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Research Article

Method Validation and Measurement Uncertainty for the Determination of Ethanol in Whole Blood

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Abstract:

Alcohol intake is known to significantly affect driving ability and there is a positive correlation between car accidents and Blood Alcohol Concentration (BAC). Alcohol intake is known to significantly affect driving ability. Therefore, many countries define and monitor the legal BAC value for drivers. Customers or legal authorities require determining and reporting the measurement uncertainty in blood alcohol analysis from laboratories in recent years. To establish the reliability and robustness of the result, the method was validated and the measurement uncertainty was calculated. A rapid, selective and quantitative gas chromatography coupled with flame ionisation detection method was developed and validated for determination of ethanol in whole blood. The method was validated for selectivity, matrix effect, recovery, linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, repeatability, reproducibility and robustness. The validation procedure was designed to be suitable for ISO/IEC 17025 accreditation. Uncertainty measurements were also determined for the validated method. LOD and LOQ were found 3.99 mg/dL and 4.30 mg/dL, respectively. The method showed good linearity in the range of 3.9 to 393.7 mg/dL ethanol with a correlation coefficient (r^2 = 0.9999). The method provides fast, precise, simple, robust and unbiased results.

1. Introduction

Ethanol is one of the best known psychoactive depressant drugs. It is consumed in beverages and food and is also one of the most abused psychoactive substances. Excessive consumption of alcoholic substances and drunkenness plays a major role in many fatal accidents, violent crimes, suicide, drowning and, traumatic deaths as proved by police reports, accident and emergency records [1,2]. According to the National Highway Traffic Safety Administration, 31% of traffic fatalities in the United States of America (USA) in 2021 were caused by alcohol-impaired driving [3]. Between 2009 and 2014, 24.7% of pedestrians at a hospital trauma centre were injured in road traffic crashes in Melbourne, Australia [4]. Alcohol analyses are traditionally performed in routine autopsy analyses. In addition, alcohol analyses are requested in every case to determine whether driving ability is impaired in traffic accidents, and analyses of narcotic stimulants are also requested for some cases. The limit value at which alcohol has an effect on driving is reported at 0.4 g/kg [5].As most countries have BAC limits for driving that are punishable by law, such as 50 mg/dL in most European countries, 0.50 mg/kg or, and 80 mg/dL in the United Kingdom (UK), USA and Canada [6,7]. The legal limit in Turkey is 50 mg/dL for car users and 0 mg/dL for commercial vehicle users by Road Traffic Law and Road Traffic Regulations [8]. Various analytical techniques have been used to determine ethanol in biological or non-biological samples. Gas chromatography (GC) [9, 10], high performance liquid chromatography (HPLC) [11,12] and infrared spectroscopy (IR) [13] have been commonly used. GC is generally the most accurate and reliable analytical method and is often the preferred method for the quantification of ethanol in human blood, vitreous humour and other biological samples in forensic toxicology [14]. Sample preparation is the most important step for reliable analysis of complex matrices such as blood, tissue or urine. Various preparation techniques are available for the determination of ethanol in biological samples, such as direct injection, static, dynamic headspace [10, 15] or headspace injection using solid phase microextraction (HS-SPME) [16, 17] and HS-GC-

FID [18]. The rapid and accurate determination of ethanol in human specimens is of great importance to analytical or forensic laboratories, and the development of novel methods and the evaluation and validation of the developed methods are required [16]. HS-GC-FID has become the most widely used technique in recent years for the analysis of volatile analytes in biological and non-biological samples due to its ability to detect low levels of analytes without the need for complex and expensive sample preparation techniques. In addition, the technique is often used in laboratories with highly routine laboratory work [17]. Headspace (HS) for alcohol analysis offers advantages such as simple sample preparation, low risk of contamination, selectivity and short analysis time compared to other sample preparation techniques [16, 18]. Therefore, this study aims to develop and validate an analytical method for the determination of ethanol in blood samples with HS-GC-FID.

2. Material and Methods

2.1 Chemicals and reagents

All chemicals and solvents were of LC-MS grade and were purchased from Merck (Darmstadt, Germany). Deionised water was provided by a Millipore® Milli-Q gradient system.

2.2. HS-GC-FID

The gas chromatography system consisted of a Perkin Elmer (Shelton, USA) Clarus 500 GC and HS module coupled to a flame ionisation detector and two columns A (Elite BAC 1(30 m×0.32 mm ID×0.6 μm)) and B (Elite BAC 2(30 m×0.32 mm ID×0.8 μm)). The transfer line , needle and oven temperature were 110˚C, 75˚C and 70˚C respectively. The injection time was 0.02 min, the hold time was 0.2 min and the cycle time was 8.5 min. The sampling rate was 12.5 points per second and the total GC run time was 10 min. The column temperature was 220 ˚C and the gas flow for each detector was 450 mL/min air and 45.0 mL/min hydrogen.

2.3 Sample Preparation

Blank human blood samples were obtained from a regional blood donation centre and were used for the validation of the method. All working solutions were prepared daily from pure solvent. The propan-1-ol internal standard (IS) solution was prepared in water (0.01 M) and stored at +4°C. For sample preparation, 200 μL of sample and 800 μL of IS were placed in a clean glass headspace vial and capped. The vials were transferred to the HS autosampler.

2.4 Method Validation

The validation of this method was performed with the parameters LOD and LOQ, linearity, intra- and inter-day precision, recovery, selectivity, matrix effect and robustness. The method was validated according to the rules of ISO/IEC 17025:2017 in accordance with international guidelines, which are common practice in clinical and forensic toxicology [19-22]. For detection and calculation of the limit of detection, the concentration in the blood sample was prepared as 3.98 mg/dL and 10 independent analyses were performed. At this concentration it was ensured that the signal to noise ratio was S/N≥3. The mean and standard deviation were calculated for ethanol. The LOD was calculated by adding 3 standard deviations to the mean and the LOQ was calculated by adding 10 standard deviations to the mean. Precision values <20% were accepted. The linear working range study was analysed separately with blood and water to investigate the matrix effect. The data obtained were evaluated by t-test and it was found that there was no significant difference between the two matrices. Therefore, calibration curves were generated using water. The linearity was assessed in the range of 3.9 to 393.7 mg/dL. The repeatability study was performed by 2 different analysts at 3 different concentrations (low: 14.0 mg/dL, medium: 72.0 mg/dL and high: 144.0 mg/dL) on the same day. For the ethanol reproducibility study, three different concentrations (low: 14.0 mg/dL, medium: 72.0 mg/dL and high: 144.0 mg/dL) were performed on six different days by two different operators. In the selectivity study, ethanol, methanol, acetone, n-butanol, propan-1-ol, propan-2-ol, formaldehyde and toluene standards at low and high concentrations of 10.0 and 400.0 mg/dL were prepared as single and mixed standards in water and blood matrix. After the retention time (RT) of each component was determined, these components were re-analysed in the mix and the effectiveness of separation was examined. It was observed that each component showed different RT in the mix. Recovery studies were performed at three different concentrations of 144.0, 72.0 and 14.0 mg/dL by two different analysts. In the robustness studies, the effects of needle temperature (75˚C, 80˚C, 85˚C), analysis time (10 min, 15 min, 20 min) and oven temperature (70˚C, 75˚C, 80˚C) on the analysis were investigated.

2.5 Uncertainty measurement

The determination of the measurement uncertainty in validated methods is very important in order to compare the results obtained by two different methods, to ensure the reliability of the results and

to reduce the controversy of the results at legal limits. In this study, the combined standard uncertainty results obtained from three different concentrations (14.0 mg/dL, 72 mg/dL and 144.0 mg/dL) were analysed according to the bottom-up approach. Calculations were performed from validation data for the HS-GC-FID method. The budget was determined in accordance with the EURACHEM uncertainty calculation guide according to the rules of ISO/IEC 17025:2017 [22, 23]. The contribution of components such as recovery, calibration curve, accuracy, repeatability and reproducibility were taken into account when determining the contribution of the distribution.

3. Results and Discussions

The LOD for the validated method was 3.99 mg/dL and the LOQ was 4.3 mg/dL. As the legal limit for driving in Turkey is 50 mg/dL, the method was found to be sufficient for the detection and quantification of alcohol. Figure 1 shows a chromatogram of ethyl alcohol at a concentration of 4.0 mg/dL and IS.

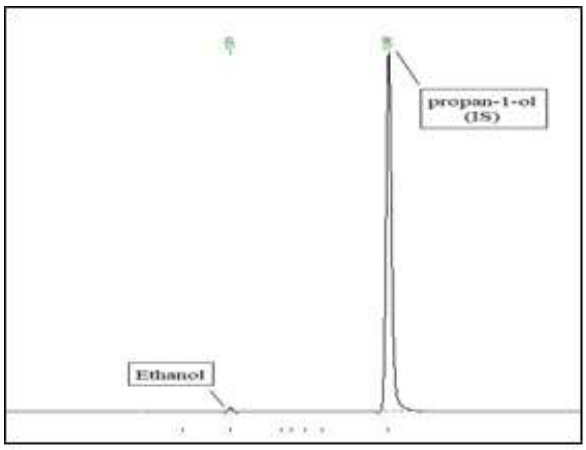

Figure 1. *The chromatogram of ethanol (RT:1.01min.) at a concentration of 4.0 mg/dL and IS (RT:1.68 min)*

Good linearity has been assessed in the range of 3.9 to 393.7 mg/dL with with a correlation coefficient (r^2=0.9999). The bias of the method was calculated from the samples used for the recovery study. For the three different concentrations analysed (low: 14.0 mg/dL, medium: 72.0 mg/dL and high: 144.0 mg/dL), the bias values were 1.8%, 1.3% and 1.7% for the first analyst and 1.6%, 1.4% and 1.3% for the second analyst. The SWGTOX guideline acceptable bias value is reported as ±10 and all bias results were within this range [20]. The recoveries for the three different concentrations analysed (low: 14.0 mg/dL, medium: 72.0 mg/dL and high: 144.0 mg/dL) were between 98.0% and 101.8%. The recovery values were found to be within acceptable limits. For the three different concentrations analysed (low: 14.0 mg/dL, medium: 72.0 mg/dL and high: 144.0 mg/dL), the repeatability RSD% values were 0.5%, 1.0% and 0.6% for the first analyst and 1.8%, 0.5% and 0.5% for the second analyst. For the three different concentrations analysed (low: 14.0 mg/dL, medium: 72.0 mg/dL and high: 144.0 mg/dL), the reproducibility RSD% values were 1.1%, 1.1% and 0.4 % for the first analyst and 0.7%, 0.7% and 0.3% for the second analyst. The acceptance criteria for % RSD value should be ≤15% [19, 24]. All calculated %RSD values for repeatability and reproducibility for two analysts were below the criteria. Table 1 presents results of different parameters in validation of method for ethanol determination.

Table 1. *Validation data of the developed method (recovery and precision values for two analyst(A1and A2))*

The Validation Parameters	A 1	A 2
Recovery (%) (Low)	98.2	98.0
Recovery (%) (Medium)	98.4	98.6
Recovery (%) (High)	101.5	101.8
Repeatability (% RSD) (Low)	0.5	1.8
Repeatability (%RSD) (Medium)	1.0	0.5
Repeatability (%RSD) (High)	0.5	0.5
Reproducibility (% RSD) (Low)	1.1	0.7
Reproducibility (%RSD) (Medium)	1.1	0.7
Reproducibility (%RSD) (High)	0.4	0.3

The selectivity study of the method was performed by injecting a sample matrix containing possible interferences and no significant interference was found. Figure 2 shows (a) negative control sample with IS. No interference was observed. Figure 2 also shows (b) the chromatographic separation of methanol, ethanol, acetone, methyl ethyl ketone, isobutanol, ethyl acetate, toluene, ethyl benzene, IS and xylene.

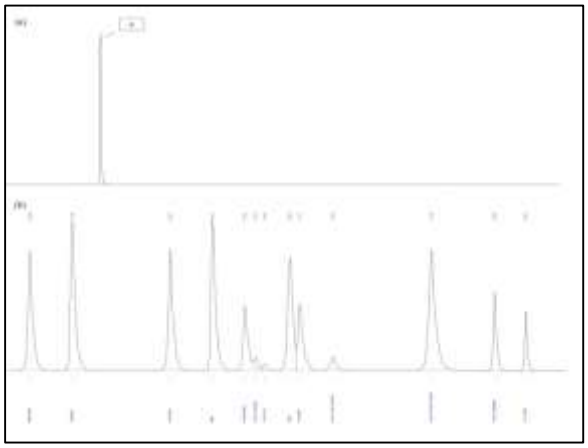

Figure 2. *(a)Negative blood sample with IS, (b) The chromatographic separations of different solvents.*

Robustness was analysed by varying the parameters needle temperature, thermostat time and oven temperature. Student's t-test showed that the changes had no significant effect on the measured value ($t_{cal} < t_{table}$). Intermediate precision was investigated by performing analyses (n=6) on the same instrument at three different concentrations (low: 14.0 mg/dL, medium: 72 mg/dL and high: 144 mg/dL) on the same day and on six different days by two different analysts. The RSD% values were then compared between the two analysts. The values were compared by F-test and Student's t-test and no significant difference was found ($t_{cal} < t_{table}$ and $F_{cal}| < F_{table}$). U_r (recovery), U_r (calibration curve), U_r (bias), U_r (repeatability) and U_r (reproducibility) values were calculated 0.005, 0.006, 0.009, 0.010 and 0,008, respectively. U_r (combined) value was calculated 0.019 (1.9%). U_r (combined) uncertainty value was found to be suitable with previous reports [25-29]. By using the Ur (expanded) value, 0.038 (3.8%) was calculated, which is based on the desired confidence level and, for an approximate confidence level of 95%, k (coverage factor) is equal to 2. By using the expanded uncertainty, it is possible to calculate the decision limits above which the blood alcohol concentration can be considered, with a certain probability, higher than the legal limits. It is the concentration above which the blood alcohol concentration can be considered, with a certain probability, to be higher than the legal limits.

4. Conclusions

The developed and validated method for blood alcohol analysis is suitable for the determination of alcohol concentration for clinical or legal purposes. The method developed using HS-GC-FID provides rapid, selective, reliable and robust results for the determination of ethanol. The validation results meet the acceptance criteria of guidelines such as EURACHEM, ICH, ISO/IEC 17025:2017 and SWGTOX. The detection limit and range of the method meet legal limits and the purpose of toxicological analysis. The measurement uncertainty calculated for the method is sufficient to eliminate controversy over the results, to obtain accurate results and to compare the results obtained. This method is suitable for all clinical and forensic laboratories producing results with ISO/IEC 17025:2017 accreditation.

Author Statements:

- **Ethical approval:** The conducted research is not related to either human or animal use.
- **Conflict of interest:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper
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