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Short Communication

Isolation of *cis*-$[PtCl(NH_3)_2(H_2O)](ClO_4)$, the monohydrated form of the anti-tumour drug cisplatin, using cation-exchange high-performance liquid chromatography

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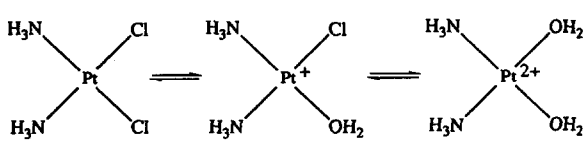
ABSTRACT

A novel procedure allowing a quantitative separation of the anti-tumour drug cisplatin and its hydration products, based on cation-exchange high-performance liquid chromatography, is presented. Thanks to this procedure, the monohydrated form of cisplatin, *cis*-$[PtCl(NH_3)_2(H_2O)]^+$, which is possibly the principal species reacting *in vivo* with DNA and thus responsible for the anti-tumour activity, could be isolated and characterized in the pure state for the first time.

INTRODUCTION

The anti-tumour drug cisplatin (*cis*-$[PtCl_2(NH_3)_2]$, **1**) binds to DNA, preferentially cross-linking GpG and ApG dinucleotides [1,2]. This selectivity is not yet well understood. Since the drug's DNA binding is under kinetic control, several groups have investigated the kinetics of the reactions of the hydrolysed, reactive forms of cisplatin, *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ (**2**) and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**), with DNA [3,4] or with oligonucleotides as models for DNA [5,6]. These studies have been hampered by the fact that **2**, which is the initial product of cisplatin hydrolysis and possibly the major species reacting with DNA *in vivo*, could not be prepared in pure form. The principal problem is the relatively rapid disproportionation of **2** in water into **1** and **3**. In this paper, we describe a method based on cation-exchange high-performance liquid chromatography that allows the separation and isolation of the monohydrated complex **2** as a frozen solution with perchlorate counterions from an aqueous solution containing **1**, **2** and **3**.



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EXPERIMENTAL

cis-$[PtCl_2(NH_3)_2]$ (**1**) was kindly provided by Rhône-Poulenc Rorer, France. Solutions of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**) were prepared by dissolving *cis*-$[Pt(NO_3)_2(NH_3)_2]$ [7] in water. Solutions of *cis*-$[PtCl_2(NH_3)_2]$ were prepared by dissolution of cisplatin in 0.1 *M* NaCl. $NaClO_4 \cdot H_2O$ and $HClO_4$ were purchased from Merck.

The HPLC system consisted of a Shimadzu LC-6A pump (Touzart & Matignon, France), connected to a Rheodyne injector with a 200-μl sample loop, and to a Spectra-Physics Focus diode array detector with a pathlength of 0.6 cm, or to a Shimadzu SPD-6A UV detector with a pathlength of 1 cm coupled to a C-R3A Shimadzu integrator.

The chromatographic conditions were optimized in initial experiments on a 5-μm Nucleosil SA column (250 × 4.6 mm I.D.) (Colochrom, France). For the subsequent separations, a 5-μm Nucleosil SA semipreparative column (250 × 7.5 mm I.D.) (Colochrom) was used. Aliquots of 20 μl (analytical column) or 200 μl (semipreparative column) of approximately 0.1 *M* solutions of the platinum complexes were injected. The mobile phase consisted of aqueous 0.25 *M* $NaClO_4$ (pH adjusted to 4.0 with 0.1 *M* perchloric acid). The flow-rate was 0.7 ml/min in analytical separations and 1.4 ml/min in preparative runs. The detection wavelengths were 302 nm, 265 nm and 254 nm on a Spectra-Physics Focus detector or 265 nm on an SPD-6A UV detector. All separations were done at room temperature.

RESULTS

When **1** is stirred with 1 equivalent of $AgNO_3$ for 24 h in water, the final solution contains an equilibrium mixture of **1**, **2** and **3**. Fig. 1 shows the chromatographic separation of such a solution. Using a semipreparative column, we quenched the fractions containing **2** in liquid nitrogen and stored them at −80°C in order to prevent redisproportionation to **1** and **3**. HPLC analyses showed that the purity of **2** was >99.8% immediately after separation and >96% after 2 weeks at −80°C.

The use of a diode array detector allowed us

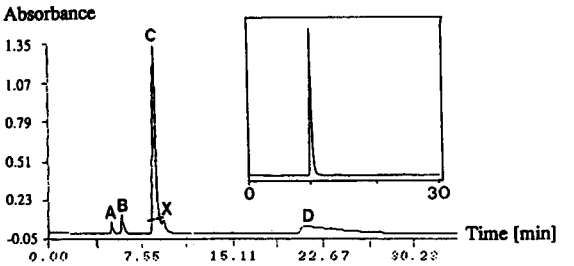

Fig. 1. Typical chromatogram of the equilibrated solution of **1**, **2** and **3** (prepared from **1** and 1 equivalent of $AgNO_3$) using UV detection at 265 nm. A = Anions (Cl^-, NO_3^-); B = **1**; C = **2**; D = **3**; X = impurity. Insert: pure **2** after separation using a semipreparative column (between the two cuts). Conditions are given in the Experimental section.

to record the absorption spectrum of each complex. The spectrum of **2**, which could be measured on a pure sample for the first time here, is shown in Fig. 2. The quantitative determination of the absorbance was carried out based on the "theoretical plates concept" [8]. According to this concept, the molar concentration of a component at the top of its peak, C_{max}, is a function of the "number of theoretical plates", N, and the injected molar fraction Q_0 [9]:

$$C_{max} = \frac{Q_0}{V_R}\sqrt{\frac{N}{2\pi}} \quad (1)$$

V_R is the retention volume, calculated as the product of the retention time t_R and the flow-rate. N is determined for each peak as $N = 5.54(t_R/\delta)^2$, δ being the half-width of the peak, measured in time units. We first recorded the chromatograms of pure authentic solutions of **1** and **3**, in order to determine precisely their quotients between peak area and concentration. Then, we analysed equilibrium mixtures of **1**, **2**

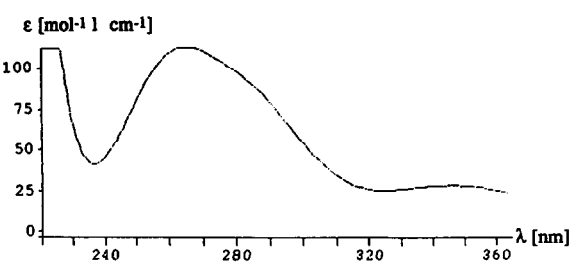

Fig. 2. UV absorption spectrum of **2** recorded at the top of its peak.

and 3 with the three detection wavelengths of 302, 265 and 254 nm, corresponding to the λ_{max} values for the first spin-allowed transition of 1, 2 and 3, respectively [10]. The chromatograms recorded at $\lambda_{max}(1)$ and $\lambda_{max}(3)$ were used to measure the molar fractions $Q_0(1)$ and $Q_0(3)$, using the quotients determined in the previous experiment. Since the sum of the molar fractions $Q_0(1) + Q_0(2) + Q_0(3)$ is equal to the total injected molar amount of platinum, $Q_0(2)$ and thus $C_{max}(2)$ can be easily calculated, giving, with the absorbance at the top of the peak of 2, the molar absorption coefficient $\varepsilon(2)$ for the wavelength used. From the chromatogram recorded at $\lambda_{max}(2)$, $\varepsilon(2)$ at 265 nm was determined as $112 \pm 8\ M^{-1}\ cm^{-1}$ (mean value from six experiments).

DISCUSSION

The first step of the reaction between cisplatin (1) and DNA is the hydrolysis of 1 to 2 [3,4,11]. Compound 2 reacts with DNA *in vitro* either directly or via further hydration to 3, the relative proportions of these pathways depending on DNA concentration [3]. Since it is doubtful whether we can regard the cell nucleus with the compactly packed DNA and the surrounding cytoplasm as a diluted system to which we could assign a DNA concentration, it is difficult to extrapolate *in vitro* results to living cells and to predict which pathway will be predominant *in vivo*. Thus, both 2 and 3 have to be considered as possible candidates for the interaction with DNA.

In previous studies on reactions of 2 with DNA, enriched solutions resulting from the reaction of 1 with 1.2 equivalents of $AgNO_3$ in dimethylformamide were used [4]. This method affords about 80% solutions of 2, with not very precisely defined concentrations of 1 and 3. The system is further complicated by the presence of the potential ligand dimethylformamide, which is not removed but only diluted with H_2O. Miller and House [12] prepared solutions enriched in 2 by passing an equilibrated solution containing 1, 2 and 3 in 0.1 *M* $HClO_4$ through an anion-exchange column charged with ClO_4^- ions. The resulting solution contains, as can be calculated from its absorption spectrum (Fig. 2 in ref. 13), approximately 85% 2, 5% 1 and 10% 3. Accurate kinetic measurements clearly require well-defined starting concentrations, and, preferably, the pure species whose reactivity is being investigated.

Separation of platinum complexes by high-performance liquid chromatography has been subject to several studies [14–20]. Most commonly, reversed-phase columns were modified with alkylsulphonate salts as ion-pair reagents and phosphate or acetate buffers were used as the mobile phase [14–17]. Sulphonate, phosphate and acetate are potential ligands for platinum, and rapid interconversions between the hydrated complexes 2 and 3 on the one hand and sulphonato, phosphato and acetato complexes on the other hand are expected [21]. These equilibria apparently do not interfere with the analytical procedure. However, since such ions as ligands alter the affinity of the complex for DNA, they would jeopardize kinetic measurements. Brandsteterova *et al.* [20] have used a Separon C_{18} packed glass column with water as mobile phase, allowing the analytical separation of 1, 2 and 3. However, the peaks overlapped significantly, rendering their integration inaccurate and making a preparative fractionation incomplete.

The principal idea of this work was to find out whether a cation-exchange column as currently used for the separation of hydrated metal ions would work for covalently bound complex cations as well. In fact, as demonstrated in Fig. 1, our simple system using a sulphonate-packed Nucleosil column does not only allow the separation of the cationic species 2 and 3, but also elutes the uncharged complex 1, affording accurate peak integration and complete separation (>99.8%) of 2. Sodium perchlorate employed as eluent is a non-invasive salt which we used in subsequent kinetic measurements in order to maintain a constant ionic strength.

Compound 2 has been recently used as an intermediate in syntheses of the anti-tumour complexes *cis*-$[PtCl(NH_3)_2(am)]Cl$ (am = aromatic amine) [22]. Enriched solutions of 2 from the reaction between 1 and $AgNO_3$ in dimethyl-

formamide were employed. The use of pure samples of **2** could considerably improve the yield of such preparations.

In conclusion, we have developed a method based on cation-exchange high-performance liquid chromatography allowing a quantitative separation of cisplatin and its hydration products. The absorption spectrum of the pure monohydrated complex **2**, given here for the first time, enables, since the spectra of **1** and **3** are known [13], a rapid determination of the molar fractions of **1**, **2** and **3** in their solution mixtures.

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