

VALIDATION OF A GC/HS METHOD FOR ETHANOL QUANTITATIVE ANALYSIS USING AS INTERNAL STANDARD TERT-BUTANOL

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Abstract

The aim of our study was to develop and validate a new gas chromatographic (GC) method coupled with headspace (HS) for ethanol quantitative analysis using tert-butanol as internal standard. Analysis was performed using Agilent GC 6890 N equipment with headspace G 1888. Ethanol was separated in two columns for ethanol analysis DB-ALC1 and DB-ALC2 with two detectors FID-1A, respectively FID-2B. The values of retention time specifically for ethanol were 1.544 (DB-ALC1; FID-1A), respectively 1.593 minutes (DB-ALC2; FID-2B). The calibration curve was linear within the range 0.5 to 4.0 g/L, and the value of the correlation coefficient was 0.99994 (DB-ALC1; FID-1A). The accuracy and the precision of method were evaluated by analysing standard ethanol solutions samples and by determining the recovery. The mean values of recovery ratio were between 100.12% (solution 3 g/L) and 102.22% (solution 1.5 g/L) when an ethanol/water solution was used. In forensic toxicology ethanol is usually determined from blood samples, thus we elaborated a calibration curve by using standard ethanol solution in blood. For DB-ALC1 column and FID-1A detector the value for the detection limit was 0.067 g/L, and 0.223 g/L for the quantification limit. The method validated in our study may be used for ethanol quantification in forensic toxicology laboratories.

Rezumat

Scopul studiului a fost de a dezvolta și valida o nouă metodă de analiză a etanolului prin gaz-cromatografie (GC) cuplată cu *headspace* (HS) utilizând terț-butanolul ca standard intern. Pentru analiză s-a utilizat un sistem Agilent GC 6890 N, cu *headspace* G 1888. Etanolul a fost analizat utilizând două coloane specifice, DB-ALC1 și DB-ALC2, cu doi detectori FID-1A și FID-2B. Timpii de retenție pentru etanol au fost de 1,544 min. (DB-ALC1; FID-1A) și 1,593 min. (DB-ALC2; FID-2B). Curba de calibrare obținută a fost liniară în intervalul de concentrație 0,5-4,0g/L, valoarea coeficientului de corelație fiind de 0,99994 (DB-ALC1; FID-1A). Pentru evaluarea acurateței și preciziei metodei au fost analizate soluții etalon ca probe și s-a determinat coeficientul de regăsire. Valorile medii ale coeficienților de regăsire la analiza soluțiilor de etanol în apă au fost între 100,12% (soluția 3g/L) și 102,22% (soluția 1,5g/L). Deoarece în toxicologia legală, etanolul este determinat din probe biologice, a fost elaborată o curbă de calibrare utilizând soluții standard de sânge cu alcool. Limita de detecție la utilizarea coloanei DB-ALC1 și a detectorului FID-1A a

fost de 0,067g/L, iar limita de cuantificare 0,223g/L. Metoda validată poate fi utilizată pentru determinarea cantitativă a etanolului în laboratoarele de medicină legală.

Keywords: ethyl alcohol, gas chromatography/headspace (GC/HS), validation

Introduction

Ethanol consumption induces direct toxic effects, affects the psychic and social status of the human being, induces driving accidents and increases the suicidal risk.

Quantitative determination of ethanol in biological samples, in Romania, is made in Forensic Departments and in Toxicology Laboratories from Emergency Hospitals. In these laboratories for ethanol quantification the Cordebard method is used. Because this method is difficult to use and the results are not so precise it is important to introduce new methods that must be fast and easy to apply in conditions of optimal features for forensic toxicology laboratories.

Based on these requirements and considering the results of previous studies, the purpose of analysis performed was to develop and validate a method for the quantitative determination of ethanol in biological samples by gas chromatography coupled with headspace (GC/HS) using as internal standard tert-butanol [1, 3, 4, 6, 7].

Materials and methods

Reagents

- standard ethanol solutions (Restek, USA) with concentrations: 0.5; 0.8; 1.0; 1.5; 2.0; 3.0 and 4.0 g/L in water;
- control blood samples (Medichem) containing ethanol with concentration: 0.5 g; 0.8 and 1.1 g/L;
- blood samples collected on anticoagulant (sodium fluoride and potassium oxalate);
- tert-butanol (Merck); 1M ammonium sulphate solution in water.

Equipment

Headspace (HS) conditions: HS 1888 G with 70 seats, oven temperature: 80°C, balance temperature: 90°C, transfer temperature: 110°C, balance time: 15 minutes, GC cycle time: 6 minutes, the injection time: 1.0 min.

Gas chromatography (GC) conditions: Agilent GC 6890 N; oven temperature 40°C (isothermal), split ratio 5:1, gas pressure in the column -

18.8 psi, carrier gas - He, make-up - He-Detector, hydrogen and synthetic air; DB-ALC1 columns (30 m x 0.32µm x 1.8µm) and DB-ALC2 (30m x 0.32µm x 1.2µm), detectors: FID-1A – 260°C and FID-2B – 260°C. The composition of the stationary phase for these columns is protected by Agilent Technologies, being now under the proprietary law [10].

Analysis and data integration was performed using ChemStation software. The calculated number of theoretical plates was between 38 000 and 45 000, which shows the efficiency of columns used.

For each concentration we performed three determinations and we present the average of these results.

The limit of detection and quantification were calculated based on the formula: $LOD = (3 \cdot SE) / a$; $LOQ = (10 \cdot SE) / a$, where LOD - limit of detection, LOQ – limit of quantification, SE - standard error and a - the slope.

Daily calibration was checked in at least three points. If checked items corresponded to known concentrations we have ran control blood samples and then samples from patients, if not, we recalibrated the equipment, and, than we have run controls and samples.

Sample preparation

In headspace vial, there were introduced: 250 µL blood sample, 1750µL of tert-butanol (internal standard) solution 0.4 g/L in 1 M ammonium sulphate solution [8].

Results and Discussion

Specificity of the method - under the mentioned conditions, the method is specific for ethanol, resulting each time a well established RT (retention time), 1.544 min (DB-ALC1, FID-1A) and 1.593min. (DB-ALC2, FID-2B). For tert-butanol the values of RT were 2.156min. (DB-ALC1, FID-1A), respectively 2.253min. (DB-ALC2, FID-2B) (Fig. 1). Our results showed the same retention times for ethanol, other than those for tert-butanol, which proves that the selectivity columns are specific to alcohol use. Resolution obtained was always > 3.5 indicating a very good separation of peaks.

To assess the *linearity* of the method, we established a calibration curve consisting of seven points, each point corresponding to a standard solution of known concentration respectively: 0.5; 0.8; 1.0; 1.5; 2.0; 3.0 and 4.0 g/L. The characteristic parameters of the curves achieved when using DB-ALC1 column and detector FID-1A, are presented in table I. The obtained calibration curve is presented in Figure 2.

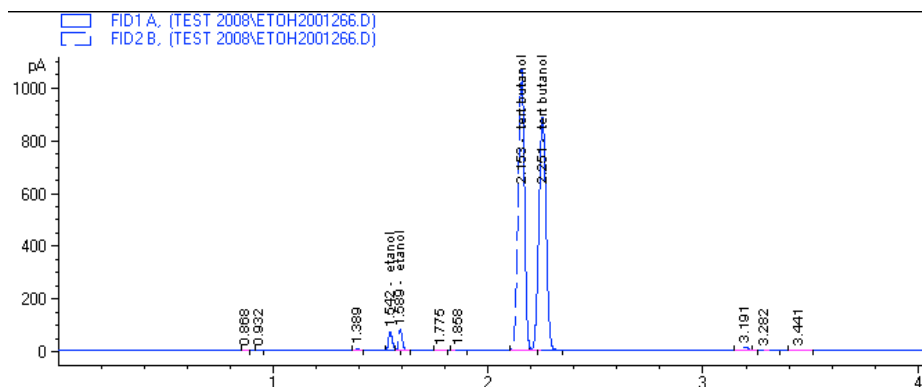


Figure 1

Chromatograms obtained after the analysis of standard solutions of ethanol 0.5 g/L

Table I

Characteristic parameters of calibration curve performed using standard ethanol solutions in water

RT* (min)	Ethanol concentration (g/L)	Average of peaks area
1.544	0.5	92.13365
	0.8	139.56987
	1.0	173.19115
	1.5	255.22137
	2.0	343.40607
	3.0	553.72424
	4.0	701.76569
SE = 3.991; a = 178.547; LOD = 0.067 g/L; LOQ = 0.223 g/L		

where: RT – Retention time; SE - standard error; a - the slope

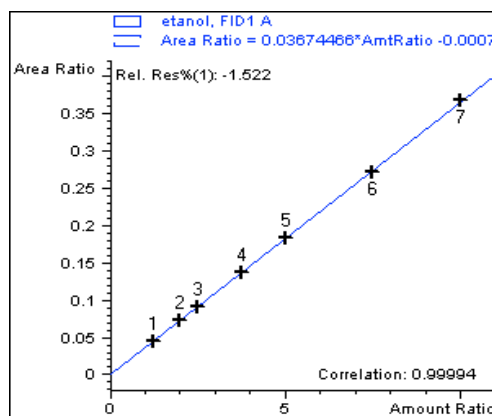


Figure 2

The calibration curve for column DB-ALC1 and FID-1A detector

The calibration curve obtained was linear, and the correlation coefficient obtained was 0.99994. This value is over 0.999, indicating a relationship of direct proportionality between the concentrations of standard solutions and the peak area.

The equation characteristic for the calibration curve is:
 $\text{area} = 178.55 \cdot \text{conc} - 3.77$.

A daily calibration curve was achieved using these concentrations, therefore the method is characterised by *reproducibility* and *repeatability*. There were no significant differences between measurements, these falling within the permissible margin of error for this type of device. So, the maximum value for RSD need to be under 5% when the values of concentrations are between 0.5 and 4 g/L.

To determine the *accuracy* of the method we ran standard solutions as samples, achieving concentrations close to the theoretical values, and the rate of recovery fitted between 100.12% and 102.22%. Also, to demonstrate both method's and device's accuracy we analysed control blood samples containing ethanol with the following concentrations: 0.25; 0.5; 0.8 and 1.1 g/L, in this case the mean values for recovery ratio were: 106.12%, 103,70% and 103.94%.

For the accuracy of the results, the calibration curve from the previous day should be checked to at least three points, on known concentrations. If the curve corresponds, we should introduce control blood samples, corresponding to a minimum of two points from the calibration curve and after that the blood samples from patients.

Data obtained from accuracy evaluation are presented in Table II.

Table II
Results on the evaluation of accuracy of the method

Average of peaks area	Concentration (g/L)		Recovery (%)
	Theoretical	Determined	
92.13365	0.5	0.50220	100.44
139.56987	0.8	0.81002	101.25
173.19115	1.0	1.00819	100.81
255.22137	1.5	1.53333	102.22
343.40607	2.0	2.03272	101.63
553.72424	3.0	3.00373	100.12
701.76569	4.0	4.06014	101.50
Statistical data			Average = 101.13% RSD = 0.72%

The percentages of recovery obtained when we analysed standardized samples of ethanol in the blood are higher than those determined from the analysis of ethanol in water solutions. These differences are caused by specific properties of blood which is an extremely complex solution compared with water.

For *precision* evaluation we analyzed three times the solutions corresponding to three points from calibrating curve, respectively with concentrations of: 0.5, 1.0 and 1.5 g/L. The values for the ratio of recovery were between 99.90% and 105.54%. The statistical data were: average = 103.195%, SD = 2.231% and RSD = 2.161%.

Measurements made on samples with different homogeneity showed a lack of uniformity matrix (blood samples with clots or micro-clots) which determined a proportional increase in RSD. This induces errors of interpretation of results. In order to determine method precision we had to analyse inhomogeneous samples. The RSD values for these samples were below 3.5%.

To prove once more method's accuracy, we elaborated a calibration curve using standard blood with ethanol concentrations: 0.25; 0.4; 0.5; 0.8 and 1.1 g/L [2, 9]. This curve was used for analysing blood samples. For such calibration curve, the correlation coefficient was 0.99921, which showed very good linearity when this type of standard was used. The calibrating curve obtained with ethanol in water solutions should be used when samples with high water content like urine and vitreous humour should be analysed. Characteristics of calibration curve for DB-ALC1 column and FID-1A detector are shown in Table III.

Table III
Characteristics of the calibration curve in blood ethanol standard

RT (min)	Ethanol concentration (g/L)	<i>Average of peaks area</i>
1.544	0.25	46.84262
	0.40	72.13791
	0.50	87.76520
	0.80	145.87405
	1.10	206.71906
SE = 4.551; a = 189.051; LOD = 0.072 g/L; LOQ = 0.240 g/L		

Following the calibration with standardized blood samples in ethanol, accuracy and precision of the method were established by checking points on the curve using solutions with the following concentrations: 0.25; 0.40; 0.50; 0.80 and 1.1 g/L. The results are shown in Table IV.

Table IV

Data obtained to assess accuracy of the method when using samples of blood standardized in ethanol

Average of peaks area	Concentration (g/L)		Recovery (%)
	Theoretical	Determined	
44.90885	0.25	0.24903	99.61%
72.68454	0.4	0.39428	98.57%
95.28483	0.5	0.51199	102.39%
141.21228	0.8	0.81539	101.92%
216.81772	1.1	1.13436	103.12%

Recovery ratio calculated at check points of the calibration curve, ranging between 98.57% and 103.12%, showed that under the used conditions, the method of separation of ethanol from biological samples with a complex composition (like blood) by using a head-space system with alcohol direct injection into the column, guaranties the obtaining of accurate results.

For *precision* evaluation we analysed three times, samples of blood standardized in ethanol corresponding to three points from calibrating curve (concentrations: 0.5, 0.8 and 1.1 g/L). The values for ratio of recovery were between 96.86% and 103.14%. The statistical data were: average= 103.195%, SD= 2.154% and RSD =2.145%.

Conclusions

The method of determination by GC-HS was validated according to the data from scientific papers and ICH guidelines [7, 11].

The obtained results showed that for the concentration range studied, the method is linear, accurate and precise ($RDS \leq 3.5\%$). This method is fast (a sample is analysed in 6 minutes) and blood samples do not require further processing, only a dilution with internal standard.

The accuracy of results is directly proportional to the homogeneity of the matrix, yielding values for these samples being 1.5% RDS.

Since this method of ethanol determination by GC-HS may be used for determining ethanol in biological fluids of deceased, tert-butanol was used as internal standard, because this compound is not synthesised in the putrefaction process of tissue or biological fluids [5]. The method can be applied in laboratories and institutes of forensic services, which thus should match international standards. It is extremely important that they can use a

working technique which can be accepted at European level, to make the test results competitive in any European court of law.

References

1. Dubowschi KM. Alcohol Determination in the Clinical Laboratory. *American Journal of Clinical Pathology*. 1980; 74: 747-750.
2. Jones A.W., Holmgren P. Uncertainty in estimating blood ethanol concentrations by analysis of vitreous humor. *J. Clin. Pathol* 2001; 54: 699-702.
3. Jones AW, Fransson M. Blood Analysis by Headspace Gas Chromatography; Does a Deficient Sample Volume Distort Ethanol Concentration? *Medicine Science and the Law*. 2003; 43: 241-247.
4. King B., Lawn R. International interlaboratory study of forensic ethanol standards. *The Analyst* 1999; 124: 1123-1130.
5. Kugelberg F.C., Jones A.W. Interpreting results of ethanol analysis in *postmortem* specimens: a review of the literature. *Forensic Science International* 2007; 165: 10-29.
6. O'Neal C.L., Wolf C.E., Levine B., Kunsman G. Gas chromatographic procedures for determination of ethanol in *postmortem* blood using t-butanol and methyl ethyl ketone as internal standards. *Forensic Science International* 1996; 83: 31-38.
7. Roman L., Bojiță M., Săndulescu R., Muntean Daniela Lucia. Validarea metodelor analitice. Editura Medicală, București; 2007.
8. Solansky AA. Effect of Different Concentration of Sodium Fluoride on Blood Alcohol Determination by Headspace Gas Chromatography Using the Internal Standard Method. *Journal of Analytical Toxicology*. 1994; Jan-Feb; 18:63.
9. Strassnig S., Lankmayr E. P. Elimination of matrix effects for static headspace analysis of ethanol. *Journal of Chromatography A* 1999; 849: 629-636.
10. * * * Comprehensive Agilent J&W Capillary GC Columns and Method Guide.
11. * * * Analytical Methods: A Statistical Perspective on the ICH Q2A and Q2B Guidelines for Validation of Analytical Methods.

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