Original Article

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Method Validation and Quantitative Estimation of Ethanol using n-Propanol as Internal Standard in whole Blood by Gas Chromatography - Headspace (GC-HS)

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Abstract

Detection and quantification of ethanol in drunken driving cases is of immense importance inforensic toxicology. Despite several analytical methods being available for identification of ethanol in blood, an accurate quantification with minimum sample preparation and rapid analysis is still an ongoing task. The present study evaluates the suitability of Headspace-Chromatography with a capillary column and Flame Ionisation Detector as a method for determining the ethanol content in whole blood samples received for the blood alcohol concentration analysis. An internal standard 'n-propanol' was added to the sample to authenticate the results. The peaks areas were measured and calculations were carried out considering peak ratios of analyte to internal standard. The total run time of GC and HS was 20.68 min. The validation study for the method resulted in linearity for a range of 7.9 mg-237 mg/100 ml, coefficient of variance (R^2) was 0.999. Recovery of more than 96% was achieved in spiked samples. LOD and LOQ were 0.20 mg/100 ml and 1.0 mg/100 ml respectively. Blood ethanol measurement by this method is an easy, simple, reliable and reproducible.

Keywords: GC-HS; Alcohol; ICH Guidelines; FID; Whole Blood etc.

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Introduction

Chemically, ethanol (C_2H_5OH) or ethyl alcohol belongsto a group of chemical compounds known as alcohols. An alcoholic beverage is a drink which contains substantial amount of this psychoactive drug, ethanol (informality called alcohol) in low doses causes euphoria, reduced anxiety and sociability and in higher doses causes intoxication (drunkenness), stupor and unconsciousness [1].

When ethanol is ingested, usually in form of an alcoholic beverage, it is readily absorbed in the blood by the process of simple diffusion by stomach lining. According to Fick's law, the rate of diffusion across a membrane is proportional to the concentration gradient on either side of the membrane [2]. Therefore more the concentration of alcohol in stomach, more with be the absorbtion. This process can be hastened by many other factors like presence of kind of food in stomach, like oily and fatty food will delay the absorbtion whereas light and sugar rich would hasten the absorbtion.

The temperature of drink, quantity of water, aerated or nonaerated nature of drink will affect the rate. With its absorbtion, majority of the alcohol is broken down or metabolised in liver through the portal venous system. Its main metabolisation is done with the "Alcohol Dehydrogenase system" yielding carbon dioxide and water as end products and acetaldehyde and acetic acid as the intermediaries. Rest of the ethanol is excreted via the kidneys unchanged in urine. With time the ethanol in blood stream is continuously removed by metabolising in liver. A formula (Widmark's) has been created to predict the amount of ethanol and individual has consumed based on time and the measured BAC [3].

Due to its use and easy abuse, ethanol is significantly associated with violent acts, drunken driving, suicides etc [4]. Due to these acts of abuse there are stringent laws associated with their consumption and conduct. Therefore determination and quantification of ethanol is perhaps the most important routine analysis done in toxicology laboratories [5]. The number of samples received and their relatively short hold time, has nowadays resulted in labs requiring methods that are fast, accurate, reliable, and have less chances of human error.

Traditional ethanol analysis is done by methods like Widmark's method, enzymatic reactions, and cuvette test, although these procedures are fast, they lack the accuracy of quantitation. In contrast, Gas Chromatography is qualitative (retention time) as well as quantitative (peak area). Latest technologies of GC with manual injection and GC with mass determination even though yield excellent separation and quantification, are known to include small inaccuracies. These inaccuracies can be in form of presence of interfering compounds and other component in biological samples during traditional methods of sample preparation and volatile extraction. On the other hand use of static headspace analysis with GC offers limited to no sample preparation, less contamination risk, higher sensitivity, complete automated analysis. Static headspace works by concentrating the volatile prior to analysis and examining the concentration of these analytes directly from the vapour phase in the sealed vial with sample. The concentration of vapours is done by sealing the vial before heating [6–10]. For laboratories pursuinghighly reliable results of volatile analysis GC-HS is the preferred technique due to its simplicity and the high number of repeated analysis in an normal daily run. This technique offers diminished inlet and column maintenance, highest sample throughput, reduced interfering artefact or sample degradation, robust and trouble free design.

In this article an attempt has been made to analyse blood samples using Gas Chromatography instrument from cases brought to laboratory for the analysis of blood ethanol concentration in cases of drunken driving, and other cases where query if for qualitative and quantitative estimation of ethanol.

Experimental

Materials and Method

Instrument: Gas Chromatography system, Model No. 7890A and Headspace Sampler, Model No. 7697A from Agilent, U.S.A. were used.

Column: DB-624 length 30m, diameter 0.530 mm, film 3.00 µm, Temperature Limits from 20^{0}C to 260^{0}C was used

Software: ChemStation® for the data analysis of the signals was used.

Reagent / Chemicals: Ethanol (GC grade) and n-propanol (GC grade) from Merck, Germany and Ultra-pure water from Rions India were used.

Glassware: 20 ml Head space vial from Agilent Technologies U.S.A. were used.

Miscellaneous: Micropipette of volume 100-1000 µl and 20-200 µl from Corning, U.S.A., septa (PTFE) and aluminium crimpfor sealing the HS vial from Agilent, U.S.A. were used.

Preparation of Ethanol Standard Solution

The stock solution of concentration, 790 mg/100 ml was prepared from absolute ethanol, by dissolving 1 ml of standard ethanol with ultrapure water in 100 ml volumetric flask.

Five working dilutions of concentration 19.75 mg/100 ml; 39.5 mg/100 ml; 79 mg/100 ml; 158 mg/100 ml; 237 mg/100 ml were prepared from the stock solution by dissolving volumes of 250 µl; 500 µl; 1000 µl; 2000 µl; 3000 µl in 10 ml volumetric flask with ultrapure water.

Preparation of Internal Standard Solution

Three hundred (300) µl ofinternal standard (n-propanol) was dissolved in 100 ml of ultrapure water in a volumetric flask.

Preparation of Calibration standard

One (1) ml of each from 19.75 mg/100 ml; 39.5 mg/100 ml; 79 mg/100 ml; 158 mg/100 ml; 237 mg/100 ml standard were taken in five different HS vial and 90 µl of internal standard was added in each vial. Each glass vial were sealed with septa and metallic crimpusing crimper.

Preparation of Sample

One (1) ml of blood sample was taken in HS vial and 90 µl of internal standard was added to it. Vial was sealed with septa and metallic crimp using crimper.

Instrumentation conditions

GC conditions: GC cycle time was set at 20.00 min. A constant Nitrogen flow of 8 ml/min was used. The injection port temperature was maintained at 250^{0}C with a 5:1 split injection of the Headspace and a septum purge flow of 3 ml/min. The initial GC oven temperature of 50^{0}C was held for 5 min and then ramped at 35^{0}C/min to a final temperature of 200^{0}C held for 1 min. Total GC runtime was for 10.286 min per sample.

Headspace conditions: Headspace oven temperature was set at 70^{0}C. The HS Loop and Transfer Line Temperature were set at 80^{0}C and 90^{0}C resp. Vial equilibration was set at 10.00 min. Injection, loop fill and total cycle time were set at 0.50 min, Default, 16.00 min respectively.

Detector conditions: Flame Ionisation detector was used for the detection of analytes. The FID temperature was maintained at 250^{0}C with Hydrogen (40 ml/min), Zero Air (400 ml/min) and makeup flow of 25 ml/min. the FID signal was zeroed at 0.01 min with data collection rate of 20Hz.

Result and Discussion

Ethanol was detected in the spiked samples of blood and DDW. GC is the routinely utilised instrumentation for ethanol estimation in forensic laboratories. Before utilisation of the developed method for quantification of ethanol in biological fluid, the developed method was fully validated for specificity, linearity, accuracy, precision, repeatability, detection limit and quantification limit according to ICH guidelines [11].

Specificity

The method demonstrated excellent chromatographic specificity with no endogenous interference at the retention times of ethanol (1.624) and n-propanol (2.900). Specificity was confirmed by analysing a known standard and overlapping its graph with that of an unknown blood sample as shown in Figure 1.

Linearity

The five serial dilution ranging from 19.5% mg to 237% mg (v/v) were selected to plot the calibration curve. Linearity of the curve was calculated using concentration (x-axis) vs peak area (y-axis) as shown

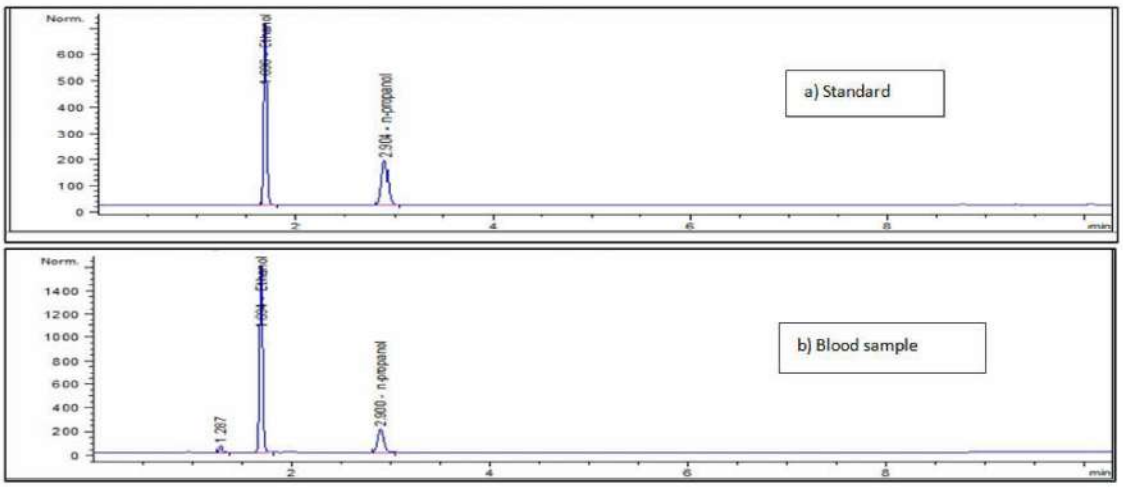

Fig. 1: GC-HS Chromatograms of a) standard sample of ethanol in DDW with n-propanol as Internal Standard, b) unknown sample of ethanol in blood with n-propanol as Internal Standard

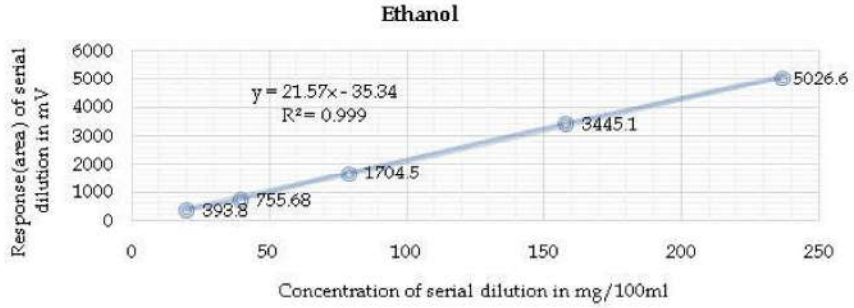

Fig. 2: Five point calibration curve of ethanol

Table 1: Inter and Intra- Day precision and accuracy of standards spiked in blood and water

Matrix	Solvent	Conc (mg/100 ml)	Intraday			Interday		
			Estm Conc. Mean ± SD (mg/100 ml)	Precision (RSD)	Accuracy	Estm Conc. Mean ± SD (mg/100 ml)	Precision (RSD)	Accuracy
Water	Ethanol	39.5 mg	39.6 ± 0.2	0.4	0.25	38.95 ± 0.70	1.81	1.39
		79.0 mg	80.15 ± 0.65	0.81	1.43	79.57 ± 0.67	0.85	0.71
		158.0 mg	159.55 ± 0.72	0.45	0.97	158.44 ± 1.21	0.77	0.27
Blood	Ethanol	39.5 mg	39.46 ± 0.11	0.29	0.10	39.22 ± 0.61	1.58	0.71
		79.0 mg	79.4 ± 0.1	0.13	0.50	79.75 ± 0.69	0.87	0.94
		158.0 mg	159.22 ± 0.16	0.10	0.76	158.85 ± 1.15	0.73	0.53

Table 2: Recovery of spiked concentration of standards in blood and water

Matrix	Concentration (mg/100 ml)	Range (mg/100 ml)	Mean	Recovery %
Water	7.9	7.9 - 8.0	7.95	100.63%
	19.75	19.50 - 19.90	19.7	99.74%
Blood	7.9	7.3 - 7.9	7.6	96.20%
	19.75	19.25 - 19.56	19.40	98.22%

in Figure 2. This resulted in correlation coefficient (R2) of 0.999. The concentration of an unknown sample was calculated using this calibration curve.

Accuracy and Precision

Intraday assays were performed using five replicates during a single day and interday assays on 3 different days. For 3 different concentrations 5 replicates were run to determine Accuracy and Precision. The results are shown in Table-1. Reproducibility of method in cases of blood sample were calculated by taking the same sample for every analysis and storage under optimal conditions to avoid degradation. Percentage accuracy is calculated as-

$$\% \text{ Accuracy} = \frac{(\text{Calculated Concentration of analyte} - \text{Actual Concentration}) \times 100}{\text{Calculated Concentration}}$$

Recovery and carry over effect

Recovery test were performed by spiking a known concentration (7.9 mg/19.75 mg) of standard to blood and water. A recovery rate of more than 96% was obtained as shown in Table 2. As evident from the data of table 2, recovery of ethanol increased with increased concentration of standard because at higher concentration the matrix effect reduces.

To study that no carryover of the previous sample remained in the head space sampler or the column after the analysis of a high concentration biological sample, a blank was run. No residual peak in the blank run confirms no carryover with this method.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

To determine the sensitivity of the method, the calibrator of the solvent with the lowest

concentration (7.9 mg/100 ml) was progressively diluted to determine the lowest limit of detection (LOD) and quantification (LOQ). The concentration to give signal to noise ratio of 3 was considered acceptable for estimating LOD. LOQ was estimated based on the signal to noise ratio of 10 obtained by diluting the standard to such extend that all compounds are detected with sharp, symmetrical chromatographic peaks. Limit of detection (LOD) was found to be 0.20 mg/100 ml and limit of quantification (LOQ) 1.0 mg/100 ml.

Discussion

Blood alcohol concentration of an case sample helps the investigator to know about the of ethanol at presence of ethanol at the time of offence. The developed method was used to analyse real life samples of blood alcohol. Quantitative method for the analysis of real samples of blood was validated for GC-HS-FID according to the ICH guidelines. To overcome the risks of any artefact during analysis an internal standard was used. Thus, the peaks areas were measured and calculations were carried out considering peak ratios of analyte to Internal Standard.The use of internal standard and flame ionisation detector in the above method for GC-HS sample testing proves effective for the proper detection of the components in the sample provided as well as in obtaining the near accurate quantity of the analyte in the sample. Using this method, desired calibration of ethanol was performed with 0.999, coefficient of variance. The peaks are obtained of the standard (GC grade ethanol) and the sample ethanol from blood with the internal standard, and is plotted against the retention time v/s the response from the FIDdetector.By evaluating the chromatograms, it is seen that the given blood sample contains ethanol as it shows similar retention (1.624 min) with that of the standard ethanol (1.624 min). Another small peak is obtained in the sample chromatogram having retention time of 1.287 min, which might be of any other analyte in the given sample or may be some impurities present in it. The quantity and the concentration of the analyte present in the sample was obtained by calculating the area of the peak.

Conclusion

The method developed is easy, economical, and rapid. It shows reasonable specificity with small sample volume. Analysis is not interfered by other volatiles in the environment. Technique is very useful in cases of driving under influence, drug-facilitated sexual assault, workplace drug monitoring, or where query is for the qualitative and quantitative estimation of ethanol in blood. Since the instrumentation is fully automated with sample preparation is done using headspace technique, manual error is significantly reduced. Therefore, this method can be routinely utilised for the said purpose of ethanol estimation in real life cases.

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