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Pharmaceutical development, quality control, stability and compatibility of a parenteral lyophilized formulation of the investigational polymer-conjugated platinum antineoplastic agent AP5346

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AP5346 is a low molecular weight polymer-conjugated platinum antineoplastic agent. The lyophilized drug product has completed a phase I clinical trial. In order to guarantee a constant quality of AP5346 pharmaceutical products, quality control and analysis of the drug substance and final product were performed. The identity of AP5346 was confirmed using ^{1}H NMR, ^{195}Pt NMR and IR spectroscopy. Furthermore, the free platinum content, platinum release characteristics, molecular size and size distribution were established. With the selected analytical techniques, AP5346 could be distinguished very well from its polymeric analogues, such as AP5280 and AP5279. Stability experiments revealed that AP5346 final product is stable for 12 months at 5 °C, in the dark. For administration to patients, AP5346 final product is reconstituted with 5% w/v dextrose and diluted in infusion containers. To investigate the influence of container materials, the stability of AP5346 after reconstitution and dilution in infusion containers was determined. The infusion containers investigated were composed of glass, polyvinyl chloride (PVC, Intraflex®) and low density polyethylene (LD-PE, Ecoflac®). AP5346 was shown to be stable after reconstitution and dilution with 5% w/v dextrose in these infusion containers for at least 96 h at 2–8 °C in the dark and at room temperature with ambient light conditions.

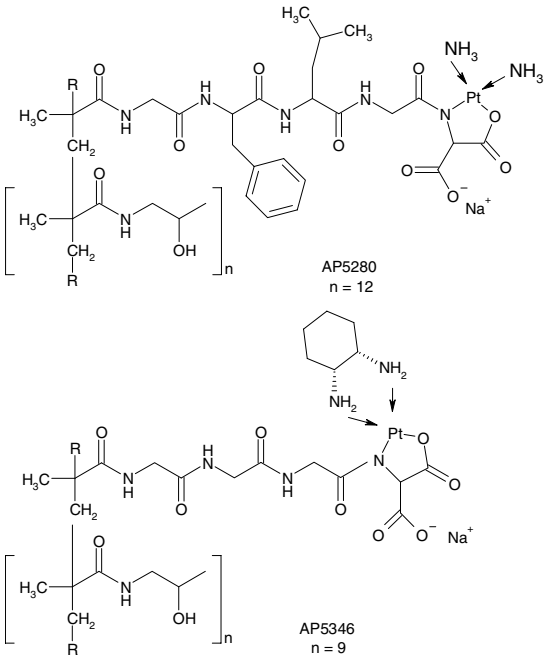
1. Introduction

Since the discovery of cisplatin (cis-diamminedichloroplatinum), platinum-containing drugs have grown to be the most widely prescribed class of anti-cancer agents for the treatment of solid tumors, especially lung, head and neck, ovarian and testicular cancers. Thus far, only cisplatin, carboplatin, and oxaliplatin have been approved for routine clinical treatment. The main problems with the conventional platinum agents are the toxic side effects, including myelosuppression, nephro-, oto-, and neurotoxicity (Kelland and Farrell 2000). In the last decade, efforts have been made to overcome these problems and the use of platinum-containing biocompatible macromolecules has been studied.

Macromolecules are targeted to tumor tissue in a passive way. Due to the enhanced permeation and retention effect (EPR) (i.e. increased permeability of the tumor vasculature and decreased lymphatic drainage) macromolecules extravasate and accumulate mainly at the tumor site (Matsumura and Maeda 1986). pHPMA (poly-*N*-(2-hydroxypropyl)-methacrylamide) is a biocompatible, water-soluble macromolecule which has been used for this purpose. In studies with doxorubicin coupled to pHPMA it was shown that this compound was highly toxic for tumor tissue and significantly less toxic for other tissues, overcame multidrug resistance, and inhibited mechanisms of cellular drug defense, contrary to free doxorubicin (Jindrich Kopecek et al. 2000).

AP5280 is the first of a series of polymer-conjugated platinum agents. It is comprised of platinum bound to pHPMA with a glycyl-phenylalanyl-leucyl-glycine (GFLG) spacer. The GFLG spacer was originally chosen because it was thought that a cleavable spacer was needed to exert its effect. This tetrapeptide is degraded by the most important lysosomal cysteine proteinase cathepsin B (Kopecek et al. 2000). The pharmaceutical development of AP5280 has been described elsewhere (Bouma et al. 2002, 2003a, 2003b).

As the DACH (diaminocyclohexane) platinum(II) moiety, exemplified by oxaliplatin, provides a different spectrum of activity compared with the diamino platinum(II) moiety, Access Pharmaceuticals conducted a research program to generate a second-generation platinum-polymer conjugate based upon DACH platinum. From the synthesis of a large number of DACH platinum-polymer conjugates, AP5346 was selected for further development based upon its excellent preclinical efficacy and therapeutic index. Access Pharmaceuticals' structure-activity studies (unpub-



lished data) had shown that a cleavable peptide link was unnecessary, but that a spacer between the polymer backbone and the chelate was required. Hence, the tetrapeptide linker of AP5280 was replaced by a simple triglycine linker. AP5346 belongs to the (1*R*,2*R*)diaminocyclohexane (DACH) platinum(II) compounds, which were shown to be active in resistant murine leukemia cells (Burchenal et al. 1977, 1979). For AP5346 prolonged tumor growth inhibition was seen in B16F10 melanoma s.c. tumor cells (Rice et al. 2003). Compared to equitoxic doses of oxaliplatin, treatment with AP5346 resulted in much greater inhibition of tumor growth (Rice et al. 2004). The total average molecular weight of AP5346 was very similar to that of AP5280 and was approximately 25 kDa.

This article describes the development of a parenteral lyophilized product of AP5346, including the quality control, establishment of specifications and performance of stability studies, all of which are needed to ensure that the drug product is stable and robust and well-suited for clinical use.

2. Investigations, results and discussion

2.1. Quality control of AP5346 drug substance

2.1.1. Hydrogen (^1H) and platinum (^{195}Pt) Nuclear Magnetic Resonance (NMR) Spectroscopy

For the quality control of AP5346 drug substance, it is important to demonstrate the specificity of the analytical techniques. To test the specificity of ^{1}H NMR and ^{195}Pt NMR, the results of the analysis of AP5346 were compared to the analogues AP5280 and AP5279. The differences between AP5346 and AP5280 are the peptidyl spacer and the platinum drug. The spacer of AP5346 is composed of three glycine amino acids. For AP5280 and AP5279, the pHPMA backbone and the platinum chelate are connected with a glycyl-phenylalanyl-leucyl-glycine (GFLG) spacer (Bouma et al. 2003b). AP5279 is an *O*,*O*-platinum chelate. AP5346 and AP5280 are both *N*,*O*-platinum chelates. Furthermore, in AP5346, a di-aminocyclohexane moiety is coupled to the platinum atom. This moiety is absent in AP5280 and AP5279. ^{1}H NMR, and ^{195}Pt NMR appeared powerful techniques to show the differences in spacer and platinum chelate, respectively.

The ^{1}H NMR spectrum provides information about the number of different types of hydrogen, the functional groups, nearby (non)hydrogen neighbours of the hydrogen atoms, relative numbers of each type of hydrogen, and the location of the hydrogens in the macromolecule.

The ^{1}H NMR spectrum of AP5346 drug substance shows 12 signals: at 1.03 ppm assigned to methyl hydrogens along the backbone, at 1.24 ppm assigned to methyl hydrogens of HPMA, at 1.67, 1.82, 1.92, and 2.13 ppm assigned to methylene hydrogens of the backbone and the cyclohexane moiety, at 3.15 and 3.22 ppm assigned to methylene hydrogens of HPMA moiety, at 3.98 ppm assigned to methylene hydrogens of the GGG-spacer and hydrogens of the methine of the HPMA moiety, at 7.61 ppm assigned to hydrogens of the amide of the HPMA moiety, and at 7.72 and 8.09 ppm assigned to hydrogens of the amides of the GGG-spacer. The ^{1}H NMR spectra of AP5346 and AP5280 are depicted in Fig. 1a. The main differences between AP5346 and AP5280 are seen at 1.5–2.5, 4.4, 7.3 and 7.4 ppm. In the first area, at 1.5–2.5 ppm, more signals are seen in the spectrum of AP5346. These signals are due to the methylene hydrogens in the cyclohexane moiety, which are absent in AP5280. At 4.4 ppm a signal is seen in AP5280, which is absent in AP5346. This signal is due to the methine hydrogen on the chiral carbon of leucine. The signals at 7.3 and 7.4 ppm, present in the spectrum of AP5280 and absent in AP5346, are due to the aromatic hydrogens of phenylalanine. Analogue AP5279 can be easily distinguished from both AP5280 and AP5346 due to the presence of a small signal at 5.6 ppm, assigned to the methine hydrogen at the 2 position of the malonato moiety (Bouma et al. 2003b).

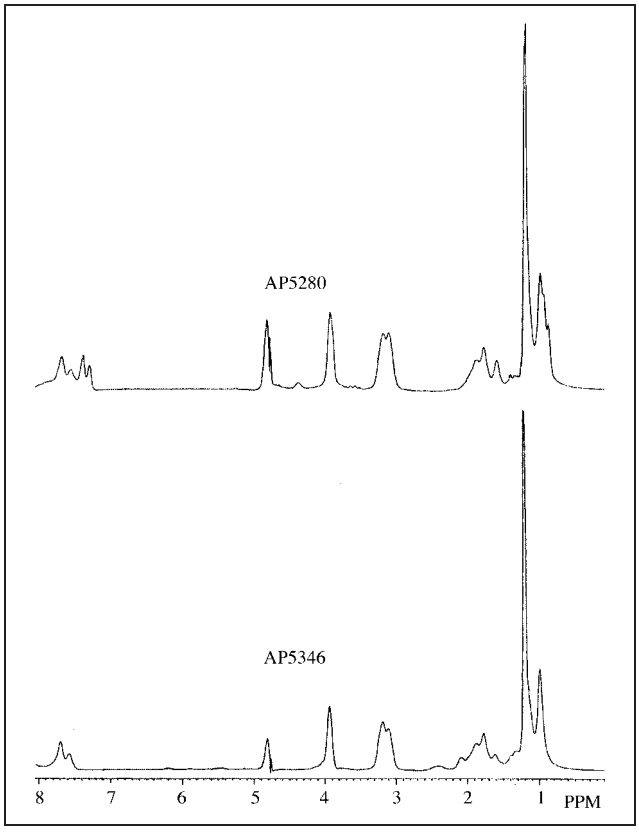


Fig. 1a: ^{1}H NMR spectra of AP5280 and AP5346 drug substance in D_2O containing 0.05% w/w TMS

Identification of AP5346 was also performed using ^{195}Pt NMR analysis. ^{195}Pt NMR analysis is a very specific method, i.e. small changes in binding of the platinum atom result in large peak shifts. For AP5346, two integrals were seen between −2200 and −2350 ppm. In AP5279 and AP5280 just one peak was seen at −1725 ppm and −2049 ppm, respectively. This large shift of AP5346 is due to the cyclohexane moiety bound to the platinum. The ^{195}Pt NMR spectra of AP5280 and AP5346 are depicted in Fig. 1b.

Besides confirmation of the identity of AP5346, ^{195}Pt NMR analysis can also be used for the determination of platinum impurities. During the manufacturing process (Stewart et al. 2004), of the DACH platinum(II) complex three isomers are formed: the *O*,*O*-chelate, *N*,*O*-chelate, and the *N*,*N*-chelate. The chelate impurities will show only minor differences in the ^{1}H NMR spectra, because the polymer and spacer are the same. In the ^{195}Pt NMR spectra however, large shifts were seen due to different bindings to the platinum atom. The two peaks between −2200 and −2350 ppm are due to the *N*,*O*-chelate (Gibson et al. 1990). The peaks between approximately −2550 and −2650 ppm are due to the *N*,*N*-chelate. Unreacted DACHPt$(OH_2)_2^{2+}$ would appear at approximately −1870 ppm. The *O*,*O*-chelate resonance would appear at approximately −1960 ppm, but neither of these *O*,*O*-species are observed. Therefore, the purity of AP5346 was determined as the percentage of ^{195}Pt NMR peaks which appear in the *N*,*O*-chelate range. The quality control data of AP5346 drug substance showed two signals, at approximately −2282 ppm and −2260 ppm, corresponding with the *N*,*O*-chelate (Table 1). The integral of these two signals was for all drug substances more than 90% of the total area.

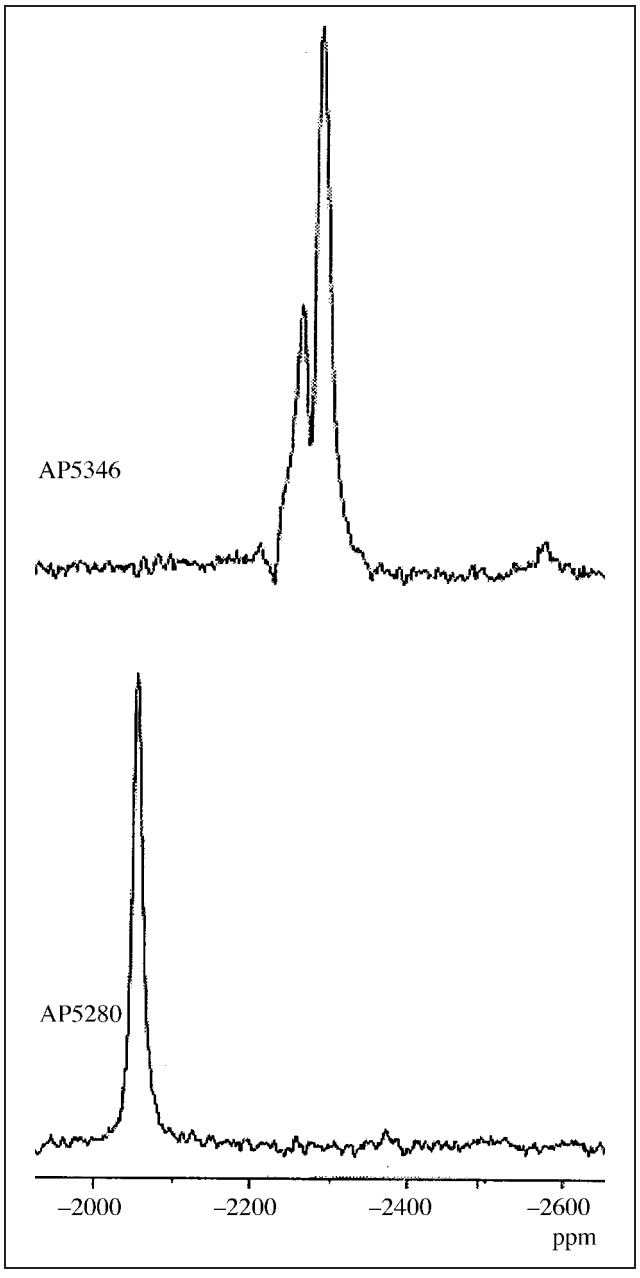


Fig. 1b: ^{195}Pt NMR spectrum of AP5280 and AP5346 drug substance in H_2O/D_2O (93 : 7)

2.1.2. Graphite Furnace Atomic Absorption Spectrometry (GFAAS)

In AP5346, the toxicity of free platinum is reduced by coupling of the platinum chelate to the pHPMA polymer. However, during the production process of AP5346 drug substance, small amounts of platinum may remain weakly associated with AP5346. These platinum species will dissociate from the macromolecule in acidic aqueous solutions and possibly increase the toxicity of the drug substance. Therefore, the amounts of "free" platinum (platinum species smaller than 3 kDa) were determined in ultrafiltrate samples from AP5346. Furthermore, the release characteristics of these small platinum species were studied in phosphate buffered saline, pH 7.4 at 37 °C to mimic the conditions after administration. The use of a furnace instead of a flame for platinum atomization in ultrafiltrate samples resulted in a much more sensitive method. Therefore, furnace AAS was used. The results are depicted in Table 1.

No significant differences in free platinum content or release of free platinum were seen for the three lots of drug substance. All lots contained 0.2–0.3% w/w free platinum. The release of free platinum in PBS at 37 °C varied from 1.0–1.3% w/w and from 3.8–4.2% w/w after 3 and 24 h, respectively.

2.1.3. Size Exclusion Chromatography (SEC)

AP5346 is a DNA crosslinking antitumour compound and exerts its cytotoxic effect by formation of platinum-DNA adducts like other platinum containing agents, e.g. cisplatin, carboplatin and oxaliplatin (Raymond et al. 2002). In AP5346 the platinum chelate is bound to a polymer backbone to obtain passive targeting (due to the EPR-effect), reducing the toxicity. Therefore, it is important for the efficacy and toxicity of AP5346 that the copolymer does not disintegrate into small fragments. Furthermore, the molecular weight of polymers is also of importance for their elimination after administration. Because pHPMA is a non-biodegradable copolymer, the molecular size must remain below the renal threshold of pHPMA (45 kDa) to prevent accumulation in the body. Therefore, the molecular size distribution was determined using SEC, which is an analytical technique based on differences in molecular size and not molecular weight. However, the molecular weight of polymers is roughly proportional to their molecular size, making SEC a very suited method for the analysis of the molecular size distribution of AP5346.

Due to the production process, synthetic polymers (such as pHPMA) are polydisperse instead of having a single molecular weight. The degree of polydispersity is calcd.

Table 1: Quality control data of AP5346 drug substance and final product

	AP5346 drug substance			AP5346 final product			
	Lot AP5346–2-100	Lot AP5346–3-325	Lot AP5346–4-325	Batch 1 Lot 300902SS2 150 mg Pt/vial	Batch 2 Lot 021002SS3 150 mg Pt/vial	Batch 3 Lot 041002SS4 150 mg Pt/vial	Batch 4 Lot 111202SS5 400 mg Pt/vial
^{1}H NMR	Peaks at 1.00, 1.21, 1.63, 1.78, 1.92, 2.10, 3.11, 3.18, 3.94, 7.59, 7.71, 8.05 ppm	Peaks at 0.99, 1.21, 1.63, 1.78, 1.92, 2.09, 3.11, 3.18, 3.94, 7.59, 7.71, 8.05 ppm	Peaks at 0.99, 1.20, 1.62, 1.78, 1.9, 2.08, 3.11, 3.18, 3.94, 7.59, 7.71, and 8.05 ppm	Peaks at 1.13, 1.34, 1.76, 1.92, 2.03, 2.23, 3.25, 3.33, 4.07, 7.54, 7.76, 8.11 ppm	Peaks at 1.00, 1.21, 1.63, 1.78, 1.88, 2.09, 3.11, 3.19, 3.94, 7.59, 7.71, and 8.05 ppm	Peaks at 1.12, 1.33, 1.76, 1.91, 2.02, 2.22, 3.24, 3.32, 4.06, 7.64, 7.75, 8.09 ppm	Peaks at 1.00, 1.22, 1.64, 1.79, 1.90, 2.10, 3.12, 3.19, 3.95, 7.60, 7.72, and 8.10 ppm
^{195}Pt NMR	Peaks at −2285 and −2260 ppm	Peaks at −2282 and $\delta = -2260$ ppm	Peaks at −2277 and −2259 ppm	Peaks at −2278 and −2260 ppm	Peaks at −2283 and −2260 ppm	Peaks at −2277 and −2260 ppm	Peaks at −2284 and −2260 ppm
Free Pt	0.20 ± 0.04%	0.26 ± 0.06%	0.33 ± 0.06%	0.15 ± 0.01%	0.15 ± 0.05%	0.15 ± 0.01%	0.30 ± 0.03%
Release of free Pt							
3 hours	1.15 ± 0.01%	1.25 ± 0.03%	1.00 ± 0.33%	1.06 ± 0.10%	0.99 ± 0.03%	1.02 ± 0.07%	1.37 ± 0.07%
24 hours	3.80 ± 0.55%	4.19 ± 0.36%	3.80 ± 0.37%	4.01 ± 0.07%	4.28 ± 0.49%	3.85 ± 0.15%	4.03 ± 0.14%
AP5346 content	*	*	*	116.9 ± 0.32%	101.4 ± 0.97%	100.0 ± 0.25%	105.9 ± 4.37%
SEC							
M_w	21.7 ± 0.86 kDa	19.5 ± 0.2 kDa	17.2 ± 0.08 kDa	21.5 ± 0.5 kDa	19.9 ± 0.09 kDa	19.9 ± 0.06 kDa	17.3 ± 0.06 kDa
M_n	10.7 ± 1.8 kDa	9.4 ± 0.6 kDa	7.8 ± 0.05 kDa	10.6 ± 0.2 kDa	8.9 ± 0.14 kDa	9.0 ± 0.21 kDa	7.9 ± 0.02 kDa
PDI	2.08 ± 0.38	2.08 ± 0.1	2.20 ± 0.58	2.04	2.23	2.21	2.20
pH	*	*	*	6.74	6.75	6.78	6.7
Moisture content	*	*	*	1.33%	1.43%	1.28%	0.74%
IR spectroscopy	Major absorption bands at app. 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}, 960–900 cm^{-1}	Major absorption bands at app. 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}	Major absorption bands at app. 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}	*	*	*	*

* Not analyzed

(M_w/M_n) as the polydispersity index (PDI). The PDI increases with increasing size distribution. The results are depicted in Table 1. The quality control results showed that the drug substances had a weight average molecular weight (M_w) of approximately 17–22 kDa and a number average molecular weight (M_n) of 7.8–10.7 kDa. The resulting PDI was 2.08–2.20.

2.1.4. Infrared Spectroscopy

Major absorption bands were seen at 3700–3100 cm^{-1} assigned to O–H and N–H stretching, 2970 cm^{-1} and 2920 cm^{-1} assigned to $-CH_2$ and $-CH_3$ stretching, 1630 cm^{-1} assigned to N–H deformation, 1520 cm^{-1} assigned to N-monosubstituted amide, 1380 cm^{-1} assigned to carboxylate ion deformation, 1290–1230 cm^{-1} assigned to C–O bending, 1190 cm^{-1} assigned to C–O stretching of esters, 1140–1050 cm^{-1} assigned to C–O stretching, and 960–900 cm^{-1} assigned to C–H bending. These assignments are the same as for AP5280 (Bouma et al. 2003b) and therefore IR spectroscopy alone is not suited to distinguish between AP5346 and its analogues. However, a combination of IR, ^{1}H NMR and ^{195}Pt NMR provides a complete identification of AP5346 drug substance.

2.2. Preformulation studies

2.2.1. Sterilization

After sterilization of a solution of AP5346 in water for injection, no changes in appearance were seen. All vials contained dark brown solutions, free from visible particles before and after sterilization. The SEC data however, showed an increase in polydispersity index (2.15 instead of 2.08) and a decrease in M_w and M_n: 17.5 kDa and 8.1 kDa instead of 21.7 kDa and 10.7 kDa, respectively. These data show that there is a change in the molecular weight and hence distribution of the polymer, but at this time what that change can be attributed to is uncertain. Due to these changes it was decided to manufacture AP5346 aseptically. In order to produce a stable product with sufficient shelf-life, a lyophilized dosage form was developed.

2.2.2. Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was performed to study the freeze drying characteristics of AP5346 formulation solution. The DSC thermogram of AP5346 showed two thermal events: a glass transition temperature (T_g) with the onset at −13.5 °C and an ice-melting endotherm. The formulation solution of AP5280 showed a T_g at −13.2 °C. This indicates that the freeze drying characteristics of both compounds are comparable. Therefore, the freezing drying program of AP5280 (Bouma et al. 2003a) was used as starting point for the development of the freeze drying program of AP5346.

2.3. Manufacturing process

Excipients are often used to improve physical and chemical characteristics of drug substances, such as solubility and stability, and to provide a solid cake with good appearance. However, AP5346 drug substance is highly soluble (due to the pHPMA moiety) in water for injection. Furthermore, freeze drying of a solution of AP5346 in water for injection revealed a voluminous cake with good appearance. Therefore, no excipients (e.g. bulking agents, cryoprotectants, solubilizing agents) were required and water for injection was chosen as dissolution vehicle.

During manufacturing of the pharmaceutical product, AP5346 drug substance was dissolved in water for injection, and subsequently freeze dried. The freeze drying program was optimized for AP5346 drug product. Vials were frozen to −40 °C in 3 h, followed by a freeze-hold lasting 5 h to ensure complete freezing of the solutions in the vials. Subsequently, the primary drying phase was started by lowering the chamber pressure to 0.15 mbar, while keeping the temperature at −40 °C. After achieving the vacuum, the shelf temperature was increased to +25 °C in 2 h. During these 2 h, the product temperature increased from −40 °C to −30 °C. The chamber pressure was held constant. This secondary drying phase was maintained for 80 h. During these 80 h, the product temperature further increased to +25 °C. This manufacturing process revealed a light brown, dry, voluminous freeze dried product, which could be reconstituted easily. Reconstitution was performed with 27.4 ml 5% dextrose solution prior to administration. No meltback of ice and hence cake collapse was seen and no vial breakage occurred. The quality control results showed that this production process did not alter the characteristics of AP5346 drug substance (Table 1).

2.4. Quality control of AP5346 final product

AP5346 final product was characterized with the same methods as used for AP5346 drug substance. Furthermore, visual inspection, reconstitution characteristics, pH after reconstitution, moisture content, and AP5346 content were determined. The results are given in Table 1. All final products were light brown, voluminous cakes. No visible contamination was seen. Reconstitution of the final products with 27.4 ml 5% w/v dextrose revealed a final volume of 30 ± 0.1 ml for all batches. The pH after reconstitution varied from 6.7–6.8. The moisture content was 0.7–1.4% w/w. For ^{1}H NMR, ^{195}Pt NMR, free platinum content, release of free platinum, and SEC analysis the results of the quality control of the final product were similar to the results of the drug substances. This indicates that the production process and freeze drying of AP5346 afforded a final product with the same physical and chemical properties as AP5346 drug substance. Therefore, this production process is suited for the manufacture of AP5346 final product.

The platinum content per vial was determined using UV/VIS analysis. These results are discussed below.

The effect of AP5346 after administration depends both on the release of platinum at the tumor site and on the platinum content (free and bound platinum) of the final product. Therefore, the dosage is calculated as mg platinum. For AP5280, analysis of the total platinum content was performed with Flame Atomic Absorption Spectrometry (Bouma et al. 2003b). However, with AP5346 a decreased signal was seen. This may be due to interference of the DACH group or the shorter distance between the DACH platinum chelate and the pHPMA backbone compared to AP5280. Therefore, a new method was developed. The total platinum content and platinum content uniformity were indirectly determined by UV/VIS spectrometry at 240 nm. UV/VIS is not a specific method, because all compounds (impurities included) which absorb at 240 nm are measured. However, if no impurities are detected with the specific analytical methods (^{1}H NMR, ^{195}Pt NMR, SEC), UV/VIS is a suitable method for the determination of the AP5346 content.

There was a small variation in platinum content of the drug substance between lots, resulting in lot-to-lot differences in the amount of total AP5346 per vial (to obtain 400 mg platinum per vial) which in turn gave rise to lot-to-lot differences in concentrations of AP5346 after reconstitution. Therefore, the solutions used for calibration had to be prepared with the drug substance from which the final product was manufactured. The platinum content of the drug substances used for calibration was determined by Inductively Coupled Plasma (ICP) by Access Pharmaceuticals. Subsequently, the platinum content of the final product (as percentage of the theoretical content of 400 mg/vial) was calculated from the AP5346 content with use of the percentage of platinum of the drug substance used for the manufacture. The first batch of final product showed a relatively high platinum content of 116.9% of the theoretical content. The platinum content of the other three batches varied form 100.0–106%. The results are listed in Table 1.

2.5. Setting of specifications

The quality control results of the 4 batches of final product resembled the quality control results of the 3 lots of drug substance, indicating that the production process did not alter the characteristics of AP5346 drug substance. Furthermore, no differences in quality control results were seen between AP5346 final product 150 mg Pt/vial and 400 mg Pt/vial, indicating that the filling volume can be varied without changing the physico-chemical characteristics of the final product. Because of similarity between the 3 lots of drug substance and the 4 batches of final product, just one set of specifications was established for both the drug substance and final product. First, preliminary specifications were set based on the means and standard deviations of the quality control results. These specifications were transformed into final specifications after completion of the stability study. The preliminary specifications are given in Table 2. Variations in the results between lots are not only due to variation in characteristics of the lots, but also due to analytical variation. This analytical variation depends on the analytical method used. Therefore, higher deviations were accepted for less robust analytical methods. Besides the analytical method, the influence of the analyzed parameter on product quality is also of importance. For example, results of SEC are less critical than the free platinum content and release of free platinum because the copolymer acts as a carrier and relative deviations in molecular size are not likely to influence the *in vivo* distribution. Furthermore, a deviation up to 10% from run to run for SEC chromatography is quite normal. Therefore, a deviation of mean ±20% was found acceptable. For the free platinum content, however, the maximum concentration is important due to the toxicity of platinum. For this parameter, a deviation of ±25% was set. The same deviation was chosen for the release of free platinum. This deviation may seem high for a critical parameter, but due to the very low free platinum content the absolute deviation remained small. For the moisture content, only an upper limit was set, because increasing water content could lead to changes in the Pt chelate. For ^{195}Pt NMR analysis, small standard deviations were seen and narrow specifications were set. For ^{1}H NMR analysis, specifications were set for 12 signals. For all signals, the accepted deviation was based on the one signal with the highest expected Rate of Failure (ROF). This was the signal at 1.03 ppm, with an expected ROF of almost 12%. The calculated expected ROF based on these specifica-

Table 2: Preliminary setting of specifications

	SEC			Free Pt	Pt release		pH	Moisture	^{195}Pt NMR	
	M_w	M_n	PDI		3h	24h			Peak 1	Peak 2
Specification limits	±20%	±20%	±20%	±25%	±25%	±25%	±7.5%	±10%	±0.5%	±0.5%
Mean	19.6	9.1	2.15	0.22	1.14	4.06	6.74	0.65	−2264	−2289
Sd	1.85	1.16	0.08	0.08	0.13	0.23	0.03	0.07	1.02	0.80
Upper Specification Limit (USL)	23.6 kDa	11.0 kDa	2.58	1.00%	1.42%	5.07%	7.25	3.00%	−2270	−2300
Lower Specification Limit (LSL)	15.7 kDa	7.3 kDa	1.72	0.00%	0.85%	3.04%	6.24	0.00%	−2250	−2280
Expected ROF (%)	3.40	11.44	$6.09 \cdot 10^{-6}$	0.16	3.14	0.00	$4.29 \cdot 10^{-50}$	$1.71 \cdot 10^{-21}$	$8.52 \cdot 10^{-8}$	$6.21 \cdot 10^{-30}$
Reliability index	2.30	1.45	$2.69 \cdot 10^{4}$	8.02	2.38	522.19	$1.44 \cdot 10^{48}$	$6.97 \cdot 10^{18}$	$5.89 \cdot 10^{6}$	$1.83 \cdot 10^{28}$

	^{1}H NMR											
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8	Peak 9	Peak 10	Peak 11	Peak 12
Specification limits	±10%	±10%	±10%	±10%	±10%	±10%	±10%	±10%	±10%	±10%	±10%	±10%
Mean	1.03	1.24	1.67	1.82	1.92	2.13	3.15	3.22	3.98	7.61	7.72	8.09
Sd	0.07	0.06	0.06	0.06	0.07	0.06	0.07	0.07	0.06	0.02	0.02	0.02
Upper Specification Limit (USL)	1.14	1.37	1.83	2.00	2.11	2.34	3.47	3.55	4.38	8.37	8.50	8.89
Lower Specification Limit (LSL)	0.93	1.12	1.50	1.64	1.73	1.92	2.84	2.90	3.58	6.85	6.95	7.28
Expected ROF (%)	11.98	4.74	0.91	0.47	0.85	0.10	$1.35 \cdot 10^{-4}$	$1.78 \cdot 10^{-4}$	$3.97 \cdot 10^{-8}$	$4.45 \cdot 10^{-294}$	$1.13 \cdot 10^{-277}$	$7.49 \cdot 10^{-239}$
Reliability index	1.43	2.00	4.41	6.37	4.60	17.07	$2.14 \cdot 10^{3}$	$1.72 \cdot 10^{3}$	$1.95 \cdot 10^{6}$	$6.06 \cdot 10^{287}$	$2.84 \cdot 10^{271}$	$6.70 \cdot 10^{232}$

tions, decreased with increasing mean value of the signal. This indicates that the expected ROF of the first signal is mainly due to variation in the analytical method.

During storage of batches of AP5346 drug substance and final product at −20 °C, analysis of these batches in time showed that the SEC parameters exceeded the limits of the preliminary specifications. An increase in M_n and the subsequent decrease in PDI, per its definition, were seen in both the drug substances and the final products, indicating that these changes are inherent to the AP5346 molecule and not the manufacturing process. The M_w remained constant, which cannot be easily explained based on the definition of M_w and M_n. The SEC data (not shown) simply indicate that the copolymer changes during storage at −20 °C. To avoid batch rejection during storage, specifications must be recalculated. Obviously, in these cases, changes of the preliminary specifications are only allowed when it can be guaranteed that the new specifications do not influence product quality. The copolymer however, is not the active part of the drug. It serves as a carrier of the DACH-platinum compound. Therefore, it is not to be expected that the toxicity or efficacy of this drug are influenced by small deviations in the size distribution of the copolymer, provided that the free platinum content and platinum release are not affected. Analyses showed that the free platinum content and platinum release were not affected during storage at −20 °C (data not shown). Therefore, the changes and recalculation of the specifications for M_n, and PDI are justified and the preliminary specifications of both SEC parameters were recalculated with the analytical data of 4 additional batches and revealed a M_n of 7.1–16.5 kDa and a PDI of 1.11–2.31. The specifications of the M_w were not changed, because this parameter remained essentially constant during storage. The change in these specifications only concerned the lyophilized product and not the drug substance.

The specifications set for AP5346 are based on a limited set of data, resulting in relative wide ranges for the specifications. While this is common for new products, it is expected in the future that the specifications will be recalculated with data from additional batches of AP5346 final product, resulting in smaller specification ranges.

2.6. Stability of AP5346 final product

The results of the stability study of the final product are given in Table 3.

During 12 months of storage at 5 ± 3 °C (ambient RH) a tendency was observed of increasing M_n and decreasing PDI. These deviations were not significant and smaller than the deviations seen at a storage condition of −20 °C. Both parameters are within the final specification (see "Setting of specifications"). Furthermore, a small increase in pH was seen. After twelve months of storage the pH of the samples stored at 5 ± 3 °C (ambient RH) was on the limit of the specification (pH 6.2–7.2). These limits seem very small, but the calcd. ROF with these specifications was 4.29×10^{-50}. However, the stability data showed a change in pH of unknown origin. Calculations based on the stability data showed that a change of specifications from pH 6.2–7.2 to pH 6.4–7.4 is required to prevent batch rejection. This minor change in specification will not influence the quality of the product and is therefore allowed. No changes were seen in the appearance, ^{1}H NMR or ^{195}Pt NMR (data not shown).

At 25 ± 2 °C/60 ± 5% RH a significant increase in pH and moisture content was seen. The free platinum content and release of free platinum remained constant, indicating that the binding of the platinum was not influenced. The increase in pH is likely due to the higher moisture content. Furthermore, an increase in M_n and a decrease in PDI were seen at 12 months. The deviations of both parameters were larger at 25 ± 2 °C/60 ± 5% RH than at 5 ± 3 °C/ambient RH.

Based on these results and the results of storage at −20 °C, a storage condition of 5 ± 3 °C/ambient RH, in the dark, is preferred. AP5346 final product was given a storage time for 12 months at a storage condition of 5 ± 3 °C, in the dark.

2.7. Stability and compatibility upon reconstitution and dilution

To investigate the stability of AP5346 final product after reconstitution and dilution, vials with final product were reconstituted with 27.4 ml 5% w/v dextrose and diluted in three kinds of infusion containers with 5% dextrose solution. Because the presence of free platinum (i.e. platinum bound to molecules smaller than 3 kDa) increases the toxicity of AP5346, the free platinum content and total platinum content were determined 96 h after reconstitution or dilution. No changes in free platinum content and total platinum content were seen after reconstitution of the product in the vials (data not shown), indicating that AP5346 is stable for at least 96 h after reconstitution and that no adsorption of platinum to the walls of the vials occurred.

The results of the analysis of the amount of free platinum after dilution in the infusion containers are depicted in

Table 3: Stability data of AP5346 final product. The given values are the means and standard deviations of 3 pilot batches

	0 months	5 ± 3 °C			25 ± 2 °C/60 ± 5% RH		
		3 months	6 months	12 months	3 months	6 months	12 months
Free Pt (%)	0.44 ± 0.24	0.22 ± 0.11	0.17 ± 0.08	0.15 ± 0.01	0.24 ± 0.07	0.21 ± 0.05	0.30 ± 0.02
Pt release (%)							
3 hours	1.01 ± 0.17	1.22 ± 0.08	1.14 ± 0.05	1.28 ± 0.07	1.29 ± 0.08	1.34 ± 0.10	1.43 ± 0.12
24 hours	*	3.81 ± 0.30	4.18 ± 0.10	4.77 ± 0.22	3.96 ± 0.34	4.18 ± 0.10	4.80 ± 0.29
AP5346 content (%)**	100.0	93.4	99.8	104.4	95.3	99.1	104.1
SEC							
M_w	20.9 ± 1.40	21.8 ± 1.10	19.6 ± 0.83	21.6 ± 1.78	21.6 ± 1.10	19.4 ± 0.81	20.7 ± 1.15
M_n	10.5 ± 0.12	10.6 ± 0.84	9.8 ± 0.59	11.5 ± 0.55	10.4 ± 0.92	9.5 ± 0.64	13.2 ± 0.75
PDI	2.01 ± 0.14	2.05 ± 0.06	2.00 ± 0.03	1.73 ± 0.27	2.08 ± 0.07	2.68 ± 1.09	1.57 ± 0.04
Moisture content % w/w	1.35 ± 0.08	*	2.50 ± 0.21	1.55 ± 0.27	*	4.20 ± 0.33	5.05 ± 0.67
pH	6.8 ± 0.06	6.9 ± 0.06	6.9 ± 0.06	7.2 ± 0.06	7.1 ± 0.06	7.3 ± 0.06	7.7 ± 0.06

* Not analyzed

** Calcd. as percentage of the initial AP5346 content

Fig. 2a to 2c. No difference was seen in compatibility of AP5346 between the different infusion containers. In all containers, the percentage of free platinum was highest for the solution of 0.034 mg Pt/ml stored at +15–25 °C. This content however, was for all containers still less than 1% after 20 h of storage. Furthermore, no differences were seen in the total platinum contents for the different containers (data not shown).

Besides the analysis of the free platinum content and release, any formation of degradation products was analyzed using ^{1}H NMR and ^{195}Pt NMR. No changes were seen in ^{1}H NMR and ^{195}Pt NMR spectra during the stability study, indicating that no structural changes occurred.

In conclusion, several different analytical methods were used to characterize AP5346 final product and drug substance. It was shown that the final lyophilization process did not alter these characteristics. Based on the analytical methods, a list of specifications was set. These specifications limits were chosen carefully, taking into account the risk of batch failure and the characteristics of the analytical method: what is the analytical variation of the analytical method and do large specification limits influence the quality of the product? These specifications were also used for the stability study. The results of the stability study of the final product showed that AP5346 final product is stable for at least 12 months at a storage condition of 5 ± 3 °C, in the dark. Furthermore, AP5346 final product was shown to be stable after reconstitution and compatible with infusion containers composed of glass, polyvinyl chloride (PVC, Intraflex®) and low density polyethylene (LD-PE, Ecoflac®).

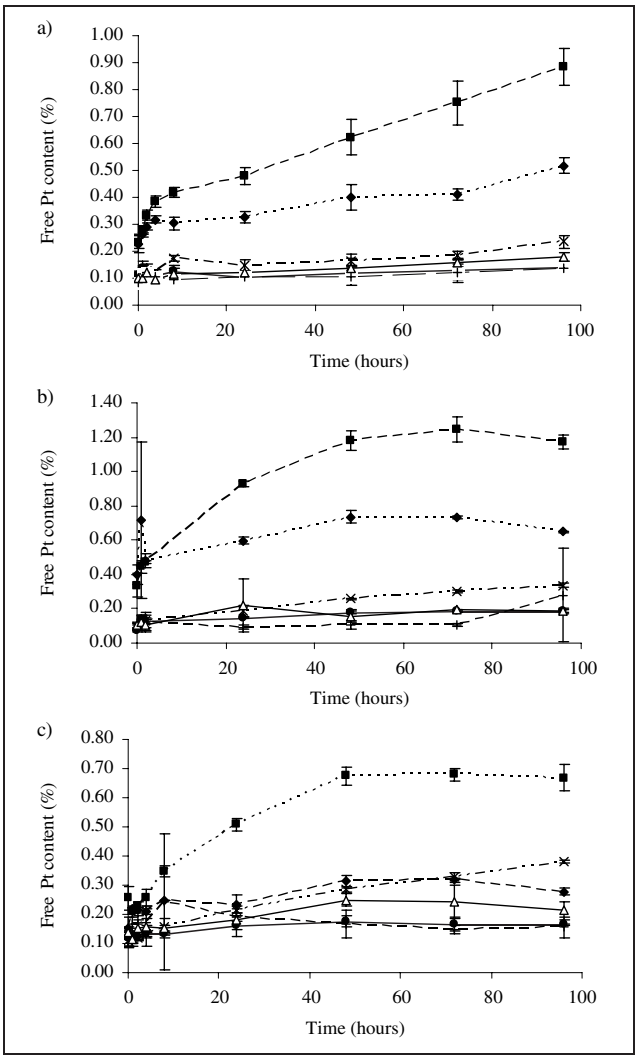


Fig. 2: Free platinum content (as % of the total Pt content) in the Ecoflac® infusion containers (a), in the Intraflex® infusion containers (b), and in the glass infusion containers (c). The containers were filled with 5% dextrose solutions containing 0.034 mg Pt/ml stored at +2–8 °C (◆), 0.034 mg Pt/ml stored at +15–25 °C (■), 0.34 mg Pt/ml stored at + 2–8 °C (●), 0.34 mg Pt/ml stored at +15–25 °C (×), 3.4 mg Pt/ml stored at +2–8 °C (+) and 3.4 mg Pt/ml stored at +15–25 °C (△)

3. Experimental

3.1. Materials

AP5346 drug substance, containing 9–12% w/w platinum, was characterized and provided by Access Pharmaceuticals, Inc. (Dallas, Texas, USA). AP5346 lyophilized pharmaceutical product was manufactured in-house (Department of Pharmacy and Pharmacology, Slotervaart Hospital, Amsterdam, The Netherlands). Sterile water for injection (Ecotainer®), 0.9% (w/v) NaCl (normal saline), 5% w/v dextrose, and the glass, Intraflex® and Ecoflac® infusion containers, all filled with 100 ml 5% w/v dextrose, were obtained from B. Braun (Melsungen, Germany). All chemicals were of analytical grade and used without further purification. Colorless CZ-resin vials (50 ml) were obtained from Daikyo-Seiko, Ltd. (Tokyo, Japan), siliconized gray bromobutyl rubber stoppers Type FM 157/1 from Helvoet Pharma (Alken, Belgium), and aluminum caps (Alu-caps 20.3 × 7.5 mm with center tear off) from Aluglas BV (Uithoorn, The Netherlands).

3.2. Quality control of AP5346 drug substance

3.2.1. Hydrogen (^{1}H) and platinum (^{195}Pt) Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H and ^{195}Pt NMR spectra were recorded on a Bruker AVANCE300 300 MHz spectrometer equipped with a z-axis gradient and a 5 mm multinuclear broad band probe.

The system for the ^{195}Pt NMR was calibrated using K_2PtCl_4 as an external reference at −1624 ppm. The temperature was nominally 22 °C. The ^{195}Pt NMR samples were prepared by dissolving 100 mg drug substance in a total volume of 650 µL H_2O/D_2O (93 : 7). Samples for ^{1}H NMR analysis were prepared by dissolving 20–30 mg in a total volume of 650 µL D_2O containing 0.05% w/w TMS. The spectra were referenced internally with respect to TMS at 0 ppm. Data acquisition was started within one hour after dissolution. This analysis was developed and performed by Access Pharmaceuticals, Inc.

3.2.2. Graphite Furnace Atomic Absorption Spectrometry (GFAAS)

The free platinum content and the release of free platinum were determined using a GFAAS system consisting of a SOLAAR MQZ Zeeman AAS spectrometer, equipped with a FS95/97 autosampler, Merlyn 33 cooler and a GF95 oven controller (all from Thermo Electron Corporation, Breda, The Netherlands). Absorbances were recorded at 265.9 nm with a bandpass of 0.2 nm. Measure time was 3 s. Argon was used to purge the graphite tube. A calibration curve of carboplatin corresponding to 0.5 to 20 µM platinum was prepared and analysis was performed using the temperature program given in Table 4. The method for determining free platinum concentration (i.e. all platinum species with a molecular weight <3 kDa) consisted of the following: AP5346 drug substance was dissolved in water for injections at a concentration of 2.0 ± 0.1 mg/ml and stored at

Table 4: Temperature program of GFAAS analysis

Phase	Temp (°C)	Time (s)[a]	Ramp (°C s^{-1})[b]	Gas flow (L min^{-1})
1	50	1.0	0	0.3
2	85	5.0	17	0.3
3	95	30.0	1	0.3
4	120	20.0	2	0.3
5	250	30.0	5	0.3
6	1400	40.0	30	0.3
7	2700	3.0	0	0
8	2800	4.0	0	0.3
9	50	10.0	0	0.3

[a] Time the temperature remains constant
[b] Velocity at which the temperature is reached

ambient temperature (+20–25 °C) for 1 h. Subsequently, 2 ml samples were ultrafiltrated (45 minutes, 41 × g) 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 the determination of the platinum release characteristics, AP5346 was dissolved in PBS at a concentration of 2.0 ± 0.1 mg/ml and stored at 37 °C. Samples were taken after 3 and 24 h and analyzed as described for the determination of the free platinum concentration. This analysis was developed by Access Pharmaceuticals, Inc and performed at the Slotervaart Hospital.

3.2.3. Size exclusion chromatography

Size exclusion chromatography was performed with an HPLC system consisting of a Spectra System P1000 pump, a Spectra Series AS3000 autosampler and a RI-150 refractive index detector (all from Thermo Separation Products (TSP), Fremont, CA, USA). The mobile phase consisted of 10 mM $LiClO_4$ in 35% methanol (aq.) at a flow rate of 1.0 ml/min. The injection volume was 100 µl and the run time 30 min. Separation was achieved using a PL aquagel-OH guard column (7.5 mm ID × 50 mm, particle size 8 µm, Varian BV, Houten, The Netherlands) and two PL aquagel-OH mixed columns (7.5 mm ID × 30 cm, particle size 8 µm, Varian BV, Houten, The Netherlands) in series kept at a temperature of +35 °C with a Croco-cil® column oven (TSP). The SEC system was calibrated using polyethylene glycol and polyethylene oxide standards (Polymer Laboratories) with molecular ranges from 1,080 to 219,300 Da. Samples were prepared by dissolving 2–3 mg AP5346 drug substance in 1.0 ml mobile phase by gentle swirling. This analysis was developed by Access Pharmaceuticals, Inc and performed at the Slotervaart Hospital.

3.2.4. Infrared spectroscopy

IR spectra were recorded with a Model PU 9706 IR spectrophotometer (Philips, Eindhoven, The Netherlands) using the potassium bromide (KBr) pellet technique. The pellet consisted of approximately 2 mg AP5346 drug substance and 300 mg KBr. The ratio recording mode was auto-smooth and the scan time 8 min.

3.3. Preformulation studies

3.3.1. Sterilization

A solution of AP5346 (Lot AP5346–2-100) 108 mg/ml in water for injection was prepared. Aliquots of 6.0 ml were filled in triplicate in 20 ml type I glass vials. Vials were closed with siliconized grey bromobutyl rubber stoppers and aluminium caps, and sterilized for 15 minutes at 121 °C in a Model 6.6.15 autoclave (Koninklijke Ad Linden B.V., The Netherlands). Subsequently, the sterilized solutions were inspected visually, and analyzed by Size Exclusion Chromatography.

3.3.2. Differential Scanning Calorimetry

Differential scanning calorimetry was performed on a Q1000 V9.0 DSC equipped with a refrigerated cooling accessory (RCS) for low temperatures in T4P mode (TA Instruments, New Castle, DE, USA). Samples of approximately 10 mg AP5346 (125 mg/ml in water for injection) were weighed into aluminium pans. Subsequently, the pans were hermetically sealed and measured against an empty pan as reference. Indium was used to calibrate the temperature and heat flux. Analysis was performed under nitrogen purge at 50 ml/min. The solutions were cooled to −40 °C at a rate of 10 °C/min, and subsequently heated to +25 °C with 1 °C/min.

3.4. Manufacturing process

The manufacturing process was performed aseptically. A formulation solution of 10 mg Pt (as AP5346)/ml in sterile water for injection was prepared. The formulation solution was sterile filtered using a sterile hydrophilic 0.22 µm filter (Millipak® 40, consisting of modified polyvinylidene fluoride (PVDF) membranes in a polycarbonate housing). Aliquots of 40 ml sterile formulation solution were filled into washed and sterilised 50 ml colorless CZ-resin vials (Daikyo-Seiko, Ltd., Tokyo, Japan). Platinum cured silicone tubing (Watson Marlow, Cheltenham, UK) was used for transportation of the formulation solution during filtration and filling processes. The vials were partially closed with grey bromobutyl rubber stoppers (Type FM 157/1, Helvoet Pharma NV, Alken, Belgium) and loaded into the freeze dryer (Model Lyovac GT4 freeze drier, STERIS, Hürth, Germany) at ambient temperature. After lyophilization, the vials were closed pneumatically under vacuum and capped with aluminium caps.

The in-process controls consisted of integrity testing of the filter, weight variation of the filling volume, and determination of the bio-burden before filtration. During freeze-drying the product temperature, shelf temperature, chamber pressure and condenser temperature were continuously monitored. Manufacturing was performed according to the Good Manufacturing Practices (GMP) guidelines (European Commission 1998).

3.5. Quality control of AP5346 final product

For the quality control of AP5346 final product, ^{1}H NMR and ^{195}Pt NMR spectroscopy, free platinum content, release of free platinum, and Size Exclusion Chromatography (SEC) were executed. Furthermore, the appearance (no visible contamination present), reconstitution characteristics, and residual moisture content were determined. The content and content uniformity were measured using UV/VIS analysis. Sample preparation and analysis of the moisture content, UV/VIS analysis and SEC analysis are described below. All other analyses mentioned above were performed as described for AP5346 drug substance.

3.5.1. Size exclusion chromatography

The product was reconstituted with 27.4 ml 5% dextrose, revealing a final volume of 30 ml and a platinum concentration of 13.3 mg Pt/ml. Samples of the final product were prepared by diluting the solution obtained after reconstitution 40 times with mobile phase. Analysis was performed as described for AP5346 drug substance.

3.5.2. Residual moisture content

The residual moisture content of the final product was determined using the Karl Fisher titration method. Immediately after opening of the vial, 500 mg lyophilized product was transferred into the titration unit of a Model 658 KF Titrino apparatus (Metrohm, Herisau, Switzerland). The moisture content was determined in triplicate.

3.5.3. UV/VIS Spectrophotometry

Analysis was performed using a Model UV/VIS 918 spectrophotometer (GBC Scientific Equipment Ld., Victoria, Australia). Calibration curves were prepared with the same batch of drug substance used for the production of the batch to be determined. Samples were prepared by diluting the solution after reconstitution (a solution of approximately 113 mg AP5346/ml 5% dextrose) with 5% w/v dextrose to a final concentration of approximately 135 µg AP5346/ml. The absorption was determined at 240 nm and AP5346 content and content uniformity were assessed.

3.6. Setting of specifications

To estimate the robustness of AP5346 drug substance and final product "The risk of batch failure" (i.e. frequency of failing to meet the specifications) was calculated. This was performed according to the method described by Stafford (1999). The risk of batch failure was estimated by using the means and standard deviations of the analytical outcomes of 3 lots of drug substance and of 4 batches of final product manufactured out of these three lots of drug substance: 3 validation batches with a batch size of 40–56 vials containing 150 mg Pt/vial, and 1 batch with a batch size of 79 vials containing 400 mg Pt/vial.

3.7. Stability of AP5346 final product

Vials of the first three pilot batches (batch 1, 2, and 3, all containing 150 mg platinum/vial) were stored at 5 ± 3 °C and 25 ± 2 °C/60 ± 5% relative humidity, both in the dark. Samples were taken after 0, 3, 6, and 12 months of storage. At each time point, the free platinum content, platinum release characteristics, AP5346 content, moisture content, pH, molecular size and size distribution were determined.

3.8. Stability and compatibility upon reconstitution and dilution

Vials of AP5346 final product were reconstituted with 5% w/v dextrose to yield a concentration of 13.3 mg Pt/ml. The reconstituted product was diluted in triplicate to final concentrations of 0.034, 0.34 and 3.4 mg Pt/ml in 5% w/v dextrose for the compatibility study with infusion containers. The compatibility of AP5346 infusion solution was investigated for three kinds of infusion containers: glass, polyvinyl chloride (PVC, Intraflex®) and low density polyethylene (LD-PE, Ecoflac®). The vials with reconstituted product and the infusion containers were stored at 5 ± 3 °C, in the dark and at room temperature, ambient light. The AP5346 concentration and the amount of free platinum were determined 0, 1, 2, 4, 8, 24, 48, 72, and 96 h after preparation using UV/VIS and GFAAS analysis, respectively. Furthermore, ^{1}H NMR and ^{195}Pt NMR spectra were recorded from the reconstituted products stored at +2–8 °C and +15–25 °C after 0 and 96 h.

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