

Isolation of the Adducts of Platinum Complexes and Nucleic Acid Bases on the Dowex 50 W Column

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The method presented here enables the isolation of three monofunctional adducts. These are: platination of cytosine, adenine and guanosine. The three detected bifunctional complexes were: guanine N7 to N7, adenine N7 to N7 and adenine N7 to N1. A mixed bifunctional complex of guanine and adenosine and the product of polymerization of three adenines and two moieties of *cis* DDP were also detected.

In the peak eluted separately from the guanine standard, no Pt was detected. The sample eluted in this peak had UV spectrum different from the standard and may represent the degraded product of guanine and polymerized Pt.

Introduction

cis-Diamminedichloroplatinum [II] (*cis*-DDP) is currently used in the treatment of human malignancies. The binding of *cis* DDP to cellular DNA is generally considered to be the major mechanism involved in its antitumor activity [1]. There has been an increasing evidence that guanine is the preferred site of reaction although a slow reaction with adenine and cytosine [1–5] also occurs. However, there are still controversies which of the possible platinated complexes are potentially most lethal to a cell [5, 6]. It is particularly important to elaborate a satisfactory method for the separation and characterization of various derivatives of the reaction between Pt compounds and DNA. Recently Eastman [7] presented the system for separation of platinated deoxynucleosides on HPLC and characterized them by NMR.

Liberation of these adducts from DNA required enzymatic digestion [7]. However, this digestion may be complicated by the refractory nature of DNA in the vicinity of the adducts [8, 9].

Recently several authors reported the use of acid hydrolysis procedure to release the Pt-nucleic acid bases adducts from DNA or *d*-nucleosides [3, 4, 10–13]. The hydrolysis caused a cleavage of the glycosidic bond but still left Pt bond in the same stoichiometric ratio to the base [10].

In the method presented here acid hydrolysis was used for the liberation of Pt-nucleic acid bases adducts from their nucleosides and the adducts were separated by means of cation exchange chromatography on Dowex 50 W columns. We also presented UV characteristics of the adducts.

Materials and Methods

All deoxyribonucleosides (*d*-nucleosides) and *cis* DDP were purchased from Sigma Chemical Company. *Trans* DDP (NSC 131 558) was a product of National Cancer Institute.

Trans- and *cis*-DDP were dissolved in 0.05 M NaClO₄ (pH 6.4) and allowed to hydrolyze for 72 h at 37 °C.

D-nucleosides were dissolved in the same solution and mixed with Pt-compound to obtain Pt/*d*-nucleoside ratio (*r*_i) 1 or 0.5. The mixtures were then incubated for 24 h at 37 °C. When the reaction had been completed the purine nucleosides were hydrolyzed with 0.5 M HCl for 30 min at 100 °C. *D*-thymine and *d*-cytosine were hydrolyzed for 2 h with 5 M HClO₄ at 100 °C and then neutralized with NaOH. The Dowex 50 W $\times$ 2/200 $\times$ 400 mesh/(Fluka) columns were applied to separate the bases and derivatives. The bases were loaded on a column (20 $\times$ 1 cm) and eluted by a gradient of 0.75–3 M HCl at a flow rate of 0.5 ml/min. For isolation platinated derivatives of purine *d*-nucleosides Sephadex G-25 columns were used [14].

Five ml fractions were collected. After evaporation and mineralization of samples, platinum concentrations were measured by the stannous chloride method, determining absorption at 405 nm [15]. The exact amounts of Pt and bases were calculated from standard curves and PT (base ratios, r_b) were counted.

Results and Discussion

Ion exchange chromatography is an useful method to isolate, nucleic acid bases from their alkylated derivatives [16]. We applied chromatography on Dowex column to isolate platinated derivatives of nucleic acid bases.

Standard of deoxyadenosine hydrolized in HCl was eluted from the Dowex column in a sharp single peak. After the reaction of deoxyadenosine and *cis* DDP ($r_i = 1$) the chromatographic profile shown on Fig. 1 contains five peaks.

Peak II corresponds to standard of adenine. No Pt was detected in this peak. Four additional peaks were associated with Pt presence.

To determine the stoichiometries of the products eluted in these peaks the absorbance at 260 nm and quantity of Pt measured by Marchenko method [15] were analyzed. The calculated molar ratios (r_b) of Pt adenine for peaks I, III, IV and V were 0.7, 1.07, 0.47 and 0.52 respectively.

Therefore peak III represents monofunctional adduct of Pt and adenine while peaks IV and V the bifunctional complexes. The reaction with the excess of the nucleoside ($r_i = 0.5$) resulted in the elution of only three additional peaks (I, IV and V)

what in turn confirms the elution of monofunctional complex in peak III. It has been reported that preferred binding sites for *cis* DDP in adenine moieties are N1 and N7 [1, 4, 5, 7]. Since under the conditions employed here we detected only one monofunctional adduct it is likely to represent the mixture of adenines platinated in both possible sites. It appears that *cis* DDP is unable to cross link two adenines, both of them through their N1 position [7]. Therefore the bifunctional complexes eluted in peak IV and V represent the adducts formed between N7 of two adenines and between N1 and N7 of adenine moieties. The adduct eluted in peak IV has $\lambda_{\max}$ at 267 nm, the same as N7 alkylated adenine, hence the complex of *cis* DDP attached to N7 positions of adenines is likely to be eluted in the peak. The complex eluted in peak V should thus represent bifunctional adduct formed between *cis* DDP and N1–N7 of adenines. It exhibits different UV spectrum with $\lambda_{\max}$ in 264 nm compared to 263 nm for N1 methylated adenine. The most unusual product of the reaction was eluted in peak I. r_b values calculated for the complex was 0.7. This product was present when equimolar ratios of deoxyadenosine and *cis* DDP were incubated and after the reaction with the excess of nucleoside. Since both *cis* DDP and adenine have two sites of reaction, this complex may be a result of polymerization in which for instance three moieties of adenine and two of *cis* DDP take part.

The porous matrix of an ion exchange resin (Dowex 50) also acts as a molecular sieve [17] so that the position of elution (before standard) may indicate that a more complex structure is eluted in this peak as well. The major product of the reaction of *cis* DDP with *d*-adenosine was eluted in peak IV. After prolonged incubation (7 days) about 60% of all platinated adenine appeared in this peak. Thus N7 position of the base seems to be the most reactive place for the reaction with *cis* DDP. It is in a good agreement with our earlier results which indicate that after interaction of DNA with *cis* DDP the major place of Pt attachment in adenine moieties is N7 position [4].

The characteristics of adenine and its platinated derivatives is presented in Fig. 2. Since UV spectra of Pt adenine complexes differ from their standard and from each other the UV description may be easily used to detect these complexes.

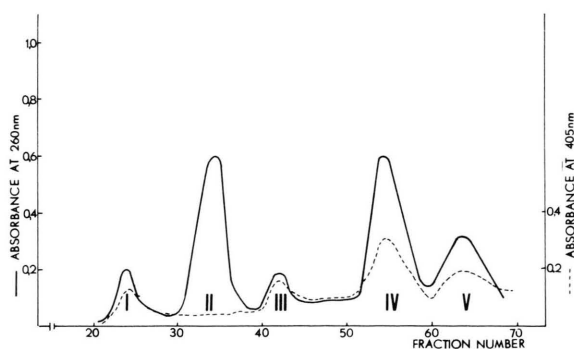


Fig. 1. Chromatography of adenine and derivatives on Dowex 50 W x 2 (200–400 mesh) after the reaction of *d*-adenosine with *cis* DDP.

When *d*-guanosine was incubated with *cis* DDP for 24 h (at *d*-guanosine/Pt ratio $r_i = 1$) the peak characteristics for the standard disappeared and yielded instead three additional peaks (Fig. 3). In two of them (peak II and III) the presence of Pt was detected. The calculated ratios of guanine (Pt/ r_b) were 1.1 and 0.53 respectively thus in peak II monofunctional adduct and in peak III bifunctional complex of the base and *cis* DDP were eluted. The formation of peak II and III was studied as a function of the reaction time ($r_i = 0.5$).

Since Cl^- ions are strong inhibitors of the reaction it is obvious that during the time course of chromatography the reaction couldn't persist. After 6 h of incubation two of the adducts were presented with almost equal quantities. After 24 h incubation peak II completely disappeared. The only product of the reaction was eluted in peak III.

These results suggest that *d*-guanosine initially reacts with *cis* DDP to form or a monofunctional adduct (which was eluted in peak II). The 1:1 adduct then interacts with another *d*-guanosine moiety to form bifunctional complex (eluted in peak III). Also the results of Marcelis *et al.* [18] pointed out that the reaction of the aquated platinum compounds with guanine moieties to form 1:1 complex was very fast. The second reaction which leads to bifunctional complex is slower [18].

Since up to now the only identified adducts of *cis* DDP and *d*-guanosine are the ones platinated at N7 position, both mono- and bifunctional complexes should be platinated at this position. The two complexes yielded UV spectra which are different from their standard (Fig. 4).

Surprisingly in the product which was eluted in peak I and had the most changed UV spectrum (Fig. 4) no Pt was detected. The lack of Pt in the product eluted in peak I may be a result of instability of the Pt-guanine complex under acid conditions of hydrolysis. As the experimental data presented here and other papers [10–13] showed that N7 guanine-Pt bonds are stable during acid hydrolysis; unstable product(s) must represent some other kind(s) of Pt attachment(s). Another possible binding site in guanine moiety is O6 [1, 5, 19, 20]. Formation of a chelate on a single guanine base with coordination at N7 and O6 has been proposed as a specific lesion only possible with *cis* DDP compounds [19, 20]. The possibility of the formation of such a N7–O6 chelate has been the subject

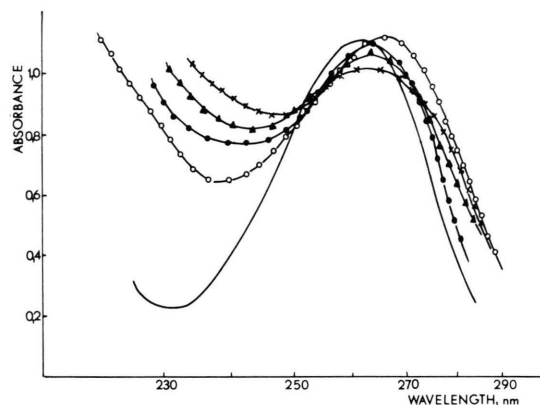


Fig. 2. UV absorption spectra of adenine and derivatives eluted from the Dowex column as individual peaks. The spectra were recorded at pH 2. peak I ▲—▲; peak II/standard of adenine —; peak III ×—×; peak IV ○—○; peak V ●—●.

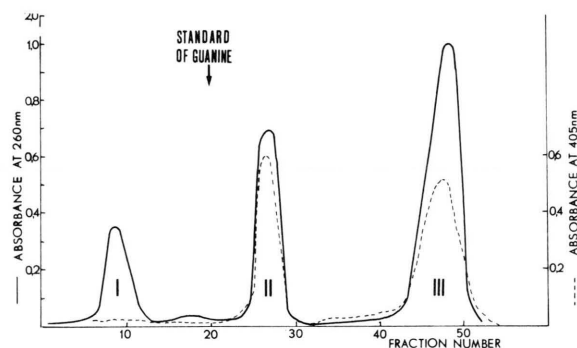


Fig. 3. Chromatography of guanine and derivatives on Dowex 50 W × 2 (200–400 mesh) after the reaction of *d*-guanosine with *cis* DDP.

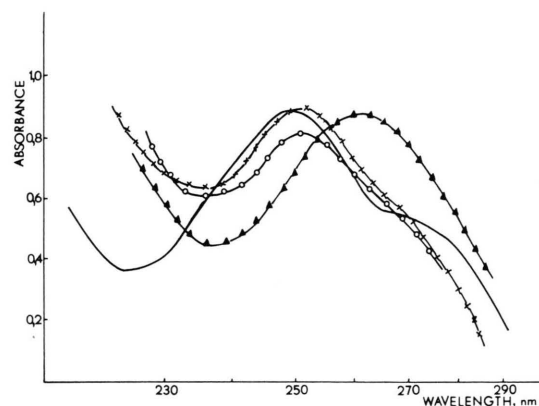


Fig. 4. UV absorption spectra of guanine and derivatives eluted from the Dowex column as individual peaks. The spectra were recorded at pH 2. peak I ▲—▲; standard of guanine —; peak II ○—○; peak III ×—×.

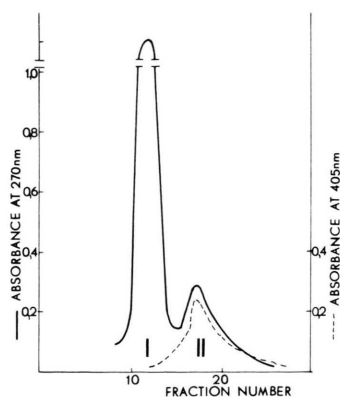


Fig. 5. Chromatography of cytosine and derivative on Dowex 50 W x 2 (200-400 mesh) after the reaction of *d*-cytidine with *cis* DDP.

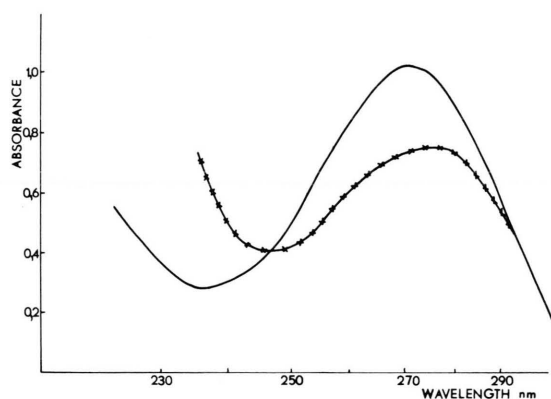


Fig. 6. UV absorption spectra of cytosine and derivative eluted from the Dowex column as individual peaks. The spectra were recorded at pH 2. peak I/standard of cytosine —; peak II x—x.

of some disputes. Several investigations have presented the results which seemed to indicate such a binding [5, 19, 20, 21]. As pointed out by others, such results do not unambiguously prove that binding occurs at the O6 atom [2, 5]. Chelation between N7 and a substituent at O6 has also been rejected on geometric grounds [5]. Therefore the product eluted in peak I is rather unlikely to represent the degradation of N7-O6 chelate. Since the peak is more distinctive in higher r_i value ($r_i = 1$) it may represent the degraded product of the reaction of *d*-guanosine with polymerized *cis* DDP. Such a complex was observed by Eastman [7].

D-cytidine was the only pyrimidine nucleoside reacting with *cis* DDP. About 6% of the loaded base was eluted after the standard in peak II following the reaction with the drug. The r_b value for peak II was 1.07 and therefore it represents monofunctional adduct. UV characteristics of the complex is presented in Fig. 6.

Similarly to Eastman [7] no bifunctional adduct of the base was detected, what may be a result of steric hindrance [7]. *D*-thymidine was completely unreactive under the conditions of our experiments.

To test the possibility of the formation of mixed bifunctional adducts the reaction with equimolar *d*-adenosine and *cis* DDP was allowed to proceed for 24 h after which time *d*-guanosine was added and the incubation was continued for a further 24 h. The result of this experiment is presented in Fig. 7. Apart from the bifunctional complexes characteristic for adenine and guanine new adduct representing A-Pt-G type of complex was detected. Further

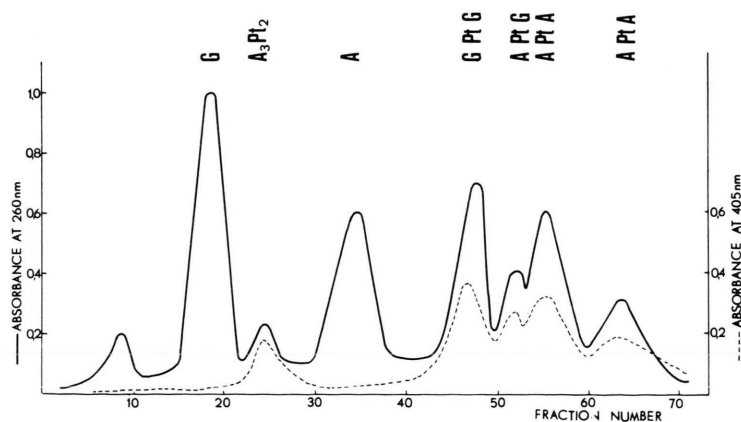


Fig. 7. Chromatography of the products resulting from the reaction of *d*-adenosine and *d*-guanosine with *cis* DDP on Dowex 50 W x 2 (200-400 mesh). For further details see description in the text.

confirmation of the character of the complex was obtained when monofunctional adducts of *cis* DDP and *d*-guanosine or *d*-adenosine, purified on Sephadex G-25 [14] were again reacted with another deoxynucleoside. When they were hydrolyzed and chromatographed on the Dowex column the same new peak which represents A-Pt-G complex appeared. As adenine has two reactive places there should exist two kinds of the mixed bifunctional complexes [7], yet we were able to detect only one peak after the reaction. However, both kinds of complexes might be eluted in this peak.

Comparative studies were performed after the reaction of the *d*-nucleosides with *trans* DDP.

The products of the reaction with *d*-guanosine were the same as with *cis* DDP, yet, the reaction with *d*-adenosine gave a different picture. No product which was eluted in peak I after the reaction of the *d*-nucleoside with *cis* DDP, was detected. The monofunctional adduct was the main product of the reaction and the two bifunctional complexes were present as well.

Alike the reaction with *cis* DDP incubation of *d*-cytidine with DDP gave rise to the monofunctional adduct.

All above characterized bifunctional and monofunctional complexes of Pt and the bases were eluted with stronger acid than their standards. This is so, since binding of platinum to nucleic acid bases caused a significant decrease in the pK_a when compared with the unmodified nucleic acid base [22].

Therefore the adducts are more positively charged and bind tightly to the column [10].

The results of the experiments reported here show that the presented method enables the isolation of different kinds of platinated derivatives of nucleic acid bases.

Studies are currently under way to apply the method for the isolation and characterization of the Pt adducts formed in DNA treated with both Pt isomers.

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