DISTRIBUTION OF ACETALDEHYDE IN HUMAN BLOOD: EFFECTS OF ETHANOL AND TREATMENT WITH DISULFIRAM

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Abstract — The distribution of free and bound acetaldehyde in human blood was studied. Fresh whole blood was precipitated with a perchloric acid (PCA) in saline solution and an aliquot of the crude sample was taken for determination of ‘total’ acetaldehyde. The remaining sample was centrifuged and the clear supernatant taken for analysis of ‘soluble’ acetaldehyde. ‘Bound’ acetaldehyde was calculated by subtracting soluble from total amounts. In samples collected from healthy control subjects, the acetaldehyde level in separated plasma was usually below the limit of detection of the method (0.2 μM), while much higher concentrations (> 2.5 μM) were detected when analyses were carried out on whole blood. In whole blood, about 70% was recovered as bound (i.e. PCA-insoluble) acetaldehyde. The soluble (i.e. free + PCA-soluble) level was higher than that found in separated plasma, suggesting that some acetaldehyde was liberated from the blood cells by PCA treatment. In blood spiked with ethanol, a spontaneous formation of acetaldehyde occurred during the analytical procedure. The artefactual formation increased only the soluble amount, while the bound level remained unchanged. Likewise, in samples drawn from intoxicated subjects, artefactual formation of acetaldehyde was observed in the soluble fraction, while the bound amount was not significantly increased. No significant differences in acetaldehyde levels were found between males and females, nor between healthy control subjects and alcoholic patients undergoing treatment with the aldehyde dehydrogenase inhibitor disulfiram (Antabuse®). However, some of the Antabuse patients possessed elevated levels of bound acetaldehyde.

INTRODUCTION

Acetaldehyde is the first metabolite of ethanol oxidation and it is formed mainly in the liver by action of the cytosolic alcohol dehydrogenase (E.C. 1.1.1.1). The acetaldehyde is further oxidised into acetic acid by a mitochondrial aldehyde dehydrogenase (ALDH; EC 1.2.1.3), and only minimal amounts normally reach the circulating blood.

The acetaldehyde concentration in blood has been a matter of controversy throughout the years, mainly due to considerable analytical problems (Eriksson, 1980; Lindros and Eriksson, 1981). Even with rapid handling of the samples, acetaldehyde may disappear prior to assay due to enzymatic oxidation and/or binding to various blood constituents. An additional problem arises if the blood samples contain ethanol, since acetaldehyde may then be formed artefactually during the analytical procedures. Around 1980, it was concluded that the acetaldehyde levels previously reported to occur in blood during alcohol intoxication were far too high and mainly reflected artefactual formation (Eriksson, 1983; Lindros, 1983). Using more reliable methods, the values in peripheral venous blood were instead reported to be at an almost undetectable level (< 1 μM). Higher concentrations were, however, found in simultaneously taken samples from the hepatic vein (Nuutinen et al., 1984), indicating that acetaldehyde was metabolised and/or bound within the blood or by surrounding tissues. In peripheral blood, elevated levels of acetaldehyde could only be detected after drinking in subjects undergoing treatment with drugs inhibiting ALDH (e.g. disulfiram) (Kit-
son, 1977) and in Oriental ‘flushers’ who possess an inactive form of the mitochondrial ALDH isozyme (Mizoi et al., 1979). However, although the free plasma level of acetaldehyde in peripheral blood is normally minute, there are now a number of reports showing that a 5- to 10-fold greater amount occurs within the erythrocytes (Di Padova et al., 1986; Peterson and Polizzi, 1987; Baraona et al., 1987), presumably associated with the hemoglobin (Stevens et al., 1981; San George and Hoberman, 1986). Since this fraction is detectable also without former intake of ethanol, it has been suggested to be of ‘endogenous’ origin. Several possible sources, both genuine and artefactual, for the endogenous acetaldehyde in blood have been proposed (e.g. pyruvate and amino acid metabolism, endogenous ethanol, protein degradation) (Ostrovsky, 1986; Eriksson, 1987; Lucas et al., 1988), but, so far, its origin remains unknown.

The present work was conducted to study further factors influencing the distribution of free and bound acetaldehyde in human blood. Experiments were carried out in the absence and presence of exogenous ethanol, and on samples drawn from alcoholic patients undergoing treatment with disulfiram (Antabuse).

MATERIALS AND METHODS

Chemicals

1,3-Cyclohexanedione, ammonium acetate (NH₄Ac), and NAD⁺ were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Disulfiram (Fluka AG, Buchs, Switzerland) was recrystallised twice from 99.5% ethanol. All other chemicals were of analytical grade from Merck AG (Darmstadt, F.R.G.). The acetaldehyde was re-distilled before use and stored in sealed glass flasks at 4°C. All solutions were made up in deionised water (Elgas-tat UHP; Elga Ltd, Lane End, U.K.).

Blood samples

Fresh blood samples were obtained from healthy control subjects (blood donors with unknown drinking history) and from voluntary alcoholic in-patients undergoing treatment with disulfiram (Antabuse®, Dumex AS, Copenhagen, Denmark) at the Detoxification Unit at Beckomberga Hospital (Bromma, Sweden). The patients had received a supervised oral dose of 400 mg Antabuse every second or third day for a period of between 2 weeks and 6 months. The blood was collected from the antecubital vein into vacutainer tubes containing citrate or EDTA as anticoagulant. For comparison, some samples were collected from the fingertip by use of heparinised microcaps. Plasma was prepared by centrifugation at 5000 x g for 10 min.

Hemoglobin was determined using the cyanomethemoglobin method. The level of endogenous ethanol was not determined in the present study but is normally below 25 μM (Ostrovsky, 1986).

Analytical procedure

Analysis of acetaldehyde in blood was performed using a slight modification of a previously published high-performance liquid chromatographic (HPLC) method (Peterson and Polizzi, 1987; Ung-Chhun and Collins, 1987; Takayanagi et al., 1989) where acetaldehyde is determined as a fluorescent adduct formed by reaction with 1,3-cyclohexanedione (CHD) and ammonium ion. In the standard assay, fresh non-hemolysed whole blood samples were vigorously mixed (1:5) with an ice-cold perchloric acid (PCA) solution (10%, w/v). The PCA was prepared in saline, since this reduces artefactual formation of acetaldehyde during protein precipitation of blood samples containing exogenous ethanol (Eriksson et al., 1982). Before the precipitated proteins were centrifuged away, an aliquot of the crude sample was added to a reaction mixture for determination of whole blood ‘total’ acetaldehyde. The remaining sample was centrifuged at 13,000 x g for 5 min and the clear supernatant taken for determination of ‘soluble’ (i.e. free + PCA-soluble) acetaldehyde. The ‘bound’ (i.e. PCA-insoluble) amount was calculated by subtracting soluble from total acetaldehyde. Of each sample, 150 μl were added to a reaction mixture (final pH 5) consisting of 150 μl NH₄Ac (20%, w/v, in water), 50 μl CHD (1.25%, w/v, in water), and 150 μl thiourea (6%, w/v, in water) in 2-
ml glass flasks with Teflon sealing. Incubation was performed under gentle shaking at 60°C for 60 min after which the samples were cooled on ice. After centrifugation at 13,000 × g for 5 min, a 10-μl aliquot of the supernatant was injected into the chromatographic system (see below).

**Chromatographic system**

The HPLC system consisted of a Pharmacia-LKB Model 2248 pump (Pharmacia LKB Biotechnology, Uppsala, Sweden), a Valco Model C6W injector with a 10-μl sample loop (Valco Instruments, Houston, TX, U.S.A.), and a 3-μm Nucleosil-120 C18 reversed-phase analytical column (75 × 4.0 mm I.D.; Scandinaviska GeneTec, Kungsbacka, Sweden). The column was eluted isocratically at a flow-rate of 0.7–0.8 ml/min at ambient temperature with a mobile phase consisting of methanol-water (27 : 73, v/v). The mobile phase was filtered and degassed before use. A Shimadzu Model RF-551 fluorescence detector (Shimadzu, Kyoto, Japan) was used with excitation and emission wavelengths of 366 nm and 440 nm, respectively. A good linear relationship ($r^2 = 0.99$) between peak height and amount of acetaldehyde was observed in the range of 0–10 μM with standard solutions of acetaldehyde prepared in water. The acetaldehyde level of unknowns was determined from the peak height in the chromatogram by reference to the standard curve. In the water blank, a small peak with the same retention time as the acetaldehyde adduct appears (Ung-Chhun and Collins, 1987), and all values were corrected by subtraction of this value. The limit of detection in blood samples was about 0.2 μM.

The intra- and inter-assay coefficients of variation of the method were below 10%, both with a standard solution containing 5.0 μM acetaldehyde and a control blood sample containing 4.1 μM total and 0.9 μM soluble acetaldehyde.

**RESULTS**

In separated plasma from nine out of ten healthy control subjects, the acetaldehyde level was below the limit of detection of the analytical method, while one sample was slightly elevated (0.35 μM). Much higher concentrations (> 2.5 μM) were found when analyses were carried out on whole blood samples. In whole blood, on an average 70% was recovered as bound acetaldehyde following precipitation with PCA. The median values for soluble and bound acetaldehyde did not differ significantly between controls and Antabuse patients (Wilcoxon two-tailed rank sum test), but some Antabuse patients possessed an elevated level of bound acetaldehyde (Fig. 1). No correlation between the bound acetaldehyde concentration and the duration of treatment was found.

When whole blood was spiked with 10 μM acetaldehyde and mixed with ice-cold PCA in saline solution with 10 sec, 60–90% of the added amount was recovered in the soluble fraction, while the bound amount remained unchanged. In blood samples treated with 100 μM disulfiram (which causes about 90% inhibition of the erythrocyte ALDH) (Helander and Tottmar, 1988) before the addition of disulfiram to a whole blood sample, 60–90% of the added amount was recovered in the soluble fraction, while the bound amount remained unchanged.

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**Fig. 1.** Soluble (S) and bound (B) acetaldehyde levels in blood from healthy control subjects (N = 18) and alcoholic patients undergoing treatment with disulfiram (Antabuse®, N = 26).

The median values did not differ significantly between patients and controls (Wilcoxon two-tailed rank sum test).
acetaldehyde, the recovery was increased but still not complete (results not shown). Moreover, if the spiked blood was left at room temperature for 30 min prior to precipitation, none of the acetaldehyde added could be recovered, not even in the presence of disulfiram. When similar experiments were carried out on hemolysed blood, only a slight disappearance of acetaldehyde was observed during the 30-min incubation period. The disappearance rate increased after addition of 0.5 mM NAD$^+$, and this effect could be inhibited by disulfiram. After 30 min at room temperature, the recovery of acetaldehyde added to plasma was about 80% and close to 100% in phosphate-buffered saline (PBS).

A dose-dependent artefactual formation of acetaldehyde was observed when whole blood samples were added with ethanol before the mixing with PCA. However, an increase was seen only in the soluble fraction, while the bound level was not affected (Fig. 2a). Likewise, in venous blood samples drawn from control subjects following oral intake of 0.4 g ethanol/kg of body weight, a marked increase in soluble acetaldehyde due to artefactual formation was seen (Fig. 2b). The bound level tended to be higher after 4 hr, but the increase was not significantly elevated from baseline (Fig. 2b). No artefactual formation of acetaldehyde was observed when ethanol was added to plasma or PBS without the presence of blood cells.

The acetaldehyde level in blood did not differ significantly between males and females, nor between samples collected from the same individual at different times of the day (results not shown). Moreover, no effect of the type of anticoagulant used was apparent. Citrated blood could be left at room temperature for up to 4 hr without any significant decrease in soluble or bound acetaldehyde levels. After 22 hr, however, the soluble level was close to the detection limit, while the bound amount remained unchanged. Precipitated blood samples (with or without exogenously added ethanol) could be stored at $-20^\circ$C for 7 days without any marked change in the acetaldehyde level (Fig. 3). In contrast, a considerable artefactual formation of acetaldehyde was observed, when assays were carried out on blood samples containing exogenous ethanol which had been stored at $-20^\circ$C without prior precipitation, and the formation was even higher if the samples were submitted to several freezing–thawing cycles (Fig. 3). The artefactual formation occurred in the soluble fraction only (data not shown).

When hemolysed whole blood was subjected to separation on a Sephadex G-25 gel filtration
Acetaldehyde in Blood

Fig. 3. Effect of different storing conditions on artefactual acetaldehyde formation in whole blood samples spiked with 20 mM ethanol. Data represent mean total acetaldehyde levels from two experiments.

The minimal or undetectable concentration of acetaldehyde in separated plasma found in the present study is in good agreement with previous observations (Eriksson, 1983; Lindros, 1983). Likewise, that higher levels are found when analyses are carried out on whole blood samples also concurs with earlier reports (Di Padova et al., 1986; Peterson and Polizzi, 1987; Baraona et al., 1987; Helander and Curvall, 1991). Following direct precipitation of whole blood by PCA, the major portion of acetaldehyde was found in the bound frac-

Fig. 4. Elution profiles of hemoglobin and acetaldehyde (AcH; total level) following separation on a Sephadex G-25 gel-filtration column.

Table: Elution profiles of hemoglobin and acetaldehyde (AcH; total level) following separation on a Sephadex G-25 gel-filtration column.
tion. Although this bound acetaldehyde was not released by PCA treatment, it was still available to the reagents during the following assay incubation. The whole blood soluble acetaldehyde level was higher than the level in separated plasma, suggesting that some acetaldehyde was liberated from the blood cells by treatment with PCA.

When hemolysed blood was spiked with acetaldehyde immediately before separation by gel filtration chromatography, a significant part of the added amount co-eluted with the hemoglobin. Since all of the added acetaldehyde would have eluted after the hemoglobin peak if it was present in free form, these results implied that some kind of reaction (i.e. binding) between the hemoglobin and acetaldehyde had taken place. Acetaldehyde is known to form both stable and unstable adducts with hemoglobin in vitro and in vivo (Stevens et al., 1981; San George and Hoberman, 1986; Lucas et al., 1988), and measurement of acetaldehyde–hemoglobin adducts, or antibodies which recognise acetaldehyde-modified epitopes, have been proposed as possible biochemical markers indicative of excessive alcohol consumption (Hoerner et al., 1986; Niemela et al., 1987). The rapid disappearance of acetaldehyde observed following its addition to whole blood is in agreement with previous studies (Stowell et al., 1978; von Wartburg and Ris, 1979). Since the recovery was increased in the presence of the ALDH inhibitor disulfiram, and no disappearance occurred in hemolysed blood unless NAD⁺ was added, and, moreover, acetaldehyde was relatively stable in separated plasma, the disappearance was probably due mainly to enzymatic oxidation by ALDH in the blood cells. However, this applies apparently only to the exogenous acetaldehyde, since the endogenous level was stable for several hours in samples left at room temperature. Taken together, the present results indicate that the major fraction of endogenous acetaldehyde in peripheral blood circulates more or less reversibly associated with the erythrocytes (i.e. hemoglobin) and is not available to oxidation by ALDH. Since acetaldehyde probably occurs in diversely bound forms, the extent to which bound acetaldehyde will be detected is most likely dependent upon the analytical procedure used.

In agreement with previous reports (Eriksson, 1980; Lindros and Eriksson, 1981), acetaldehyde was formed spontaneously from exogenous ethanol during the analytical procedure, when assays were carried out both on control blood spiked with ethanol and on samples drawn from intoxicated subjects. Therefore, a procedure involving control blood spiked with different concentrations of ethanol has to be employed to correct for artefactual formation. With the present analytical method, however, the artefactual formation interfered only when acetaldehyde was expressed as total or soluble amounts, while the bound fraction was not significantly affected. Furthermore, once the samples had been mixed with the PCA in saline solution, they could be stored frozen prior to analysis without further artefactual formation of acetaldehyde.

Due to the inhibition of ALDH, it was possible that acetaldehyde originating from endogenous sources would accumulate in the body during Antabuse treatment. However, the median level in blood was not significantly higher in the Antabuse patients compared to healthy controls. This may correspond to the fact that exposure to acetaldehyde normally is very low in blood, even during ethanol intoxication (Lindros, 1983; Eriksson, 1983). However, apparently independent of the period of treatment, some of the Antabuse patients possessed a markedly elevated level of bound acetaldehyde. This could not be explained by artefactually formed acetaldehyde due to presence of exogenous ethanol, since this, as already mentioned, would have increased only the soluble level. Elevated levels of bound acetaldehyde after treatment with ALDH inhibitors have been found in a study on rats (Eriksson, 1985), and the level was increased further if the rats were given ethanol at the same time. In spite of the aversive ‘disulfiram–ethanol reaction’, it is known that some alcoholics continue to drink while taking Antabuse, and are thereby exposed to high levels of acetaldehyde (Kitson, 1977). However, whether this causes accumulation of bound acetaldehyde and could explain why some of the Antabuse patients in the present...
study possessed elevated levels is unknown. It should be noted that elevated acetaldehyde levels have also been found in alcoholics who were not undergoing treatment with Antabuse (Lucas et al., 1988).

In conclusion, the present results suggest that the major portion of human blood 'endogenous' acetaldehyde occurs associated with hemoglobin. What this bound acetaldehyde represents, and whether it is available to peripheral tissues and of physiological importance, remains to be elucidated. From the present data, it seems unlikely that endogenous ethanol would interfere with the bound acetaldehyde level, unless endogenous and exogenously added ethanol behave in a totally different way. However, the possibility of artefactual acetaldehyde formation from a non-ethanol source (e.g. blood cell components) still remains and will constitute a potential source or error as long as the bound acetaldehyde is not measured directly but following its release.

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REFERENCES


Takayanagi, M., Goto, S., Kokubo, Y., Suzuki, M. and...
