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ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 1358–1363
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Validation of an *in vitro* method for the determination of cyanide release from ferric-hexacyanoferrate: Prussian blue

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Received 21 August 2006; received in revised form 30 October 2006; accepted 3 November 2006
Available online 14 December 2006

Abstract

Prussian blue (PB), ferric hexacyanoferrate, $Fe_4[Fe(CN)_6]_3$ is indicated for the treatment of known or suspected internal contamination with radioactive cesium, radioactive thallium, or non-radioactive thallium. Owing to the molecular properties, cyanide is likely dissociated from PB under physiologically relevant pH conditions, thus raising a concern for the safety of the product. The objective of this study was to calibrate and validate a cyanide assay over a wide pH range (from 0.5 to 12) on the basis of Spectroquant cyanide test method (Merck). Merck's photometric method requires that the measurement solution be within pH 5.5–6.0, hence samples and standards need to be adjusted to this pH range. Since the process of pH adjustment may have significant impact on the determination of cyanide, the analysis method needs to be optimized, calibrated and validated under each pH condition in the study. The validation characteristics included accuracy, precision, quantification limit, linearity, and stability. The intra-day accuracy ranged from 90% to 109% for the deionized water and solutions of pH 0.5–12. The intra-day precision (R.S.D.) ranged from 2.4% to 8.1% for the deionized water and solutions of pH 0.5–12. The analytical range was linear from 0.05 to 0.5 ppm (mg/L). The R^2 ranged from 0.9925 to 0.9998. This validated method was successfully implemented to determine cyanide release from PB under various pH conditions (from 1.0 to 12) at different time-points (from 1 to 24 h).
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Keywords: Cyanide; Prussian blue; Spectroquant cyanide-test kit; Method validation

1. Introduction

Prussian blue (PB) also know as ferric-hexacyanoferrate [1,2], $Fe_4[Fe(CN)_6]_3$ shown in Fig. 1 has been used as an investigational agent for several decades to enhance the excretion of cesium-137 and thallium from the body [3–7]. Orally administered PB absorbs cesium and thallium in the gut thereby interfering with their enterohepatic circulation causing a reduction in radioactive body burden [8]. The Chernobyl nuclear reactor accident in 1986 caused the widespread contamination to many areas of Europe with both cesium-137 and cesium-134, which led to extensive studies on the use of PB as a countermeasure for reduce radiocesium levels in humans as well as in a wide range of animals in the countries affected by Chernobyl fallout [9–13]. Tragically, the Goiânia accident in Brazil in September 1987 resulted in approximately 250 individuals being exposed to radiocesium [14,15]. Although this event was a terrible radiation disaster it provided the opportunity to carry out the first large human trial on PB for treatment of radiocesium poisoning. The results showed that PB therapy reduced the biologic half-life of cesium by approximately 43–69% [16].

In response to the need for medical countermeasures in the event of a terrorist attack such as "dirty bomb" the FDA

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[1] 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.

0731-7085/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jpba.2006.11.010

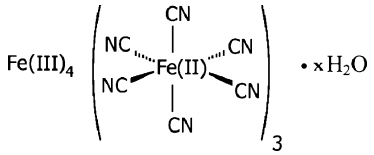

Fig. 1. The chemical structure of Prussian blue.

requested submissions of New Drug Application (NDA) for PB drug products. In March 2003, the FDA received its first marketing application in response to the agency's call. On 2 October 2003, the FDA approved an NDA for Radiogardase, also known as PB, giving the nation the first drug that can be used as a medical countermeasure to the threat of radioactive cesium. Radiogardase capsules contain ferric-hexacyanoferrate, $Fe_4[Fe(CN)_6]_3$. It is currently manufactured by HEYL Chemisch-pharmazeutische Fabrik GmbH & Co. KG. of Berlin, Germany.

Oral administration is the only dosing route for PB. Toxicological studies of PB in variety of animals, found no specific toxicity but determined that cyanide can be released from the parent drug [17,18]. Chemically, free cyanide may be released as a result of protonation of CN on the surface of the metal–ligand complex. Additional exposure time may result in decomposition of the compound under certain physiologically relevant pH conditions, especially under very low pH conditions found in the stomach. This raises a concern for the safety of the product. Under fasting conditions, the pH ranges in the gastro-intestinal tract vary from 1.5 to 2.0 in the stomach, 4.9 to 6.4 in the duodenum, 4.4 to 6.4 in the jejunum, 6.5 to 7.4 in the ileum and 7.4 in colon [19]. There is evidence of pH levels being as high as 8.9, in some cases Brunner's gland (i.e. duodenal gland) secretions may have pH values between 8.0 and 8.9 [20]. Thus, physiological conditions may range from low pH to slightly alkaline conditions.

Several factors have hampered analysis of cyanide-related toxicity from PB exposure. First, the *in vivo* determination of cyanide concentration during PB treatment in the setting of cesium or thallium poisoning seems not practical. Second, it is difficult to differentiate between the toxicity associated with cyanide released from PB from that attributed to cesium or thallium poisoning itself in clinical practice. Given the above facts, it is necessary to develop an *in vitro* method to monitor the PB cyanide release profile under the physiologically relevant conditions to serve as an *in vivo* surrogate.

Most determinations of cyanide are based on acidification of the sample and liberation of the formed hydrocyanic acid (HCN). The evolved HCN may be analyzed by different methods, such as the Conway microdiffusion cell, the detector Dräger HCN tube, or the head-space technique [21,22]. The methods are not only cumbersome and complex for routine use, but the acidification procedure during analysis makes them unsuitable for our simulation studies. The use of the Spectroquant cyanide-test kit from Merck to determine cyanide appears to be a simple and fast analytical method for the determination of free cyanide. However, this method requires that the measurement solution be within pH 5.5–6.0, hence samples and standards need to be adjusted to this pH range. The process of pH adjustment has significant impact on photometric measurement of cyanide, especially when the starting pH values are below 3 or above 7. The objective of this study was to validate a cyanide assay over a wider pH range (from 0.5 to 12) on the basis of Spectroquant cyanide test method while addressing the validation characteristics of accuracy, precision, specificity, linearity and analytical range and limit of quantitation to provide a fast and simple method to monitor the PB cyanide release profile under both physiologically relevant conditions and extremely high pH conditions that may be encountered in some environmental wastes streams to provide a complete pH profile for cyanide release from Prussian blue.

2. Experimental

2.1. Chemicals and reagents

Cyanide standard (1000 ppm, 1 mL = 1 mg CN) was purchased from LabChem Inc. (Pittsburgh, PA). Potassium cyanide (certified), Fisher certified buffer solutions (pH 1.0–11), hydrochloric acid and sodium hydroxide (10N) were purchased from Fisher Scientific (Fair Lawn, NJ). Sodium chloride was purchased from Sigma (St. Louis, MO). Monobasic potassium phosphate and potassium hydroxide were purchased from J.T. Baker Inc. (Phillipsburg, NJ). Spectroquant® Cyanide Test Kit (1.09701.0001) was obtained from Merck KGaA (Darmstadt, Germany). PB active pharmaceutical ingredient (API) was provided by HEYL Corporation. All other chemicals were of reagent grade.

2.2. Preparation of pH solutions

The solution of pH 0.5 was prepared by adding concentrated hydrochloric acid into deionized water. Since purchased pH 1.0 buffer solution strongly interferes with the cyanide measurement, pH 1.0 solution was also prepared by adding concentrated hydrochloric acid into deionized water. The five times diluted pH 2.0–11 solutions were prepared from corresponding certified buffer solutions. The pH 12 solution was prepared by adjustment of pH 11 buffer solution with sodium hydroxide, since it was not commercially available.

2.3. Preparation of calibration standards and quality control standards

Cyanide standard (1000 ppm CN, 1 mL = 1 mg CN) was used as standard stock solution. Seven standard working solutions were prepared by diluting the stock solution with deionized water to yield 1, 2, 4, 6, 8 and 10 ppm CN, respectively. The calibration standard solutions were prepared by transferring 0.5 mL of each working solution to a 10-mL volumetric flask and then adding deionized water or the corresponding pH solution to 10 mL resulting in the final concentrations of 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 ppm CN, respectively. If necessary, the pH of solution was adjusted to a range of 5.5–6.0 with hydrochloric acid or sodium hydroxide as required by Merck Kit test. An aliquot

of 5 mL solution was transferred to a 10-mL plastic tube to be analyzed by Merck Kit.

The quality control (QC) cyanide stock solution (1000 ppm CN, 1 mL = 1 mg CN) was prepared by dissolving 2.51 g of potassium cyanide and 2 g of potassium hydroxide in 1000 mL of deionized water. Four QC working solutions of 1, 2, 5 and 8 ppm were prepared from QC stock solution. Subsequently, lower limit of quantification (LLOQ) of 0.05 ppm, low QC of 0.1 ppm, intermediate QC of 0.25 ppm and high QC of 0.4 ppm were prepared by transferring 0.5 mL of each working solution to a 10-mL volumetric flask and then adding deionized water or the corresponding pH solution to 10 mL. The pH of solution was adjusted to a range of 5.5–6.0, if necessary. An aliquot of 5 mL solution was used for CN analysis by means of the Spectroquant cyanide-test kit.

2.4. Preparation of samples

1.0 g of PB API was accurately weighed and transferred to a 100-mL glass flask containing 50 mL of corresponding pH solution. The flask was tightly stopped and incubated in a shaking water bath at 37 °C with 75 shake/min for 1, 4, or 24 h, respectively. During the incubation, the pH of sample solution was verified and adjusted if necessary. When incubation was completed, the PB 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 test tube. The pH of sample was adjusted to a range of 5.5–6.0 and diluted with deionized water, if necessary. Then 5 mL of sample was used for CN analysis.

2.5. Analytical method

All standards and samples were analyzed using Spectroquant® Cyanide Test Kit following the instruction sheet. Briefly, to 5.0 mL of solution prepared above, one level green microspoon of reagent CN-3 was added and dissolved by vortex, and then one level blue microspoon of reagent CN-4 was added and dissolved by vortex vigorously. After 10 min reaction at room temperature, the samples were measured with a Hewlett Packard 8452A UV–vis Spectrophotometer (Wilmington, DE) at detection wavelength of 606 nm.

3. Results and discussion

3.1. The influence of the formation of hydrogen cyanide on the determination of CN ions

The principle of Spectroquant cyanide test kit is based on the pyridin-free König's reaction. CN ions react with a chlorinating agent to form cyanogen chloride which in turn reacts with 1,3-dimethylbarituric acid to form a violet dye that is determined photometrically in the visible region. To yield optimal result, this color reaction requires that the pH of the measurement solution be within the range 5.5–6.0. Since the pH levels of testing solutions in our study ranged from 0.5 to 12, adjusting the pH to a required range was the first step as well as a very critical procedure for the CN analysis. To test whether the starting pH of solution itself has any effect on the determination of CN, the acidic solutions (pH 0.5–3.0) were spiked with standard CN working solution to yield the final CN concentration of 0.2 ppm for all samples. The samples were then pH adjusted to 5.5–6.0 using sodium hydroxide in order to determine the cyanide content by the test kit. The results are shown in Fig. 2. For each sample, the absorbance was lower than the absorbance for 0.2 ppm CN at pH 6.0 (Blank bar in Fig. 2) and this effect was more pronounced at the lower pH values (pH 0.5 and 1.0). The absorbance values ranged from 39% (pH 0.5) to 86% (pH 3.0) of the absorbance at pH 6.0 (100%). This may be due to loss of cyanide and/or interference with the color reaction due to addition of NaOH to form NaCl. It is well known that hydrogen cyanide (HCN) can be readily formed when cyanide is added to an acidic solution. The chemical reaction of HCN formation is

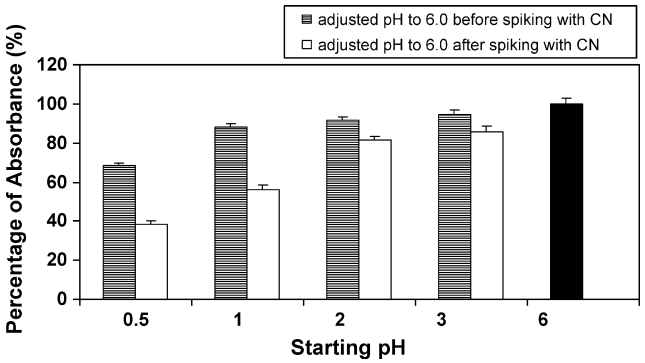

Fig. 2. The influence of the formation of hydrogen cyanide on the determination of cyanide ions. The pH solutions were spiked with standard cyanide solution either before (hatched) or after (open bars) pH adjustment. The concentration of cyanide was 0.2 ppm for all samples. The absorbance was measured photometrically at 606 nm following a colorimetric reaction. The data represent the mean and standard deviation of triplicate samples.

$$HCl + KCN \rightarrow HCN\uparrow + KCl$$

HCN can be volatilized from the solution when it is warmed up. During the process of pH adjustment to 5.5–6 from low pH, the neutralization reaction following NaOH can raise the temperature of the solution which may result in HCN volatilization. Consequently, the absorbance is reduced as a result of the loss of the CN ion. The amount of HCN formed and volatilized largely depends on the pH level of a solution. The lower the initial pH, the greater HCN formation and subsequent volatilization. Therefore, the formation and volatilization of HCN appear to be the primary reason why the sequence of pH adjustment has a significant impact on the cyanide determination. Assuming that similar amount of HCN would be formed if the samples, standards and QCs are prepared in a similar way, maintaining the consistency of experimental conditions is a key parameter in the CN analysis.

3.2. The impact of the formation of sodium chloride on the color reaction of CN ions

To isolate the effect of the addition of NaOH, samples were pH adjusted to 5.5–6.0 before spiking with cyanide. In these cases cyanide would not be lost due to the formation and

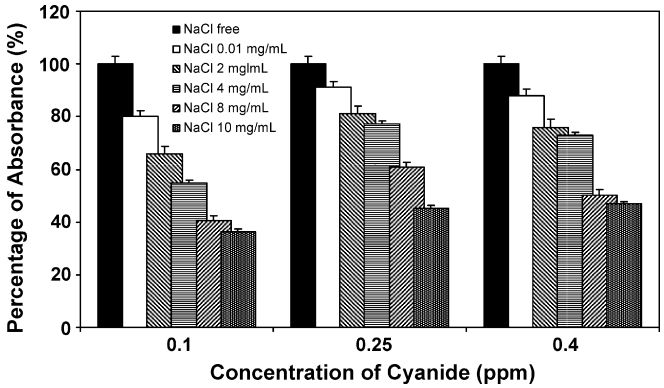

Fig. 3. The impact of formation of sodium chloride on the colorimetric cyanide reaction. The deionized water was spiked with standard cyanide yielding concentration of 0.1, 0.25 or 0.4 ppm, respectively, and then added different amounts of sodium chloride. The absorbance was measured photometrically at 606 nm following a colorimetric reaction. The data represent the mean and standard deviation of triplicate samples.

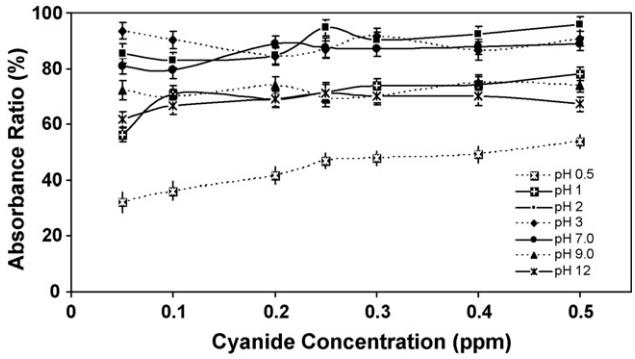

Fig. 4. The recovery of absorbance under different pH conditions. The deionized water and various pH solutions were spiked with standard cyanide resulting in different concentrations of cyanide. After spiking the pH of each solution (except for deionized water) was adjusted to range of 5.5–6.0. The absorbance was measured photometrically at 606 nm following a colorimetric reaction. Assuming the absorbance from deionized water spiked with cyanide was 100%, the percentage was calculated for each pH solution against deionized water. The data represent the mean and standard deviation of triplicate samples.

volatilization of HCN. The results from this parallel study are shown in Fig. 2. The absorbance values for these samples ranged from 69% (pH 0.5) to 95% (pH 3.0) of the absorbance generated in pH 6.0 solution. Based on the mechanism of cyanide assay of the Spectroquant Test Kit, some foreign substances such as cationic and anionic ions, and oxidizing and reducing agents may interfere with the CN color reaction and influence the cyanide determination. Following the pH adjustment, the final concentrations of NaCl ranged from 8 mg/mL (pH 0.5) to 0.01 mg/mL (pH 3.0). To test the hypothesis, inhibition experiments were performed. The inhibition effect for sodium chloride was determined at the cyanide concentrations of 0.1, 0.25 and 0.4 ppm in deionized water titrated with sodium chloride of 0.01–10 mg/mL. The solution free of sodium chloride served as a positive control. The photometric absorbance was measured as described above. Comparison of the photometric absorbance for the solutions containing various amount of sodium chloride provided evidence that the cyanide color reaction can be inhibited by sodium chloride (Fig. 3). Even with very low concentration of sodium chloride (0.01 mg/mL), the absorbance displayed a significant decrease by 12–20% for the solutions with cyanide concentrations of 0.1, 0.25 and 0.4 ppm, compared with the positive control solution. Moreover, the results demonstrated a sodium chloride dose-dependent inhibition effect on the absorbance regardless of cyanide concentration.

3.3. The optimization and validation of cyanide assay

The data presented above indicated that the influence of pH adjustment on the cyanide analysis is primarily related to the formation of sodium chloride and volatilization of HCN. To further demonstrate the effect of pH adjustment on the cyanide analysis method, the solutions with a wide range of pH from 0.5 to 12 were systematically evaluated by the photometric absorbance. As seen in Fig. 4, the absorbance for same concentration of cyanide varied considerably from one pH condition to another. Assuming that the absorbance was 100% for deionized water spiked with cyanide without experiencing the process of pH adjustment, the absorbance ratio of each individual pH solution verses deionized water was calculated. By comparison, the absorbance of a solution with pH 0.5 was only 32–53% at the cyanide concentrations of 0.05–0.5 ppm, which increased as the pH increased and reached about 90% at pH 3.0, followed by a gradual decline to 85%, 72%, and 68% at pH 7.0, 9.0 and 12, respectively. The mechanism of the formation of hydrogen cyanide and sodium chloride also holds for the high pH since hydrochloric acid was added to basic solution to do pH adjustment. The results strongly suggest that the cyanide assay method under our experimental conditions should be carefully controlled and calibrated to ensure the method validity. Therefore, in the present study Spectroquant cyanide test method was optimized and validated under various pH conditions. The method validation included assessment of the stability of the solutions, linearity, precision, accuracy and quantification limit.

3.3.1. Stability of the solutions

The response factor of potassium cyanide standard solutions was found to be unchanged for up to 60 days. Less than 0.2% concentration difference was found between the solutions freshly prepared and those aged 60 days. The solutions can therefore be used within this period without the results being affected.

3.3.2. Linearity

The cyanide test method was calibrated over a range of pH values to account for the reductions in absorbance due to HCN losses and pH adjustments. The calibration curve was linear over the validated range of 0.05–0.5 ppm for spiked deionized water and the solutions with pH level of 0.5–12. Coefficient of determination was greater than 0.99 on 10 different days performed over a period of 6 months (Table 1).

3.3.3. Accuracy and precision

The accuracy is the closeness of mean test results obtained by the method to the true value of analyte, calculated as the percentage of recovery. The precision describes the degree of

Table 1
Parameters of calibration curve ($n \geq 5$)

Solutions	Linear range (ppm)	Calibrators	R^2 value	Slope
Deionized water	0.05–0.5	8	0.9991	4.1995
pH 0.5	0.05–0.5	8	0.9941	2.2360
pH 1.0	0.05–0.5	8	0.9940	2.5096
pH 2.0	0.05–0.5	8	0.9976	3.1077
pH 3.0	0.05–0.5	8	0.9988	3.3638
pH 5.0	0.05–0.5	8	0.9984	3.4899
pH 7.0	0.05–0.5	8	0.9981	3.5660
pH 9.0	0.05–0.5	8	0.9955	2.7758
pH 12	0.05–0.5	8	0.9925	3.3730

Table 2
Quality control: accuracy (%, $n \geq 10$)

Quality control standards	Cyanide concentration (ppm)			
	0.05	0.1	0.25	0.4
Deionized water	101.8	103.4	104.9	103.0
pH 0.5	103.4	90.0	96.4	95.8
pH 1.0	103.3	90.9	94.8	99.6
pH 2.0	96.6	97.2	102.7	99.8
pH 3.0	109.4	107.1	103.9	105.0
pH 5.0	90.3	91.4	91	99.4
pH 7.0	100.2	96.8	101.1	96.5
pH 9.0	99.6	109.3	107.0	101.5
pH 12	96.0	105.6	96.9	107.3

agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample and calculated as relative standard deviation (R.S.D.). The results of intra-day accuracy and precision in the study are compiled in Tables 2 and 3. The mean accuracies ranged from 90% to 109% for deionized water and eight levels of pH solutions at the cyanide concentrations of 0.05, 0.1, 0.25 and 0.4 ppm of the quality control standards. The mean precision ranged from 0.9% to 8.9% for deionized water and eight levels of pH solution at the cyanide concentrations of 0.05, 0.1, 0.25 and 0.4 ppm. Both accuracy and precision meet the guidance criteria. The inter-day accuracy and precision of the method were studied by analyzing five identical samples containing 0.1, 0.25 and 0.4 ppm. The inter-day accuracy varied from 90% to 113%, while precision ranged from 0.5% to 12.0% for both deionized water and pH solutions.

Table 3
Quality control: precision (R.S.D. %, $n \geq 10$)

Quality control standards	Cyanide concentration (ppm)			
	0.05	0.1	0.25	0.4
Deionized water	2.61	2.71	2.00	1.46
pH 0.5	3.04	8.9	3.62	2.27
pH 1.0	3.01	6.53	2.43	2.44
pH 2.0	3.85	5.08	4.46	2.49
pH 3.0	1.36	4.25	4.46	2.73
pH 5.0	2.35	1.36	3.69	2.11
pH 7.0	2.61	3.95	2.72	1.73
pH 9.0	2.75	4.82	2.30	1.84
pH 12	3.25	5.43	1.41	0.80

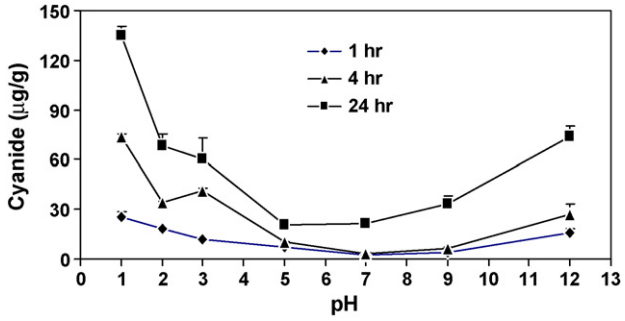

Fig. 5. The pH-dependent cyanide release profile. PB was incubated in corresponding pH solution at 37 °C for 1–24 h. When the incubation completed, sample was filtered and 10 mL aliquot was used for pH adjustment and cyanide analysis. The absorbance was measured photometrically at 606 nm following a colorimetric reaction. The data represent the mean and standard deviation of triplicate samples.

3.3.4. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD is the lowest amount of drug that can be detected, but not necessarily quantitated based on a signal-to-noise ratio (S/N) of at least 6:1. The LOD was evaluated from five independent samples, which were spiked with cyanide in order to produce a peak height close the six times of the base line noise. Conversely, the LOD for all the testing conditions was estimated as 0.01 ppm. The LOQ is the lowest amount of analyte in the sample that can be determined with acceptable precision and accuracy. In our case, LOQ was determined as 0.05 ppm under the study conditions.

3.4. Applications

The validated method was applied to determine the amount of cyanide released from five lots of PB APIs and three lots of PB drug products under different pH conditions (pH 1.0–12), over a incubation period of 1–48 h. A typical cyanide release profile from API is shown in Fig. 5. Cyanide release from both API and drug product demonstrated a pH-dependent profile. The greatest release occurred at pH 1.0, followed by a gradual decline as pH increased to pH 7.0. The lowest release was observed at pH 7.0, thereafter cyanide release increased again as the pH rose to 12. The highest CN concentration was found when PB was incubated in pH 1.0 solution for 48 h. The variation of cyanide release from batch to batch was not significant among the APIs, but was remarkable among the drug products. This will be investigated in a subsequent publication.

4. Conclusions

The method of Spectroquant cyanide test has been optimized and validated over a wide range of pH conditions in our study. The method addressed each of the analytical validation characteristics such as linearity, accuracy, precision and stability, and met the acceptance criteria defined in the guidance. The usefulness of this method is demonstrated by successful application for the *in vitro* determination of cyanide-release from PB.

Acknowledgements

The authors wish to acknowledge the contribution of Dr. Mansoor Khan for his helpful comments and insightful review of this manuscript. The authors also wish to thank Dr. Moheb M. Nasr for his valuable scientific and project administration of this study.

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