Acetaldehyde–Hemoglobin Adducts: An Unreliable Marker of Alcohol Abuse

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Acetaldehyde reacts with hemoglobin in vitro to produce acetaldehyde–hemoglobin adducts, but we could not detect these in the blood of 20 alcoholics, either by cation-exchange chromatography or agar gel electrophoresis. Thus, contrary to previous reports, this test is not a reliable marker of alcohol abuse. Nuclear magnetic resonance and gas chromatographic–mass spectroscopic analysis of acetaldehyde revealed the presence of aldehydeic impurities. Formation of adducts in vitro may thus result from the reaction of hemoglobin with these impurities or with acetaldehyde condensation products (aldol) or from acetaldehyde condensation/dehydration products (crotonaldehyde).

Additional Keyphrases: alcoholism · acetaldehyde condensation products · cation-exchange chromatography · agar gel electrophoresis

Detection of alcoholism by laboratory tests is difficult because of the lack of a reliable marker of alcohol abuse (1, 2). Currently used tests such as mean corpuscular volume (MCV) and activities of γ-glutamyltransferase (EC 2.3.2.2) and aspartate aminotransferase (EC 2.6.1.1) in serum, either individually or in combination, are neither sensitive nor totally specific (3). Recent reports of substances formed by the reaction of acetaldehyde, the first metabolite of alcohol, with hemoglobin (acetaldehyde–hemoglobin adducts) raised hopes that at last a reliable marker of alcohol abuse had been discovered (4, 5). Because our laboratory has a particular interest in laboratory methods for detecting alcohol abuse (6, 7), we undertook this study to evaluate the reliability of the new test.

Materials and Methods

Reagents

Acetaldehyde (99.0% purity) and toluene were purchased from British Drug Houses, Poole, Dorset, U.K.; crotonaldehyde, from Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.; (bis-2-hydroxyethyl)aminotrihydroxymethyl) methane (Bistria) and reagents for the colorimetry of total hemoglobin from Sigma Chemical Co., Poole, Dorset, U.K.; and cation-exchange resin (Bio-Rex 70, 200–400 mesh, sodium form) from Bio-Rad Labs Ltd., Watford, Herts, U.K.

Patients

We studied 16 men and four women (ages 24–60 years), admitted to the Drug Addiction Unit, All Saints Hospital, Birmingham, because of their heavy drinking habits. All admitted to drinking more than 60 g of alcohol per day for at least three years. None of the patients was cirrhotic or suffering from any obvious vitamin deficiency. Within 24 h of admission and before any vitamin supplements had been administered, blood was collected into tubes containing EDTA as anticoagulant. We also studied seven healthy subjects, four men and three women, ages 21–60 years, from the laboratory staff. All were considered to be social drinkers (consuming less than 40 g of alcohol per day).

Procedures

Hemoglobin concentration. We determined the hemoglobin concentration in erythrocyte lysates by Drabkin's method (8).

Separation of hemoglobins by cation-exchange chromatography. We used a 1 × 30 cm Bio-Rex 70 column to separate 30 mg of hemoglobin into "fast" and major hemoglobins, according to the method of Trivelli et al. (9). We monitored the absorbance of the eluate simultaneously at 280 and 405 nm with an Ieco UA-5 spectrophotometer (Measuring and Scientific Equipment Ltd., Crawley, Sussex, U.K.).

We also used cation-exchange chromatography with 50-μL samples of erythrocyte lysate, applied to BCL mini-columns (Test Combination Hemoglobin A1; The Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K.). The absorbance of the eluate was measured at 415 nm with a Gilford 240 spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH).

Separation of hemoglobins by gel electrophoresis. We used agar gel plates developed for hemoglobin analysis and supplied by Corning Medical, Halstead, Essex, U.K., subjecting a 1-μL sample of lysate to electrophoresis for 40 min at 60 V in a Corning AC1 cassette electrophoresis cell. The gel was then scanned with a Corning 720 densitometer at 420 nm.

Biochemical and hematological measurements. We determined serum aspartate aminotransferase activity according to the method of Kessler et al. (10) by continuous-flow analysis (SMA 12/60; Technicon Instruments Corp., Ltd., Basingstoke, Hants., U.K.), serum γ-glutamyltransferase activity by the method of Szasz (11) with a Vitatron-Akes analyzer (Measuring and Scientific Equipment Ltd.), and MCV with a "Coulter S Plus" (Coulter Electronics Ltd., Luton, Beds., U.K.).

Spectroscopic analysis. For nuclear magnetic resonance spectroscopy we used a 100-MHz instrument (Model R14; Perkin-Elmer, Beaconsfield, U.K.), with tetramethylsilane as internal standard. For mass spectroscopy–gas chromatography we used a Model MS 80 RF mass spectrometer (Kratos Ltd., Scientific Instruments, Manchester, U.K.).

In vitro studies of the reaction of acetaldehyde with hemoglobin. Whole blood drawn from healthy subjects, or lysate corresponding to 2 to 3 mmol of hemoglobin, was incubated in a sealed glass tube at 37 °C for 2 to 24 h with various concentrations of acetaldehyde, according to the method of Stevens et al. (4). The reaction mixtures were assayed for hemoglobin by cation-exchange chromatography and by agar gel electrophoresis. Results were expressed as the percentage of the total hemoglobin that was "fast" hemoglobin. In similar experiments we substituted aldon or crotonaldehyde for acetaldehyde.

Clinical studies. Hemoglobins in anticoagulated blood from alcoholics and healthy controls were measured both by cation-exchange chromatography and by agar gel electrophoresis.
Results and Discussion

In Vitro Studies

We confirmed the findings of Stevens et al. (4) that hemoglobin undergoes a concentration-dependent reaction with acetaldehyde to produce an increase in the "fast" hemoglobin fraction. This increase was attributed to the formation of acetaldehyde-hemoglobin adducts.

The cation-exchange chromatographic method used to isolate the adducts was lengthy and required the use of cyanide. We therefore investigated simpler methods, more suitable for routine use. Because the adduct behaved analytically like a glycated hemoglobin, we examined whether existing methods for determining glycated hemoglobin would be useful. Cation-exchange chromatography with pre-packed mini-columns and agar gel electrophoresis proved faster and more convenient, and both gave results similar to those obtained with the Bio-Rex columns (Table 1). We therefore used these methods in the subsequent clinical studies.

Clinical Studies

We could not detect an increase in the "fast" hemoglobin fraction in blood from alcoholics, either by the chromatographic (mini-columns) or agar gel electrophoretic methods. The mean percentages of the total hemoglobin that was "fast" hemoglobin in blood from alcoholics were 6.0% (SD 0.98%; reference interval for a normal population, 5–8%) for the BCL column method and 6.4% (SD 0.76%; reference interval for a normal population, 5.6–7.6%) for the agar gel electrophoresis. We confirmed these findings by re-analyzing the blood by the Bio-Rex column method. The high-pressure liquid chromatography method used by Stevens et al. (4) for analyzing blood for these adducts was not attempted, because those authors indicated in a subsequent publication (5) that with the more convenient Bio-Rex 70 cation-exchange chromatography they were also able to detect an increase in the "fast" hemoglobin fraction in alcoholic patients as compared with controls.

In view of the discrepancy between our results and those of other workers (4, 5), we investigated possible confounding variables. The alcoholics we studied were not atypical because conventional tests of alcohol abuse gave positive results in all subjects: mean serum γ-glutamyltransferase 107.3 U/L (SD 104.5; reference interval 6–28 U/L); mean serum aspartate aminotransferase, 73.3 U/L (SD 93.3; reference interval 5–30 U/L); and mean MCV, 98.6 fL (SD 8.16, reference interval 75–90 fL). The subjects represented a range of drinking histories, amount of alcohol, duration of abuse, and time since last drink; thus, if adduct formation was either a transient or a long-term phenomenon, it would have been detected in at least some of the subjects.

Increased acetaldehyde concentrations in the blood of alcoholics after ingestion of large doses of alcohol have been reported by many authors (12–14). However, this increase in acetaldehyde concentrations has been attributed to artefactual acetaldehyde formation from ethanol during protein precipitation (15, 16). Thus it seems unlikely that sufficient acetaldehyde accumulates for a subsequent reaction with hemoglobin.

The reaction of acetaldehyde with amino groups of proteins is a reversible reaction (Schiff base formation); consequently, the formation of a stable adduct is unexpected. An Amadori rearrangement, as in the case of glycated hemoglobin formation (from glucose and hemoglobin), cannot occur because there is no α-hydroxyl group in acetaldehyde.

Examination of the acetaldehyde we used for the in vitro studies by both nuclear magnetic resonance and gas chromatography–mass spectroscopy revealed the presence of 1% impurities, one of which was tentatively identified as aldol (CH3-COH-CH2-CHO). Aldol arises by acid/base-catalyzed condensation of acetaldehyde (17), and readily loses water on warming to form crotonaldehyde (CH3-CH=CH-CHO). Incubation of hemoglobin with aldol and crotonaldehyde produced adducts with chromatographic and electrophoretic properties identical with those produced with acetaldehyde. We therefore suspect that the adducts observed in vitro are artefacts arising from impurities in acetaldehyde or from the products of acetaldehyde condensation or acetaldehyde condensation and dehydration.

Thus, even though a test for alcohol abuse based on the detection of acetaldehyde–hemoglobin adducts (like glycated hemoglobins in diabetes) is an attractive proposition, we have found no evidence of the presence of such adducts in blood from alcoholics. Adducts were detected in vitro, but this may be an artefact resulting from the reaction of hemoglobin either with impurities present in acetaldehyde or with products (aldol or crotonaldehyde) that may be formed from acetaldehyde under mild conditions.

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References
Effects of Residual Liquid Junction Potential in Direct Potentiometry of Potassium

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To further the accurate direct potentiometry of plasma electrolyte concentrations, we investigated the effects of solution composition on the residual liquid junction potential (RLJP) during measurement of K+. Assuming that the binding constant between K+ and proteins or bicarbonate is no greater than with Na+, we calculate that the amount of bound K+ can be neglected. A significant RLJP exists between simple solutions containing Na+, K+, and Cl− ions and solutions containing Na+, K+, Cl−, and HCO3− ions. Replacing Cl− with HCO3− leads to an increase in the RLJP, which in turn contributes to a negative error in K+ analysis. A small decrease in RLJP is observed as the ionic strength is increased. The Henderson equation gives a reasonable estimate of the magnitude of the observed RLJP, even though the liquid junction does not meet the conditions under which the equation is rigorously applicable. Errors attributed to RLJP may be substantially minimized by using a calibrator solution that contains an anion with mobility similar to that of HCO3−.

There is considerable debate currently as to the accuracy with which sodium and potassium ion concentrations in serum can be determined by direct potentiometry. A major question is the role of the residual liquid junction potential (RLJP). Several published studies (1–3) deal with the influence of the composition of the calibrator solution on the observed sodium ion concentration. The same RLJP effects observed for Na+ have been predicted for K+, but such studies have not been reported. Our work was focused on the variations in observed potassium ion concentrations that are attributable to RLJP.

The difference between results by direct potentiometry and by flame photometry has been attributed in part to some binding of Na+ by proteins and bicarbonate (4), although there is no direct evidence for such binding (5). The effects of cation binding in serum would be even less evident for K+ because of its lower concentration, but the RLJP effect on direct potentiometry should be observable.

We were particularly concerned with the RLJP when test solutions with and without HCO3− ions are compared. We examined the magnitude of RLJP as a function of ionic strength and of the concentration of the filling solution used in the reference electrode. These values were then compared with RLJP as calculated by the Henderson equation.

Materials and Methods

Reagents. We prepared solutions of KCl, NaCl, and NaHCO3 (all AR grade) in distilled, de-ionized water that was stored in air to ensure a constant concentration of dissolved carbon dioxide.

Apparatus. For direct potentiometry of potassium, we used a potassium ion-selective electrode (Model 93-19; Orion Research Inc., Cambridge, MA 02139) and a standard Ag/AgCl reference electrode with a ceramic frit (Corning Scientific Products, Medfield, MA 02052). The internal filling solutions were 0.1 mol/L KCl and 2.0 mol/L KCl. The leak rate of the junction was approximately 8 μL per hour.

Sodium and potassium concentrations were verified by use of a flame photometer (Model 643; Instrumentation Laboratory Inc., Lexington, MA 02173), calibrated with 2/120 and 5/140 mmol/L KCl/NaCl standards. Chloride and bicarbonate concentrations were verified with a CI−/total CO2 analyzer (Model 643 IL; Instrumentation Laboratory Inc.) calibrated with 80/25 mmol/L Cl−/HCO3− standards.

Voltage readings, made with a digital millivolt meter, were recorded with a sensitive continuous multi-pen recorder. An opposing voltage source was placed between the millivolt meter and the recorder to permit operation of the recorder at high sensitivity.

Procedure. We prepared groups of solutions having constant ionic strengths ranging from 120 to 200 mmol/L and