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Short communication

# Determination of free acetaldehyde in total blood for investigating the effect of aspartate on metabolism of alcohol in mice

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**Abstract**

To explore the effect of sodium L-aspartate monohydrate (aspartate) as a $NAD^+$ regenerating agent for acetaldehyde in alcohol metabolism, a simple HPLC method has been developed for the measurement of free acetaldehyde in total mice blood digested with alcohol and aspartate. The blood samples were collected in EDTA Vacutainer tubes, and treated with 2,4-dinitrophenylhydrazine (DNP hydrazine) reagent in total blood. Acetaldehyde DNP hydrazone was extracted from total blood and analyzed by HPLC using an Ultrasphere ODS column. The compounds were separated using acetonitrile–water (50:50, v/v) as mobile phase and detected at 356 nm. The detection limit for acetaldehyde DNP hydrazone was 0.1 ppm. A blank determination was carried out for each analysis and subtracted from the results. The amount of acetaldehyde in blood has been determined as a function of time lapse after sole alcohol administration and aspartate ingestion followed by alcohol administration, respectively. This comparative analysis demonstrates that the ingestion of aspartate before the administration of alcohol dramatically decreases the aldehyde level in blood, and aspartate may be utilized as a prospective antagonist for acceleration of ethanol metabolism and prevention of acetaldehyde toxicity. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Acetaldehyde; Aspartate; Alcohol

## 1. Introduction

Acetaldehyde which is produced during ethanol metabolism is known to be extremely toxic and to cause alcoholic disease [1]. Therefore, the identification of a means of quickly and safely antagonizing the acute effects of acetaldehyde could provide a treatment for acute intoxication and could lead to a better understanding of its mechanism of action [2,3]. Thus, a simple method for the determination of free acetaldehyde is very important in investigating a treatment for acute intoxication as well as the understanding of the alcohol digestion mechanism.

The earlier methods to determine the concentration of acetaldehyde in blood were concerned with plasma acetaldehyde which does not reflect total blood acetaldehyde because acetaldehyde binds reversibly/irreversibly to protein and is rapidly metabolized by enzymes [4–9]. In the present study, we describe the preparation of acetaldehyde DNP hydrazone in total blood, and the concentration change of acetaldehyde in total blood after alcohol administration measured by a high-performance liquid chromatography (HPLC) method with UV detection using 2,4-dinitro-

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phenylhydrazine (DNP hydrazine) reacted with acetaldehyde.

It has been known that aspartate plays an important role in mediating alcohol oxidation in alcohol metabolism [10]. To explore the additional efficacy of aspartate as a $NAD^+$ regenerating agent for acetaldehyde oxidation, we have determined the acetaldehyde concentration in total mice blood drawn at 20, 40, 80, 120, 240 and 360 min, respectively after aspartate ingestion followed by ethanol administration. The overall aim of the present study was to utilize this HPLC analytical method to better characterize the role of aspartate during alcohol metabolism in mice.

## 2. Experimental

### 2.1. Chemicals

Sodium L-aspartate monohydrate was purchased from Sigma (St. Louis, MO, USA). All solvents and chemicals were purchased from Aldrich (Milwaukee, MI, USA). The following reagents were used: DNP hydrazine; hexane, HPLC grade; HCl, acetaldehyde; acetonitrile, HPLC grade; anhydrous $Na_2SO_4$, acetaldehyde DNP hydrazone. Acetaldehyde DNP hydrazone in total blood was synthesized as described by Vogel [11]. Water was deionized and distilled once. Glassware was soaked overnight in chromic acid and washed with ultrapure water.

### 2.2. Chromatography

The HPLC system consisted of a pump (Model 510, Waters, CA, USA), a UUK injector (Rheodyne, Cotati, CA, USA), Lambda Max detector (Model 481, Waters), a Millipore Waters injector, Capcell Pak $C_{18}$ Type UG 120 column (5 μm, 250×4.6 mm I.D.; Shiseido, Tokyo, Japan) and a Data Module integrator (Millipore, Waters, CA, USA). The eluent used was acetonitrile–water (50:50, v/v). The chromatographic system was operated at ambient temperature. The flow-rate was set at 1.5 ml/min and the pressure was 120.7 bar. A UV detector (Model 481, Waters) was set at 356 nm for the detection of acetaldehyde DNP hydrazone. Under these conditions, HPLC retention time for aldehyde DNP hydrazone was 6.85 min.

### 2.3. Subjects and blood specimens

Male and female mice were housed individually in wire-bottom cages in a controlled environment at 23°C and 95% relative humidity. The number of mice used in this experiment was 42. The mice were injected with alcohol. The dose of ethanol was 1.5 g/kg, administered as a 10% (v/v) solution of 95% ethanol in saline. The volume injected was 20 ml/kg. Half of the mice group were injected with sodium L-aspartate monohydrate (12 mg/kg) just before ethanol was injected. Venous blood was taken at 20, 40, 80, 120, 180, 240, 360 min, respectively after alcohol and aspartate/alcohol injection, and collected in EDTA Vacutainer tubes.

### 2.4. Procedure

A 0.5-ml volume of total blood at 4°C was immediately treated with 0.01 *M* DNP hydrazine in 3.6 *M* HCl solution and shaken for 30 min at room temperature and 20 ml *n*-hexane added. The organic phase was separated and washed three times with 10 ml water. The trace water remaining in the hexane phase was removed by anhydrous $Na_2SO_4$. The organic phase was separated and the solvent was evaporated to dryness using vacuum rotary evaporator. The residue was then dissolved in 1~5 ml $CH_3CN$–water (50:50, v/v) depending on their concentrations and analyzed by HPLC as described above.

Blood used for measurement of acetaldehyde basal levels and for calibration curve was collected from untreated mice which were not injected with alcohol, and was immediately transferred to ice cold tubes.

A calibration curve was prepared by determination of the amount of DNP hydrazone synthesized by adding acetaldehyde as aqueous solution (1, 2, 3 and 4 ppm) to blood aliquots. The samples were then processed as described above for the measurement of blood levels.

## 3. Results and discussion

A HPLC chromatogram of DNP hydrazone of acetaldehyde in total blood was obtained on a reversed-phase analytical column (UG-120, Shiseido). Aqueous acetonitrile was used as eluent; a

preliminary experiment showed that acetonitrile–water (50:50, v/v) gave good separation of the DNP hydrazone of acetaldehyde.

Fig. 1A identifies that the authentic standard DNP hydrazone of acetaldehyde generated a 6.85 min retention time in the HPLC chromatogram. As shown in Fig. 1B, a small peak corresponding to that of the DNP hydrazone of acetaldehyde was obtained with the reagent blank; this peak probably originated from the environmental aldehyde trace and could not be eliminated.

The calibration curve was obtained by increasing amounts of acetaldehyde to total mice blood. A linear relationship ($r=0.9997$) between peak area (DNP hydrazone of acetaldehyde) and the concentration of acetaldehyde added was obtained and regression analysis showed a linear relationship between added and recovered acetaldehyde. The linear regression equation was $y=1637.5$ $(\pm 0.001)x-72.5$ $(\pm 0.02)$; where $y$ is the relative peak area at a detector sensitivity of AUFS (absorbance unit of full scale)$\times 0.05$ and $x$ is the concentration of acetaldehyde (ppm) added in blood. The accuracy was tested by adding acetaldehyde to samples of blood in the concentration range 1~4 ppm. The method has been further validated by intra-day and inter-day precision measurement. The intra-day precision (relative standard deviation, R.S.D.) for five replicates of the same extracted sample was 0.49%. The reproducibility of this method was assessed on five samples. The inter-day precision (R.S.D.) for the experiment performed for three days with 24-h intervals was found to be 0.88%.

The comparison of peak areas of the DNP hydrazone synthesized in the total blood with those in the authentic standards allowed one to calculate a recovery from the blood of 86.3~90.4% for acetaldehyde at all tested concentrations. The analytical result of the samples where DNP hydrazone of acetaldehyde was obtained from aqueous solution of acetaldehyde indicated the yield of the derivative formation and of its extraction. The much higher recovery of acetaldehyde from blood in comparison to that reported earlier by Pezzoli et al. [4] seems to confirm that acetaldehyde bound to erythrocyte is

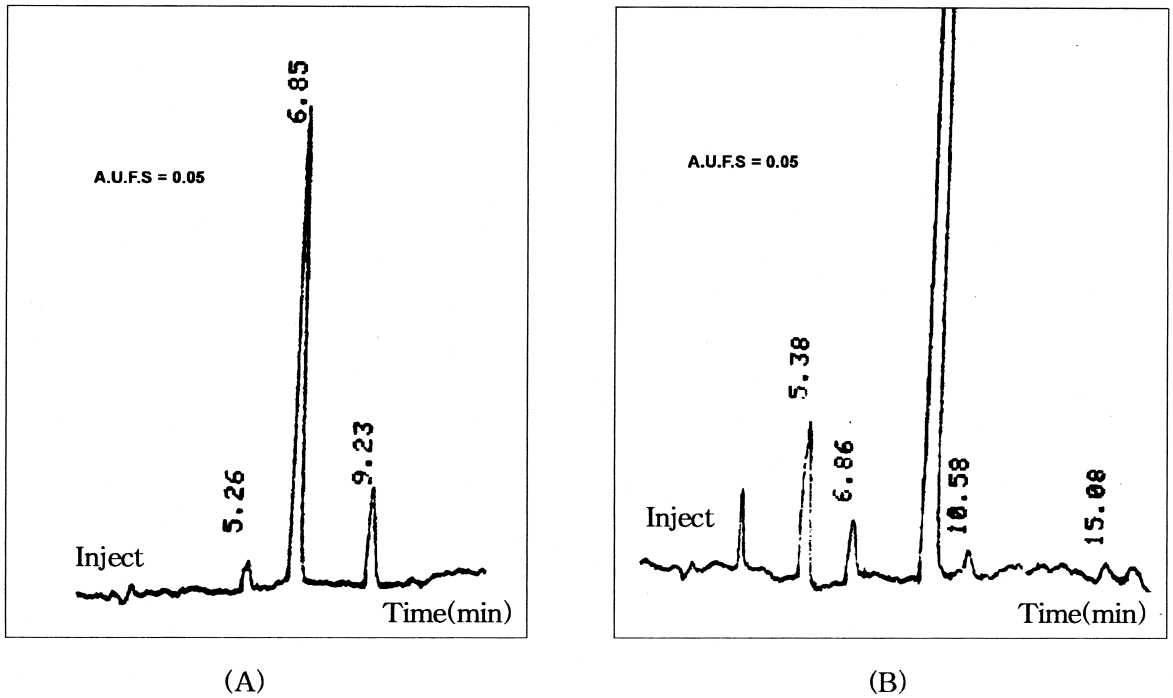


Fig. 1. (A) HPLC trace of an authentic standard solution for DNP hydrazone of acetaldehyde (for identification of compound). (B) Chromatogram of blank blood sample without injection of aspartate and alcohol.

almost released through three-times solvent extraction from total blood rather than plasma acetaldehyde which does not reflect total blood acetaldehyde.

Acetaldehyde DNP hydrazone can also be identified and determined by gas chromatography (GC) [or GC–mass spectrometry (MS)], and the detection limit (0.2 ppm) of GC was higher than that (0.1 ppm) of HPLC with UV detection. The retention times of the GC chromatogram for the HPLC eluate were identical to those of authentic standard sample. It was also confirmed that the base peaks for the HPLC eluate and standard sample were identical at $m/z$ 224, which corresponded to the molecular ion peak of DNP hydrazone of acetaldehyde using the electron-impact (EI) ionization mode in GC–MS (not shown).

As described above, it is known that sodium L-aspartate monohydrate plays an important role in accelerating the alcohol oxidation in alcohol metabolism. To explore the additional efficacy of aspartate as a $NAD^+$ regenerating agent for acetaldehyde oxidation, we have determined the acetaldehyde concentration in total mice blood drawn at 20, 40, 80, 120, 240 and 360 min, respectively after aspartate ingestion followed by ethanol administration.

As an application of the earlier developed method, acetaldehyde levels were determined in mice blood, of which one group was injected with aspartate (12 mg/kg) just before ethanol was administered and the other was solely injected with ethanol. The dramatic decrease of acetaldehyde levels in whole blood samples with aspartate injection is shown in Fig. 2. Levels were measured as a function of time lapse after injection of alcohol. The variation of acetaldehyde concentration in blood as a function of time lapse after alcohol and/or aspartate digestion was tabulated and is displayed in Table 1 and Fig. 3, respectively; the peak corresponding to DNP hydrazone of acetaldehyde significantly decreased for blood ingested with aspartate. Aldehyde levels for the mice blood without and with injection of aspartate were $7.36 \pm 0.51$ ppm and $0.48 \pm 0.02$ ppm,

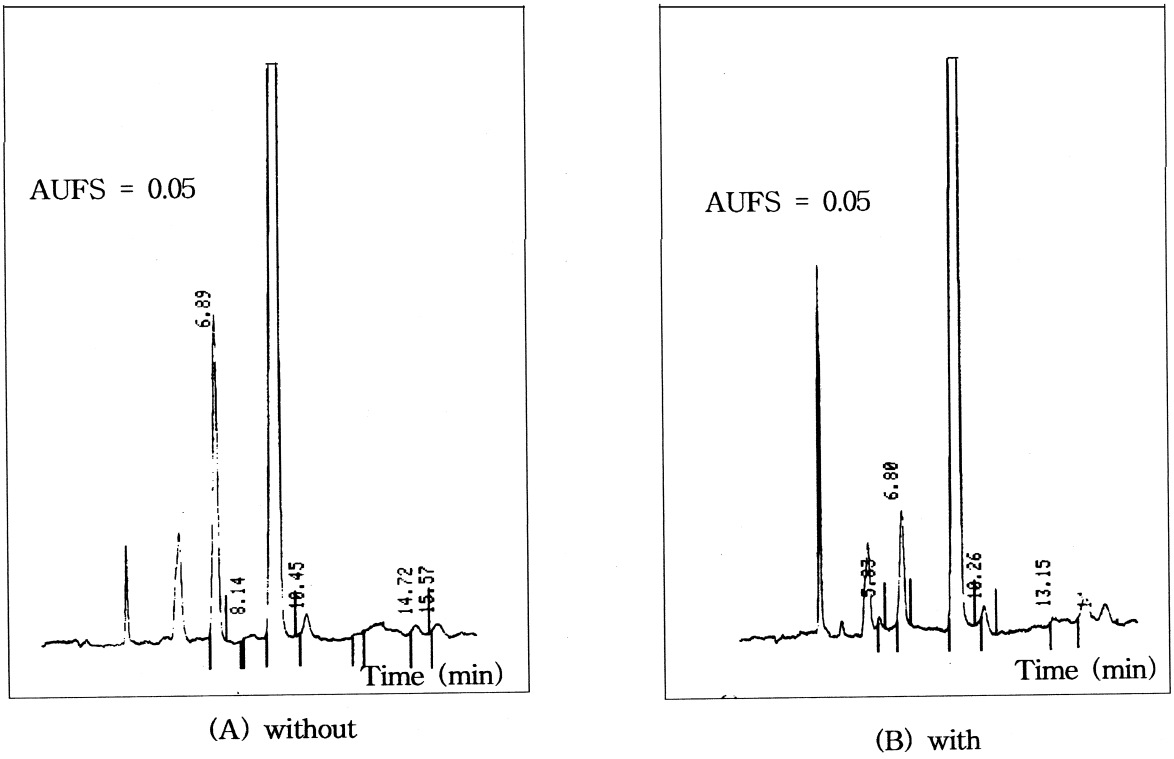


Fig. 2. Chromatograms of whole blood samples after alcohol administration without (A) and with (B) aspartate injection.

Table 1
The concentration of acetaldehyde in blood after sodium L-aspartate monohydrate was administered

| Compound | Dose (mg/kg) | $n$[a] | Concentration of acetaldehyde in total blood after alcohol was administered (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 20 min | 40 min | 80 min | 120 min | 180 min | 240 min | 360 min |
| Vehicle[b] | – | 3 | 1.83±0.11[d] | 1.86±0.29 | 3.13±0.47 | 7.36±0.51 | 5.41±0.53 | 5.90±0.66 | 0.72±0.11 |
| Aspartate[c] | 12 | 3 | 0.53±0.03 | 1.11±0.29 | 1.21±0.63 | 0.48±0.02 | 0.78±0.22 | 0.82±0.23 | 0.62±0.11 |

[a] Number of measurements for acetaldehyde concentration.
[b] Blood sample for which alcohol was solely administered.
[c] Blood sample for which aspartate (12 mg/kg) and alcohol (1.5 g/kg) was administered.
[d] Each value represents the mean±standard deviation.

respectively in 2 h after ingestion of ethanol. These results indicate that aspartate is able to activate the acetaldehyde oxidation during alcohol metabolism.

It was not clear that aspartate activates the oxidation of acetaldehyde during the process of ethanol metabolism. However, as it is known that aspartate is oxidised to oxaloacetate by transaminase and it is reduced to malate by malate dehydrogenase, $NAD^+$ produced in the reduction process of oxaloacetate seems to be involved in the oxidation pathway of acetaldehyde as well as alcohol [10]. Therefore, aspartate could be utilized as a prospective antagonist for acceleration of ethanol metabolism and prevention of acetaldehyde toxicity.

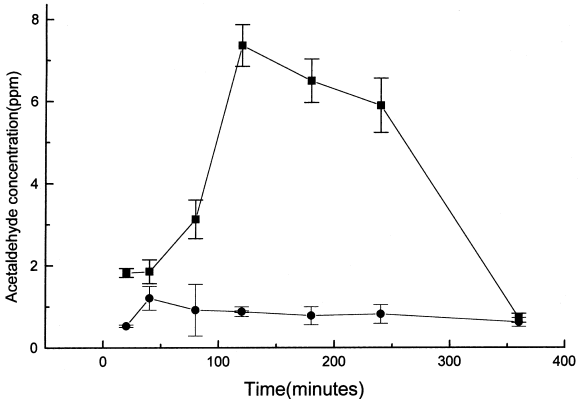


Fig. 3. Variation of acetaldehyde concentration in blood as a function of time lapse after (■) the administration of alcohol (●) the administration of aspartate (12 mg/kg) and alcohol (1.5 g/kg).

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