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Quantitative determination of cesium binding to ferric hexacyanoferrate: Prussian blue☆

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Abstract

Ferric hexacyanoferrate ($Fe_4^{III}[Fe^{II}(CN)_6]_3$), also known as insoluble Prussian blue (PB) is the active pharmaceutical ingredient (API) of the drug product, Radiogardase®. Radiogardase® is the first FDA approved medical countermeasure for the treatment of internal contamination with radioactive cesium (Cs) or thallium in the event of a major radiological incident such as a "dirty bomb". A number of pre-clinical and clinical studies have evaluated the use of PB as an investigational decorporation agent to enhance the excretion of metal cations. There are few sources of published *in vitro* data that detail the binding capacity of cesium to insoluble PB under various chemical and physical conditions. The study objective was to determine the *in vitro* binding capacity of PB APIs and drug products by evaluating certain chemical and physical factors such as medium pH, particle size, and storage conditions (temperature). *In vitro* experimental conditions ranged from pH 1 to 9, to cover the range of pH levels that PB may encounter in the gastrointestinal (GI) tract in humans. Measurements of cesium binding were made between 1 and 24 h, to cover gastric and intestinal tract residence time using a validated atomic emission spectroscopy (AES) method. The results indicated that pH, exposure time, storage temperature (affecting moisture content) and particle size play significant roles in the cesium binding to both the PB API and the drug product. The lowest cesium binding was observed at gastric pH of 1 and 2, whereas the highest cesium binding was observed at physiological pH of 7.5. It was observed that dry storage conditions resulted in a loss of moisture from PB, which had a significant negative effect on the PB cesium binding capacity at time intervals consistent with gastric residence. Differences were also observed in the binding capacity of PB with different particle sizes. Significant batch to batch differences were also observed in the binding capacity of some PB API and drug products. Our results suggest that certain physiochemical properties affect the initial binding capacity and the overall binding capacity of PB APIs and drug products during conditions that simulated gastric and GI residence time. These physiochemical properties can be utilized as quality attributes to monitor and predict drug product quality under certain manufacturing and storage conditions and may be utilized to enhance the clinical efficacy of PB. Published by Elsevier B.V.

Keywords: Prussian blue; Cesium binding; pH-profile; GI model; Particle size; Moisture; Atomic emission spectroscopy; Product quality

☆ This scientific contribution is intended to support regulatory policy development. The views presented in this article have not been adopted as regulatory policies by the Food and Drug Administration at this time.

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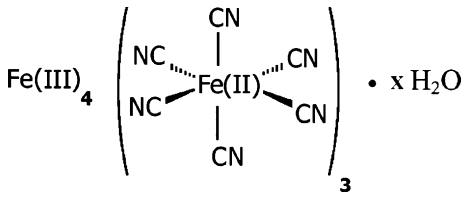

Fig. 1. The chemical structure of Prussian blue.

1. Introduction

Prussian blue (PB) was first prepared in 1704 as a prospective dye and is considered to be the first synthetic coordination compound [1]. PB is currently used as an inorganic pigment (pigment blue 27) whose international color index number is CI 77510 [2]. PB is also known as iron blue, Chinese blue, Hamburg blue, mineral blue, Paris blue as well as a variety of less commonly known names, Brunswick blue, Turnbull's blue and Milori's blue [2,3]. All of these names refer to the blue colored complex of ferric hexacyanoferrate(II) with the empirical formula of $Fe_4^{III}[Fe^{II}(CN)_6]_3$. There are a number of related hexacyanoferrate compounds such as potassium hexacyanoferrate $KFe_3^{III}[Fe^{II}(CN)_6]_3$, $K_2Fe_2^{III}[Fe^{II}(CN)_6]_3$ and ammonium hexacyanoferrate $NH_4Fe_3^{III}[Fe^{II}(CN)_6]_3$ and other metal substituted $MFe_3^{III}[Fe^{II}(CN)_6]_3$ where M = Li, Na, K, Rb, Cu, and Ni that are also called although imprecisely and inaccurately, Prussian blue [4].

The PB crystal structure is a cubic lattice [5,6] with the Fe^{II} and Fe^{III} atoms occupying the corners of the cube [7] and the cyanide group is placed at the sides (Fig. 1). The Fe^{II} atoms are bonded to the carbon atom in the cyano group and the Fe^{III} atoms are bonded to the nitrogen atom in the cyano group. Additionally there are typically 14–16 water molecules coordinated to the PB molecule [1,8]. The cubic structure is retained in aqueous media and due to the stability of the crystal lattice, $Fe_4^{III}[Fe^{II}(CN)_6]_3$ is insoluble.

When given orally insoluble PB is able to bind metal ions and enhance the excretion of radioactive isotopes of cesium (Cs) and thallium ions that are enterically cycled into the gastrointestinal tract, preventing their reabsorption, and thereby reducing the radioactive burden to the body. The mechanism of cesium and thallium binding by insoluble PB is not yet known in full detail although PB has been used since the 1960s to enhance the excretion of cesium and thallium from the body [9–15]. Chemical ion exchange, physical adsorption and ion trapping may all be involved [16,17]. The primary metal binding mechanism for PB is believed to be the monovalent cesium ion exchange with hydrogen ion (H^+) or from water bound (hydronium ion-H_3O^+) in the PB crystal lattice [17]. Additionally if monovalent cations are present within the lattice as a result of different synthetic reagents and routes, cesium may exchange with alkali metal cation impurities such as sodium or potassium or possibly ammonium [18,19]. Interestingly, ion exchange with alkali cations may play a greater role or may even be the primary exchange mechanism in metal hexacyanoferrate analogs such as $KFe_3^{III}[Fe^{II}(CN)_6]_3$ or $NH_4Fe_3^{III}[Fe^{II}(CN)_6]_3$. Additional mechanisms of exchange typically discussed are the physical adsorption of cesium onto the crystal lattice resulting from electrostatic forces and mechanical trapping of cesium ions within the cavities of the crystal lattice.

Cs-133 is a naturally occurring element found throughout the environment. It is an alkali metal that has 32 radioactive isotopes. The two isotopes with the longest radioactive decay half-lives are Cs-137 (30.17 years) and Cs-134 (2.06 years). Both radioactive isotopes decay by emitting beta particles with the resultant decay products (Ba-137 and Ba-134) emitting gamma radiation. Cs-137 is the major source of concern primarily, because it is a nuclear fission fragment that is produced from uranium and plutonium and has a relatively long environmental half-life [20]. It is widely used in industry for thickness and moisture-density gauges and in medicine as a source of gamma radiation used in the treatment of various forms of cancer.

Due to the extensive atmospheric nuclear testing in the 1950s and 1960s, Cs-137 was released into the environment and this radioactive isotope was subsequently detected in humans in 1956 [21]. Early studies were carried out in the 1960s to evaluate the efficacy of various forms of PB as a potential decorporation agent for cesium and thallium [9,22,23]. Early work with PB focused on reducing internal radiocesium contamination in humans following acute exposure [9,10,24,25]. The ubiquitous nature of radiocesium has heightened recent concern about long-term exposure since it is readily transferred throughout the environment [26–33] and into the food chain, remaining for many decades [34–37].

The three most feasible modes of internal contamination of radiocesium are inhalation, oral ingestion and percutaneous absorption. Inhalation [38,39] is the most probable mode of acute radiocesium contamination [40]. Cesium is highly water soluble and readily absorbed into human tissues. It is distributed widely and uniformly throughout the body's soft tissue and eliminated through the kidneys with a small amount excreted in the feces [20,41–43]. Cs-137 is absorbed rapidly and completely with about 10% of the total radioactive body burden excreted by the kidneys primarily through the urine in the first 2 days [20]. Oral treatment with PB results in cesium being bound to the PB and being primarily excreted in the feces [20] Following treatment with PB the physiological cesium urine to feces ratio is reversed from 4:1 to 1:4 [41]. Cs-137 elimination in humans seems to be related to age and gender [44,45] as well as to height and weight [46,47]. The average biological half-life of radiocesium in adult humans is between 50 and 150 days [20,41]. Children have the shortest half-life of approximately of 25–30 days [47]. It is theorized that cesium retention may be related to the mass of skeleton muscle [44].

The Chernobyl nuclear reactor accident in 1986 caused widespread environmental contamination with Cs-137 and Cs-134, which led to extensive studies on the use of PB as a countermeasure for acute and long-term internal radiocesium contamination in humans and animals throughout Europe. The Goiânia tragedy in Brazil in the fall of 1987 provided the opportunity to carry out the first large human trials of PB for the treatment of radiocesium poisoning under temporary IND clearance by the U.S. FDA. The overall results showed that PB

therapy reduced the biological half-life of radiocesium by 43% [41].

Although a number of *in vivo* studies have been published to support the historical use of PB as a decorporation agent, the scientific literature has little information on *in vitro* studies that assess the *in vivo* efficacy of cesium or cesium metal binding to PB under various chemical and physical conditions. Additionally, many of the *in vitro* studies and animal investigations utilized various types of hexacyanoferrates resulting from different starting reagents, synthetic routes and manufacturing drying conditions known to affect binding [17]. In many cases it is not clear which hexacyanoferrates were used. Therefore the applicability of previous results for predicting clinical outcome without a clearer understanding of which hexacyanoferrate analogs were used and an evaluation of their physiochemical properties may be imprecise. Therefore, we have systematically studied the *in vitro* binding capacity of insoluble PB, ferric hexacyanoferrate ($Fe_4^{III}[Fe^{II}(CN)_6]_3$), to cesium under certain physical and chemical conditions.

We report PB tested under various gastrointestinal (GI) pH conditions and transit times based on physiologically relevant conditions. Additionally, this study evaluates the effect of such factors as particle size and storage conditions on the cesium binding capacity of PB, active pharmaceutical ingredients (APIs) and drug products. Cesium binding to different batches of PB API and drug product was studied to identify product quality differences and correlate these data to physiochemical properties that may be utilized as quality attributes to ensure a more standardized and optimum clinical outcome.

2. Material and methods

2.1. Chemicals and reagents

Cesium single element standard (1000 ppm, 1 mg/mL Cs) certified was purchased from High-Purity Standards (Charleston, SC). Cesium chloride was purchased from Aldrich (Milwaukee, WI). Certified buffer solutions (pH 1–9), hydrochloric acid and sodium hydroxide (10N) and desiccant (Drierite) were purchased from Fisher Scientific (Fair Lawn, NJ). Dibasic and monobasic potassium phosphate and potassium hydroxide were purchased from J.T. Baker Inc. (Phillipsburg, NJ). PB active pharmaceutical ingredient was provided by Sigma–Aldrich (St. Louis, MO) and HEYL Corporation (Berlin, Germany). Drug products (500 mg/capsule) were provided by HEYL Corporation (Berlin, Germany) and Oak Ridge Institute for Science and Education (ORISE) (Oak Ridge, TN). Filtered 18 MOhm water was supplied in house by a Millipore Milli-Q System (Bedford, MA). All other chemicals were of reagent grade.

2.2. Preparation of media solutions and standards

2.2.1. Preparation of pH solutions

The solutions of pH 1, 2, 5 and 9 were prepared by diluting corresponding certified buffer solutions fivefold with DI water. The phosphate buffer solution of 40 mM with pH 7.5 was prepared by using dibasic potassium phosphate and monobasic potassium phosphate.

2.2.2. Preparation of calibration standards

Cesium standard (1000 ppm Cs) was used as standard stock solution. Six calibration standard solutions were prepared by transferring 1.5, 2.5, 5.0, 7.5, 10.0 and 15.0 mL of stock solution to 50-mL volumetric flasks and then adding the corresponding pH solution to 50 mL resulting in the final concentrations of 35, 50, 100, 150, 200 and 300 ppm Cs, respectively. Two standard curves, which included standard blanks, were prepared daily.

2.2.3. Preparation of quality control standards

The quality control (QC) cesium stock solution (30 mg/mL Cs) was prepared by dissolving 3.795 g of cesium chloride in 100 mL of DI water. The lower limit of quantification (LLOQ) of 30 ppm, low QC of 50 ppm, intermediate QC of 100 ppm and high QC of 200 ppm were prepared by transferring 0.05, 0.83, 0.167 and 0.334 mL of stock solution to 50-mL volumetric flasks and then adding the corresponding pH solution to 50 mL. Five standards at each QC level were prepared daily.

2.3. Analytical method and validation

All standards and samples were analyzed by atomic emission spectroscopy (AES) using a PerkinElmer (Shelton, CT) AAnalyst 800 atomic absorption/emission spectrometer equipped with an air–acetylene burner. The sample solution was aspirated into an air–acetylene flame for a time period of 5 s. Each sample was analyzed in triplicate by AES. Sample blanks were run between every five samples or standards to assess carry over. Cesium was detected at an emission line of 455.5 nm. The method was validated according to USP Category I requirements. The following validation characteristics were addressed: precision, accuracy, linearity, range and specificity.

Student's *t*-test was performed on both API and drug product data, respectively for comparison applying 4 degrees of freedom at the 95% confidence level ($p < 0.05$).

2.4. pH-profile (600 ppm)

One API batch (API-1) and one drug product batch (DP-1) were selected for analysis. A 0.1 g amount of each PB API or drug product sample batch was added individually to a 100-mL glass flask, 49 mL of corresponding pH solution and 1.0 mL of cesium QC stock solution (30 mg/mL Cs) was added resulting in a final concentration of 600 ppm. The flask was tightly stopped and incubated in a shaking water bath at 37 °C with 75 shakes/min for 1, 4 and 24 h, respectively. Samples were tested at pH 1, 2, 5, 7.5 and 9. All sample batches were prepared in triplicate.

2.5. Concentration profile (600, 750, 900, 1200, 1500 ppm)

Corresponding pH solution (49 mL) and 1.0 mL of cesium stock solution (30, 37.5, 45, 60, or 75 mg/mL Cs) were added

to a 100-mL glass flask, followed by the addition of 0.1 g of PB API to produce a final cesium concentration of 600, 750, 900, 1200 or 1500 ppm. The flask was tightly stopped and incubated in a shaking water bath at 37 °C with 75 shakes/min for 1, 4 and 24 h, respectively. Upon completion of incubation, sample was filtered with a 0.2 μm Acrodisc® syringe filter (Gelman Laboratory, MI), and then 10 mL aliquot of sample was transferred to a glass test tube. The sample was diluted, if necessary. All samples were prepared in triplicate.

2.6. Quality profile-batch to batch variability (600 ppm)

Five different APIs batches (API-1, API-2, API-3, API-4 and API-5) and three different drug product batches (DP-1, DP-2, DP-3) were selected for analysis. A 0.1-g amount of each PB API or drug product sample batch was added individually to a 100-mL glass flask, and 49 mL of corresponding pH solution and 1.0 mL of cesium stock solution (30 mg) was added to produce a final cesium concentration of 600 ppm. The flask was tightly stopped and incubated in a shaking water bath at 37 °C with 75 shakes/min for 1, 4 and 24 h, respectively. Samples were prepared in triplicate and tested at pH 7.5.

2.7. GI profile (cesium binding test following API exposure to low pH solutions sequentially)

PB was sequentially exposed to increasing pH of 1.0, 2.0, 3.0, 5.0 and 7.5. API-1 was selected in this test. For pH 1.0 condition, 0.1 g API-1 was mixed with 49 mL of pH 1.0 buffer solution and 1.0 mL of cesium stock solution (30 mg) at the reference binding concentration (600 ppm) in a 100-mL glass flask. It was incubated in a shaking water bath at 37 °C with 75 shakes/min for 1 h. The test procedure for sample preparation was the same as above. For pH 2.0 condition, 0.1 g API-1 was mixed with 10 mL of pH 1.0 buffer solution and incubated in a shaking water bath at 37 °C with 75 shakes/min for 1 h. It was centrifuged and the solution supernatant was removed. The PB pellet was transferred to a glass flask, and 49 mL of pH 2.0 buffer solution and 1.0 mL of cesium stock solution (30 mg) was added to produce a final cesium concentration of 600 ppm. The same procedure was followed for the remainder of the sample preparation step. For pH 3.0 condition, PB was exposed to pH 1.0 and 2.0 for 1 h each in order and then the sample was prepared in pH 3.0. For pH 5.0 condition, PB was exposed to pH 1.0, 2.0 and 3.0 for 1 h each sequentially and then the sample was prepared in pH 5.0. For pH 7.5 condition, PB was exposed to pH 1.0, 2.0, 3.0 and 5.0 for 1 h each progressively and then the sample was incubated with cesium in pH 7.5 for 1 and 4 h. To ensure that the entire sample preparation procedure did not cause a significant loss of PB, a control experiment was conducted. In the control experiment, PB was sequentially exposed to deionized water for 3 cycles of 1 h each and the sample was prepared in pH 7.5 buffer.

2.8. Drying and storage conditions

2.8.1. Effect of storage conditions (35 °C)

API-1 was selected for analysis. API-1 was placed in drying oven VWR 1305U and dried for 0, 2, 3, 5 and 7 days at 35 °C. The samples were dried to mimic storage conditions in a non-climate controlled warehouse. Samples were removed from the oven and immediately placed in a desiccator with calcium sulfate desiccant until immediate use. A 0.1-g amount of each dried API-1 sample was added individually to a 100-mL glass flask, 49 mL of corresponding pH solution and 1.0 mL of cesium stock solution (30 mg) was added to produce a final cesium concentration of 600 ppm. The flask was tightly stopped and incubated in a shaking water bath at 37 °C with 75 shakes/min for 1, 4 and 24 h. Samples were prepared in triplicate and tested at pH 7.5.

2.8.2. Effect of drying on APIs and drug products (105 °C)

API-1, API-2, DP-1 and DP-2 were selected for analysis. The PB APIs and drug products were placed in a drying oven VWR 1305U, VWR Corporation (West Chester, PA). The samples were dried according to standard USP criteria of 3 h at 105 °C. The PB sample batches were also analyzed in a non-dried state to determine if drying (i.e. the loss of moisture) affects binding. Samples were removed from the oven and immediately placed in a desiccator with calcium sulfate desiccant until immediate use. A 0.1-g amount of each PB API or drug product sample batch was added individually to a 100-mL glass flask, and 49 mL of corresponding pH solution and 1.0 mL of cesium stock solution (30 mg) was added to produce a final cesium concentration of 600 ppm. The flask was tightly stopped and incubated in a shaking water bath at 37 °C with 75 shakes/min for 1, 4 and 24 h, respectively. Samples were prepared in triplicate and tested at pH 7.5.

2.8.3. Effect of drying on API (105 °C)

API-1 was selected for analysis. API-1 was placed in drying oven VWR 1305U and dried for 0, 2, 4 and 24 h at 105 °C. The samples were tested to evaluate cesium binding following systematic drying conditions that may mimic drying time following manufacturing. Samples were removed from the oven and immediately placed in a desiccator with calcium sulfate desiccant until immediate use. A 0.1-g amount of each dried API-1 sample was added individually to a 100-mL glass flask, 49 mL of corresponding pH solution and 1.0 mL of cesium stock solution (60 mg) was added to produce a final cesium concentration of 1200 ppm. The flask was tightly stopped and incubated in a shaking water bath at 37 °C with 75 shakes/min for 1, 4 and 24 h. Samples were prepared in triplicate and tested at pH 7.5.

2.8.4. Moisture loss from Prussian blue

Samples of API-1 were pre-dried at 105 °C for 0, 2, 4 and 24 h and were evaluated for moisture content by thermal gravimetric analysis (TGA) with a TA Instruments-TA 2950 Thermal Gravimetric Analyzer (New Castle, DE). Samples of undried API-2, DP-1 and DP-2 were also evaluated for moisture content. Platinum pans were tared individually, and approximately 10 mg of PB sample was evenly distributed onto each pan. The pans were

Table 1
Parameters of calibration curve ($n \geq 3$)

Solutions	Linear range (ppm)	Calibrators	R^2 value	Slope
pH 1.0	30∼300	7	0.9894	35.47
pH 2.0	30∼300	7	0.9838	31.42
pH 5.0	30∼300	7	0.9990	43.09
pH 7.5	30∼300	7	0.9978	46.36
pH 9.0	30∼300	7	1.0000	43.66

placed in the calibrated instrument under atmosphere of nitrogen flowing at 60/40 mL/min. The heating rate was 20 °C/min from ambient room temperature to 300 °C to determine the weight loss due to moisture.

2.9. Particle size experiments

2.9.1. API particle size distribution determination

PB API-1 was tested to determine the particle size distribution using an ATM Sonic Sifter ATM Corporation (Milwaukee, WI). Nine sieves were used to sift API-1. The sieve fractions were: <20, 20–32, 32–45, 45–63, 63–90, 150–212, 212–300 and >300 μm. One gram of PB was sieved from API-1. Following each sieving step the sieve fraction was weighed on an AE-100 Mettler–Toledo analytical balance (Columbus, OH) and the percentage of particles was determined for each sieve fraction. A mean particle size distribution was calculated for the d_{10}, d_{50} and d_{90} fractions of API-1.

2.9.2. Cesium binding to API particle size fractions experiment (1200 ppm)

Two particle size fractions, <90 and >212 μm that bracketed the major particle size distribution d_{10}, d_{50}, and d_{90} fractions were collected by sonic sifting from API-1 and tested for cesium binding at a cesium incubation concentration of 1200 ppm cesium to evaluate the effect of particle size on the cesium binding capacity of PB. Each particle size fraction and API-1 were prepared in triplicate and tested at 1, 4 and 24 h at pH 7.5.

3. Results

3.1. Analytical method validation

The atomic emission analytical method was found to be selective with no interfering atomic spectral lines or background noise in the reference blanks or controls. The lower limit of quantitation was determined to be 30 ppm cesium. Calibration standard curves were found to linear over the analytical range of 30–300 ppm cesium for pH of 1, 2, 5, 7.5 and 9, respectively (Table 1). Accuracy of the quality control standards at the LLOQ QC-1 (30 ppm), QC-2 (50 ppm), intermediate QC (100 ppm) and high QC (200 ppm) each met the acceptance criteria of <15% for pH 1, 2, 5, 7.5 and 9, respectively (Table 2). Precision of the quality control standards each met the acceptance criteria of <2% for pH of 1, 2, 5, 7.5 and 9, respectively (Table 3). Cesium stock solutions for both calibration and quality control standards were found to be stable for at least 10 weeks.

Table 2
Quality control: accuracy (%, $n \geq 18$)

Solutions	30 (ppm)	50	100	200
pH 1.0	111.8	99.3	97.2	105.3
pH 2.0	108.3	98.40	97.1	106.2
pH 5.0	110.5	112.8	109.9	111.9
pH 7.5	102.7	106.4	109.4	102.1
pH 9.0	99.6	102.3	101.9	105.7

Table 3
Quality control: precision (% R.D.S., $n \geq 18$)

Solutions	30 (ppm)	50	100	200
pH 1.0	0.2231	0.8463	0.8065	0.3696
pH 2.0	0.6226	1.0463	1.0244	0.4667
pH 5.0	1.3826	0.5875	1.0050	0.4938
pH 7.5	0.8724	1.1878	1.3700	0.6686
pH 9.0	0.4442	0.7647	0.6531	0.3145

3.2. The pH-dependent cesium binding profile (600 ppm)

For comparison, the cesium binding was studied using API-1 and DP-1. For both API and drug product, the cesium binding to PB displayed a clear pH-dependent profile as indicated in Fig. 2A and B. The results show that the lowest amount of cesium binding occurred at pH 1.0, followed by a gradual increase in cesium binding as pH increased and was found to be maximal at pH 7.5 for both API and drug product. Cesium binding decreased as the pH increased from 7.5 to 9. For all pH values, the cesium binding increased with

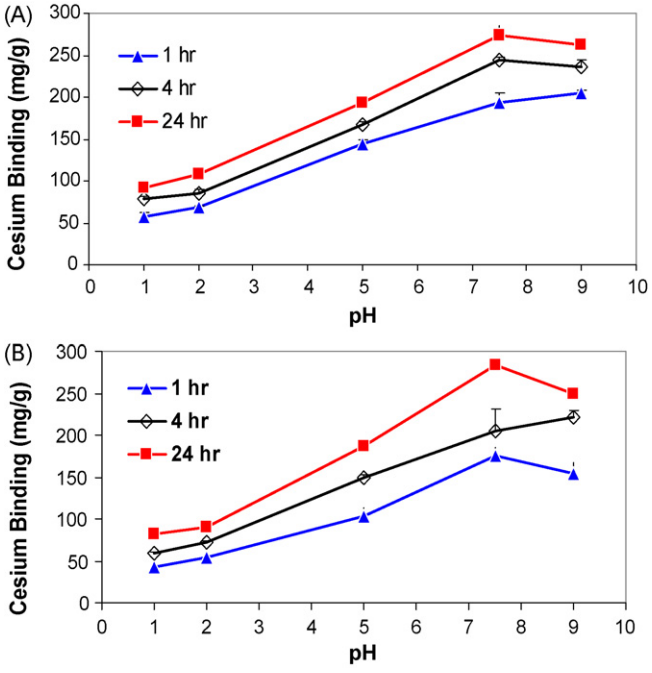

Fig. 2. The pH-dependent cesium binding profile of API-1 (A) and DP-1 (B) were incubated with 600 ppm cesium in corresponding pH solutions 1, 2, 5, 7.5 and 9 at 37 °C for 1, 4 and 24 h, respectively.

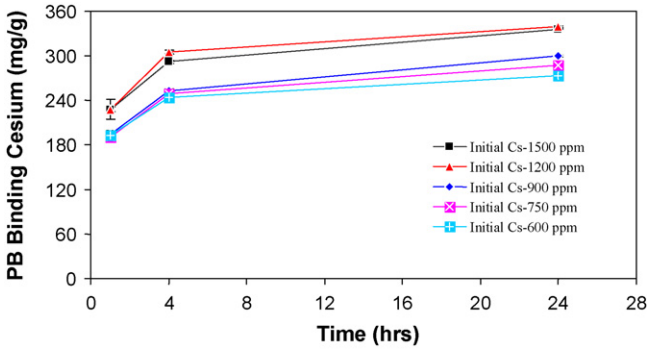

Fig. 3. Concentration dependent profile of API-1. API-1 was incubated 1 at 600, 750, 900, 1200 and 1500 ppm cesium in the pH 7.5 solution at 37 °C for 1, 4 and 24 h, respectively.

incubation time. The highest amount of cesium binding was found at human physiological pH 7.5 for both API and drug product at 24 h, to be 273.5 and 283.4 mg Cs/g PB, respectively.

3.3. Concentration- and time-dependent profile of cesium binding

Results shown in Fig. 3A indicated that increasing the initial incubation concentration of cesium from 600 to 1500 ppm and the exposure the time (1–24 h) resulted in increased amounts of cesium bound to PB API-1. The cesium binding capacity of PB API-1 at each cesium incubation concentration was tested at the maximal binding pH of 7.5. The cesium binding was similar for cesium concentrations of 600, 750 and 900 ppm. Significant concentration dependent effects of cesium bound to PB were observed at higher cesium concentrations of 1200 and 1500 ppm. For 24 h incubation, the highest amount of cesium binding to PB was 339.2 mg Cs/g PB at 1500 ppm cesium compared to 273.6 mg Cs/g PB at 600 ppm. A 2.5-fold increase in concentration (600–1500 ppm) resulted in 20% increase in cesium bound to the same amount of PB (1 g) over 24 h.

3.4. Determination of maximal binding capacity (MBC)

To determine the maximal binding capacity of PB to cesium, binding data from the five cesium concentrations 600–1500 ppm were used. Cesium binding data shown in Fig. 3 to 24 h and was also supplemented with additional longer timepoint experiments (600 ppm) extended to 48 h with API-1 (no statistical difference $p > 0.1245$ between 24 and 48 h 276.3mg/g versus 276.7 mg/g, respectively) to verify that the cesium binding approached equilibrium at 24 h. Therefore, the binding data at 24 h was used for plotting the Langmuir isotherm (Fig. 4). According to the Langmuir equation:

$$C_{xm} = \frac{1}{k_1 k_2} + \frac{C}{k_2}$$

where C_{xm} is the ratio of free to bound cesium, C is the free cesium concentration in mg/L, k_1 is the affinity constant, and k_2 is the MBC in mg Cs/g PB, which is calculated from the slope

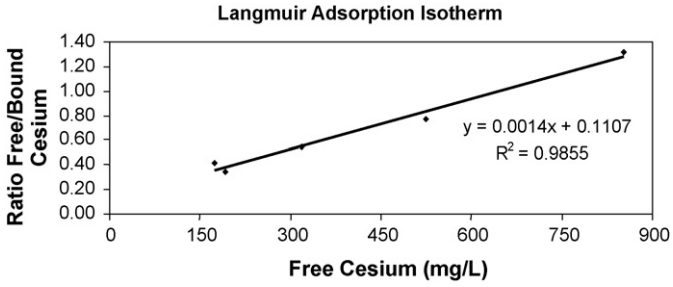

Fig. 4. The concentration-dependent cesium binding profile of API-1 (A). The Langmuir isotherm was plotted based on 24 h binding data with best straight line calculated by linear regression using the least squares method. The *X* axis represents the concentration of free cesium (mg/L) in the solution near equilibrium. The *Y* axis represents the ratio of the concentration of free cesium (mg/L) versus the bound cesium (mg/g) near equilibrium (B).

($1/k_2$) using least squares linear regression. In this study the MBC was found to be approximately 715 mg Cs/g PB.

3.5. Quality profile of cesium binding among batch to batch for PB APIs and products

Five PB API batches and three drug product batches were tested to determine batch to batch variability or quality differences in cesium binding at pH 7.5. Mean results with 95% confidence intervals are shown for APIs in Fig. 5A and drug products in Fig. 5B. For the API batches, API-2 had the lowest and API-5 had the highest cesium binding at all time points. Based on pairwise application of the Student's *t*-test (95% confidence level) each batch of API bound a statisti-

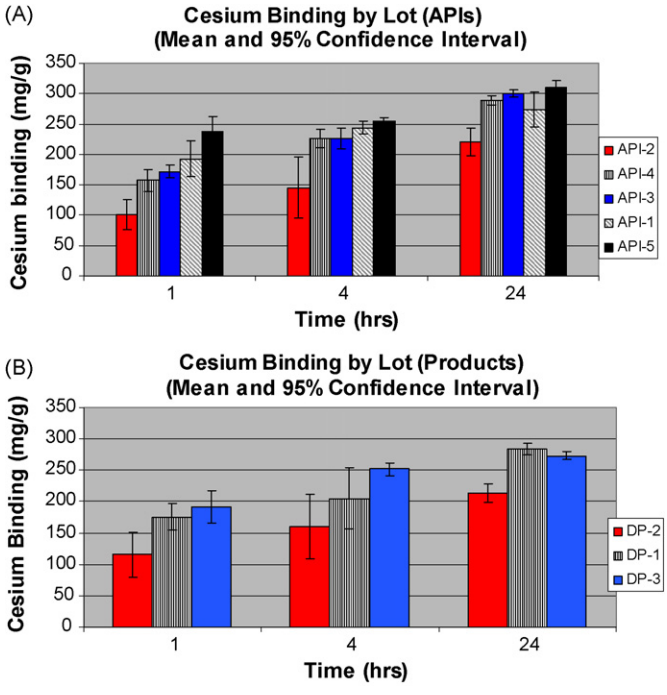

Fig. 5. Analytical comparison and statistical assessment of cesium binding among batch to batch for both APIs and products. API–1, API-2, API-3, API-4 and API-5 (A) and DP-1, DP-2 and DP-3 (B) were incubated with 600 ppm cesium in the pH 7.5 solution at 37 °C for 1, 4 and 24 h, respectively.

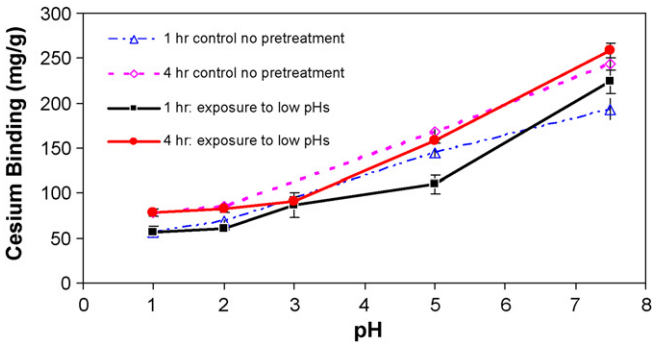

Fig. 6. Effect of increasing pH on the cesium binding of Prussian blue API-1 (*Samples exposed to previous lower pH level for 1 h and then incubated at next pH level for 1 and 4 h). All samples were incubated with 1200 ppm cesium at 37 °C.

cally different amount of cesium ($p < 0.0421$), except that API-3 was not significantly different from API-4 at 4 h ($p > 0.9299$), and API-1 was not significantly different from API-4 at 24 h ($p > 0.0843$). Each DP bound a statistically different amount of cesium ($p < 0.0156$), except that no statistical difference was observed between DP-1 and DP-3 at 1 h ($p > 0.1238$) or DP-1 and DP-2 at 4 h ($P > 0.0562$).

3.6. GI profile (cesium binding test following API exposure to low pH solutions sequentially)

To mimic physiological conditions that would be experienced during residence and transition processes in the GI tract, PB was sequentially exposed to increasing pH of 1.0, 2.0, 3.0, 5.0 and 7.5. As shown in Fig. 6, pre-treatment at lower pH values had no significant effect on the cesium binding at pH 2.0, 3.0 and 7.5. PB pre-treatment at lower pH levels tended to lower the cesium binding at pH 5.0 (25% at 1 h).

3.7. Effect of drying and storage conditions

3.7.1. Effect of storage conditions (35 °C)

API-1 stored at 35 °C over a 7-day period had a negative effect on the cesium binding (Fig. 7). The samples were

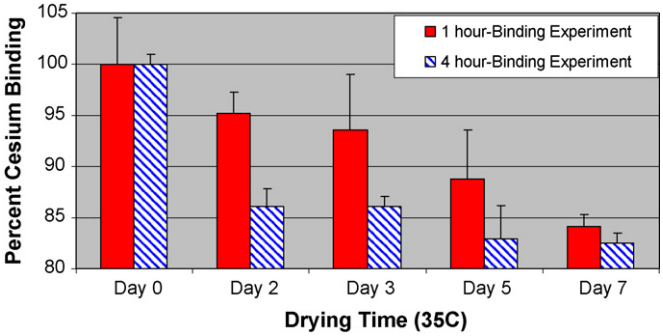

Fig. 7. Effect of drying (35 °C). API-1, was dried for 0, 2, 3, 5, and 7 days at 35 °C and then incubated with 600 ppm cesium in the pH 7.5 solution at 37 °C for 1, 4 and 24 h, respectively. When the incubation was completed, sample was filtered and 10 mL aliquot was used for cesium binding analysis by AES. Day 0 is the control and is designated as 100% binding.

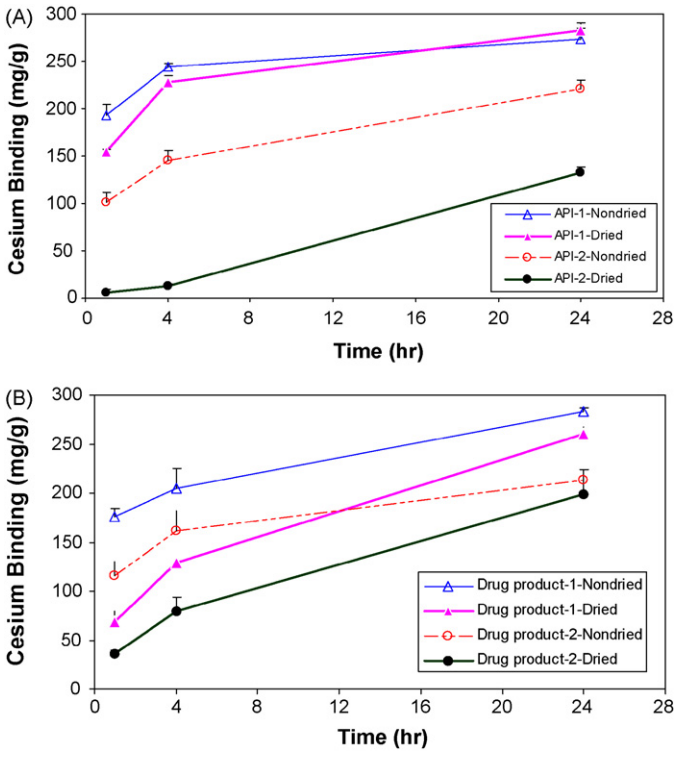

Fig. 8. Effect of drying (105 °C). API-1, API-2 (A) and DP-1, DP-2 (B) were dried at 105 °C for 24 h and then incubated with 600 ppm cesium in the pH 7.5 solution at 37 °C for 1, 4 and 24 h, respectively.

dried to mimic storage conditions in non-climate controlled warehouse. Compared to baseline samples (day 0), the cesium binding following 1 h incubation progressively decreased from 2 to 7 days. For 4 h incubation, most of the decrease in cesium binding (approximately 13–16%) occurred after 2-days of drying.

3.7.2. Effect of drying on APIs and drug products (105 °C)

Fig. 8A indicates that drying the API at 105 °C for 3 h drastically decreased the cesium binding (94% reduction at 1 h incubation) to API-2, but had little effect on API-1 except at 1 h of cesium incubation. Drying the drug product for 3 h decreased the cesium binding to both DP-1 and DP-2 (Fig. 8B) at 1 and 4 h of incubation with 600 ppm cesium. In both cases the effect of drying on cesium binding had diminished by 24 h of incubation with cesium.

3.7.3. Effect of drying on APIs (105 °C)

Fig. 9 indicates that drying API-1 at 105 °C for 2, 4 and 24 h significantly decreased the cesium binding following 1 and 4 h incubation with 1200 ppm, but had a less significant effect on the 24 h dried sample following of incubation with 1200 ppm cesium. Drying API-1 for 24 h decreased the cesium binding by approximately 70 and 45% at 1 and 4 h of incubation with 1200 ppm cesium. In general, a negative effect was observed for cesium binding following of the systematic drying of API-1 over 24 h.

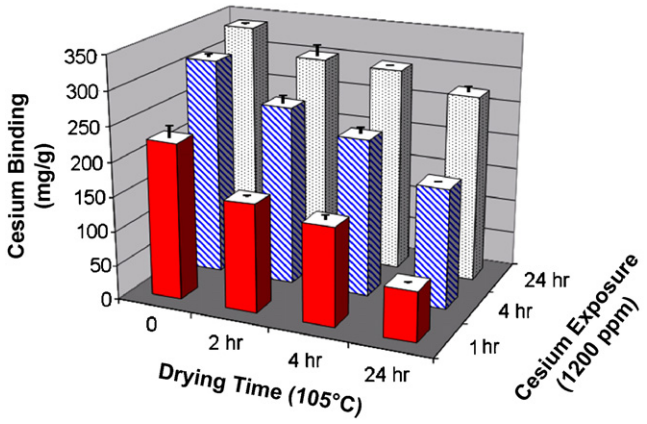

Fig. 9. Effect of drying (105 °C). API-1 was dried for 0, 2, 4 and 24 h at 105 °C and then incubated with 1200 ppm cesium in the pH 7.5 solution at 37 °C for 0, 1, 2, 4 and 24 h, respectively.

3.7.4. Moisture content

Shown in Fig. 10 are the moisture content and cesium binding results for API-1 and a series of API-1 pre-dried samples to achieve lower moisture content levels. TGA determined the undried control API-1 had a moisture content of 26.2%. The prepared pre-dried API-1 samples were determined to have a moisture content of 15.2, 10.5 and 4.75%. A clear trend was observed with the loss of moisture accompanied by a reduction in cesium binding following moisture loss. Samples of undried API-1 with a moisture content of 26.2% bound 227 mg Cs/g PB while the API-1 pre-dried samples with a moisture content of 4.75% bound only 72 mg Cs/g PB following 1 h incubation with 1200 ppm cesium. Overall, cesium binding decreased exponentially as moisture content decreased.

3.8. Particle size

3.8.1. Particle size distribution (API-1)

The mean particle size distributions was calculated for the distribution of the d_{10}, d_{50}, and d_{90} fractions of API-1, and were determined to be 45, 166 and 288 μm, respectively [48].

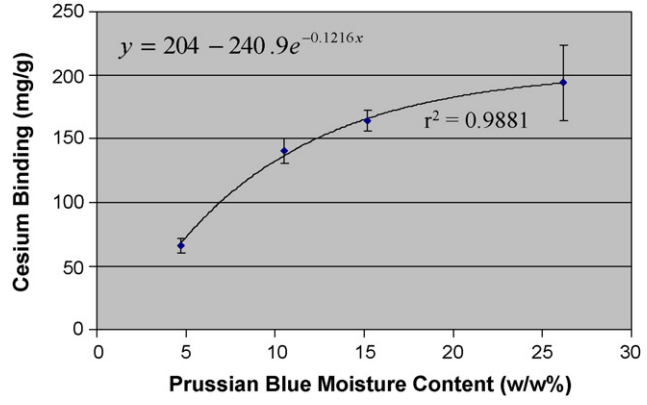

Fig. 10. Effect of moisture content on cesium binding of PB samples containing 4.75, 10.5, 15.2 and 26.2% moisture was determined following incubation with 1200 ppm cesium in the pH 7.5 solution at 37 °C for 1 h. The data represent the mean and standard deviation of triplicate samples fitted with an exponential function.

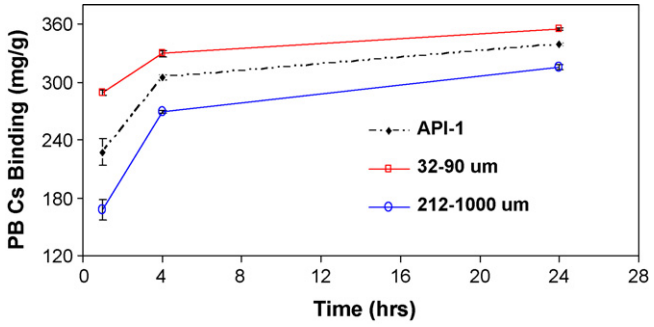

Fig. 11. Comparison of cesium binding among API-1 and API-1 particle size fractions (32–90 um and 212–1000 μm) were incubated with 1200 ppm cesium in the pH 7.5 solution at 37 °C for 0, 0.5, 1, 2, 3 4 and 24 h, respectively.

3.8.2. Cesium binding to different particle size fractions (1200 ppm)

The results shown in Fig. 11 for the two particle size fractions 32–90, and >212 microns and API-1 (non-fractionated control-d_{50} 166 μm) at time 1, 4 and 24 h, indicate cesium binding increases as particle size fraction size decreases.

4. Discussion

Since PB was first introduced as a treatment for internal radiocesium contamination or thallium poisoning in the 1960s, numerous studies have been carried out to evaluate the *in vivo* efficacy of the compound. Few studies however have systematically focused on factors such as pH [49], gastric residence time, GI transit time, particle size [50,51] and storage conditions [52,53]. The results of our study suggest that physiochemical factors such as pH, exposure time, temperature (drying and storage conditions), moisture content and particle size may play important roles in the clinical efficacy of cesium binding to PB.

The pH of the human gastrointestinal tract under fasting conditions, normally ranges from 1.4 to 2.1 in the stomach, 4.9 to 6.4 in the duodenum, 4.4 to 6.6 in the jejunum, and 6.5 to 7.4 in the ileum [54–56]. In some cases Brunner's gland (i.e. duodenal gland) secretions may have pH values between 8.0 and 8.9 [55] and the pediatric colon may reach 8.5 associated with certain disease states. For normal subjects, the average gastric emptying time (80% of content) is approximately 30 min for a low-calorie bland meal, and 3.5 h for a high-fat liquid meal [57,58]. The intestinal residence time in normal subjects varies from 20 to 30 h [57]. Based on documented human GI physiology, pH levels ranging from 1.0 to 9 and incubation times that extend from 1 to 24 h used in the current *in vitro* system it effectively modeled the *in vivo* pH, residence and transit time in the human gastrointestinal track.

Based on our *in vitro* pH-profile data (Fig. 2A and B), the *in vivo* efficacy of PB APIs and drug products may be significantly affected by low pH in the stomach. Cesium binding

at a gastric pH (1–2) was approximately 60% lower than the highest cesium binding at pH 7.5 at all timepoints. It is likely that cesium binding is reduced at low pH due to the greater availability of hydronium ions (H_3O^+) ions, which compete with Cs^+ ions in an effort to bind cyanide in the PB lattice. Interestingly, cesium binding at pH 9 is approximately 15% lower than the maximal cesium binding at pH 7.5 at all timepoints. This may be a result of hydroxide ions binding any hydrogen or hydrodium ion further reducing any cation competition. It is also important to note that longer exposure times throughout the pH range of 1–9 do not significantly increase the amount of cesium binding after 4 h (Fig. 2A and B). These data highlight an important issue that the greatest amount of cesium binding to PB (approximately 80%) occurs rapidly, following the initial period of cesium exposure. Taken together, this information may prove valuable in optimizing dosing schedules.

A notable concern is the effect of gastric pH on the extent of PB binding capacity throughout the GI tract. In the current study, we showed that low pH can have a negative affect on the cesium binding to PB. Therefore, a GI profile experiment was designed to mimic gastric exposure and progressively higher pH present along the GI tract by sequentially exposing PB to low pH solutions and assessing cesium binding at a progressively higher pH. This GI experiment was also conducted to address a longstanding assertion the PB decomposes in acid, thus losing its ability to bind metals such as cesium The GI profile experiment (Fig. 6) demonstrated that following exposure to gastric pH; PB can recover its maximal binding capacity of its average GI *in vivo* pH 5 within 4 h. These data resolve the concerns that PB would be unstable at low gastric pH and would not be able to recover its binding capacity in the GI tract. It is important to note that the significant reduction in PB binding capacity in gastric pH may be controlled through formulation approaches (e.g. pH-specific controlled release) that minimize exposure during gastric transit times. This would ensure a more efficient binding immediately following the dosing of PB. Additionally dosing of PB with bland meals will raise the gastric pH while facilitating the rate of gastric emptying (30–60 min) versus typical high fat meals (1–3 h) and would also ensure a greater initial binding capacity in the gut where PB would bind cesium while minimizing the physiochemical effect of gastric pH on PB.

The concentration profile provides strong evidence that PB cesium binding continues to increase as the cesium concentration increases. These data reveal a truly surprising phenomenon where a 2.5-fold increase in cesium concentration results in a 20% increase in cesium bound to PB over 24 h. Using a classical approach to describe this phenomena the Langmuir adsorption isotherm indicates that the maximum cesium binding capacity is approximately 700 mg/kg. It is important to note that the MBC is an approximate value since equilibrium is never truly reached in vivo and therefore provides an estimate of the maximum binding capacity that has been traditionally useful for toxicology purposes. Overall, insoluble PB has a remarkable capacity to bind cesium over a wide range of pH and concentration conditions.

The quality profile assessment (Fig. 5A and B) revealed a significant variability in cesium binding between the studied lots of PB API and drug product. Because PB is used in the treatment of radiation exposure, it may not be sufficient to just focus the therapeutic value of the product on reducing a maximal amount of cesium over 24 h. As we noted the chemical and physiological properties of radiocesium are especially pernicious due to its very high water solubility and rapid widespread distribution in the body. In addition radiocesium follows the potassium metabolic pathway with a metabolic discrimination ratio of only about 1.5. Therefore, it is essential that the initial rate of cesium binding be as efficient as possible to minimize the radiocesium material available for systemic distribution. Based on *in vivo* studies that evaluated the biological half-life of radiocesium following PB treatment, significant variation has been also observed based in age, gender and weight. These previous *in vivo* study findings coupled with batch to batch variations of cesium binding observed in this study may provide an opportunity to identify quality attributes that may assist with the optimization of the API and dosage form.

These data may facilitate the development of treatments for targeted subpopulations (pediatric, geriatric gender and certain disease states) and thus provide a better overall clinical outcome. For example, the overall extent of PB binding capacity may be greatly reduced in cystic fibrosis patients due to very low gastric pH, relatively low upper GI pH coupled with longer gastric and intestinal residence times. Clinical circumstances as described may require greater attention to dosing with appropriate meals to raise the gastric pH, facilitate gastric emptying and the employment of pH site-selective formulations. Other populations such as adult males or patients with greater body mass may require additional treatment regimes due to the greater retention of radiocesium. Interestingly other subpopulations such as the elderly may actually have overall, a higher therapeutic PB binding capacity as a result of their relatively higher gastric pH levels and generally a higher pH throughout the GI tract. Thus, identifying and ultimately understanding the scientific basis for product variability may better ensure product quality and clinical outcome for a diverse patient population.

The state of PB hydration was shown to have a significant effect on cesium binding. This is most clearly illustrated by the direct relationship between cesium binding and moisture content (Fig. 10). This study highlighted what may be a crucial quality attribute to ensure PB product quality. In general, drying different PB APIs and drug products resulted in a reduction in cesium binding capacity. It should be noted that there was almost full recovery of the cesium binding capacity at 24 h incubation with cesium for the rapidly dried API-1, DP-1 and DP-2 (Fig. 8), indicating that the overall binding capacity was not affected. In contrast, API-2 lost almost 90% of its binding capacity (measured after 1hr cesium incubation) and was unable to fully recover its binding capacity, the maximum recovery, reaching only 70% of the non-dried control at 24 h. This may indicate that besides losing water, the PB lattice of API-2 may have undergone additional physical or chemical changes. TGA measurements (data not shown) determined the moisture content of API-2 and DP-2 to be about 25% less than API-1 and

DP-1, which may account for the reduced binding capacity but this does not fully address the inability of API-2 to fully recover its binding capacity fully at 24 h. These data suggest that there may be a variety of physiochemical factors that may affect the PB binding capacity both *in vitro* and its therapeutic outcome *in vivo*.

Although the cause for the reduced binding is not directly known, it is assumed based on the mechanism of cesium exchange with the hydronium ion (H_3O^+) that these protonated waters are removed from the binding site and not available for cesium ion exchange resulting in binding. Preliminary thermal analysis data and stoichometric calculations determined that API-2 had only 12 bound water molecules versus approximately 16 bound waters for the other APIs. The dramatic loss of binding capacity both before and after drying of API-2 which was manufactured almost 20 years before the other APIs, may be a result of storage conditions or long-term stability (i.e. loss of internal waters of hydration). These data imply a need for a more through understanding of the mechanism of cesium binding and physical factors such as temperature that may significantly affect the overall binding capacity and drug product efficacy.

In order to more fully evaluate the effect of storage temperature on PB, a model storage condition of 35 °C (95 °F) typical for extended periods in various parts of the US (ICH zone III conditions), was selected. Experiments (Fig. 7) over a 7-day period showed a steady reduction in cesium binding at day 2–7 at, 1 and 4 h of cesium exposure. The binding reduction although only 13–16% lower than nominal may indicate that even moderate short-term storage conditions may affect both the initial binding capacity and the short-term efficacy of PB. As noted previously the low binding results for API-2 may be an indication that even moderate long-term storage at less dramatic ambient conditions, may affect PB efficacy. There is only one reference identified in the literature that refers to the binding capacity of PB being unaffected following long-term storage, although no storage conditions or *in vitro* data are provided [53]. Our laboratory is currently evaluating the effect of various long-term storage conditions to further understand the impact of moderate conditions on *in vitro* cesium binding and product quality.

The particle size of PB is a physical property that is typically a result of crystallization and post-synthetic processing. Variations in these processes can result in significant variability in the particle size distribution within the API and dramatic differences from batch to batch. Using sieve fractions from the distribution of API-1, it was demonstrated that the cesium binding capacity is inversely related to particle size. The greater surface area provided by the small particles may enhance cesium binding capacity immediately following dosing while additionally compensating for the negative effect of gastric exposure. API-1 and the large particle size fraction bound only 80% and 60% as much cesium as the small particle size fraction. This illustrates that physical properties such as particle size will also impact product quality of PB. Product performance could be more appreciably impacted during gastric residence if a significant number of PB particles are larger than 160 μm. These data suggest that the particle size of PB should be controlled to ensure the efficient *in vivo* binding of PB.

Overall these data provide a more fundamental understanding of certain physiochemical factors and how they may affect PB metal binding capacity. To ensure both efficient and maximal metal binding and the recognized possibility of minimizing clinical variability for a more consistent clinical outcome, the use of process monitoring tools (e.g. near infra-red spectroscopy; chemical imaging) may rapidly assess quality attributes (i.e. moisture content and particle size, respectively) and allow process control of the manufacture of the API and the long-term stability assessments of the DP. This study provides the scientific basis for utilizing certain physiochemical properties as quality attributes for evaluating drug product quality with the potential for optimizing the *in vivo* efficacy of PB.

5. Conclusions

Physiochemical factors such as, pH, exposure time, moisture content and particle size play important roles in cesium binding to PB APIs and drug products. A clear pH-profile has been established from low to high pH that models the stomach and GI tract. Gastric pH does not decompose the Prussian blue molecule but negatively affects the initial binding capacity (consistent with gastric exposure time) but may not significantly affect the extent of the *in vivo* binding capacity of PB as it travels through the GI tract (consistent with GI residence and transit time). Interestingly, the pH-profile for cesium binding is the inverse of the pH-profile for cyanide release (a side effect) from PB (hexacyanoferrate) observed by Yang et al. [59,60]. Taken together, the results from both studies provide evidence that PB is optimally safe and efficacious at pH 7.5.

Increasing concentrations of cesium and increasing exposure time demonstrated that PB has a seemingly elastic capacity to bind increasing amounts of cesium. Prussian blue was determined to have a maximum *in vitro* binding concentration (MBC) for cesium of approximately 715 mg/g indicating it has a significant intrinsic capacity to bind radiocesium to effectively address a serious radiological threat like a “dirty bomb”.

Drying or storage at elevated temperatures significantly reduces cesium binding to PB. This phenomenon was shown to be a related to the moisture content of the PB molecule. Therefore, the long-term stability of PB in suboptimal storage conditions or manufacturing processes may reduce the moisture in the PB molecule thus having a significant negative effect on the PB binding capacity. This may pose a serious product quality issue if proper storage conditions are not observed for the drug product. This highlights the need to periodically monitor the moisture content of PB products in long-term storage such as the national stockpiles to ensure their product quality.

The significant variations in cesium binding observed from batch to batch for both API and DP may indicate that further control of the manufacturing process is necessary to optimize the physiochemical properties (moisture content and particle size) of PB to better ensure product quality. This PB variability may have a minimal impact on the general population, but pose a greater risk to certain subpopulations creating a significant therapeutic

variability affecting the *in vivo* PB efficacy and the long-term clinical outcome. Therefore the use of certain physiochemical properties as quality attributes may optimize the dosage form to better ensure the product quality and standardize the therapeutic benefit of PB.

In conclusion, this systematic study of cesium binding to insoluble PB has provided a more comprehensive understanding of certain physiochemical properties. The monitoring and control of these properties through manufacturing (particle size and moisture content) and storage (moisture content), alternate formulations (pH), dosing of PB relative to meals (pH) and exposure time may provide the scientific and therapeutic basis for more effective clinical treatment for diverse patient populations in the event of a radiological incident.

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