Chapter 2.1

Pharmaceutical quality control of the investigational polymer-conjugated platinum anticancer agent AP 5280

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Submitted for publication.
Abstract

AP 5280 is a novel polymer-conjugated platinum anticancer agent currently in Phase I clinical trials. In order to guarantee the quality of AP 5280 drug substance for use in the manufacture of a drug product for intravenous human use, an array of tests was utilized for its quality control. Proton nuclear magnetic resonance ($^1$H NMR) spectroscopy and infrared (IR) spectroscopy were employed for structural identification. The molecular weight (MW) and MW distribution, which play a large role in the distribution of AP 5280 in vivo, were determined by Size Exclusion Chromatography (SEC). Platinum binding assessment was performed using platinum nuclear magnetic resonance ($^{195}$Pt NMR) spectroscopy. The free platinum content and release profile of small platinum species, measured using Flameless Atomic Absorption Spectroscopy (F-AAS), were determined as a measure of molecular integrity, a very important aspect of its assumed mechanism of action. The total platinum content of the copolymer was determined employing flame Atomic Absorption Spectroscopy (AAS). The combined results of the analyses performed on AP 5280 drug substance provided a meaningful picture of its structure, size, and integrity, and thus an excellent basis for its quality control.
Introduction

AP 5280 (poly(N-(2-hydroxypropyl) methacrylamide)-GFLG-Ama=Pt(NH$_3$)$_2$, Figure 1) is a novel polymer-conjugated platinum compound, designed for tumor targeting. In this copolymer, platinum is attached to poly-N-(2-hydroxypropyl) methacrylamide (pHPMA) via a tetrapeptide spacer (glycine-phenylalanine-leucine-glycine, or GFLG), and an amidomalonic acid (Ama) chelating agent. Due to the hyperpermeable nature of the neovasculature of tumors in combination with their limited lymphatic and/or capillary drainage, it is expected that AP 5280 will preferentially accumulate in tumor cells as compared to normal tissue. This phenomenon is called the Enhanced Permeation and Retention (EPR) effect [1-4]. Subsequently, platinum is released from the polymer intratumorally by lysosomal thiol-dependent proteinases, enzymes known to be elevated in human tumors [5]. Theoretically, intravenous AP 5280 administration will lead to higher intratumoral platinum concentrations and therefore potentially greater efficacy than the currently marketed non-polymer platinates cisplatin, carboplatin, and oxaliplatin. Preclinical studies show that AP 5280 indeed has a higher therapeutic index than cisplatin and carboplatin when administered to mice implanted with several different types of tumor [6].

Pharmaceutical quality control of a drug substance is a pivotal step in its use and the development of its formulation [7]. The quality of a drug substance has to be ensured in order to manufacture a drug product of guaranteed quality. Therefore, great attention should be paid to the development of structural and analytical characterization methods of each new drug substance. At our laboratory, experience has been gained in the development of assays and the formulation of several novel anticancer agents of low molecular weight [8,9,10] as well as of (marine-derived) polypeptides [11,12]. Characterization and pharmaceutical development of a macromolecule like AP 5280 (20-25 kDa) provided a novel challenge.

Although pHPMA conjugates of several cytotoxic agents (e.g. doxorubicin [13], paclitaxel [14], methotrexate [15], and (9-amino)camptothecin [16,17]) have been developed, their complete characterization and quality control have not been published to date. Furthermore, no pharmacopoeial methods are available for macromolecules like AP 5280. Therefore, it was necessary to develop our own procedures for the pharmaceutical quality control of AP 5280.
Chapter 2.1

This paper describes the development of a series of analytical methods for the quality control of AP 5280 drug substance, utilizing several regulatory guidelines [18,19,20] as starting points.

Figure 1. Chemical structure of AP 5280 and AP 5279. Molecular weight 24 ± 3 kDa, polydispersity index 1.2-2.3
Materials and methods

Chemicals

Access Pharmaceuticals, Inc. (Dallas, Texas, USA) developed and provided the methods to determine the free platinum content, release of free platinum, Size Exclusion Chromatography (SEC) and interpretation of the $^1\text{H}$ NMR spectra, and provided AP 5280 drug substance, AP 5279 (the O,O-Pt chelate precursor of AP 5280), and pHMA homopolymer. Phosphate-buffered saline (PBS) and distilled water were manufactured in-house (Department of Pharmacy and Pharmacology, Slotervaart Hospital, Amsterdam, The Netherlands). Platinum atomic absorption standard and lithium perchlorate ($\text{LiClO}_4$) were purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Hydrochloric acid 37% was purchased from Merck (Darmstadt, Germany), methanol from Biosolve Ltd. (Amsterdam, The Netherlands), and Water for Injections from B.Braun Medical (Melsungen, Germany). All chemicals were of analytical grade and used without further purification.

Proton ($^1\text{H}$) and platinum ($^{195}\text{Pt}$) nuclear magnetic resonance (NMR) spectroscopy

$^1\text{H}$ and $^{195}\text{Pt}$ NMR spectra were recorded with a Bruker DPX 300 spectrometer with a 5 mm multi-nucleus probe. A variable temperature unit was used to maintain the temperature at 293 K for $^1\text{H}$ NMR spectroscopy and at 298 K for $^{195}\text{Pt}$ NMR spectroscopy. The apparatus was calibrated using tetramethylsilane (TMS) at $\delta=0$ ppm for $^1\text{H}$ NMR spectroscopy and $\text{K}_2\text{PtCl}_4$ at $\delta=-1614$ ppm for $^{195}\text{Pt}$ NMR as external references. During $^1\text{H}$ NMR spectroscopy, the water signal was minimized using the presaturation technique. Samples were prepared by dissolving 80-100 mg AP 5280 in a total volume of 750 µl water with 5% $\text{D}_2\text{O}$ added for locking the signal.

Infrared (IR) spectroscopy

IR spectra of AP 5280 were recorded with a Model PU 9706 IR spectrophotometer (Philips Nederland B.V., Eindhoven, The Netherlands) using the potassium bromide (KBr) pellet technique. The pellet consisted of 2 mg analyte and 300 mg KBr. The ratio recording mode was auto-smooth and the scan time 8 minutes.
Size Exclusion Chromatography (SEC)
Chromatographic analyses were performed using a Spectra System P1000 pump, Spectra Series AS300 autosampler and RI-150 refractive index detector (all Thermo Separation Products (TSP), Fremont, CA, USA). Separation was achieved using a PL aquagel-OH guard column (7.5 mm ID x 50 mm, particle size 8 µm, Polymer Laboratories, Shropshire, UK), and two PL aquagel-OH MIXED columns (7.5 mm ID x 30 cm, particle size 8 µm, Polymer Laboratories) in series kept at a temperature of 308 K with a Croco-cil® column oven (TSP). The mobile phase consisted of 10 mM LiClO₄/methanol 35%/65% (v/v) at a flow of 1.0 ml/min. The injection volume was 100 µl and the run time 30 minutes. The SEC system was calibrated using polyethyleneoxide (PEO) and polyethyleneglycol (PEG) standards (Polymer Laboratories) with molecular weights ranging from 1,080 to 219,300 Da. Samples were prepared by weighing 2-3 mg of AP 5280 into an autosampler vial and adding 1.0 ml mobile phase to dissolve the drug substance by gentle swirling.

Atomic Absorption Spectroscopy (AAS)
Total platinum concentrations were measured using a Perkin Elmer 3100 Flame Atomic Absorption Spectrometer (Perkin Elmer Inc., Boston, MA, USA). A slit width of 0.7 nm, wavelength of 266 nm, and air-acetylene flame were employed. Platinum standards (39.20, 31.36 and 23.52 mg/l) and quality control samples (35.28, 31.36 and 23.52 mg/l) in 0.4 mg/ml pHPMA in 0.11 M hydrochloric acid/methanol 50/50% (v/v) were used for quantification of total platinum concentrations. Samples were prepared by dissolution of AP 5280 in 0.11 M hydrochloric acid/methanol 50/50% (v/v) to yield a theoretical total platinum concentration of approximately 30 mg/l.

Flameless Atomic Absorption Spectroscopy (F-AAS)
F-AAS (or graphite furnace AAS) analysis was performed using a SpectrA-A 30/40 Zeeman Graphite Furnace Atomic Absorption Spectrometer (Varian, Techtron Pty Ltd, Victoria, Australia), consisting of a spectrometer, GTA-75 autosampler and a DS-15 data station equipped with the Quality Control Protocol software package (Varian). Absorbances were recorded at 265.9 nm, a slit bandwidth of 0.2 nm and a time constant of 0.05 s. Argon was used to purge the graphite tube. Analysis of platinum concentrations was carried out as previously described [21].
For determination of the free platinum concentration (all platinum species with molecular weight < 3 kDa), AP 5280 was dissolved in Water for Injections (WFI) at a concentration of 2.0 ± 0.1 mg/ml and stored at ambient temperature (+20-25°C) for one hour. Subsequently, 2 ml samples were ultrafiltered (45 minutes, 41 x g, Eppendorf 5403 centrifuge) through a Centricon YM-3 filter (3 kDa cut-off, Millipore, Milford, MA, USA). The platinum concentration in the ultrafiltrate was analyzed in duplicate.

For determination of the release profile of small platinum species, AP 5280 was dissolved in PBS at a concentration of 2.0 ± 0.1 mg/ml and stored at 37°C. After 3 and 24 hours, samples were prepared in the same manner as described for the free platinum content determination.

Results and discussion

In comparison to most other anticancer agents, which are relatively “simple” molecules of low molecular weight, AP 5280 is a complex agent. AP 5280 comprises four components: pHpMA copolymer, a tetrapeptide spacer, a chelating agent, and a platinum moiety. The pHpMA copolymer provides the complex with its molecular weight (a prerequisite for the EPR effect [4]) and high aqueous solubility. The tetrapeptide spacer and chelating agent connect the platinum moiety to the pHpMA copolymer, and are responsible for binding the platinum during systemic transport, but releasing the biologically active platinate intratumorally through enzymatic cleavage in the lysosomes of the cells [22]. Finally, the structure of the conjugated drug and the way it is bound to the chelator influences interactions of the copolymer with the lysosomal enzymes inside the tumor tissue and in this manner affects release of the biologically active drug [15, 22].

For pharmaceutical quality control of an active pharmaceutical ingredient (API), methods to structurally characterize it, determine its content and purity, and provide data on any other aspect considered important to its biological activity have to be developed. The size of AP 5280 and its integrity in the blood stream are of extreme importance to its mechanism of action, as they can affect both activity and toxicity of the compound. Furthermore, the platinum content needs to be determined in order to properly dose the drug. Lastly, the analytical methods for the characterization of AP 5280 must be able to distinguish AP 5280 from related substances (specificity must be proven) [20]. Test methods were developed using both an AP 5280 reference substance and two related compounds: AP 5279, and pHpMA
homopolymer. AP 5280 is manufactured from a copolymer intermediate consisting of a 90:10 monomer ratio of HPMA and HPMA substituted with the GFLG linker. The latter is further substituted with a terminal amidomalonate chelating group for binding of the diammineplatinum(II). The initial (kinetic) product of the platinum addition reaction is the O,O-platinum chelate (Figure 1), which is designated AP 5279. Subsequently, this complex is rearranged to yield the thermodynamically stable N,O-platinum chelate that is present in AP 5280. It thus becomes important to confirm that this conversion has essentially progressed to completion.

\textbf{\( ^1 \)H NMR spectroscopy}

\( ^1 \)H NMR spectroscopy is probably the most widely used method for structural elucidation of any substance containing protons. Table 1 summarizes the results of the characterization of AP 5280 reference drug substance using \( ^1 \)H NMR spectroscopy. To test the specificity of this technique in the structural identification of AP 5280, AP 5279 and pHMA homopolymer were also analyzed. The \( ^1 \)H NMR spectrum of AP 5279 shows a small extra signal at 5.6 ppm, assigned to the methine proton at the 2 position of the malonato moiety, which is not present in the \( ^1 \)H NMR spectrum of AP 5280. This is a small but very distinctive difference in the \( ^1 \)H NMR spectrum, clearly showing which chelate is present. The pHMA homopolymer does not display the signals at 0.8, 1.6, 4.4, 7.3 and 7.4 ppm, all of which are related to the tetrapeptide spacer, which is not present in the pHMA homopolymer.

\( ^1 \)H NMR spectroscopy thus is a useful analytical method to detect structural changes in AP 5280. Even the way of coordination of platinum to the chelator (O,O instead of N,O chelate) is detected. However, \( ^1 \)H NMR spectroscopy will not detect low levels of many impurities and thus is primarily used as an identification method. As each absorbance peak corresponds so clearly to a specific proton (group) of the entire molecule, the specification for the \( ^1 \)H NMR spectrum of AP 5280 was set to include all of these peaks, without allowing the presence of others. To date, all batches of AP 5280 drug substance received at our laboratory have shown identical \( ^1 \)H NMR spectra.

\textbf{IR}

The assignments of the IR peaks of AP 5280 drug substance are presented in Table 1. All the peaks in the IR spectrum correspond to specific moieties within the molecule.
Table 1. Results of IR and $^1$H NMR characterization of AP 5280 drug substance.

<table>
<thead>
<tr>
<th>Analytical method</th>
<th>Results</th>
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<tbody>
<tr>
<td>$^1$H NMR spectroscopy</td>
<td>Proposed assignments: 0.8 ppm = isopropyl moiety of leucine: $\text{RCH(CH}_3\text{)}_2$; 1.0 ppm = methyl protons along backbone: $\text{CH}_2\text{C(CH}_3\text{)}_2\text{C=OR}$; 1.1 ppm = methyl of $\text{RCH(OH)CH}_3$; 1.6 ppm = methylene protons between chiral methine and isopropyl methine moiety of leucine: $\text{RCH(}R'\text{)CH}_2\text{CH(CH}_3\text{)}_2$; 1.7-2.0 ppm = methylene protons on the backbone and methine of isopropyl moiety of leucine: $\text{RCH(CH}_3\text{)}_2$; 3.0-3.3 ppm = diastereotropic methylene protons of the side chain: $\text{RNHCH}_2\text{CH(OH)CH}_3$ and those between the chiral methine and phenyl ring of phenylalanine: $\text{RCH(}R'\text{)CH}_2\text{Phi}$; 3.9 ppm = enantiomeric methine of the N-2-hydroxypropyl side-chain of the pHMA component of the copolymer: $\text{RNHCH}_2\text{CH(OH)CH}_3$; 4.4 ppm = methine on the chiral carbon of leucine; 7.3 ppm and 7.4 ppm = aromatic protons of phenylalanine; 7.5 ppm and 7.7 ppm = amide protons</td>
</tr>
<tr>
<td>IR spectroscopy</td>
<td>Characteristic absorption bands (approximately): 3700-3100 cm$^{-1}$: O-H and N-H stretching; 2970 cm$^{-1}$ and 2920 cm$^{-1}$: -CH$_2$ and -CH$_3$ stretching; 1630 cm$^{-1}$: N-H deformation; 1520 cm$^{-1}$: N- monosubstituted amide or benzene group of phenylalanine; 1380 cm$^{-1}$: carboxylate ion deformation; 1290-1230 cm$^{-1}$: C-O bending; 1190 cm$^{-1}$: ester (in the platinum moiety); 1140-1050 cm$^{-1}$: C-O stretching and 960-900 cm$^{-1}$: C-H bending</td>
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</table>
IR analysis of the related substances revealed that for AP 5279 the peak at 1380 cm\(^{-1}\) changes to a broad absorption band between 1410-1350 cm\(^{-1}\). The IR spectrum of the pHPMA homopolymer shows four extra peaks at 1480, 1450, 1420, and 1372 cm\(^{-1}\) in its IR spectrum. Thus, the region between 1350 and 1500 cm\(^{-1}\) should be scrutinized closely during analysis of AP 5280, as it is the region that will show small structural changes of the copolymer. These changes are small, however, and structural changes are more evident in the \(^1\)H NMR spectra.

**SEC**

AP 5280 did not show a distinct ultraviolet/visible light (UV/VIS) spectrum, except for some aspecific absorption at 200-230 nm. The lack of a strongly absorbing chromophore precludes the use of UV/VIS spectrophotometry and high performance liquid chromatography (HPLC) with UV/VIS detection as identity tests. However, AP 5280 in solution was found to display a distinct refractive index that was concentration-dependent. Most anticancer agents conjugated to pHPMA are analyzed by SEC to determine their molecular weights, usually employing a RI detector [13, 15-17].

The molecular weight (MW) of copolymers to be administered to humans influences their distribution and elimination. Many polymers, including pHPMA, are non-degradable and must therefore have a MW lower than the renal excretion threshold, otherwise they would be retained in the body. The renal threshold for pHPMA copolymers was determined to be 45 kDa [23].

Synthetic polymers, instead of having a single MW, show a distribution in MW and are considered polydisperse. The shape, width, and magnitude of the MW distribution are characterized in terms of statistical averages of the distribution: the number-average molecular weight \(M_n\), the weight-average molecular weight \(M_w\), and the polydispersity index PI. \(M_n\) is the average molecular weight per molecule, influencing properties that are affected by chain ends, such as colligative properties, refractive index, density and the specific heat capacity [24]. \(M_w\) is a weighted average for the molecular weight of a molecule and affects polymer properties that are influenced by large chains, such as melting point and solution viscosity [24]. The width of the distribution, or polydispersity, of a sample is the ratio of the \(M_w\) to the \(M_n\), \(M_w / M_n\). For a monodisperse or single molecular weight system, the polydispersity index (PI) is 1 [24].

SEC is an analytical technique used to determine MW distributions based on separation according to the differences in hydrodynamic volume (molecular size), not their MW.
However, as molecular sizes of copolymers are roughly proportional to their MW, SEC provides a rapid and simple means to determine MW averages of copolymers. Initially, a SEC system as previously described by Mendichi et al. for the analysis of pHPMA-conjugated doxorubicin and paclitaxel [25, 26] was set up, employing columns filled with a polystyrene-divinylbenzene matrix at 50°C and 10 mM lithium bromide (to prevent molecular aggregation) in dimethylformamide (DMF) as mobile phase. Universal calibration was performed using polyethylene oxide (PEO) and polyethylene glycol (PEG) standards, as it has been shown that this mode of calibration generally provided more accurate MW values than calibration using pHPMA fractions [26]. However, use of this system led to secondary interaction of AP 5280 with the gel phase of the column, causing variable distortion of peaks and late elution of AP 5280 leading to inaccurately calculated MW values. Different SEC systems were thus investigated, resulting in the system as described above. This method has produced reproducible and accurate results, with a (negative) reference peak produced by lithium perchlorate at a retention time of approximately 21 minutes (Figure 2A).

AP 5280 shows a normal, mono-modal distribution of MW. A sample chromatogram of AP 5280 drug substance is shown in Figure 2B. The chromatogram produced by injecting AP 5279 into the SEC system resulted in an identical SEC chromatogram as produced by injection of AP 5280 (Figure 2B). As the MW characteristics of AP 5279 are not different from those of AP 5280, it is not surprising that a change in chelator binding is not detected by SEC.

SEC of pHPMA homopolymer produced a chromatogram as depicted in Figure 2C. It shows a much broader peak for pHPMA than obtained for AP 5280. As the pHPMA employed in these tests did not undergo extensive purification, a wider range of molecular weights is present, resulting in a larger polydispersity index (5.5 for pHPMA vs. 1.2-2.3 for AP 5280). Figure 2D shows a chromatogram of AP 5280 dissolved in 5% dextrose instead of water. A second peak is observed, which is formed by dextrose, as was confirmed by injection of 5% dextrose itself. This shows that the SEC method is able to detect the presence of low MW compounds as separate peaks. All in all, the presence of related substances and other agents with different MW characteristics than AP 5280 will be detected by the SEC system.
Figure 2. 
A. Sample SEC chromatogram of mobile phase. 
B. Sample SEC chromatogram of AP 5280 drug substance. 
C. Sample SEC chromatogram of pHPMA. 
D. Sample SEC chromatogram of AP 5280 in 5% dextrose.
To date, all AP 5280 drug substance batches received at our lab have shown $M_w$’s in the range of 21.8 to 26.5 kDa, $M_n$’s between 11.8 and 17.0 kDa, and PI’s in the range of 1.54 to 1.89. This means that AP 5280 should be easily cleared from the body, as its molecular weight is lower than the renal threshold [23], but that accumulation in tumor tissue should take place, according to the EPR hypothesis. Specifications were set at 24 ± 3 kDa for the $M_w$, 14.5 ± 3 kDa for the $M_n$, and 1.2-2.3 for the PI.

**$^{195}$Pt NMR spectroscopy**

$^{195}$Pt NMR spectroscopy is a technique excellently suited for assessment of platinum binding. Because differences in chemical environment of platinum cause resonance shifts, changes in platinum binding can be detected. The batches of AP 5280 drug substance obtained thus far have shown single signals in the $^{195}$Pt NMR spectrum ranging from $\delta = -2045$ to $-2053$ ppm (mean: $\delta = -2048$ ppm, standard deviation: 2.4 ppm).

AP 5279 produced a $^{195}$Pt NMR spectrum with a single signal at $\delta = -1725$ ppm. The combined presence of AP 5280 and AP 5279 led to formation of two signals in the $^{195}$Pt NMR spectrum, in a ratio according to the relative amount of each chelate present.

The pHPMA homopolymer was not analyzed by $^{195}$Pt NMR spectroscopy, as no platinum is present in this molecule.

Although $^{195}$Pt NMR spectroscopy is not a very sensitive method (i.e. the presence of up to 5% of a differently bound platinum species will not be detected, depending on the measuring time), it apparently is a very specific method. Changes in platinum binding will be detected by large changes in the peak position, as was illustrated by comparison of the $^{195}$Pt NMR spectra produced by AP 5280 and AP 5279. The specification for the $^{195}$Pt NMR spectrum was set at $\delta = -2048 \pm 10$ ppm and absence of other signals.

**Total platinum analysis**

For most pHPMA-conjugated anticancer agents, the content of the anticancer agent is measured after acid hydrolysis followed by a suitable HPLC or spectroscopic technique [13-17]. HPLC-UV analysis of AP 5280 after acid hydrolysis employing a previously described method for cisplatin analysis [27] showed the presence of several different platinum species, making quantification of the total platinum content impossible. Therefore, another approach to determine the platinum content was required. AAS is a widely employed method for
determination of metal concentrations, and it was investigated for its use in determination of the platinum content of AP 5280.

Initially, a flameless AAS (F-AAS) method was set up to determine the total platinum content of AP 5280, but matrix interference limited its use. Therefore, a flame AAS (AAS) method was developed to quantitate the total platinum content of AP 5280 drug substance. Again, matrix interference at first did not lead to complete recovery of the theoretical amount of total platinum (approximately 75%). This problem appeared to originate from the presence of pHHPMA and was solved by dissolving the standards and samples in identical matrices: pHHPMA homopolymer was added to the standards in the same concentration as present in AP 5280 samples. Furthermore, methanol was added to both samples and standards to make the solution less viscous, thus facilitating transportation into the flame. Thus, the solvent of both samples and standards was prepared to contain 0.4 mg/ml pHHPMA (either as homopolymer for the standards or as AP 5280 for the samples) in 0.11 M hydrochloric acid/methanol 50/50% (v/v). This method led to complete recovery of the theoretical total platinum content. Each batch of AP 5280 drug substance produced thus far contained 8.0 ± 0.5% (w/w) platinum.

**Free platinum content**

Polymer therapeutics offers the opportunity to inactivate the bound cytotoxic drug during transport to the tumor and thus reduce its systemic toxicity. In this manner, tumor targeting may be improved by the EPR effect. In addition to the platinum bound as the N,O-platinum chelate, very small amounts of platinum may remain weakly associated with the AP 5280 macromolecules during the manufacturing process. These platinum species will readily dissociate from AP 5280 in aqueous solution, and this unbound ("free", low molecular weight) platinum may be separated from AP 5280 by passage through a 3 kDa ultrafiltration device. It is important to quantitate this characteristic of the drug product and to establish an acceptance limit.

F-AAS was chosen for determination of free platinum, as the free platinum concentrations are very low and this method is more sensitive than e.g. AAS. As the samples were ultrafiltered before analysis, no matrix interference due to the presence of pHHPMA was experienced.

AP 5280 reference substance was tested for its free platinum content in water at several points in time after dissolution, for a duration of 6 days. It was shown that the free platinum content did not increase in time. Therefore, water was chosen as the medium in which to determine
the free platinum content of each batch of AP 5280 drug substance. Of the lots of AP 5280 drug substance received thus far, free platinum levels ranged between 0.12 to 0.86% with respect to the total platinum content. The acceptance limit was set at 1.0%.

**Release of small platinum species**

Apart from free platinum present in AP 5280 drug substance, some release of small platinum species (“liberated platinum”) will take place upon dissolution of AP 5280 in many media due to hydrolysis and other exchange mechanisms of platinum with medium components [28]. This phenomenon is also likely to take place upon intravenous administration. In order to get an indication of the degree of platinum release after intravenous infusion, AP 5280 and related compounds were dissolved in PBS (pH 7.4, 37°C). AP 5280 and AP 5279 showed characteristic release profiles of liberated platinum, as depicted in Figure 3. Although the free platinum contents of AP 5279 and AP 5280 were found to be approximately the same, AP 5279 showed a release profile with liberated platinum contents being a factor 2-5 higher than those of AP 5280. As a reference, platinum release from AP 5280 dissolved in water, the medium used for the determination of free platinum, stored at room temperature, was also determined. AP 5280 in water does not show any measurable release of platinum. Platinum release thus appears to be a process driven by the presence of chloride ions (possibly along with other components of the dissolution medium), as was observed previously [27]. The liberated platinum contents 3 and 24 hours after dissolution of each different compound showed very distinct values and can thus be used as a measure of identity and platinum binding stability of AP 5280 drug substance.

![Graph](image)

**Figure 3.** Liberated platinum content of AP 5280 (♦) and AP 5279 (▲) in PBS at 37°C and of AP 5280 in water at room temperature (○).
To date, the batches AP 5280 drug substance produced for clinical trials have shown mean liberated platinum contents of 0.88% ± 0.18% of the total platinum content after 3 hours, and of 2.70% ± 0.75% after 24 hours of dissolution in PBS (pH 7.4, 37°C). Acceptance limits were set at 1.5% of the total platinum content being present as small platinum species 3 hours after dissolution and at 3.5% after 24 hours.

Conclusions

A broad spectrum of analytical techniques was used to characterize AP 5280 drug substance and thus gain a meaningful picture of the structure, size, and integrity of this complex molecule for quality control purposes. Identification was performed employing ¹H NMR and IR spectroscopy, and SEC was applied for determination of the molecular weight characteristics. The size of AP 5280 is an important aspect in view of its proposed mechanism of action. Because the way platinum is bound to the chelator determines how easily it is released as a biologically active platinate, platinum binding assessment was performed using ¹⁹⁵Pt NMR spectroscopy. F-AAS was employed to determine the free platinum content and the release profile of liberated platinum of AP 5280. This was performed as a measure of the integrity of the molecule, which influences the pharmacokinetics of AP 5280. The assay consisted of total platinum content determination by AAS. The identification methods (¹H, ¹⁹⁵Pt NMR, IR spectroscopy, and SEC), were tested for their specificity. The presence of related substances was detected by these methods. Based on the results obtained in the analysis of the first batches of AP 5280 drug substance, specifications were set. Table 2 summarizes the methods chosen for the quality control of AP 5280 drug substance, the results obtained thus far, and the specifications defined on the basis of these results.
Table 2. Overview of the tests performed, results obtained, and specification set of multiple batches of AP 5280 drug substance.

<table>
<thead>
<tr>
<th>Test method / item</th>
<th>Results</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Appearance</td>
<td>Light brown, flaky substance, free from visible signs of contamination</td>
<td>Light brown, flaky substance, free from visible signs of contamination</td>
</tr>
<tr>
<td>2. $^{195}$Pt NMR spectroscopy</td>
<td><strong>A. Identity</strong>&lt;br&gt;A. A single peak at $\delta$ = -2048 ± 2 ppm&lt;br&gt;B. Absence of other signals than at $\delta$ = -2048 ± 2 ppm.</td>
<td>A. A single peak at $\delta$ = -2048 ± 10 ppm&lt;br&gt;B. Absence of other signals than at $\delta$ = -2048 ± 10 ppm.</td>
</tr>
<tr>
<td></td>
<td><strong>B. Purity</strong>&lt;br&gt;A. Presence of other signals than at $\delta$ = -2048 ± 2 ppm.</td>
<td>B. No other accompanying signals are present.</td>
</tr>
<tr>
<td>3. $^1$H NMR spectroscopy</td>
<td><strong>A. Identity</strong>&lt;br&gt;A. Peaks are present at 0.8-1.0, 1.1, 1.6, 1.7-2.0, 3.0-3.3, 3.9, 4.4, 7.3, 7.4, 7.5 and 7.7 ppm.</td>
<td>A. Peaks are present at 0.8-1.0, 1.1, 1.6, 1.7-2.0, 3.0-3.3, 3.9, 4.4, 7.3, 7.4, 7.5 and 7.7 ppm.</td>
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<tr>
<td></td>
<td><strong>B. Purity</strong>&lt;br&gt;B. No other accompanying signals are present.</td>
<td>B. No other accompanying signals are present.</td>
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<tr>
<td>4. F-AAS</td>
<td><strong>A. Free platinum</strong>&lt;br&gt;A. 2.0±0.1 mg/ml AP 5280 in WfI contains 0.12-0.86% free Pt (with respect to the total Pt content) at room temperature (20-25°C).</td>
<td>A. 2.0±0.1 mg/ml AP 5280 in WfI contains $\leq$1.0% free Pt (with respect to the total Pt content) at room temperature (20-25°C).</td>
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<td></td>
<td><strong>B. Release of small platinum species</strong>&lt;br&gt;B. 2.0±0.1 mg/ml AP 5280 in PBS at 37°C releases 0.88 ± 0.18% small Pt species after 3 hours and 2.72 ± 0.85% free Pt after 24 hours (with respect to the total Pt content)</td>
<td>B. 2.0±0.1 mg/ml AP 5280 in PBS at 37°C releases $\leq$1.5% small Pt species after 3 hours and $\leq$3.5% free Pt after 24 hours (with respect to the total Pt content)</td>
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<tr>
<td>5. AAS</td>
<td><strong>Total platinum content</strong>&lt;br&gt;8.0 ± 0.5 % (w/w)</td>
<td>8.0 ± 0.5 % (w/w)</td>
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<tr>
<td>6. Size-Exclusion</td>
<td><strong>M_w</strong>&lt;br&gt;A. 21.8 - 26.5 kDa</td>
<td>A. M_w = 24 ± 3 kDa</td>
</tr>
<tr>
<td>Chromatography</td>
<td><strong>M_n</strong>&lt;br&gt;B. 11.8 - 17.0 kDa</td>
<td>B. M_n = 14.5 ± 3 kDa</td>
</tr>
<tr>
<td></td>
<td><strong>polydispersity index</strong>&lt;br&gt;C. PI = 1.5 - 1.9</td>
<td>C. PI = 1.2-2.3</td>
</tr>
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<td></td>
<td><strong>shape of the peak</strong>&lt;br&gt;D. Mono-modal</td>
<td>D. Mono-modal</td>
</tr>
<tr>
<td>7. IR spectroscopy</td>
<td>AP 5280 exhibits major absorption bands at approximately 3700-3100 cm$^{-1}$, 2970 cm$^{-1}$, 2920 cm$^{-1}$, 1630 cm$^{-1}$, 1520 cm$^{-1}$, 1380 cm$^{-1}$, 1290-1230 cm$^{-1}$, 1190 cm$^{-1}$, 1140-1050 cm$^{-1}$ and 960-900 cm$^{-1}$.</td>
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Chapter 2.1

References


