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# Method development and quantitative estimation of Prussain Blue in bulk drug and Radiogardase-Cs by UV spectroscopy

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Insoluble Prussian Blue (PB) is a well established antidote for removal of cesium and thallium and its radioisotopes. Radiogardase-Cs is the only commercially available, capsules formulation of PB. Although PB is an intense blue colored powder but being an insoluble drug, quantitative estimation of PB is a challenge for researchers. Therefore, present research article is focused on the development of a new, simple and sensitive spectrophotometric method for determination of PB in bulk drug samples as well as formulations like Radiogardase. The method is based on two-step dissolution process. PB reacts with dilute base to form iron(III) hydroxide which reforms ferric hexacyanoferrate on reacting with acid. The resulting solution forms nanosuspension of PB which do not aggregate, and remain suspended in the aqueous solution and shows an absorption maxima at 710 nm. The reformed PB was characterized by infrared spectroscopy and particle size distribution analysis. Based on the UV-Visible spectral studies, analytical method for the determination of PB was developed and requisite validation parameters were performed. A linear response was observed in the range of 0.1–100 μg/ml with a regression coefficient of 0.9997. The LOD and LOQ were found to be 0.099 and 0.330 μg/ml, respectively.

Keywords: PB, spectrophotometry, method development, method validation, decorporating agent.

## Introduction

Prussian Blue, $Fe_4[Fe(CN)_6]_3$, is a solid pigment that was first synthesized in 1704 by Berlin artist Diesbach[1]. The compound has since been used by artists around the world as a source of deep blue color. Chemists use the formation of PB as a qualitative test for the presence of iron in solution, and numerous biological research procedures employ it as a stain. PB have drawn the attention of researchers due to its magnetic properties[2]. PB and its synthetic analogues have been used in the preparation of ferronanomagnetic particles[3]. PB has the ability to form a wide range of metal hexacyanoferrates and its ion exchange properties, play an important role in trapping other heavy metal ions[4].

Since 1960, it has become the drug of choice for the removal of radioactive cesium from nuclear waste and in treatment of thallium poisoning[5]. PB was used as a decorporating agent in humans for the removal of $^{137}Cs$ in 1987 Goinia tragedy[6]. The proposed mechanism of action of PB involves ion exchange, mechanical trapping or physical adsorption. World Health Organization included PB in Model List of Essential Medicines as a specific antidote for radionuclide poisoning. The U.S. Food and Drug Administration approved the PB under the name Radiogardase® as a decorporation agent for radioactive cesium ($^{137}Cs$) and thallium ($^{201}Tl$). The recommended daily dose of Radiogardase® (PB insoluble capsules or ferric hexacyanoferrate(II) capsule) is 3–10 g for adults. PB insoluble, after oral ingestion, is not absorbed through the intact gastrointestinal wall. It acts by ion-exchange, adsorption, and mechanical trapping within the crystal structure. The insoluble complex is excreted without being absorbed from the intestinal walls (Radiogardase (PB) US-FDA)[7]. Although, this formulation of PB is available commercially, but it is difficult to quantitatively estimate the PB content in it. So far no analytical method has been reported for the quantitative estimation of insoluble PB in bulk drug and its formulations which is an extremely important aspect of formulation development. Therefore, the present study is

aimed to develop a UV spectrophotometric method of determination of PB in bulk drug and pharmaceutical dosage forms.

*Materials and method:*

*Materials and instrumentation:*

All absorbance and spectral determinations were carried out on a Biomate single beam UV-Visible spectrophotometer (model UV-1601) using glass cuvette of 10 mm path length. The Visionlite software was used for absorbance measurements and spectral determinations. The baseline correction was made by the built-in baseline memory at the initializing period while auto-zero adjustment was made by one-touch operation[8]. PB insoluble (iron(III) hexacyanoferrate) was procured from Tokyo Chemical Industry (TCI) Co. Ltd., Tokyo, Japan. Analytical grade NaOH was obtained from Sigma Aldrich, Sweden. General purpose $H_2SO_4$ was obtained from Merck Chemicals Limited, Worli, Bombay.

*Method development:*

*Preparation of stock solution and working solution:*

Stock solution of PB was prepared by adding 1 ml of 4 *N* NaOH in 10 mg of PB. The mixture was incubated for 10 min and then 200 μL of conc. $H_2SO_4$ was added. This solution was diluted to make a stock solution of 1 mg/ml nano-suspension of PB. Working solution in the range of 0.1–100 μg/ml solutions were prepared.

*Determination of $\lambda_{max}$ and preparation of calibration curve:*

The working solutions were scanned with UV-Visible spectrophotometer in the range 400–1000 nm against water as blank and $\lambda_{max}$ corresponding to maximum absorbance was found at 710 nm. The calibration curve of PB was plotted by taking concentration on X-axis and absorbance on Y-axis[9].

*Method validation:*

*Linearity:*

The linearity of the method was determined by preparing calibration curves of absorbance versus the concentration of PB. Various aliquots ranging from 0.01–100 μg/ml were prepared from the stock solution (100 μg/ml). The samples were scanned in UV-Vis spectrophotometer against blank and absorbance at 710 nm was measured[10].

*Accuracy:*

Accuracy of the method is determined by standard addition method at 3 levels. Standard quantity equivalent to 80%, 100% and 120% of the test concentration was added in sample using PB working solution as per the method[11]. The absorbance of each solution was measured using UV spectrophotometer in triplicate.

*Repeatability, intra-day and inter-day precision:*

Precision of the method was demonstrated by repeatability, intra-day and inter-day variation studies. The repeatability of the method for PB was determined in range of 5–100 μg/ml for six times. In intra-day variation study three different solutions of concentration 20, 40 and 60 μg/ml were analyzed three times in a day i.e. at morning, afternoon and evening. In the inter-day variation studies, solution of same concentration i.e. 20, 40 and 60 μg/ml were analyzed three times for the three consecutive days[11]. The concentration was calculated using the calibration curve. The absorbance, mean, standard deviation and %RSD were calculated.

*Robustness:*

Robustness of the method was determined by carrying out the analysis under temperature condition i.e. at 5 and 35°C. The respective absorbances of 40 μg/ml were noted and the result was indicated as %RSD[12].

*Ruggedness:*

Ruggedness of the method was determined by carrying out the analysis of sample containing 20 μg/ml test drug for six times. The absorbance was measured on two different instruments Biomate and Beckman Coulter DU730 UV/Vis spectrophotometer under same operational and environmental conditions, and the respective absorbance was noted. The result was expressed as %RSD[13].

*Limit of detection (LOD) and limit of quantification (LOQ):*

The limit of detection (LOD) and limit of quantification (LOQ) of the developed method was determined by the standard deviation of the y-intercept and slope of the calibration curve[14]. The following formulas were used to calculate the limit of detection and limit of quantification value

$LOD = 3.3\ \sigma/S$

$LOQ = 10\ \sigma/S$

where '$\sigma$' is the standard deviation of the intercept and 'S' is the slope of the calibration curve.

*Infrared spectroscopy:*

10 mg of PB was reacted with 1 ml of 4 *N* NaOH at room temperature. A reddish brown precipitate of iron(III) hydro-

xide was formed. After an incubation of 10 min 200 µL of conc. sulphuric acid was added. This mixture was dried at 80°C. The dried powder was analysed using Perkin-Elmer FTIR 2000 spectrophotometer.

*Particle size analysis:*

A dilute suspension of the native PB was made in distilled water. The particle size distribution was studied in Zeta particle size analyzer, Malvern Instruments Limited, Worcestershire WRCS. The mean diameter and polydispersity were calculated by subjecting the data to Particle Sizing Software Ver. 3.42. Similarly the particle size analysis of reformed PB was calculated.

*Drug assay in Radiogardase-Cs capsules:*

10 mg of Radiogardase-Cs capsules content was incubated with 1 ml of 1 *N* NaOH for 24 h followed by the addition of 200 ml of conc. $H_2SO_4$. The drug content in this solution was estimated UV spectrophotometrically using the above method.

## Results

*Method development and optimization:*

PB is completely insoluble in aqueous and organic solvents. During the method development phase, PB was made soluble for UV analysis by reacting with 4 *N* NaOH and conc. $H_2SO_4$. The solutions containing PB were scanned in the wavelength range of 400 to 1000 nm and the maximum absorbance ($\lambda_{max}$) was shown at 710 nm. The spectra of PB at different concentrations were depicted in Fig. 1. Calibration curve (Fig. 2) was obtained in the range of 0.1–100 µg/ml and their optical characteristics were represented in Table 1.

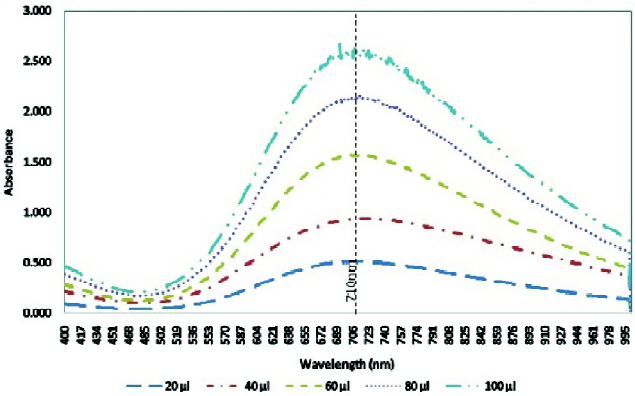


**Fig. 1.** UV spectra of PB at different concentrations.

*Validation of method:*

*Linearity:*

The calibration curve obtained was evaluated by its correlation coefficient. It was found that the drug shows linearity between the selected ranges of 0.1–100 µg/ml with a correlation coefficient ($R^2$) of 0.9992.

*Accuracy:*

The recovery result showed that the proposed method has an acceptable level of accuracy for PB which is from 80–

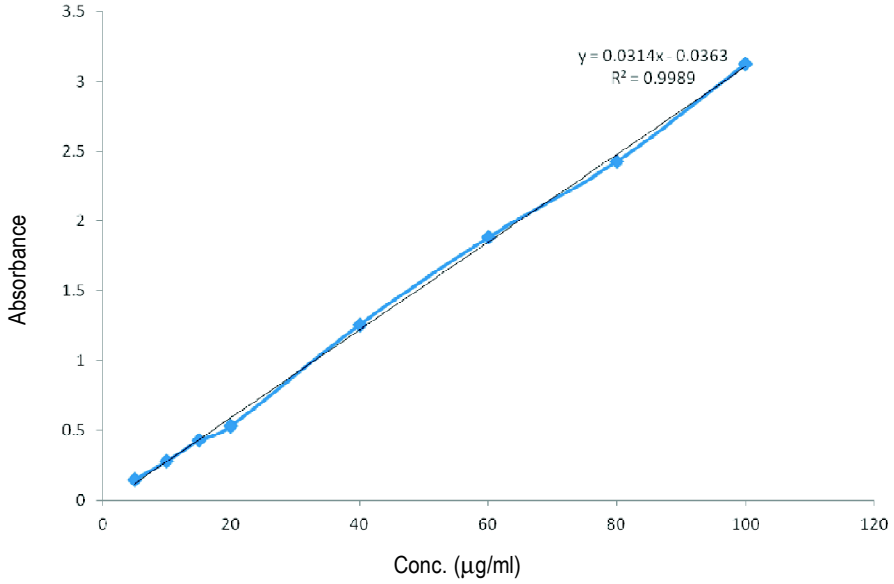


**Fig. 2.** Calibration curve for PB at 710 nm.

**Table 1.** Recovery, intra-day and inter-day accuracy, robustness and precision of PB determination

| PB concentration (μg/ml) | % Recorvery (n = 3) %±SD | Intra-day (n = 6) Accuracy | Intra-day (n = 6) Precision RSD | Inter-day (n = 6) Accuracy | Inter-day (n = 6) Precision RSD | Robustness 5°C (%±SD) | Robustness 40°C (%±SD) |
|---|---|---|---|---|---|---|---|
| 20 | 98.85±0.190 | 99.51 | 0.2432 | 100.34 | 0.154 | 98.45±1.29 | 98.95±0.911 |
| 40 | 102.09±0.177 | 102.94 | 0.446 | 102.67 | 0.447 | 102.87±0.0478 | 102.95±0.336 |
| 60 | 99.73±0.1749 | 103.3 | 0.779 | 102.59 | 0.769 | 102.45±0.396 | 101.78±0.346 |

120%. The percentage recovery of each sample was written against each concentration. The best recoveries of test concentration are from 98 to 102%. These results indicate that the method was accurate.

*Precision:*

The precision of the developed method was calculated by performing repeatability, intra-day and inter-day precision study (Table 1). Repeatability was determined by analyzing the samples in the range of 5–100 μg/ml three times. Repeatability of the method was evaluated by its correlation coefficient ($R^2$). The $R^2$ was found to be 0.997, 0.998 and 0.998 indicated the method was repeatable each time.

Inter-day and the intra-day precision study were performed in nine determinations at three concentrations in each study covering the specified range. The precision was determined by calculating relative standard deviation (%RSD) of the mean recoveries. The developed method confirmed adequate sample stability and method reliability where all the RSDs were <2% (Table 1).

*Ruggedness:*

The ruggedness of the proposed method was evaluated by applying the developed procedures to assay 20 μg per ml of PB using two different instruments under the same optimized conditions on different days. No significant difference was observed in both the instruments. The relative standard deviation was found to be 1.37111 which is less than 2. Thus, the proposed methods could be considered rugged.

*Robustness:*

The robustness of the method was evaluated by analyzing the solution containing 40 μg/ml of the drug at two different temperatures. The %RSD was found to be less than 2. So we can consider the method to be rugged (Table 1).

*Limit of detection:*

The limit of detection and limit of quantification of PB by the proposed method was determined using standard deviation method with calibration standards. The LOD and LOQ of the proposed method were found to be 0.099 μg/ml and 0.330 μg/ml respectively indicating that the method developed is sensitive and without the interference of any other substances.

*Infrared spectroscopy:*

IR spectrum of pure PB gave a high intensity peak at 2083 $cm^{-1}$, which is characteristic of the CN stretching vibration[3,15] as shown in Fig. 3a. Other low intensity peaks at 3746 and 1416 $cm^{-1}$ were also observed. The reformed PB's IR spectrum also showed the CN stretching vibration peak at 2076.06 (Fig. 3b). However, the broadening and increase in the intensity of the peaks indicate that water for crystallization has increased in the reformed PB.

*Particle size analysis:*

The particle size analysis of native PB by laser light scattering gave a mean particle diameter of 14.095 μm with a polydispersity of 0.600. The reformed PB has a mean particle size diameter of 193.9 nm and 0.267 polydispersity. Reformed PB when suspended in water results in the formation of an aqueous solution and this suspension of nanometric particles is called colloid suitable for estimation by UV spectrophotometery. The particles of PB nano-suspension were found to remain suspended for 24 h thus enabling the detection feasible till 24 h.

*Drug assay in Radiogardase-Cs capsules:*

Drug assay in bulk drug and formulations was carried out. Radiogardase-Cs capsule (carried out in triplicate) were found to contain 67.058± 3.456% which indicated more than 98% accuracy of the developed analytical method. Drug assay in bulk drug was carried out in two more samples purchased from Avra Chemicals and Senova Pharmaceuticals. The percentage content of PB was found to be between 98–102% accuracy.

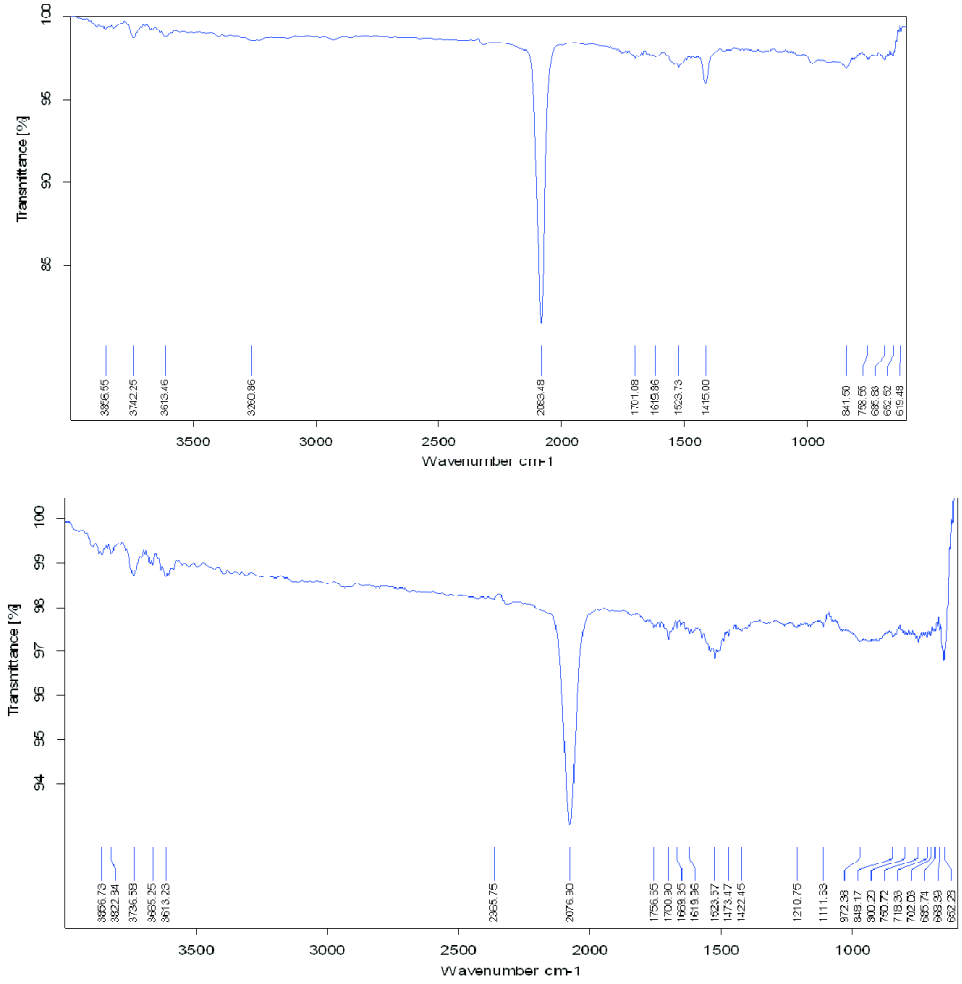


**Fig. 3.** IR spectra of (a) PB and (b) reformed PB.

## Discussion

PB is insoluble in water, organic or inorganic solvents. It is sparingly soluble in 1 *M* oxalic acid[16]. It is characterized by infrared spectroscopy and X-ray powder diffraction studies. Sebastein *et al.* (2000) prepared and characterized the nanoparticles of PB of various particle sizes by transmission electron microscopy and electron diffraction pattern[17]. The absorption band at around 2087 $cm^{-1}$ is a CN stretching vibration, typical of solid PB[3]. Scholz *et al.* have used the decadic absorbance at 2083 $cm^{-1}$ for the quantitative analysis of PB load in goethite[3]. Nagaraja *et al.* in 2000 have determined phenothiazines spectrophotometerically by oxidation of the drug with iron(III) and subsequent chelation with ferricyanide to form a PB colored product.

Although PB is insoluble in organic and inorganic solvents, the two-step dissolution process resulted in the formation of a nano-suspension of PB. When dilute base (sodium hydroxide) is added to PB, a brown precipitate of ferric hydroxide is formed.

$$Fe_4[Fe(CN)_6]_3 + 12NaOH \rightarrow 4Fe(OH)_3 + 12Na^+ + 3[Fe(CN)_6]^{4-}$$

On addition of acid to this, a blue color appears, if enough pigment is present. This is due to the dissolution of iron(III) hydroxide, followed by reformation of the ferric ferrocyanide:

$$(1)\ 2Fe(OH)_3 + 3H_2SO_4 \rightarrow 2Fe^{3+} + 6H_2O$$

$$(2)\ Fe^{3+} + [Fe(CN)_6]^{4-} \rightarrow Fe_4[Fe(CN)_6]_3$$

In the present work, the aqueous solution of the reformed PB was analysed spectrophotometrically with absorption maxima at 710 nm. Based on this, an analytical method was

developed and validated as per ICH guidelines for the determination of PB in bulk drug and pharmaceutical dosage forms. All the parameters including linearity, accuracy, precision, ruggedness and robustness were found in satisfactory range thereby establish this method as an appropriate method for the quantitative estimation of PB in bulk drug and pharmaceutical dosage forms. The LOQ and LOD were also found to be 0.099 μg/ml and 0.330 μg/ml respectively showing the method being highly sensitive.

Additional parameters to characterize and compare the reformed PB with PB were carried out by IR spectroscopy and the particle size analysis. The IR spectrum of the PB showed a high intensity peak at 2087 $cm^{-1}$, which is characteristic of the CN stretching vibration[3,14]. The reformed PB's IR spectrum also showed the CN stretching vibration peak at 2076. However, the broadening and increase in the intensity of the peaks at 3257 and 1616.88 $cm^{-1}$ indicated that water for crystallization has increased in the reformed PB thereby resulting in a nano-suspension of PB. Further the particle size analysis of native PB by laser light scattering gave a mean particle diameter of 14.095 μm with a polydispersity of 0.600. The reformed PB showed a mean particle size diameter of 193.3 nm and 0.267 polydispersity resulting in a nano-suspension of PB nano-suspension which was found to remain suspended for 24 h thus enabling the detection.

## Conclusion

The IR and UV-Visible spectroscopic studies of reformed PB suggest that the acid-base treatment results in the formation of PB with a decrease in the particle size of 100 folds. The nanometric particles of the PB when estimated spectrophotometrically at 710 nm was quantified over a concentration range of 0.1–100 μg/ml with correlation coefficient of 0.9997. The developed method was found to be precise as the %RSD values for intra-day and inter-day were found to be less than 2%. The method was also found to be specific, indicated by the % recoveries ranging from 98 to 102%. The LOD and LOQ were found to be in the microgram level indicating the sensitivity of the method. The method was also found to be robust and rugged as indicated by the %RSD values, which were less than 2%. Further the quantitative estimation of PB content in Radiogardase-Cs with more than 98% established the developed method for detection of insoluble PB in different pharmaceutical dosage forms and for bulk drug assay.

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