Fluorous and Fluorogenic Derivatization for Selective Liquid Chromatographic Analysis of Cyanide in Human Plasma

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Abstract

A liquid chromatographic (LC) method with fluorous derivatization for the determination of cyanide in human plasma is described. In this method, the cyanide was transformed to fluorous and fluorogenic compound by derivatizing with 2,3-naphthalenedialdehyde and perfluoroalkylamine reagent under the mild reaction conditions (a reaction time of 5 min at room temperature). The obtained derivative was successfully retained on the perfluoroalkyl-modified LC column in the use of high concentration of organic solvent in the mobile phase conditions, whereas non-fluorous derivative was hardly retained, followed by fluorometric detection at excitation and emission wavelengths of 420 nm and 490 nm, respectively. Under the optimized conditions, the limit of detection and the limit of quantification for cyanide in a 5 µL injection volume were 1.3 µg/L ($S/N = 3$) and 4.4 µg/L ($S/N = 10$), respectively. The recovery from spiked human plasma was achieved in the range of 54–90% within a relative standard deviation of 3.5%. The feasibility of this method was further evaluated by applying it to analysis of human plasma samples.

Keywords
Cyanide, Fluorous and fluorogenic derivatization, Liquid chromatography, Human plasma
Introduction

Cyanide is known to be exposed to people by many circumstances of occurrence, such as industrial sources, automobile exhaust, smoke inhalation from tabaco or fires.\textsuperscript{1-4} Because the acute and/or chronic toxicity of cyanide causes detrimental effects on human health, the measurement of cyanide in the human body is of great importance in the research involving clinical and forensic science fields.

For the determination of cyanide in biological samples, chromatographic methods in combination with chemical derivatization have been often utilized.\textsuperscript{5} For example, cyanide has been analyzed by headspace gas chromatographic method with nitrogen-phosphorus detection\textsuperscript{6} or mass spectrometry (MS) detection.\textsuperscript{7,8} In addition, liquid chromatographic (LC) method with fluorescence (FL)\textsuperscript{9-11} and MS\textsuperscript{12-14} detection have been also reported. In these reports, cyanide was derivatized with \textit{o}-phthalaldehyde\textsuperscript{11} or 2,3-naphtalenedialdehyde (NDA).\textsuperscript{9,10,12-14} Although these methods are reliable, some interference derived from complex biological samples may cause unaccurate peak identification and determination of target analyte on the chromatograms.

Recently, the highly selective LC analysis method for biogenic-related compounds using fluorous chemistry has been developed.\textsuperscript{15} The “fluorous” means the fluorophilicity, not hydrophobicity or hydrophilicity, which is the unique affinity of highly fluorinated compounds.\textsuperscript{16} In order to utilize the fluorous affinity, target analytes are derivatized with perfluoroalkyl-containing (fluorous) reagent. Thereafter, the fluorous-derivatized analytes are selectively analyzed by LC system with perfluoroalkyl-modified stationary phase column because of extreme retention \textit{via} fluorous affinity. This fluorous derivatization technique is proposed as the useful
method for eliminating matrix-effects in analysis with LC-mass spectrometry.\textsuperscript{17-20} In addition, the combination with fluorescent-based detection approaches were also utilized as selective analysis method to focus on some biogenic compounds.\textsuperscript{21-23}

The aim of this study is to develop the fluorous and fluorogenic derivatization method for the highly selective and sensitive analysis of cyanide with LC-FL detection. To this end, NDA and 4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11-heptadecafluoroundecylamine (HFUA) were used as derivatization reagents (Fig. 1). The resulting derivative can be analyzed selectively and sensitively via both fluorous affinity and fluorogenic property. After optimization studies for the derivatization and the LC analysis conditions, this method was applied to the analysis of cyanide in the spiked human plasma samples without any other enrichment and/or clean-up procedures.

**Experimental**

**Reagents and chemicals**

Unless stated otherwise, all chemicals mentioned below were of the highest purity available and were used as received. Potassium cyanide, NDA, HFUA, and \(n\)-undecylamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pooled human plasma was obtained from Cosmo Bio (Tokyo, Japan). All organic solvents were of LC grade. It should be noted that these reagents and solvents are toxic to the eyes, lungs, and skin, and should be used carefully according to guidelines specified in the latest material safety data sheets. Ultrapure water purified using a Milli-Q gradient system (Merck Millipore, Darmstadt, Germany), was used to produce all aqueous solutions. A standard stock solution (10 mg/L) of cyanide was freshly prepared by
dissolving in water. A working standard solution was used by diluting further with ultrapure water to the required concentrations before use. Both 0.5 mM NDA and 1 mM HFUA solutions were prepared by 65% ethanol and used within 1 d.

**Derivatization procedure**

To the sample solution (50 µL) placed in a polypropylene 1.5-mL vial were added 250 µL of methanol. After vortex mixing for a few seconds, the solution was centrifuged at 16,000 × g for 5 min at 4°C. An aliquot of the supernatant (30 µL) was transferred to another polypropylene vial, and 30 µL of 0.5 mM NDA and 40 µL of HFUA were added. The vial was sealed, and the mixture was left at room temperature for 5 min. Thereafter, the entire reaction solution was placed in the autosampler of an LC system.

**Instrumentation and conditions**

The analyses of fluorous and fluorogenic derivatized cyanide by LC were performed using a Waters Alliance™ 2695 system (Waters, Milford, MA, USA) consisting of a binary pump, an on-line degasser, an autosampler, and a column oven connected to a fluorous phase PFA-modified LC column (150 × 2.1 mm i.d., particle size 3 µm; GL Sciences, Osaka, Japan) and Waters 2475 fluorescence detector equipped with an 8-µL flow cell. The injection volume was 5 µL. The fluorescence detector was operated at an excitation wavelength of 420 nm and emission wavelength of 490 nm. A mixture of acetonitrile and water (85:15, v/v) was used as a mobile phase for isocratic elution. The flow rate and column oven temperature were set at 0.2 mL/min and 30°C, respectively.
Comparison of fluorous and non-fluorous derivatives

We performed the derivatization with the non-fluorous reagent (n-undecylamine) using the same procedure as outlined above (expect for the type of amine reagent used) for confirmation of the selectivity of fluorous interaction. The non-fluorous derivative of cyanide was also analyzed using the same LC conditions.

Validation study

We investigated the sensitivity, linearity, and repeatability of the determination of cyanide using the fluorous derivatization in combination with LC-fluorescence analysis for method validation. The concentration range of the calibration standard solutions was 6.5–650 μg/L (6.5, 32.5, 65, 325, and 650 μg/L). These standard solutions were derivatized with NDA and HFUA using the procedure described above and were injected onto the LC system by the autosampler. The intra-day precisions of the method were evaluated using standard solutions (6.5, 65, and 650 μg/L), by repeated analysis 6 times. The limit of detection (LOD) and the limit of quantification (LOQ) were defined as the concentrations that gave signal-to-noise ratios (S/N) of 3 and 10, respectively.

Results and Discussion

Derivatization conditions

The fluorogenic derivatization of cyanide with NDA is known to easily proceed in the presence of an amine reagent. In this study, for introduction of the fluorophilicity to fluorogenic derivative of cyanide, HFUA as the amine reagent in the derivatization was used (Fig. 1). The derivative of cyanide with NDA and HFUA exhibited the fluorescence with excitation wavelength of 420 nm and emission
wavelength of 490 nm. We examined the optimization studies for the reaction conditions using HFUA to obtain the most intense fluorescence peak area of the derivative on LC analysis. By varying the concentration of NDA over a concentration range 0.01–5 mM under a fixed HFUA concentration (1 mM), the maximum peak area of the derivative of cyanide was obtained by adding more than 0.5 mM of NDA. Therefore, we selected 0.5 mM NDA for the derivatization. The HFUA concentration was also found to affect the fluorescence peak area of the derivative and was also optimized. We examined various HFUA concentrations in the range 0.01–5 mM. As a result, more than 1 mM HFUA was found to produce the maximum peak area for derivative. This derivatization reaction could be performed at room temperature. In order to optimize the reaction time, the progress of the reaction was examined over the course of 120 min. A reaction time over 5 min was required to obtain the maximum peak area with constant value. Consequently, we chose concentrations of 0.5 mM for NDA and 1 mM for HFUA, as well as a reaction time of 5 min, as optimal for the derivatization of cyanide in this study.

**Fluorous LC separation**

In this study, the cyanide was successfully transformed to fluorous and fluorogenic derivative with NDA and HFUA. Therefore, the obtained derivative could be detected with high sensitivity by fluorescence detector after strong retention on the fluorous LC column. To tune the chromatographic behavior of the derivative on the fluorous LC column, the mobile phase compositions were optimized. Among the organic solvents examined for mixing with water in the mobile phase (acetonitrile, methanol, or tetrahydrofuran), acetonitrile was the most effective to obtain good peak shape and appropriate retention of the fluorous derivative. In contrast, when a mixture of water
and methanol was used as the mobile phase, overly-long retention time and peak broadening of the derivative were observed. Although the use of tetrahydrofuran improved the peak shape, higher concentration of it caused weak retention of the fluorous derivative on the column. Consequently, the composition of mobile phase was decided to use a mixture of acetonitrile and water (85:15, v/v) at a flow rate of 0.2 mL/min in the isocratic elution for this study. The selectivity of fluorous derivative via fluorophilicity could be confirmed by analyzing the non-fluorous derivative of cyanide obtained with NDA and n-undecylamine. As shown in Fig. 2, the fluorous derivative was strongly retained on the fluorous LC column under high concentration of acetonitrile in the mobile phase, while the non-fluorous derivative was not in spite of its adequate hydrophobicity. These results demonstrated that only the fluorous derivative could be retained selectively on the fluorous LC column owing to its fluorophilicity not hydrophobicity.

**Analysis of standards**

Validation data for the standard solutions are shown in Table 1. The correlation coefficient \( (r^2) \) of the calibration curve was 0.9981. The relative standard deviations (RSDs) of the intra-day precision values of this method were within 5.3%, as established by repeated determinations \( (n = 6) \) using standard solutions at concentrations of 6.5, 65, and 650 µg/L. The LOD \( (S/N = 3) \) and LOQ \( (S/N = 10) \) for cyanide in this method were 1.3 and 4.4 µg/L (corresponding to 5.1 and 17 fmol on column), respectively. The obtained LOD value of cyanide was lower than those obtained using previously reported LC-FL analysis\(^9,11\) and almost the same with LC-MS\(^{12-14}\) analysis with conventional NDA derivatization methods.
Analysis of spiked human plasma samples

We applied this method to analysis of cyanide standard-spiked and non-spiked human plasma samples for confirmation of the applicability of this method to biological samples. Figure 3 illustrates typical chromatograms obtained from spiked and non-spiked human plasma samples. The fluorous and fluorogenic derivative of cyanide was successfully analyzed by fluorous LC with clearly distinguishing from matrix components of plasma that eluted earlier than the derivative under the present LC conditions, indicating that this method enables the selective analysis of cyanide without interference from plasma matrix. The recovery obtained for the spiked human plasma samples (6.5, 65, and 650 ng/mL plasma) were in the range of 54–90% within 3.5% as RSDs (Table 2). The recovery from human plasma sample was relatively low. This may be because free cyanide is trapped with some proteins, such as human serum albumin and immunoglobulin G in plasma.\textsuperscript{24,25} However, it is considered that the quantitative analysis of cyanide with this method would be no problem because of the good reproducibility. We successfully applied to analysis of endogenous cyanide in human plasma (Fig. 3B), and the found concentration is almost same as previous reports.\textsuperscript{26-30}

Conclusions

We developed the fluorous and fluorogenic derivatization method for the selective analysis of cyanide. In this study, cyanide was derivatized with NDA and HFUA, and the obtained derivative could be analyzed with strong retention on the fluorous LC column followed by sensitive fluorescence detection. Furthermore, this method was applicable to the analysis of cyanide in human plasma without interference from sample matrix, indicating that the fluorophilicity of derivative contributes to selective analysis
of cyanide. We believe that this method will make it useful for evaluating cyanide contamination in various biological samples in the fields of clinical and forensic sciences.
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Table 1  Validation data for standard samples.

| Linearity\(^a\) \((r^2)\) | LOD\(^b\), \(\mu g/L\) | LOQ\(^c\), \(\mu g/L\) | RSD, \(\%^d\) |
|------------------------|-----------------|-----------------|--------------|
|                        |                 |                 | 6.5 \(\mu g/L\) | 65 \(\mu g/L\) | 650 \(\mu g/L\) |
| 0.9981                 | 1.3             | 4.4             | 5.3          | 1.4           | 2.6           |

a. Correlation coefficient of the calibration curves in the range from 6.5 to 650 \(\mu g/L\).

b. Limit of detection, defined as signal-to-noise ratio of 3.

c. Limit of quantification, defined as signal-to-noise ratio of 10.

d. Relative standard deviations of peak areas \((n = 6)\).

Table 2  Mean recovery \((n = 6)\) of cyanide for human plasma.

| Concentration, ng/mL plasma | Mean recovery, % | RSD, % |
|-----------------------------|-----------------|--------|
| 6.5                         | 54              | 3.5    |
| 65                          | 63              | 2.4    |
| 650                         | 90              | 2.3    |
**Figure Captions**

Fig. 1  Fluorous and fluorogenic derivatization for cyanide with NDA and HFUA in this study.

Fig. 2  Typical chromatograms of (A) standard of cyanide (650 µg/L) and (B) blank derivatized with NDA and HFUA and (C) standard obtained from derivatization with NDA and n-undecylamine.

Fig. 3  Typical chromatograms of (A) cyanide spiked (32.5 ng/mL plasma) and (B) non-spiked human plasma samples (endogenous cyanide was 72 ng/mL plasma).
Figure 1

cyanide + NDA $\rightarrow$ fluorogenic and fluororous derivative

\[ \text{HFUA, Rf = C}_{17} \text{F}_{17} \]
Figure 2
Figure 3
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