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Alkylation of DNA by melphalan: investigation of capillary liquid chromatography–electrospray ionization tandem mass spectrometry in the study of the adducts at the nucleoside level

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Abstract

Nitrogen mustards are among the oldest cancer chemotherapeutic agents and remain the drugs of choice for treatment of many human cancers. A serious complication of treatment with nitrogen mustards is the increased risk of a secondary leukaemia in long-term survivors because not all alkylating agent interactions with DNA result in cell death. In an earlier study 2′-deoxy-5′-mononucleotide/melphalan adducts have been analysed by us by LC–ES MSMS. In this work we want to present the first results of the analysis of the corresponding 2′-deoxynucleoside/melphalan adducts from DNA hydrolysates by column switching/capillary LC–ES tandem mass spectrometry. Nucleosides, compared to nucleotides, give better chromatographic results and show a good sensitivity under electrospray (+) [ES(+)] ionisation. Several adducts were identified under ES(+) conditions. Mono-alkylated nucleoside adducts alkylated at the base moiety were identified for dGuo, dCyd and dAdo. Structures were identified by recording the low-energy CAD product ion scans. Also a mono-alkylated nucleotide pdA with alkylation position at the phosphate moiety could be detected. This proves that in the case of phosphate alkylation the enzymatic dephosphorylation reaction was inhibited. A Jurkat cell suspension was treated with melphalan (1 m*M*) and incubated at 37 °C (5% CO_2). After 6 and 48 h, the DNA was isolated and enzymatically hydrolysed. The corresponding nucleoside pool was evaluated with the developed LC–MS method. In the 48-h experiment, one adduct could be identified as a N-7 alkylated dGuo. In the 6-h experiment, no adducts could be found. Additional experiments were done wherein Jurkat-DNA, isolated from a non-treated cell culture, was treated with melphalan. These results were analogous with the data found in melphalan-treated calf thymus DNA. Additionally, we tried to determine the exact alkylation position by interpreting high-resolution fragmentation spectra.
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Keywords: DNA; Melphalan; Nucleosides

1. Introduction

Humans are exposed to a wide variety of carcinogens by both endogenous and exogenous sources [1]. These DNA-binding agents can induce mutations and the relationship with cancer was already a topic

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of many papers [1–4]. The most versatile method used in this field undoubtedly is the ^{32}P-postlabeling method. However, this method does not provide detailed structural information, although, compared to liquid chromatography hyphenated to mass spectrometry (LC–MS), it has a better sensitivity. Within the different approaches used in cancer research the chemical characterisation and quantitation of DNA-adducts in in vivo sources is one of them [5]. There are several techniques to investigate DNA adducts, such as immunoassays (ELISA, RIA) [6], ^{32}P-post-labeling [7], tandem mass spectrometry [8] and miniaturised LC–MS techniques [9,10].

Alkylating agents are widely used for treatment of many human cancers, including Burkitt's lymphoma, multiple myeloma [11], Hodgkin's disease and various other carcinoma's [12]. Therefore they are considered as a group of clinically important antineoplastic compounds. Actions of these agents can result in cell death because of the inability of DNA to duplicate successfully during the cell cycle. Unfortunately, not all such interactions result in cell death. It is possible that damage occurs that causes alterations in the cell that are mutagenic rather than lethal [13]. As such, a serious complication of treatment with alkylating agents is the increased risk of a secondary leukaemia in long-term surviving cancer patients [14].

One of these agents, melphalan, is a bifunctional alkylating chemotherapeutic compound (Fig. 1) that reacts via an aziridinium intermediate [15–17] which is the result of a spontaneous dechlorination of melphalan in solution. The cytotoxicity of melphalan and other nitrogen mustards like chlorambucil, cyclophosphamide and mechlorethamine, is thought to be due to monoalkylation and cross-linking at nucleophilic sites in DNA (Fig. 2) and in particular at the N-7 positions of guanosine residues [18]. The idea that cross-linked adducts play an important role in producing the cytotoxic effects is a common theme throughout the literature [14,19].

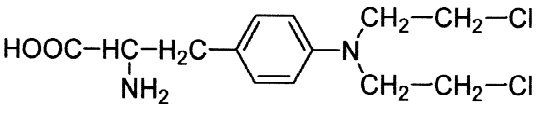


Fig. 1. Structure of melphalan.

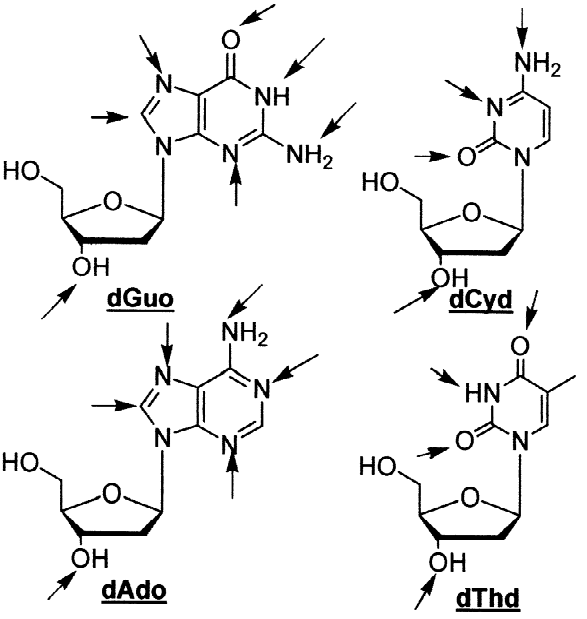


Fig. 2. Structure of 2′-deoxynucleosides with indication of possible alkylation sites.

DNA cross-link adducts of melphalan have not been identified by us until now using miniaturised LC–MS [9,10], nor in calf thymus DNA, neither in a treated Jurkat cell line. Nevertheless cross-links are thought to represent the lethal lesion following exposure of cells to bifunctional alkylating agents [20]. Only in in vitro reactions of 2′-deoxynucleoside-5′-monophosphates with melphalan, we detected in very low concentration a cross-linked adduct (dGMP–melphalan–dGMP), using nano-LC ES–MS–MS [10]. Also, Vu et al. [21] elucidated the structure of a cross-linked adduct of phosphoramide mustard after reaction with guanosine-5′-monophosphate at the N-7 position.

Using an antibody specific for melphalan-alkylated DNA, Tilby and co-workers [22,23] characterised several adducts. Also, Osborne and co-workers [24,25] claimed the presence of Salmon testes DNA–melphalan cross-linked adducts between guanine and adenine and between two guanine residues. Detailed structural information, however, was not available. Hansson et al. [26] investigated DNA cross-linking in human melanoma cells by the alkaline elution technique described by Ross et al. [27]. Maximum DNA cross-linking in L1210 cells was occurring

6–12 h following 30-min melphalan exposure. Bauer et al. [12] performed gel electrophoresis experiments to determine DNA cross-linking at the G–G–C sequence by mechlorethamine, phosphoramide mustard and melphalan. According to these authors, the mustards selectively formed interstrand cross-linking adducts but secondary alkylation was much slower for melphalan than for other mustards and the resulting cross-link was more stable. Phosphoramide mustard and mechlorethamine also induced intrastrand cross-links in double-stranded DNA but melphalan did not. A possible explanation of this phenomenon was given in terms of molecular dynamic simulations: the monofunctional bond intermediates of phosphoramide mustard and mechlorethamine form thermodynamically stable conformations with the second chloro-ethyl arm in a position appropriate for intrastrand cross link formation, while the corresponding melphalan monoadduct did not.

In view of the aforesaid our research focuses on the development of capillary- and nano-LC coupled to electrospray tandem mass spectrometry (LC–ES MS–MS), as a sensitive technique enabling to provide structural information. The implementation of miniaturised LC–MS was done to improve the mass-sensitivity of the system. Concentration sensitivity was enhanced by installing a column-switching system. Using this approach we could obtain an analytical tool to measure adducts from both in vitro and in vivo sources [9].

In this paper we wish to report on the LC–ES MS–MS analysis of melphalan DNA-adducts at the nucleoside-level with emphasis on the development of the methodology and the mass spectrometric behaviour of the different compounds under low energy CAD conditions. Accurate mass measurements were used to support the interpretation of low-energy CAD fragmentation schemes.

2. Experimental

2.1. Materials

Melphalan, 2′-deoxyguanosine (dGuo), 2′-deoxycitidine (dCyd), 2′-deoxyadenosine (dAdo), thymidine (dThd), calf thymus DNA, nuclease P1 (EC 3.1.30.1), phosphodiesterase 1 (EC 3.1.4.1), alkaline phosphatase (EC 3.1.3.1), ammonium acetate, ammonium bicarbonate and deuterated solvents (D_2O and MeOD) were purchased from Sigma (Sigma–Aldrich, Belgium). Tris(hydroxymethyl)aminomethane (Tris–Cl) was obtained from Fluka (Sigma–Aldrich, Belgium), sodium acetate, methanol and ethanol from Acros (Geel, Belgium). Jurkat-cells ($\pm 10^6$ cells/ml) were cultured in a growth-medium (Iscove's modified Dulbecco's medium, Gibco), containing 10% foetal bovine serum, purchased from Biochrom (Berlin, Germany). A GenomicPrep™ blood DNA isolation kit (Amersham Pharmacia, New York, USA) was used to isolate the cellular DNA. Millipore Milli-Q water was used in all experiments. All samples and mobile phases were filtered over a 0.2-μm nylon syringe filter (Alltech, Lokeren, Belgium) just before analysis. The drug used in this study, melphalan, is listed as a carcinogen to humans. Appropriate precautions are necessary when handling this drug.

2.2. Methodology

2.2.1. Interaction of 2′-deoxynucleosides with melphalan

In order to prepare nucleoside–melphalan adducts, 0.5 ml of a 20 m*M* melphalan solution in methanol was added to a solution of 2 mg of a 2′-deoxynucleoside (dGuo, dAdo, dCyd and dThd) in 1.5 ml NH_4Ac (0.01 *M*). The solution was stirred at 37 °C for 3 h. Afterwards the samples were dried under a nitrogen-stream in order to remove the methanol. Sample-residues were redissolved in 2 ml of water. The resulting solution was used for analysis without further treatment.

2.2.2. Interaction of calf thymus- and Jurkat-DNA with melphalan

Jurkat-DNA was first isolated from a cell-culture with the method described in Section 2.2.3. Calf thymus DNA was commercially available. These two different types of DNA were evaluated in adduct-formation in different experiments. To a mixture of 2 mg DNA in 1 ml water, 0.5 ml of a 20 m*M* melphalan solution in methanol was added. This was incubated for 3 h at 37 °C after which the DNA was precipitated in ice-cold ethanol and NaAc (3 *M*, pH

5.2). This was followed by centrifugation at 9000 rpm for 10 min. The DNA-pellet was dried under a nitrogen flow after removal of the supernatant.

2.2.3. Treatment of a Jurkat cell-line and isolation of the DNA

Jurkat-cells were cultured at 37 °C under 5% CO_2 atmosphere. When the cell-suspension contained $\pm 10^6$ cells/ml, a solution of melphalan in methanol was added to a final melphalan-concentration in the suspension of 1 m*M*. In two different experiments the cells were incubated at 37 °C for 6 and 48 h. After these periods the cells were isolated from the suspension by centrifugation (3000 rpm, 10 min) and washed with PBS (phosphate-buffered saline). In each isolation experiment 15 ml of the cell suspension or approximately 15×10^6 cells were used. The amount of isolated DNA was determined using UV and calculated at a value between 10 and 50 μg.

2.2.4. Hydrolysis of DNA

In order to start the hydrolysis procedure [28], the DNA was redissolved in 0.5 ml 1 m*M* Tris–Cl (pH 7.4). 50 μl 0.1 *M* NH_4Ac (pH 5.3) was added, together with 20 Units nuclease P1. The resulting solution was incubated for 2 h at 45 °C. Afterwards 55 μl 1 *M* ammonium bicarbonate (pH 7.8) and 0.02 units phosphodiesterase 1 were added. The solution was further incubated at 37 °C for 2 h. In a last step, 5 Units alkaline phosphatase were added and the mixture was kept at 37 °C for another 1 h. At this stage we have a nucleoside-pool with both adducts and unmodified nucleosides that was used for further analysis.

2.2.5. Deuterated experiments

In order to confirm the exact structure of the adducts or their product ions, all in vitro experiments were re-evaluated using deuterated solvents. Reactions were carried out as described above after which the samples were freeze-dried. The obtained products were redissolved in D_2O–MeOD and analysed in the same way as their non-deuterated equivalents. The results of these experiments are marked in the presented figures with a symbol (**).

2.3. Mass spectrometric conditions

Electrospray (+) mass spectra were recorded on a Quattro II triple quadrupole mass spectrometer and a Q-TOF 2 instrument (Micromass, Manchester, UK), both equipped with a pneumatically assisted Z-spray source. The system was hyphenated with a capillary LC-system (CAP-LC, Micromass, Manchester, UK). A 10-way stream select valve (Micromass, Manchester, UK) was used allowing column-switching experiments. The capillary column was coupled to a standard electrospray probe. The voltage on the stainless-steel capillary was set between 2.5 and 3.5 kV. Low-energy collision activated dissociation (CAD)-spectra were recorded using argon as collision-gas (gas-cell pressure: $\pm 4\times 10^{-3}$ mbar). The collision energy was varied between 5 and 30 eV. Accurate mass measurements were recorded on a Q-TOF 2 mass spectrometer. Polyethylene glycol, added post-column at a flow-rate of 1 μl/min, was used as a lock-mass in the accurate mass measurements. The exact masses of product ions were determined using the parent ion as lock-mass. The capillary LC experiments required a source temperature of 80 °C. In the full-scan mode a mass range from m/z 100 to 1000 was scanned. Scan times were 1.5 s for LC–MS measurements on the Quattro- and 1 s on the Q-TOF-instrument, both with an interscan delay of 0.1s. Data were acquired and processed with Masslynx 3.4 software (Micromass, Manchester, UK). Mass spectra were recorded in the continuum mode. The flow-rate of the nebulizing gas (N_2) and the drying gas (N_2) was 20 and 300 l/h, respectively.

2.4. Chromatographic conditions

Pre-columns (500 μm×5 mm, C_{18}) and analytical columns (Inertsil ODS-3, C_{18}, 3 μm particles, 300 μm×150 mm) were purchased from LC-Packings (Amsterdam, The Netherlands). A column-switching technique was developed to capture the adducts selectively, leaving the unmodified nucleosides unretained and discarded. This approach improves the concentration sensitivity of our system and prevent overloading of the capillary column. Therefore, 10 μl of the sample[1] was injected on a pre-column and eluted with an isocratic buffer consisting of 0.8:99.2 (v/v) MeOH–NH_4Ac (0.25 *M*) at a flow-rate of 10

[1]Starting from whole DNA, the sample also contains the proteins and salts from the hydrolysis procedure.

μl/min. Unmodified nucleosides were washed to a waste, which was verified by coupling the end of the pre-column with the mass spectrometer during the first 3.5 min of analysis. This verification also proved that no adducts eluted from the pre-column during the loading, although these compounds have little retention. After these 3.5 min, the captured adducts were backflushed to the analytical column (Fig. 3).

In order to obtain well-shaped and well-resolved chromatographic signals, adducts were separated using a gradient elution at 5 μl/min starting at 99.2:0.8 (v/v) NH_4Ac (0.25 *M*)–MeOH. The composition of the mobile phase was altered to 92:8 (v/v) NH_4Ac (0.25 *M*)–MeOH in 1.5 min going to 40% MeOH in 20 min. The latter conditions were maintained for another 35 min, after which initial conditions were restored.

3. Results

3.1. Adduct formation of melphalan with different 2′-deoxynucleosides

In order to obtain a better insight in the chromatographic and mass spectrometric behaviour of the different melphalan nucleoside adducts, the reaction mixtures of each of the 2′-deoxynucleosides common in DNA (dGuo, dCyd, dAdo and dThd) were analysed using LC–ESMS (Fig. 4). Prior to LC–MS analysis, unmodified nucleosides were removed from the reaction mixture as described in Section 2.4. Next to the adducts, melphalan derivatives were found (Table 1). Although these compounds dominate the TIC (Fig. 4) these products were of minor importance. Melphalan–nucleoside adducts were further characterised by using accurate mass measurements and low-energy CAD-spectra (collision energy: 5–30 eV). Accurate mass measurements of the product ions were recorded with the parent ion as lock-mass (Table 3). In each of the samples, only mono-alkylated melphalan–DNA adducts could be found as shown in Table 2. There is still some doubt about the structures of the adducts of thymidine. Because of the low abundant $[MH]^+$ ions no low energy CAD spectra could be recorded. A general feature observed in all low energy CAD product ion spectra of the $[MH]^+$ ion of the corresponding adduct was the presence of the alkylated base moiety $[BH_2\text{-mel}]^+$ in the product ion spectrum.

In order to avoid an overload of the capillary column, an on-line column switching procedure was

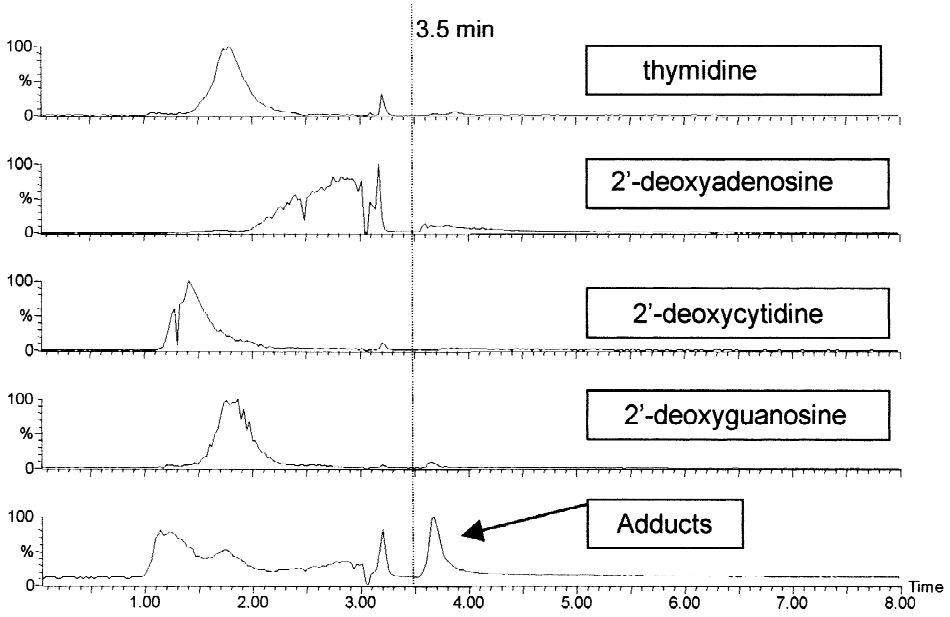


Fig. 3. Column-switching after 3.5 min in order to improve the concentration sensitivity of the LC–MS system and to separate the unmodified nucleosides from the adducts prior to HPLC analysis.

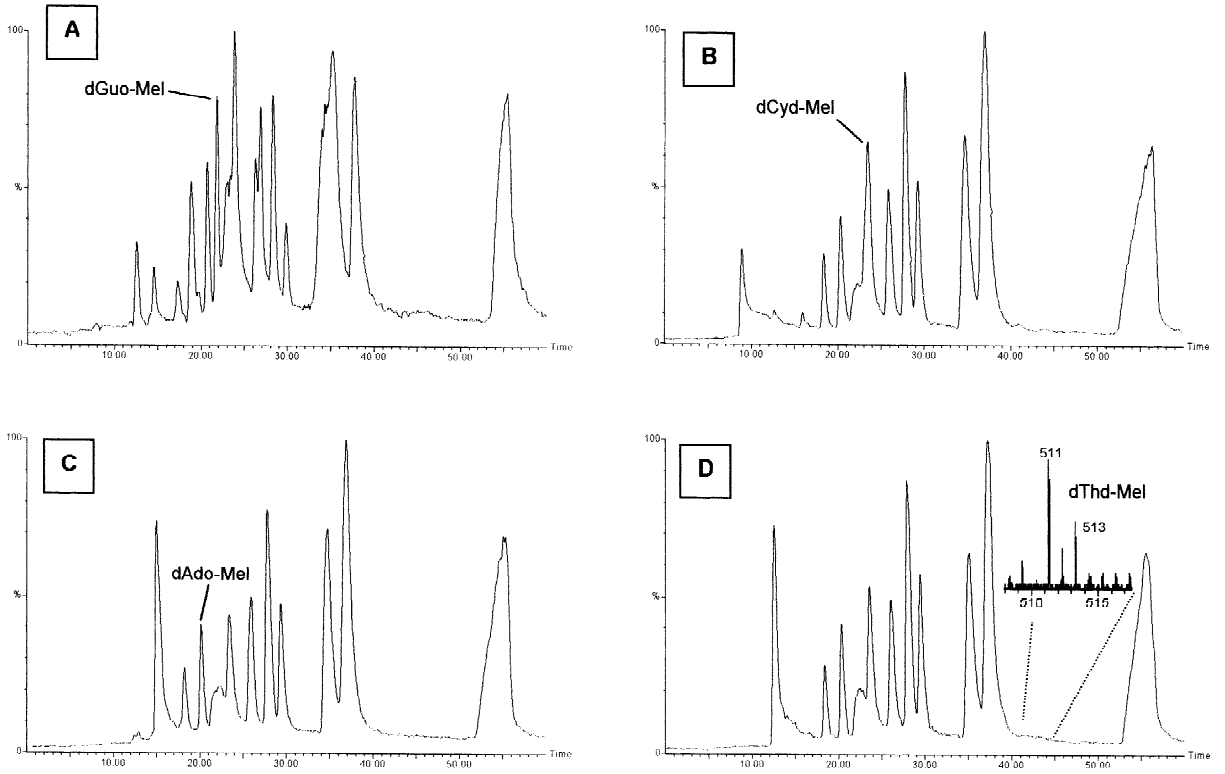
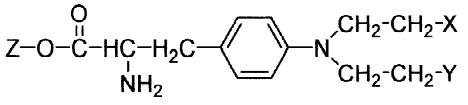


Fig. 4. LC analysis of the reaction mixtures of melphalan and the different 2′deoxynucleosides on a capillary LC column (300 μm×15 cm, C_{18}, 3 μm) at a flow-rate of 5 μl/min. Unmodified nucleosides were sent to a waste prior to LC analysis by injecting 10 μl of the sample on a pre-column (500 μm×5 mm) and eluting it with 0.8:99.2 (v/v) MeOH–NH_4Ac (0.25 *M*) at a flow-rate of 10 μl/min; column-switching after 3.5 min. (A) 2′-Deoxyguanosine+melphalan, (B) 2′-deoxycytidine+melphalan, (C) 2′-deoxyadenosine+melphalan, (D) thymidine+melphalan.

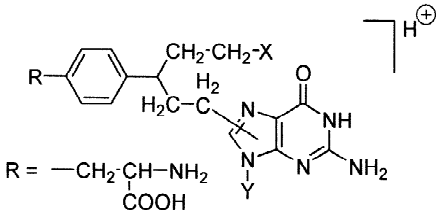
Table 1
Melphalan derivatives found in the reaction mixture of melphalan with 2′-deoxynucleosides: analysis with column-switching capillary LC–MS



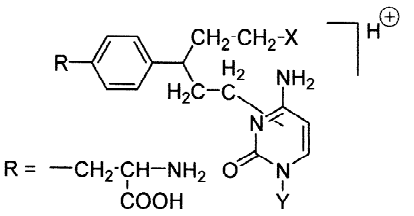
X	Y	Z	t_r (min)
$-OCH_3$	$-OCH_3$	$-CH_3$	20.1
-Cl	-OH	-H	23.4
$-OCH_3$	$-OCH_3$	-H	25.9
$-OCOCH_3$	$-OCH_3$	-H	27.7
$-OCOCH_3$	$-OCOCH_3$	-H	29.2
$-OCH_3$	-Cl	-H	34.8
-Cl	$-OCOCH_3$	-H	36.8
-Cl	-Cl	-H	55.0

developed which allowed the removal of unreacted compounds prior to LC–MS analysis. At the same time, the concentration sensitivity of the system was enhanced. To achieve this, a 500-μm×0.5-cm pre-column was mounted and equilibrated with 0.8:99.2 (v/v) MeOH–NH_4Ac (0.25 *M*) at a flow-rate of 10 μl/min. After equilibration 10 μl of the reaction mixture was injected on the pre-column by a loop-injection. After 3.5 min the pre-column was back-flushed and the captured adducts were separated on a 300-μm×150-mm capillary column. In the case of the analysis of the in vitro nucleoside reaction mixtures also the melphalan derivatives are present. If adducts from whole DNA (calf thymus DNA and DNA from a Jurkat cell-line) were analysed, these latter compounds were absent due to the isolation procedure of the DNA prior to enzymatic cleavage.

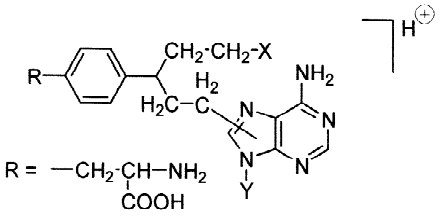
Table 2
Chromatographic results of the different adducts found in reaction mixtures of melphalan with (A) dGuo, (B) dCyd, (C) dAdo and (D) dThd. Analysis by capillary LC ES–MS, column switching after 3.5 min; 10 μl injections on a pre-column (500 μm×5 mm); separations of the adducts on a capillary column (300 μm×150 mm) using gradient-elution



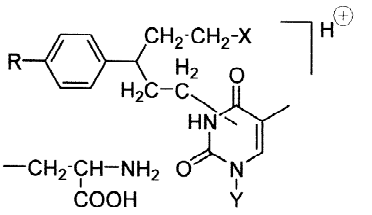
X	Y	t_r(min)
-OH	-dRib	14.5
-OH	-H	17.3
$-OCH_3$	-dRib	19.1
-Cl	-dRib	21.8
-Cl	-H	26.8



X	Y	t_r(min)
-OH	-dRib	15.9
-OH	-H	---
$-OCH_3$	-dRib	20.1
-Cl	-dRib	23.1
-Cl	-H	24.6



X	Y	t_r(min)
-OH	-dRib	12.9
-OH	-H	---
$-OCH_3$	-dRib	17.7
-Cl	-dRib	20.1
-Cl	-H	23.0



X	Y	t_r (min) Isomer 1	t_r (min) Isomer 2
-OH	-dRib	23.9	25.7
-OH	-H	---	---
$-OCH_3$	-dRib	29.9	31.3
-Cl	-dRib	39.7	44.2
-Cl	-H	---	---

It was shown by Hoes and co-workers [9,10] that nucleotide-adducts of melphalan could be separated on a reversed-phase Hypersil column using a gradient of H_2O (2% HOAc) and MeOH. Results of the latter study showed alkylation positions at the phosphate—and the ribofuranosyl—as well as on the base-moiety in the mononucleotide reaction mixtures. In this work we developed a method in which the different nucleoside adducts could be separated on a Inertsil ODS-3 column using a gradient of NH_4Ac (0.25 *M*) and MeOH. A complete analysis was done within 60 min. After each analysis the analytical column was re-equilibrated for a period of 30 min. As shown in this work, rather complex mixtures originate when mixing melphalan with 2′-deoxynucleosides. Sensitivity studies will therefore be carried out in the near future when a general method is developed that allows preparative separation of a melphalan nucleoside adduct.

3.1.1. dGuo–melphalan adducts

In the reconstructed ion-chromatogram of the reaction mixture of melphalan with dGuo, one unmodified melphalan adduct could be found (theoretical: $[MH]^+$ 536.2024, ^{35}Cl) at t_r=21.8 min. Mono-alkylated adducts in which the remaining chlorine atom was replaced by a hydroxyl- or methoxy-group were also found at t_r=14.5 and 19.1 min, respectively. The alkylation site was tentatively assigned to the N-7 position, because of the presence of a depurinated adduct which was formed in solution. Cleavage of the anomeric bond often occurs

when position N-7 of the guanine base is alkylated. Depurinated adducts eluted in between the nucleoside adduct at t_r=17.3 min (Gua–Mel–OH) and t_r=26.8 min (Gua–Mel–Cl). Low-energy CAD spectra of $[MH]^+$=536, with a collision energy below 25 eV, showed product ions at m/z 420, 269 and 152, which were attributed to the $[gua–mel]^+$ ion (alkylated base), ion A (representing the melphalan side chain) and the $[gua]^+$ ion (free base moiety), respectively (Fig. 5). Product ion spectra at a collision energy higher than 25 eV only yielded more fragmentation of the melphalan side chain as discussed. An accurate mass was determined for the monoalkylated dGuo–melphalan (^{35}Cl) adduct at m/z 536.2015; a difference of 0.9 mDa with the theoretical value.

3.1.2. dCyd–melphalan adducts

In the reaction mixture of melphalan with dCyd a monoalkylated adduct was found (theoretical: $[MH]^+$ 496.1963, ^{35}Cl) at t_r=23.1 min. Other types of monoalkylated adducts were detected in which the chlorine of the remaining melphalan side-chain was replaced by a hydroxyl- (t_r=15.9 min) or a methoxy-group (t_r=20.1 min). Furthermore, a monoalkylated Cyt adduct (m/z 380), formed in solution, eluted at t_r=24.6 min (Cyt–Mel–Cl). Recording the low-energy CAD (collision energy <25 eV) product ion spectrum of $[MH]^+$=496 gave ions at m/z 380, 269 and 138. These ions could be attributed to the $[Cyt–mel]^+$ ion, the melphalan side-chain A and an ion B as represented in Fig. 5. An accurate mass was determined for the monoalkylated dCyd–melphalan (^{35}Cl) adduct at m/z 496.1945; a difference of 1.8 mDa with the theoretical value.

3.1.3. dAdo–melphalan adducts

During LC–MS analysis of the dAdo–melphalan reaction mixtures one monoalkylated dAdo adduct (theoretical: $[MH]^+$ 520.2075, ^{35}Cl) was detected at

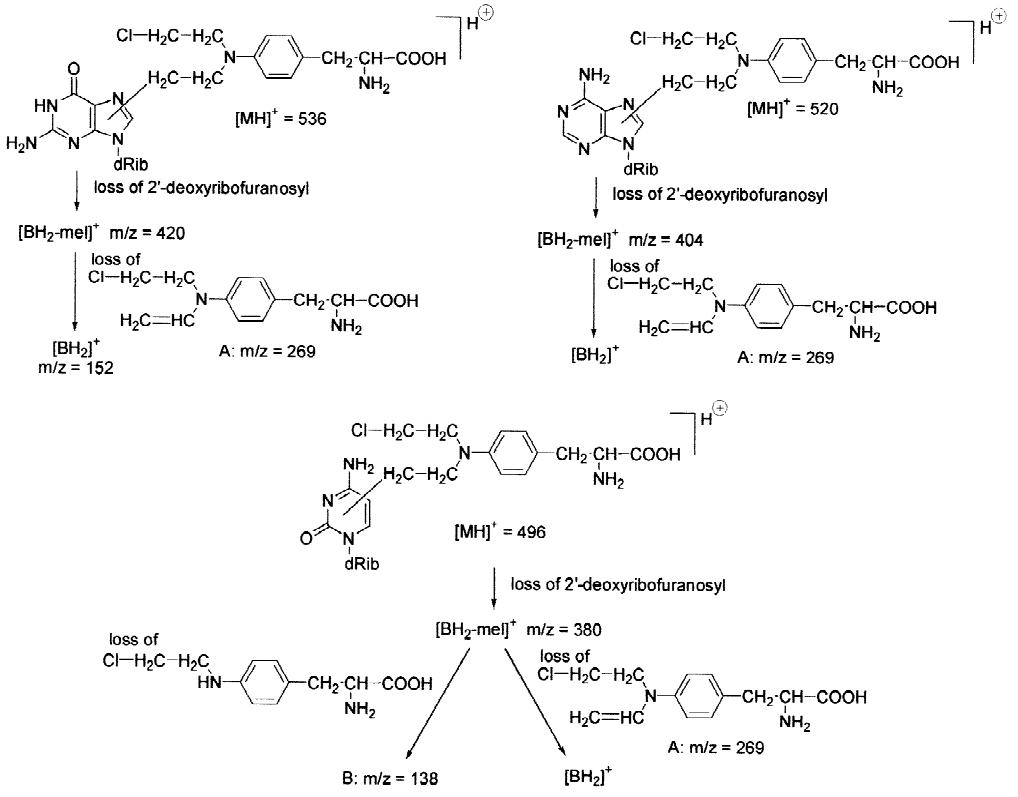


Fig. 5. Low-energy CAD product ion fragmentation scheme of the different nucleoside adducts (collision energy <25 eV) of melphalan.

t_r=20.1. Low-energy product ion spectra (collision energy <25 eV) of the protonated molecule $[MH]^+$=520 showed ions at m/z 404, 269 and 252, identified as the $[Ade–mel]^+$ ion, the melphalan side chain A and the nucleoside moiety, respectively. An accurate mass was determined for the monoalkylated dAdo–melphalan (^{35}Cl) adduct at m/z 520.2059; a difference of 1.6 mDa with the theoretical value. Other types of mono-alkylated adducts could be found: dAdo–mel–OCH_3 (t_r=17.7) and dAdo–mel–OH (t_r=12.9).

3.1.4. dThd–melphalan adducts

As expected, dThd was one of the less reactive and less nucleophilic nucleosides. Indeed adducts were hardly detected. Two signals with very low intensity (theoretical: $[MH]^+$ 511.1960, ^{35}Cl) were found and assigned to two isomers of dThd–mel (^{35}Cl). Their retention times were t_r=39.7 and 44.2 min, respectively, both with an intensity too low to obtain product ion scans or accurate mass measurements. Base-adducts, due to decomposition in solution, were not found.

3.2. Adduct formation in calf thymus- and Jurkat-DNA

The developed technique was used for the detection and identification of adducts, formed in whole DNA-strands. Similar results were obtained if commercially available calf thymus DNA and cell-extracted Jurkat-DNA was treated in vitro with melphalan. In earlier studies [9] involving the interaction of melphalan with calf thymus DNA at the nucleotide level, the presence of a monoalkylated dGMP was proven. Also monoalkylated dinucleotides, such as pdApdT/melphalan and pdGpdC/melphalan, were present in the hydrolysates. Alkylation at G and A occurred predominantly. However, in the aforementioned study no phosphate-alkylated adducts were found in the DNA-pellet. Although DNA-alkylation was studied at the nucleoside level in this paper, i.e. DNA was hydrolysed to the nucleosides prior to analysis, it was possible to detect phosphate-alkylation for dAMP (t_r=24.6 min). Most likely the alkaline phosphatase is disturbed in its activity due to the presence of the melphalan moiety on the phosphate. Low-energy CAD product ion spectra gave diagnostic ions for phosphate-alkylation at m/z 447 and 349 (Fig. 7). As shown in Fig. 6, monoalkylation adducts, with the alkylation positioned at the nucleobase, could be detected for dGuo, dCyd, dAdo and dThd. The typical presence of a chlorine isotope pattern, low-energy CAD product ion spectra and accurate mass measurements (Table 3) identified the nature of the alkylation products. In the reconstructed ion chromatrograms of dThd–mel–OH, dCyd–mel–OH, dAdo–mel–OH and dAdo–mel–Cl, more signals can be seen but they do not represent melphalan adducts. In some cases they are isotope signals of compounds with a lower mass (1 or 2 units), in other cases they were also found in blank samples which only contained the products of the hydrolysis procedure. No further research was done to characterise this type of signals, because they were of minor importance to us.

3.3. Adduct formation in melphalan-treated Jurkat cell-lines

Two experiments were performed on melphalan-treated Jurkat cell-lines. In a first experiment Jurkat-cells were incubated with melphalan during 6 h, a value which was arbitrary chosen. After this period, DNA was extracted and enzymatically hydrolysed to the nucleoside level by using nuclease P1, snake venom phosphodiesterase and alkaline phosphatase successively. The samples were analysed with the developed column-switching LC ES–MS system. However, no adducts could be found in this hydrolysate neither by single ion recording (SIR) or single reaction monitoring (SRM). Therefore, it was concluded that either the concentration of the adducts was below the detection limit of the system or that no adducts had been formed at all. Earlier studies by Hoes et al. [9] showed a detection limit of 4 pg for a dAMP–melphalan adduct under SIR using a column-switching capillary LC system. In a second experiment Jurkat-cells were incubated with melphalan for 48 h and these results were compared with the 6 h experiment. In these DNA hydrolysates (Fig. 8), only monoalkylated dG adducts could be detected with alkylation position on the nucleobase. The dGuo–Mel–OH and dGuo–Mel–Cl were identical to the adducts detected in the nucleoside reaction mixtures and were identified by interpretation of the

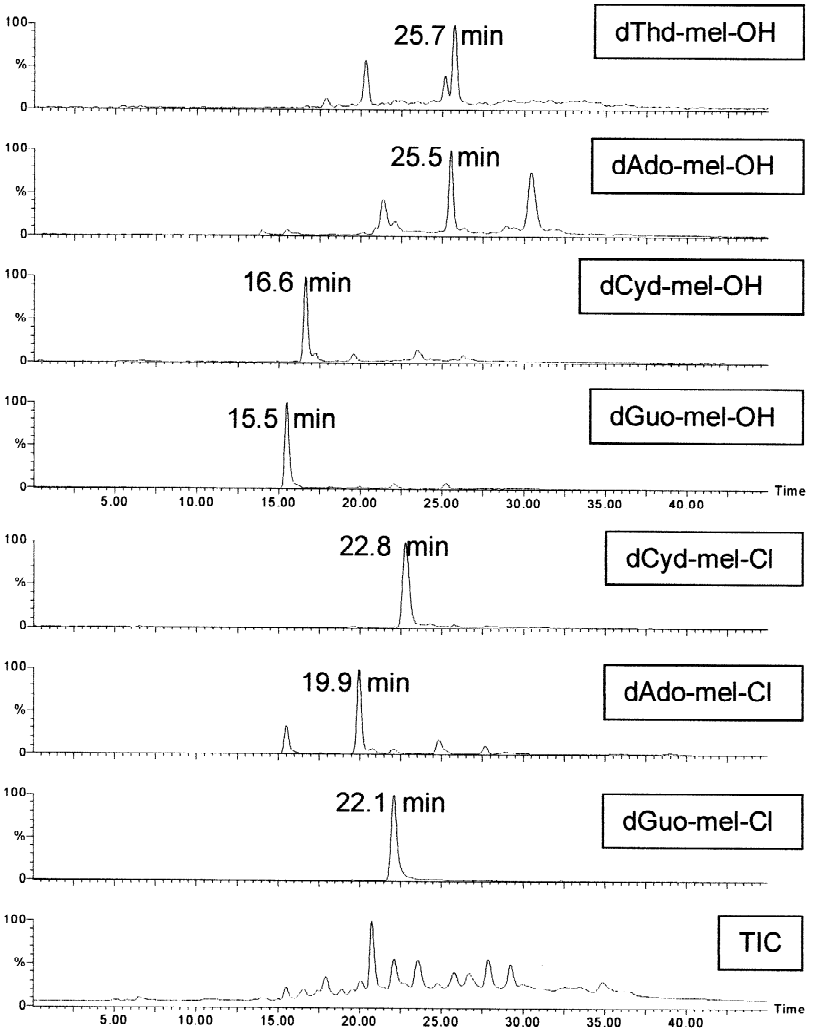


Fig. 6. TIC and RIC of the $[MH]^+$ of the different monoalkylated adducts found in calf thymus- and Jurkat-DNA hydrolysates after column-switching procedure. Analysis by capillary LC ES–MS.

low-energy CAD product ion scans of the $[MH]^+$ ion. As can be seen from the corresponding total ion chromatogram (TIC) (Figs. 6 and 11) a lot of other compounds eluted during this analysis. In order to find out whether these are relevant compounds i.e. melphalan adducts, several blanks were run which only contained the products used in the hydrolysis procedure. From these results we could conclude that these signals can be attributed to compounds of a non-nucleosidic and non-melphalan nature. No special effort was done to characterize the latter compounds. Because cellular DNA is situated in the cell nucleus or in the mitochondria, interacting agents have to travel through the cell membrane, the cytoplasm and the mitochondrial membrane or the cell nucleus. This process is time-dependent and might explain the differences we found between the 6- and the 48-h experiments.

3.4. Study of the fragmentation pattern of the melphalan–nucleoside adducts

When studying product ion spectra of the melphalan–nucleoside adducts, one could observe that

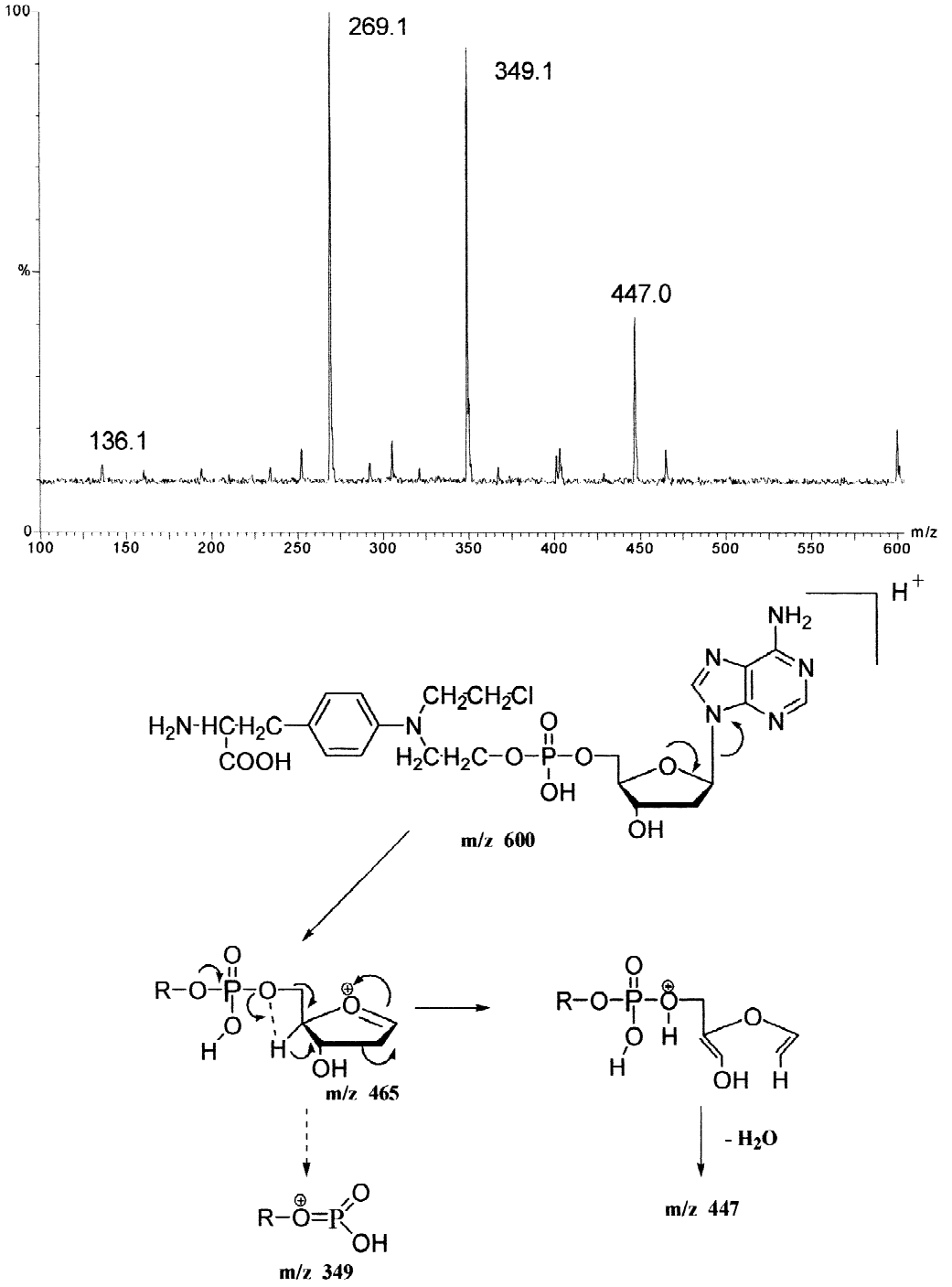


Fig. 7. Tentative fragmentation scheme of the phosphate-alkylated dAMP adduct, found in calf thymus DNA hydrolysates. Analysis by capillary LC ES–MS.

Table 3
Exact mass measurement of the molecular ion $[MH]^+$ and fragment ions as presented in Fig. 5

Product ions of	Theoretical	Experimental	*D* (mDa)	*D* (ppm)	(**)
dGuo–Mel–Cl	536.2024	536.2015	0.9	1.7	544
[Gua–mel]+	420.1551	420.1538	1.3	3.1	427
A	269.1057	269.1065	0.8	3.0	272
[Gua]+	152.0572	152.0578	0.6	3.9	157
dGuo–Mel–OH	518.2363	5118.2358	0.5	1.0	527
[Gua–mel]+	402.189	402.1884	0.6	1.5	410
A′	251.1396	251.1400	0.4	1.6	255
[Gua]+	152.0572	152.0570	0.2	1.3	157
dCyd–Mel–Cl	496.1963	496.1945	1.8	3.6	503
[Cyt–mel]+	380.1489	380.1489	0.0	0.0	386
A	269.1057	269.1072	1.5	5.6	272
B	138.0667	138.0681	1.4	10.1	140
dCyd–Mel–OH	478.2302	478.2296	0.6	1.3	486
[Cyt–mel]+	362.1828	362.1866	3.8	10.5	369
A′	251.1396	251.142	2.4	9.6	255
B	138.0667	138.0685	1.8	13.0	140
dAdo–Mel–Cl	478.2302	478.2296	0.6	1.3	486
[Ade–mel]+	404.1602	404.1594	0.8	2.0	410
A	269.1057	269.1066	0.9	3.3	272
[dAdo]+	252.1097	252.1108	1.1	4.4	257
[Ade]+	136.0623	136.0648	2.5	18.4	140
dAdo–Mel–OH	478.2302	478.2296	0.6	1.3	486
[Ade–mel]+	386.1941	386.1962	2.1	5.4	393
[dAdo]+	252.1097	252.1114	1.7	6.7	257
A′	251.1396	251.1406	1	4.0	255
[Ade]+	136.0623	136.0632	0.9	6.6	140

Low-energy CAD product-ion spectra, collision energy <25 eV); A′=remaining chlorine atom on the side-chain is replaced by a hydroxyl group. (**) indicates deuterated experiments.

the major fragment ion was formed by loss of the sugar moiety as a result of the cleavage of the anomeric C–N bond, a process often encountered in the mass spectra of nucleosides in general. Nucleoside samples were introduced under flow-injection (FIA) conditions. This introduction method was a more rapid procedure of analysis which could be applied because analysis of the in vitro nucleoside mixtures by LC–MS showed the presence of only one isomer. In order to get more fragmentation of the $[BH_2–mel]^+$ ion, the 2′-deoxyribofuranosyl moiety was removed by in source CAD. The optimal cone-voltage for this process was determined for each adduct: 60 V for dGuo–mel, 50 V for dCyd–mel and 55 V for dAdo–mel. Once the correct cone voltage was applied, the $[BH_2–mel]^+$ ion of the alkylated base was selected by the first MS and fragmented in the collision cell (argon pressure: 4×10^{-3} mbar). The elemental composition of the different product ions was determined by accurate mass measurements. The $[BH_2–mel]^+$ ion was used as the lock-mass (Fig. 9). Since at a collision energy >25 eV the $[BH_2–mel]^+$ ion disappeared, measurements were done using multiple collision energy settings. In order to obtain the necessary data product ion spectra were recorded by combining scans at a given collision energy together with a scan containing the parent ion using low collision energy settings (<25 eV). Structures for the identified product ions of Gua–Mel are proposed in Table 4. Energy-resolved mass spectra using in source formation of the $[BH_2]^+$, as shown in Fig. 10 for dGuo–Mel, indicate a maximum in the presence of the alkylated base (m/z 420) at a low collision-energy of 10 eV, as

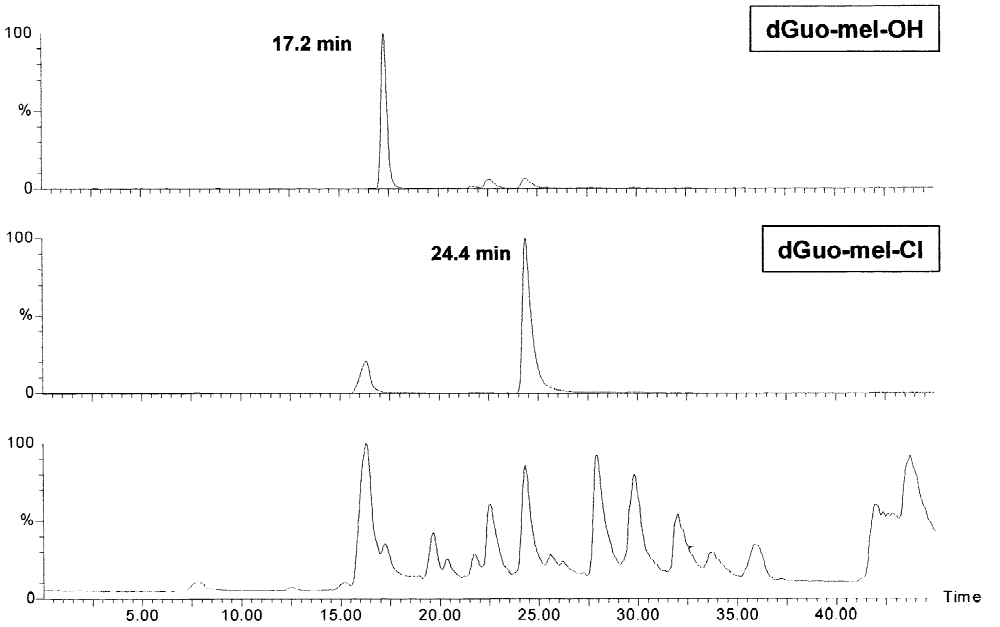


Fig. 8. TIC and RIC of the monoalkylated dGuo adduct found in a treated Jurkat cell-line. Column-switching after 3.5 min, analysis by capillary LC ES–MS.

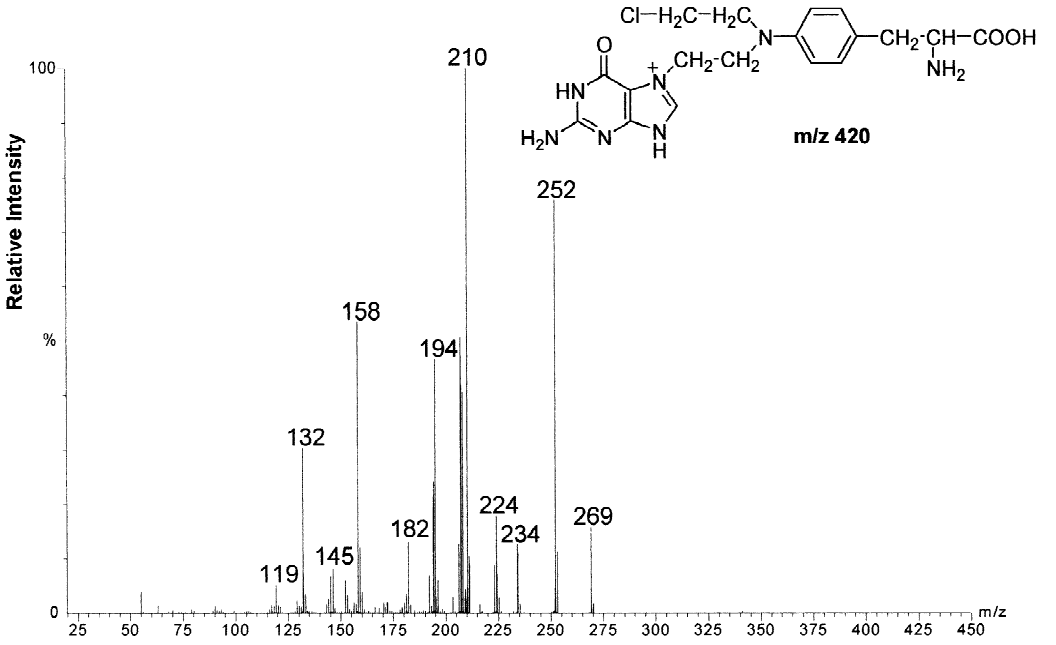
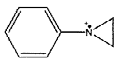


Fig. 9. Low-energy product ion scan (collision energy: 35 eV) of the $[MH]^+$ ion (m/z 420) of Gua–mel.

Table 4
Tentative structures for the product ions of of the $[MH]^+$ ion (m/z 420) of Gua–Mel

A		m/z 119

expected. This results were very similar for the other nucleoside adducts. At a slightly higher collision energy, the covalent bond between melphalan and the heterocyclic moiety was broken (20 eV) and product ions at m/z 269, derived from the melphalan, and an ion representing the free heterocyclic base (m/z 252 for Gua) were found. At a higher collision energy (30 eV), the product ions were explained by further fragmentation of the melphalan side-chain and the free base moiety. However, this fragmentation could not pinpoint the exact alkylation site of the melphalan moiety. Furthermore it is worthwhile mentioning that the product ion of the different base-adducts showed great similarity (except m/z 138 for dCyd–mel). This indicates that the product ions found at m/z 269, 252, 234, 224, 210, 194, 182, 158, 145, 132 and 119 were due to fragmentation of the melphalan side-chain since this is the only common part in all adducts. Experimental proof for the presence of these fragments is given in Table 5.

We already mentioned that at a collision energy >25 eV, a great resemblance in the product ion spectra of the different melphalan adducts was observed. This is explained by the rupture of the covalent bond between the melphalan side chain and the heterocyclic moiety. However, one major difference was observed in the product ion spectrum of dCyd–mel–Cl compared to the spectra of the other nucleoside adducts. A specific product ion at m/z 138 (deuterated m/z 140) was observed. This could be explained by the partial loss of the melphalan side-chain as explained in Fig. 11. Alkylation position is possible at the N_3- or the N^4-position of the cytosine moiety.

3.5. Cross-link adducts of melphalan

Although generally accepted to be present in the reaction mixtures of nitrogen mustards with nucleos-(t)ides or DNA, cross-linked adducts could not be detected in the experiments mentioned above. These are surprising results since LC–MSMS provides both the sensitivity and structure information needed for the positive identification of these products. In search for cross-linked adducts, we tried to synthesize a di-alkylated adduct of melphalan with 2′-deoxyguanosine according to the method of Vu et al. [21] in order to test its behaviour in our developed

Table 5
Experimental data of the product ions of Gua–Mel

	Experimental (Da)	Theoretical (Da)	*D* (mDa)	*D* (ppm)	Deuterated (**)	Elemental composition
A	119,0778	119,0778	4.3	36.1	119	$C_8H_9N^{+\cdot}$
B	132,0797	132,0797	1.6	12.3	133	$C_9H_{10}N^+$
C	145,0883	145,0883	0.8	5.9	145	$C_{10}H_{11}N^{+\cdot}$
D	158,0965	158,0965	0.5	3.0	158	$C_{11}H_{12}N^+$
E	182,0730	182,0730	0.7	3.6	182	$C_{10}H_{13}N^+$
F	194,0720	194,0720	1.7	8.5	194	$C_{11}H_{13}NCl^+$
G	210,0666	210,0666	2.0	9.4	210	$C_{11}H_{13}NOCl^+$
H	224,0833	224,0833	0.9	4.1	224	$C_{12}H_{15}NOCl^+$
I	234,0687	234,0687	0.1	0.6	234	$C_{13}H_{13}NOCl^+$
J	252,0775	252,0775	1.6	6.5	253	$C_{13}H_{15}NO_2Cl^+$
K	269,1055	269,1055	0.2	0.7	272	$C_{13}H_{18}N_2O_2Cl^+$
L	Lockmass	420,1551	–	–	427	$C_{18}H_{23}N_70_3Cl^+$

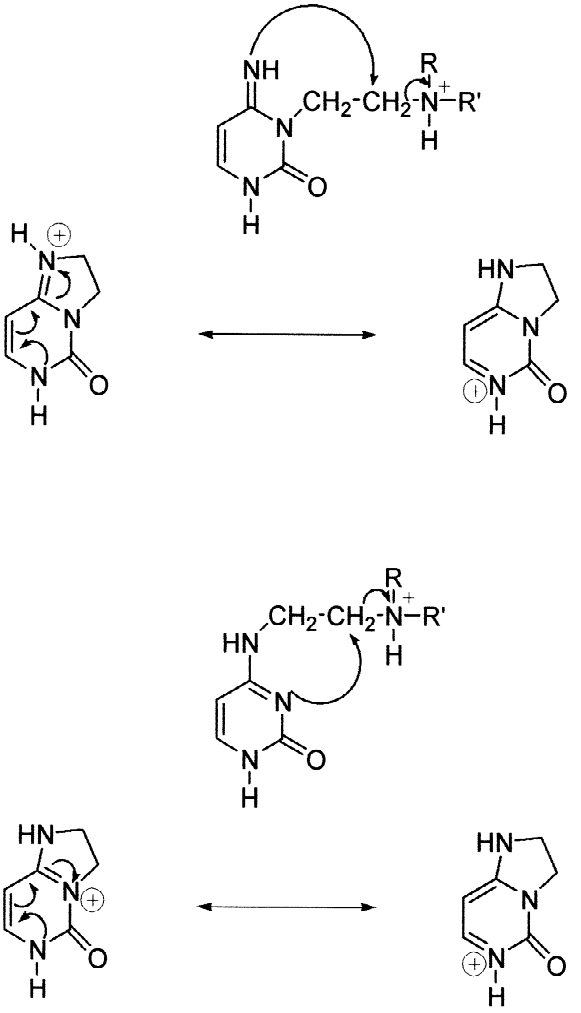


Fig. 11. Explanation of the presence of a product ion with m/z 138 in dCyd–mel–Cl with alkylation at either the N_3 or the N^4 position.

CP–dGuo, Gua–CP–dGuo and Gua–CP–Gua. These results indicate that cross-linking does not seem to be a straightforward feature for every type of nitrogen-mustard. According to Vu et al. [21], using the same procedure, cross-link adducts could be obtained for phosphoramide mustard. The lack of detection of cross-linked products in our melphalan-treated cell cultures or in calf thymus DNA might therefore be explained by the absence of these cross-linked adducts.

4. Discussion

We have shown that the developed miniaturized LC ES–MS system in combination with a column-switching technique is capable of detecting melphalan–DNA adducts in in vitro as well as in cell culture samples. The analytical method developed for the analysis of adducts in in vitro mixtures can be used without any modification in the study of in vivo formed adducts. No changes had to be made to the chromatographic and mass spectrometric conditions when analysing these two types of samples. Struc-

Table 6
Occurrence of mono- and di-alkylation adducts for different nitrogen mustards in their in vitro reaction with dGuo and dGMP

Adducts for	Mono-alkylation	Cross-link
Mechloroethamine	Yes	No
Melphalan	Yes	No
Cyclophosphamide	No	No
Phosphoramide mustard [21]	Yes	Yes

tures were assigned by recording low-energy CAD spectra and by interpreting data obtained from accurate mass measurements. Using capillary LC ES−MS only monoalkylated adducts were found. Cross-linked adducts could not be detected. In the future we will optimise a nano-LC–ES−MS(−MS) technique with column-switching to improve the sensitivity of the method. The method described in this paper and the more sensitive nano-LC ES−MS method will be evaluated for the detection of melphalan adducts formed in rats and in human blood from cancer patients, treated with melphalan.

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