ORIGINAL ARTICLE

Development of a rapid method for blood alcohol concentration levels and its application in a population group exposed to controlled doses

Arthur de Mello Prates[1,4]; Ana Cristina Simões Rosa[1]; Paula de Novaes Sarcinelli[1]; Jefferson Jose Oliveira-Silva[2,4]; Claudio Cerqueira Lopes[3] and Sergio Rabello Alves[1,4]

1- Laboratory of Toxicology/Center of Studies of Worker's Health and Human Ecology/Sergio Arouca National School of Public Health/Oswaldo Cruz Foundation;
2 – Biochemistry Department/Center of Health and Biological Sciences /Federal University of State of Rio de Janeiro; 3- Chemistry Institute, Federal University of Rio de Janeiro; 4- Civil Police of the Rio de Janeiro State/Security Bureau of the Rio de Janeiro State/Government of Rio de Janeiro State.

ABSTRACT

In Brazil that each year, 38,000 people die by injuries in traffic incidents. A considerable portion of these records is associated with the recreational use of ethyl alcohol. Therefore, the aim of this study was the development of a method for blood ethyl alcohol concentration levels as well as its application in a population group exposed to controlled doses. Gas chromatography system coupled with a flame ionization detector and the headspace as extraction was the technique used under the following final conditions: 1) extraction at 110°C/2 min.; 2) analytical conditions: Injection at 250°C; detector temperature at 280°C; Injection mode: "split" 1/20; carrier gas: N_2, 50Kpa of constant pressure. Run time: 4.0 minutes. DBALC-1™ column; isotherm at 40°C. Volunteers ingested a total volume of 2.4 L of beer distributed in three monitoring phases every 0.8 L. At each step, the volunteers passed by a blood sampling to determine the ethyl alcohol concentration level. The rapid extraction by headspace and run demonstrated good selectivity with mean retentions times for methanol, acetaldehyde, ethyl alcohol, 2-propanol, acetone and isobutanol of 0.925, 1.028, 1.177, 1.450, 1.768 and 3.154 minutes, respectively. The healthy volunteers who participated in the protocol of controlled exposure to alcohol study had mean ethyl alcohol concentrations in $dg.L^{-1}$ of 1.9 ± 0.7 (1st), 3.4 ± 1.5 (2nd) and 6.0 ± 1.6 (3rd sampling battery). This method had the good analytical characteristics for its use in wide range of cases involving the monitoring of the human performance under drunk.

Keywords: Blood; alcohol, chromatography; method; human

1. Introduction

According to the World Health Organization (WHO), five million people have died due to injuries in 2000, approximately 520,000 people died of homicides, 815,000 from suicide and 1.2 million of road traffic incidents. In addition to the considerable number of deaths, in Brazil, 38,000 people die by injuries in traffic incidents with social and economic impacts reaching R$ 24.6 billion, including the loss of production due to the death or business interruption victims (1).

Whatever the cause of death, an important portion of these records are associated with the recreational use of ethyl alcohol by oral route. This compound is routinely used as antiseptic, disinfectant and vehicle for some pharmaceutical products. However, the biggest impact is determined by the non-medical use, both by the high frequency of use as the numerous health problems and public safety associated with it (1-2).

Regarding the political surveillance for driving under influence of alcohol, the world legal limits have important differences. These can range from zero to 8 decigrams per liter ($dg.L^{-1}$) of blood. Despite a trend in decreased of blood alcohol concentration levels (BAC), supported on a paradigm of harm reduction, customs and religion are variables that can interfere with the establishment of such laws, without taking into account the scientific (toxicological and epidemiological) studies associated with the theme. In fact, there are few studies involving ethanol exposure in humans under controlled conditions (3-5). In Brazil, there is only one study about the recreational exposure to alcohol under controlled conditions (6).

The Federal Law number 11.705/08 is the brazilian norma to driving under influence of alcohol, which determines that drivers must be caught with a BAC of 2.0 $dg.L^{-1}$ (tolerance limit). Police across Brazil tests BAC levels of suspected offenders at the site using breathanalysers (7).

In addition to the devices mentioned above, that use the exhaled air as a matrix, having as a principle an electric current generation proportional to the ethyl alcohol concentration

d) Corresponding author: 55-21-2598-2820 (Toxicology Lab/ENSP/FIOCRUZ).
Fax: 55-21-2270-3219; E-mail: alvessr@ensp.fiocruz.br and Skype™: sergio.rabello.alves.

by oxidation of the molecules in an electrochemical cell (8), numerous methods are available for the determination of BAC in intoxication occurrences. The use of gas chromatography for such analysis is not new; several such methods are already in use (2, 6 and 9). However, many analytical methods still prefer the time-consuming headspace (HS), considerable time of chromatographic development, small linear work range, with limited applications in clinical and *postmortem* analysis without care with the application in laboratories in developing countries or with basic instrumental structure.

Therefore, the aim of this work was the development of a rapid method for BAC and other volatile compounds as well as its application in a population group exposed to recreative controlled doses.

2. Material and Methods

2.1. Standards and reagents

Ethanol, acetaldehyde, 2-propanol and 2-methyl-1-propanol standards used in this study had at least 99% pure purchased from Sigma-Adrich co. Acetone and methanol used had at least 99.5% pure and were purchase from Tedia Brazil co. Deionized water (type 2) prepared by reverse osmosis system purchase from Milli-Q (Millipore) co., Direct 8 model. All analytical reagents and standards showed appropriate expiration date, until the time of conducting the study.

2.2. Instrumental used

Gas chromatography system coupled with a flame ionization detector (GC-FID) and the headspace as extraction technique, purchase from Thermo Scientific co. - Models Focus-GC and Triplus (Headspace). This equipment was qualified by brand office with certificate number 0044/2011 on February, 2011. In this study, we used the two columns with the following characteristics of size X internal diameter (i.d.) X film thickness: Carbowax 20M (60 m X 0.25 mm X 1.0 μm) and DB-ALC1 (30m X 0.53 mm X 3.0 μm).

2.3. Validation procedures, quality policy and initial chromatographic conditions

The Guidelines of the National Institute of Metrology, Quality and Technology (INMETRO) were used for analytical validation assays (10). Due to a lab quality policy (Accreditation by the Joint International Commition in 2012) and start training and initial procedures for the adoption of the norms ABNT NBR ISO/IEC 17025:2005 (11), all precision instruments (e.g.: glassware, automatic pipettes and analytical balances) were calibrated and certified previously.

The intra-assay deviation was evaluated by 5 replicates of calibration curve in different days and the inter-assay deviation was evaluated by 5 replicates of calibration curve in the same day, both for ethyl alcohol concentrations between 0.1 to 10.0 dg.L^{-1} (11-12).

To specific parameters for the development of the analytical method, and evaluation of separation efficiency by calculating the values of *Trennzahl* (TZ or separation number) and calculation of Response Factor (RF) for later comparison with the concentrations obtained from the calibration curve (quantification step), were extracted from Aquino-Neto and Nunes (12).

As a starting point for analytical procedures, the conditions used were extracted from Corrêa (13), a classic methodology developed in São Paulo University and routinely used in brazilian forensic toxicology labs (e.g. Tox Lab of Forensic Medical Institute of Rio de Janeiro State), and our reference method (RM) (2). These RM were used for purposes of comparison with the new analytical methodology developed in this paper.

2.4. Ethical aspects, subjects and sampling

The study was performed at the Federal University of Rio de Janeiro State (UNIRIO) and Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, on ten healthy volunteers of both sexes aged 18-47, who previously signed informed consent about participation in this study, approved by the Ethics Committee of Sergio Arouca National School of Public Health/FIOCRUZ, under de number 0279.0.031.000-11. The participants had not consumed alcoholic beverages for at least 24 h before the exposure protocol. This work was a subproject of a big population-based exposure design with a representative sample for the population of the Rio de Janeiro city. For this study, volunteers ingested a total volume of 2.4 L of beer (standard bland – 5.0% volume) distributed in three monitoring phases every 0.8 L. At each step, the volunteers passed by a clinical and neurobehavioral evaluation, in addition of exhaled air, blood and urine sampling to determine the ethyl alcohol concentration.

The blood was taken from the cubital vein using a vacuum sterile device. The disinfection of the skin before venepuncture was performed by ethanol at 70%. A volume of 3 ml of blood was collected to a sample tube with 1% sodium fluoride, filled up to the top and shaken gently.

2.5. Management data, statistical and softwares employed

For the development of datasheets, quantification procedures and text editing was used the package Microsoft Office™ 2007. For graphics was used the Sigma Plot™ version 10.0.0.54 which was purchased from Jandel Scientific Co. and EndNote™ X1.0.1 was used for the organization of references. Statistical tests as One-Way Analysis Of Variance (ANOVA) in analytical procedures were performed using the Primer™ software, version 3.0 from McGraw-Hill Inc. (14-17).

3. Results

The headspace vapour concentrations of aqueous ethyl alcohol in blood samples, above a series of 1.0 dg.L-1, were measured in quintuplicate in different times (2, 5 and 10 minutes) and temperatures (80, 95 and 110ºC) and a multivariate analysis

performed in order to evaluate the best recoveries (areas). The results presented in Table 1, showed that 110ºC for 2 minutes showed better recovery results. This condition is shown associated with a better resolution of the chromatographic peaks obtained, closely related to specificity, as presented in Table 1.

The Figure 1 shows a typical chromatogram obtained by injecting 0.1 µL of blood sample containing 0.01 dg.L^{-1} of standards mixture and IS (isobutanol − 1.6 dg.L-1). As can be seen, all peaks are sharps and clearly distinguishable. The mean retention time for methanol, acetaldehyde, ethyl alcohol, 2-propanol, acetone and isobutanol were respectively 0.925, 1.028, 1.177, 1.450, 1.768 and 3.154 minutes. The chromatographic separation efficiency showed differences by calculating the TZ values (0.453 to acetaldehyde/ethanol, 1.058 to ethanol/isopropanol and 8.485 to ethanol/isobutanol) to the targets compounds, giving a good selectivity to the method.

The final analytical conditions showed separation efficiency in a short period of extraction (2.0 min) and chromatographic development (4.0 min.). Comparing between the developed method and the reference method (RM), the order of elution of the peaks showed some differences. The first showed the following order of elution: methanol (0.925 min.), acetaldehyde (1.027 min.), ethyl alcohol (1.177 min.), isopropanol (1.450 min.), acetone (1.768 min), and isobutanol (3.150 min.). To RM, acetaldehyde eluted first, followed by acetone, methanol, isopropanol, ethanol and isobutanol (Figure 1 and Table 2).

The curves constructed with five calibrators in 10 replicates for the two lowest concentrations and 5 replicates for the other concentrations, indicated a satisfactory ethyl alcohol correlation coefficient (R) of 0.99995. The regression equation was y = ax + b, where a=1024849.8 and b=4284.0. The assay sensitivity were 0.5 and 0.1 dg.L^{-1} for quantification (LOQ) and detection (LOD) limits, respectively. The representative calibration curve for ethyl alcohol is shown in Figure 2.

For this method, no matrix effect was observed in complex samples (blood and urine). These results are shown in Figure 3, where no statistical differences (NS) of recovered ethyl alcohol areas, compared with deionized water as matrix to the concentrations tested (0.5 to 250.0 dg.L^{-1}).

In relation to precision parameter, the percentages of coefficients variation presented a range of 0.08 to 3.11 (intra-assay) and 0.39 to 3.42 (inter-assay) for ethyl alcohol concentrations between 0.1 to 10.0 dg.L^{-1} (Table 3).

Analysing samples in the day after of blood collection, we performed a simple stability test in order to assess if the quantitative response remained constant for up five days storage. There were no significant changes in the areas of blood samples fortified with 1.0 dg.L^{-1} of ethyl alcohol, for up two days under refrigeration conditions (2 to 8ºC) (data not shown).

For the quantification procedure, the raw data (areas) were processed in two different ways (curve calibrators and response factor) and the final concentrations were compared. The BAC levels showed systematically higher values when compared to concentrations obtained by calculating the Response Factor (RF) obtained (1.1771). The concentrations obtained by the calibration curve (CC) were 0.50 ± 0.04, 0.01 ± 0.86 and 1.96 ± 0.09 dg.L^{-1} to three sampling steps, respectively. The final values for BAC levels obtained by the RF were 0.40 ± 0.03, 0.72 ± 0.02 and 1.53 ± 0.08 dg.L^{-1} for sampling steps 1, 2 and 3, respectively. These differences in concentrations varied between 16.36 and 21.98% for three batteries of sampling. The results showed significant differences in the final concentrations obtained by two quantification methods (Table 4).

The healthy volunteers (n=10) who participated in the protocol of controlled exposure to ethyl alcohol study had mean BAC levels (in dg.L^{-1}) of 1.9 ± 0.7 (1st), 3.4 ± 1.5 (2nd) and 6.0 ± 1.6 (3rd sampling battery). Typical chromatogram (a) and respective plot generated in blood samples (b) is presented in the Figure 4. The first peak corresponds to ethyl alcohol followed by Isobutanol (1.6 dg.L^{-1}) as internal standard (IS). For this oral exposure case, the volunteer presented BACs levels of 2.1, 2.8 and 6.9 dg.L^{-1}, for batteries one (0.8 L), two (1.6 L) and three (2.4 L), respectively.

4. Discussion

The choice regarding which method of ethanol determination should be chosen for a given analytical application (e.g. *in vivo* vs. *postmortem* samples; toxicokinetic characteristics - absorption or elimination phases) must take into consideration the application, if simultaneous measurement of ethyl alcohol and/ or another organic volatile compounds (OVCs) of toxicological relevance is desirable, the accuracy of the sample processing and assay technique. Technical procedures should include adequate sensitivity, linearity over the selected concentration range, reproducibility, and adequate stability of the compounds

Table 1: Headspace multivariate treatments and analyses in the blood specimen fortified with 1.0 dg.L-1 of ethyl alcohol (final concentration in the vial).

Treatment		Ethyl alcohol area ± SD
T. (ºC)	TIME (min.)	
80	2	2131888 ± 161946,6 (*)
80	5	2504160 ± 110053,3 (*)
80	10	2200565 ± 33487,16 (*)
95	2	2586773 ± 365788,2 (*)
95	5	3351499 ± 228894,7 (*)
95	10	3229680 ± 123071,7 (*)
110	2	3973944 ± 46213,9
110	5	3918667 ± 43268,89 (NS)
110	10	3533114 ± 249208,1 (*)

Cromatographic conditions initially extracted from (11) and (2). Mean of three experiments;
SD = Standard deviation;
(*) *p* ≤ 0.05 by One way analysis of variance in relation to the biggest area (110ºC/2 min. treatment)
(NS) = No statistical significance

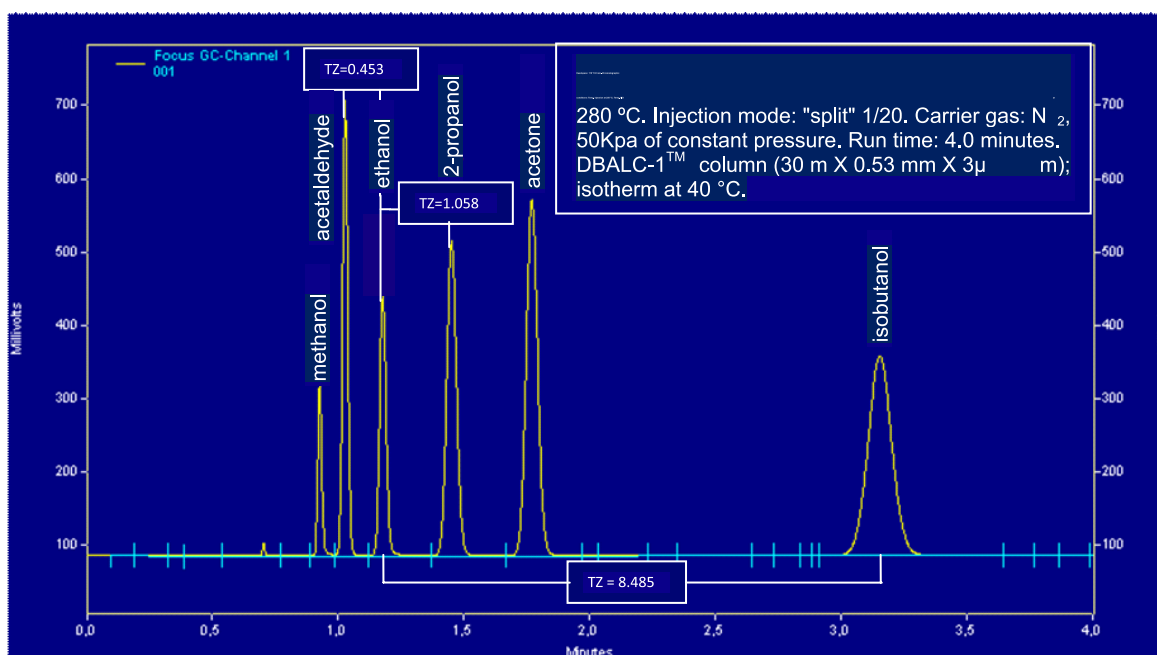**Figure 1:** Typical chromatogram generated by DB-ALC1 column in blood samples fortified with 0.1 dg.L^{-1} of each analyte: methanol, acetaldehyde, ethyl alcohol, 2-propanol, acetone, and 1.6 dg.L^{-1} of isobutanol as internal standard (IS).

Table 2: Mean retentions times of the target analytes studied in two chromatographic systems (our method X RM) and elution order from columns by two chromatographic systems.

	Mean Retention Time (in min. ± SD)		Elution order from column (our method) /(RM)
	Our method	**Reference method (RM)**	
Ethyl alcohol	1,1775 ± 0,0005	10,7560 ± 0,0221	(3)/(5)
Acetaldehyde	1,0275 ± 0,0005	4,8760 ± 0,0044	(2)/(1)
Isobutanol	3,1537 ± 0,0046	18,3380 ± 0,0075	(6)/(6)

Note: SD = Standard deviation.

Table 3: Precision results expressed by coefficient of variation (CV) intra and inter-assay.

[EtOH] (dg.L^{-1})	Intra-assay CV (%)	Inter-assay CV (%)
0.10	1.94	3.43
0.50	0.08	0.70
1.00	3.11	2.47
5.00	0.19	0.39
10.0	2.99	0.77

Table 4: Comparison between two different ways of quantifying ethyl alcohol from blood sample of a volunteer who participated in the protocol of controlled exposure study.

SAMPLING	[BAC] in dg.L^{-1} ($\chi \pm$ SD)		
	1	2	3
CC	0.50 ± 0.04 (*)	0.86 ± 0.01 (*)	1.96 ± 0.09 (*)
FR	0.40 ± 0.03	0.72 ± 0.02	1.53 ± 0.08

Notes: 1) $\chi \pm$ SD = mean concentration and the standard deviation to at least three replicates for each determination of BAC;
2) CC = Curve calibrators and RF = response factor (11);
3) FR = 7.1771;
4) (*) Statistical significance ($p < 0.05$) between CC and FR groups by One Way Analysis of Variance (ANOVA) test.
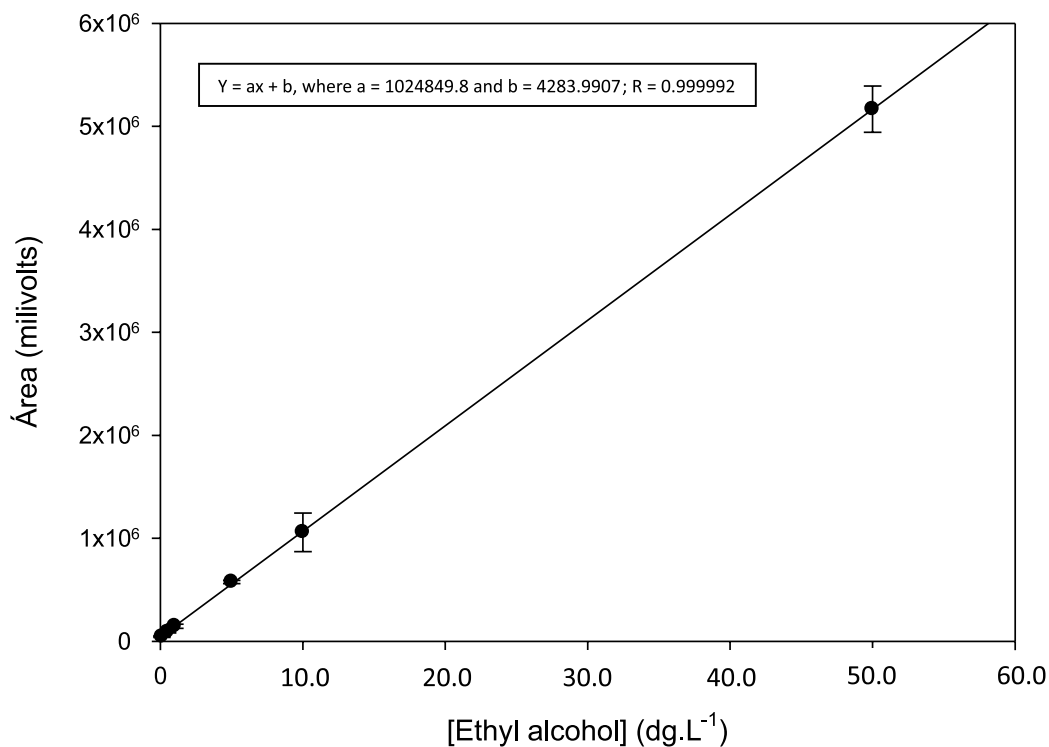
measured over the time necessary for the assay performance. For practical reasons, the ease of use of the method in a given application must be considered (18-19).

The classic technology of HS injection eliminates the extensive sample preparation, which decreases technician

Y = ax + b, where a = 1024849.8 and b = 4283.9907; R = 0.999992

Figure 2: Representative curve calibration of ethyl alcohol in blood sample. The concentrations ranged from 0.1 to 50.0 dg.L^{-1}. Minimum of three experiments.
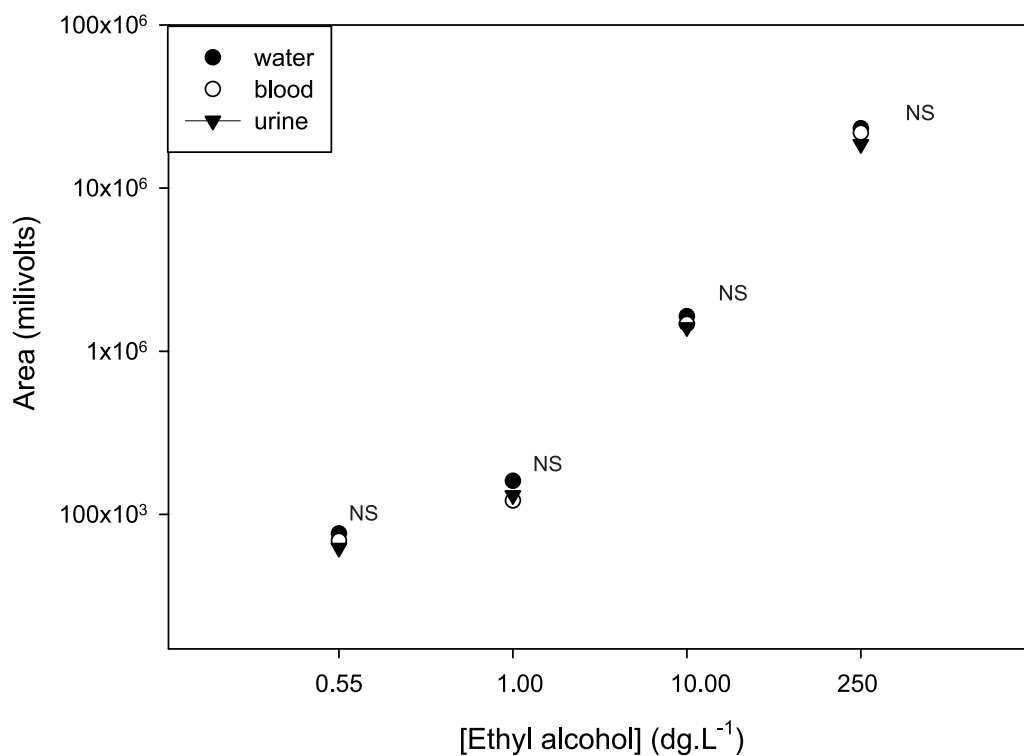

Figure 3: No serial fortified samples (water distillated, blood and urine) with ethyl alcohol at concentrations ranging from 0.50 to 250.00 dg.L^{-1}. Minimum of three experiments. NS = no statistical significance between tested matrices by One-Way analysis of variance in relation to matrices (water, blood and urine).
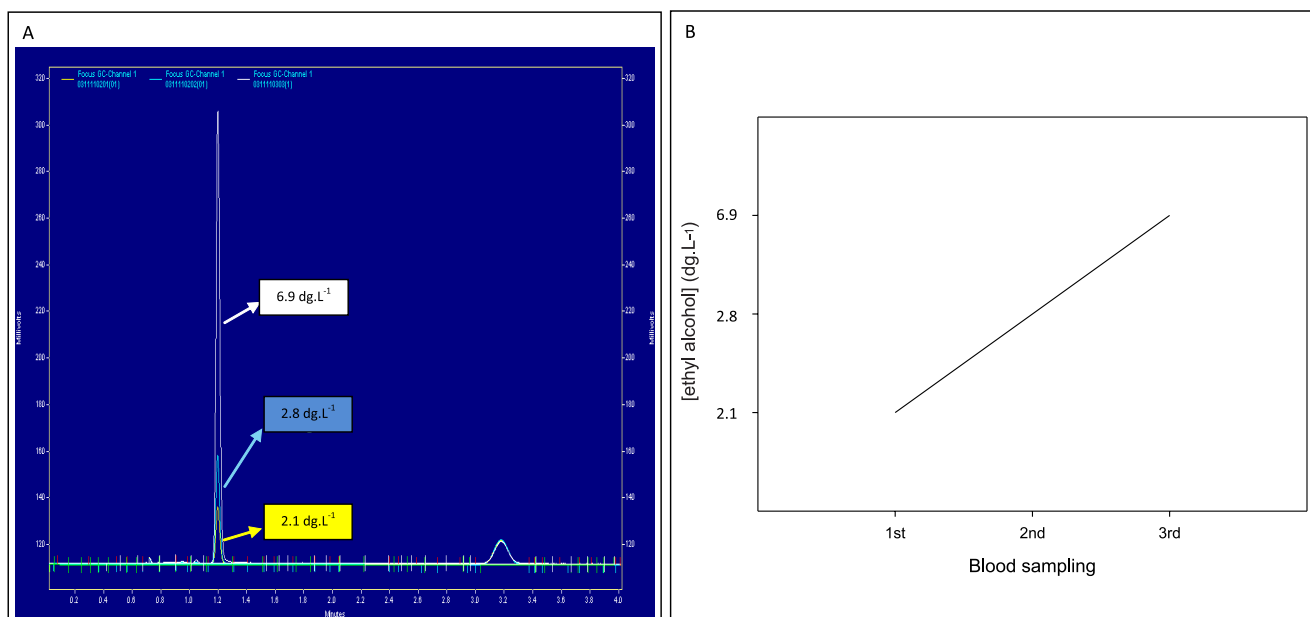
Figure 4: Typical chromatogram (A) and respective plot (B) from blood samples of another volunteer who participated in the protocol of controlled exposure study. The second peak is isobutanol (1.6 dg.L^{-1}) as internal standard (IS).

analysis time and provide more "clean" injections, without interferents or analytical artifacts. The automation and our short run times results (110ºC/2.0 min.) enhance convenience, velocity (5 times faster and when compared with RM) and efficiency of use. A similar principle using partition ratios was described for urine ethanol determinations (RM), but our method presented best areas results (2, 12 and 20).

The method presented is a rapid (7.0 minutes: 2.0 min. – HS and 5.0 min. - chromatographic development), accurate (CVs< 3.50%), sensitive (0.5 and 0.1 dg.L^{-1} to LOQ and LOD, respectively) and automated technique for the simultaneous measurement of whole BAC *in vivo* human studies as demonstrated in the volunteers evaluated, plus other OVCs. In relation of RM (2 and 11), the total analysis (HS extraction and chromatographic development) was 4.3 times faster and 10 times more sensitive (by RM confront). According to these findings, this method has higher sensitivity (10 times – LOD) and best correlation coefficient (R) if compared to the similar Serbian controlled *antemortem* study. Together with the absence of matrix effect, all these analytical properties contribute to the implementation of this method (13 and 21).

The stability of blood samples with preservative (1% sodium fluoride) did not alter BAC levels when compared with the initial measurements, until analysis (24 hours). These results increase the degree of certainty that alcohol did not suffer significant changes in the concentration with respect to time and storage temperature, due to a possible increase in bacterial putrefaction and its endogenous production. Again, our findings were superior to the latter authors who mentioned stability problems to blood samples without preservatives, time and temperatures storage (22).

The method developed in this study was successfully applied in the exposed volunteers to controlled doses, and due to its large range of work concentrations (0.500 to 250.0 dg.L-1), together with other analytical characteristics described above, this work presents versatility to different applications in toxicological analysis as forensic (in vivo and post mortem cases), social, and emergency procedures (22 and 23).With respect to quantification, important differences were observed at final concentrations by the construction of curve calibrators (CC) compared to the response factor (RF). The analytical chemistry/toxicology papers only describe just one criteria used. However, depending on the method quantification criteria and their application in toxicology areas (e.g.: forensic vs. emergency toxicological analysis). This step is very important, because it may have different implications that may affect the guarantee of wide legal defense procedures in cases that involve driving under ethyl alcohol influence and any cases of substances subject to regulation at different toxicology areas. Thus, this result opens a "new front in analytical toxicology discussion". The volunteers showed an increase in average levels of BAC of 0.19 ± 0.07 (1st), 0.34 ± 0.15 (2nd) and 0.60 ± 0.16 (3rd) and considering the zero-order toxicokinetic process of ethanol, we observed the occurrence of initial intoxication on the absorption phase, at concentrations above the tolerance limit established by current legislation in Brazil of 2.0 dg per liter of blood (tolerance limit). In fact, the project proposal was not characterizing the alcohol acute intoxication, but identifies the lowest BAC level that may already be associated to an alteration of the clinical and neurobehavioral efficiency. To help answer the question of this project, the developed method was sensitive to identify and quantify the BAC levels (7 and 24).

A zero-tolerance Law for Driving Under the Drug Influence

(DUDI) in Sweden led to a 10-fold increase in the number of cases submitted by the police for toxicological analysis. This phenomenon can probably be happening in Brazil with the enactment of law 11,705/08. Therefore, it is very important that the government provide accurate methodologies to identify and quantify the BAC levels in drivers (7 and 25).

The federal law 11,705/08, article 277 in its second paragraph that says, "the traffic officer can characterize the act of driving under alcohol influence by obtaining other evidence legal admitted, about the notorious signs of intoxication presented by the driver". However, the literature point to the need to strengthen the testing strategy, given the high subjectivity degree of the inspection agent (7 and 26). This subjectivity of diagnosis of alcohol exposure impacts the guarantee of wide legal defense. Therefore, it is essential that governments use technology and scientific methods to assess the BAC levels and its relationship to the act of driving. This method is a small contribution to this process.

In Brazil, in fact, beyond the likely subjectivity, there is no integrated health and security public policy for systematic collection of monitoring of BAC in driver occurrences and fatalities, and thus no reliable method to assess prevalence in toxicology emergency labs of public and particular health units and forensic institutes. This research is a contribution to reversing this situation considering that the method developed consorts satisfactory analytical properties with the simplicity and robustness of technology (GC-FID-HS), besides being one of the inexpensive instrumental strategies. Therefore, it is possible that it can be used in developing countries toxicology labs with low instrumental technology.

6. Conclusions

This method had the good analytical characteristics for its use in a wide range of cases involving the monitoring of the human performance under drunk. All analytical findings described previously, corroborate our developed method is viable and it is real application in various toxicology labs as in emergency or social toxicological analyses (simplicity, high velocity to produce results and sensitivity), ante and postmortem samples in forensic toxicology (specificity, sensitivity and wide working range).

Further studies will be conduct to evaluate the behaviour of this biomarker in a representative population, the comparison between the results produced by this biomarker (BAC), urine and breath in this controlled dose protocol, the inclusion of biomarkers of chronic alcohol use in the discussion of analytical strategies for different labs applications.

Acknowledgements

References

1. WHO (World Health Organization). Global Status Report on Road Safety. Geneve: WHO, 2013. Eng.

2. Alves, SR. Toxicologia forense e saúde Pública: desenvolvimento e avaliação de um sistema de informações como ferramenta para a vigilância de agravos decorrentes da utilização de substâncias químicas. [Tese] Rio de Janeiro: Escola Nacional de Saúde Pública, FIOCRUZ; 2005.

3. WHO (World Health Organization). Global status report on alcohol and health. Geneve: WHO, 2011. Eng.

4. Holmgren, A, Holmgrenm, P, Kugelberg, FC, Jones, AW, Ahlner, J. High re-arrest rates among drug-impaired drivers despite zero-tolerance legislation. Accident Analysis and Prevention 2008; 40: 534-540.

5. Paul Marques, P, Tippetts, S, Allen, J, Javors, M, Alling, C, Yegles, M, Pragst, F, Wurst, F. Estimating driver risk using alcohol biomarkers, interlock blood alcohol concentration tests and psychometric assessments: initial descriptives. Addiction 2009, 105: 226-239.

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. Analytica Chimica Acta 2004, 522: 163-168.

7. Brasil. Law 11,705/08 of june 19, 1988.

8. Jones, AW. A Rapid Method for Blood Alcohol Determination by Headspace Analysis Using an Electrochemical Detector. Journal of Forensic Sciences, 1978, 23(2): 283-291.

9. Tagliaro, F, Lubli, G, Ghielmi, S, Franchi, D, Marigo, M. Chromatographic methods for blood alcohol determination Journal of Chromatography B: Biomedical Sciences and Applications 1992, 580 (1-2):161-190.

10. INMETRO (INSTITUTO NACIONAL DE METROLOGIA, QUALIDADE E TECNOLOGIA). DOQ-CGCRE-008. Orientação sobre validação de métodos analíticos. 3ª ed. Rio de janeiro: INMETRO, 2010. Port.

11. ABNT (ASSOCIAÇÃO BRASILEIRA DE NORMAS TÉCNICAS). Requisitos Gerais para competência de Laboratórios de Ensaio e Calibração. Brasília: ABNT, 2005. Port.

12. Aquino-Neto, FR e Nunes, DSS. Cromatografia – Princípios Básicos e Técnicas Afins. 1ª Ed. Rio de Janeiro: Interciência. 2003.

13. Corrêa, CL. Validação da urina para análise toxicológica do etanol em programas de controle e prevenção do uso de álcool e drogas no local de trabalho. [Tese] São Paulo: Faculdade de Ciências Farmacêuticas. Universidade de São Paulo, USP: 1997.

14. Microsoft Office Package [computer program] version 2007. Redmond, WA, USA: Microsoft Corporation, 2007.

15. Sigma Plot for Windows™ [computer program] version 10.0.0.54. Chicago, Illinois. USA: Jandel Scientific co., 1997.

16. EndNote for Windows™ [computer program] version 1.0.1. New York, USA: Thompson Reuters Inc., 2011.

17. Primer for Windows™ [computer program] version 3.0. New York, USA: McGraw-Hill Inc.,1992.

18. Moreau, RLM. e Siqueira, MEPB. Ciências Farmacêuticas – Toxicologia Analítica. 1ª Ed. Rio de Janeiro: Guanabara Koogan. 2011.

19. Aquino-Neto, FR e Nunes, DSS. Cromatografia: Princípios básicos e técnicas afins. 1ª Ed. Rio de Janeiro: Interciência. 2003.

20. Jones, AW. A Rapid Method for Blood Alcohol Determination by Headspace Analysis Using an Electrochemical Detector. Journal of Forensic Sciences 1978, 23 (2): 283-291.

21. Jain, NC. Direct Blood-Injection Method for Gas Chromatographic Determination of Alcohols and Other Volatile Compounds. Clinical Chemistry 1971, 17(2): 82-85.

22. Stojan Petković, S., Savić, S., Zgonjanin, D. and Samojlik, I. Assessment and detection: ethanol concentrations in antemortem blood samples under controlled Conditions. Alcohol & Alcoholism 2008, 43 (6):658-660.

23. Büyük, Y., Eke, M., 2, Cagdir, AS. and Hicran K. Karaaslan, HK. Post-mortem alcohol analysis in synovial fluid: an alternative method for estimation of blood alcohol level in medico-legal autopsies? Toxicology Mechanisms and Methods, 2009, 19(5): 375-378.

24. McCarver-May, DGM and Lubica Durisin, L. An Accurate, Automated, SimultaneousGas Chromatographic Headspace Measurement of Whole Blood Ethanol and Acetaldehyde for Human In Vivo Studies. Journal of Analytical Toxicology 1997, 21:134-41.

25. McCarthy, ML., Sheng, P., Baker, SP., Rebok, GW. and Li, G. Validity of police-reported alcohol involvement in fatal motor carrier crashes in the United States between 1982 and 2005. Journal of Safety Research 2009, 40:227-232.