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# Validation of an analytical method for cyanide determination in blood, urine, lung, and skin tissues of rats using gas chromatography mass spectrometry (GC-MS)

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**Abstract** This study was conducted to establish the analytical method for the determination of cyanide in blood, urine, lung and skin tissues in rats. In order to detect or quantify the sodium cyanide in above biological matrixes, it was derivatized to Pentafluorobenzyl cyanide (PFB-CN) using pentafluorobenzyl bromide (PFB-Br) and then reaction substance was analyzed using gas chromatography mass spectrometer (GC/MS)-SIM (selected ion monitoring) mode. The analytical method for cyanide determination was validated with respect to parameters such as selectivity, system suitability, linearity, accuracy and precision. No interference peak was observed for the determination of cyanide in blank samples, zero samples and lower limit of quantification (LLOQ) samples. The lowest limit detection (LOD) for cyanide was 10 μM. The linear dynamic range was from 10 to 200 μM for cyanide with correlation coefficients higher than 0.99. For quality control samples at four different concentrations including LLOQ that were analyzed in quintuplicate, on six separate occasions, the accuracy and precision range from -14.1 % to 14.5% and 2.7 % to 18.3 %, respectively. The GC/MS-based method of analysis established in this study could be applied to the toxicokinetic study of cyanide on biological matrix substrates such as blood, urine, lung and skin tissues.

**Key words:** Cyanide, Sodium cyanide, Method validation, GC/MS

## 1. Introduction

Cyanide exists as a single-constituent substance or a mixture, in the solid, liquid, or gaseous form. Because it is known to be a highly toxic substance, very few cases of poisoning occur from intentional ingestion of cyanide. However, there is a risk of cyanide poisoning from chemical processes such as electroplating and precious metal refinement, industrial accidents during manufacture, and everyday activities and events such as smoking, automobile exhaust, and fire.[1-3] Cyanide ions ($CN^-$) are generated by pyrolysis when products containing cyanide are burned, and if inhaled, they may lead to carbon monoxide and cyanide poisoning in humans.[3] The time of manifestation of cyanide toxicity differs depending on the route of

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exposure; toxic effects of cyanide are exhibited when it enters the body through inhalation, ingestion, absorption through skin, or injection.[2,3] When cyanide is introduced into a living organism, it exhibits toxic effects by mechanisms that include inhibition of cytochrome c oxidase activity; it can also cause cellular hypoxia and cytotoxic anoxia, which may even lead to death in severe cases.[4] Cyanide is highly volatile and reactive and has a very short half-life, which makes it difficult to measure cyanide exposure when a certain amount of time has elapsed after the exposure.[5] Therefore, it is important to identify the route of cyanide exposure and estimate the cyanide concentration in different types of living tissues so that hazards associated with cyanide exposure can be assessed.

Previous studies that measured cyanide concentrations used ion chromatography (IC),[6] spectrophotometry,[7] gas chromatography (GC),[8-10] gas chromatography-mass spectrometry (GC-MS),[1,11-13] and high-performance liquid chromatography-mass spectrometry (HPLC-MS).[14] Analytical methods that use mass spectrometry typically include a derivatization step to enhance the physicochemical attributes for sensitivity and specificity of the analytes.[15] Analytical methods reported in previous studies also included GC-MS with a derivatization reagent called pentafluorobenzyl bromide (PFBBr) as an alkylating agent to introduce alkyl groups into organic compounds. Recently, this analytical method has been used to identify the cause of death in vehicle fire victims.[16] PFBBr dissolves in organic solvents, is thermally stable, and is highly volatile, which makes it an ideal derivatization reagent for high-sensitivity GC-MS.[4,5]

The present study used a PFBBr-based derivatization method[1,11-13] for derivatization of $CN^-$ to obtain PFB-CN (*Fig*. 1), which was analyzed by GC-MS. The study also validated an analytical method for determination of cyanide in blood, urine, lung, and skin tissues, which could have been absorbed owing to cyanide exposure, in order to assess hazards associated with cyanide exposure.

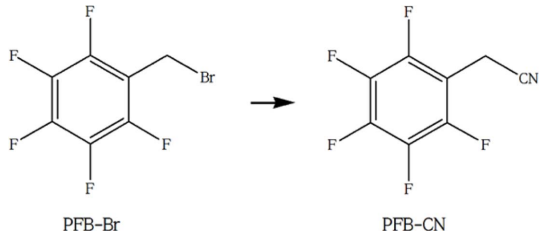


*Fig.* 1. Chemical structures of before and after derivatization.

## 2. Experimental Methods

### 2.1. Standards and reagents

The standard material used in the experiment, sodium cyanide (NaCN; 95.5 %), was purchased from Sigma-Aldrich (Saint Louis, MO, USA). The internal standard (IS), 2,5-dibromotoluene (2,5-DBT; 99.7 %), was purchased from Acros Organics (New jersey, USA). Other products used were purchased from the following sources: sodium hydroxide concentrate (NaOH, 0.1 mol/L in water) and tetrabutylammonium sulfate solution (50 wt.% in water) from Sigma-Aldrich (Saint Louis, MO, USA); PFBBr (99.1 %) from Thermo Fisher Scientific (Haverhill, MA, USA); borate and 0.5 M buffer solution (pH 8.0) from Alfa Aesar (Haverhill, MA, USA); and ethyl acetate (99.9 %) and water from Burdick & Jackson (Brooklyn, NY, USA).

Blood, urine, lung, and skin tissue samples obtained from Sprague-Dawley rats and stored at temperature below -20 °C were used in the validation of the analytical method. An anticoagulant, EDTA-2K, was used for the blood samples.

### 2.2. Preparation of standards and reagents

The stock standard solution of NaCN was prepared by weighing sodium cyanide and using 10 mM NaOH to bring it to 100 mM, after which it was stored in a refrigerator until use. The working standard solutions were prepared to concentrations of 200–4000 μM using 100 mM stock standard solution and 10 mM NaOH. NaOH (10 mM) used to prepare the standards was prepared by 10-fold dilution of 0.1 M sodium hydroxide concentrate using distilled water. The IS, 2,5-DBT, was prepared to a concentration of 0.2 μM using ethyl acetate, after which it was stored in a refrigerator until use.

Borate (0.5 M buffer solution), and distilled water were used to prepare a 0.1 M borate buffer solution. Tetrabutylammonium sulfate and 0.1 M borate buffer solutions were used to prepare a 10 mM tetrabutylammonium sulfate solution, whereas PFBBr and ethyl acetate were used to prepare 20 mM PFBBr.

### 2.3. Preparation of calibration standard and quality control (QC) samples

Calibration standards for the blood, urine, lung, and skin tissue samples were prepared by mixing blank blood, blank urine, blank lung, or blank skin tissue, respectively, with the working standard solution at a ratio of 95:5 (v/v) to bring the calibration concentration to 10–200 μM. The blank and zero blank were prepared using 10 mM NaOH instead of working standard solution. The blank lung and blank skin tissue samples were prepared by adding 1 mL of 10 mM NaOH per 0.1 g of sample and homogenizing in a homogenizer. The blood, urine, lung, and skin tissue samples for QC were prepared by mixing blank blood, blank urine, blank lung, or blank skin tissue, respectively, with the working standard solution to bring the concentration to 10 μM (lower limit of quantitation; LLOQ), 20 μM (low QC; LQC), 50 μM (mid QC; MQC), and 150 μM (high QC; HQC).

### 2.4. Pretreatment of samples

For pretreatment of blood, urine, lung, and skin tissue samples, 100 μL of IS was added to 100 μL each of calibration and QC samples (blood, urine, lung, and skin tissue), including the blank and zero blank, and mixed for 1 minute. After mixing 800 μL of 10 mM tetrabutylammonium sulfate solution and 500 μL of 20 mM PFBBr for 10 minutes, derivatization reaction was carried out at 70 °C for 1 hour. The derivatized sample was centrifuged (14,000 *g*, 4 °C, 5 minutes), and the supernatant was used in subsequent analysis.

### 2.5. Gas chromatography-mass spectrometry

For analysis of PFB-CN derivatized after pretreatment, SCION SQ (Bruker, Billerica, MA, USA) was used. The mass values m/z for quantification of PFB-CN and 2,5-DBT were set to 207.0 and 249.8, respectively. GC column used was BR-5ms (Bruker, Billerica, MA, USA), and the parameters for GC-MS were as shown in *Table* 1.

*Table* 1. GC/MS operating conditions and parameters

| **GC condition** | | | | |
|---|---|---|---|---|
| Column | BR-5ms (30 m × 0.25 mm, 0.25 μm, BRUKER, USA) | | | |
| Column oven | Temperature (°C) | Rate (°C/min) | Hold (min) | Total (min) |
| | 60.0 | 0.0 | 1.00 | 1.00 |
| | 100.0 | 5.0 | 0.00 | 9.00 |
| | 140.0 | 20.0 | 0.00 | 11.00 |
| | 280.0 | 50.0 | 2.00 | 15.80 |
| Carrier gas / Flow rate | Helium / 1.0 mL/min | | | |
| Injector temperature | 210 °C | | | |
| Injection volume | 1 μL, Split Ratio 1:5 | | | |
| **MS condition** | | | | |
| Ionization | Electron ionization (EI) | | | |
| Source temperature | 200 °C | | | |
| Transfer line temperature | 250 °C | | | |
| Parameters | Cyanide | | 2,5-Dibromotoluene (IS) | |
| Quan Ions | 207.0 | | 249.8 | |
| Qualifier | 157.0 | | 168.9 | |
| Retention time (min) | 6.78 | | 11.10 | |

### 2.6. Validation of analytical method

For validation of the analytical method for determination of cyanide in blood, urine, lung, and skin tissues of rats, the selectivity, system suitability, linearity, accuracy, and precision of the method were tested. Validation of the analytical method was carried out in accordance with the Guideline on Bioanalytical Method Validation provided by the FDA and EMA.[17,18]

Selectivity indicates the ability of an analytical method to isolate and quantify the target substance when other substances are coexistent within a biological sample. Accordingly, selectivity was assessed by analyzing whether the peaks of blank, zero blank, and LLOQ samples of blood, urine, lung, and skin tissues obtained from six different individuals are affected by interference peaks. In addition, system suitability was examined by assessing the precision for peak area on the basis of six repeated injections of LLOQ samples.

Linearity was determined on the basis of calibration curve results obtained using standard samples with at least six different concentrations, including LLOQ and upper limit of quantitation, along with the blank and zero blank. Accuracy and precision were assessed on the basis of six repeated measurements of at least four different concentrations, including LLOQ, with respect to the entire operation of the test method. Accuracy was compared using relative error (%RE),

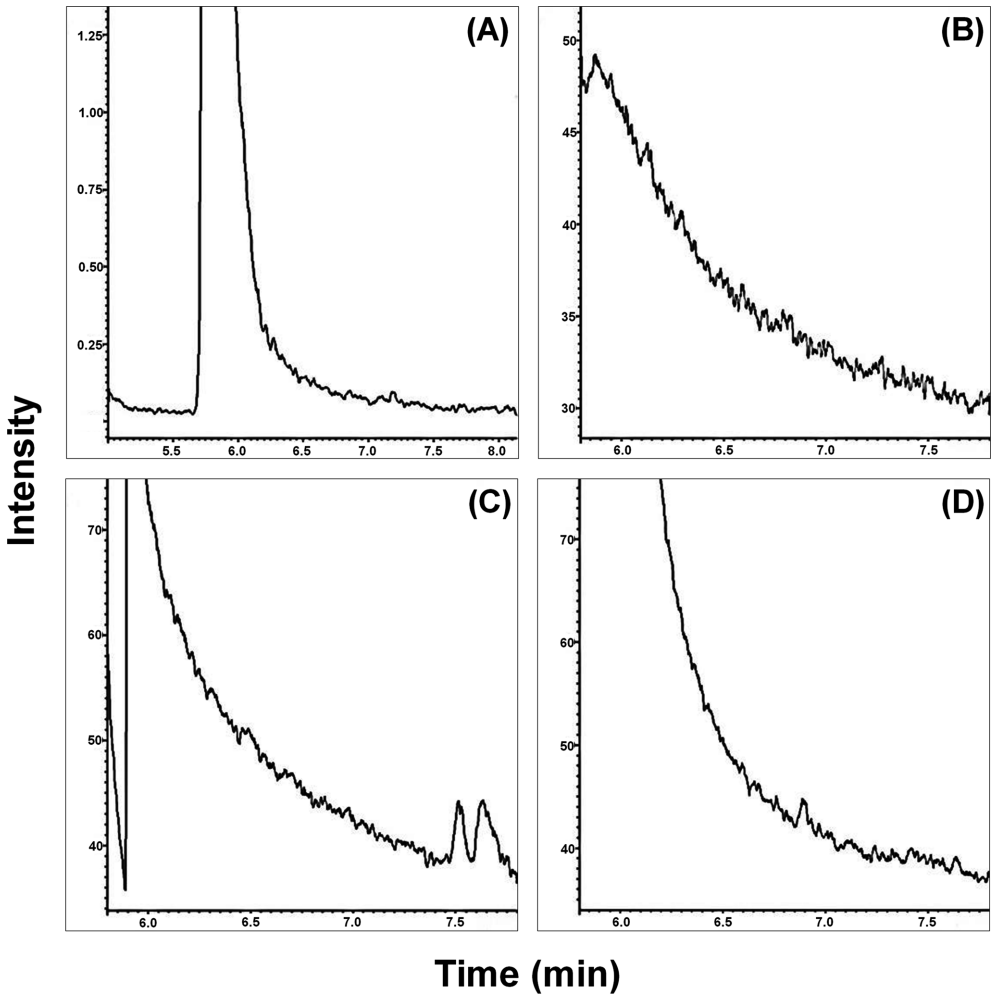


*Fig.* 2. Representative blank chromatogram of sodium cyanide in rat (A) blood, (B) urine, (C) lung and (D) skin tissue.

whereas precision was compared using coefficient of variation (%CV). %RE and %CV were calculated using the equations shown below.

%RE = [(Measured concentration − Theoretical concentration)/Theoretical concentration] × 100

%CV = [SD of measured concentration/Mean of measured concentration] × 100

## 3. Results and Discussion

### 3.1. Selectivity

To test the selectivity of the analytical method using GC-MS for determination of cyanide in blood, urine, lung, and skin tissues, blank and zero blank samples using blood, urine, lung, and skin tissues obtained from six individuals and LLOQ sample treated with NaCN and IS were assessed (*Fig.* 2, 3). No interference peaks were observed in the interval in which cyanide and IS were eluted. The results demonstrated that the analytical method exhibits good selectivity for cyanide analysis and is not affected by interfering substances.

### 3.2. System suitability

The test the system suitability of the analytical method, biological samples at the LLOQ concentration were injected six times into GC-MS. The results

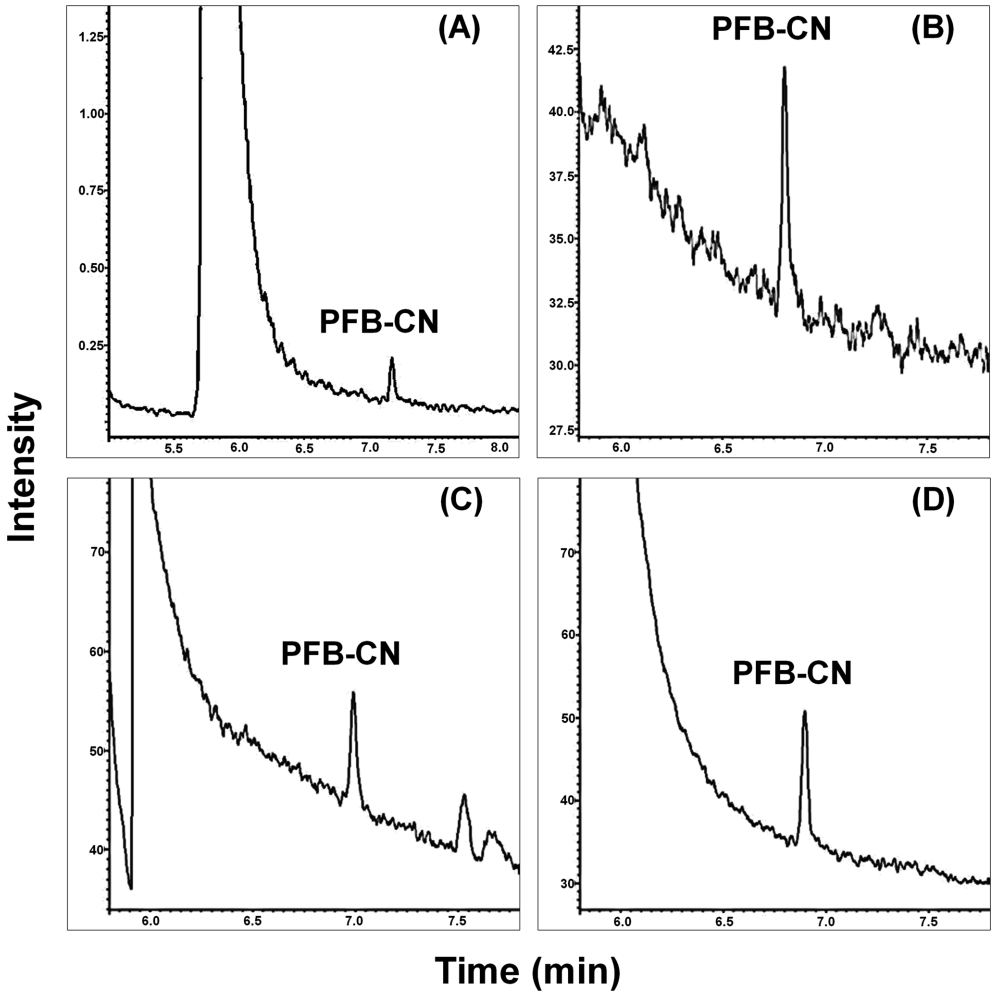


*Fig.* 3. Representative LLOQ chromatogram of sodium cyanide in rat (A) blood, (B) urine, (C) lung and (D) skin tissue.

*Table* 2. System suitability of sodium cyanide in blood, urine, lung and skin tissues in rats

| | Peak area | |
|---|---|---|
| | PFB-CN | 2,5-DBT |
| Blood | | |
| (%) CV | 6.8 | 3.3 |
| Urine | | |
| (%) CV | 15.4 | 6.0 |
| Lung | | |
| (%) CV | 6.7 | 1.3 |
| Skin | | |
| (%) CV | 3.8 | 2.6 |

*Table* 3. Linearity of calibration curves of sodium cyanide in blood, urine, lung and skin tissues in rats

| | Nominal concentration (μM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 10 | 20 | 35 | 50 | 75 | 100 | 150 | 200 |
| Blood | | | | | | | | |
| (%) RE | -4.8 | 10.7 | 3.3 | -10.4 | 3.0 | 1.1 | -4.8 | 2.5 |
| Urine | | | | | | | | |
| (%) RE | 2.3 | -4.9 | 1.7 | -8.2 | 10.6 | -1.6 | 3.8 | -3.8 |
| Lung | | | | | | | | |
| (%) RE | 2.3 | -5.7 | -3.3 | 6.9 | 4.8 | -4.3 | -2.0 | 1.2 |
| Skin | | | | | | | | |
| (%) RE | 4.8 | -9.5 | -6.9 | 6.0 | 8.2 | -3.6 | 0.1 | -0.7 |

showed that the precision values for the analyte PFB-CN and IS was 3.8–15.4 % and 1.3–6.0 %, respectively, which satisfied the acceptance criterion of <20 % (*Table* 2). Accordingly, it was determined that GC-MS used in the testing of the analytical method was properly maintained and managed.

### 3.3. Linearity

To test the linearity, the ratio of PFB-CN to IS concentrations (x axis) and ratio of PFB-CN to IS peak area (y axis) were used to construct a calibration curve with a weight of $1/x^2$. The correlation coefficient ($r^2$) of each biological sample was within the range of 0.9943–0.9984 (*Fig.* 4), and the accuracy of the calibration curve was found to be -10.4–10.7 % (*Table* 3). These values were consistent with the criteria for assessing the calibration curve, that is, correlation coefficient ≥0.99 and concentration within 15 % (20 % for LLOQ) of the theoretical concentration, which were determined to be the

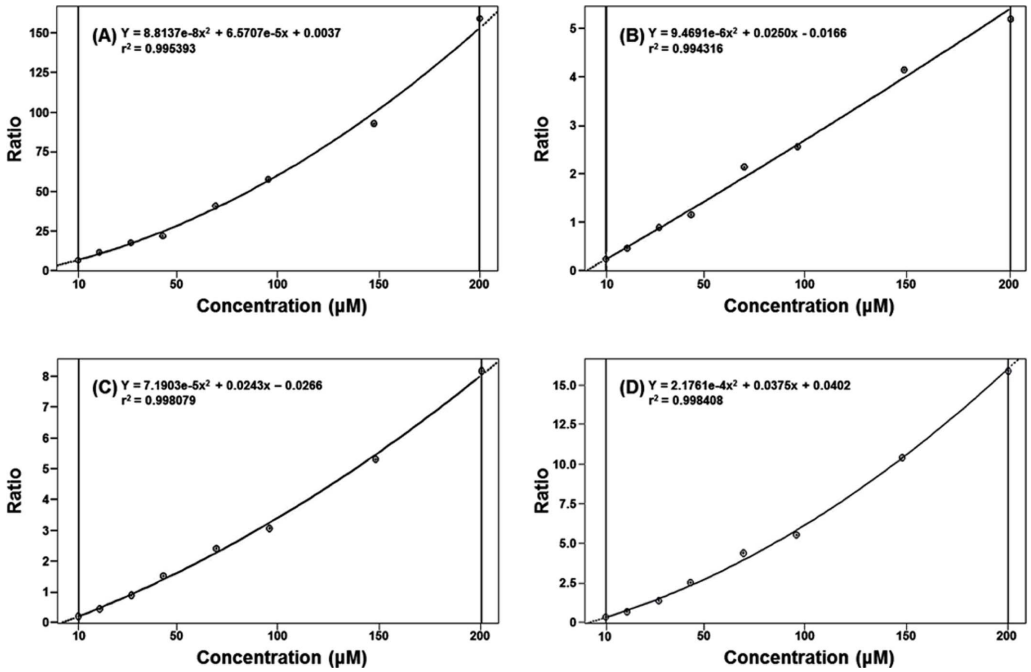


*Fig.* 4. Calibration of sodium cyanide in rat (A) blood, (B) urine, (C) lung and (D) skin tissue.

*Table* 4. Accuracy and precision of sodium cyanide in blood, urine, lung and skin tissues in rats

| | Nominal concentration (μM) | | | |
|---|---|---|---|---|
| | 10 | 20 | 50 | 150 |
| Blood | | | | |
| (%) RE | -0.7 | 6.8 | 0.4 | -7.9 |
| (%) CV | 18.3 | 10.5 | 6.6 | 2.7 |
| Urine | | | | |
| (%) RE | 14.5 | -0.7 | -9.2 | 9.5 |
| (%) CV | 10.5 | 13.5 | 13.9 | 9.6 |
| Lung | | | | |
| (%) RE | 13.2 | -14.1 | 10.8 | 5.4 |
| (%) CV | 10.0 | 3.2 | 3.0 | 8.2 |
| Skin | | | | |
| (%) RE | 3.7 | 1.2 | -7.2 | 1.0 |
| (%) CV | 15.2 | 12.5 | 9.7 | 3.4 |

levels suitable for cyanide determination.

### 3.4. Accuracy and precision

Accuracy represents the proximity between the theoretical value and mean value obtained by analysis, whereas precision represents the proximity of values measured repeatedly when analyzing a test article at the same concentration. *Table* 4 shows the results of analyzing %RE and %CV from six repeated measurements of LLOQ, low QC, mid QC, and high QC samples. The accuracy and precision of each biological sample was within -14.1–14.5 % and 2.7–18.3 %, respectively, which satisfied the accuracy criterion of mean concentration being within 15 % (20 % for LLOQ) of the theoretical concentration and precision criterion of %CV <15 % (20 % for LLOQ). The results confirmed that the analytical method is suitable for the determination of derivatized cyanide.

## 4. Conclusions

In the present study, the analytical method using GC-MS for cyanide determination in blood, urine, lung, and skin tissues was validated by assessing its selectivity, system suitability, linearity, accuracy, and precision. In the analytical method, the quantitation range of derivatized cyanide PFB-CN was 10–200 μM, and within this range, the correlation coefficient ($r^2$) of the calibration curve of each biological sample containing PFB-CN was ≥0.99, showing good results. The results of accuracy (%RE) and precision (%CV) from LLOQ QC, Low QC, Mid QC, High QC samples were within ±15 % (20 % for LLOQ) of the values specified in the FDA and EMA guidelines. Validation of the analytical method for targeted biological samples confirmed that the method is capable of cyanide determination in blood, urine, lung, and skin tissues. These results indcated that could be able to use as a useful basic data for application in the research related to routes of exposure by cyanide inhalation.

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