WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 7.421
Volume 7, Issue 4, 849-858 Research Article ISSN 2278 – 4357

VALIDATION OF A GAS CHROMATOGRAPHY-HEADSPACE METHOD FOR QUANTITATIVE ESTIMATION OF METHANOL IN WHOLE BLOOD USING ACETONITRILE AS INTERNAL STANDARD

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Article Received on
19 Jan. 2018,
Revised on 10 Feb. 2018,
Accepted on 07 March 2018
DOI: 10.20959/wjpps20184-11232

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ABSTRACT

The present study evaluates the efficacy of a Headspace Gas-Chromatography with a capillary column as a method for determining the methanol content in Whole Blood. This procedure allows the use of GC-HS for quantitative and qualitative estimation of methanol. The extensive review literature in this field yielded methods with extensive instrumentation and method preparation. Aim of the study was to determine a methodology using Gas Chromatography- Headspace that was suitable for different biological matrix; minimum sample preparation; optimized internal standard and small sample volume. Analysis was performed on whole blood using acetonitrile as internal standard. Peaks were suitably resolved for methanol (1.342min); acetonitrile (2.148min) (IS) within 8.889 min. Linearity was achieved across a concentration of 7.92 mg to 237.6 mg/100 ml with a correlation coefficient of 0.999 for whole blood and standard in DDW. LOQ and LOD were within the range of 2.37-6.33mg% Reproducibility of samples and standards resulted in precision and accuracy of 0.27-6.51%.

KEYWORDS: Methanol, Poisoning; Alcohol, Gas Chromatography, GLC etc.

INTRODUCTION

All the substances where hydroxyl group is the functional group attached to a saturated carbon chain, can be termed an alcohol. Pure methanol is a colourless liquid, having a specific gravity of 0.792, a boiling point of 65^0C and a slight odour distinctly different from that of ethanol. It belongs to the category of aliphatic alcohols with chemical formula;

CH_3OH and molecular wt. 32.04 and commonly known as Wood alcohol, wood-naphtha, carbinol, methylated spirit. It is widely used as a solvent for extraction, antifreeze, fuel, and denaturant to render alcohol (industrial ethanol) unfit for consumption.[1] Methanol is an alcohol, toxic to humans and majority of cases of poisoning happen due to unintentional drinking of industrial ethanol or malicious intent of poisoning.[2] The term 'alcohol poisoning' is sometimes used to describe the most serious and life-threatening complications of alcohol overdose, such as slowed breathing; loss of consciousness and even death.[3] These symptoms result from the accumulation of formate in the bloodstream and may progress to death by respiratory failure. Poisoning can occur by over-consumption of alcohol or unintentional consumption of spurious alcohols.

Methanol can be exposed to body by inhalation; dermal application; eyes and oral route. It is readily absorbed in the blood stream from gastrointestinal tract, respiratory tract and percutaneous route. Concentrations above 0.2g/L are toxic and values higher than 0.5g/L indicate severe poisoning, concentrations above 0.9g/L are potentially deadly.[4] After absorption, methanol is widely distributed in total body water, the volume of distribution is 0.6-0.7L/kg (methanol) and 0.5L/kg (formic acid) in methanol poisoning cases. Metabolisis in liver, follows a chemical pathway to oxidize methanol to formaldehyde by alcohol dehydrogenase and to formic acid (formate) by aldehyde dehydrogenase. Upon ingestion, methanol initially has narcotic effect followed by an asymptomatic period of approx. 10-15 hours. It is rapidly absorbed from the gastrointestinal tract with peak absorption in 30 to 60 minutes. Acute intoxication of methanol causes headache, vertigo, fatigue, nausea, vomiting, blurred vision, blindness and even death.[5] Methanol in itself is an nontoxic compound, and its toxicity is due to its metabolites rather than methanol itself.[6] The quantification of methanol in body fluids is very important for confirmation of methanol intoxication- related deaths. The quantification of methanol in biological fluids can also be a biomarker of intentional, accidental, or occupational hazard.

Blood alcohol testing is one of the most accurate methods for measuring alcohol toxicity, because it presents the physiopathological chemistry of compound in body. The analysis of blood and other body fluids for alcohol is most commonly performed using "Headspace-Gas Chromatography" due to its simplicity in operation and the number of matrices that can be analysed with one instrument.[7-10] Although various methods have been described for estimation of methanol from blood they have utilised higher instrumentations and sample

preparations.[1,8,11,12] But the present methodology validated, utilises 1ml of sample, addition of internal standard and 18.99min of total analysis time, with minimum sample preparation for quantitative and qualitative estimation of methanol in matrix.

MATERIALS AND METHODS

Instrument

Gas Chromatography system: Model No. 7890A from Agilent, U.S.A.

Headspace Sampler: Model No. 7697A from Agilent, U.S.A.

Column: J&W 125-1334; 30mX530µmX3µm.

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

Reagents: Methanol and Acetonitrile of purity, ≥99.80%, from Merck, India, Ultra-pure water from Rions, India were used.

Glassware: 20 ml Headspace glass vials from Agilent Technologies U.S.A; 100ml and 10ml volumetric flasks from Merck, India were used.

Miscellaneous

Micropipette of volume 100-1000 µl and 20-200 µl from Corning, U.S.A., septa (PTFE) and Aluminium crimp cap and Crimper for sealing the HS vial from Agilent, U.S.A. were used.

Blood Sample: Fresh blood samples were obtained from individuals. The blood collected was mixed with (EDTA) anticoagulant and stored at 4^0C.

Preparation of Methanol Standard Solution

The Stock solution of concentration 792mg/100ml, was prepared by dissolving 1 ml of Methanol with ultrapure water in 100 ml volumetric flask.

Five working dilutions of concentration 7.92 mg/100 ml; 19.8 mg/100 ml ; 79.2 mg/100 ml; 158.4 mg/100 ml; 237.6 mg/100 ml were prepared from stock solution by dissolving volumes of 100 µl; 250 µl ; 1000 µl; 2000 µl; 3000 µl in 10 ml volumetric flask with ultrapure water.

Preparation of Internal Standard Solution

Internal standard of concentration 39.15mg/100ml was prepared by dissolving 1ml Acetonitrile in 100 ml of ultrapure water in a 100ml volumetric flask.

Preparation of Calibration standard vial

1 ml of methanol standard of concentration 7.92 mg/100 ml; 19.8 mg/100 ml ; 79.2 mg/100 ml ; 158.4 mg/100 ml ; 237.6 mg/100 ml each, was taken in HS vial and 50 µl of internal standard was added to ach vial. Vial was sealed using septa and cap with crimper.

Preparation of Sample for Precision and Recovery

From the stock solution of Methanol of concentration, 792mg/100ml three working dilutions of concentration 39.60mg/100ml; 79.2 mg/100 ml; 237.6 mg/100 ml were prepared by dissolving volumes of 500 µl; 1000 µl; 3000 µl in 10 ml volumetric flask with Distilled water. Similar sample preparation was done for Blood samples.

Preparation of Sample: 1 ml of blood sample was taken in HS vial and 50 µl of internal standard was added to it. Vial was sealed using septa and cap with 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°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°C was held for 5 min and then ramped at 45°C/min to a final temperature of 180°C held for 1 min. Total GC-HS run time was for 20.88 min per run.

Headspace conditions: Headspace oven temperature was set at 70°C. The HS Loop and Transfer Line Temperature were set at 80°C and 90°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 Ionization detector was used for the detection of analytes. The FID temperature was maintained at 250°C with Hydrogen (40ml/min), Zero Air (400ml/min) and makeup flow of 25ml/min. the FID signal was zeroed at 0.01 min with data collection rate of 20 Hz.

RESULTS AND DISCUSSION

The developed method was fully validated for specificity, linearity, accuracy, precision, recovery, carryover effect, detection limit and quantification limit according to ICH guidelines.[13]

Specificity: Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. To match the qualitative estimation, individual standards of Methanol and Acetonitrile were processed same as the optimized and graphs of Negative Blood; Positive Blood and Standard were overlapped. No interference was observed at any retention times of either standard or internal standard.

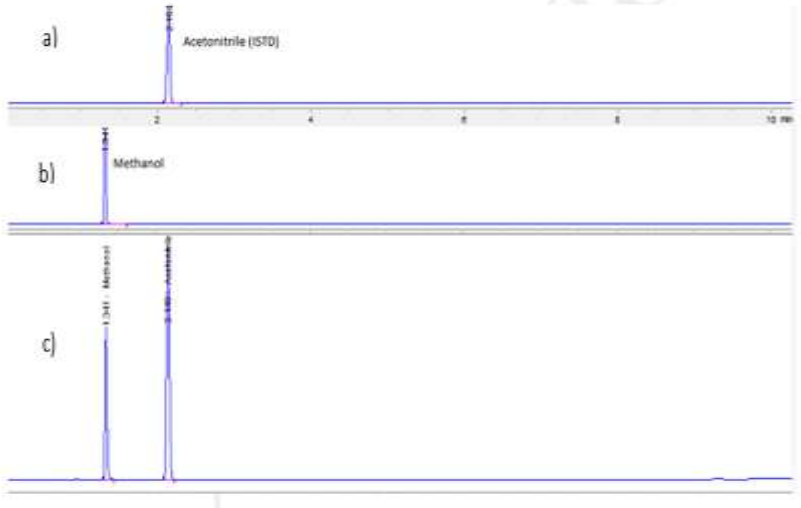

Figure. I: Chromatogram overlaps of a) Acetonitrile Standard; b) Methanol Standard; c) Mixture of Standard and Internal Standard in Water.

Analytes, Methanol (1.341min) and Acetonitrile (2.154min) were detected in the matrix of standard; whole blood as confirmed by the comparison of Rt_{sam} with that of Retention times of individual analytes as shown in Figure-I and II.

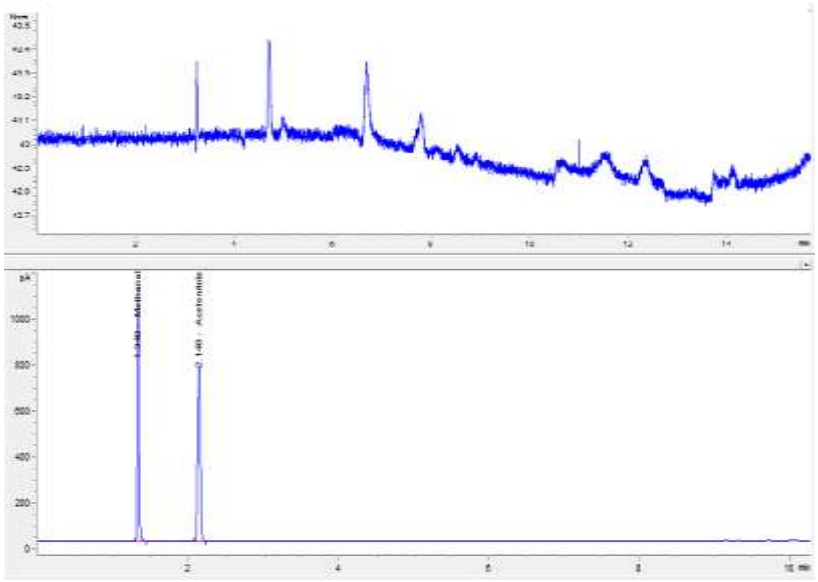

Figure. II: Chromatogram overlaps of a) Negative Blood; b) Spiked Blood.

Calibration curve and Linearity: Regression study of calibration data was attempted over the considered range of 7.92mg/dl to 237.6mg/dl for matrix and Standards. The linearity curve was plotted by calculating the ratios of peak area of the analyte with that of peak area of the internal standard and relating these ratios to that of successive concentrations of serial dilutions as shown in Table-I. All analytes in the sample matrix followed a linear graph with the regression coefficient of 0.999 within the range of 7.92mg/dl to 237.6mg/dl as shown in figure-III.

Table. I: Concentration and area of successive dilutions for plotting Linearity curve.

Dilution	Methanol in Water		Methanol in Blood	
	Concentration	Area	Concentration	Area
100µl	7.92mg/dl	93.3	7.92mg/dl	80.1
250µl	19.80mg/dl	233.35	19.80mg/dl	214.2
1000µl	79.20mg/dl	898.67	79.20mg/dl	897.58
2000µl	158.40mg/dl	1899.4	158.40mg/dl	1790.73
3000µl	237.60mg/dl	2525.01	237.60mg/dl	2724.41

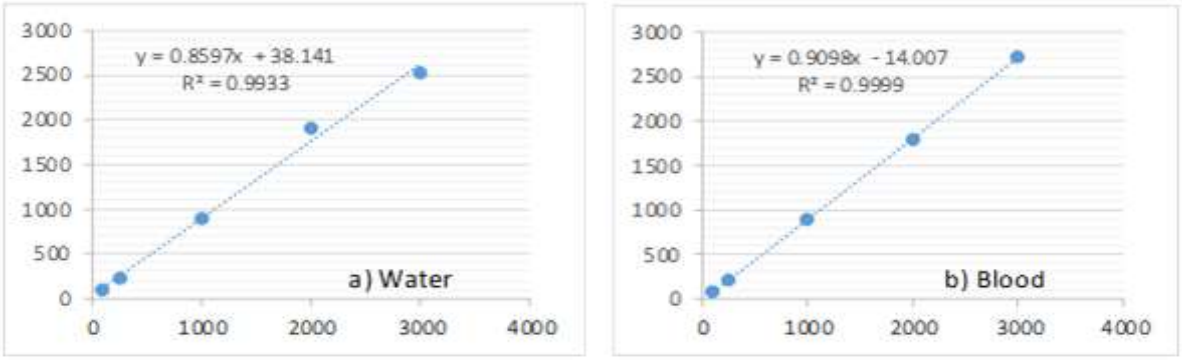

Figure. III: Calibration curve of a) Methanol in Water, b) Methanol in Blood.

Accuracy: Interday and Intraday accuracy were calculated, as the closeness of the mean test concentration obtained by the method to the actual concentration of analyte expressed as % accuracy. Three concentration of the matrix was evaluated for determining accuracy. Percentage accuracy is calculated by using following formula

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

Precision

Intraday assays were performed by analysing 5 replicates of 3 serial concentrations (low, mid, high) on one day. Interday precision was performed by analysing 5 replicates of 3 serial concentrations on five different days. The precisions so determined were within the range of coefficient of variance and depicted as % Relative Standard Deviation (%RSD). Inter and

Intraday precisions were expressed as Relative Standard Deviation (%RSD) for all matrixes and found to be within the acceptable range of 1.92- 5.6% with acetonitrile as Internal Standard as shown in Table-II.

Table II: Interday and Intraday Precision and Accuracy of analysed samples.

MATRIX	SOLVENT	CONC	INTRADAY			INTERDAY		
			Estm Conc. Mean ± SD (mg/100ml)	Precision (%RSD)	Accuracy (%Bias)	Estm Conc. Mean ± SD (mg/100ml)	Precision (%RSD)	Accuracy (%Bias)
Water	Methanol (n=5)	(39.6mg/dl)	39.36±0.75	1.92	0.60	39.22±0.65	1.66	0.94
		(79.2mg/dl)	78.98±1.22	1.55	0.27	78.63±1.12	1.42	0.71
		(237.6mg/dl)	223.56±5.51	2.46	5.90	229.21±7.04	3.07	3.52
Blood	Methanol (n=3)	(39.6mg/dl)	37.02±0.50	1.37	6.51	37.19±1.28	3.46	6.06
		(79.2mg/dl)	77.61±0.92	1.19	2.00	77.97±0.85	1.09	1.55
		(237.6mg/dl)	232.60±2.36	1.01	2.10	233.95±2.21	0.94	1.53

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 (7.9mg/100ml) 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. The values of LOQ and LOD are shown in Table-III.

Table. III: LOQ and LOD of the method.

Matrix	LOD (mg/100ml)	LOQ (mg/100ml)
Water	2.37	5.54
Blood	3.96	6.33

Recovery: Three different concentrations were spiked individually in known matrix, mean was calculated of the 5 replicates of each concentration. Recovery was calculated by comparing the results of spiked samples with that of pure standard of corresponding concentration as shown in Table-IV. % Recovery was calculated by using following formula

$$Recovery\,\% = \frac{Observed\ value}{Known\ Concentration} \times 100$$

Table. IIV: Recovery % of Blood.

Matrix	Conc	Mean	Recovery%
Water	39.6mg/dl	39.36±0.75	99.39%
	79.2mg/dl)	78.98±1.22	99.72%
	237.6mg/dl)	223.56±5.51	94.09%
Blood	39.6mg/dl)	37.02±0.50	93.48%
	79.2mg/dl)	77.61±0.92	97.99%
	237.6mg/dl)	232.60±2.36	97.89%

Carry-over Effect

Carryover is the appearance of an analyte, in a run when a blank containing no analyte is injected. To calculate if any carryover is observed in the run, a blank is injected immediately after a high concentration of the analyte. With appearance of no carryover in the subsequent blank runs, it can be concluded that the methodology observes no carryover even with high concentrations of 200mg/100ml.

CONCLUSION

Methanol can be encountered in clinical and forensic cases, the goal was to create a method of quantification of methanol that could be useful in both clinical and toxicological setting. A number of chromatographic procedures were reported for the determination of methanol in different matrix, however no chromatographic methods for analysis of methanol in whole blood using same analytical conditions without pre-treatment of the samples are reported. As methanol is a volatile, use of Headspace sampler ensures vaporisation of volatiles within the sealed vial, standardized temperature, and pressurization and injection time. The analysis only required 1 ml of sample and addition of internal standard, total runtime of analysis (1 analytes, 1 IS) was achieved within 20min as shown in Figure-I. Method validation was attempted according to the conditions as prescribed by ICH guidelines (13). Limit of Detection (LOD) and Limit of Quantification (LOQ) in the matrix was suitable for routine analysis. During all procedures samples and standard were stored at 4°C. If the standards required prolonged storage, samples were kept frozen at -4°C. The proposed methodology serves to be competent enough to analyse the compound, quantitatively and qualitatively in research , clinical and medicolegal samples. Since the analytical method was fully automated with the use of GC System and HS sampler, human interaction and error was significantly reduced.

ACKNOWLEDGEMENT

The authors express their sincere gratitude to Dr Sudhir Kumar Gupta, Professor and Head of Department of Forensic Medicine and Toxicology All India Institute of Medical Sciences, New Delhi for his support in use of resources in the laboratory.

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