

Determination of Acetaldehyde in Human Blood by High-Performance Liquid Chromatography Using Fluorometry

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A method was developed for the measurement of acetaldehyde in human blood by high-performance liquid chromatography (HPLC). The method was based on a pre-column reaction; a fluorescent substance was formed by a coupling reaction between 2 mol of cyclohexan-1,3-dione and 1 mol of acetaldehyde with ammonium acetate. The coupling compound was analyzed by HPLC and the concentration of acetaldehyde was obtained from the calibration curve drawn from the results using standard solutions. The determination was sensitive and reproducible with a range of 0.2–10 μM and a precision (coefficient of variation) of 2.43 %.

Keywords acetaldehyde; cyclohexan-1,3-dione; fluorometry; high-performance liquid chromatography; human blood

In blood, acetaldehyde is produced from the metabolism of ethanol and shows a high reactivity towards proteins.^{1,2)} It is commonly studied in relation to the pathogenesis of alcoholism.^{3–5)} However, the determination of acetaldehyde in human blood is not easy, because of its binding to proteins both reversibly and irreversibly, rapid metabolism by enzymes,⁶⁾ and the production of acetaldehyde from ethanol during sample pretreatment.^{6–8)}

Most methods for the determination of blood acetaldehyde employ deproteinization and analysis by gas chromatography and high-performance liquid chromatography (HPLC). Some methods with improved sensitivity were reported,^{9–13)} because blood acetaldehyde levels in normal subjects were very low. Recently, Suzuki¹⁴⁾ reported a sensitive method for the determination of several kinds of aliphatic aldehydes in whisky using cyclohexan-1,3-dione (CHDO). We, therefore, examined the application of this method for the determination of acetaldehyde in human blood.

Experimental

Reagents CHDO (Tokyo Kasei, Tokyo, Japan), a kit for acetaldehyde analysis (Boehringer Mannheim, W. Germany), and thiourea, ammonium acetate, trichloroacetic acid solution (TCA solution, 100%), and acetaldehyde solution (about 90%) (Wako, Osaka, Japan) were purchased from the indicated sources. Other solvents and inorganic chemicals were of reagent grade. Acetaldehyde standard solutions were obtained diluting the acetaldehyde solution (about 90%) with water by 3×10^4 times, and diluting it with additional water to yield a concentration from 1 to 10 μM .

Chromatographic Conditions Chromatography was performed with a Tri-roter VI liquid chromatograph (JASCO, Tokyo, Japan). The system included a manual injector, a DG-3510 degasser, a Tri-roter VI pump, and a 250×4.6 mm Finepak SIL C₁₈T-5 analytical column (JASCO). The eluent was continuously monitored with an FP-210 variable-excitation-and-emission-wavelength spectrophotometer (JASCO).

We used 30% methanol as the mobile phase, at a flow-rate of 1.0 ml/min. Fluorescence was measured at 366 nm (excitation) and 440 nm (emission). The wavelengths were discussed in a recent report.¹⁴⁾

Measurement Procedure A 0.5 ml aliquot of each sample and 0.1 ml of 100% TCA solution were put into a test tube and mixed well. After standing at 4°C for 30 min, the mixture was centrifuged at $1500 \times g$ for 5 min, and 0.15 ml of the supernatant was added to a mixture of 0.15 ml of 20% ammonium acetate solution, 0.06 ml of 2.5% CHDO solution and 0.15 ml of 6% thiourea solution and mixed well. The mixture was incubated at 60°C for 30 min, and then cooled in water. This reaction mixture was analyzed by HPLC and the concentration of acetaldehyde in each sample was calculated from a standard curve obtained using standard solutions.

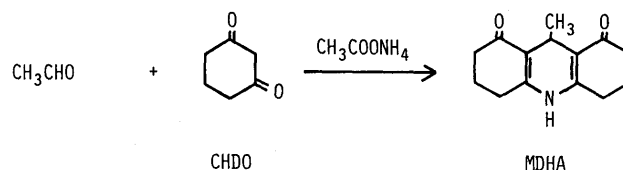


Fig. 1. Scheme of Formation of the Fluorescent Substance

CHDO, cyclohexan-1,3-dione; MDHA, 9-methyl-decahydroacridin-1,8-dione.

Results

Optimal Concentrations of the Reagents The optimal concentration of each reagent in the reaction mixture, suitable for the measurement of acetaldehyde concentrations up to 10 μM , was first determined. The optimal concentration of ammonium acetate and CHDO were 20% and 6%, when they were tested between 0–50% and 0–10%. The concentration of the thiourea solution was set at 2%, because the solubility of thiourea in water was sufficient at 10% or less. When the volume of thiourea solution was tested between 0 and 0.60 ml using a blood sample with 9.0 μM acetaldehyde and 50 μM ethanol, a recovery rate of 100% of the added acetaldehyde was obtained upon addition of 0.15 ml or more of this solution, and therefore the amount was set at 0.15 ml to prevent interference by ethanol.

Study for HPLC Conditions The method for the analysis of several aldehydes in whisky, reported by Suzuki,¹⁴⁾ was carried out using a linear gradient from 50 to 100% aqueous methanol as a mobile phase. However, blood contains many components that show a native fluorescence. As shown in the chromatogram in Fig. 2, some of them were found as small peaks. Thus, 30% methanol was used (tested between 25 and 45%), because it provided better separation of the objective compound from other blood components with shorter retention times.

Method Validation With the present method, good linearity was obtained between peak height and acetaldehyde concentration (up to 10 μM). The lower limit of concentration detectable was 0.2 μM , which gave a signal-to-noise ratio of 2.0.

The accuracy was tested by adding acetaldehyde to blood samples in the concentration range of 0–10 μM . The recovery was 94.4–102.0% ($n=15$).

The intra-assay study was undertaken using a blood

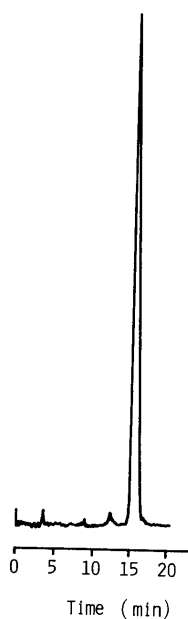


Fig. 2. Chromatogram of a Reaction Mixture under the Proposed Conditions

Acetaldehyde concentration in the blood sample was $9\text{ }\mu\text{M}$.

sample with an acetaldehyde concentration of $4.5\text{ }\mu\text{M}$ in five test tubes. The intra-assay precision, that is, the coefficient of variation (C.V.) was 2.43%.

Five human blood samples obtained from normal subjects were tested and the acetaldehyde concentrations were $0.60 \pm 0.08\text{ }\mu\text{M}$ (mean \pm S.D.).

Discussion

In this method, the object was to form a fluorescent substance with CHDO and acetaldehyde¹⁵⁾ (Fig. 1). This could be assayed with high sensitivity (detectable range; $0.2\text{--}10\text{ }\mu\text{M}$) and specificity (HPLC analysis). The sensitivity of this method was the same as that of other sensitive methods reported recently.^{10,12)}

Stowell *et al.*⁸⁾ reported that acetaldehyde was formed from ethanol spontaneously in blood. As presented in this

paper, interference by ethanol in sample blood was eliminated in the proposed method, because thiourea was used as reported by Iversen and Damgaard.¹⁶⁾

During the HPLC analysis of the reaction mixtures, the eluent used was 30% methanol, and perfect separation was obtained. In the report by Suzuki,¹⁴⁾ a 50—100% methanol gradient was used, because several aldehydes, formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, and others had to be analyzed. In our method, however, the target aldehyde was only acetaldehyde, so a more separative eluent, 30% methanol, was chosen as the mobile phase.

The normal range of acetaldehyde in healthy human blood is $1\text{ }\mu\text{M}$ or less, but alcoholics' blood contains higher levels of acetaldehyde. Thus, the analytical range of the proposed method, $10\text{ }\mu\text{M}$ or less, is very useful in the clinical field.

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