cancer Res. 52 (1992) 4328.
[19] J.H.M. van Delft, E.J.M. van Weert, M.M. Steeliness, E. Claassen, R.A. Baan, Carcinogenesis 12 (1991) 1041.
[20] J.H.M. van Delft, M.J.M. van Winden, A. Luiten-Schuite, L.R. Ribeiro, R.A. Baan, Carcinogenesis 15 (1994) 1867.
[21] T.L. Young, Y. Habraken, D.B. Ludlum, R. Santella, Carcinogenesis 11 (1990) 1685.
[22] M. Saha, A. Abushamaa, R.J. Giese, J. Chromatogr. A 712 (1995) 345.