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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC INVESTIGATIONS ON THE TIME-DEPENDENT REACTION OF *cis*-$Pt(NH_3)_2Cl_2$, $Pt(en)Cl_2$ AND $Pt(pn)Cl_2$ WITH RNA FRAGMENTS

B. WENCLAWIAK*, W. KLEIBÖHMER and B. KREBS
Anorganisch Chemisches Institut der Universität, Corrensstr. 36, D-4400 Münster (F.R.G.)

SUMMARY

The reaction of three platinum compounds, $Pt(NH_3)_2Cl_2$, $Pt(en)Cl_2$ and $Pt(pn)Cl_2$ with nucleic acid fragments (Ade, Gua; GpG, GpA and ApA), both reactants (1:1, $1 \cdot 10^{-2}$ *M*; $1 \cdot 10^{-3}$ *M*) being dissolved in water, has been studied. UV measurements have been used to characterize the products. Kinetics and adduct formation in the nucleoside–Pt compound system were studied by reversed-phase high-performance liquid chromatography [gradient 0–30% methanol in 30 min, ammonium acetate buffer (0.1 *M*) at pH 4.25] at fixed intervals of time and the product formation was examined by measuring the peak heights. After 10 h, the reaction was complete for guanosine and showed a steady state for adenosine. The increasing lipophilicity in the sequence of the platinum compounds resulted in a shift to longer retention times.

INTRODUCTION

The discovery of the biological activity of *cis*-dichlorodiammineplatinum(II) (*cis*-DDP) by Rosenberg and co-workers in 1965[1,2] has led to extensive effords to understand its mechanism of action as a cancer chemotherapeutic agent. In the cell the active form of the compounds implies the aquation of one or both chloride ligands[3]. Most theories on the antitumour activity of *cis*-DDP and analogous compounds suggest that DNA is the primary target of the platinum(II) drugs[4–6]. Further, there is much evidence for a coordination to special bases of the DNA. Guanine is the preferred site of action, although a slower coordination also occurs to adenine and cytosine[7,8].

In order to obtain a better understanding of the complexity of the Pt–DNA interaction, it is essential to learn some details at a basic level, *i.e.*, the nucleosides and dinucleotides level. The bases are the fundamental building units of nucleic acids and, in this case, nitrogen is the preferred coordination atom for platinum(II)[9]. The chromatographic behaviour in solvent-generated anion exchanges of halogen-substituted *cis*-platinum compounds is known[10]. The common *cis*-dichlorodiammineplatinum(II) contains two NH_3 ligands. Both can be replaced by either one ethylenediamine (en) or one propylenediamine (pn) ligand.

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We substituted the non-halogenated NH_3 ligands by en and pn and the three synthesized Pt compounds, $Pt(NH_3)_2Cl_2$, $Pt(en)Cl_2$ and $Pt(pn)Cl_2$, were reacted with nucleosides and nucleotides. We used reversed-phase gradient liquid chromatography to separate the reaction products and in this paper we discuss the time-dependent reaction of the three platinum compounds with guanosine and adenosine. Additionally, we examined the chromatography of GpG, GpA and ApA, after reaction with *cis*-DDP and DEP. For the abbreviations used for the compounds, see Table I.

TABLE I

ABBREVIATIONS, NAMES AND FORMULAE OF COMPOUNDS

Abbreviation	*Name*	*Formula*
cis-DDP	*cis*-Dichlorodiammineplatinum(II)	$Pt(NH_3)_2Cl_2$
DEP	Dichloroethylenediamineplatinum(II)	$Pt(en)Cl_2$
DPP	Dichloropropylenediamineplatinum(II)	$Pt(pn)Cl_2$
Ade	Adenosine	$C_{10}H_{13}N_5O_4$
Gua	Guanosine	$C_{10}H_{13}N_5O_4$
GpG	Guanylyl-(3'–5')guanosine	$C_{20}H_{25}N_{10}O_{12}P$
GpA	Guanylyl-(3'–5')adenosine	$C_{20}H_{25}N_{10}O_{11}P$
ApA	Adenylyl-(3'–5')adenosine	$C_{20}H_{25}N_{10}O_{10}P$

EXPERIMENTAL

Apparatus

The module chromatograph comprised of two Altex 100 pumps, a Model 420 Altex gradient programmer, a Uvikon 720 LC variable-wavelength detector with a 2.8-μl, 10 mm path length flow-through cell and a Uvikon recorder. The 5-μl syringe injections were made with a Rheodyne 7126 injection valve fitted with a 20-μl loop.

A column (250 × 4.6 mm I.D.) was slurry packed with 5-μm Polygosil 60 C18 (Machery, Nagel & Co., Düren, F.R.G.). The gradient was 0–30% methanol within 30 min, with a further 10 min at 30% methanol, in an ammonium acetate buffer (0.1 *M*) at a flow-rate of 0.5 ml/min. Additionally we used a gradient 0–60% methanol within 30 min.

The chromatograms were recorded at 254 nm. For the determination of the λ_{max} values characterizing the peaks the detector was used in the scan mode (450–200 nm). The flow was stopped at the top of the peak and the scan was recorded at a chart speed of 10 cm per 100 nm. All UV spectra were baseline corrected.

Reagents

Analytical-reagent grade chemicals and quartz doubly distilled water were used. *Cis*-DDP and DEP were synthesized according to Dhara's method[11]; DPP was synthesized via propylene hydrochloride[12]. Gua, Ade, GpG, GpA and ApA were purchased from Sigma Chemie, Munich, F.R.G.

1-l volume of 0.1 *M* ammonium acetate solution was adjusted with concentrated acetic acid to pH 4.25, which was commonly used for our chromatographic experiments; for measuring the pH dependence of the retention time, the values were set at 5.0 and 5.6, respectively.

In a solution of 10 ml of water and 0.2 mmol sodium perchlorate equimolar ratios of nucleoside–Pt compound (0.1 mmol) or nucleotide–Pt compound (0.01 mmol)[13] were allowed to react in dark vials in a thermostated water-bath (37°C), while the solution was stirred constantly. The dinucleotides reacted for 15 h before being injected.

To investigate the time-dependent formation of Ade/Gua–Pt compounds we injected 5 μl of the reaction solution every hour. The heights of the peaks on the chromatograms determine the corresponding amounts of products formed; the ratios of peak height and reaction time were taken as the basis of the kinetic curves.

RESULTS

Nucleosides and Pt compounds

The chromatograms after a reaction time of 10 h are shown in Fig. 1 (Ade–*cis*-DDP/–DEP/–DPP) and Fig. 2 (Gua–*cis*-DDP/–DEP/–DPP). The chromatograms with a gradient of 0–30% methanol did not differ from those with a gradient of 0–60% methanol, *i.e.*, no further peaks were eluted. With adenosine five peaks were obtained, for the products (I–IV) and the unreacted adenosine. These measurements are in agreement with those of Eastman[13], who characterized the products I–IV as $AdePt_2$ (I), AdePt (II,III) and AdePtAde (IV). AdePt (II) and AdePt (III) are the monoadducts, peak II representing a coordination to the N-1 (atom) of adenosine and peak III representing a coordination to N-7. Ade_2Pt (IV) includes three possibilities of coordination (N-7–N-7, N-7–N-1 = N-1–N-7, N-1–N-1), two of which are indicated with Ade–*cis*-DDP. Coordination of N-7 is slightly more preferred than

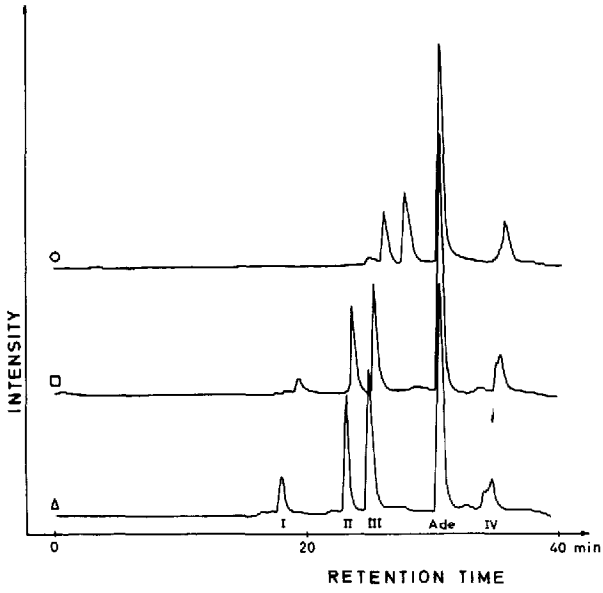

Fig. 1. Reversed-phase gradient chromatogram of adenosine after reaction with *cis*-dichlorodiammineplatinum(II) (△), dichloroethylenediamineplatinum(II) (□) and dichloropropylenediamineplatinum(II) (○) for 10 h at 37°C.

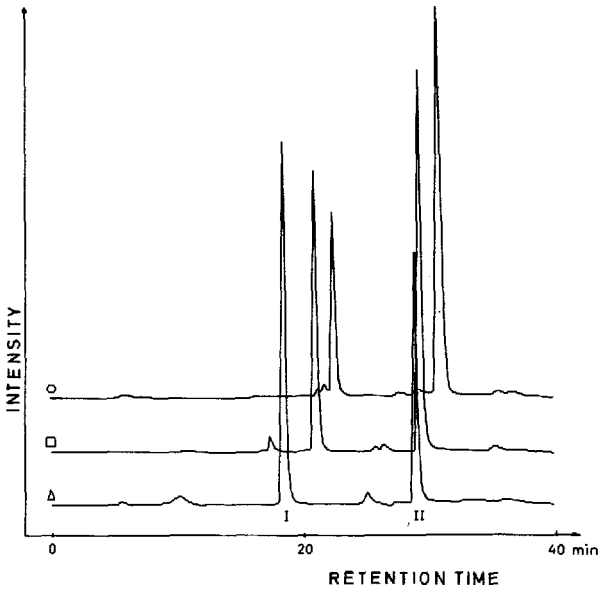

Fig. 2. Reversed-phase gradient chromatogram of guanosine after reaction with *cis*-dichlorodiammineplatinum(II) (△), dichloroethylenediamineplatinum(II) (□) and dichloropropylenediamineplatinum(II) (○) for 10 h at 37°C.

shown in the chromatogram: detection at 254 nm scales down the intensities, especially for AdePt (III) which has its maximum absorption at 268 nm.

After *ca.* 10 h the presence of adenosine indicates a seemingly incomplete reaction, but the adduct concentration remains almost constant. We did not observe an increasing peak height with longer reaction times, *i.e.*, the reaction is close to equilibrium conditions. The chromatograms of the reaction solutions of guanosine with the three Pt compounds showed in each instance that the reaction is complete after 10 h; no starting material could be measured. We therefore used an incubation

TABLE II

PEAK IDENTIFICATION AT pH 4.25

λ_{max} (nm) of each peak obtained by peak scanning under stopped-flow conditions (as described in the text). Peak numbers are identical with the numbers in the chromatograms. Pure guanosine at the pH used (4.25) showed an absorption maximum at 254 nm.

Type	*Compound*	*Peak*				
		I	*II*	*III*	*Ade*	*IV*
Adenosine–Pt compound	*cis*-DDP	268	260	268	260	260
	DEP	–	258	268	260	261
	DPP	–	262	266	260	–
Guanosine–Pt compound	*cis*-DDP	258	259			
	DEP	258	260			
	DPP	258	259			

time of 10 h at 37°C. With DEP and DPP the retention times, the peak II:III ratios and UV absorptions correspond to the adducts of *cis*-DDP (Table II).

For adenosine and guanosine a stronger shift of the monoadduct peaks was noted than for the biadduct peaks of *cis*-DDP, DEP and DPP. Regarding *cis*-DDP, DEP and DPP as a sequence, we observed that the peaks of products of adenosine and guanosine are shifted to longer retention times if the platinum compound is chelated to diamines. The shift may be caused by the greater lipophilicity of DPP compared with DEP and, certainly, with *cis*-DDP.

Kinetics of nucleosides and Pt compounds

A large difference in the kinetics between the Pt adducts of Gua and those of Ade was noted. First, under the chosen conditions guanosine reacts faster and more completely than adenosine with all Pt compounds, and second all Pt compounds form the monoadduct faster with guanosine than with adenosine. The better solubility of *cis*-DDP in water may be one reason for its accelerated reaction with nucleosides, another reason could be the S_n2 transition states for replacing the first ligand (see curve △ in Figs. 1 and 2).

For the formation of bis-adducts the two-step reaction is greatly favoured, whereas the mono-adducts are formed earlier in higher yield. This was confirmed by NMR spectroscopy[14] and by our chromatographic experiments. The yield of mono-adducts decreases (Fig. 3, I; Fig. 4, II and III) in the sequence *cis*-DDP > DEP > DPP, whereas the opposite sequence occurs for the bis-adducts (Fig. 3, II; Fig. 4, IV).

The mono-adducts of *cis*-DDP and Gua are formed so rapidly and in such high yields that the bis-adduct formation is restricted for lack of excess of Gua. In all instances mono-adduct formation with DEP > DPP proceeds more slowly, which can be explained by the lower solubility of the platinum compounds. All bis-adducts of Gua and Ade with DEP and DPP are formed as fast as with *cis*-DDP.

Influence of pH on retention

We investigated the influence of pH on the retention time. Increasing pH values (5.0, 5.6) moved the peaks to higher retention times, but we could find no special

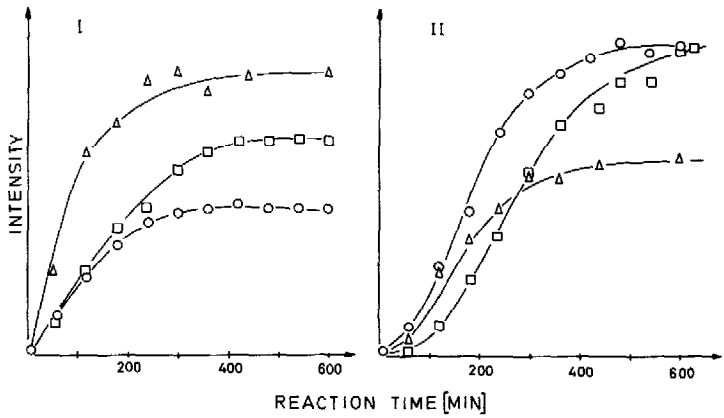

Fig. 3. Peak heights of guanosine–Pt compounds correlated with the reaction time. I, GuaPt; II, GuaPt-Gua. △, *cis*-DDP + guanosine; □, DEP + guanosine; ○, DPP + guanosine.

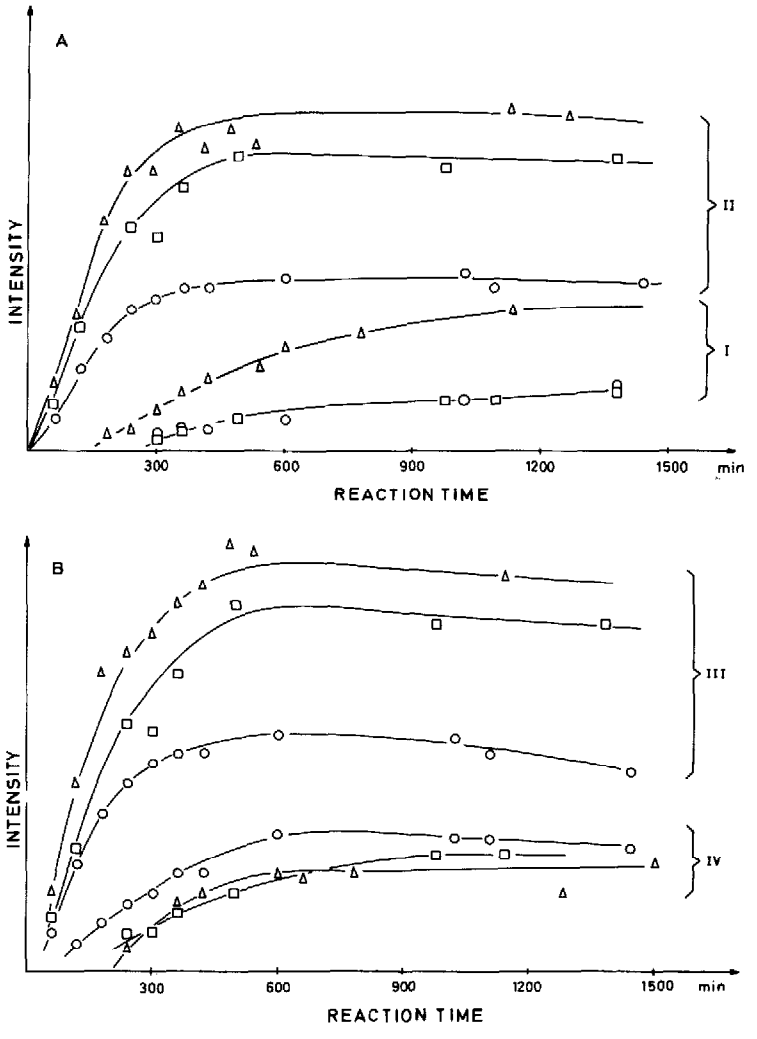

Fig. 4. Peak heights of adenosine–Pt compounds correlated with the reaction time (scaled up about 4 times). △, *cis*-DDP + adenosine; □, DEP + adenosine; ○, DPP + adenosine. (A) I, $AdePt_2$; II, AdePt. (B) III, AdePt; IV, AdePtAde.

shift for the three different platinum(II) adducts or for adenosine or guanosine. At the lower pH level (4.25) of the eluent a better peak resolution was obtained but no influence on the products was observed.

Dinucleotides and cis-DDP and DEP

The three dinucleotides ApA, GpA and GpG were chromatographed after reaction with *cis*-DDP and DEP. For the nucleic acid bases, being natural multi-site ligands, we found a number of product peaks, as expected. ApA, which contains adenine with two well established coordination nitrogens (N-1 and N-7), after its reaction with *cis*-DDP and DEP showed the largest number of peaks, as expected.

Substitution of one adenine by guanine, which prefers only N-7 coordination, changed the variety of products, whereas only one main product was formed in the presence of GpG.

The kinetics and retention times of the dinucleotides are comparable to those of the nucleosides. After a reaction time of 10 h, the chromatogram of the reaction mixture of GpG and *cis*-DDP showed one (early) eluted peak and no starting material was left. The completeness of the reaction is similar to that observed with Gua, whereas the reactions with ApA and GpA showed different behaviour: here some unreacted starting material could be observed.

CONCLUSION

We have observed the kinetics and the chromatographic behaviour in a sequence of adducts of the Pt(II) compounds *cis*-DDP, DEP and DPP with nucleosides and nucleotides. The investigation of the kinetics with the selected reaction mixture (1:1 ratio) demonstrates one important application of LC. It is possible, in one experiment, to obtain quantitative information about the adducts and about several products. Considering the results of our kinetic experiments we propose that a reaction time of 10 h is sufficient. Decreasing adenosine concentration does not explain the completeness of the reaction, because a longer reaction time does not improve the yields. The increasing lipophilicity of adducts of Gua and Ade with *cis*-DDP, DEP and DPP can be deduced from a shift to longer retention times. At pH 4.25 sharper peaks and shorter retention times were observed than at pH 5.0 or 5.6.

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