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Liquid chromatographic determination of ethyl alcohol in body fluids

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Abstract

A high-performance liquid chromatographic technique for ethyl alcohol determination in body fluids is proposed. Ethyl alcohol is quantitatively converted into acetaldehyde-phenylhydrazone by oxidation in the presence of alcohol dehydrogenase, nicotinamide–adenine dinucleotide and phenylhydrazine. The derivative is suitable for reversed-phase liquid chromatography and ultraviolet detection at 276 nm. The limits of linearity, detection and quantification as well as accuracy and reproducibility were investigated in water, serum and whole blood. Analytical responses were linear within the 0.008 to 5 g/l range, and the limit of quantification was 0.02 g/l both in aqueous standard and in biological matrix assays. Mean analytical recovery of ethyl alcohol in blood serum averaged 98.2±4.2%, imprecision (CV%) at 0.80 g/l was 2.2%, and the limit of quantification was 0.02 g/l. Serum concentrations of persons that avoided alcoholic beverages for a week were less than the limit of quantification. Ethyl alcohol concentrations in serum and whole blood compared well with those obtained by headspace gas chromatography. This simple and reliable procedure, which was also used for a urine assay, could be suitable for validation of the screening procedures used to monitor ethanol abuse.

Keywords: Ethyl alcohol

1. Introduction

The determination of ethyl alcohol in blood, urine, saliva and breath in man has become increasingly important for clinical, forensic and administrative purposes; in particular, accurate ethanol determination is required in law enforcement, with specific application to automobile traffic violations. It should be emphasised that blood ethanol determinations carried out in the hospital laboratory for diagnostic purposes can subsequently assume forensic relevance as evidence of intoxication.

Enzymatic–spectrophotometric or gas chromatographic procedures are currently employed. The alcohol dehydrogenase (ADH, EC 1.1.1.1) spectrophotometric method is commonly used for measuring ethanol in biological samples. In the presence of nicotinamide–adenine dinucleotide (NAD) and ADH, the oxidation of ethanol produces acetaldehyde and reduced NAD (NADH), which is de-

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tected spectrophotometrically. An alkaline pH and the presence of a carbonyl-trapping agent (i.e. semicarbazide) allow the oxidation to proceed [1]. This method is used for routine screening purposes, however, to achieve legal defensibility, 'positive' results should be confirmed using a second, more specific test [2].

Gas chromatography (GC), which is widely used in the forensic medicine environment, is particularly advisable for the confirmation of enzyme-reaction-based assays [3]. However, GC has encountered only limited favour in clinical chemistry and emergency toxicology.

High-performance liquid chromatography (HPLC) is widely employed in clinical chemistry and clinical toxicology laboratories, and the availability of a reliable application for the confirmation of ethanol screening data would be appreciated in these environments.

The first application of a HPLC assay for the determination of ethyl alcohol in blood used electrochemical detection to determine precolumn-generated NADH from ADH-catalyzed ethyl alcohol oxidation [4]. Because the inherent selectivity limit of the enzymatic assay, i.e., nonspecific production of NADH, could not be obviated by the use of this HPLC procedure, its accuracy and reliability do not differ greatly from that of the spectrophotometric method. A further application of a HPLC assay for ethyl alcohol in blood used a post-column enzymatic reaction with electrochemical detection. The alcohol, separated on the column, is converted by immobilised alcohol oxidase from *Candida boidinii* into acetaldehyde, with the formation of a stoichiometric amount of hydrogen peroxide, which was detected via oxidation at a platinum electrode [5]. This procedure can hardly be used for routine purposes, mainly because of the limited life of the enzymatic reactor [2]. In a recently described HPLC procedure with pre-column enzyme-mediated derivatisation of ethanol, deproteinised blood was reacted with ADH–NAD and then with cyclohexanedione reagents, to obtain a fluorescent derivative of acetaldehyde [6]. The procedure is laborious and time-consuming: it requires the deproteinisation of blood, the enzymatic conversion of ethanol into acetaldehyde, a 60-min-long derivatisation reaction at 70°C and a complex injection system.

In this paper, a HPLC procedure for the quantitative determination of ethyl alcohol in biological fluids is described. It is based on the ADH–NAD-mediated conversion of ethanol into acetaldehyde that, in the presence of phenylhydrazine, is converted in turn into acetaldehyde-phenylhydrazone. This is suitable for reversed-phase liquid chromatographic separation with detection by UV absorption [7].

2. Experimental

2.1. Reagents and chemicals

Alcohol dehydrogenase (ADH, EC 1.1.1.1) from bakers yeast (300–400 kU/g protein, lyophilised powder), phenylhydrazine and anhydrous sodium pyrophosphate were purchased from Sigma (St. Louis, MO, USA). NAD lithium salt was purchased from Boehringer (Mannheim, Germany). Aqueous standard solutions of ethyl alcohol (0.5–4.0 g/l) were from Merck (Darmstadt, Germany). Deionised–distilled water was used.

Solutions containing 480 mmol/l phenylhydrazine were prepared fresh before use by diluting 50 μl of concentrated amine with 1.0 ml of water. The following reagents were stored at 4°C until use: ADH suspension, 7500 U dissolved in 5 ml of water (stable for one month); aqueous solution of NAD (50 mmol/l, prepared weekly); aqueous solution of $Na_4P_2O_7$ (75 mmol/l), brought to pH 9.2 with concentrated hydrochloric acid (prepared monthly). Aqueous KH_2PO_4 (14.70 mmol/l; A) and $K_2HPO_4^{\cdot}\cdot 3H_2O$ (8.76 mmol/l; B) solutions and HPLC-grade acetonitrile (C) were used in the preparation of the mobile phase. The work-environment was checked for the absence of ethanol solutions before reagent preparation and assays.

2.2. Samples

Blood serum and heparinised whole blood samples, drawn into vacuum tubes (Becton Dickinson Vacutainer Systems, NJ, USA), and postmortem whole blood samples were collected, stored at −20°C and analyzed within two weeks. Ethyl alcohol concentrations in samples from 14 healthy subjects who had been free from alcohol consump-

tion for at least one week were taken as 'negative' control data. Forty-eight serum specimens from persons that ingested alcoholic beverages and eight postmortem blood samples were also analyzed with the proposed method, in parallel with headspace gas-chromatography.

2.3. Sample preparation

To 1.0 ml of pyrophosphate buffer, placed in a 1.5-ml capped glass vial, 40 μl of phenylhydrazine solution, 80 μl of NAD solution and 20 μl (30 U) of ADH suspension were added and the mixture was shaken for 10 s. A 20-μl volume of sample, standard or water was added, the mixture was mixed again and incubated at room temperature. After an incubation time of 30 min, 10 μl aliquots of the mixture were injected into the column.

Endogenous acetaldehyde, when needed, was analyzed by following the same procedure, but omitting the addition of both ADH and NAD to the otherwise complete reaction mixture.

2.4. Chromatographic conditions

The chromatographic apparatus was from Hitachi (Tokio, Japan) and consisted of an L-6200 solvent delivery module, an L-4250 UV–Vis detector, an AS-2000 autosampling injector and a D-2500 Chromato-Integrator. The detector was set at 276 nm, 0.002 a.u.f.s., and the integrator input was set at 128 mV, full scale. An octadecylsilyl (250×4 mm) LiChrospher 100 RP-18 5 μm column (Merck) was used throughout, with a 4×4 mm LiChrospher 100 RP-18 5 μm (Merck) guard column. The isocratic elution was performed at a flow-rate of 1.5 ml/min, at room temperature, using a ternary mixture of A–B–C (15:45:40, v/v). Peak area was used to quantify the analytical response. Results were calculated by using a five-point external standard calibration, including a reagent blank. Samples with ethanol concentrations exceeding 4.0 g/l were diluted four-fold and reanalysed.

2.5. Analytical validation

The experimental approach to the analytical validation is essentially based on schemes reported elsewhere [8,9]. In order to investigate the lower and upper limits of linearity (LOL), detection (LOD) and quantitation (LOQ) of ethanol in water, a series of aqueous standard solutions containing progressively lower concentrations of ethyl alcohol, ranging between 10.0 and 0.002 g/l, was prepared. Each specimen was processed in triplicate and the analytical responses were corrected for the mean of the reagent blanks.

The corrected mean analytical responses of aqueous matrix against theoretical ethanol concentrations were plotted. A range of concentrations that gave a linear curve was selected and a linear equation was computed by least squares regression. The criterion to determine the LOL in water required that the first point beyond the range of linearity should deviate by more than 10% (arbitrarily selected) from a target value predicted by the linear regression.

Likewise, the LOD was the lowest observed mean concentration that did not deviate from the target value by more than 10%.

Once the lowest detectable concentration was determined, ten replicates of this concentration were analyzed and the mean and standard deviation were determined. The limit of quantification (LOQ) was calculated as the LOD plus ten times the SD [9]. Subsequently, a set of aqueous ethanol concentrations, selected within the range of linearity, was used as the calibrating standards for the assays of biological samples.

Selected negative (i.e. no detectable difference with reagent blank) serum and postmortem whole blood samples were used to obtain specimens containing known and progressively lower concentrations of ethanol in the range between 10.0 and 0.002 g/l: the LOL, LOD and LOQ were determined by following the procedure described above for a water matrix.

2.6. Comparison procedure

A Fractovap GI 450 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a flame-ionisation detector, a head-space autosampler and an integrator was used. Gas-chromatographic conditions were as follows: glass column (2 m×4 mm I.D.) packed with 60/80 mesh Carbopack B coated with 5% Carbowax 20M; carrier gas, nitrogen; flow-rate, 50 ml/min;

column temperature, 95°C; injector and detector temperatures, 150°C. Serum samples and standards were diluted with an equal volume of water containing *n*-propanol, 10 g/l (internal standard), and were allowed to sit in the autosampler for 3 h at 60°C before the injection of 100 μl of vapor [3].

3. Results

3.1. Enzyme reaction

Phenylhydrazine reacts with activated carbonyl groups to form the corresponding UV-absorbing phenylhydrazones [7,10]; reaction with acetaldehyde, in the presence of the pyrophosphate buffer at pH 9.2, produces two *syn*- and *anti*-diastereoisomers that are suitable for reversed-phase liquid chromatography. Using the above chromatographic conditions, they were resolved, giving two distinct peaks. The UV absorption spectrum for each isomer was assessed by repeating runs at different wavelengths and plotting the corresponding peak areas. Although shifted by some 5–10 nm, the two spectra were of similar shape: λ_{max} of the less-retained isomer was 272 nm, whereas that of the more retained one was 276 nm (Fig. 1).

Because of the high absorption of the derivative and the virtual absence of interfering peaks, any wavelength in the range between 250 and 320 nm could be suitable for the assay. In this work, the peak area of the more retained isomer, measured at 276 nm, was chosen as the analytical signal because of its higher response.

The conversion of ethyl alcohol into acetaldehyde-phenylhydrazone in the presence of ADH and phenylhydrazine was found to take place, giving raise to the above isomers (Fig. 2). Apart from some additional peaks eluting within the first 3 min of the chromatographic run, the chromatographic traces of serum, whole blood or urine samples did not differ from that of aqueous standards. The peak area ratios of the two isomers were the same as that measured with the reaction of acetaldehyde, and were independent of the different kind of matrix analyzed. No carryover was observed after consecutive runs of samples containing elevated ethanol concentrations. The time-course of the derivatisation was monitored by analyzing a serum sample containing ethanol, 1.52 g/l, and by varying the amount of enzyme between 7.5 and 75 U. Apart from the amount of enzyme, the operative conditions were those described above. By using 15 or more units of enzyme, the reaction rates had similar shapes; during the first 30 min, the derivative concentration increased, but afterwards, it remained stable for at least 3 h. No

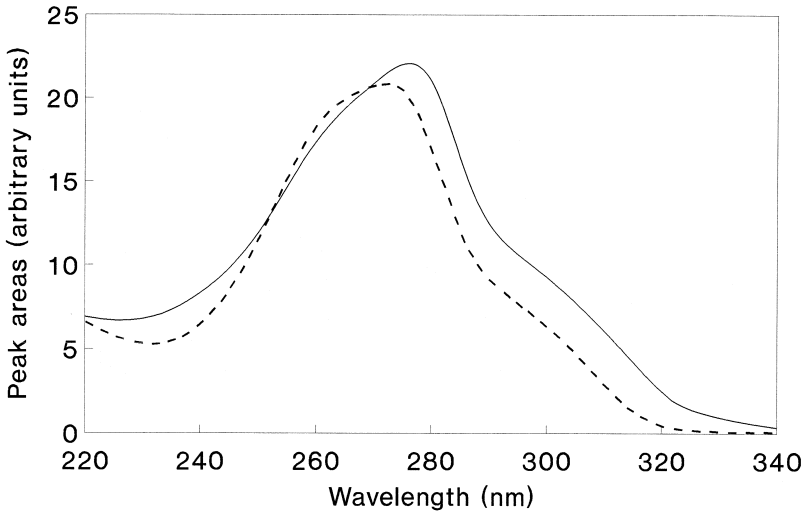

Fig. 1. Ultraviolet absorption spectra of the less retained (dashed line) and more retained (unbroken line) acetaldehyde-phenylhydrazone stereoisomers.

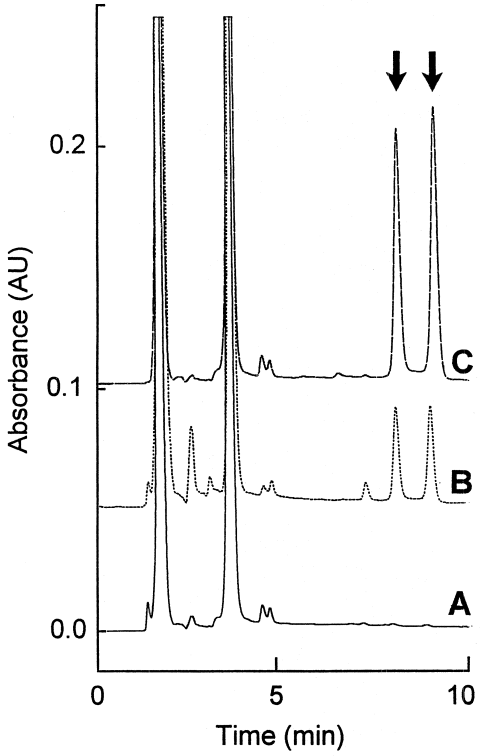

Fig. 2. Chromatograms of an ethyl-alcohol-free serum sample (A), a serum sample containing ethyl alcohol at 0.71 g/l (B), an aqueous solution of ethyl alcohol at 2.00 g/l (C). Arrows indicate the acetaldehyde-phenylhydrazone peaks.

substantial increase in the reaction rate was achieved when the phenylhydrazine concentration was doubled. Using the standard conditions, the reactivities of ethyl alcohol in aqueous solution and in serum were compared: quantitative conversion was achieved within 30 min (Fig. 3). Progress curves for the derivatisation of ethanol in whole blood samples had similar shapes (not shown).

3.2. Stability of the derivative

After storage at room temperature in the dark, the concentration of the derivative obtained from the assay of a 2.0-g/l aqueous standard decreased by some 3, 9 and 14% in 3, 8 and 24 h, respectively. Similar trends in stability were observed when serum or whole blood was analyzed.

3.3. Analytical validation

The LOL, LOD and LOQ, determined in water, blood serum and whole postmortem blood matrices, are summarised in Table 1.

Ethanol aqueous standard solutions containing 0, 0.1, 0.5, 2.0 and 4.0 g/l were used as calibrators in the assays of biological matrices. The least squares regression equation of a typical calibration curve was $y=50.4(\pm 0.7)x-0.4(\pm 1.2)$, where y=peak area (arbitrary units) and x=ethyl alcohol concentration (g/l); $(S_{y/x})=0.7$; $r=0.999$; $n=10$.

The within- and between-run reproducibilities of serum and whole blood assays, at different ethanol concentrations, are indicated in Table 2.

Ten different serum samples (ethanol: 0–2.9 g/l) and six whole blood samples (0–3.2 g/l) were reanalyzed after the addition of 0.5 g/l of ethyl alcohol; the measured recoveries averaged 98.2 ± 4.2 and 99.2 ± 5.1%, respectively.

Application of the method to the assaying of urine was also investigated: the analytical performances were similar to those obtained for blood (not shown).

3.4. Interferences

Endogenous acetaldehyde may represent a potential cause of overestimation in this assay for ethyl alcohol. Its concentration in blood, which is normally undetectable, increases after ethanol intake and remains steady over a wide range of falling ethanol levels. Indeed, due to its instability in the blood, it remains some orders of magnitude lower than ethanol and, subsequently, well below the LOQ of the present procedure. The same holds true if some observed in-vitro generation from ethanol in the blood is considered [11]. Nevertheless, in the cases of ethanol intoxication or aged samples, in order to prevent any possible acetaldehyde interference, the assay of endogenous acetaldehyde may be performed by simply omitting the ADH/NAD addition from the otherwise complete reaction mixture. Nevertheless, four postmortem blood samples (ethanol, 0.8–3.2 g/l) and 21 serum samples (0–2.9 g/l) were analyzed for acetaldehyde. Measured concentrations never exceeded 0.01 g/l, which is negligible compared to the ethanol signal in ethanol-free serum samples (0.01–0.02 g/l). The addition of methyl,

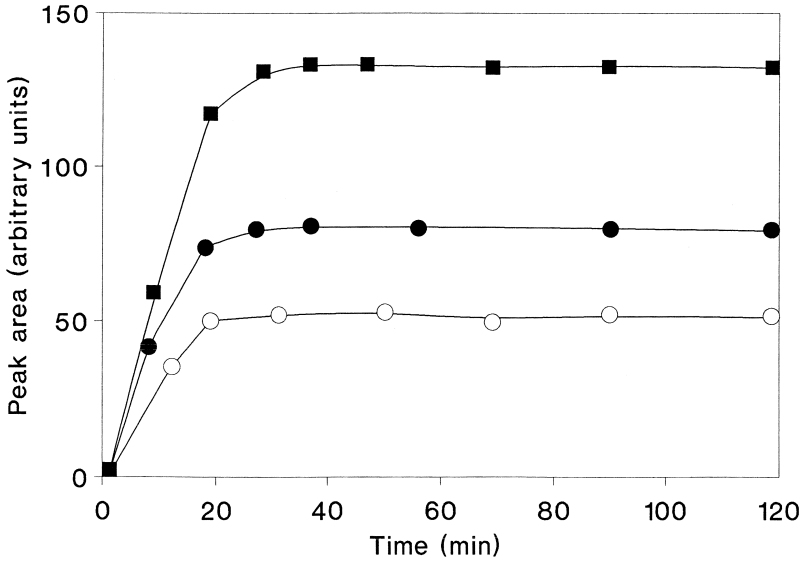

Fig. 3. Progress curves for ethyl alcohol derivatisation. The reaction was carried out as described in the text. Analysis of an aqueous standard solution, 1.0 g/l (○), a serum sample containing 1.52 g/l of ethyl alcohol (●) and of the same serum supplemented with 1.00 g/l of ethyl alcohol (■).

Table 1
Ranges of linearity ($n=3$) and limits of detection and quantification ($n=10$)

Sample matrix	Investigated conc. range (g/l)	Range of linearity (g/l)	LOD			LOQ (g/l)
			(g/l)	SD (g/l)	CV (%)	
Water	0.002–10.0	0.008–6.0	0.0082	0.0012	14.7	0.020
Blood serum	0.002–10.0	0.008–5.0	0.0074	0.0010	13.7	0.017
Whole blood	0.002–10.0	0.008–5.0	0.0076	0.0009	11.9	0.017

Table 2
Reproducibility for the replicate assays[a] of serum and whole blood samples spiked with known concentrations of ethanol

Theoretical concentration (g/l)	Blood serum			Whole blood		
	Found (g/l)	Within-run CV (%)	Between-run CV (%)	Found (g/l)	Within-run CV (%)	Between-run CV (%)
0.02	0.02	8.1	9.4	0.02	9.1	9.5
0.20	0.20	3.7	4.1	0.21	4.0	4.2
0.80	0.80	2.2	2.8	0.78	2.3	2.7
1.00	1.01	1.9	2.4	1.02	2.0	2.3
5.00	4.86	4.2	5.3	4.90	3.9	5.1

[a] Replicate assays comprised five independent assays of frozen stored samples carried out within the batch and on different days.

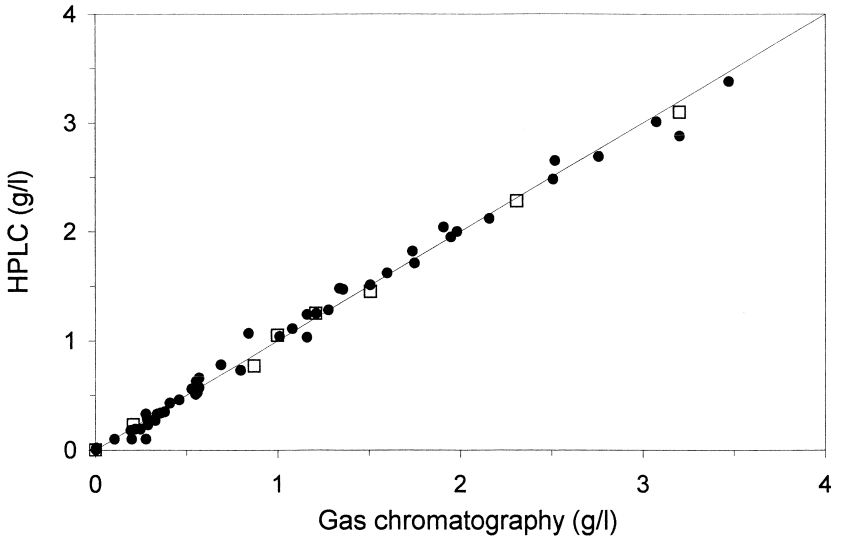

Fig. 4. Comparison between ethanol concentrations in serum (●) and in postmortem whole blood (□) measured by HPLC (y) and headspace GC (x) techniques. Least-squares regression equations for serum [$y=0.986(\pm0.014)x+0.013(\pm0.020)$; $S_{y/x}=0.088$ g/l; $r=0.995$; $n=48$) and whole blood ($y=0.970(\pm0.020)x+0.016(\pm0.020)$; $S_{y/x}=0.055$ g/l; $r=0.999$; $n=8$) specimens. Straight line is $y=x$.

n-propyl, isopropyl and *n*-butyl alcohols (0.5 g/l each) to a pooled serum sample did not affect the ethanol assay. The same held true when serum was spiked with the most common short chain aldehydes or α-keto acids (formaldehyde, glyoxylic acid, pyruvic and β-hydroxy pyruvic acids, α-ketobutyric and α-ketovaleric acids).

3.5. Comparison procedure

Forty-eight serum samples from persons that had consumed alcohol and eight postmortem blood samples were analysed by using the HPLC procedure, and were reanalysed using headspace gas-chromatography: there was a close correspondence (Fig. 4).

4. Discussion

Previous liquid chromatographic applications for assaying ethanol in biological fluids were affected by either inaccuracy [4] or costly and tedious reagent preparation [5–6]. The present liquid chromatographic determination of ethyl alcohol is more specific than the former HPLC method that used precolumn-generated NADH as the analytical marker [4], by which any inherent unspecificity of the enzyme reaction could not be obviated. Nevertheless, other HPLC procedures, which used immobilised enzyme for post-column reaction, are not very useful for routine purposes, mainly because of the need for periodical enzyme reaction coil preparation and its limited stability [2], whereas our procedure uses stable reagents and reproducible conditions of reaction.

When the reliability of the chromatographic method is compared to that of the enzyme–spectrophotometric one, it can be observed that both use a specific enzyme reaction, but, with the former method, the analytical marker is resolved chromatographically from any potential interference, which is an additional selectivity factor.

A further factor substantiates the reliability of the procedure, i.e., the reproducibility, recovery data and extension of the linear ranges for water, serum and whole blood assays are similar.

Although the criterion adopted for establishing the LOLs and LODs [8,9] is restrictive, the dynamic range of linearity is extended enough (the upper LOL is some 300-fold higher than the LOQ): the upper LOLs exceed the highest ethanol blood concentrations that are usually encountered in a toxicol-

ogy laboratory and the LOQs are well below the legislative and symptomatology thresholds [12].

The proposed method, however, does not allow the determination of other alcohols, and this may be a limitation in situations that are clinically similar to ethanol intoxication.

In conclusion, although the present procedure is not properly based on different analytical methods, as it should be for a confirmatory method, it fulfils the need for a simple and reliable liquid chromatographic assay for ethyl alcohol in body fluids and can be proposed as a valid alternative to the traditional GC techniques used in clinical toxicology.

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