Review

Determination of ethanol in human fluids — I. Determination of ethanol in blood

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Abstract: A review of the methods for determination of ethanol in human fluids is presented. This first part of the review deals with the methods for determination of alcohol in blood, which have been divided into chemical, enzymatic, chromatographic and miscellaneous for clearer exposition and discussion.

Keywords: *Ethanol; blood; automatic methods; non-automatic methods; review.*

Introduction

The production of alcoholic beverages dates back to prehistoric times (beginning of cereal cultivation). Although the ingestion of alcohol is a serious hazard for man, economic conditions have moved to lessen the importance of the problem and yet to induce an increase in alcohol consumption. Taking into account the world figure of alcoholics, alcohol consumption can be said to have reached an alarming level. Alcohol is, directly or indirectly, one of the main causes of death in present-day society. It has been shown that about 30% of all fatal road accidents involve drivers whose alcohol level in blood exceeded 0.8 g l^{-1} [1], and that industrial accidents are more frequent in alcoholics. Likewise, most criminal offences and suicides are committed under the influence of alcohol.

The increasing demand for alcohol determinations in recent times arises from the growing demands in the toxicological and forensic fields, which require accurate, fast and selective analyses of ethanol in blood (one of the most frequent determinations in clinical and toxicological laboratories). The great interest in this subject shows in the reviews produced in the last few years [2–8].

The influence exerted by different ethanol concentrations on drivers permits the establishment of three ranges associated with different symptoms [9]: concentrations between 0.2 and 0.5 g l^{-1} do not impair body reflexes, but, increase aggressiveness, thus

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favouring traffic offences; the first intoxication symptoms are detected at 0.5 g l^{-1} and further increases result in a progressive deterioration of visual alertness, a considerable risk being involved with alcohol concentrations above 0.8 g l^{-1}; above 1.5 g l^{-1} alcoholic intoxication is evident, with clear external symptoms of depression of the central nervous system.
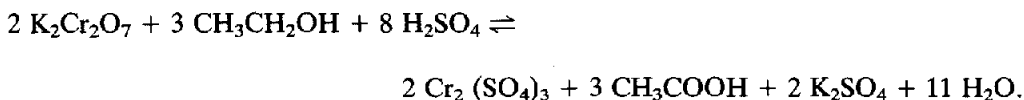
An important aspect requiring comprehensive study is the distribution of ethanol within the body (capillaries, arterial and venous blood) as well as its distribution over time. Ethanol diffuses rapidly across cellular membranes because of its solubility in water and lipids, so that ethanol ingested orally is instantaneously absorbed from the stomach and small intestine, and rapidly distributed throughout the body's water. Over this distribution period the ethanol concentration is higher in arterial than in venous blood [6]. This gradient promotes the diffusion from the tissues to venous blood until equilibrium is attained. Studies [10, 11] have inferred that the amount of ethanol ingested by a person can be determined by analysis of blood, breath, urine and saliva [5], the most trustworthy of which is analysis of blood. In this first part of the present review, the authors deal with the various methods for the determination of ethanol in blood (chemical, enzymatic, chromatographic) suggested so far.

Chemical Methods for Determination of Ethanol in Blood

The reducing and ester-formation capabilities of ethanol have been used but in all cases an initial separation by distillation, aeration, diffusion or extraction is necessary. Understandably, these methods have become obsolete owing to their inherent disadvantages which include large sample volumes required, slowness of analysis, difficulty with automation, large errors and poor reproducibility.

(a) *Methods based on the reducing character of ethanol*

In general, these methods are based on the quantitative oxidation of ethanol by an oxidizing agent (usually potassium dichromate) according to the scheme:

$$2 \, K_2Cr_2O_7 + 3 \, CH_3CH_2OH + 8 \, H_2SO_4 \rightleftharpoons$$

$$2 \, Cr_2(SO_4)_3 + 3 \, CH_3COOH + 2 \, K_2SO_4 + 11 \, H_2O.$$

The conditions under which the oxidation is carried out are responsible for the fact that acetic acid is not the only product yielded. The reaction may only proceed up to the formation of acetaldehyde or advance to the formation of carbon dioxide. Different stoichiometric ratios may therefore be involved and it is advisable to calculate an empirical factor which takes this into account. Two types of application have been involved as follows:

Titrimetric. The first quantitative determination of ethanol by this technique was carried out in 1865 [12]. Subsequently it was applied to the determination in blood [13–19], which has been performed by direct [20] or back titration, using Fe(II) [21–25] or $I_2/S_2O_3^=$ [22, 26–30].

Photometric. A large number of methods for the direct measurement of non-reacted dichromate [31–33] and for monitoring the product of a reaction in which dichromate

takes part have been reported in the literature [31–33]. The reagents most frequently used in this last case have been diphenylcarbazide [34, 35] and brucine [36]. The use of a coupled reaction involving acetaldehyde which results in the displacement of the oxidation reaction towards completion offers greater advantages. In this sense reagents such as thiosemicarbazide [37], thiobarbituric acid and nitroprusside [38] have been utilized as trapping agents. Other photometric methods exploit the reducing character of ethanol with vanadium (V) oxide as oxidizing reagent, and involve the monitoring of the blue colour of the VO^{2+} ion formed (λ = 730 nm) [39–43].

The most common sources of error in these oxidation methods are leakage of ethanol in the preliminary separation process, non-stoichiometric oxidation reaction and low selectivity. The most serious interference is caused by other reductants present in blood, such as acetone, ketoacids and sugars [44], or those arising from an exogenous source (chloroform, isopropyl alcohol, ethyl bromide, formaldehyde, etc. [19]).

(b) *Methods based on other properties of ethanol*

Determinations based on ester formation have been suggested in which reagents such as vanadium-8-hydroxychinolate [45] and vanadium-8-hydroxy-5,7-di-iodochinolate [46] are used to obtain coloured compounds (λ_{max} = 600 and 480 nm, respectively).

Enzymic Methods

Enzymic methods are considered to be superior to those mentioned above because of their selectivity, simplicity and speed. They involve both equilibrium and, more usually, kinetic measurement. The monitored product is generated either by a single reaction or by coupled reactions.

Generally, the enzymic determination of ethanol in biological fluids is based on its oxidation by nicotinamide–adenine dinucleotide (NAD^+) catalysed by alcohol dehydrogenase enzyme (ADH) in a reaction whose basic scheme is:

$$CH_3CH_2OH + NAD^+ \overset{ADH}{\rightleftharpoons} CH_3CHO + NADH + H^+.$$
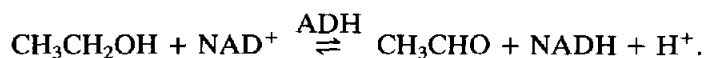
This reaction does not go to completion on its own ($K = 10^{-11}$), and coupled processes are used to favour completion by reducing the acetaldehyde concentration with the aid of trapping agents such as semicarbazide, aminoacetic acid or hydrazine, or NADH concentration by coupling a second enzymic reaction. The system is generally monitored photometrically, fluorimetrically or voltammetrically through the reduced form of the coenzyme. In some instances the formation of a dye in the coupled reaction is monitored. Tables 1 and 2 list the enzymic determinations used by non-automated (Table 1) and automated or easily automatable methods (Table 2). The features of the method are indicated in each case: detection technique, physical form of the enzyme, type of sample and pre-treatment, sample volume, range of concentration covered, both in the extract (linear range of calibration curve) and in the original blood, and several other characteristics.

Considering the importance of the determination of blood ethanol in legal terms and for clinical and forensic medicine enzymic methods would appear to fit best with present needs. They are rapid, require lower reagent and sample, and no sample pretreatment and little labour are required. Both continuous flow and discrete [47] automation have been applied.

Table 1
Non-automatic-enzymic methods for determination of ethanol in blood

Detection system	Enzyme		Sample			Linear range of calibration curve	Determination range in blood	Error (%)	Features	Ref.
	Disol.	Immob.	Treatment	Volume	Other reagents					
Photometry	X	—	Dilution	—	—	20–200 ppm	—	4	—	45
Photometry	X	—	Diffusion	10 µl	—	—	0.5–3.0 g l^{-1}	—	Diffusion process: 90 min	46
Photometry	X	—	Deproteinization	0.10 ml 0.25 ml	— —	30–150 ppm	0.6–3.0 g l^{-1} 0.1–0.6 g l^{-1}	3	Variable time kinetic method	53
Photometry	X	—	Deproteinization	—	—	—	—	—	Equilibrium measurements (70 min)	159
Photometry	X	—	Deproteinization	100 µl	yes	17–120 ppm	0.5–3.5 g l^{-1}	—	Formation of formazan	55
Photometry	X	—	Serum	—	yes	—	—	—		54
Amperometry	X	—	Serum	—	—	—	—	—	Direct measurement of NADH	73
Amperometry	X	—	Blood	20 µl	yes	—	—	—	Coupled reaction (diaphorase)	77
Photometry	X	—	Serum	10 µl	yes	—	0.1–1.5 g l^{-1}	6	Formation of formazan	57
Amperometry	X	—	Serum	5 µl	yes	1–25 ppm	0.2–5.0 g l^{-1}	—	Measurement depletion oxygen	84
Fluorimetry		X	Deproteinization	50 µl	—	—	0.5–3.0 g l^{-1}	—		68

Table 2
Automatic-enzymic methods for determination of ethanol in blood

Detection system	Enzyme		Sample		Sampling frequency (h⁻¹)	Linear range of calibration curve (ppm)	Determination range in blood (g l⁻¹)	Error (%)	Features	Ref.
	Disol.	Immob.	Treatment	Volume						
Photometry	X	—	Blood	25 µl	40	—	0.40–1.60	5	Segmented flow	61
Photometry	X	—	Blood, serum	2 µl	30	7.5–80.0	0.15–1.60	3	ΔA/Δt measurements	52
Photometry	X	—	Deproteinization	20 µl	—	—	0.10–3.00	—	Simultaneous determination	160
Photometry	X	—	Serum, plasma	10 µl	15	—	0.20–1.70	—	Centrifugal analyser	161
Photometry	X	—	Serum	0.5 ml	—	—	0.75–2.25	—	Centrifugal analyser	
Photometry	X	—	Plasma	2.5 µl	96	—	0.05–3.00	—	ABA-100 analyser	60
Photometry	X	—	Blood	30 µl	70	12.8–32.0	1.20–3.20	—	FIA/stopped-flow	62
Photometry	X	—	Blood	50 µl	40	5.0–30.0	0.5–3.0	0.5	FIA/stopped-flow/merging zones	63
Fluorimetry	X	—	Blood	20 µl	30	—	0.10–3.00	—	Segmented flow	67
Fluorimetry	X	—	Blood	50 µl	40	1.25–20.0	0.125–2.0	1.5	FIA/stopped-flow/merging zones	69
Amperometry	X	—	Serum	10 µl	30	4.6–18.2	0.90–3.60	—	Continuous flow	79
Amperometry	—	X	Plasma	50 µl	12	—	0.60–1.80	1	Enzymatic electrode	74
Amperometry	—	X	Serum	—	12	5.0–100.0	0.25–5.0	—	Measurement oxygen depletion	85
Voltammetry	X	—	Blood	50 µl	50	5.0–30.0	0.5–3.0	0.9	FIA/stopped-flow/merging zones	75

(a) *Photometric-enzymic methods*

Non-automated photometric methods can be based on the use of the previously mentioned reaction with monitoring of the reduced form of the coenzyme (λ_{max} = 340–360 nm) [45, 46, 48–50]; the phosphorylated form of the coenzyme and its reduced form (λ_{max} = 365 nm) can be monitored [51]. The application of coupled reactions provides these methods with higher sensitivity. For example, aldehyde dehydrogenase can be used to displace the oxidation reaction of ethanol by eliminating the acetaldehyde formed; two moles of the monitored product (NADH) are yielded per mole of ethanol [52]; or a semicarbazide, variable-time kinetic method has been suggested by Malmstadt [53]. The formation [54–57] or disappearance [58] of a dye provides a better wavelength measurement, and favours the above-mentioned reaction displacement. An unfortunate common feature of photometric methods is their need for an often laborious sample pre-treatment stage.

Discrete automatic methods; Malmstadt *et al.* [57] have suggested a kinetic determination using a centrifugal analyzer (aldehyde/semicarbazide coupled reaction) which requires only 2 μl of sample and allows a rate of analysis of up to 30 samples per hour. The same type of analyser has also been applied in conjunction with a coupled reaction involving acetaldehyde/aldehyde dehydrogenase [59]; a method proposed by Whitehouse [60] affords a rate of 192 samples h^{-1} and a consumption of 2.5 μl of sample per analysis.

Continuous flow automation; the only method described so far [61] requires the use of 25 μl of untreated blood and affords sampling rates of 40 samples h^{-1}. The apparatus involved is rather expensive.

Flow injection automation (FIA); the coupled reaction between acetaldehyde and semicarbazide has been applied to the stopped-flow technique either without using [62] or using [63] the merging zones mode [64, 65]. The method features low sample and reagent consumption (50 μl per analysis), no sample pre-treatment, sampling frequency 70 per hour and good precision. Inexpensive instruments are characteristic of this technique [64, 65].

(b) *Enzymic fluorimetric methods*

Fluorimetry has been little used as a detection system for alcohol measurement despite its sensitivity and specificity compared to photometry. Sample pre-treatment is not required.

Only four methods (all automatic) have been proposed. All use a trapping agent for acetaldehyde (semicarbazide) and the alcohol dehydrogenase/NAD$^+$ system. They involve monitoring the reduced form of the coenzyme (λ_{ex} = 340 nm, λ_{em} = 460 nm). Following the methods proposed by Technicon [66], Ellis and Hill [67] used segmented flow analysis to produce a method which requires only 20 μl of untreated blood, achieves good sampling rates and has a wide linear range (Table 2). Less expensive enzymic methods have been proposed by Kuan and Guilbault [68] using enzyme immobilization on cellulose. Plasma only may be used possibly because blood allows adsorption of proteins and other macromolecules on the enzyme support. Enzyme immobilization is tedious, but once performed, the reactor may be used for at least a week with no loss of sensitivity. The reactor is located on a stirrer in a special cell into which 50 μl of 1:4

plasma diluted with a buffer solution containing the coenzyme and the trapping agent are introduced. The increase in fluorescence is monitored over a 2-min period.

A method using FIA has recently been suggested [69] based on the stopped-flow/merging zones principle and requiring little whole blood (50 µl) and reagents. The range of measurement is 1.25–20.00 µg ml^{-1} and up to 40 analyses can be performed per hour.

(c) *Electroanalytical enzymic methods*

Voltammetry is the electroanalytical technique most frequently applied to the determination of ethanol in blood. Most of the methods described have been based on the ethanol–ADH/NAD$^+$ reaction with or without coupling to another reaction and with or without enzyme immobilization. The type of sensor used depends on the product monitored. These methods have been classified according to the reaction on which they are based.

Measurement of the reduced coenzyme. The electrochemical oxidation of NADH at solid electrodes has been extensively studied [70–72]. Thomas and Christian [73] have developed an automatic method for the determination of ethanol in samples of pooled serum with the aid of a glassy carbon electrode vs saturated calomel as reference ($E = 0.75$ V). The difference between the sample and blank signals integrated for 8 s is related to the ethanol concentration. Blaedel and Engstrom [74] have designed an electrode by constraining ADH and NAD$^+$ onto the surface of a platinum electrode (reagentless enzyme electrode). This has been applied to the analysis of ethanol in plasma. A voltammetric FIA method (glassy carbon electrode) has also been proposed for determination of ethanol in whole blood [75] with low sample consumption (50 µl), good sampling frequency and an excellent linear range (Table 2).

Indirect measurement of NADH. In this, a high sensitivity is achieved by coupling another enzymic reaction between the reduced coenzyme and an oxidant. Either the reagents or products of the coupled reaction are measured. The redox agents most frequently used are Bindschedler's green [76], 2,6-dichlorophenolindophenol [77], and exacyaneferrate (III) [78]. In this way the alcohol concentration in whole serum is measured by enzymic differential amperometry in flowing streams, using alcohol dehydrogenase as the enzyme catalyst, 2,6-dichlorophenolindophenol as the redox mediator and electroactive species (diaphorase is the catalyst of the coupled reaction [77]). The apparatus required is simple and inexpensive and sample consumption is low (10–20 µl). The sensing system is formed by two tubular electrodes. An automatic system with similar principles but using hexacyaneferrate (III) as the redox mediator and the enzyme immobilized on several supports has been developed by Christian *et al.* [79].

Clark's electrode has been used for the automatic-amperometric determination of ethanol in blood. Oxygen depletion is measured during the aerobic oxidation of NADH in the presence of peroxidase and Mn(II) [80]. The reaction is accelerated by the presence of some phenols [81, 82]; it has low sample consumption and an excellent sampling rate [84]. A similar method uses a single reaction in the presence of immobilized alcohol oxidase [85]. This paper includes an interesting study on the different forms of immobilization of ADH on various supports [86, 87].

In view of the large number of enzyme electrodes constructed using different techniques for enzyme immobilization on the sensor [93–95], it is not surprising that

several electrodes of this type have been developed for ethanol determination. One of the most interesting of these is that reported by Blaedel and Engstrom [74], who use a solution of ADH and NAD$^+$ separated from the sample or standard external solution by a selective membrane supported on the platinum sensor. The membrane is permeable to ethanol and acetaldehyde but non-permeable to the enzyme and coenzyme. This electrode has been used in a continuous flow system in which ethanol diffuses across the membrane to form acetaldehyde, which in turn is diffused across the membrane into the stream. The NADH formed is quantitatively oxidized at the working electrode yielding an anodic current which is measured. Although the range of measurement is not very wide, the method features major advantages such as a small sample volume and considerable reduction in enzyme and coenzyme consumption.

From Table 2 it can be observed that almost all methods for voltammetric determination of ethanol use continuous flow automation with good measurement range and low detection limits. Generally sample pre-treatment is necessary owing to the risk of the adsorption of impurities on the electrode surface. In some methods the pre-treatment merely entails a simple dilution.

In summary, enzymic methods are tending towards the use of smaller sample volumes with minimum or no pre-treatment, the use of immobilized enzymes to lower the cost per analysis and high sampling rates.

Chromatographic methods

Chromatographic methods are widely accepted for the determination of several types of organic compounds such as alcohols, ketones and aldehydes in biological specimens, and have been used for over 20 years in the determination of ethanol in blood. Although some of the earlier techniques have become obsolete, recently the incorporation of advances such as head-space chromatography, have extended the popularity of chromatography. The literature has been reviewed in two papers [3, 4].

The following classification of the methods used has been taken from Jain and Cravey [3].

(a)*Direct injection*

Methods using direct injection of whole blood suffer from the adsorption of undesirable compounds (proteins and other macromolecules) on the column and consequently only few have been published [96–102] but in others prior dilution or centrifugation have been used [103–112].

(b) *With extraction*

The general features of chromatographic methods involving a prior extraction step are summarized in Table 3 [113–117]. Usually they are more accurate than standard chromatographic [118, 119] or enzymic [113, 110] methods.

(c) *With distillation*

In these methods the sample and internal standard (*n*-propanol) in sodium tungstate/sulfuric acid are subjected to distillation. The distillate is injected into the column (generally of stainless steel) and detection is performed by thermal conductivity [111, 112] or flame ionization [123, 124].

Table 3
Chromatographic methods for determination of ethanol in blood with prior extraction

| Organic solvent | Column | | Carrier rate | Detection system | Instrument | Ref. |
	Packed	Temperature (°C)				
n-Propylacetate	2 g Flexol + 100 g Firebic	110	60 ml min^{-1}	Thermal conductiv.	Beckman GC-2	113
n-Butanol	15 g Flexol + 3 g polyethylenglycol + 100 g Chromosorb	130	40 ft s^{-1}	Thermal conductiv.	Beckman GC-2	114
Dioxane	2 g Flexol + 100 g Firebic	105	65 ml min^{-1}	Flame ionization	Beckman GC-2A	115
n-Propylacetate	Hallenonid M-18 in HMDS	—	—	Flame ionization	—	116
n-Butanol	10% Igepan in Diatoport S	—	—	Flame ionization	HP 402	117

(d) *Head-space methods*

The methods are at present, possibly, the most frequently used for clinical, toxicological and forensic purposes. Many authors have described methods using head-space gas chromatography [125–135]. These offer distinctive advantages over direct injection methods, the most important of which is the prevention of column contamination. However, most head-space procedures equilibrate blood ethanol samples at room temperature (25°C) and, therefore, are useful only at ethanol concentrations in excess of 0.1 mg ml^{-1}. Ozeris and Bassette [126, 127] have reported methods for the measurement of concentrations below 0.1 μg ml^{-1} utilizing an equilibrium temperature of 60°C.

Table 4 lists the most widely used methods of this type [136–143], with their different features. Natelson and Stellate [144] describe a complex apparatus in which the carrier gas is used to drag the vapour off a partially dehydrated sample. Savory *et al.* [145] have introduced a modification in Natelson's procedure by using a chassis thermostatically controlled by a hot water stream, and a hydrogen flame ionization detector. Liebich *et al.* [146] describe a method to analyse the different constituents of human serum or plasma by this technique which involves their identification with a mass spectrometer.

Other Methods of Analysis

In this section are summarized different methods which cannot be included in the above categories.

(a) *Osmometric methods*

Several authors [147–150], such as Redetzk [151], have considered the possibility of determining the ethanol concentration after verifying that an increase results in increased serum osmolality. The contribution of alcohol to the serum osmolality can be directly related to its concentration in blood, and good correlation has been found between the values obtained by enzymic methods and those calculated from osmolality measurements using the freezing point method. The method is not specific because of significant errors which may arise from subjects suffering from diabetic acidosis, renal failure, hyper-natremia, etc.

(b) *Catalytic methods*

Three catalytic methods of very different nature have been applied. In that suggested by Pfeil [152] ethanol is driven by an air stream through a copper spiral at 280°C. The acetaldehyde produced is absorbed into a solution of 0.85% sodium nitroprusside, 8% morpholine and 1 N HCl. A blue compound is formed and monitored photometrically. Another method is based on the thermal decomposition of ethanol into ethylene in the presence of a catalyst. Ethylene is collected into a standard solution of bromine and the excess is titrated iodometrically [153]. The method is subject to little interference because of the specificity of the thermal-catalytic decomposition of ethanol into ethylene and subsequent bromination. A catalytic method developed using FIA has been suggested by Huber *et al.* [154]. After separating ethanol from the sample (30 μl) in an air stream, this compound is injected into a carrier stream that leads it to a flow-cell with a nickel oxide electrode (0.55 V vs mercury/mercury(II) oxide electrode as reference) which catalyses the reduction [155–157], yielding a current intensity proportional to the concentration of ethanol in the sample over the range 0.2–50.0 mmol l^{-1}.

Table 4
Features of the determination of ethanol in blood by head-space chromatography

Test tube Temperature (°C)	Time	Column Packed	Column Temperature (°C)	Detection system	Sensitivity*	Instrument	Ref.
60	—	15% polyethylenglycol in celite + 15% polyethylenglycol in Chromosorb	100	Flame ionization	d.l. $\mu g\ ml^{-1}$	Perkin–Elmer 116E	136
85	5 min	2 g Flexol + 100 g Firebic	100	Thermal conduct.	d.l. 0.01%	Beckman GC-2A	137
60	3 min	Styrene-divinyl benzene	165	Flame ionization	$0.0030–1.2\ mg\ ml^{-1}$	Varian 2100 GCL	138
45	2.5 h	Polyoxiethylenglycol in celite C-22	80	Flame ionization	dm.l. 0.03%	Varian 2100 GCL	139
20	—	Carbowax in Chromosorb	110	Flame ionization	dm.l. 0.05%	—	140
—	—	Porapak Q	130	Flame ionization	dm.l. 2.7 μM	—	141
60	10 min	Carbomak	100	Flame ionization	dm.l. 0.3%	—	142
132	—	Porapak S	132	Thermistors	—	Alco-Analyzer	143

* d.l. detection limit; d.m.l. determination limit.

(c) *Infra-red absorption method*

This requires the prior extraction of ethanol from the sample into carbon disulfide and measurement at λ = 3.39μ, corresponding to the molecular C–H stretching bond vibration. The absence of water is essential because, in addition to its interference in IR absorption the cell windows used (NaCl or NaF) are water soluble [158].

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