Rom J Leg Med [30] 53-59 [2022]
DOI: 10.4323/rjlm.2022.53

DEVELOPMENT AND VALIDATION OF A QUANTITATIVE HS-GC/FID METHOD FOR ALCOHOL ANALYSIS IN BIOLOGICAL SAMPLES: STABILITY ASSESMENT

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Abstract: Determination of blood alcohol concentration is one of the most common analyses in forensic toxicology laboratory practice. With reference to this, the aim of the present work was to develop a method for quantitative analysis of ethyl alcohol in blood by head space gas chromatography, with n-propanol as an internal standard and at the same time ensuring good resolution of ethyl alcohol and acetone using one capillary column. Validation parameters comprising selectivity, specificity, linearity, accuracy and precision have indicated that the method could be applied in routine analysis of ethyl alcohol in post-mortem and ante-mortem blood samples as well as in other biological fluids, in line with the set criteria based on the revised international guidelines. In post-mortem samples there are many interfering substances due to various reasons (as a result of acidosis, produced in vitro on account of an improper storage of the samples or during the autolysis / putrefaction phase of the body). Because of this occurrence, within the validation procedure, more attention was imparted to the stability of the samples, including processed sample, freeze-thaw and long-term stability estimation. The results of the additional stability tests have shown that applying of preservative significantly improved the stability of the samples by inhibiting the in vitro ethyl alcohol production.

Keywords: ethyl alcohol, blood, validation, stability.

INTRODUCTION

Widespread alcohol use and abuse makes the determination of blood alcohol concentration one of the most important analyses in clinical and forensic toxicology [1, 2]. This fact points out the need of accurate and rapid quantitative analyses. Commonly used enzymatic methods have a number of weaknesses whereupon are almost completely replaced by headspace gas chromatography with flame ionization detector (HS-GC/FID) that is "golden standard" for this type of analyses [3-5]. The critical step in a headspace GC method development for analysis of alcohol is to obtain good specificity. With respect to analytical separation, the main problem occurs due to *in vitro* production of ethyl alcohol and other volatiles as a result of glucose metabolism by microorganisms present in the body/corps [6-8]. For example, acetone is often present in post-mortem samples as a consequence of various reasons (as a result of acidosis, produced *in vitro* due to improper storage of the samples or during the autolysis / putrefaction phase of the body). Hence, within the validation procedure of a HS-GC/FID method developed for alcohol blood analysis, sufficient attention should be paid to the parameter stability of the samples. Although very important, the available data related to the stability of the samples as an integral part of the validation process of published methods are incomplete [4, 9, 10]. As a result, this study was aimed to develop and validate a simple and reliable HS-GC/FID method for quantitative determination of alcohol in blood, taking into account the stability of the samples.

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METHOD DEVELOPMENT AND VALIDATION

Materials

Blood samples were obtained from real cases (Institute of Forensic Medicine, Criminology and Medical Deontology, RN Macedonia) previously confirmed negative for ethyl alcohol presence, and human pool blood samples (Institute for Transfusion Medicine, RN Macedonia). All samples were stored at + 4 °C, in tubes with NaF/oxalate. For standard solutions and quality control samples (QCs) were used ethanol absolute, methanol ≥ 99.9%, 2-propanol 99.9%, ethyl acetate 99.5%, dichloromethane 99.9%, acetonitrile 99.8%, n-hexane ≥ 99% (Merck, Darmstadt, Germany), acetone ≥ 99.5% (Sigma Aldrich, St. Louis, USA) and n-propanol (Park Scientific, Northampton, UK). Aqueous analytical standard of 4 mg/dL (LGC Standards, Germany) was used for preparation of calibrators. Samples were analyzed by Gas Chromatograph with flame ionization detector – GC/FID, GC 2010 Plus (Shimadzu, Japan). The chromatographic separation was performed using a capillary column with dimensions 30 m x 0.53 mm x 3 μm (InertCap 624, GL Science, Japan).

Samples and preparation of standard solutions

Samples were prepared by mixing of 200 μL internal standard (IS) and 300 μL of the sample (calibrator, QC or real sample) in 20 mL glass vials with magnet screw cap.

To plot the calibration curve, aqueous standard solutions in following concentrations: 0.1, 0.2, 0.5, 1, 2, 3 and 4 mg/dL were used. As an IS n-propanol at a concentration of 0.5 mg/dL was used. Control samples of whole blood spiked with ethyl alcohol standard solution were prepared in three concentration levels (low – QC1, medium – QC2 and high – QC3). Applying the same procedure, adequate samples for analyses of both selectivity and specificity (spiked blood samples with mixed standard solution containing methanol, acetone, acetonitrile, 2-propanol, ethyl acetate, dichloromethane and n-hexane) were prepared.

HS-GC/FID method

Quantitative blood alcohol analysis includes pretreatment of the sample, chromatographic separation and identification and quantification of the analyte. Pretreatment of the samples consisted of incubation at 90°C (thermo shaker) and conversion to a gas phase. After incubation, 1 mL of the gas sample is injected into the instrument where substances are separated by medium polar capillary column bonded 6% cyanopropylphenyl and 94% dimethylpolysiloxane under the pressure of helium and controlled temperature. Injector, GC and FID parameters are shown in Table 1. An IS method and weighting factor inverse square of concentration "1/x2" were used for plotting of the calibration curve.

Method validation

Validation was performed according to the previously adopted validation plan based on the revised international guidelines [11-13] considering selectivity, specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, carry over, accuracy (inter- and intra-day) precision (inter- and intra-day), dilution integrity and samples stability.

Selectivity: six blind samples of different origin (one human blood pool sample, two post-mortem samples, two ante-mortem samples taken one month before and one ante-mortem sample taken immediately before the analyses) were prepared. Acceptance criteria include an absence of the analytical response at the retention time (RT) of ethyl alcohol and IS or analytical response to be lower than 10% of IS and LOD for ethyl alcohol.

Specificity: the same set of samples as for the selectivity test were prepared, spiked with mix standard solution of eventually interfering substances (methanol, 2-propanol, ethyl acetate, acetone, dichloromethane,

Table 1. Instrument and operating parameters of the GC/FID-HS method

Autosampler	Injector	Column	Detector
Incubation temperature (°C): 90	Temperature: 250 °C	Temperature: 40.0 °C	Temperature: 280.0 °C
Incubation Time (m:ss): 4:00	Injection Mode: Split	Equilibration Time: 0.5 min	Sampling rate: 40 msec
Agitator speed (rpm): 600	Carrier Gas: Helium		Delay Time: 0.00 min
Fill Speed (μL/s): 250	Flow Control Mode: Pressure		Subtract Detector: None
Pullup Delay (ms): 1000	Pressure: 21.6 kPa		Flow Program: Makeup
Injection Speed (μL/s): 500	Total Flow: 89.9 mL/min		Makeup Gas flow: 40 mL/min
Flush Time (m:ss): 0:45	Column Flow: 4.14 mL/min		H2 Fow: 40.0 mL/min
	Linear Velocity: 30.3 cm/sec		Air Flow: 400.0 mL/min
	Purge Flow: 3.0 mL/min		
	Split Ratio: 20.0		

acetonitrile and n-hexane) at a concentration of 5 mg/dL. The acceptance criteria were identical as for the selectivity.

LOD: for LOD determination blood samples spiked with ethyl alcohol standard solution at following concentrations: 0.2, 0.1, 0.05, 0.02 and 0.01 mg/dL were prepared. Six repetitions of each concentration were analyzed (two post-mortem, two ante-mortem and two human blood pool samples). Acceptance criterion was set as the lowest concentration that provides repetitive analytical response in ratio 3:1, compared to the background noise of the blind samples.

LOQ: for LOQ determination, the same set of samples as described in the previous subsection (LOD) was used. Acceptance criterion was set as the lowest concentration that ensures repetitive analytical response in ratio 10:1, compared to the background noise of the blind samples.

Linearity: to test the linearity, standard solutions at seven concentration levels were prepared and analyzed in duplicate, together with blind and negative probe. Analyses were repeated in five consecutive days. Acceptance criteria were set as correlation factor (R2) > 0.99 and the results of all standard samples to be within ± 10% of the theoretical value (back-calculation).

Carry over: this test was performed by analyzing negative sample immediately after sample with high concentration (10 mg/dL ethyl alcohol), in three repetitions. The acceptance criteria include an absence of the analytical response at the retention time (RT) of ethyl alcohol and IS or analytical response to be lower than 10% of IS and LOD for ethyl alcohol.

Accuracy (inter- and intra-day): to test the accuracy, six QCs in three concentration levels were prepared. Concentrations of the QCs were: 0.5 mg/dL for QC1, 1.2 mg/dL for QC2 and 3 mg/dL for QC3. The set of six samples of each control level was analyzed in the same batch with calibrators, and a calibration curve was plotted. This procedure was repeated in five consecutive days. The acceptance criterion was set as the standard error (Er) for each QC to be within 10% of its theoretical value.

Precision (inter- and intra-day): this test was conducted using the same samples as for the accuracy test. The highest value for RSD % (for each level separately) in the same batch was used for intra-day (within-run) precision determination. The highest value for RSD % (for each level separately) in all five batches was used to determine intra-day (between-run) precision. Acceptance criterion was set as a maximum of 10% RSD for each concentration level. Additionally, precision of the measurement can be shown via RSD % value of the slope of consecutive five days calibration curves, and the RSD should not exceed 5%.

Dilution integrity: whole blood sample spiked with standard solution of ethyl alcohol to a final concentration of 8 mg/dL (above the upper limit of quantification, ULOQ), and diluted in a ratio 1:3 with blank whole blood was prepared. At least five repetitions of diluted samples were analyzed with an acceptance criterion set ± 10% of Er and RSD.

Stability: (1) On-tray (processed) sample stability: twelve QC1 and QC3 samples were prepared. Six samples of both control levels were analyzed at the beginning of the batch, and the rest set of six samples were analyzed after 10 hours. (2) Freeze thaw stability: six samples of QC1 and QC3 were prepared and then treated in three freeze thaw cycles. Each cycle has involved at least 20 hours freezing and at least one hour thawing. After three complete cycles, samples were reanalyzed and the results were compared with the results obtained by the analysis of freshly prepared QC samples. (3) Long-term stability: six QC1 and QC3 samples were prepared, and were analyzed one month later. Until analysis, the samples were kept at + 4 °C and – 20 °C. For all stability tests, the difference in concentration between the treated samples and the freshly prepared ones to not exceed 10% can be regarded as an acceptance criterion.

RESULTS

The results of the validation procedure carried out pointed to an efficient use of the described method for analysis of ethyl alcohol in blood. Selectivity and specificity of the method were confirmed by the absence of an analytical response in the blank probe (matrix effect) and in the samples spiked with mixture of methanol, acetone, acetonitrile, 2-propanol, ethyl acetate, dichloromethane and n-hexane (potentially interfering substances) at the RT of ethyl alcohol (4.05 min) and IS (7.75 min). The lowest ethyl alcohol

Table 2. Results of LOD and LOQ tests

Concentration (mg/dL)	0.01	0.02	0.05	0.1	0.2
Nº of samples (n)	6	6	6	6	6
Analitical response ratio	4.97	10.44	27.12	60.42	124.67
RSD (%)	22.28	15.86	4.73	3.47	4.75

concentration that meets the acceptance criteria set for LOD was 0.02 mg/dL. Although this concentration fulfills the LOQ requirements, concentration of 0.1 mg/dL was selected to be used as LOQ (Table 2).

The developed method was linear in a range of 0.1-4 mg/dL, with correlation factor of 0.999 (Table 3). The linearity of the method was additionally confirmed using the mode of "back calculation" of each standard solution (calibrator) level. Since the highest Er was 5.02%, one can conclude that this result meets the acceptance criterion of ± 10% variation compared to the theoretical value.

The data of analyzing QCs have confirmed accuracy and precision of the method i.e., all results for intra- and inter-day accuracy were within the criteria set for these parameters (Table 4). Precision was also certified by the RSD% of the slope value (Table 3). A dilution in a ratio 1:3 has been found to provide accurate and precise results (the highest individual Er – 6.67%, RSD – 0.92%). Stability test has shown that samples stored at appropriate conditions (tubes containing NaF/oxalate) are stable in a period of one month at + 4 °C and – 20 °C, with better stability noticed at – 20 °C (Table 5). The results for on-tray and freeze thaw stability have been observed to be within defined ranges of the acceptance criteria.

As given above, the described method meets criteria for accuracy, selectivity, specificity and precision

Table 3. Results of the linearity test

Nº of calibration	**Slope (a)**	**Intercept (b)**	**(R^2)**
1	0.96144	2.56E-02	0.999
2	0.94955	3.16E-02	0.999
3	0.953197	-2.36E-02	1.000
4	0.978726	-2.52E-02	0.999
5	0.961703	-2.97E-02	0.999
av (average)	0.961	-0.004	0.999
SD (standard deviation)	0.011	0.030	
RSD (%)	1.17		

Table 4. Results of both accuracy and precision tests

Day of analyses	**[1]QC**	**[2]n**	**[3]av[3]**	**[4]Er (%)**	**[5]RSD (%)**
	QC1	6	0.4863	2.73	1.24
1 (intra-day)	QC2	6	1.209	1.05	0.98
	QC3	6	2.99	1.41	1.55
	QC1	6	0.487	2.69	2.18
2 (intra-day)	QC2	6	1.154	3.83	2.34
	QC3	6	3.016	0.99	1.16
	QC1	6	0.495	1.78	2.33
3 (intra-day)	QC2	6	1.181	1.55	1.49
	QC3	6	3.071	2.37	0.85
	QC1	6	0.5008	1.03	1.47
4 (intra-day)	QC2	6	1.205	1.68	1.97
	QC3	6	3.111	3.71	1.78
	QC1	6	0.4865	2.71	1.49
5 (intra-day)	QC2	6	1.194	1.37	1.83
	QC3	6	3.023	1.24	1.63
	QC1	30	0.491	2.19	2.07
1-5 (inter-day)	QC2	30	1.189	1.93	2.36
	QC3	30	3.044	1.94	1.92

1QC – control sample; 2n – number of samples; 3av – average value; 4Er – standard error; 5RSD – relative standard deviation.

Table 5. Results of on-tray and long-term stability tests

	10 h at room temperature		**1 month at + 4 °C**		**1 month at - 20 °C**	
	[5]QC1	[5]QC3	[5]QC1	[5]QC3	[5]QC1	[5]QC3
[1]n	6	6	6	6	6	6
[2]av	0.4741	3.040	0.5070	3.0799	0.4949	3.0316
[3]Er (%)	3.57	1.98	2.65	2.09	0.62	0.85
[4]RSD (%)	1.40	1.90	1.97	2.21	0.84	1.22

1n – number of samples; 2av – average value; 3Er – standard error; 4RSD – realtive standard deviation; 5QC – control sample.

and is applicable for determination of the ethyl alcohol concentration in ante-mortem and post-mortem blood samples, as well as for analysis of all other volatiles analyzed during the specificity test. This method was applied for quantitative determination of alcohol in other body fluids too, including humor vitreous, urine and gastric content (with or without dilution, depending of the ethyl alcohol concentration). Based on the results have been obtained in samples other than blood (data not shown and in a compliance with the previously described quality assurance criteria), it may be assumed an effective use of the developed method for quantification of alcohol in various biological samples.

Method application

In addition, a study of the preservative influence on the sample's stability was conducted using a described method. In this study randomly selected post-mortem blood samples (collected from 34 consecutive autopsies) were included. The samples were analyzed immediately after autopsy (Group 1), then each sample was divided into sterile tube stoppered air tight with a screw cup without any preservative (Group 2) and sterile tube containing NaF/ oxalate (Group 3). Samples of group 2 and 3 were stored at + 4 °C and blood alcohol concentrations were re-analyzed after three months. Summary results of the three tested groups of samples are shown in Table 6. The second analysis of the samples (Group 2 and 3 both) indicated variable concentration of ethyl alcohol. Significant differences between the mean values of the ethyl alcohol concentration determined in samples of groups 1 and 2 were confirmed (Wilcoxon match pair test: $Z = 2.139$, $p = 0.0324$). However, no statistically significant difference between the mean values of the ethyl alcohol concentration in samples of groups 1 and 3 was observed (Wilcoxon match pair test: $Z = 1.408$, $p = 0.1589$). The stability study apparently showed that samples with added preservative remained stable in a period of three months at + 4 °C. Samples stored at the same conditions, but without preservative have been shown *in vitro* production of ethyl alcohol in concentrations up to 0.31 mg/dL.

DISCUSSION

The developed method combines the simplicity and accuracy of an HS-GC/FID together providing effectiveness and efficiency for routine blood alcohol analysis. An additional advantage of the HS-GC/FID method is the high specificity i.e., the ability to separate all interfering substances, especially acetone. An accurate determination of ethyl alcohol blood concentration in post-mortem samples from the autopsy is an important analysis in forensic toxicology laboratories, in order to determine the ethyl alcohol intoxication or the stage of alcoholism at the time of death. Often, there is a need for analyzing ante-mortem samples (taken or delivered to the laboratory) for various reasons, work place testing, traffic control, etc.

The results of all analyzed (ante- and post-mortem) samples using the developed method were further subjected to a legal procedure. It is necessary appropriate protocols to be followed, which will ensure accurate, unambiguous and reliable results (evidence). A serious problem toxicology forensic laboratories are facing with is a lack of information about eventual presence of a preservative in the samples which are delivered to the laboratory. Therefore, *in vitro* ethyl alcohol production was quantified to examine the effect of the preservation of samples on their stability.

There is a lot of different blood alcohol determination methods described in the literature, depending on the laboratory needs or resources. Numerous methods use one column for chromatographic separation [4, 9, 14-16] that is specially designed for alcohols (BAC1 and/or BAC2), but also there are described methods that use two columns for analytes separation [10, 17]. Combination of two columns is used to enhance the acetone and ethyl alcohol separation. This method using one column with semi polar stationary phase, has an advantage for efficient separation of the two volatiles (acetone and ethyl alcohol) with a resolution factor higher than 2. As an IS, n-propanol [4, 16, 18] and t-butanol [5] are usually used; however 2-propanol has been also

Table 6. Distribution of examined samples according to presence of ethyl alcohol

Samples	Ethanol not detected	Ethanol detected	Total
First analysis (group 1)	19 55.88%	15 44.12%	34
Second analysis after 3 months storage without NaF (group 2)	9 26.47%	25 73.53%	34
Second analysis after 3 months storage with NaF (group 3)	19 55.88%	15 44.12%	34
Total	47	55	102

reported [17]. In our study as an IS n-propanol was chosen at a concentration of 0.5 mg/dL. Validation parameters and acceptance criteria are also choice of the laboratory, depending of the adopted guidelines. The range of the method (for which linearity should be confirmed) mainly depends on the laboratory needs. Range of calibration should include toxic and lethal concentrations of ethyl alcohol (4 mg/dL), necessary for forensic toxicology laboratory [4, 9], although there are many methods with ULOQ of 2.5 mg/dL [9, 15, 16]. General recommendations for the error of the control samples are ± 20% (± 30% for concentrations near the LOD) [19]. However, stricter rules are suggested for blood alcohol analyses i.e., an error within ± 10% is considered acceptable [13]. This criterion was set in a few more recent published methods [4, 9, 15, 16, 18]. Stability test is rarely performed as an integral part of validation of methods for alcohol determination. In the literature, three tests for 24 hours stability [10], freeze thaw stability [9] and processed sample stability [4] have been reported. As mentioned before, the stability is a critical parameter when developing such a method because of forensic-toxicology specific nature, especially when samples from other institutions are delivered to the laboratory. For this purpose, a long-term stability test was performed. This test will provide a high level of certainty that the sample is stable and the analyst can accept the result of the analysis of ethyl alcohol in a period of one month after sampling, if stored at a temperature of + 4 °C or – 20 °C. In addition, the results of studying the influence of NaF application on the stability of samples and ethyl alcohol itself are in line with the literature generally recommending sample's preservation [6, 20] and may have a significant impact on the creation of protocols in forensic practice in our country. Apart these, in a case of autopsy samples, it is recommended to be analyzed other, alternative samples, for determination of alcohol concentration (as well as other volatiles produced by putrefaction), in order to determine the ethyl alcohol origin in the body. Best choice for alternative sample is humor vitreous, because it is protected from microorganisms and is more stable sample than the blood [21, 22] and the described method was found to be eligible for quantification of ethyl alcohol in various biological samples.

In conclusion, the method for determination of alcohol in blood samples using HS-GC/FID was validated according to the latest internationally established guidelines comprising selectivity, specificity, linearity, accuracy and precision. According to the results, the developed method using only one capillary column is simple, rapid and reliable and hence may be easily applicable in routine analysis of ethyl alcohol concentration in ante- and post-mortem blood samples. Besides, compared with other published methods, this method offered solid resolution of ethyl alcohol and acetone using one column. In addition, it can be applied to other biological fluids in a range of 0.1 to 4 mg/dL, as well as to unknown liquids (evidence materials) with or without dilution. More important is that the extensive stability tests within the method validation have confirmed the stability of ethyl alcohol concentration after 10 hours of sample processing, after one month storage under appropriate conditions as well as after three freeze-thaw cycles. This finding ensured the analyst in the validity of the obtained results comprising this way treated samples, concomitantly legitimating their use in legal procedures. The method was also used to study the influence of NaF application on the sample stability. Despite the low concentration of an *in vitro* produced ethyl alcohol which was not higher than 0.31 mg/dL in the samples without preservative, the use of the preservative is recommended. This conclusion encompassing the low level of *in vitro* produced ethyl alcohol in preservative free samples is insignificant in cases with ethyl alcohol intoxication, but can markedly affect all other cases, especially where there is a limit for the concentration of ethyl alcohol (for example 0.5 mg/dL in the blood of drivers or 0.0 mg/dL in the blood of professional drivers and other regulated professions).

Conflict of interest

The authors declare that they have no conflict of interest.

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