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Analysis of melphalan adducts of 2′-deoxynucleotides in calf thymus DNA hydrolysates by capillary high-performance liquid chromatography–electrospray tandem mass spectrometry

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Abstract

Melphalan is a bifunctional alkylating agent that covalently binds with intracellular nucleophilic sites. A methodology using electrospray mass spectrometry was developed to detect and identify DNA adducts. Alkylation sites within a particular nucleotide were examined using electrospray tandem mass spectrometry hyphenated to capillary liquid chromatography in combination with a column switching system. In the reaction mixtures resulting from the interaction of 2′-deoxynucleotides and melphalan several base-aklylated adducts were found. In the case of 2′-deoxyadenosine monophosphate, thymidine monophosphate and 2′-deoxyguanosine phosphate alkylation was observed in the mononucleotide reaction mixtures but not in the DNA-hydrolysates. Calf thymus DNA was reacted in vitro with melphalan. The DNA pellet was isolated and enzymatically hydrolyzed with the aid of Nuclease P_1. In this hydrolysate both mono-alkylated 2′-deoxynucleotides and dinucleotides were found. The most important adduct found was identified as the N-7 alkylated dGMP adduct. The alkylated dinucleotides were identified as a pdApdT/melphalan and pdGpdC/melphalan the latter being the most important.

Keywords: Melphalan; Nucleotides; DNA adducts

1. Introduction

Chemotherapeutic compounds used in the treatment of cancer reduce tumoral cell growth by interacting with different biomolecules. As such they disturb or block cell replication.

One of these compounds, melphalan (L-phenylalanine mustard, 4-(bis(2-chloroethyl)amino)-L-phenylalanine, is a bifunctional alkylating agent that covalently binds to the nucleophilic sites present in DNA [1] (Fig. 1). However, unlike other mustards melphalan does not require metabolic activation [2] in order to become cytotoxic: it dechlorinates sponta-

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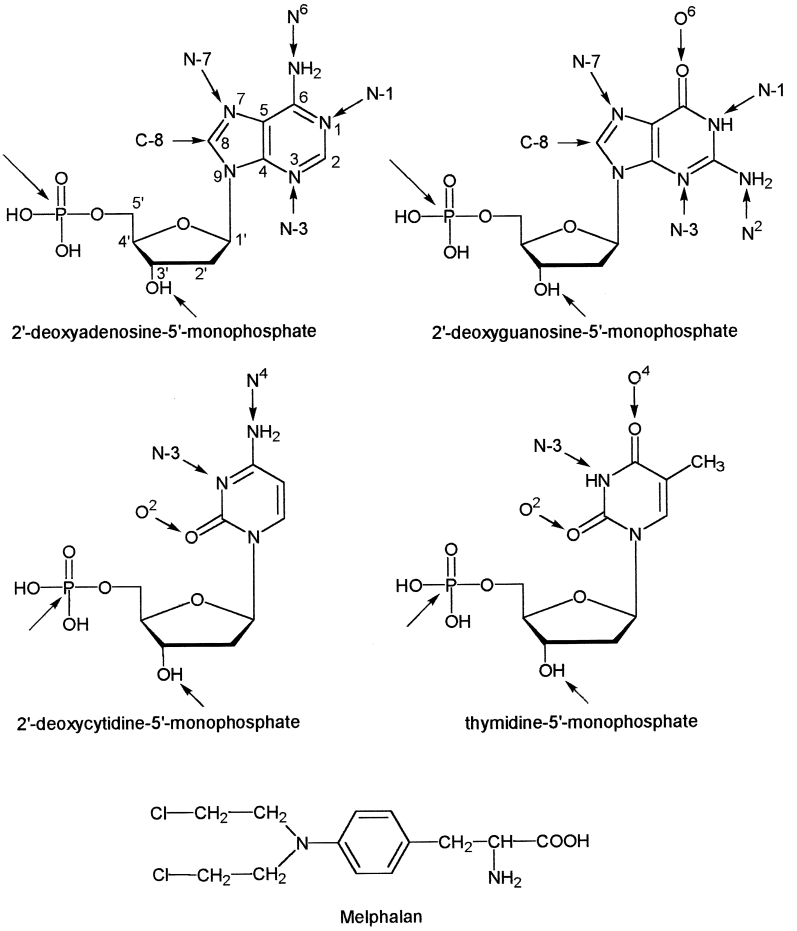

Fig. 1. Structure of 2′-deoxynucleotides indicating possible alkylation sites and the structure of melphalan.

neously and forms a highly reactive intermediate aziridinium ion [3] which immediately reacts with DNA with subsequent formation of melphalan adducts. This appears to be the major mechanism responsible for both cell death and mutagenesis. In the process of evaluating the effects of adduct formation on cell proliferation the identification of the formed adducts is of utmost importance.

Alkylating agents can interact with DNA in a variety of ways, each of which has specific implications on DNA replication. While mono-adduct formation on the nitrogen atoms or exocyclic oxygens may cause inhibition of DNA replication or base mispairing, intra- and interstrand cross-links which are possible with bifunctional alkylation agents, also will prevent DNA replication and transcription.

In the case of melphalan, termination of DNA replication did not occur at the G-rich sites where most of the adducts (N-7 alkylation) occurred [4,5] and where one could assume intra- and interstrand cross-links likely to occur. Therefore, it is postulated that other less frequently alkylated sites must be the cause of the inhibition of DNA replication, however, these sites remain to be identified.

Recently some progress has been made in the detection [6,7] and characterization [8–10] of these adducts. Immunological assays have been developed by Tilby [6]. Monoclonal antibodies have been

produced that specifically bind to sites on DNA that have been modified by alkylation and these antibodies have enabled quantitation of melphalan–DNA adducts. This has resulted in the determination of the extent of DNA alkylation occurring in peripheral blood cells of patients during therapy with high-dose melphalan [11]. Adducts were investigated in a human soft tissue sarcoma cell line RD and in bacteria. In hematopoietic cells experiments were performed on the single cell level [12]. However some negative aspects have to be considered: several factors may affect the antibody adduct and these techniques used do not give unambiguously structural information. Many interesting studies are hampered by the low availability of cellular material obtained from patients. It is, therefore, very important to develop new techniques that allow the simultaneous detection and identification of very small amounts of adducts.

In view of this our research focuses on the development of methods based on hyphenating CZE [13–15], capillary- and nano-HPLC [16,17] to electrospray tandem mass spectrometry (ES-MS–MS). Especially capillary HPLC and nano-HPLC in combination with a column-switching technique seems to provide the sensitivity required to measure adducts both from in vitro and also in vivo sources at the nucleoside or nucleotide level [16]. Therefore, DNA is enzymatically hydrolyzed to the nucleotide level prior to LC–MS analysis. This is important in our approach because at this stage all functional parts of the DNA molecule, i.e., purine-pyrimidine ring, the 2′-deoxyribofuranosyl system and the 5′-phosphate moiety are still present. This is in contrast to other sensitive mass spectrometric approaches where the derivatized purine or pyrimidine bases are analyzed by electron capture GC–MS [18].

In this paper we wish to report on the analysis of melphalan adducts of 2′-deoxynucleotides and calf thymus DNA hydrolysates by capillary HPLC–electrospray tandem mass spectrometry in combination with a column switching system. The low-energy collision-activated decomposition (CAD) product ion spectra allow differentiation between base- and phosphate-alkylated adducts in the case of the mono-alkylated 5′-nucleotides and give additional structural information in the case of the mono-alkylated dinucleotides.

2. Experimental

2.1. Materials

Calf thymus DNA (Sigma, St. Louis, MO) was used without further purification. Melphalan, 2′-deoxyadenosine-5′-monophosphate (dAMP), thymidine-5′-monophosphate (TMP) and 2′-deoxyguanosine-5′-monophosphate (dGMP) were purchased from Sigma. Methanol, glacial acetic acid (analytical grade), ammonium acetate (analytical grade), $ZnCl_2$, NaOAc and 2′-deoxycytidine-5′-monophosphate (dCMP) were purchased from Acros Chimica (Geel, Belgium). Millipore Milli-Q purity water was used in all experiments. The Chromabond® HR-P packing material used for the isolation of the dAMP-melphalan adduct was obtained from Macherey-Nagel (Düren, Germany).

Caution: melphalan used in this study has toxic properties and is listed as a carcinogen to humans. Appropriate precautions were taken and melphalan was handled with great care.

2.2. Preparation of melphalan/2′-deoxynucleotide adducts

To a solution of 2 mg of a 2′-deoxynucleotide (dAMP, dCMP, dGMP and TMP) in 1.5 ml NH_4OAc (0.01 *M*, pH 6.9), 0.5 ml of a melphalan solution (0.6%, w/v, melphalan in methanol) was added. The solution was stirred at 37°C. Aliquots were taken after 3 h and used for analysis without further treatment.

2.3. Preparation of melphalan/DNA adducts and the enzymatic hydrolysis to 2′-deoxynucleotides

Melphalan-treated calf thymus DNA was prepared according to the following protocol. To a solution of 2 mg calf thymus DNA in water, 0.5 ml of a melphalan solution (0.6%, w/v, melphalan in methanol) was added. The solution was incubated at 37°C. After 3 h the DNA was precipitated with 1 ml ice cold ethanol and 150 μl NaOAc (3.0 *M* pH 5.2) and the solution was centrifuged at 12000 *g* for 15 min, and dried under a stream of nitrogen gas. The resulting pellet was dissolved in 150 μl NaOAc (0.1 *M*, pH 5.2) and 125 μl $ZnCl_2$ (2 m*M*). One-ml

aliquots were hydrolyzed with nuclease P_1 at 37°C for 5 h. This was based upon the procedure described by Crain et al. [19]. The enzymatic hydrolysis of unmodified DNA was followed by (−)ES-MS–MS (multiple reaction monitoring) as a function of the amount of nuclease P_1 added (Fig. 2, Table 1). Aliquots were analyzed after 5 h by flow injection analysis (FIA) under (−)ES conditions using the specific conditions summarized in Table 1. From this data one could conclude that an optimum was reached at 7.9 units nuclease P_1/0.5 mg DNA. Analogous conditions were then used for the melphalan-treated DNA.

Table 1
MRM transitions used for monitoring the enzymatic hydrolysis of calf thymus DNA under (−)ES-MS conditions

	MRM parameters $[M-H]^- \Rightarrow B^-$ transition		
	$[M-H]^-$	Product ion $[B]^-$	Collision energy (eV)
dCMP	306	110	19
TMP	321	125	22
dAMP	330	134	19
dGMP	346	150	20

2.4. Mass spectrometric conditions

(+)Electrospray mass spectra were recorded on a Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Kontron HPLC system (Kontron Instruments, Milan, Italy). This system consisted of a 325 HPLC pump and a 332 HPLC detector (λ_{max}=260 nm; capillary U-shaped flow cell (LC-Packings, Amsterdam, The Netherlands). Aliquots of 5 μl were injected with a FAMOS (Fully Automated Micro Sampling Workstation) auto-sampler allowing column switching experiments. The flow-rate of the drying gas (N_2) was 250–300 and 15 l/h for the nebulising gas (N_2). The voltage on the capillary was between 2.3 and 3 kV. Low-energy collision-activated decomposition (CAD) spectra were obtained using argon as collision gas. The gas pressure was ca 3.0×10^{-3} mbar. The collision energy was optimized for each component. The optimal collision energy varied between 5 and 40 eV. The source temperature was set at 80°C. The mass spectra were recorded in the continuum mode. Scan times were 2.5 s for LC-MS experiments and 1.5 s for LC-MS–MS experiments. Data were acquired and processed by the Masslynx data system.

2.5. Chromatographic conditions

2.5.1. Column switching

In order to improve the concentration sensitivity of the LC-MS system and at the same time to separate

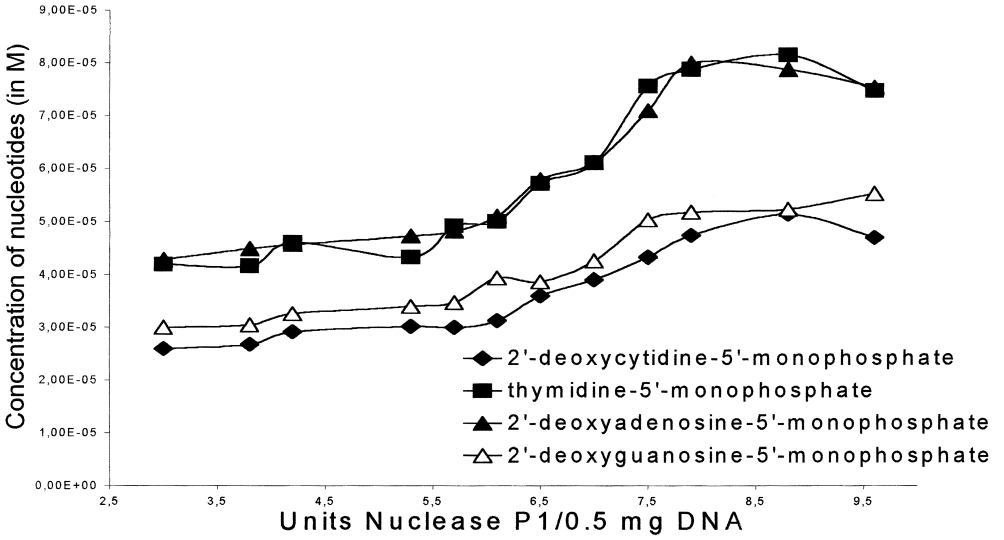

Fig. 2. Enzymatic hydrolysis of calf thymus DNA as a function of the amount of nuclease P_1 added. Aliquots were taken after 5 h.

unmodified nucleotides from the adducts prior to HPLC analysis a column switching procedure was developed. Five-μl samples, dissolved in 99/1 H_2O (HOAc 2%)/MeOH were injected on a 5 mm×500 μm I.D. column filled with Hypersil C_{18}. Adducts were captured by eluting the pre-column with an isocratic buffer consisting of 1/99 MeOH/HOAc (2%, v/v) at a flow-rate of 20 μl/min delivered by a second pump (HPLC pump 422, Kontron). Unmodified nucleotide material was sent to a waste (monitored by a second UV system equipped with a Capillary U-shaped flow cell). After 1.5 min the adducts were backflushed to the analytical column.

The column-switching system, used for the analysis of the melphalan-treated calf thymus DNA was adapted because of the presence of an additional unknown impurity. After 5.0 min, the adducts were backflushed to the analytical column and separated using a gradient elution starting at 80% HOAc (2%, v/v)/20% methanol and going to 50% HOAc (2%, v/v)/50% methanol in 30 min. With this solvent composition the column was eluted for 10 min. Then the solvent composition was altered to 100% MeOH in 15 min. These conditions were maintained for 5 min after which initial conditions were restored in 15 min.

2.5.2. HPLC analysis

The melphalan adducts were separated on a capillary column (Hypersil C_{18} BDS 300 μm×15 cm, LC Packings) using a gradient elution starting at 85% HOAc (2%, v/v)/15% methanol and going to 80% HOAc (2%, v/v)/20% methanol in 12 min. Then the solvent composition was altered to 50/50 in 28 min. These conditions were maintained for 20 min, after which initial conditions were restored in 15 min. The flow-rate was 7 μl/min.

2.6. Isolation of a dAMP adduct

The major adduct (see Section 3) found in a dAMP–melphalan reaction mixture prepared as described above was isolated using a self-packed column (0.85 mm×4.5 cm) filled with Chromabond® HR-P Packing material. The column was eluted at 0.5 ml/min. The column was equilibrated with 20 ml MeOH, 30 ml MeOH/H_2O (50/50) and 60 ml H_2O. Then 500 μl of the sample were loaded and the column was eluted, respectively, with 20 ml H_2O, 2.5 ml MeOH (33%) and 10 ml H_2O. The fraction eluting between 42 and 45 min was collected. This experiment was repeated eight times and the combined fractions were dried in a Speedvac™ (Speedvac Plus SC 110A, Savant Life Sciences Int., Leuven, Belgium) at 0°C and kept away from light. The isolated adduct (1.1 mg) was dissolved in 1/99 MeOH/HOAc (2%) and further diluted for sensitivity measurements.

3. Results and discussion

In order to get a better insight in both the chromatographic and mass spectral behavior of the different melphalan adducts, the mixtures resulting from the separate treatment of each of the 2′-deoxynucleotides (dGMP, dAMP, dCMP and TMP) with melphalan were investigated by capillary LC-ESMS. FIA analysis of these mixtures clearly showed that adducts were formed in very low quantities and that the presence the large amount of unmodified nucleotide material hampered their detection via the electrospray ionization process. Therefore, separation of unmodified 5′-mononucleotides prior to mass spectral analysis of the DNA adducts was a prerequisite not only in terms of sensitivity but also in view of the identification of possible isomeric DNA adducts. Based on earlier experiments [16] we decided to elaborate an automatic on-line enrichment method by installing a 500-μm I.D. pre-column in front of the analytical column. As stated above the goal was to elute the unreacted 2′-deoxynucleotides while capturing the adducts and hence enhance the concentration sensitivity of the system (Fig. 3).

3.1. Sensitivity

In order to get an idea about the detection limits using this LC-MS approach and more specifically in the analysis of melphalan adducts both unmodified dAMP and an isolated melphalan–dAMP adduct (see Section 2) were investigated under (+)ES ionization. Compounds were introduced without (FIA) and with the installation of a capillary HPLC column.

As shown in Table 2 for dAMP, the best sensitivity was obtained under single ion recording (SIR of

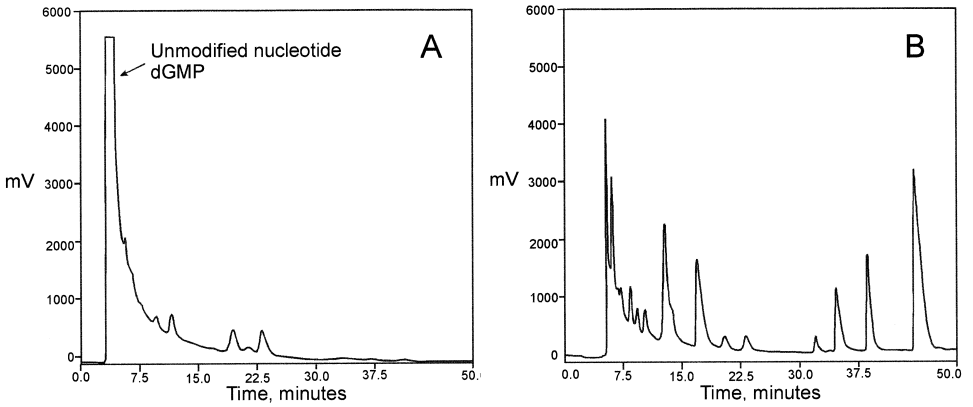

Fig. 3. Effect of column switching on the LC analysis of dGMP adducts on a capillary column (15 cm×300 μm I.D.; UV detection, λ=260 nm; mobile phase, 15/85 MeOH/HOAc (2%); flow-rate, 7 μl). (A) Without column switching: injection volume, 500 nl of a dGMP–melphalan reaction mixture. (B) With column switching: injection volume, 5 μl of a dGMP–melphalan; column switching after 90 s.

$[MH]^+$ at m/z 331.22) (21 fg; S/N=5). When the 300-μm I.D. analytical capillary column was installed (isocratic elution with 80/20 MeOH/HOAc (2%)) this meant an injection of 100 nl of a 6.5×10^{-10} M dAMP solution. Under single reaction monitoring (SRM) (transition m/z 331 ⇒ m/z 136) 43 fg could be detected. The detection limits in full-scan modes were also determined. In the capillary LC-MS full scan mode, a spectrum of 2 pg dAMP could be recorded with a S/N of 3 in the total ion chromatogram (TIC). The S/N ratio in the corresponding mass spectrum was 4. The relative standard deviation (RSD) value of the peak of dAMP (6.5×10^{-10} M; three injections), which reflects the reproducibility, was 4%.

For the dAMP–melphalan adduct (see Section 2) detection limits were investigated using the capillary HPLC column in combination with a pre-column in a column switching technique. Different concentrations of the adduct were injected under the conditions described in Section 2. Under SIR conditions (dwell time, 1s; cone voltage, 23 V; resolution, 15.0),

Table 2
Detection limits of dAMP and a base-alkylated mono-dAMP melphalan adduct under different (+) ES MS/MS conditions

Compound	Injection method	Mass spectral condition	Concentration (M)	Calculated amount injected	S/N
dAMP	FIA	Full scan	6.5×10^{-8} M	2 pg	3 (mass spectra: 4)
		SIR	6.5×10^{-10} M	21 fg	5
		SRM	1.3×10^{-9} M	43 fg	3
	Capillary LC (300 μm)	Full scan	6.5×10^{-8} M	2 pg	3 (mass spectra: 14)
		SIR	6.5×10^{-10} M	21 fg	3
		SRM	1.3×10^{-9} M	43 fg	3
dAMP adduct	FIA	SIR	6.6×10^{-8} M	4 pg	10
		SRM	6.6×10^{-8} M	4 pg	10
	Capillary column switching	SIR	1.1×10^{-9} M	4 pg	9
		SRM	8.3×10^{-9} M	30 pg	6

monitoring $[MH]^+$ (m/z 599.96), a detection limit of 4 pg ($S/N=8$) was found. The detection limits in SRM mode (dwell time, 1s; cone voltage, 42 V; resolution MS1, 14.0 and MS2,14.7; collision energy, 28 eV; transition m/z 600 $\Rightarrow$ m/z 404) were also explored. Here the detection limit was 30 pg, considering that the next run of the blank gives a response less than 0.1% of the signal. The RSD value of the peak of dAMP–melphalan (1.1×10^{-9} M; three injections), which reflects the reproducibility was 1%.

When going to lower concentrations, the signal of the blank became more important. Therefore, we want to emphasize that when using a column switching system, memory and matrix effects must be taken into consideration. Therefore, every analysis was followed and proceeded by a blank injection of the same eluant in which the adduct was dissolved.

3.2. LC–ES-MS–MS results

In the past several DNA adducts resulting from the interaction of potential genotoxic/cytotoxic industrial epoxides such as phenylglycidyl ether and bisphenol A-diglycidyl ether were characterized by us using LC–ES–MS [16,20,21]. These adducts could be separated by RP-HPLC using mixtures of 0.01 *M* NH_4OAC/CH_3OH and both (+)ES- and (−)ES-recordings gave good results. Especially in the case of 2′-deoxynucleotide adducts (−)ES recordings were recommended and their MS–MS data allowed differentiation between phosphate and base alkylation [22]. However, in the case of melphalan adducts the situation is quite different since here a much less lipophilic side chain is introduced. Hence completely different chromatographic conditions had to be elaborated [HOAc (2%, v/v)/CH_3OH]. This change in solvent composition had, as could be expected, its effect on the ES process in such a way that (+)ES gave better quality mass spectra compared to (−)ES ionization.

In all reaction mixtures several metabolites of melphalan were identified as a result of hydrolysis and solvolysis reactions with H_2O and CH_3OH. Since these reaction products were of minor importance to us their structure is not discussed. Nevertheless it is important to mention that these compounds eluted in between or after the DNA adducts. This makes their removal, prior to LC–MS analysis, via the column switching system quite impossible. However, this is not in the case of melphalan-treated calf thymus DNA. Here, melphalan and its metabolites are lost as the DNA-pellet is isolated.

3.2.1. dAMP–melphalan adducts

In the reconstructed ion chromatogram for the $[MH]^+$ of the mono-alkylated melphalan adduct of dAMP (m/z 600, ^{35}Cl)) three signals were observed at, respectively, $t_r=10$, 15 and 25 min. The most important adduct occurred at 15 min. In order get more insight in the structure of these isomeric melphalan–dAMP adducts, the low-energy collision-activated dissociation (CAD) product ion spectra were recorded of $[MH]^+$ (Fig. 4).

The product ion spectrum of adduct eluting at $t_r=10$ min (isomer A) showed a minimum of fragment ions (m/z 136, 269 and 332) compared to the adduct eluting at $t_r=25$ min (isomer C). In the latter spectrum ions were found at m/z 582, 447, 349, 269, 252 and 136. The melphalan–dAMP adduct eluting at $t_r=1$ min (isomer B) showed ions at 404 and 269. When the mass spectra of these three isomers were examined some diagnostic ions were found in isomers B and C. In the spectrum of isomer B m/z 404 can be explained by the $[BH_2]^+$ ion of the alkylated adenine moiety, while in isomer C the presence of an ion at m/z 136 corresponds to the $[BH_2]^+$ ion of adenine. This suggested that the melphalan moiety resides somewhere on the sugar moiety.

Two alkylation sites are possible in the 2′-deoxy-D-ribofuranosyl moiety, i.e., the 3′-hydroxy group or the 5′-phosphate function. In the case of 5′-phosphate alkylation some typical ions are observed in the low-energy CAD product ion spectra of the $[MH]^+$ of the corresponding melphalan adducts of all mononucleotides studied (see below). These ions are summarized and explained in Fig. 5a,b. At higher collision energy also the $[BH_2]^+$ ions (m/z 112, 127, 136 and 152) are present.

The ion at m/z 269 is observed in all spectra and is derived from the melphalan side chain. The spectrum of isomer A is characterized by product ions at m/z 136 and 332. The former ion pleads for alkylation at the 2′-deoxyribofuranosyl moiety because of the absence of m/z 349 (phosphate alkyla-

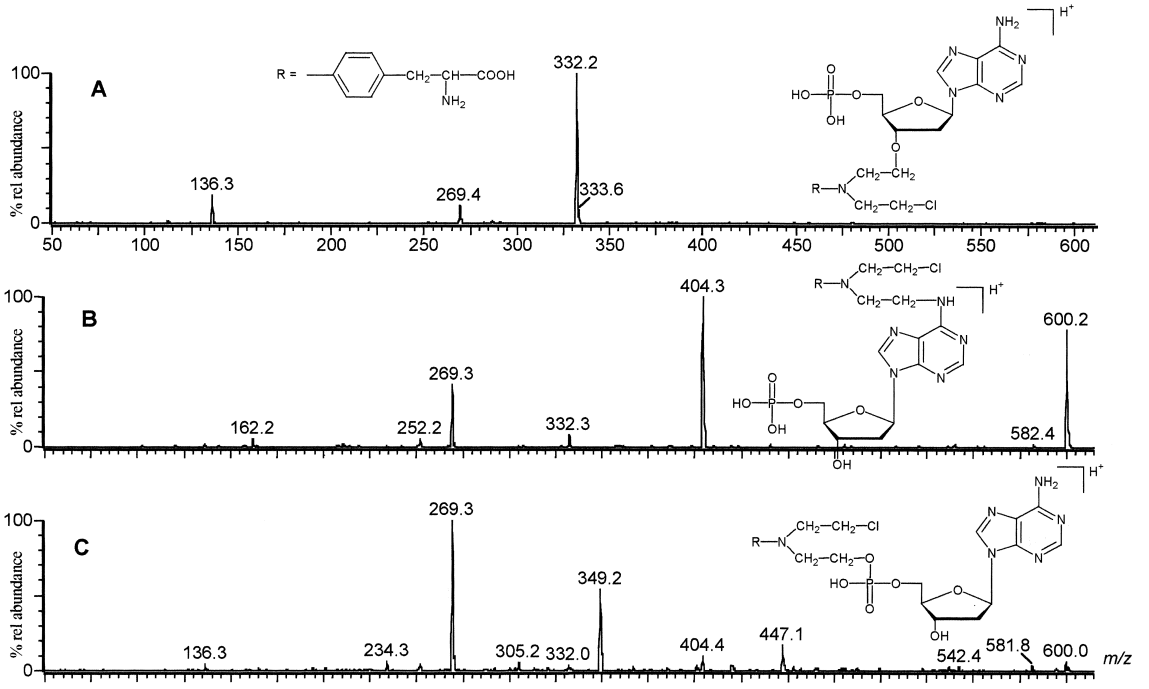

Fig. 4. Low-energy CAD product ion scan of $[MH]^+$ (m/z 600) of the different mono-alkylated melphalan adducts of dAMP. (A) 2′-Deoxyribofuranose alkylated dAMP; (B) mono-dAMP adduct alkylated at the base moiety; and (C) tentatively assigned to an alkylation at the 5′-phosphate moiety.

tion) and the presence of m/z 136 ($[BH_2]^+$ of the free adenine base). Therefore, alkylation of the 3′-OH function is put forward. Typical for this spectrum is the occurrence of an ion at m/z 332 corresponding to the protonated molecule of dAMP.

Other compounds characterized by a protonated molecule $[MH]^+$ at m/z 582 were chromatographically not completely resolved and eluted between t_r=10 min and t_r=11 min. This m/z value corresponds with monoalkylated melphalan/dAMP adducts in which the remaining chlorine atom in the melphalan side chain was replaced by an hydroxyl group. Product ion spectra of the pure isomers could not be obtained but it was logical to assume that the 'hydroxyl'-analogs of the isomers A, B and C were present. This was supported by the presence of the following ions: m/z 386 (base-alkylated $[BH_2]^+$), m/z 331 (phosphate alkylation, cf. m/z 349 if ^{35}Cl is present) and a product ion at m/z 332 (protonated dAMP, probably 3′-OH alkylation). An ion was also found at m/z 162 in all base-alkylated adducts. An analogous ion was observed in the base-alkylated dCMP adducts at m/z 138 which allowed the assignment of the alkylation position (see Section 3.2.2).

3.2.2. dCMP–melphalan adducts

For the protonated molecule $[MH]^+$ of the mono-alkylated melphalan adduct of dCMP (m/z 576, ^{35}Cl) four signals were found. One of very low intensity elates at 19 minutes. The other isomers eluted at t_r=8 (isomer A), 16 (isomer B) and 18 min (isomer C), of which B and C were the most important. The corresponding low-energy CAD product ion spectra are shown in Fig. 6.

Isomers B and C were readily identified as the base-alkylated and the phosphate-alkylated adducts of dCMP. Indeed the product ion spectrum of the former was characterized by an ion at m/z 380 (mono-alkylated $[BH_2]^+$). In the latter, as explained for the dAMP analog, m/z 349 pointed to 5′-phosphate alkylation. This is substantiated by the presence of m/z 112 ($[BH_2]^+$ of cytosine).

Isomer A gave ions at m/z 112 and 308. The presence of these ions and the absence of m/z 349

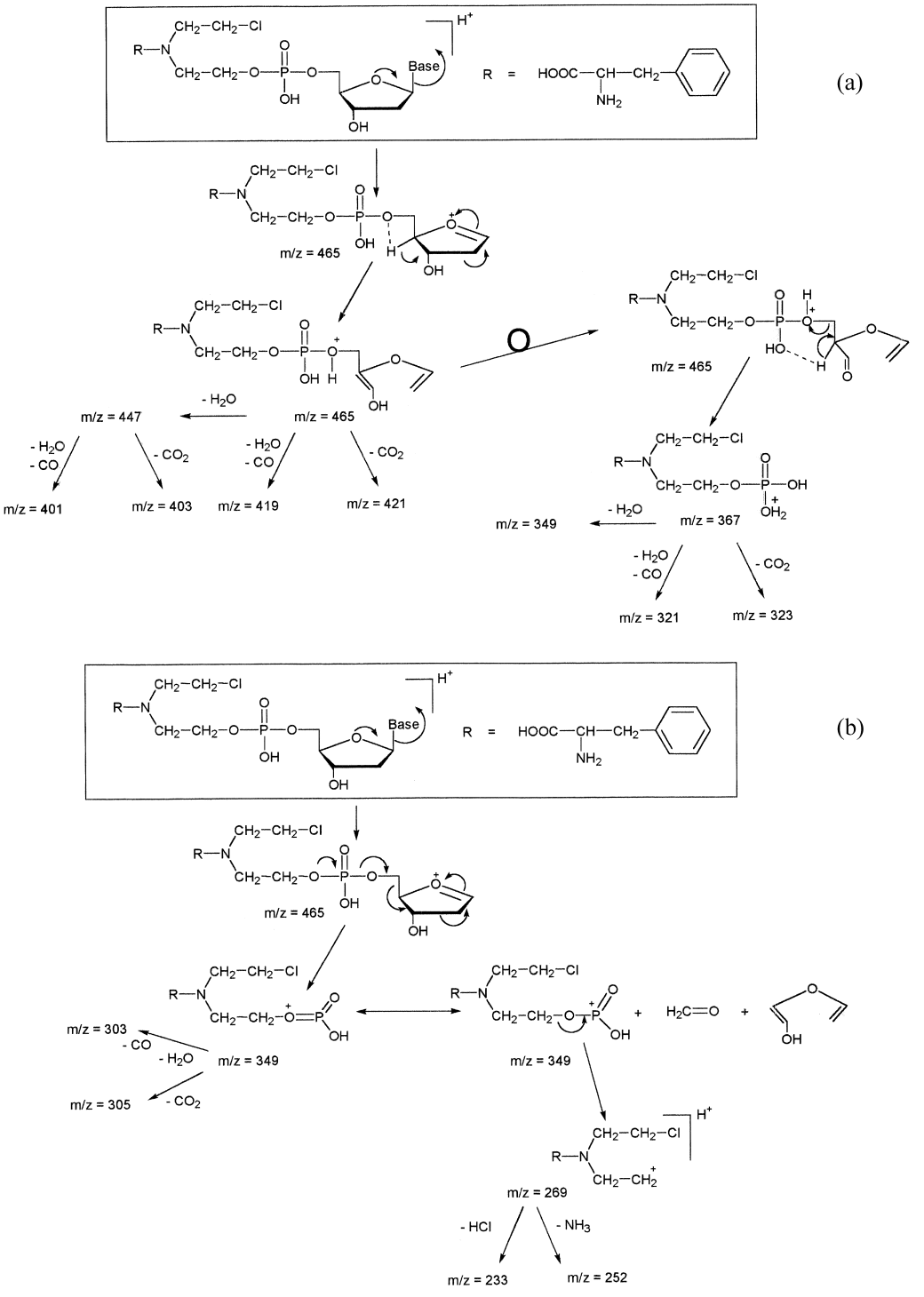

Fig. 5. Tentative fragmentation scheme explaining the different phosphate alkylated product ions.

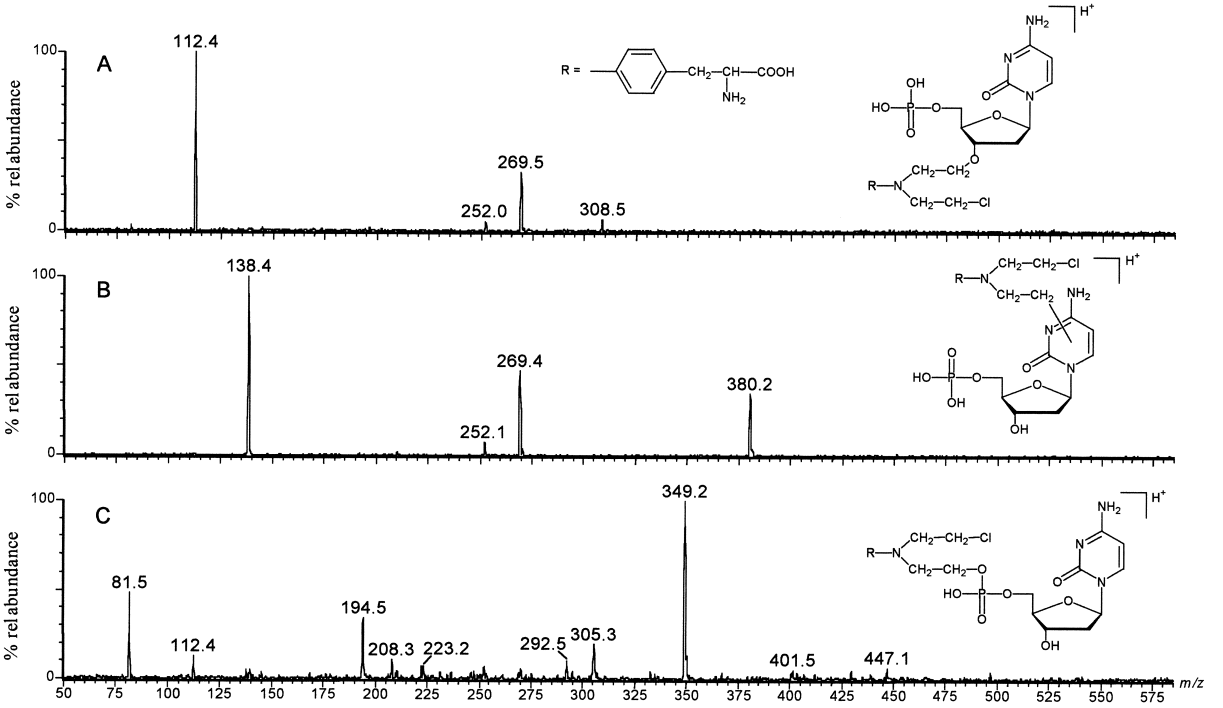

Fig. 6. Low-energy CAD product ion scan of $[MH]^+$ (m/z 576, ^{35}Cl) of the different mono-alkylated melphalan adducts of dCMP. (A) Tentatively assigned to an alkylation of the 2′-deoxyribofuranose moiety; (B) mono-dCMP adduct alkylated at the base moiety; and (C) 5′-phosphate-alkylated dCMP.

and 380 suggests a 3′-OH alkylated adduct. Furthermore in analogy with the dAMP adduct and ion at m/z 308 was found corresponding to protonated dCMP.

In analogy with the dAMP-mixture hydrolyzed mono-alkylated melphalan dCMP adducts ($[MH]^+$ at m/z 558) also could be detected. These isomers were found at t_r=11 (isomer D) and 12.5 min (isomer E). The corresponding CAD spectra are shown in Fig. 7. The base-alkylated adduct (eluting at 11 min) was characterized by the ion at m/z 362 which corresponded to the $[BH_2]^+$ of a mono-alkylated cytosine moiety as a result of the cleavage of the anomeric bond. The adduct eluting at t_r=12.5 min was the phosphate-alkylated adduct characterized by the diagnostic ion at m/z 331.

In the low-energy CAD spectra of the $[MH]^+$ ion of isomers B and D an ion at m/z 138 was observed. This ion was also found in the product ion spectra of separately prepared adducts of dCyd and cytosine. Based on these observations we concluded that this product ion was generated out of $[BH_2]^+$ and that it was independent of Cl⇒OH substitution in the melphalan side chain. Consequently m/z 138 can be explained by the elimination of $XCH_2CH_2NH\Phi CH_2CH(NH_2)COOH$ out of m/z 380 (isomer B) and m/z 362 (isomer D), respectively.

In order to prove this product ion spectra of m/z 138 were generated. This was done by producing m/z 138 first by in source CAD (cone voltage, 74–81 V) followed by a low-energy CAD product ion scan. A major ion was found at m/z 95 which corresponded to the loss of 43 u (HNCO). This observation pinpointed the melphalan chain at the exocyclic N^4 position. Additional proof was gathered from the low-energy CAD spectra from a deuterated dCyd/melphalan (MD^+=503 (d-5 analogue, ^{35}Cl)) and cytosine–melphalan adduct (MD^+=385 (d-4 analogue, ^{35}Cl)). In the spectrum of the nucleoside adduct m/z 138 was shifted to m/z 140. Again m/z 140 was formed by in source CAD and subjected to low-energy CAD. Prominent product ions were

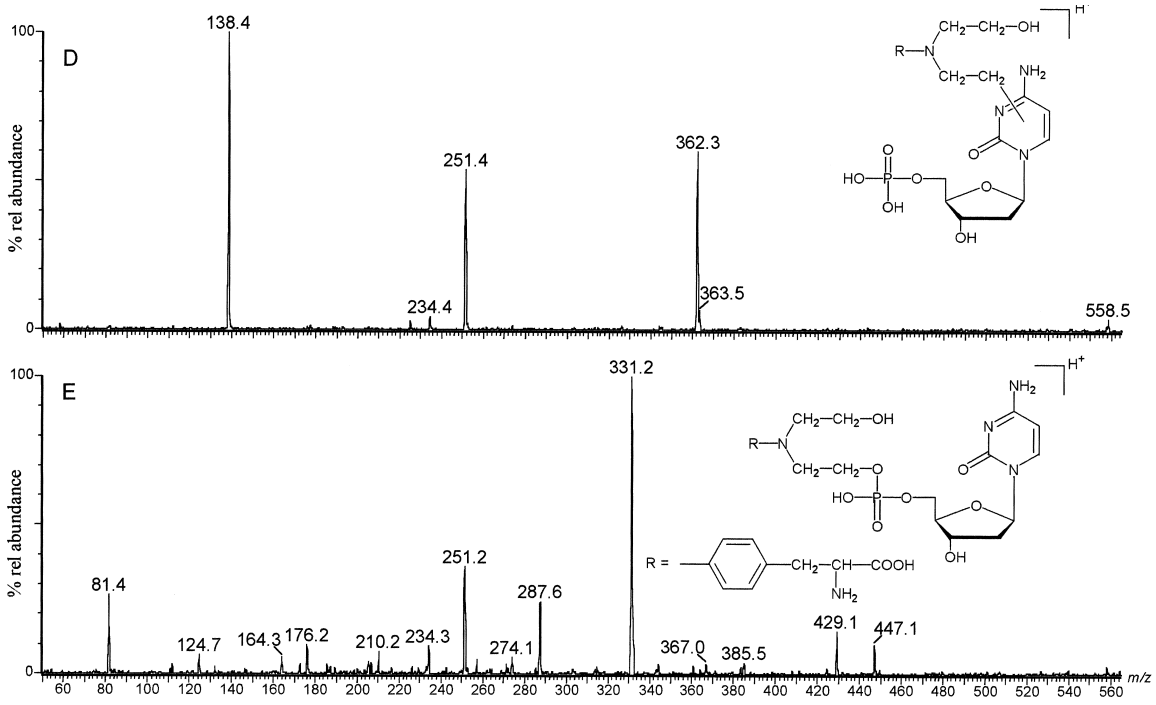

Fig. 7. Low-energy CAD product ion scan of $[MH]^+$ (m/z 558) of the different hydrolysed mono-alkylated melphalan adducts of dCMP. (D) Mono-alkylated dCMP adduct alkylated at the base moiety and (E) 5′-phosphate mono-alkylated dCMP.

found at m/z 96 and 46, which can be explained by the loss of DNCO and the occurrence of $[DNCO+D]^+$, respectively.

3.2.3. dGMP–melphalan adducts

Three mono-alkylated dGMP adducts ($[MH]^+$ at m/z 616, ^{35}Cl) were detected at t_r=6 (isomer A), 20 (isomer B) and 23 min (isomer C), the B isomer being the most prominent compound. As can be seen in Fig. 8, the low-energy CAD product ion scan of the $[MH]^+$ of isomer C showed the diagnostic ions indicating 5′-phosphate alkylation (see Section 3.2.1). In contrast the product ion spectrum of isomer B only showed a strong ion at m/z 420 which pointed to a guanine-alkylated dGMP ($[BH_2]^+$). The isotope pattern of m/z 616 (from a full scan mass spectrum) is shown in the insert. The alkylation site was tentatively assigned to the N-7 position of the guanine moiety not only because of the intensity of m/z 420 but also because of the presence in the reaction mixture of a compound eluting at t_r=18 min. This molecule was characterized by a $[MH]^+$ at m/z 420, which corresponds to a Gua–melphalan adduct generated in solution by deglycosylation of the dGMP/melphalan adduct. Such deglycosylation reaction often occurs if position N-7 in the guanine base is alkylated. Comparison of the low energy CAD spectrum of the in source CAD generated $[BH_2]^+$ ion at m/z 420 of isomer B with the low-energy CAD product ion spectrum of $[MH]^+$ (m/z 420) of the Gua–melphalan adduct showed both spectra to be analogous. This can be considered as a plea for N-7 alkylation in isomer B.

Isomer A eluting at t_r=6 min probably corresponds to a 3′-OH alkylated adduct. The major ions are at m/z 152 ($[BH_2]^+$), 252, 269, 348 ($[dGMP+H]^+$) and 429. The latter product ion can be explained by the combined loss of guanine (Δ 151 u) and HCl out of m/z 616 (^{35}Cl).

Hydrolyzed adducts, i.e., those in which the chlorine atom was substituted by an hydroxyl group were were present at t_r=13.5 and 14.5 min ($[MH]^+$ at m/z 598, no chlorine isotope pattern). The isomer eluting at 13.5 min corresponded to the phosphate-

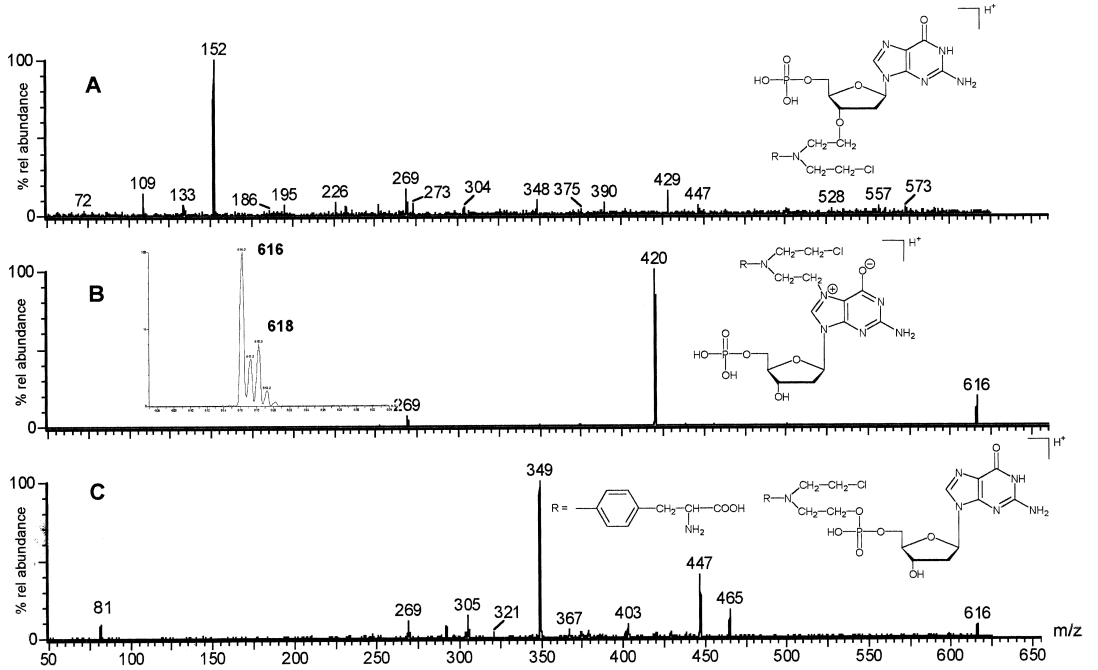

Fig. 8. Low-energy CAD product ion scan of $[MH]^+$ (m/z 616) of the different mono-alkylated melphalan adducts of dGMP. (A) Tentatively assigned to an alkylation at the 2′-deoxyribofuranose moiety; (B) tentatively assigned to an alkylation at N-7 position; and (C) 5′-phosphate alkylated dGMP.

alkylated adduct (m/z 331), while the compound eluting at t_r=14.5 min was identified as the base-alkylated adduct because of the presence of an ion at m/z 402.

3.2.4. TMP–melphalan adducts

In this mixture, two products with $[MH]^+$ at m/z 591 (mono-alkylated adduct of TMP) were found (t_r=7.5 and 22 min). Thymidine is the less nucleophilic nucleotide so alkylation was expected in very low yield. The isomer eluting at t_r=22 min was occurring less and identified as the phosphate-alkylated adduct (product ion at m/z 349). The base peak in the low-energy CAD product ion spectrum was found at m/z 269, a non-specific ion already explained in the foregoing sections. In the product ion spectrum of the compound eluting at t_r=7.5 min. No diagnostic ions were found. Two other compounds were found at m/z 573 (t_r=8.5 and 10 min), which corresponded to the $[MH]^+$ of a hydrolyzed melphalan adduct. Both showed an ion at m/z 331 pointing to phosphate alkylation suggesting in this case the separation of two diastereomers.

3.2.5. Melphalan-treated calf thymus DNA hydrolysates

The next step was to apply the data acquired above in order to search for different melphalan adducts in melphalan-treated calf thymus DNA. As described earlier this DNA was enzymatically hydrolyzed to a pool of modified an unmodified 2′-deoxymononucleotides by treatment with nuclease P_1. Furthermore it was observed that the column-switching system, used in the analysis of the melphalan-treated mononucleotide mixtures had to be slightly adapted (see Section 2) because of the presence of an additional undefined impurity. As such, aliquots of 5 μl were injected on the pre-column which was now eluted with an isocratic buffer consisting of 1/99 MeOH/HOAc (2%) at a flow-rate of 20 μl/min. Under these conditions not only the nucleotides but also this impurity were sent to the waste prior to LC–MS analysis. (column switching after 5 min).

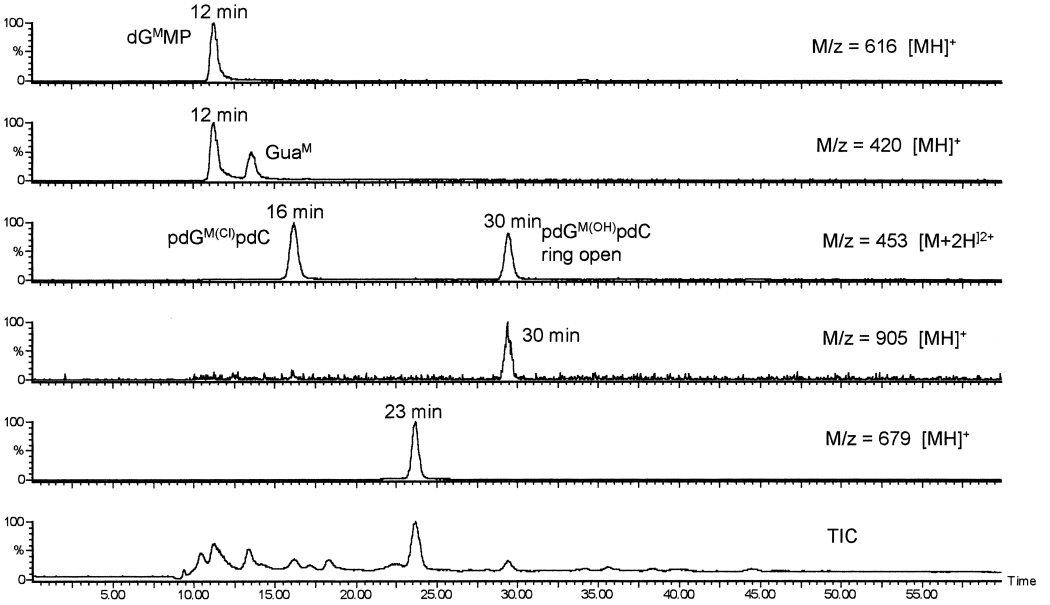

Fig. 9. RIC of the protonated molecules of the different adducts found in calf thymus DNA hydrolysate after column switching (5.00 min). Analysis by capillary LC–ES-MS.

This approach revealed the presence of several additional adducts (Fig. 9). In view of the results described earlier the chromatograms were reconstructed for the different mono-alkylated adducts which could be expected. The most important adduct was found at t_r=12 min. It was characterized by the presence of a protonated molecule $[MH]^+$ at m/z 616 (^{35}Cl) with a characteristic chlorine isotope pattern and corresponds to a mono-alkylated dGMP. As shown in Fig. 10, the low-energy CAD mass spectrum of the adduct in the hydrolysate (trace B) matched the spectrum of dGMP–melphalan adduct produced in the reference mixture (trace A). At t_r=13 min a very small amount of a mono-alkylated dGMP adduct was found in which the remaining chlorine atom on the melphalan moiety has been replaced by a hydroxyl function. This corresponds to a $[MH]^+$ at m/z 598. Additional proof for this was the absence of the typical chlorine isotope pattern in the latter ion. The corresponding deglycosylated adducts were found at t_r=14 min ($[MH]^+$=420 (^{35}Cl)) and t_r=10 min ($[MH]^+$=402).

At t_r = 26 min a compound was found characterized by a $[MH]^+$ at m/z 597 and a doubly charged ion $[M+2\ H]^{2+}$ at m/z 299. The molecular mass of this compound corresponded to the dinucleotide pdCpdC. Also other unmodified dinucleotides namely pdGpdG ($[MH]^+$=677), pdGpdC ($[MH]^+$=637), pdGpdA ($[MH]^+$=661), pTpT ($[MH]^+$=627) and pdCpdA ($[MH]^+$=621) could be found in the chromatogram but were not of interest to us. In addition to these, the mono-alkylated dinucleotides: pdGpdC/melphalan ($[MH]^+$=905), pdGpdA/melphalan, ($[MH]^+$=929), pdCpdC/melphalan, ($[MH]^+$=865), pdCpdA/melphalan ($[MH]^+$=889) and pdApdA/melphalan ($[MH]^+$=913) were found. The most prominent dinucleotide adduct amongst them was the pdGpdC–melphalan adduct eluting at 16 min. The low-energy CAD product ion spectra for m/z 453 (^{35}Cl) ($[M+2\ H]^{2+}$ ion) of this adduct is shown in Fig. 11. The spectrum was characterized by an ion at m/z 112, corresponding to the free $[BH_2]^+$ ion of the cytosine moiety. An isomeric compound was found at 30 min. It is interesting to note that the molecular mass of a genuine pdGpdC/melphalan adduct, i.e., in which the second chlorine atom is still present in the melphalan side chain of the adduct, is isobaric with the isomer in which simultaneously the chlorine atom has been substituted by an hydroxyl group, and the imidazole ring of the guanine base

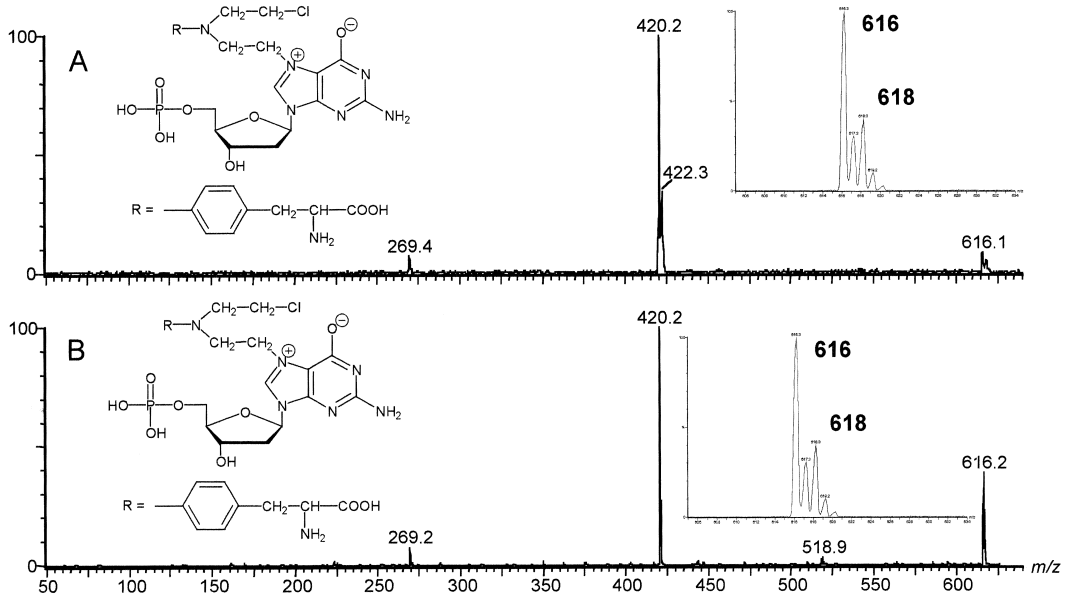

Fig. 10. Comparison of the low-energy CAD product ion scans of $[MH]^+$ (m/z 616): (A) in a reference mixture (collision energy, 18 eV; cone voltage, 28 V) and (B) in calf thymus DNA (collision energy, 12 eV; cone voltage 25 V).

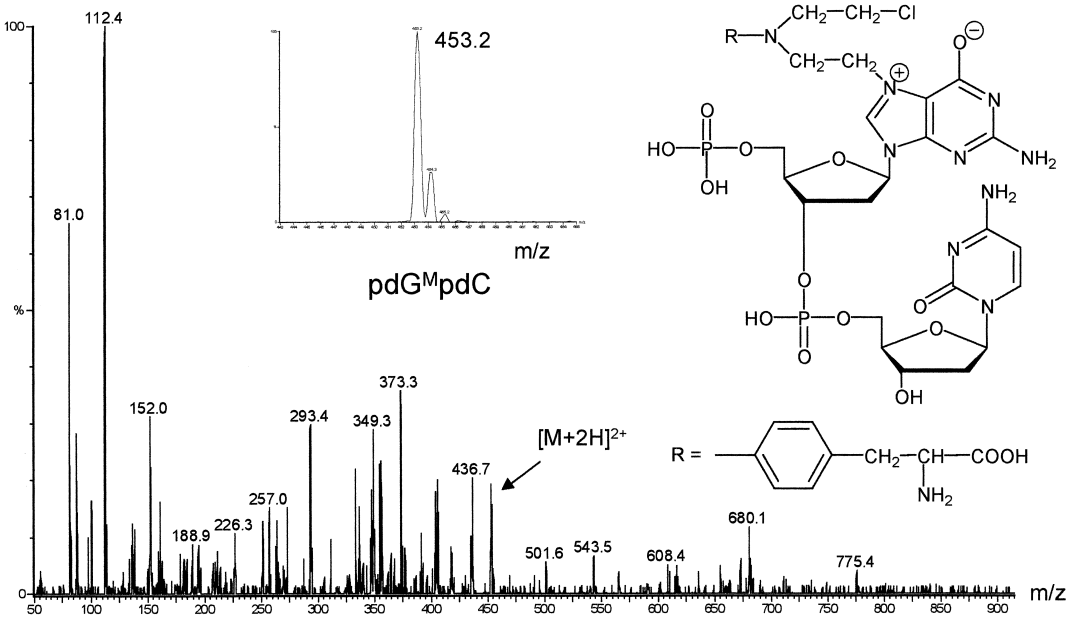

Fig. 11. Low-energy CAD product ion spectrum of $[M+2H]^{2+}$ (m/z 453) of mono-alkylated pdG(Mel)pdC dinucleotide. Isomer eluting at 17 min (collision energy, 15 eV).

has been opened by the attack of a molecule of H_2O. These two isomers can be readily distinguished by looking at the isotope pattern of the $[M+2H]^{2+}$ ion, as shown in the inserts of the low-energy CAD spectra (Figs. 11 and 12). From the latter data, more in particular because of the presence of an ion at m/z 210.3 the alkylation site was assigned to the base moiety of the guanine part defining the structure as pdG(MOH)pdC. In view of the arguments already mentioned earlier it was reasonable to assume that alkylation has occurred at the N-7 position. For this assignment no real mass spectrometric evidence is present but the presence of the imidazole ring opened analogue can be put forward as an argument in favor for it is known N-7-alkylated guanosine adducts to undergo ring opening and depurination reactions.

In the low-energy CAD product ion spectra for m/z 452.5 ($[M+2H]^{2+}$ (^{35}Cl)) which refers to the pdApT–melphalan-modified dinucleotide ($t_r=12$ min) is shown in Fig. 13. Not too much structural information was present: m/z 404 corresponds to the mono-alkylated $[BH_2]^+$ ion of adenine suggesting the pdA(Mel)pT structure.

Other modified dinucleotides gave such a weak diprotonated molecule that no product ion spectra could be recorded. A more sensitive method is will be a prerequisite. Therefore, further experiments are planned in which nano-HPLC will be coupled to nano-electrospray.

Finally we wish to comment on the occurrence of a compound eluting at 23 min which is characterized by a protonated molecule at m/z 679 [m/z 677 in (−)ES] and a prominent ion at $[M+2H]^{2+}$ at m/z 340 both ions without any chlorine isotope pattern. The low-energy CAD product ion scan of m/z 340 gave ions at 453 and 227. These ions seemed to suggest the presence of a trimer of a compound with MM 226: $(226)_3H^+=679\Rightarrow453\Rightarrow227$. These findings were confirmed by the (−)ES low-energy product ion scan of $[M-H]^-$ at m/z 677. Here, ions were found at m/z 451 and 225. Next to these also ions were observed at m/z 659, 433 and 207, which can be explained by the loss of 17 u out of m/z 677, 451 and 225, respectively. Prolonged treatment of the mixture with nuclease P_1 (8 h) had, in contrast to the other alkylated dinucleotides in the mixture, no

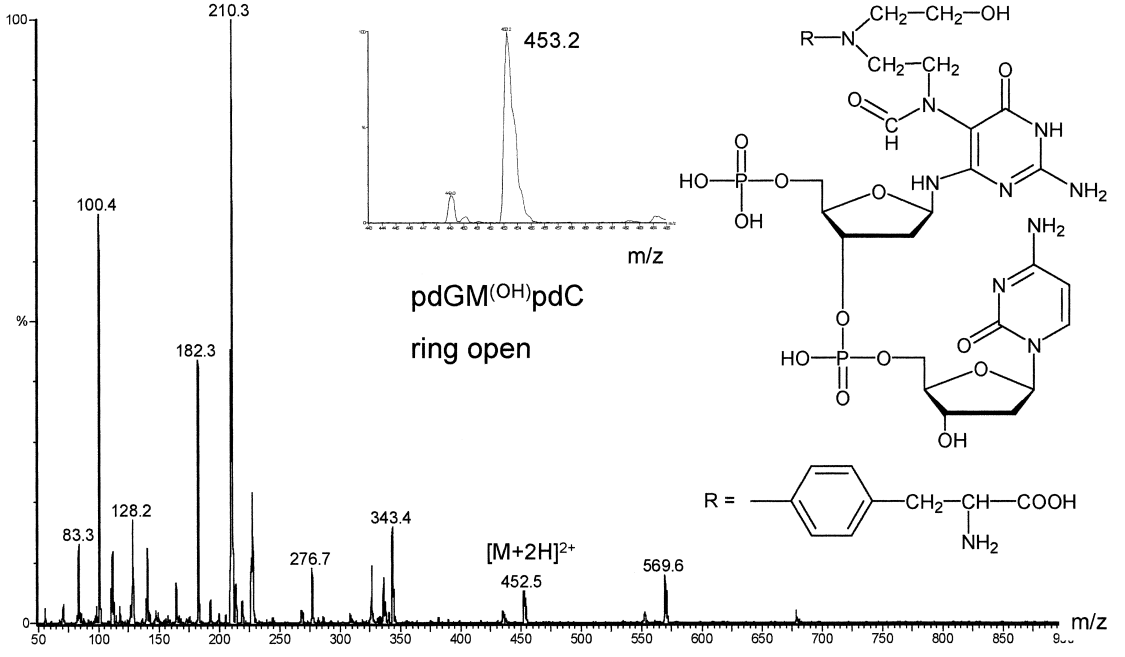

Fig. 12. Low-energy CAD product ion spectrum of $[M+2H]^{2+}$ (m/z 453) of mono-alkylated pdGpdC dinucleotide. Isomer eluting at 31 min (collision energy, 25 eV). Structure was assigned to the ring opened hydrolysed dinucleotide.

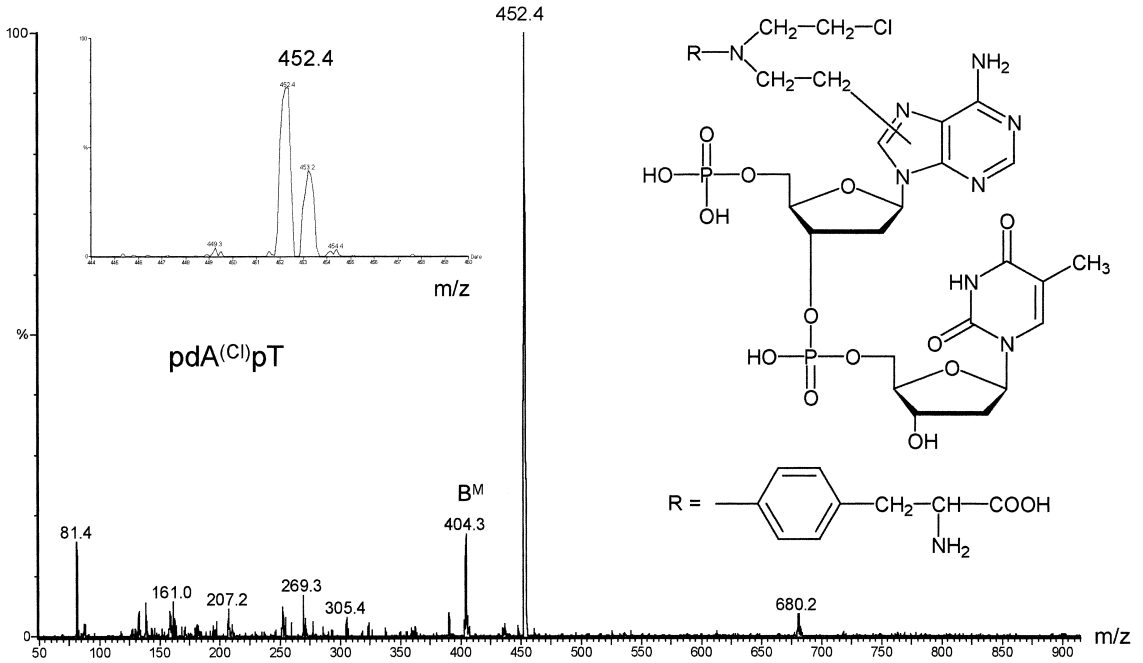

Fig. 13. Low-energy CAD product ion spectrum of $[M+2H]^{2+}$ (m/z 452.5) of a mono-alkylated pdA(Mel)pT-dinucleotide.

effect on the compound. If we look at the possible 'nucleot(s)idic' combinations we cannot find a satisfactory solution to the problem. In the (−) ES low-energy CAD spectrum also ions are found which differ 126 u. This could suggest the presence of a thymine moiety. We feel that a complete structure elucidation from mass spectral data alone seems difficult; therefore, attempts will be made to isolate the compound by semi-preparative HPLC in order to perform NMR experiments which hopefully in combination with the data presented above may solve the problem.

4. Conclusion

In order to be able to identify DNA adducts in biological material an approach based on the hyphenation of capillary HPLC to ES-MS using column switching has been developed. The sensitivity of the system allows the detection and structural identification of these adducts at the nucleotide level in the low pg range. From the melphalan treated calf thymus DNA we can conclude both G and A alkylation predominantly to occur. At the moment no phosphate-alkylated adducts were found in the pellet. However, their formation cannot be excluded since phosphate-alkylated adducts are residing in the supernatant as a result of early DNA cleavage in solution [15].

Future experiments, both qualitative and quantitative are needed and will be conducted towards the analysis of DNA hydrolysates of DNA pellets isolated from melphalan-treated Jurkat cell lines and finally from DNA isolated from the blood of patients treated with melphalan. Meanwhile the development of a LC-MS method based upon the coupling of nano-LC to nano-ES will be started in order to increase the mass sensitivity of the LC–MS system. This will allow the analysis of even more minute amounts of sample which from a medical point of view is an important feature especially if blood samples from patients treated with melphalan have to be analyzed.

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References

[1] T.L. Loo, E.J. Freireich, P.L. Munson (Editor-in-chief), Principles of pharmacology, in: Basic Concepts and Clinical Applications, Chapman and Hall, New York, Ch. 10, p. 1479.
[2] D.T. Vistica, Pharmacol. Ther. 22 (1986) 1163.
[3] M.H.N. Tattersall, N. Jarman, E.S. Newlands, Eur. J. Cancer 14 (1978) 507.
[4] R.O. Pieper, B.W. Futscher, L.C. Ericson, Carcinogenesis 10 (1989) 1307.
[5] R.O. Pieper, L.C. Ericson, Carcinogenesis 11 (1990) 1739.
[6] M.J. Tilby, J.M. Styles, C.J. Dean, Cancer Res. 47 (1987) 1542.
[7] M.J. Tilby, H. McCartney, J. Cordell, A.J. Frank, C.J. Dean, Carcinogenesis 16 (1995) 1895.
[8] M.R. Osborne, P.D. Lawley, Chem. Biol. Interact. 84 (1992) 189.
[9] M.R. Osborne, P.D. Lawley, Chem. Biol. Interact. 89 (1993) 49.
[10] M.J. Tilby, P.D. Lawley, P.B. Farmer, Chem. Biol. Interact. 73 (1990) 183.
[11] M.J. Tilby, D.R. Newell, C. Viner, P.J. Selby, C.J. Dean, Eur. J. Cancer 29A (1993) 681.
[12] A.J. Frank, S.J. Proctor, M.J. Tilby, Blood 88 (1996) 977.
[13] D.L.D. Deforce, F.P.K. Ryniers, E.G. Van den Eeckhout, F. Lemière, E.L. Esmans, Anal. Chem. 68 (1996) 3575.
[14] D.L.D. Deforce, F. Lemière, E.L. Esmans, A. De Leenheer, E.G. Van den Eeckhout, Anal. Biochem. 258 (1998) 331.
[15] D.L.D. Deforce, F. Lemière, I. Hoes, R.E.M. Millecamps, E.L. Esmans, A. De Leenheer, E.G. Van den Eeckhout, Carcinogenesis 19 (1998) 1077.
[16] K. Vanhoutte, W. Van Dongen, I. Hoes, E.L. Esmans, H. Van Onckelen, E. Van den Eeckhout, R.E.J. van Soest, A.J. Hudson, Anal. Chem. 69 (1997) 3191.
[17] E.L. Esmans, D. Broes, I. Hoes, F. Lemière, K. Vanhoutte, J. Chromatogr. A 794 (1998) 109.
[18] R.W. Giese, Chem. Res. Toxicol. 10 (1997) 255.
[19] P. Crain, in: Preparation and enzymatic hydrolysis of DNA and RNA for mass spectrometry, J. McCloskey (Ed.), Methods in Enzymology, Vol. 193, Academic Press, New York, 1990, pp. 782–824.
[20] F. Lemière, E.L. Esmans, W. Van Dongen, E. Van den Eeckhout, H. Van Onckelen, J. Chromatogr. 647 (1993) 211.
[21] F. Lemière, P. Joos, K. Vanhoutte, E.L. Esmans, M. Claeys, E. Van den Eeckhout, J. Am. Soc. Mass Spectrom. 7 (1996) 682.
[22] K. Vanhoutte, W. Van Dongen, E.L. Esmans, E. Van den Eeckhout, H. Van Onckelen, Eur. Mass. Spectrom. 2 (1996) 181.