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High-performance liquid chromatographic–mass spectrometric analysis of *cis*-dichlorodiamineplatinum–DNA complexes using an ionspray interface

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ABSTRACT

An efficient RP-HPLC separation technique was used in combination with mass spectrometric detection with an ionspray ionization source to analyse complexes between nucleosides and *cis*-dichlorodiamineplatinum(II). Conventional detection techniques (UV and atomic absorption spectrometry) were also used as starting points for the setting-up of this HPLC–MS approach. The method was developed using complexes obtained either by reaction of free deoxynucleosides with *cis*-dichlorodiamineplatinum or by reaction *in vitro* of DNA samples with the same drug. DNA samples before HPLC–MS were completely depolymerized by digestion with nuclease P1 and alkaline phosphatase, in order easily to separate and determine the complexes formed. The sensitivity obtained makes this technique very suitable for future application in biological studies. The detection level, defined as the detector response with a signal-to-noise ratio of 2, corresponds to 2 pmol injected. In DNA samples treated with *cis*-dichlorodiamineplatinum, a series of *cis*-dichlorodiamineplatinum–deoxynucleoside complexes not previously described were also detected.

INTRODUCTION

Several platinum derivatives have cytotoxic activity. Among others, *cis*-dichlorodiamineplatinum (II) (CDDP) is one of the most effective drugs in cancer therapy [1] and the theory of its mechanism of action suggests that DNA is its main intracellular target [2]. NMR studies of the *in vitro* reaction between CDDP and DNA have shown [3] that deoxyguanosine (dGua) and deoxyadenosine (dAde) are the nucleosides involved in the adduct formation. Fig. 1 shows the structures of the possible complexes.

The sensitivity of the NMR technique was far too inadequate, however, to study *in vivo* situations. In fact, in order to reach an adequate sensitivity for the study of DNA platination in biological samples, mainly alkaline elution [4], atomic absorption spectrometry (AAS) [5] and immunochemical techniques [6] have been used.

The biological effects of platinum derivatives are different and depend on the structure of the complexes with nucleosides that can be formed. None of

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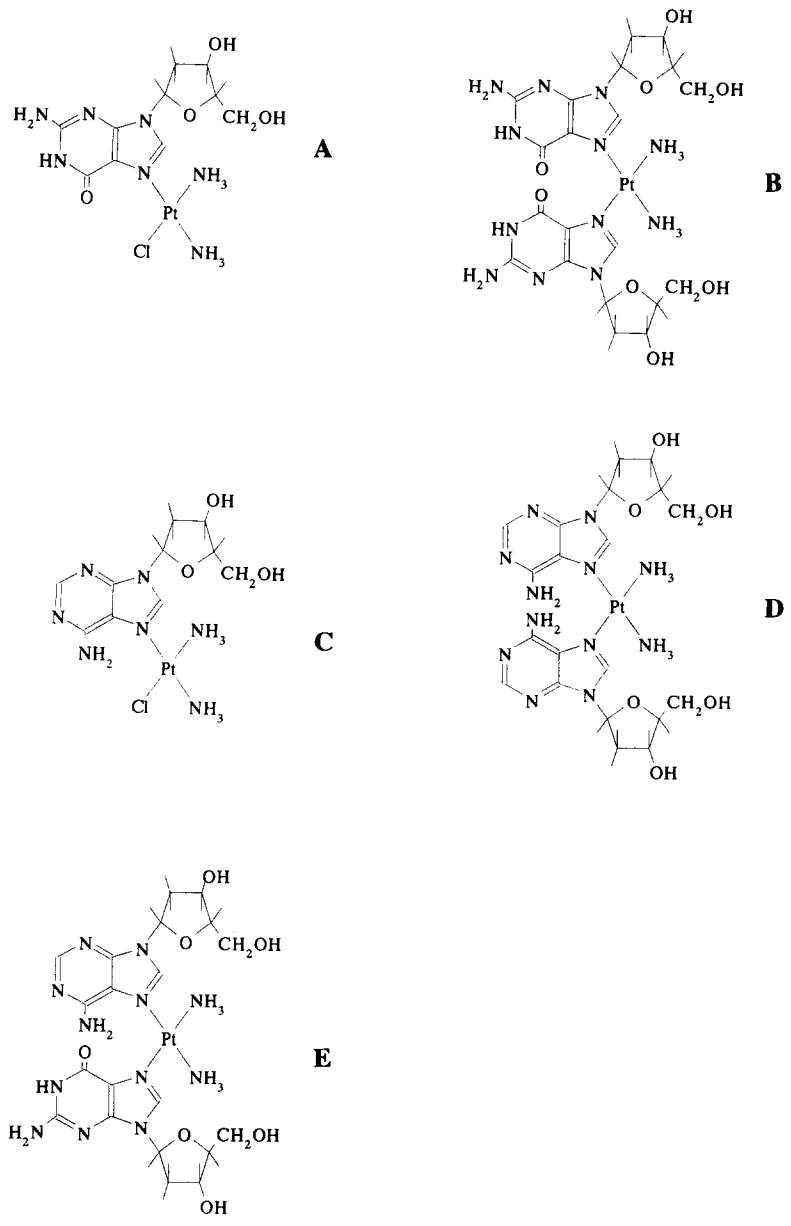


Fig. 1. Structures of the known nucleoside–CDDP complexes. A = dGua–CDDP; B = dGua–CDDP–dGua; C = dAde(N-7)–CDDP; D = dAde–CDDP–dAde; E = dGua–CDDP–dAde.

the aforementioned techniques, except the immunochemical type, allow a complete identification of the complexes present in the cells. Unfortunately, the immunochemical techniques are not of general application because they are tailored for the specific platinum compound under study, so they cannot be easily adapted to investigate different platinum derivatives with different antigenic properties. Since, at present, one of the main tasks of pharmacological research is to carry out studies on new cytotoxic

anticancer drugs, there is a need for a technique of general applicability and high sensitivity.

Mass spectrometry (MS) is a sensitive technique that is able to provide relevant structural information and, at the same time, flexible enough to be used in many different analytical situations. Therefore, in combination with an efficient separation mode, it may play an important role in extending to biological samples the study of the interactions between nucleosides and platinum compounds. The aim of this work was to check the possibilities of this technique by studying under *in vitro* conditions the products of the reaction between CDDP and DNA.

The efficiency of reversed-phase HPLC for the separation of the various complexes of CDDP with nucleosides has already been reported [7]. In previous work [8] we adopted, in the analysis of such complexes, an HPLC–MS technique using a plasma thermospray interface, but poor results were obtained. In this study we have combined a microbore RP-HPLC separation procedure and mass spectrometric detection with a pneumatically assisted ionspray ionization source, particularly suitable for polar compounds. In this way we obtained structural information about various CDDP–nucleoside complexes of different stoichiometry and composition. Structures already known were confirmed and at the same time some evidence for the presence of new complexes was obtained.

EXPERIMENTAL

Chemicals

All the reagents of the purest grade available and HPLC-grade acetonitrile were purchased from Fluka, except nuclease P1, which was obtained from Pharmacia. Ultrapure water was prepared with a Milli-Q purification system (Millipore).

Synthesis of nucleoside–CDDP complexes

To obtain complexes of CDDP with dGua and dAde, 10.0 μmol of each nucleoside were reacted separately for 24 h at 37°C in the dark with an equimolar amount of CDDP in 2.5 ml of distilled water. To synthesize the complexes of CDDP with both nucleosides (dGua–CDDP–dAde), 5.0 μmol each of the two nucleosides were combined and made react with 1.0 μmol of CDDP, dissolved in 2.5 ml of water, for 24 h at 37°C in the dark.

Synthesis of platinated DNA

A 1.0-mg amount of extractive DNA from calf thymus was incubated for 24 h at 37°C in the dark dissolved in 2.5 ml of distilled water containing 1.0 mg of CDDP.

Chromatographic separations

Separations were performed with an Applied Biosystems Model 140A syringe pump HPLC system and a Perkin-Elmer ISS-101 autosampler. UV detection was performed at 260 nm with an Applied Biosystems 1000S diode-array detector and the data were treated with a Varian 4270 computing integrator. When necessary, fraction collections were performed using a FRAC 100 fraction collector (Pharmacia). Separations were performed with a reversed-phase column [Hypersil MOS, 5 μm, 25 cm × 1 mm I.D. (Shandon)]. The mobile phase was 0.05 *M* ammonium acetate buffer (pH 4.0) (solvent A) and methanol–0.1 *M* acetate buffer (pH 4.0) (50:50) (solvent B). The elution conditions adopted were as follows: after 10.0 min of isocratic condition elution with 100% A, a linear gradient was started with a slope of 2.0% B min^{-1} up to 10% B and then 3.33% B min^{-1} up to 60% B, this final composition being maintained for 25.0 min. The flow-rate was 50 $\mu l/min^{-1}$. The samples were collected with the fraction collector just after injection in 30 fractions for 60 min.

Atomic absorption spectrometric assay

Platinum determinations by AAS were performed with a Varian SpectraAA 10 atomic absorption spectrometer equipped with a Varian GTA 96 graphite furnace. Aliquots (20.0 μl) were injected into the graphite tube and vaporized at 2700°C after ashing at 1200°C in a nitrogen atmosphere.

Enzymatic degradation of DNA samples

To the DNA samples after the reaction with CDDP were added solid NaCl up to a final concentration of 0.1 *M* and subsequently 2.5 volumes of cold ethanol. The resulting solution was kept overnight at −20°C in order to obtain complete precipitation of DNA. DNA pellets, recovered by centrifugation (20 min at 10 000 *g* in Corex tubes), before enzymatic treatment were dried under vacuum. Depolymerization of DNA was achieved by treating the samples, dissolved in 50.0 m*M* sodium acetate

buffer (pH 6.0) containing 3.0 m*M* $ZnCl_2$, with nuclease P1 (5 U for 1 mg of DNA) for 6 h at 37°C. To remove terminal phosphate groups the samples were then basified, by addition of 50.0 m*M* Tris–HCl buffer (pH 10.55) containing 3.0 m*M* $MgCl_2$, and 5 U (for 1 mg of DNA) of alkaline phosphatase were added and the incubation was continued at 37°C for 12 h. After these treatments, enzymes and residual undigested materials were removed from nucleosides by ultrafiltration with an Ultrafree-MC 10K NMWL (Millipore) with a molecular mass cut-off of 10 000 operated in a fixed-angle centrifuge (3000 *g* for 45 min).

Mass spectrometry

Mass spectra were acquired using a Perkin-Elmer–Sciex API III triple quadrupole tandem mass spectrometer equipped with an ionspray atmospheric pressure ionization source. The mobile phase flow was introduced, without splitting, to the source. Mass spectra were acquired in the positive-ion mode scanning, in a range including the expected molecular masses of nucleoside–CDDP complexes.

RESULTS

The first step in this work was a detailed HPLC study with different detection system (UV, AAS, MS) of the products of the reaction between CDDP and dGua and dAde free nucleosides.

The chromatographic separation and determination with UV detection of the products of the reaction between deoxyguanosine and CDDP is illustrated in Fig. 2. The higher peak at t_R = 11.3 min (peak 2) corresponds to the unreacted deoxyguanosine, as confirmed by injection of a pure standard of the nucleoside. The assignment of the peaks eluting at t_R = 9.7 min (peak 1) and 14.9 min (peak 3) was performed by MS detection (Fig. 2B and C). Their MS peaks corresponded to *m/z* values typical of the protonated molecular ions $[M + H]^+$ of the complexes CDDP–dGua (Fig. 1A) and dGua–CDDP–dGua (Fig. 1B). In complete agreement with these results, platinum was detected by AAS in the fractions collected from the same peaks (data not shown).

In Fig. 3 are summarized analogous results obtained analyzing the products of the reaction of de-

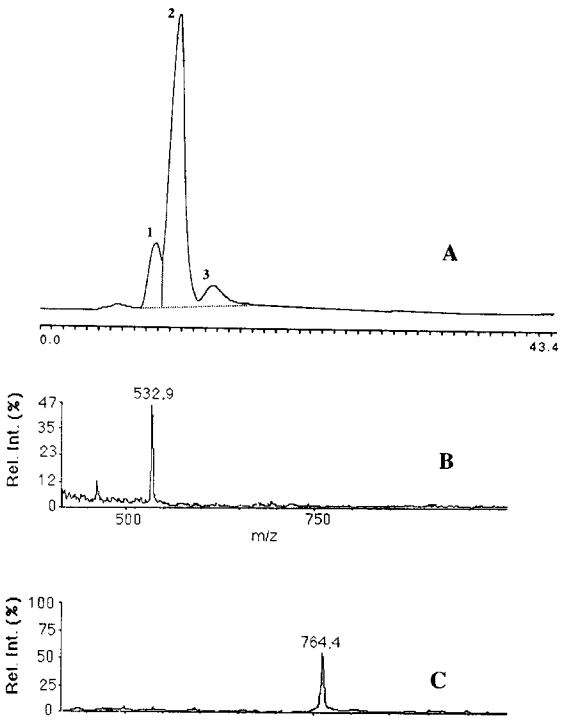


Fig. 2. (A) RP-HPLC separation with UV detection at 260 nm of a standard mixture of dGua–CDDP complexes. For chromatographic conditions, see Experimental. 1 = dGua–CDDP; 2 = free dGua; 3 = dGua–CDDP–dGua. (B) Mass spectrum of peak 1. (C) Mass spectrum of peak 3.

oxyadenosine with CDDP. The free nucleoside, as confirmed by injecting the pure standard, elutes at t_R = 18.5 min (peak 3). The first two eluting peaks in Fig. 3A (t_R = 13.3 and 14.9 min), analysed by MS (spectra reported in Fig. 3B and C), were identified as dAde–CDDP complexes, on the basis of the presence of *m/z* values corresponding to the masses of the protonated molecular ions. However, both peaks show identical mass spectra owing to the possibility of *cis*-CDDP coordinating with the nitrogen atom in either position 7 or 1 of the dAde molecule, as already proposed by Wenclawiak *et al.* [7] (Fig. 1C shows one of the possible structures). For the same reason, three different types of dAde–CDDP–dAde complex (one of them is depicted in Fig. 1D) are formed, eluting at t_R = 36.6 min (peak 4), 37.5 min (peak 5) and 38.9 min (peak 6) with identical mass spectra, exhibiting the most relevant peak at

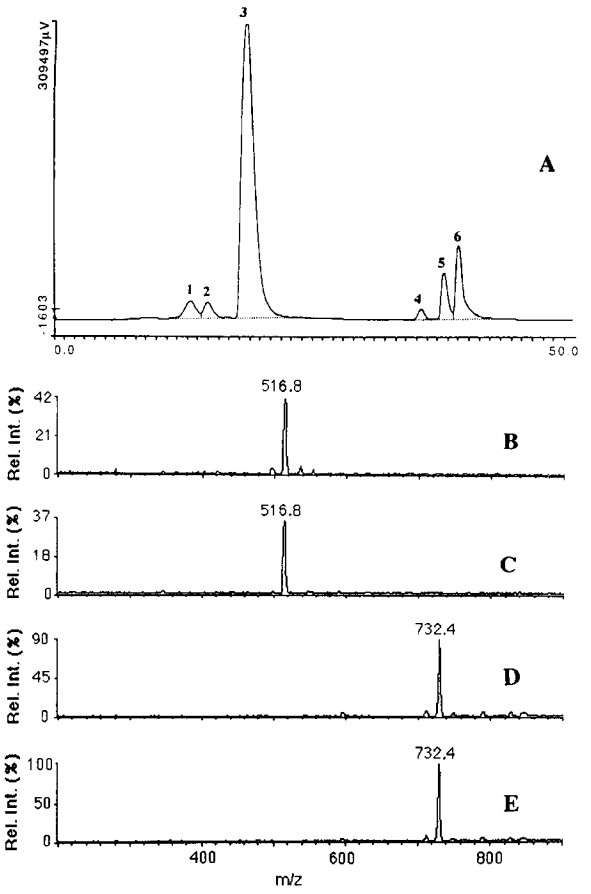


Fig. 3. (A) RP-HPLC separation with UV detection at 260 nm of a standard mixture of dAde–CDDP complexes. Chromatographic conditions as in Fig. 2. 1, 2 = dAde–CDDP complexes differing in the N atom involved in the coordination; 3 = free dAde; 4, 5, 6 = dAde–CDDP–dAde complexes differing in the N atoms involved in the coordination (N-1–N-1, N-7–N-1, N-7–N-7). (B,C) Mass spectra obtained from peaks 1 and 2; (C,D,E) Mass spectra obtained from peaks 4, 5 and 6.

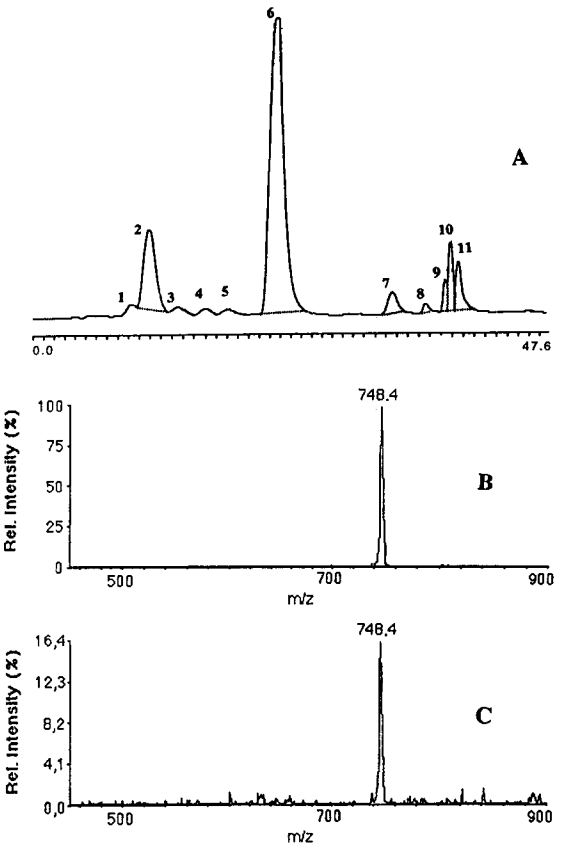


Fig. 4. (A) RP-HPLC separation with UV detection at 260 nm of the products of the reactions of dGua, dAde and CDDP. Chromatographic conditions as in Fig. 2. 1 = dGua–CDDP; 2 = free dGua; 3 = dGua = dGua–CDDP–dGua; 4 = dAde(N-1)–CDDP; 5 = dAde(N-7)–CDDP; 6 = free dAde(N-1)–CDDP-dGua; 8 = dAde(N-1)–CDDP–dAde(N-1); 9 = dAde(N-1)–CDDP–dAde(N-7); 10 = dAde(N-7)–CDDP–dGua; 11 = dAde (N-7)–CDDP–dAde(N-7). (B,C) Mass spectra of peaks 7 and 10.

the m/z value corresponding to the mass of the $[M + H]^+$ ion (Fig. 3D and E for peaks 5 and 6, respectively). As before, the AAS results (data not shown) obtained from fractions collected during this separation are in agreement with the proposed interpretation.

Fig. 4 shows the results obtained after reaction of both nucleosides with CDDP. In the UV trace (Fig. 4A) are present the chromatographic peaks previously observed and identified either as nucleoside–CDDP complexes or free nucleosides. The MS data (spectra not reported) supported this interpretation. In addition, two new peaks containing platinum (confirmed by AAS) were observed at t_R = 33.6 min (peak 7) and 38.8 min (peak 10) and identified as dAde–CDDP–dGua mixed complexes differing in the nitrogen atom (N-1 or N-7) of dAde involved in the complexation (Fig. 1E shows one of the possible cases) on the basis of the corresponding mass spectra (Fig. 4B and C) showing relevant signals at the m/z value corresponding to the $[M + H]^+$ ion.

The next step was an analogous chromatographic

study of the products of the *in vitro* reaction between CDDP and intact DNA in order to verify the type and number of complexes formed and to compare them to those examined previously.

Fig. 5A shows the separation of the digestion products obtained from a pure DNA sample and monitored by UV detection. Four large peaks can be observed at retention times comparable to deoxycitydine, deoxyguanosine, thymidine and deoxyadenosine. The slight shifts in the observed retention times are probably due to co-elution problems. Fig. 5B reports the UV trace of the chromatographic separation of the products obtained after reaction of the pure intact DNA sample with CDDP. The four major peaks corresponding to the free nucleosides are still present, but there is a decrease in those corresponding to dGua and dAde, clearly owing to their reaction with CDDP. By comparing the peak areas of the two separations it was calculated that 85.3% and 36.5% of dGua and dAde, respectively, have reacted. Some of the peaks corresponding to the complexes whose formation was previously assessed were hidden by the peaks of deoxycitydine and thymidine and several of the adducts involving dAde were not found. On the other hand, new peaks undetected in the preceding study can be observed, eluting at very long retention times.

In Fig. 5C are reported the results, obtained by AAS, for platinum contents in the fractions collected during the chromatographic run of the same sample. As expected, platinum was found in the fractions corresponding to the retention times of dGua–CDDP, dGua–CDDP–dGua and dGua–CDDP–dAde. However, large amounts of platinum were unexpectedly found either in a fraction corresponding to the elution time of dAde or in the group of the later eluting peaks, suggesting the formation of nucleoside–CDDP complexes of unknown structure.

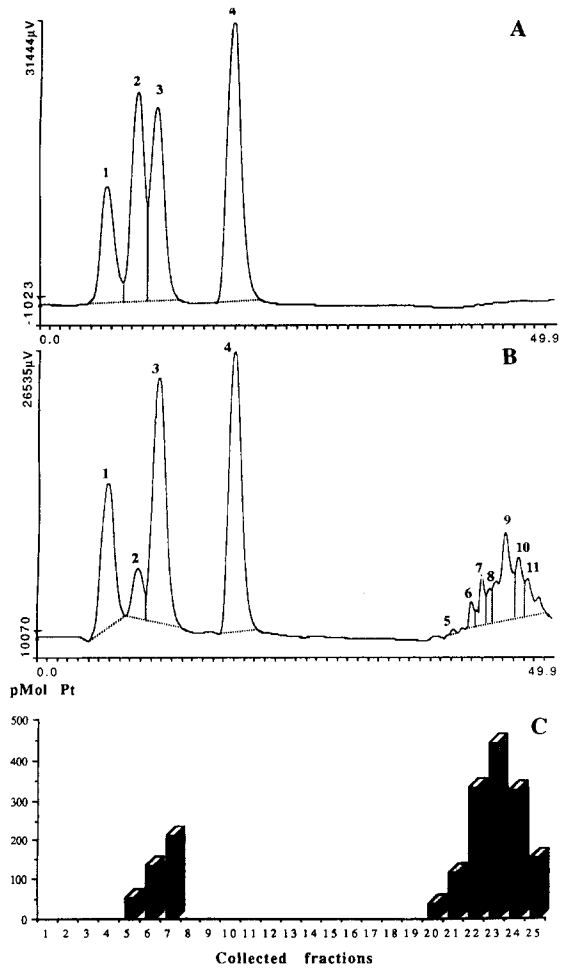


Fig. 5. (A) RP-HPLC separation with UV detection at 260 nm of a standard mixture of (1) deoxycitydine, (2) deoxyguanosine (3) thymidine and (4) deoxyadenosine. (B) Chromatographic separation under the same conditions as above of the products of reaction between pure intact DNA and CDDP. (C) Pt contents determined by AAS of the fractions collected during the run of chromatogram B.

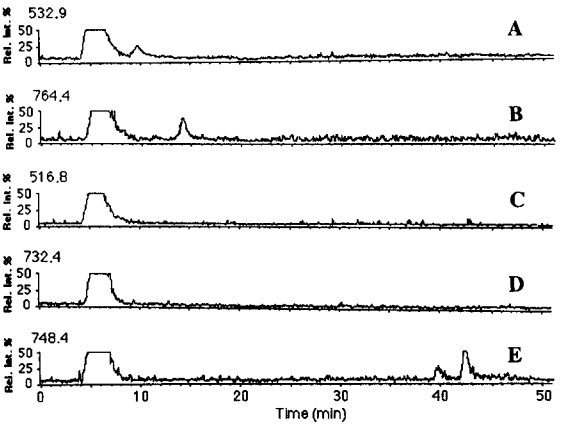


Fig. 6. Chromatogram of Fig. 5B retraced by MS detection with single-ion monitoring at the *m/z* values corresponding to the protonated ions of (A) dGua–CDDP (532.9), (B) dGua–CDDP–dGua (764.4), (C) dAde–CDDP (515.8), (D) dAde–CDDP–dAde (732.4) and (E) dAde–CDDP–dGua (748.4).

The chromatogram in Fig. 5B was retraced, with MS detection, in a number of chromatograms detected at the m/z values corresponding to the protonated ions of dGua–CDDP, dAde–CDDP, dGua–CDDP–dGua, dAde–CDDP–dAde and dGua–CDDP–dAde. Such reconstructed chromatograms are shown in Fig. 6. All of the traces exhibit a large peak, eluting at the void volume of the column, corresponding to ions produced by ionization of salts present in the sample. The chromatograms in Fig. 6A and B, detected at the m/z values of dGua–CDDP and dGua–CDDP–dGua molecular ions, respectively, show peaks with the expected retention times. In traces C and D, corresponding to ion monitoring of dAde–CDDP and dAde–CDDP–dAde complexes, respectively, no peaks can be detected according to previous studies which had already shown that these types of complexes are hardly formed in platinated DNA. Trace E, corresponding to the monitoring of dGua–

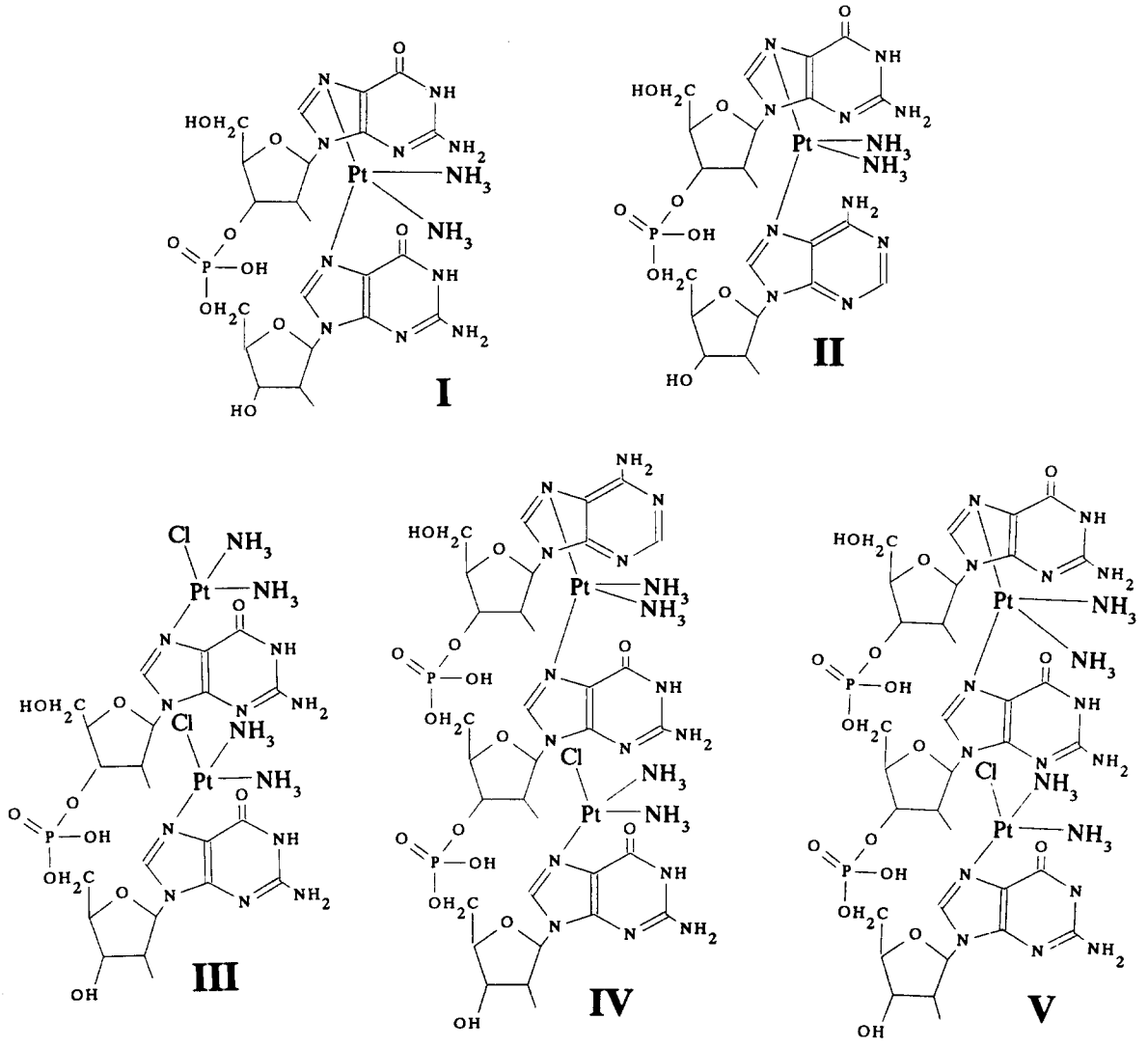


Fig. 7. Structures of possible nucleoside–CDDP complexes proposed in order to explain the presence of the last-eluting peaks of Fig. 5B containing platinum.

CDDP–dAde molecular ion, clearly shows a peak at the expected retention time along with a larger peak at a retention time corresponding to one of the later eluting substances of unknown structure.

Fig. 7 illustrates some proposed structures of possible nucleoside–CDDP complexes which could correspond to the last-eluting peaks. The formation of such molecules may be hypothesized as a consequence of the steric hindrance exerted by adjacent nucleoside–CDDP complexes on the phosphorus bonds, not allowing correct cleavage by nuclease P1. In particular, the complex

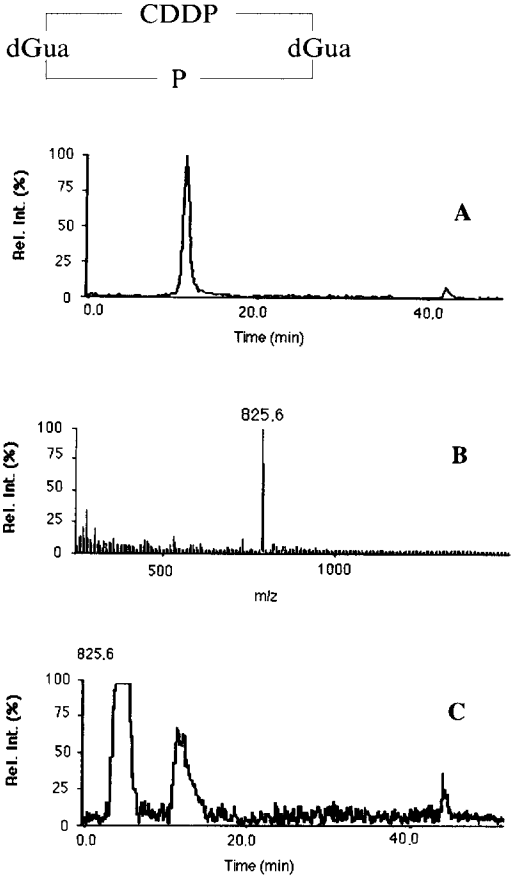
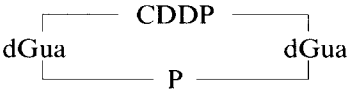


Fig. 8 (A) Chromatographic profile with MS detection (total ion current) of the products or reaction between dGua–P–dGua and CDDP. (B) Mass spectrum of the first peak of chromatogram A. (C) Chromatogram A retraced with single-ion monitoring at the m/z value corresponding to the $[M + H]^+$ ion from the complex

where –P– indicates the phosphorus bonds between the carbohydrates of the nucleosides (I, Fig. 7) is a structure already proposed [9]. We did not find this type of complex before because its formation is impossible in the reaction of free nucleosides with CDDP.

To elucidate this aspect better, 1.0 mg of synthetic dGua–P–dGua (Sigma, Rochester, NY, USA) was reacted with 1.0 mg of CDDP under the same conditions as reported for the nucleoside–CDDP reaction. In Fig. 8A is presented the chromatographic trace obtained in the total ion current mode for the product of this reaction under the same HPLC–MS conditions as described earlier. Platinum amounts were detected by AAS (data not shown) mainly in the second large peak (t_R = 12.1 min) and much less in the peak at 43.3 min. Fig. 8B shows the mass spectrum obtained from the peak at 12.1 min, characterized by an intense signal at m/z corresponding to the $[M + H]^+$ ion. As expected, in the reconstructed chromatogram at m/z 825.6 (Fig. 8C), corresponding to the complex



present in the DNA samples, two peaks are observed at retention times of 12.3 and 43.4 min, respectively.

As regards the last-eluting peaks in the chromatogram with UV detection in Fig. 5B, whose identification remained undefined at that stage of the study, an examination of their mass spectra was

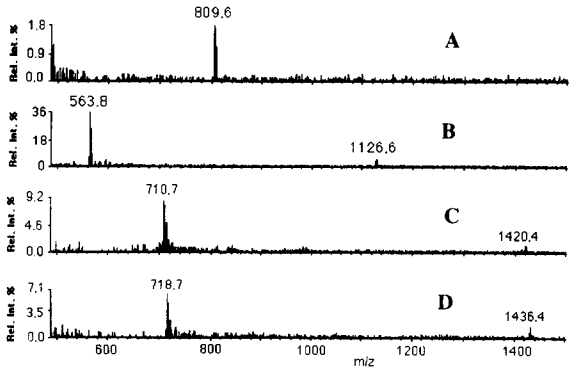


Fig. 9. Mass spectra of some of the later eluting peaks in the chromatogram in Fig. 5B. See text for explanations.

accomplished with the aim of finding significant mass spectral peaks compatible with the structures proposed in Fig. 7 (II–V). Fig. 9A shows the mass spectrum of the peak at t_R = 44.2 min. It is characterized only by an ion at m/z 809.6 corresponding to the protonated ion of the complex

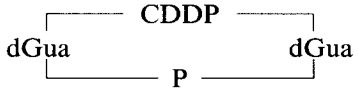
(II, Fig. 7). Also in this instance we tried to prepare such a type of complex starting from dGua–P–dAde as described previously for dGua–P–dGua. The chromatographic analysis of the product of this reaction gave several peaks whose mass spectra were similar to that reported in Fig. 9A.

The mass spectrum illustrated in Fig. 9B (peak at t_R = 47.1 min) includes two relevant ions that seem to be singly and doubly charged ions derived from the same molecule; the m/z values correspond to the hypothetical complex (III, Fig. 7) formed by two dGua–CDDP linked by a phosphorus bond. We could not obtain this type of complex from the synthetic dinucleotide and therefore we could not verify this hypothesis definitively.

The spectra presented in Fig. 9C (peak at t_R = 47.8 min) and D (peak at t_R = 48.9 min) are again characterized by mass spectral peaks due to singly and doubly charged ions. The molecular masses calculated from these two spectra may be related to complexes made up of a sequence of three nucleosides, two dGua and one dAde (M_r = 1435.4) or three dGua (M_r = 1419.4) with intact phosphorus bonds and two molecules of CDDP (IV and V, Fig. 7). Further reasonable interpretations in terms of mass spectral analysis could not be obtained. About fifteen peaks can be observed in the UV trace between 40 and 50 min and at present we can propose an interpretation for only five of them.

DISCUSSION

The conditions adopted for the chromatographic analysis provided separations of limited resolution (Fig. 5A and B). Peaks of thymidine, dGua, dGua–CDDP, dAde–CDDP,



and dGua–CDDP–dGua elute very closely, without a complete separation. However, with MS detection, the separation was good enough to obtain a reliable identification and determination of the different CDDP–nucleoside complexes and nucleosides. In fact, as regards the quantitative aspect, analyses performed with single-ion monitoring at the m/z values in Fig. 6 gave a limit of detection, defined as the detector response with a signal-to-noise ratio of 2, of 2 pmol injected for each of the complexes of defined structure analysed alone or in a DNA digestion.

By analysing the products of the reaction of free nucleosides with CDDP, all of the expected complexes were identified, and also those involving deoxyadenosine that cannot be formed by reaction inside DNA. The results achieved for DNA reacted with CDDP confirmed the findings obtained by other workers [7]; moreover, an interesting series of peaks corresponding to complexes not yet described were also found.

These results do not conflict with previous studies, as most of these complexes seems to be always dGua–and/or dAde–CDDP complexes, even if with much more complicated structures, owing to a linkage of the constituents in sequences resistant to enzyme degradation.

It is worth noting that the reaction between synthetic dGua–P–dGua and CDDP produced two products having the same mass spectra but very different chromatographic properties (Fig. 8A–C). No attempts have made to interpret these results, which seem suggest the existence of two isomers.

Some peaks could be characterized, because their mass spectra were not interpretable with reasonable combinations of CDDP, nucleosides and phosphoric acid molecules. Under our experimental conditions (molecular mass exclusion limit of the ultrafiltration step = 10 000, presence of proteic materials coming from several sources), complexation between CDDP and protein fragments may not be excluded and DNA–CDDP–protein or amino acid–CDDP complexes have been extensively described in other studies.

If these more complex structures are really present in the cells, it is possible that they can play a relevant role in determining the biological effects of CDDP because, as they are resistant to the action of nuclease P1, they can be more resistant also to the

action of the enzymes involved in the mechanisms of DNA repair. Experiments on DNA extracted from cells treated with CDDP will be necessary in order really to understand the role of these complexes. Moreover, other techniques such as NMR spectroscopy, must be used to clarify their structures.

The sensitivity of the present procedure is very satisfactory and, by comparison with the results of other workers regarding the amount of nucleoside–CDDP complexes present in cells treated with CDDP, may well be considered suitable for the analysis of biological samples. In conclusion, we believe that the present HPLC–MS analysis under the proposed conditions is a promising technique for chemical and biological studies on platinum drug–DNA interactions.

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