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A. W. Jones, [1] *Ph.D.*

A Rapid Method for Blood Alcohol Determination by Headspace Analysis Using an Electrochemical Detector

The introduction of electrochemical sensors for the detection and analysis of volatile substances, in particular low concentrations of ethyl alcohol in the vapor phase, has led to the development of a range of breath alcohol instruments based on this principle [*1*]. The Alcolmeter Evidential Device is a breath-testing instrument that aspirates a sample of breath or vapor into an electrochemical cell for quantitative oxidation of ethanol [*2*]. The same device could well be used for headspace analysis of ethanol in biological materials, enabling rapid, on-the-spot screening of samples and even quantitative blood alcohol determinations.

The technique is based on headspace analysis of the vapor phase above a blood sample and a known-strength aqueous ethanol solution, maintained under identical equilibrium conditions. From the partition coefficients for ethanol distribution between air-water and air-blood and the headspace concentrations found by analysis, the blood alcohol concentration can be calculated [*3*].

The aims of this work are (1) to adapt a breath alcohol testing device as an instrument for blood alcohol determination by means of headspace analysis; (2) to assess the optimum conditions of analysis and determine the linearity of detector response; (3) to ascertain the precision of the headspace analysis; and (4) to determine the accuracy of this method of blood alcohol determination by comparison with a reference (alcohol dehydrogenase) technique by using aliquots of the same specimen for analyses.

Materials and Methods

Alcolmeter Evidential Device

The Alcolmeter Evidential Device (Lion Laboratories Ltd., Cardiff, United Kingdom) is based on an electrochemical ("fuel cell") principle for ethanol oxidation. The unit consists of a sampling head, thermostated at 60 °C between tests, containing the electrochemical detector and the aspirating sampling valve. A "SET" button serves to activate the sampling valve and discharge the fuel cell and a "READ" button releases the sampling valve (that is, causes aspiration of the 0.6-ml test sample) and activates the fuel cell. The detector signal is displayed on a digital voltmeter in d-c voltage, which may be calibrated to blood alcohol units, in mg/ml,[2] allowing the blood alcohol concentration to be read down to 0.01 mg/ml. The result is kept displayed until reset to zero by the operator in preparation for a new test. The detector may be calibrated with dry alcohol-in-gas mix-

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[1] Senior research scientist, Department of Alcohol Research, Karolinska Institutet, Stockholm, Sweden.

[2] 1.0 mg/ml = 1.0 promille = 100 mg/dl = 0.1% w/v = 21.7 mmoles/litre.

tures or air-ethanol mixtures generated from a simulator by equilibration of aqueous solutions [3].

Headspace Analysis

For headspace analysis, the sampling head of the Alcolmeter is fitted with a special nozzle connection to hold a Luer-fitting hypodermic needle in place of the mouthpiece nozzle normally used for breath alcohol analysis. The sample to be analyzed is contained in a 10-ml serum bottle.

Sampling Technique

A headspace analysis is carried out according to the following procedure:

1. Remove the sampling head from its heated holder and attach a 21-gage Luer-fitting hypodermic needle.
2. Press READ button and check that the digital voltmeter reads zero. If a positive reading is observed, press SET button and repeat this test until reading is zero.
3. Take the sampling head with hypodermic needle attached and pierce the rubber septum of the serum bottle containing the sample.
4. Press SET button to activate the sampling mechanism. Immediately afterwards press READ button to aspirate the headspace vapor sample for analysis.
5. Remove the hypodermic needle from the serum bottle, detach the sampling head, and replace the head into the heating jacket.
6. Record the final reading on the digital voltmeter; this will be proportional to the alcohol concentration in the headspace vapor.
7. Press SET button to discharge the detector in preparation for a new test.
8. Flush out the hypodermic needle several times with room air.
9. For a new determination, wait 2 min and repeat the procedure, beginning at Step 1.

A schematic diagram of the sampling arrangement is shown in Fig. 1. The sampling procedure should be completed as quickly as possible so that the detector temperature does not materially change from 60 °C. Step 4 introduces 0.6 ml of air into the serum

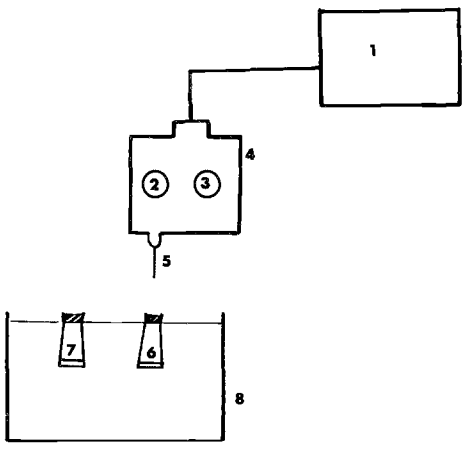

FIG. 1—*Block diagram of equipment for headspace analysis using an Alcolmeter device*; (1) *Alcolmeter unit*; (2) *READ button*; (3) *SET button*; (4) *sampling head*; (5) *Luer-fitting hypodermic needle*; (6) *blood sample*; (7) *aqueous alcohol standard*; *and* (8) *constant temperature water bath.*

bottle, so subsequent aspiration of 0.6 ml of headspace will not alter equilibrium conditions to any significant extent.

Preparation of Samples for Headspace Analysis

The blood sample to be analyzed (in the present work 0.1 ml) is transferred into a 10-ml serum bottle and made air-tight with a self-sealing rubber septum and an aluminum pressure seal cap. Aqueous standards are treated similarly.

The blood samples and aqueous standards are then equilibrated in a water bath at 25 °C to maintain standard conditions for serial determinations. Equilibration may also be carried out at room temperature, the critical factor being that the blood samples and standards are at the same equilibrium temperature; temperatures higher than 25 °C may be used if desired. Fifteen minutes were found to be the minimum time for equilibration when samples were initially at room temperature and subsequently thermostated at 25 °C. The vapor phase above each blood sample is adjusted to atmospheric pressure prior to headspace sampling.

Calculation of Blood Alcohol Concentration

The blood alcohol concentration is derived from the headspace concentrations above the blood sample and above an aqueous ethanol standard based on the partition ratios for the distribution of ethanol between air-water $k_{a/w}$ and air-blood $k_{a/b}$. The quotient $k_{a/b}/k_{a/w}$ is constant and independent of equilibrium temperature between 10 and 40 °C (Fig. 2). It is also independent of aqueous ethanol concentration between 0 and 10% w/v [3]. With a liquid to air ratio within the range of 1:100 to 1:5, which corresponds to a range of 0.1 to 2.0 ml of the sample in a 10-ml flask, the headspace vapor concentration is constant, as illustrated in Fig. 3. The quotient $k_{a/b}/k_{a/w}$ will, however, be affected by abnormal hematocrit and anticoagulant additives. In the present series of experiments the anticoagulant used was an oxalate-fluoride mixture, 5 mg/ml blood, the same concentration used by Harger et al [3], whose results were used to derive the quotient $k_{a/b}/k_{a/w}$ used in the present experiments. The hematocrits of blood samples used in the present experiments were within normal ranges.

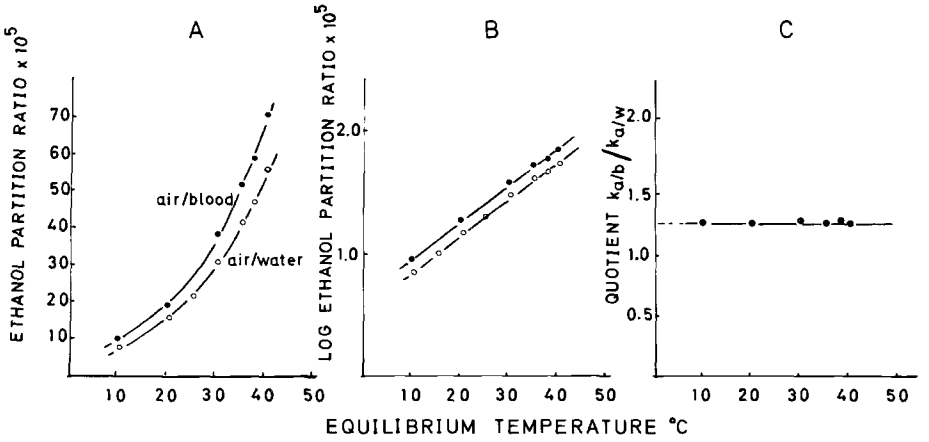

FIG. 2—*Relationship between air/water and air/blood alcohol partition ratios at various equilibrium temperatures.*

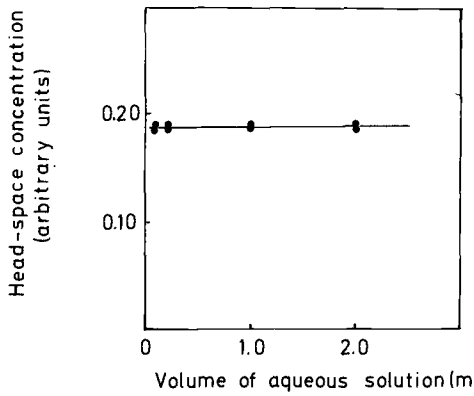

FIG. 3—*Relationship between headspace vapor concentration and volume of aqueous ethanol solution at equilibrium in a 10-ml flask.*

The headspace concentrations above blood samples and aqueous standards are proportional to the liquid phase concentrations. The blood alcohol concentration c is therefore calculated from the formula:

$$c = (C/A)B(1/1.25) \text{ mg/ml} \quad (1)$$

where

C = concentration of aqueous ethanol solution (mg/ml),
A = Alcolmeter reading for headspace analysis above the aqueous standard (arbitrary digital voltmeter units dc),
B = Alcolmeter reading for headspace analysis above the blood sample (arbitrary digital voltmeter units dc), and
1.25 = quotient $k_{a/b}/k_{a/w}$.

The blood alcohol results may be read directly from the Alcolmeter if the unit is precalibrated with known-strength aqueous standards and the 1.25 to 1 correction factor. This factor, 1.25 to 1, does not apply universally to all blood samples; as emphasized earlier, it is dependent on the condition of the blood sample and primarily on the nature and concentration of anticoagulant used.

Subjects and Conditions

Nine male subjects took part in the experiments. They had a mean age of 41 years (range, 27 to 52 years) and a mean body weight of 75 kg (range, 68 to 80 kg).

The subjects were given a standard dose of whisky as the alcoholic beverage. Six received 2 ml/kg body weight (0.72 g alcohol/kg) and three received 2.5 ml/kg body weight (0.90 g alcohol/kg). The calculated volume was taken neat after the subjects had fasted and was consumed over a 20-min period.

Ethanol Standards

Aqueous ethanol solutions were prepared from absolute alcohol, 99.8% w/v (Vin och Spritcentralen Ltd.), by first making a 10% w/v solution by weighing and volumetric dilution and further diluting from this stock solution to concentrations between 0.05 and 3.0 mg/ml.

Blood Sampling

Capillary blood samples were taken from a fingertip at 30-min intervals from the start of drinking for the first 2 h, and thereafter at 1-h intervals for a period of 7 to 9 h. One set of samples for comparison purposes was taken in triplicate with 10-μl disposable blood pipettes and diluted with 1 ml of 0.05*M* sodium fluoride in AutoAnalyzer® cups. From the same specimen of blood 0.1 ml was taken into a Widmark capillary tube containing a 50:50 mixture of potassium oxalate and sodium fluoride as anticoagulant (0.5 mg). The samples were stored in a refrigerator at +4 °C and analyzed 24 h later. A separate study had shown that no losses of ethanol occurred during storage (unpublished work).

Blood Analysis

The triplicate 10-μl blood samples were analyzed with an automated enzymatic method as the reference procedure. The standard deviation of a single determination increased with increase in concentration, being ±0.0164 mg/ml at a mean concentration in blood of 0.51 mg/ml [*4*]. The blood from each Widmark tube was blown into a 10-ml serum bottle and the bottle sealed. After equilibrium, the headspace vapor was sampled by using the modified Alcolmeter device. Duplicate samples were analyzed from each bottle.

Results

Partition Ratios

The partition ratios for the in-vitro distribution of ethanol between air/water and air/blood are shown in Fig. 2 (data from Harger et al [*3*]).

The partition ratios increased with increase in temperature. In a log scale, however, the $k_{a/b}$ and $k_{a/w}$ temperature relationships are parallel (Fig. 2*B*). This implies that the quotient $k_{a/b}$ to $k_{a/w}$ is also constant and independent of temperature (Fig. 2*C*). This quotient varies between 1.23 to 1 and 1.27 to 1 over the temperature range 10 to 40 °C, being 1.25 to 1 on average, which is the factor used in Eq 1 to calculate the blood alcohol concentration from headspace analysis above a blood sample, as compared to that of a known-strength aqueous ethanol standard when maintained under the same equilibrium conditions.

Influence of Volume of Aqueous Phase on Headspace Analysis

The headspace vapor concentrations above a series of 0.50 mg/ml aqueous ethanol solutions were measured in duplicate after being equilibrated at 25 °C. The volumes chosen were 0.10, 0.20, 1.0, and 2.0 ml, which were equilibrated in 10-ml serum bottles (that is, a 20-fold aqueous volume range), corresponding to a 100:1 to 5:1 range in the air/liquid volume ratio.

The results are presented in Fig. 3, showing that under the experimental conditions used the headspace vapor concentration is independent of aqueous phase volume. This was confirmed by regression analysis. The regression equation was $y = 0.1869 + 0.00075x$, where x = volume of aqueous phase and y = ethanol vapor equivalent concentration measured by headspace analysis.

The regression coefficient that measures the change in vapor concentration with increase in volume was not significantly different from zero, 0.00075 ± 0.00353 (Student's t = 0.2133; degrees of freedom df = 6; probability $P > 0.05$).

Linearity of Detector Response

Aqueous ethanol solutions ranging in concentration from 0.05 to 3.0 mg/ml were equilibrated in 10-ml serum bottles at 25 °C. Four measurements of the headspace vapor above each solution were made. The responses as a function of aqueous concentration are shown in Fig. 4.

The rectilinear relationship was proved by regression analysis. The regression equation was $y = 0.0117 + 0.5729x$, where y = instrument reading (arbitrary units) being proportional to the ethanol vapor concentration and x = aqueous ethanol concentration (mg/ml). The variates were highly correlated, $r = 0.999 \pm 0.0079$ ($P < 0.001$), and, moreover, the y-intercept (the instrument response at zero ethanol concentration), 0.0117 ± 0.0066, was not significantly different from zero ($t = 1.757$; $df = 30$; $P > 0.05$). This means that the detector gives no false positive results when the true ethanol concentration is zero.

Precision of Headspace Analysis

When the headspace concentrations of blood samples were analyzed, the determinations were always made in duplicate. The differences between the duplicate results of the analysis may be used to estimate the standard deviation of a headspace ethanol determination.

Fifty-three blood samples ranging from 0.0 to 1.40 mg/ml were each analyzed in duplicate. The mean difference plus or minus the standard error between duplicates was -0.0045 ± 0.00274 mg/ml. This mean difference was not significantly different from zero ($t = -1.64$; $df = 52$; $P > 0.05$). This indicates unbiased estimates and a random distribution of the differences around zero.

The standard deviation of the differences s_d was ± 0.01996. Hence the standard deviation of a single determination was ± 0.0141 mg/ml ($s_d/2^{-2}$), indicating a high precision of this method of analysis.

To test if the variability (here equal to the standard deviation) varied with concentration, the absolute differences y between the duplicate determinations were correlated with the concentration, in mg/ml, in the samples x. The regression equation was $y = 0.0115 + 0.0049x$. The regression coefficient showed no trend ($P > 0.05$) and the correlation coefficient was not significantly different from zero, $r = 0.0971 \pm 0.1304$ ($t = 0.697$; $df = 51$; $P > 0.05$). This implies that the standard deviation is independent of blood

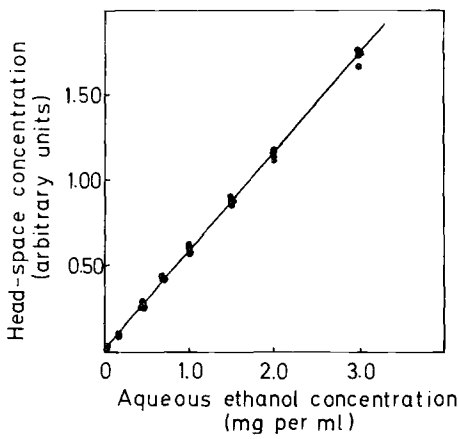

FIG. 4—*Relationship between headspace vapor concentration (detector response) and aqueous ethanol concentration.*

alcohol concentration within the range studied of 0.0 to 1.40 mg/ml, which covers the most important forensic range (0.50, 0.80, and 1.0 mg/ml, the legal blood alcohol limits for driving in most countries). Higher blood alcohol concentrations were not encountered during the present experiments.

Accuracy of Blood Alcohol Determination by Headspace Analysis

The accuracy of the headspace technique was assessed by making determinations on the same blood samples (n = 52) by headspace analysis and by an automated enzymatic method [*4*], the samples ranging in concentration from 0.0 to 1.40 mg/ml.

The differences between the results were analyzed by using Student's t test, based on intra-individual differences. The mean difference plus or minus the standard error was -0.0125 ± 0.0068 mg/ml and was not statistically different from zero ($t = -1.827$; $df = 51$; $P > 0.05$). The standard deviation of the difference was ± 0.0493 mg/ml.

If the enzymatic analyses of blood samples are considered the best estimate of the true concentrations (mean, 0.572 mg/ml), then the accuracy expressed as estimated analytical bias (that is, as recovery) is 102.2% since the mean blood alcohol concentration based on the headspace analyses was 0.585 mg/ml.

The differences between the results by the two methods were not correlated to the blood alcohol concentration, as shown by regression analysis. The regression equation was $y = 0.0225 + 0.0266x$, where y is the absolute difference between the two methods (enzymatic and headspace) and x is the blood alcohol concentration. The regression coefficient 0.0266 ± 0.0150 was not different from zero ($t = 1.77$; $df = 50$; $P > 0.05$), that is, there was no trend in the relationship.

As a further check on the accuracy of the headspace method a correlation regression analysis has also been made based on the results of the two methods. The scatter diagram is shown in Fig. 5.

The regression equation was $y = 0.0192 + 0.9883x$, where y = results from headspace

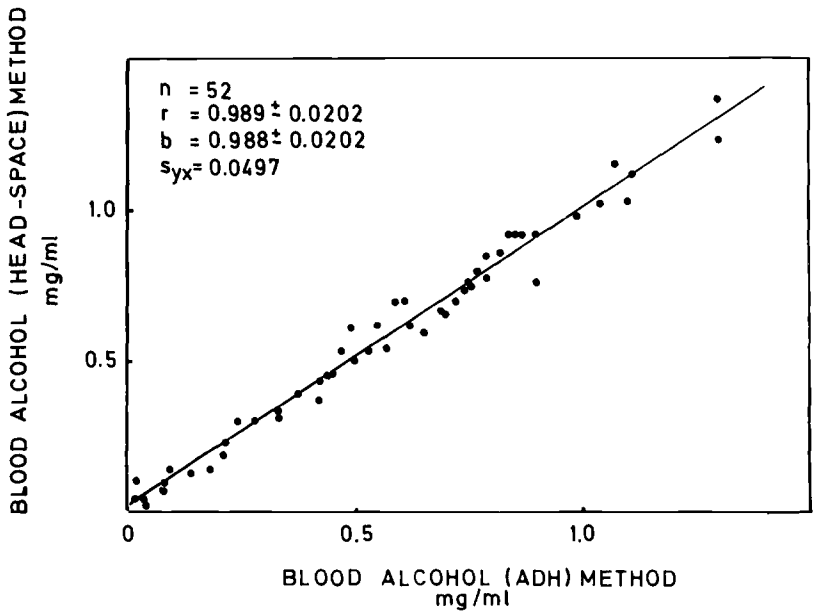

FIG. 5—*Results of blood alcohol analysis by the Alcolmeter headspace technique and by enzymatic analysis.*

analysis and x = results from enzymatic analysis. The regression coefficient was not significantly different from unity ($t = 0.5792$; $df = 50$; $P > 0.05$) and the y-intercept 0.0192 ± 0.01346 was not significantly different from zero ($t = 1.42$; $df = 50$; $P > 0.05$), proving that there are no systematic differences between the two methods.

The standard error estimate s_{yx}, a measure of the scatter of the data points around the least squares regression line, was ± 0.0497 mg/ml. Since there were no statistically significant differences between the results by the two methods, this statistic represents the standard deviation, a measure of the accuracy of the headspace method when compared with the enzymatic method. This value is in excellent agreement with the figure found earlier by evaluating the differences between determinations by Student's t test. The standard deviation of the differences was ± 0.0493 mg/ml. Thus, 5% of the results of random blood analyses will lie outside a range of 90 to 110% from the true values ($\pm 2s_{yx}$).

Discussion

The aspirating property of the sampling valve used in the Alcolmeter instrument has been used to convert the instrument into a reliable headspace analytical device. Determination of ethanol in blood samples by electrochemical oxidation is a new procedure. The technique makes use of the quotient between the air/blood and air/water partition ratios, which defines the ethanol equilibrium between the blood samples and the aqueous standards. The ethanol partition to the vapor is very low at room temperature, which means that the equilibrium vapor phase may be sampled without materially affecting the aqueous concentration. The exact volume of the blood sample used for analysis therefore does not have to be known.

A similar principle using partition ratios was described for urine ethanol determinations, although this was a macro-chemical method requiring 100 to 200 ml of sample and the volume of vapor analyzed needed to be measured accurately with a gas burette [5].

A novel headspace method for blood alcohol analysis has recently been described with a solid-state sensor [6]. This method involved saturating the blood sample with sodium chloride, causing a "salting-out" of alcohol prior to exposure of headspace to the sensor by means of a spring-loaded syringe. The salting-out step was thought to be necessary to overcome the variable solid contents of blood samples with different history and when pretreatment is unknown. The same technique could be used with the headspace procedure described in the present paper and is recommended when blood samples of unknown origin are used. The blood samples used in this work were, however, of normal composition and the pretreatment was standardized. The solid composition of blood samples analyzed is less important if only a qualitative screening of samples is required; the modified Alcolmeter device described would be ideal for this purpose.

The technique of headspace analysis using partition ratio data and a modified breath alcohol device requires only 0.1 ml of blood sample, that is, it is a micro method. The detector used appears to be highly specific for ethyl alcohol; with several hundred ethanol-free blood samples analyzed, the Alcolmeter has given in every case a zero reading. The response to a range of commonly encountered volatiles is presently being studied.

The method is highly accurate and precise and compares favorably with conventional techniques of blood analysis used in forensic and toxicology laboratories [7]. There were no systematic differences between the results by the headspace method and an automated alcohol dehydrogenase technique used for comparison.

The rapidity of the method (no sample pretreatment necessary and only 1 or 2 min recovery time between replicate determinations) makes it possible to be used for mass screening of biological materials for the presence of ethyl alcohol.

Summary

A method for the determination of blood alcohol concentration by headspace analysis using an electrochemical detector is described. A determination can be made within 2 min, and only 0.1 ml of blood is required for each analysis. The detector response was linearly related to ethanol concentrations up to 3.0 mg/ml. The standard deviation of a single determination was ±0.014 mg/ml. The accuracy of the method based on comparison with an enzymatic (alcohol dehydrogenase) technique was high, the mean recovery being 102.2% of the attributed concentration.

The ease of the operation and fast analysis time make the method ideal for serial determinations, for example during mass screening of biological samples for ethyl alcohol in forensic and toxicology laboratories.

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Address requests for reprints or additional information to
A. W. Jones, Ph.D.
Department of Alcohol Research
Karolinska Institutet
S-104 01 Stockholm
Sweden