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Rapid determination of cyanide in human plasma and urine by gas chromatography–mass spectrometry with two-step derivatization

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ABSTRACT

Cyanide (CN) is a powerful poison and rapidly toxic agent. Because of its wide availability and high toxicity, quantification of CN in blood and urine is frequently required in clinical and forensic practice. We present a sensitive and less time consuming method based on solid-supported liquid–liquid extraction (SLE) and gas chromatography–mass spectrometry (GC–MS) with two-step derivatization for determination of CN in plasma and urine. Buffer solution, 1,3,5-tribromobenzene (internal standard) and benzaidehyde were added to sample to complete the first-step derivatization. Then the analytes were poured onto the column of diatomaceous earth, eluted with *n*-hexane containing 0.4% of heptafluorobutyryl chloride (HFB-Cl) to complete the second-step derivatization forming the final analyte, heptafluoro-butyric acid-alpha-cyanobenzyl ester. This method was linear (r^2 = 0.9988, 0.9993), reproducible (intra-day RSD = 4.37–7.24%, 3.19–5.74%; inter-day RSD = 5.13–7.63%, 4.31–6.69%), accurate (recoveries = 90.58–115.56%, 93.01–114.6%) and sensitive (LOD = 0.04, 0.01 μg/mL) for plasma and urine, respectively. The total time was about 25 min. This method was successfully applied to the analysis of blood sample and urine sample collected from a victim who died as a result of ingestion of potassium cyanide.

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1. Introduction

Cyanide (CN) is known as one of the most rapidly acting and powerful poisons. There are many ways in which people can be exposed to cyanide, such as smoke given off during fires, automobile exhaust, electroplating, refining of precious metals, fumigation, cassava plants, etc. [1–5]. CN is also produced endogenously in patients treated with the sodium nitroprusside, a hypotensive agent. CN exerts its toxic effects by inhibits cytochrome oxidase of the mitochondrial respiratory chain and then results in a rapid impairment of vital functions [6,7]. Because of its wide availability and high toxicity, quantification of CN in human biological fluids, especially in blood and urine, is frequently required in clinical examination and forensic identification.

Several methods, including spectrometry [8–11], electrochemistry [12,13] and chromatography [14–24] have been reported for the determination of CN. Gas chromatography–mass spectrometry (GC–MS) appears most efficient because of the enormous abilities of high-resolution capillary column together with high selective detection system. Headspace gas chromatography–mass spectrometry (HS-GC–MS) has been described for the determination of CN by the several research groups [15–20]. Most procedures involve the liberation of CN^- from the samples as gaseous HCN by acidification, and the headspace gas was injected directly or transferred by solid-phase microextraction (SPME) to GC system for detection. However, the acidification may cause the artificial formation of CN from thiocyanate (SCN) in biological samples [25]. In some studies, ascorbic acid has been added in the sample to prevent the interference from SCN. However, the effect of ascorbic acid was small at the toxic concentration of CN and suppression in the peak area measured for HCN was observed with the use of ascorbic acid [15,19]. On the other hand, the molecular ion of HCN at *m*/*z* 27 is too small to suitable for mass spectrometry detection and it is similar to molecular mass of N_2 (*m*/*z* 28) which abounds in air, and the interference in mass spectrometry is inevitable, even though under the condition of high vacuum. To overcome the disadvantages, CN should be derived to a major mass molecule, which can be detected in an excellent mass range by GC–MS and can prevent the interference from SCN. In most derivatization procedures, CN was extracted from aqueous solution by the phase-transfer catalyst and derived to another molecule which can be detected [21–24]. Tetradecyldimethylbenzylammonium chloride was used as the phase-transfer catalyst and pentafluorobenzyl bromide was used as the alkylating agent [24]. However, the preparations of samples were laborious and time consuming.

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Here we present a new method for the determination of CN in plasma and urine based on solid-supported liquid–liquid extraction (SLE) and gas chromatography–mass spectrometry (GC–MS) with two-step derivatization. In this procedure, CN is first derived directly with benzaidehyde in aqueous matrix samples to form a relatively stable chemi-intermediate, then poured onto diatomaceous earth column, following derivatizing with heptafluorobutyryl chloride (HFB-Cl) and extraction simultaneously to form the final derivative. This procedure proved to be a more available and rapid for sample preparation. In this paper, higher sensitivity, accuracy and speed were obtained through optimizing analytical conditions. This method is suitable for the detection and quantification of CN in clinical examination and forensic identification.

2. Materials and methods

2.1. Materials

Potassium cyanide, benzaidehyde, isopropanol, sodium borate, sodium hydroxide and *n*-hexane were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai China). Diatomaceous earth and 1,3,5-tribromobenzene (internal standard, I.S.) were obtained from Wako Pure Chemical Ind., Ltd. (Tokyo, Japan). Diatomaceous earth was dried for 3 h at 150 °C to reserve. Heptafluorobutyryl chloride (HFB-Cl) was purchased from Kanto Kasei Chemical Co., Inc. (Tokyo, Japan). Human plasma and urine were collected from nonsmoker volunteers.

Other chemicals and solvents were purest grade.

2.2. Solutions

A stock solution of cyanide (10 mg/mL) was prepared by dissolving potassium cyanide in 0.5 M sodium hydroxide solution. The concentration of the stock solution was standardized by titration against silver nitrate using P-dimethylaminobenzalrhodanine as an indicator. The I.S. solution was prepared by dissolving 1,3,5-tribromobenzene at 0.1 mg/mL in isopropanol. The sodium borate buffer solution of pH 10 was prepared by mixed 0.05 M sodium borate solution with 0.2 M sodium hydroxide solution. All the solutions prepared were stored at 4 °C to reserve.

2.3. Derivatization and extraction

Sodium borate buffer solution (pH 10) of 0.5 mL and 5 μL I.S. (0.1 mg/mL) were added to 0.5 mL CN-fortified plasma or urine sample. To complete the first-step derivatization, 5 μL benzaidehyde was added directly to plasma or urine sample by micro-syringe and vortex-mixed for 30 s, and then maintained for 2 min at room temperature. After that the sample was poured onto the column filled with 1.5 g desiccated diatomaceous earth. Ten minutes later, the analytes were eluted with 5 mL *n*-hexane containing 0.4% HFB-Cl to complete the second-step derivatization forming the final analyte, heptafluoro-butyric acid-alpha-cyanobenzyl ester. The eluent was evaporated to nearly drying under stream of nitrogen and 1 μL followed by GC–MS analysis.

2.4. Investigation of the reaction time for the first-step derivatization

CN-fortified plasma of 10 μg/mL was used for the investigation of the reaction time for the first-step derivatization in which benzaidehyde was used as an alkylating agent. The plasma added benzaidehyde was kept for 0, 1, 2, 5 and 10 min at room temperature separately before poured onto the diatomaceous earth column.

2.5. Instrumental analysis and data acquisition

GC–MS analyses were performed with a Shimadzu GC–MS QP 2010 (70 eV, electron impact mode) by the scan and the selected ion-monitoring (SIM). Chromatographic separation was achieved on a Rtx-5 fused-silica capillary column (10 m × 0.18 mm I.D., 0.2 μm film thickness) using helium as carrier gas at 1.01 mL/min in a constant flow rate mode. The GC oven temperature was initially held at 60 °C for 1 min, and then elevated at a rate of at 15 °C/min up to 180 °C with a hold at 180 °C for 1 min. The temperatures of injector, interface and ion source were 250, 270 and 200 °C, respectively. The GC–MS was programmed to perform a 1 μL splitless injection.

In the scanning mode, the mass range was 40–900 u at a rate of 0.5 s/scan. In the SIM mode, the peaks of cyanide derivative and I.S. for plasma and urine were identified by matching the retention time and the relative abundance of characteristic ions *m*/*z* 116, *m*/*z* 329 for cyanide derivative and *m*/*z* 235, *m*/*z* 314 for I.S. Cyanide was quantified in plasma or urine by measuring the area ratios of cyanide derivative (*m*/*z* 116) vs I.S. (*m*/*z* 314) and comparing these ratios with the area ratios obtained from known cyanide concentrations with internal standards.

2.6. Method validation

2.6.1. Assay calibration and linearity

Calibration standards were prepared with concentrations of 0.02, 0.1, 0.2, 1, 2, 10, 20 and 100 μg/mL from the CN stock solution by diluting in plasma or urine. All standard solutions prepared were stored at 4 °C. The calibration samples together with fixed amount of I.S. (0.1 mg/mL × 5 μL) were subjected to the reaction as described above. The peak-area ratios of cyanide derivative (*m*/*z* 116) to I.S. (*m*/*z* 314) were plotted against the known added amounts of CN. Linearity was evaluated by linear regression and expressed as r^2. To check for possible interferences of plasma and urine samples used to obtain the calibration curve, blank plasma and urine samples were processed along with the above samples.

2.6.2. Limit of detection (LOD)

LOD was calculated by progressing decreasing the analyte concentration in the standard sample by GC–MS-SIM such that signal was clearly discerned at signal-to-noise ratio (S/N) greater than 3.

2.6.3. Precision

The precision of the method was evaluated by analyzing the samples fortified with a known amount of CN at low (0.1 μg/mL), medium (1 μg/mL) and high (10 μg/mL) concentrations. Intra-day precision was determined by assaying four replicates of each concentration on one day. Inter-day precision was determined on 20 different days over a period of 6 weeks by assaying refrigerated urine or plasma samples fortified with each concentration. All samples were freshly prepared by adding the appropriate amount of CN so as not to confound the results with stability issues of CN in stored samples.

2.6.4. Accuracy

The accuracy was determined by calculating analytical recoveries of added cyanide. Urine and plasma samples tested were divided into three portions. Low, medium and high concentrations of cyanide were added separately. Each specimen was analyzed in triplicate. The recovery was calculated as: (final concentration − initial concentration)/added concentration.

2.6.5. Stability

All stability experiments were performed on the samples of 1 μg/mL CN in plasma and urine. Stability experiment included evaluation of the stability of CN during multiple freeze–thaw cycles

and stability of CN derivative at 4 °C. Freeze–thaw stability was determined by completing five freeze–thaw cycles with analysis of CN after each individual cycle. For CN derivative stability experiment, CN derivative was allowed to stand at 4 °C and analyzed at 0, 2, 4, 6, 8, 12 and 24 h post-derivatization. The stabilities were demonstrated by analysis of the relative standard deviation of measured samples.

2.7. Quantification of CN in blood and urine sample

We examined a blood sample and a urine sample collected from a 47-year-old man who had died after ingestion of potassium cyanide. Urine sample of 0.5 mL was derivatized directly in the same manner as described above. Blood sample of 1.5 mL was centrifuged for 10 min at 3500 r/min, and then 0.5 mL aliquot of the supernatant was submitted to the procedure of derivatization. The derivatives were analyzed by GC–MS in SIM mode separately. CN was quantified by measuring the area ratios of cyanide derivative (*m*/*z* 116) vs I.S. (*m*/*z* 314) and comparing these ratios with the area ratios obtained from known cyanide concentrations with internal standards.

3. Results and discussion

3.1. Two-step derivatization

At pH > 9.21, cyanide exists mostly as cyanide ion in sample. When benzaidehyde is added, a nucleophilic addition reaction occurs. The nucleophilic electron pair donated by cyanide ion attacks the positive carbon of the polarized C=O bond, forming a CC bond. The π electron pair of the original C=O bond moves onto the oxygen to give it a whole negative charge, forming $C_6H_5CH(CN)O^-$, which is a strong conjugate base and will abstract a proton from water to give the alpha-cyanobenzyl alcohol product. This product reacts with HFB-Cl by nucleophilic addition–elimination and forms heptafluoro-butyric acid-alpha-cyanobenzyl ester as shown in Fig. 1.

For the reaction time of the first-step derivatization, the result shows that after vortex-mixing for 30 s, reaction of cyanide ion and benzaidehyde completes within 1 min. The product, alpha-cyanobenzyl alcohol, is proved to be relatively constant by maintaining for 1, 2, 5, 10 min at room temperature separately before poured onto diatomaceous earth column, and as a result, 2 min was chosen in this procedure. The result proves that the first-step derivatization is quickly completed in aqueous matrix samples.

The second-step derivatization completes on diatomaceous earth column. The product of the first-step derivatization distributes itself in the form of a thin film over the diatomaceous earth (chemically inert matrix and thus acting as a stationary phase), following derivatizing with HFB-Cl and extraction by *n*-hexane which extracts the final derivative from the aqueous into the organic phase. In this process, the second-step derivatization completes without any phase-transfer catalyst which was used in most studies [21–24]. The eluate was free from emulsions and can be evaporated for further analysis.

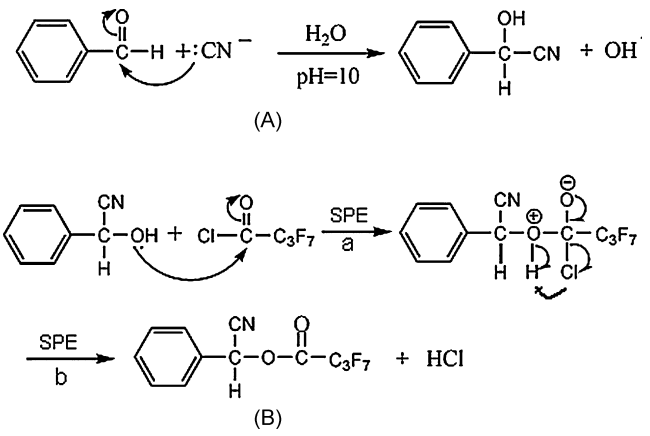

Fig. 1. Mechanism: (A) nucleophilic addition reaction; (B) nucleophilic addition–elimination reaction. (a) The positive carbon of carbonyl is attacked by the nucleophilic alcohol to form a highly unstable ionic intermediate. (b) The lone pairs of electrons shift to complete C=O carbonyl bond, simultaneously the C–Cl bond pair moves onto the chlorine atom and leaves as a chloride ion which abstracts a proton to form the ester product.

3.2. GC–MS analysis

Some chromatograms are shown in Fig. 2 (plasma) and Fig. 3 (urine) in TIC (A) and SIM mode (B, C) for the spiked (A, B) and the blank (C). The retention time was 4.277 and 6.424 min for CN

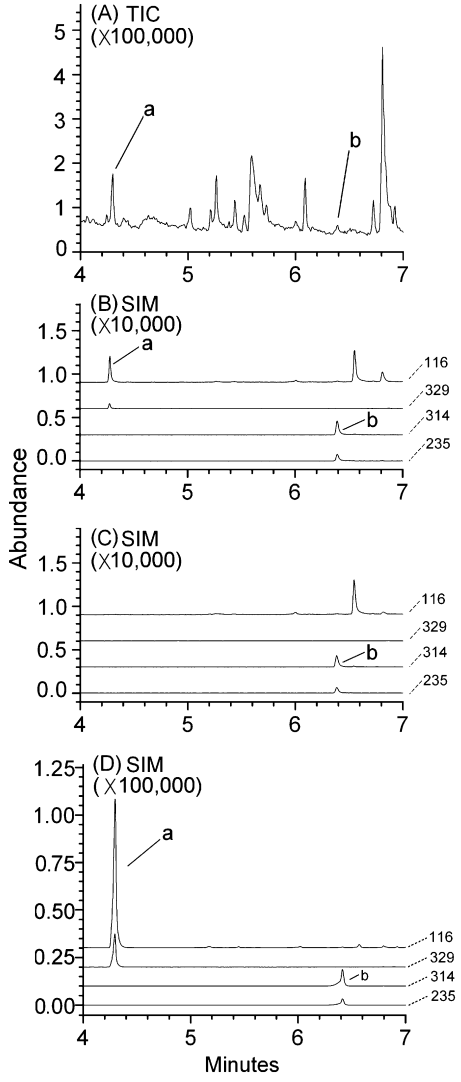

Fig. 2. GC–MS chromatograms of cyanide derivative for plasma samples: (A) total ion chromatogram (TIC) and (B) SIM chromatogram for plasma (nonsmoker) spiked with 1 μg/mL CN^-. (C) SIM chromatogram for blank plasma (nonsmoker). (D) SIM chromatogram for plasma sample collected from a victim who died as a result of ingestion KCN. All samples were spiked with fixed amount of I.S. (0.1 mg/mL × 5 μL). SIM chromatogram obtained at *m*/*z* 116, *m*/*z* 329 for cyanide derivative and *m*/*z* 235, *m*/*z* 314 for I.S. a = cyanide derivative; b = 1,3,5-tribromobenzene (I.S.).

derivative and I.S., respectively. Sharp, no interfering peaks and a very low background were observed across the whole linear range. No signal at the retention time for CN derivative was ever detected in blank plasma and urine samples which used to obtain the calibration curve. Mass spectral patterns are displayed in Fig. 4 for CN derivative (A) and I.S. (B). The mass-to-charge ratio, *m*/*z* 329 and *m*/*z* 314, correspond to the molecular ion of the cyanide derivative and I.S., respectively. The fragment ion at *m*/*z* 116 (base peak), *m*/*z* 132 and *m*/*z* 169 for cyanide derivative correspond to $[C_6H_5CHCN]^+$, $[C_6H_5CH(CN)O]^+$ and $[C_3F_7]^+$, respectively. The fragment ion at *m*/*z* 74 and *m*/*z* 235 for I.S. correspond to $[C_6H_3]^+$ and $[C_6H_3Br_2]^+$. In the SIM mode, peaks of cyanide derivative and I.S. were identified by matching the retention time, and the relative abundance of characteristic ions *m*/*z* 116, *m*/*z* 329 for cyanide derivative and *m*/*z* 235, *m*/*z* 314 for I.S. Cyanide was quantified by measuring the area ratios of cyanide derivative (*m*/*z* 116) vs TBB (*m*/*z* 314) and comparing these ratios with the area ratios obtained from known cyanide concentrations with internal standards. The total time for one analysis cycle including sample preparation and GC–MS run time was about 25 min which is superior to the method of Kage [24].

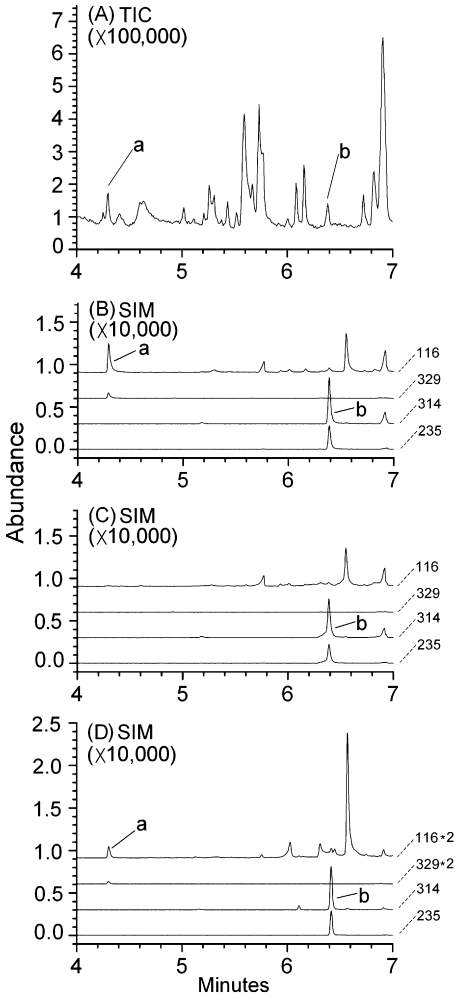

Fig. 3. GC–MS chromatograms of cyanide derivative for urine samples: (A) total ion chromatogram (TIC) and (B) SIM chromatogram for urine (nonsmoker) spiked with 1 μg/mL CN^-. (C) SIM chromatogram for blank urine (nonsmoker). (D) SIM chromatogram for urine sample collected from a victim who died as a result of ingestion KCN. All samples were spiked with fixed amount of I.S. (0.1 mg/mL × 5 μL). SIM chromatogram obtained at m/z 116, m/z 329 for cyanide derivative and *m*/*z* 235, *m*/*z* 314 for I.S. a = cyanide derivative; b = 1,3,5-tribromobenzene (I.S.).

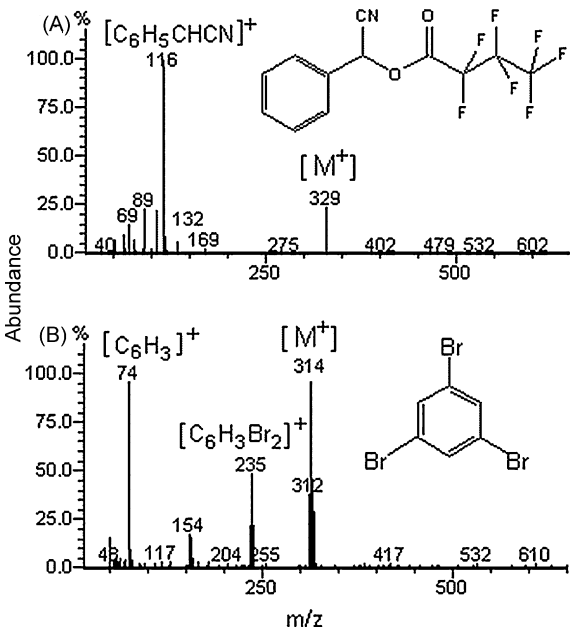

Fig. 4. Electron-impact mass spectra of CN derivative (A) and I.S. (B) obtained in the scanning mode at a rate of 0.5 s/scan with a mass range of *m*/*z* 40–900.

3.3. Method validation

The concentration of CN derivative in plasma and urine matrix showed excellent linearity with the least-squares linear regression analysis. For plasma samples, the equation for the regression line was $y = 1.5784x - 0.211$ ($r^2 = 0.9988$), linear range was 0.1–20 μg/mL, LOD was 0.04 μg/mL, intra-day precision (Table 1) were between 4.37–7.24%, inter-day precision ($n = 20$) were between 5.13–7.63% and the recoveries (Table 2) were between 90.58–115.56%. For urine samples, the equation for the regression line was $y = 2.4407x - 0.1442$ ($r^2 = 0.9993$), linear range was 0.02–10 μg/mL, LOD was 0.01 μg/mL, intra-day precision (Table 1) were between 3.19–5.74%, inter-day ($n = 20$) precision were between 4.31–6.69% and recoveries (Table 2) were between 93.01–114.60%. The stabilities were demonstrated by analysis of the relative standard deviation of measured samples. The stabilities were found to be excellent, with RSD 7.04% and 8.04% for the freeze–thaw stability in urine and plasma samples, as well as 1.63% and 2.01% for the derivative stability in urine and plasma samples, respectively.

Pentafluorobenzyl bromide has been reported as the derivatizing reagent of CN in a literature [24]. This study included precipitating protein, extracting CN from aqueous solution by the phase-transfer catalyst, deriving to pentafluorobenzyl-CN which needed to be maintained at 55 °C in a water bath for 30 min and then centrifuged for 15 min. The detection limit of cyanide was 0.26 μg/mL. The calibration curve was linear from 0.52 to 26 μg/mL with analysis times of 60 min and the recovery was 80%. The preparations of samples seem to be laborious and time consuming. Compared with them, our assay is a simple, rapid, more sensitive and accurate method for the determination of CN in biological matrices.

The minimal adult lethal dose has been estimated as 100 mg for hydrocyanic acid and 200 mg for potassium cyanide [26]. Lethal cases have shown a blood cyanide concentration ranges from 1.1 to 53 μg/mL due to ingestion of cyanide and from 1.0 to 15 μg/mL due to inhalation of cyanide, while urine cyanide concentration ranges from 0.5 to 1.1 μg/mL due to ingestion of cyanide and 2.0 μg/mL due to inhalation of cyanide [26]. Our method, for urine samples, linear from 0.02–10 μg/mL, the LOD 0.01 μg/mL, for plasma samples,

Table 1
Intra-day precision of the assays.

Urine sample concentration (μg/mL)	Low 0.1	Medium 1	High 10	Plasma sample	Low 0.2	Medium 2	High 20
1	0.1073	1.0652	10.4463	1	0.2153	2.1263	21.0325
2	0.0951	1.0323	9.7925	2	0.2093	2.0452	19.8734
3	0.0973	1.0278	10.2931	3	0.1825	1.8610	20.7641
4	0.1046	0.9836	9.8704	4	0.2102	1.9145	19.0951
Intra-day RSD[a]%	5.74	3.26	3.19	Intra-day RSD[a]%	7.24	6.09	4.37

[a] RSD, relative standard deviation.

Table 2
Recoveries of CN added to urine and plasma.

	Added (μg/mL)	Expected (μg/mL)	Measured (n = 3, mean) (μg/mL)	Recovery (%)
Urine tested at 0.0492 μg/mL	0.0000	0.0492	0.0492	
Low	0.0500	0.0992	0.1065	114.60
Medium	0.5000	0.5492	0.5691	103.98
High	7.0000	7.0492	6.5602	93.01
Plasma tested at 0.1891 μg/mL	0.0000	0.1891	0.1891	
Low	0.2000	0.3891	0.4202	115.56
Medium	5.0000	5.1891	5.4235	104.69
High	80.0000	80.1891	72.6521	90.58

linear from 0.1–20 μg/mL, the LOD 0.04 μg/mL, is suitable for the detection and quantification of CN in clinical and forensic practice.

3.4. Quantification of CN in blood and urine sample

The blood and urine collected from a 47-year-old man who had died after ingestion of potassium cyanide were examined and SIM chromatogram is shown in Figs. 2(D) and 3(D) separately. By the equations for the regression line, the concentration of cyanide is 0.15 μg/mL in urine and 11.4 μg/mL in plasma.

4. Conclusion

In summary, we have developed a new SLE-GC–MS method with two-step derivatization for rapid quantitative detection of CN in plasma and urine. Our assay is simple, sensitive, and accurate. It is also significantly less labor intensive and less time consuming than previously reported GC–MS methods with derivatization. This simple and accurate method is suitable for the detection and quantification of CN in most clinical and forensic practice.

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