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High-performance liquid chromatographic determination of cyanide in human red blood cells by pre-column fluorescence derivatization

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ABSTRACT

A method for the determination of cyanide in human red cells has been developed. Cyanide was extracted from red cells by adding water and methanol, and then derivatized with 2,3-naphthalenedialdehyde and taurine to give a fluorescent product, which was determined by reversed-phase high-performance liquid chromatography with fluorescence detection. The recovery of cyanide from red cells was *ca.* 83%, and the limit of detection was 100 pmol/ml. The mean concentrations of red cell cyanide from ten smokers and from ten non-smokers were 705 and 466 pmol/ml, respectively. The method was also applicable to whole blood.

INTRODUCTION

Traces of cyanide have been found in human blood and urine, suggesting that humans are frequently exposed to cyanide from dietary, industrial, environmental and other sources. Toxicological problems then arise owing to its chronic toxicity and the rapid lethal effect [1]. Exposure to cyanide had usually been evaluated by using red cells or whole blood as samples, because most of the cyanide in blood is trapped in red cells [2,3] and bound to methaemoglobin [3]. Various methods have been employed for this purpose, which involved conventional methods such as spectrophotometric [3–6], fluorimetric [7–9] and electrochemical methods [10,11], and chromatographic methods such as high-performance liquid chromatography (HPLC) with photometric [12] or fluorimetric [13] detection, and gas chromatography with electron-capture [14] or nitrogen–phosphorus detection [15]. However, these methods are applicable to red cells or whole blood only after separation of the cyanide as hydrogen cyanide by acidification of samples and most are not sensitive enough to detect cyanide in blood from healthy persons. Therefore, a practical method suitable for monitoring the cyanide levels in biological fluids, especially whole blood and red cells, is desirable.

Recently, we developed an HPLC method for the determination of cyanide in urine [16]. The method is based on the pre-column derivatization of cyanide with 2,3-naphthalenedialdehyde (NDA) and taurine to give a fluorescent 1-cyano-2-substituted benzoisoindole derivative [17,18] (Fig. 1) for reversed-phase HPLC separation and fluorescence detection, by which urine was analysed directly after dilution with water. Taking into account the excellent features of the HPLC method regarding sensitivity, selectivity

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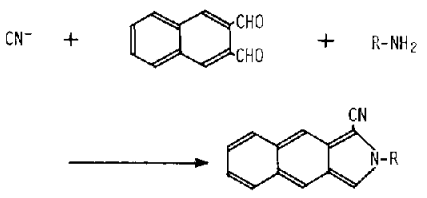

Fig. 1. Fluorogenic reaction of cyanide with NDA and primary amines.

and resolution, it seems that cyanide in red cells or whole blood can also be determined by this method without the acidification step required in previous methods.

This paper describes an HPLC method for the determination of cyanide in red cells using the fluorogenic reaction described above. The method was successfully applied to red cells from smokers and non-smokers, and also permitted the analysis of whole blood.

EXPERIMENTAL

Chemicals

All chemicals used were of analytical-reagent grade, unless stated otherwise. Water was purified on a Milli RO–Milli Q system (Millipore, Bedford, MA, USA). HPLC-grade methanol was purchased from Kanto Chemical (Tokyo, Japan). A stock standard solution of cyanide was prepared with potassium cyanide (Kanto Chemical) at 10 m*M* concentration in 0.1 *M* sodium hydroxide. Working standard solutions were prepared by dilution of the stock solution with water. A borate–phosphate buffer (pH 8.0) was prepared by mixing 535 ml of a 0.05 *M* sodium borate solution and 465 ml of a 0.1 *M* potassium dihydrogenphosphate solution. A stock 4 m*M* solution of NDA (Tokyo Kasei, Tokyo, Japan) was prepared in methanol, and this was diluted with the borate–phosphate buffer to give a 1 m*M* solution in an amber glass bottle. The diluted solution could be used for at least seven days when stored in a refrigerator. Taurine (Nacalai Tesque, Kyoto, Japan) was used as a 5 m*M* solution in the same buffer.

HPLC conditions

The HPLC system consisted of a Shimadzu LC-6A pump (Shimadzu, Kyoto, Japan), a Rheodyne Model 7125 injector with a 20-μl sample loop (Rheodyne, Cotati, CA, USA) and a 5-μm Hibar LiChrosorb RP-18 (125 mm × 4 mm I.D.) (Merck, Darmstadt, Germany) with a guard column (LiChroCART RP-18) (Merck). The mobile phase was 0.05 *M* potassium phosphate buffer (pH 6.8)–methanol (58:42 v/v). The column temperature was ambient and the flow-rate was 1.0 ml/min. Detection was carried out with a Hitachi F-1000 fluorescence spectrophotometer equipped with a flow cell (12 μl) and a xenon lamp (Hitachi, Tokyo, Japan), operated at 418 nm excitation and 460 nm emission.

Determination of cyanide in red cells

Blood was taken by venipuncture into a heparinized tube. Haematocrit was determined using a Wintrobe's tube. The blood (1 ml) was centrifuged at 1000 *g* for 10 min at room temperature, and the plasma and buffy coat were removed with a micropipette. Red cells were washed twice with 0.9% (w/v) sodium chloride and re-suspended in 0.9% sodium chloride to give a volume of 1 ml.

To a 100-μl aliquot of the red cell suspension, 500 μl of water was added followed by addition of 2 ml of methanol. After vortex-mixing, the mixture was centrifuged at 1000 *g* for 10 min at room temperature. A 500-μl aliquot of the supernatant was taken into a 1.5-ml brown-coloured test-tube, and 100 μl each of 5 m*M* taurine and 1 m*M* NDA solutions were added. After standing for more than 30 min at room temperature, a 20-μl aliquot of the mixture was injected into the chromatograph. The cyanide concentration was ascertained from the peak height of a cyanide standard solution (20 or 50 pmol/ml).

RESULTS AND DISCUSSION

Extraction of cyanide from red cells

Prior to the HPLC analysis of red cells for cyanide, separation of the cyanide from solid matrix in red cells was necessary. Therefore, extraction

with organic solvents (2 ml), such as methanol, ethanol or propanol, was examined first, using 100-μl aliquots of red cell suspension containing known amounts of added cyanide. Methanol was the preferred solvent because red cells were most efficiently haemolysed and denaturated by methanol, and also it gave the highest recovery of the cyanide. Next, in order to check the possibility that cyanide might be an artefact from some compounds structurally related to cyanide, methanol was added to a red cell suspension to which 50 nmol/ml potassium thiocyanate, or 500 pmol/ml methyl thiocyanate, benzyl thiocyanate, potassium hexacyanoferrate(II), potassium hexacyanoferrate(III) or amygdalin had been added. Artifactual formation of cyanide was observed from the first three compounds, and the concentrations of cyanide formed in the suspension were 114, 51 and 146 pmol/ml, respectively. This cyanide formation is considered to be due to the oxidative degradation of these compounds by the action of oxygen generated from oxyhaemoglobin in the cells through direct destruction by methanol, and must be avoided because thiocyanates are known to occur in red cells [2,13,19] and may remain in the cells even after washing with 0.9% sodium chloride solution.

Hence we examined a stepwise treatment of red cells with water and methanol, on the assumption that if red cells were haemolysed gently with water, oxyhaemoglobin in the red cells would be spontaneously converted into methaemoglobin, which does not release oxygen when denaturated with methanol. As described below, this approach was found to be highly effective. As shown in Fig. 2, when red cell suspensions (100 μl) with and without added thiocyanate were treated with more than 300 μl of water followed by addition of methanol (2 ml), the cyanide concentration appeared to level off, and the artifactual formation of cyanide from thiocyanate was suppressed effectively.

The effect of the amount of methanol was examined using the haemolysate prepared from a red cell suspension (100 μl) by adding 500 μl of water. Changes in the amount of methanol from 1 to 3.5 ml had little effect on the concentration

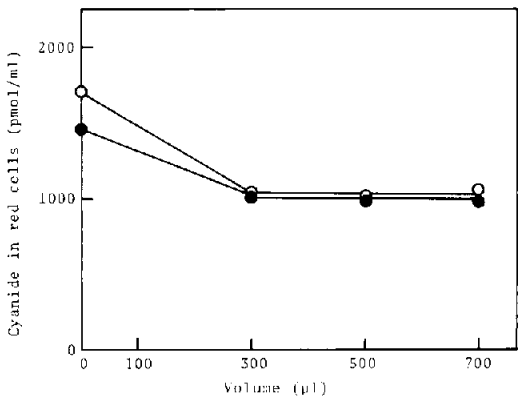

Fig. 2. Effect of addition of water on the determination of cyanide in red cells: concentrations of cyanide found in (●) red cells and (○) red cells spiked with 106 nmol/ml thiocyanate. Various amounts of water were added to the red cell suspension, which was then treated as described in Experimental.

of cyanide in red cells, and more than 80% recoveries were obtained for cyanide added to the red cells. Therefore, the successive treatment of red cell suspensions (100 μl) with 500 μl of water and 2 ml of methanol was recommended. This procedure also completely prevented the undesirable formation of cyanide from the organic thiocyanates.

Derivatization and HPLC

Although 2 m*M* NDA and 30 m*M* taurine were used for the derivatization of urinary cyanide [16], the effect of reagent concentrations on the determination of cyanide in red cells was reexamined. The endogenous cyanide concentration in red cells reached a constant value at taurine and NDA concentrations above 5 and 1 m*M*, respectively, and the recovery of cyanide added to the red cells (1042 pmol/ml in red cells) was more than 80% at the same concentration ranges for taurine and NDA. Therefore, 5 m*M* taurine and 1 m*M* NDA were used.

Fig. 3a shows a typical chromatogram obtained from red cells. The chromatographic conditions used were the same as those reported previously [16]. The fluorescence peak for cyanide was detected at the retention time of 13 min, which was exactly the same as that of the standard cyanide. The identification of cyanide was

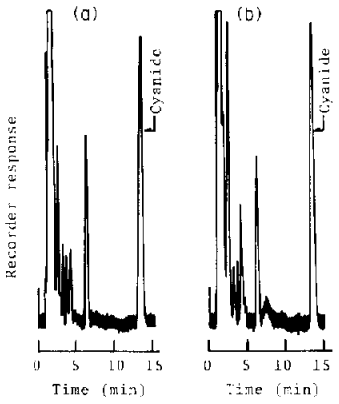

Fig. 3. Chromatograms of cyanide in (a) red cells from a smoker (containing 787 pmol/ml cyanide) and (b) the original whole blood (containing 398 pmol/ml cyanide: haematocrit 0.48). Whole blood was treated as described for the analysis of red cells.

further verified as follows. When the derivatization was carried out using alanine as an amine instead of taurine, the peak for cyanide was found at the retention time of 15 min, which was also identical with that of the reaction product resulting from the reaction of standard cyanide with NDA and alanine. This peak did not appear when the amine or DNA was omitted from the derivatization procedure. These results clearly indicate that the HPLC method permits satisfactory resolution of peaks for cyanide and other endogenous substances in red cells.

Furthermore, Fig. 3b shows a typical chromatogram obtained from whole blood. Judging from the chromatogram, in which cyanide could be detected without any interference, this method also proved to be applicable to the determination of cyanide in whole blood. The cyanide concentration in plasma is known to be extremely low [3], and therefore the concentration found in the whole blood (398 pmol/ml in whole blood) seems to be quite reasonable because it is nearly equal to the product of the concentration in the red cells (787 pmol/ml) and the haematocrit (0.48).

Recovery

Recovery tests were performed using red cells spiked with known amounts of cyanide. As shown in Table I, which represented the intra-assay results, the recovery of cyanide in the concentration range 425–2128 pmol/ml in red cells was *ca.* 83%, with a standard deviation (S.D.) of 1.5–8.8%. The inter-assay recovery of cyanide (mean ± S.D.) at a concentration of 1064 pmol/ml in red cells was 82.5 ± 7.6% (n = 14). The limit of detection of cyanide in red cells, estimated on the basis of the chromatogram shown in Fig. 3a is *ca.* 100 pmol/ml in red cells.

Most of the cyanide added to red cells is known to bind to methaemoglobin in the cells [3]. However, based on the above findings, it can be assumed that the treatment of red cells with water and methanol liberates the binding cyanide because the recovery of cyanide added to red cells is more than 80% over the concentration range examined.

Determination of cyanide in red cells

The determination of cyanide in red cells from healthy persons, ten smokers (men) and ten non-smokers (seven men and three women), was attempted. The concentrations of cyanide (mean ±

TABLE I

RECOVERY OF CYANIDE ADDED TO RED CELLS (n = 5)

Cyanide added (pmol/ml in red cells)	Found (mean ± S.D.) (pmol/ml in red cells)	Recovery (mean ± S.D.) (%)
0	743 ± 32	–
425	1096 ± 37	83.1 ± 8.8
1064	1623 ± 45	82.7 ± 4.3
2128	2516 ± 34	83.3 ± 1.5

S.D.) were 466 ± 72 pmol/ml for non-smokers and 705 ± 112 pmol/ml for smokers. These values are similar to those reported by Lundquist *et al.* [3] (240 ± 220 pmol/ml for the former and 680 ± 200 pmol/ml for the latter), and also there is a significant difference between the concentrations of cyanide in red cells from smokers and non-smokers (Student *t*-test, $p < 0.001$) as suggested previously [3,7].

In order to check the reliability of the results, eleven of the twenty red cell samples were also analysed by a microdiffusion fluorimetric method for the determination of cyanide using 10% sulphuric acid as an acidifying agent [4] and NDA and taurine as fluorogenic reagents [18]. The cyanide concentrations (pmol/ml in red cells) obtained by the HPLC method (y) agreed well with those obtained by the microdiffusion method (x): $y = 0.79x + 110$ ($r = 0.89$).

Previously, we reported a method for the determination of cyanide in whole blood and urine [18], which was based on the separation of cyanide by microdiffusion using 1 *M* acetate buffer (pH 5.2) as an acidifying agent [9], and subsequent determination of the cyanide by fluorimetry or HPLC with electrochemical detection. The utility of the acetate buffer for the separation of cyanide from urine samples has been confirmed in our preceding study [16]. On the other hand, in the application to whole blood, it is noted that the cyanide concentrations determined by such procedures (below 20 pmol/ml for non-smokers and below 100 pmol/ml for smokers [18]) are much lower than those obtained here and reported accurately by Lundquist *et al.* [3] (130 ± 80 pmol/ml for the former and 330 ± 120 pmol/ml for the latter). This may be because the acetate buffer inadequately dissociates the cyanide bound to methaemoglobin, and therefore it seems that mainly free cyanide in plasma and/or red cells can be determined by the previous microdiffusion method [18].

Although some fluorimetric methods [7,18] are capable of determining 5–30 pmol/ml cyanide in whole blood or red cells, and the detectability is superior to that of the HPLC method described here, these methods require the acidification of samples for the separation of cyanide prior to the determination and this step is troublesome. The HPLC method eliminates the acidification step and consequently the method is easy to perform. In addition, its sensitivity is sufficient to determine accurately cyanide in red cells from healthy persons, and the method is similarly applicable to whole blood. Furthermore, in the HPLC method, if desired, the sample size of whole blood can be reduced to 100 μl or less, though previous methods usually require more than 1 ml of whole blood. Therefore the proposed HPLC method should be useful for clinical and toxicological studies.

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