Pharmaceutical Sciences, 2021, 27(2), 297-301
doi:10.34172/PS.2020.40
https://ps.tbzmed.ac.ir/

Short Communication

A Simple Colorimetric Method for Determination of Ethanol in Exhaled Breath Condensate

Fariba Pourkarim[1], Elaheh Rahimpour[2,3*], Maryam Khoubnasabjafari[4], Vahid Jouyban-Gharamaleki[5], Sara Farhang[6], Abolghasem Jouyban[2,7]

[1]*Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*
[2]*Pharmaceutical Analysis Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran*
[3]*Food and Drug Safety Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*
[4]*Tuberculosis and Lung Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*
[5]*Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*
[6]*Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran*
[7]*Digestive Diseases Research Institute, Tehran University of Medical Sciences, Tehran, Iran*

Article Info

Article History:
Received: 22 April 2020
Accepted: 17 May 2020
ePublished: 2 September 2020

Keywords:
-Ethanol
-Exhaled breath condensate
-Redox reaction
-Colorimetry

Abstract

Background: Ethanol is considered as a toxic compound when used in excess amounts. The toxic concentration for ethanol was reported to be 1000 – 2000 μg.mL^{-1} in plasma and serum samples. The aim of the current study was to develop a rapid and catalyst free colorimetric method for determination of ethanol in exhaled breath condensate (EBC) sample.

Methods: A redox reaction with dichromate -based colorimetric method was used for determination of ethanol in EBC.

Results: The proposed method shows a good sensitivity and selectivity for ethanol in compared with other compounds and biomarkers existing in EBC. The color change can be easily observed by the naked eye in the presence of ethanol in the range of 300 - 8000 μg.mL^{-1}. The quantitative detection of ethanol was fully validated and used for determination of ethanol in EBC of alcohol administrated individuals.

Conclusion: This catalyst free colorimetric method has great potential for ethanol determination owing to many desirable properties such as high reliability, high sensitivity, and fast response time.

Introduction

Ethanol is considered as a toxic compound when is used in excess amounts. Chronic alcohol abuse has some negative consequences, including poor grades, alcohol addiction, and car accidents[1] and can be harmful for the liver, brain, and other organ systems, and result in certain types of cancers, and fetal alcohol syndrome.[2] The toxic concentration for ethanol is reported to be 1000 – 2000 μg.mL^{-1} in plasma and serum samples.[3]

The detection of ethanol is a crucial analytical procedure in biomedicine and clinical applications. Various analytical techniques were reported for determination of ethanol in various biological samples. Macchia *et al.*[4] used a headspace - gas chromatography with flame ionization detection method (GC–FID) with a capillary column for determining of the spiked ethanol content in urine, serum, plasma, blood and saliva in the range of 500 – 3000 μg.mL^{-1}. Pontes *et al.*[5] validated a GC–FID with direct injection, using a capillary column, for determination of spiked ethanol in various human body fluids including whole blood, vitreous humour, and urine in the range of 75 – 2400 μg.mL^{-1}. De Martinis *et al.*[6] developed a headspace solid-phase microextraction (HS-SPME) – GC-FID for determination of ethanol in blood and urine in the range of 5 – 8000 μg.mL^{-1}. Jones *et al.*[7] used a GC- mass spectroscopy for determination of endogenous ethanol in breath condensates. Kucherenko and Moiseev[8] used a ^{1}H-NMR spectroscopy and refractometry for studying of the distribution of nonelectrolytes n-alcohols series between extracellular medium and human red blood cells. Jones[9] used an automated enzymatic method based on photometry in the present of yeast alcohol dehydrogenase and the coenzyme nicotinamide adenine dinucleotide for ethanol determination in saliva in the range of 0.0 – 1900 μg.mL^{-1}. Gibson and Blotner[10] used a photoelectric colorimetery for quantification of ethanol in blood and urine. They reported 0 - 6.5 mg. per cent for normal

*Corresponding Author: Elaheh Rahimpour, Email: rahimpour_e@yahoo.com

blood and 0-2.4 mg. per cent for normal urine. Jetter,[11] Anderson,[12] Shapiro,[13] and Kozelka and Hine[14] described the same method with some modification for ethanol determination in urine, blood and tissue samples. Another colorimetric method based on dichromate in the presence of a catalyst for ethanol determination in the exhaled breath was reported in the literature.[15] However, no values from ethanol measurement were reported in these studies. Diskin *et al.*[16] used a selected ion flow tube mass spectrometry (SIFT-MS) for determination of endogenous ethanol in breath samples. They were reported a mean concentrations in the range of 27–153 ng.mL^{-1}. Despite these methods, the necessity of validating a faster and more precise method for determination of ethanol in the biological fluids is still of great importance.

In the present study, a simple colorimetric method based on a redox reaction with dichromate was used for determination of ethanol. Ethanol is oxidized to ethanoic acid by reacting with dichromate in acid media according to the following reaction:

$$3\ C_2H_5OH + 2\ K_2Cr_2O_7 + 8\ H_2SO_4 \rightarrow 2\ Cr_2\ (SO_4)_3 + 3\ CH_3COOH + 2\ K_2SO_4 + 11\ H_2O$$

The colorimetric distinction of various concentration of ethanol is based on the formation of green colored chromate ions that in mixed with the excess yellow colored dichromate ions produce a differentiable colors. This method is fully validated for the qualitative and quantitative detection of ethanol in exhaled breath condensate (EBC), as a simple biological sample, of alcohol administrated individuals. The novelty of the present work is mainly related to the development of a catalyst free colorimetric method for EBC samples that have a subtle difference with exhaled breath. In the exhaled breath analysis, the measurement of analytes (with high vapor pressure and low melting point) in gaseous form is performed,[17] while in EBC analysis, approximately all components volatile than water (*e.g.* ethanol with boiling point of 78.37 ˚C) can be easily condensed in the liquid phase. It is well known that measurement in the liquid phase is simpler and more reliable than in the gaseous phase.

Materials and Methods

Reagents and solutions

All reagents were of analytical-reagent grade and the ultrapure deionized water were purchased from Ghazi Pharmaceutical Co. (Tabriz, Iran). Potassium dichromate (Merck, Darmstadt, Germany), and sulfuric acid (Scharlau Chemie, Spain) were used in this study.

Apparatus and instruments

The UV–Vis absorption spectra were recorded on a double-beam UV–Vis spectrophotometer model UV-1800 (Shimadzu, Japan) with 0.5 cm quartz cells. For weighing the solid materials, an electronic analytical balance model AB204-S (Mettler Toledo, Switzerland) was employed.

Exhaled breath condensate (EBC) collection

EBC samples were collected by using a lab-made cooling trap system.[18,19] The EBC collection device comprises a cooling trap that can be set to temperature from 0 to -25 ˚C. The device works by rapidly cooling exhaled air with consequent condensation of water vapor, as well as the sedimentation of aerosol particles onto a cold surface. EBC samples used for method validation were a pool of samples collected from healthy subjects. Sample donors signed a written consent form approved by the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1398.755).

General procedure

For ethanol concentration range of 300 – 1500 µg.mL^{-1}, 0.092 g of potassium dichromate was accurately weighed into a 5-mL screw cap tube and dissolved in 4.0 mL of sulfuric acid: water (3:1). 250 µL of standard or sample solution containing ethanol was transferred into a 2 mL vial and then 100 µL of prepared reagent was added. The reaction was proceed by placing in a Ben-Marie water bath at 80 ºC. After completing the reaction for 8 min, the absorbance intensity was measured at 580 nm.

For ethanol concentration range of 1600 – 8000 µg.mL^{-1}, 500 µL of standard or sample solution containing ethanol was transferred into a 2 mL vial, 85 µL of dichromate solution (100 g.L^{-1}) and 100 µL of sulfuric acid were added. After incubation for 5 min at room temperature, the absorbance intensity of samples was measured at 580 nm.

Results and Discussion

Optimization of reaction conditions

To optimize the conditions of redox reaction, parameters

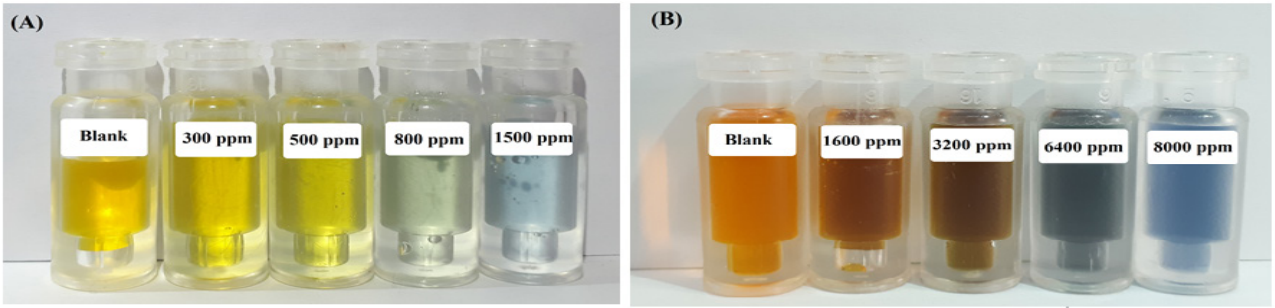

Figure 1. A selected photograph of colorimetric response of redox reaction with dichromate to the different concentration of ethanol: (A) 300 – 1500 µg.mL^{-1} and (B) 1600 – 8000 µg.mL^{-1}.

such as concentration of dichromate and sulfuric acid and reaction time were examined. For this purpose, several trials (different ratios of reagents in various reaction times) were performed. On the basis that distinctive colors were obtained (Figure 1) with 100 µL of dichromate solution (23 $g.L^{-1}$) in sulfuric acid: water (1:1) for ethanol with the concentration ranges of 300 – 1500 $\mu g.mL^{-1}$ and 85 µL of dichromate solution (100 $g.L^{-1}$) and 100 µL of sulfuric acid for ethanol with the concentration ranges of 1600-8000 $\mu g.mL^{-1}$. The color obtained with the reagents is dependent on the time and it devastated with time as the samples stands more than desired time reported in "general procedure" section.

Method validation

Validation was performed for the presented method using FDA guidelines.[20] System linearity, sensitivity, accuracy, precision, selectivity, stability and robustness were investigated.

Linearity

Linearity was investigated by preparing standard solutions of ethanol at various concentration levels over the range of 300 - 1500 $\mu g.mL^{-1}$ and 1600 - 8000 $\mu g.mL^{-1}$. The absorbance of each sample against respective concentration of analytes was found to be linear at 581 nm. The regression coefficients were 0.978 and 0.984. Linearity results were presented in Figure 2.

Sensitivity

The sensitivity was expressed as limits of detection (LOD) and quantitation (LOQ). LOD and LOQ, which are obtained from $3S_b/m$, and $10S_b/m$ (in which S_b is the standard deviation of the blank and m is the slope of the calibration curve), were 82.5 $\mu g.mL^{-1}$ and 275.0 $\mu g.mL^{-1}$, respectively.

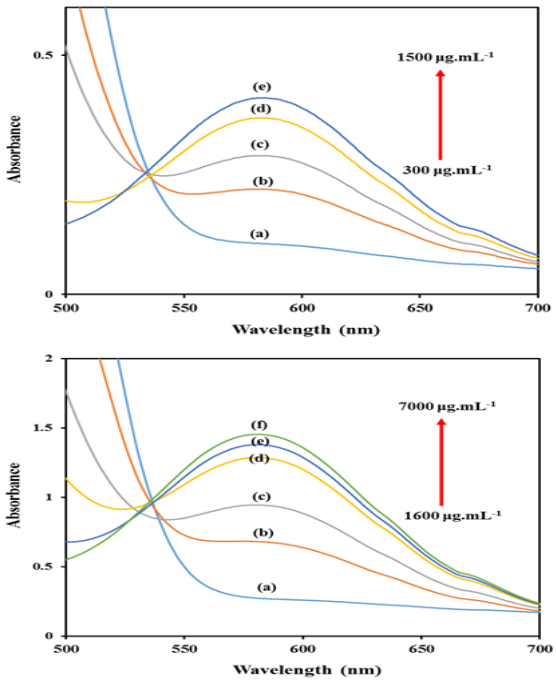

Figure 2. Absorbance spectrum of reagents of redox reaction in the absence (a) and presence (b-f) of ethanol.

Precision

For precision studies, the standard solution having concentrations 600, 800, 3200 and 4800 $\mu g.mL^{-1}$ of ethanol were analyzed during the course of experimentation on the same day and on different days. For both inter-day and intra-day variations, analyte solutions were measured in triplicates and the results are summarized in Table 1.

Selectivity

The tolerance of the validated method against some used drugs by various individuals was also examined. For this purpose, these compounds in the concentration found in serum of subjects receiving desired drugs and five time higher than these concentrations [3,21] were added in to EBC samples spiked with standard solution of 800 $\mu g.mL^{-1}$ ethanol. The obtained results are presented in Table 2. As can be seen, all drugs tested had no interference on the determination of ethanol (the tolerance limit was set as the amount of interfering species to cause ± 10% error in the determination of ethanol). Furthermore, the tolerable limit

Table 1. Inter-day and intra-day relative standard deviations (%*RSD*) for replicated determinations for different levels of ethanol for redox reaction with dichromate in EBC.

[Ethanol] $\mu g.mL^{-1}$	%RSD	
	Intra-day	Inter-day
600	1.1	4.3
800	2.9	4.8
3200	1.5	2.9
4800	1.6	3.2

Table 2. Tolerance amount of interfering species in the determination of 800 $\mu g.mL^{-1}$ of ethanol with absorbance (*A*) of 0.369.

Drug	Plasma conc. ($\mu g.mL^{-1}$)	Tolerance limit conc. $\mu g.mL^{-1}$ (ΔA)
Nifedipine	0.025 – 0.100	0.31 (0.052)
Ibuprofen	10 – 30	100.00 (-0.044)
Chlordiazepoxide	0.4 – 2	6.00 (-0.062)
Sildenafil citrate	0.05 – 0.5	1.38 (0.023)
Oxazepam	0.2 – 1.5	4.25 (0.084)
Pantoprazole	2 – 4.6	16.50 (0.075)
Losartan	0.2 – 1.2	3.50 (0.153)
Clonazepam	0.01 – 0.08	0.22 (0.040)
Diazepam	0.02 – 2	5.05 (-0.102)
Celecoxib	0.05 – 0.5	1.38 (0.041)
Alprazolam	0.005 – 0.08	0.21 (0.070)
Carvedilol	0.02 – 0.16	0.45 (0.137)
Methadone	0.05 – 1.00	2.62 (0.066)
Budesonide	0.05 – 0.100	0.38 (0.04)
Paracetamol	2.50 – 25.00	68.75 (-0.020)

of the H_2O_2 as an oxidative biomarker present in EBC was 8000 µg.mL^{-1} which very higher than reported level in EBC of the healthy or patient subjects.[22,23] These results show that the developed method is selective for quantification of ethanol in EBC.

Stability

Stability studies were performed by four concentrations of ethanol. EBC samples containing ethanol were freezed in -20 ºC and thawed for 3 cycles with interval of 24 hours between cycles and finally analyzed. The statistically differences from initial absorbance intensity value was found to be 1.0 – 11.0 %. The results are summarized in Table 3.

Robustness

To examine the potential variability in the reaction conditions when a method is transferred from one laboratory to another or performed by another analyst, the robustness of the method was investigated and the results are shown in Table 4. It can be seen that the results of this study show no significant changes on the analytical results indicating the robustness of the method.

Real samples analysis

In order to investigate the applicability of the developed method on the real samples, the method was used to determine ethanol in EBC of five alcohol administrated individuals. The accuracy of the established method was evaluated by comparing the analytical results of the method with results obtained from a commercially available test kit (Home health, UK) designed to measure alcohol content in exhaled breath. The obtained results are shown in Table 5. As can be seen, our method shows 800 and 500 µg.mL^{-1} for samples 1 and 3, respectively which is compatible with the results obtained from test kit. For samples 2, 4 and 5 which test kit show 200 µg.mL^{-1}, our method with lower limit of quantification of 300 µg.mL^{-1} cannot detect any ethanol for them. These results show that the developed colorimetric method is accurate and has a great potential for determination of alcohol in EBC samples.

Table 3. Stability study for different levels of ethanol for dichromate based redox reaction.

[Ethanol] µg.mL^{-1}	Freeze-thaw stability (%RE)		
	After 24 h	After 48 h	After 72 h
600	2.6	5.8	11.0
800	1.9	6.6	7.9
3200	4.3	8.9	9.8
4800	3.2	6.5	8.8

%RE = [($A_{Measured}$) – ($A_{Expected}$)) / ($A_{Expected}$)] × 100.

Table 4. Robustness study for different levels of ethanol for dichromate based redox reaction.

No.	RE%[a] for three level of ethanol (µg.mL^{-1})			
	600	800	3200	4800
1[b]	3.3	4.1	9.0	6.2
1[c]	3.1	3.8	1.4	2.5
2[c]	4.2	10.1	2.8	2.2
3[c]	4.3	4.1	6.0	7.4
4[c]	6.5	4.8	9.8	6.8

[a]%RE = [($A_{Measured}$) – ($A_{Expected}$)) / ($A_{Expected}$)] × 100, [b]One laboratory to another, [c]One researcher to another

Table 5. Details of the real samples and found concentration of ethanol in EBC samples of alcohol administrated individuals by developed method and commercially available test kit.

No.	Gender	Weight (Kg)	Age (year)	Concentration, µg.mL^{-1} (± SD[a])	
				Presented method	Test kit
1[b]	Male	80	41	800 ± 20	800
2	Male	48	40	<300	200
3	Male	79	41	500 ± 15	500
4[c]	Female	65	41	<300	200
5	Male	70	47	<300	200

[a] Standard deviation for n=2 replicative determinations by the developed method. The measurements by test kit are performed one time per each sample. [b]co-administrated drug: Sertraline, [c]co-administrated drug: S- Citalopram, Lamotrigine, Pregabalin

Conclusion

In this work, a simple colorimetric method was validated for the detection and quantification of ethanol in EBC of alcohol administrated individuals. Advantages of this method utilizing a simple detection method include the minimal time required to analyze samples without any sample preparation or preconcentration steps which make it useful method for clinical applications.

Authors' Contributions

FP: Methodology, ER: Interpretation of data for the work and drafting the work, MK: Investigation, VJ: Investigation, SF: Investigation, AJ: Writing - review and editing. All authors read and gave approval of the final manuscript.

Ethical Issues

EBC sample donors signed a consent form which was approved by the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1398.755).

Acknowledgements

This work was supported by Research Affairs of Tabriz university of Medical Sciences, under grant number 64109. Authors would like to thank Dr Jafar Soleymani for collecting the patients' real samples.

Conflict of Interest

The authors have declared no conflict of interest.

References

1. Brook JS, Pahl K, Brook DW, Morojele NK. Risk and Protective Factors for Substance Use and Abuse. El-

Guebaly N, Carrà G, Galanter M, editors. Textbook of addiction treatment: International perspectives. Milano: Springer Milan; 2015. p. 2279-305. doi:10.1007/978-88-470-5322-9_101
2. Brooks P. DNA damage, DNA repair, and alcohol toxicity—a review, Alcohol Clin Exp Res. 1997;21(6): 1073-82. doi:10.1111/j.1530-0277.1997.tb04256.x
3. Schulz M, Schmoldt A. Therapeutic and toxic blood concentrations of more than 800 drugs and other xenobiotics. Pharmazie. 2003;58(7):447-74.
4. Macchia T, Mancinelli R, Gentili S, Lugaresi EC, Raponi A, Taggi F. Ethanol in biological fluids: headspace GC measurement. J Anal Toxicol. 1995;19(4):241-6. doi:10.1093/jat/19.4.241
5. Pontes H, Guedes de Pinho P, Casal S, Carmo H, Santos A, Magalhães T, et al. GC determination of acetone, acetaldehyde, ethanol, and methanol in biological matrices and cell culture. J Chromatogr Sci. 2009;47(4):272-8. doi:10.1093/chromsci/47.4.272
6. De Martinis BS, Ruzzene MAM, Martin CCS. Determination of ethanol in human blood and urine by automated headspace solid-phase microextraction and capillary gas chromatography. Anal Chim Acta. 2004;522(2):163-8. doi:10.1016/j.aca.2004.07.007
7. Jones A, Mårdh G, Änggård E. Determination of endogenous ethanol in blood and breath by gas chromatography-mass spectrometry. Pharmacol Biochem Behav. 1983;18(Suppl 1):267-72. doi:10.1016/0091-3057(83)90184-3
8. Kucherenko U, Moiseev A. The use of 1H-NMR spectroscopy and refractometry for investigation of the distrubution of nonelectrolytes N-alchols series between human red blood cel ls and extracellular medium V. Membr Cell Biol. 1999;13(5):633-44.
9. Jones A. Assessment of an automated enzymatic method for ethanol determination in microsamples of saliva. Scand J Clin Lab Inv. 1979;39(3):199-203. doi:10.3109/00365517909106094
10. Gibson JG, Blotner H. The determination of ethyl alcohol in blood and urine with the photoelectric colorimeter. J Biol Chem. 1938;126(2):551-9. doi:10.1016/S0021-9258(18)73861-1
11. Jetter WW. Modified dichromate method for determination of ethyl alcohol in biologic tissue. A J Clin Pathol. 1950;20(5):473-5. doi:10.1093/ajcp/20.5_ts.473
12. Anderson RK. A simple method for determination of alcohol in blood. Am J Clin Pathol. 1942;12(6):85-9. doi:10.1093/ajcp/12.6_ts.85
13. Shapiro J. An adaptation of the photoelectric colorimeter to the determination of alcohol in blood and urine. Am J Clin Pathol. 1942;12(6):66-68. doi:10.1093/ajcp/12.6_ts.66
14. Kozelka F, Hine C. Method for determination of ethyl alcohol for medicolegal purposes. Ind Eng Chem Anal Ed. 1941;13(12):905-7. doi:10.1021/i560100a014
15. Levine B, editor. Principles of forensic toxicology. Amer. Washington, D.C.: Assoc. for Clinical Chemistry; 2003. p. 164-5.
16. Diskin AM, Španěl P, Smith D. Time variation of ammonia, acetone, isoprene and ethanol in breath: a quantitative SIFT-MS study over 30 days. Physiol Meas. 2003;24(1):107. doi:10.1088/0967-3334/24/1/308
17. Ghosh C, Singh V, Grandy J, Pawliszyn J. Recent advances in breath analysis to track human health by new enrichment technologies. J Sep Sci. 2020;43(1):226-40. doi:10.1002/jssc.201900769
18. Jouyban A, Khoubnasabjafari M, Ansarin K, Jouyban-Gharamaleki V. Breath sampling setup, Iranian patent 81363. 2013.
19. Pourkarim F, Rahimpour E, Khoubnasabjafari M, Jouyban-Gharamaleki V, Jouyban A. Direct monitoring of verapamil level in exhaled breath condensate samples. Pharm Sci. 2019;25(1):50-6. doi:10.15171/PS.2019.8
20. Bansal S, DeStefano A. Key elements of bioanalytical method validation for small molecules. AAPS J. 2007;9(1):E109-14. doi:10.1208/aapsj0901011
21. Regenthal R, Krueger M, Koeppel C, Preiss R. Drug levels: therapeutic and toxic serum/plasma concentrations of common drugs. J Clin Monit Comput. 1999; 15(7):529-44. doi:10.1023/a:1009935116877
22. Jouyban A, Rahimpour E, Jouyban-Gharamaleki V, Khoubnasabjafari M, Abdolmohammad-Zadeh H. Development and validation of a novel fluorometric sensor for hydrogen peroxide monitoring in exhaled breath condensate. Anal Methods. 2017;9(30):4371-4379. doi:10.1039/C7AY01535F
23. Montuschi P, Barnes PJ. Analysis of exhaled breath condensate for monitoring airway inflammation. Trends Pharmacol Sci. 2002;23(5):232-237. doi:10.1016/S0165-6147(02)02020-5