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Development and validation of a quantitative determination method of ethanol in Human Plasma: Experience of Laboratory of Toxicology and Pharmacology, Moroccan Poison Control and Pharmacovigilance Center

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ABSTRACT: Acute intoxication and chronic ethanol is recognized by specific symptoms and needs to be confirmed by blood or plasma ethanol dosage. Although, the aim of our paper is to develop and validate a method in the order to determinate the amount of blood ethanol, using gas chromatography with flame ionization detection (FID), after a Headspace sampling. We used butanol as an internal standard, our method is specific and linear at the range amount from 0,1g/L to 5 g/L. the coefficient of correlation was 0.9998. Using the quality control from standard solution we determined the coefficient of variation and recovery percent, compared to the theoretical amount (25%; 50% and 75% of the maximum amount). The coefficient of variation was within 4.046%; 9.682% and 3.553 % at concentration of 0.3; 2.5 and 4 g/L, respectively. The limit of detection was 0.063 g/L. In the same conditions, limit of quantification was 0.085 g/L. The storage's and transport's samples were at +4°C for one week as a maximum. The accuracy of our method was 96.75 % for a concentration of 2 g/L. The overall results show that the proposed bio-analytical procedure is acceptable and it is purpose for which it is intended, determination of ethanol in plasma.

KEYWORDS: Ethanol; plasma; blood; Morocco, chromatography; Headspace; validation.

1 INTRODUCTION

The effects of alcohol on health today are a heavy public health problem. Alcohol is often implicated in fatal road accidents in Morocco [1], [2]. The alcohol abuse is one of the most commonly encountered poisonings [3], [4]. The consequences of the use of alcoholic beverages on health, however, depend on the susceptibility of the consumer as well as the way and especially of its consumption.

Ethanol is the most common analyte identified in forensic toxicology laboratories [5]. In Morocco, the Laboratory of Toxicology and Pharmacology Poison Control and Pharmacovigilance Centre of Morocco (CAPM) contributes via its laboratory of toxicology and pharmacology to the orientation of the clinician in different intoxication's situation it faces, through the development of protocols for detection metering and xenobiotic. Among these protocols is found that the ethanol in plasma.

There are numerous analytical methods for determination of alcohol content already available. These techniques vary greatly in their preparation, accuracy/precision, use, cost, environmental and overall practicality[6], [7].The Headspace Gas Chromatographic (GC-HS) analysis has become a worldwide modern method for determination of ethanol in blood or other body fluids (urine, vitreous humor). Direct injection and headspace analysis are the most used techniques, but headspace analysis has important advantages: protected column and injector so that contamination will not occur [8].

However, the initiation of such types of analytical method requires a validation. To prove that the protocol is sufficiently accurate and reliable, and to have confidence in the results for a specific purpose.

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Indeed, the present work has the objective to develop and validate a technique to quantify ethanol in human plasma by gas chromatography with a flame ionization detector and using the Headspace as an injection technique [9], [10].

2 MATERIAL AND METHOD

2.1 APPARATUS

Experimentation was done using a gas chromatograph Agilent 6890 Series GC System GC System(USA), coupled with a Flame Ionization Detector (FID), Autosampler (Agilent 7683 Series Injector)and hydrogen generator Agilent S184-3503, equipped HP5 Agilent capillary column (30 m x 0.32 mm id, 1.5 µm film). The result analysis and data integration have been done with ChemStation soft.

2.2 REAGENTS

- Ethanol (Reide-deHaen 0,788g/ml).
- Butanol (Probus de 0,807 g/ml).
- Purified water
- Blank Human plasma
- Methanol (SIGMA-ALDRICH 0.790g/ml)
- Propanol (SIGMA-ALDRICH 0.804 g/ml)

2.3 GAS CHROMATOGRAPHY ANALYSIS

Standard solutions of ethanol and butanol (internal standard) were prepared. The relative response factor (RRF) between ethanol (EtOH) and butanol (BuOH) was determined by mixing 1000 µl of the standard solution or the calibrator with 150 µl of the internal standard (4g/L). Samples were directly injected into the GC-FID using the Headspace. The curve calibration was determined by plotting the ratios of the peak area for EtOH to BuOH in each sample (y-axis) against the standard concentration of EtOH (x-axis).

2.4 GAS CHROMATOGRAPHY AND HEADSPACE CONDITION

2.4.1 HEADSAPACE

2.4.1.1 HEATING

- Vial : 70°C
- Loop (: 80°C
- transfert line: 90°C

2.4.1.2 TIME

- Cycle time : 13.5min
- Vial equilibrate Time : 20min
- Pressurization time : 0.15min
- Loop fill time : 0.15min
- Loop equilibrate time : 0.05min
- Injection time : 0.50min

2.4.1.3 PRESSURE

- vial pressure : 15 Psi
- Carrier gas pressure : 2,2 Psi

2.4.2 GAS CHROMATOGRAPHY

The GC run time was 6 minutes. Oven temperature was isothermal 40 °C and pressure was isobaric 30 psi. Azote was used as the Makeup gas. FID was used on each column with a temperature of 250 °C. Gas flow through detector was 400 mL/min of air 40.0 mL/min of hydrogen and 30 ml/min of Azote.

3 RESULTS AND DISCUSSIONS

3.1 SPECIFICITY AND SELECTIVITY

3.1.1 SELECTIVITY

Under the given conditions, the method is specific for ethanol, every time we obtained The same retention time (RT); 1.665 min for specific peak of ethanol, 1.598 min for Methanol and 1.727min for the peak of Propanol (fig1). The same retention times prove the selectivity of columns.

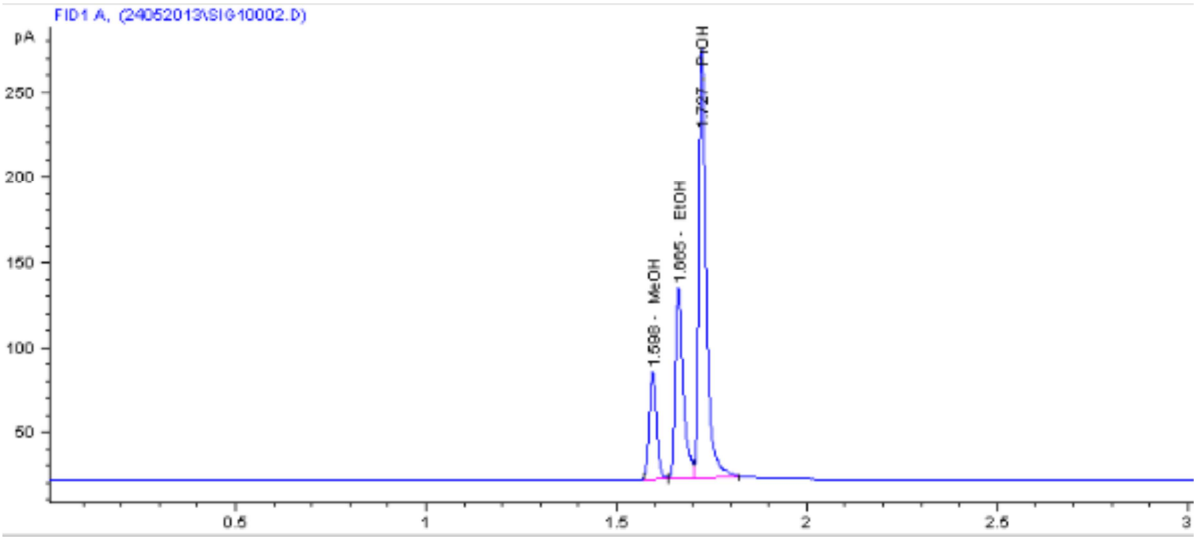

Fig. 1. Chromatogram of a mixture of ethanol, methanol and propanol.

The resolution factors (R) and the selectivity factors (α), calculated for the two couples (EtOH/MeOH and EtOH/PrOH) was greater than 3 and 1 , respectively proving a very good separation of the peaks (table 1).

Table 1. Results of the study of selectivity

	RT(min)	T0 (min)	ω(min)	Selectivity α	Resolution R
methanol	1,598	0,024	0,0204	1,042 1,038	3 ,199 3,017
ethanol	1,665		0,0215		
propanol	1,727		0,0196		

3.1.2 SPECIFICITY

The specificity of the method was studied, to determine the matrix effect on the determination of ethanol. A comparative analysis was carried out for the blank sample (Blank plasma + IS). On the basis of the chromatograms obtained no significant response at the retention time of ethanol (1.665 min) Fig2.

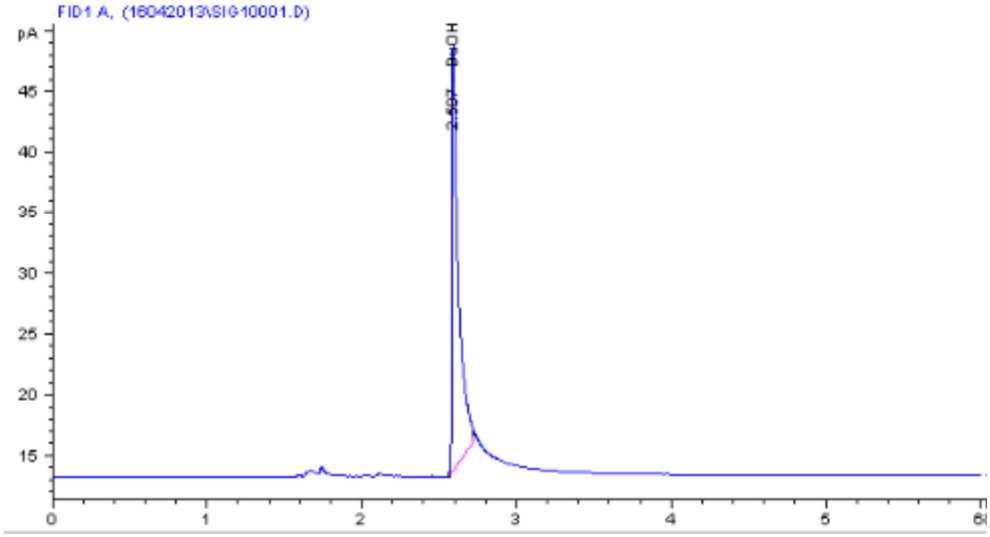

Fig. 2. Chromatogram of blank sample (blank plasma + IS).

3.2 LINEARITY

The Linearity was evaluated by analyzing the prepared ethanol standards. Six levels of ethanol solution witch concentrations of 0; 0.1; 0.5; 1; 2 and 5 g/L, are prepared in blank plasma. Then 150 µl of Butanol solution water as internal standard with concentration 4g/l is added into 1000 µl of each level in 10 ml headspace vial.

Samples were directly injected into the GC-MS using a 0.2 µL split injection 10:1 at 250˚ C. Each sample was repeated 3 times. The calibration curve was determined by plotting the ratios of the peak area for ethanol to butanol in each sample (y-axis) against the standard concentration of ethanol (x-axis). Fig3.

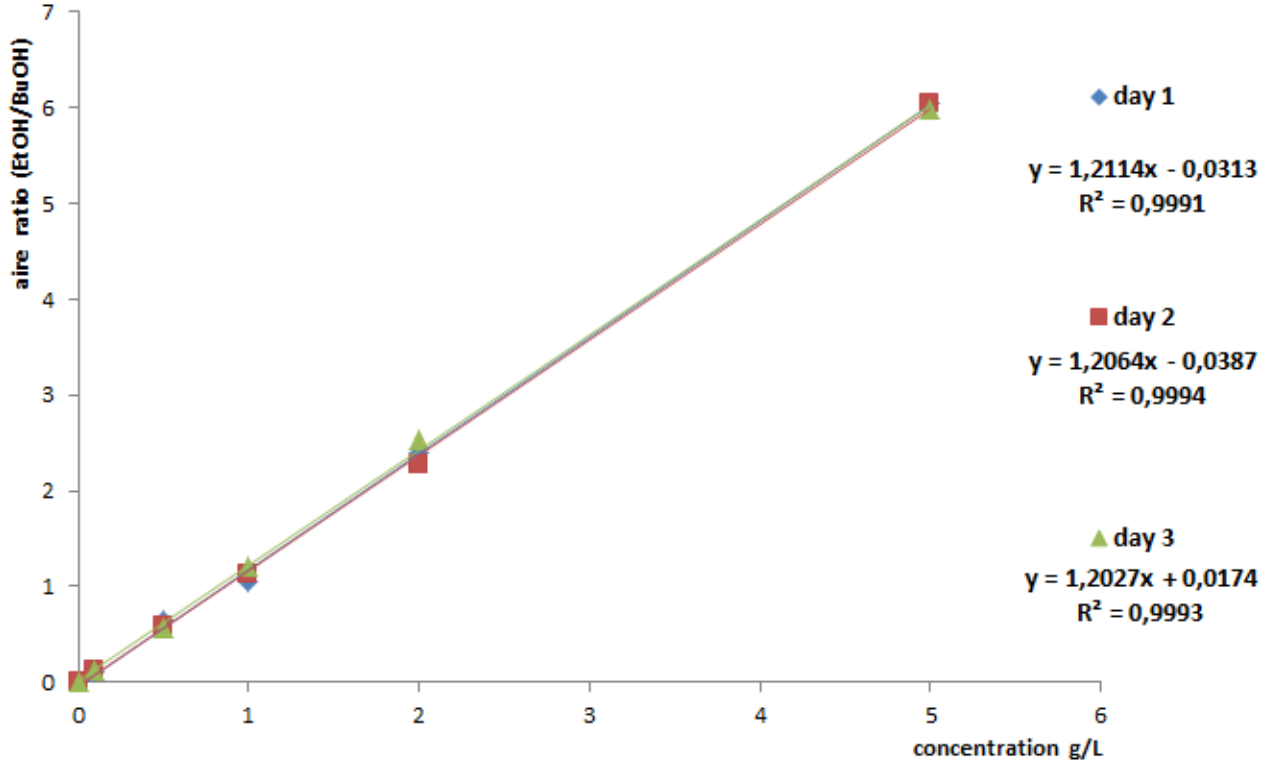

Fig. 3. individual calibration curves obtained for the three injections

Figure 3 shows that there is no significant difference between the three calibration curves. This continued Linearity allows us to use the equation of the average of the three calibration curves (Fig4).

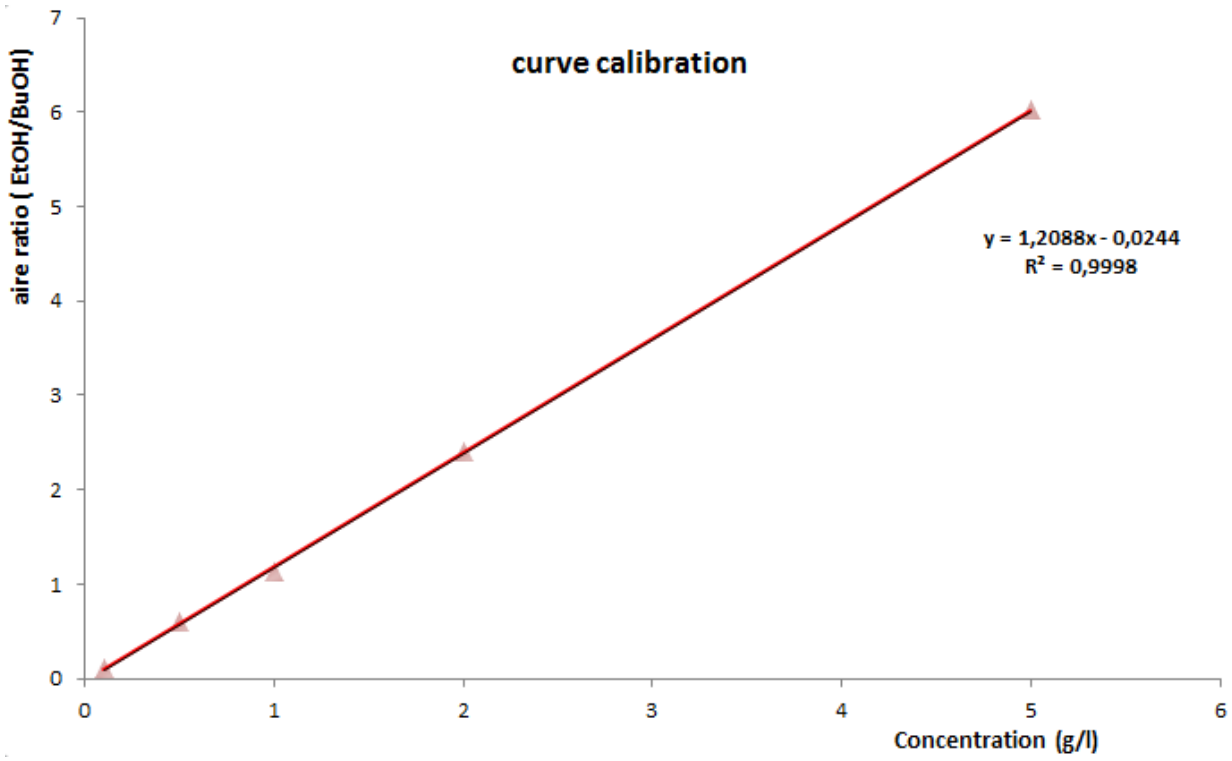

Fig. 4. Calibration curve average of 3 injections

The method was shown to be linear with a coefficient of correlation (r^2) of 0.9998 for ethanol from 0.01to 5.0 g/L.

3.2.1 QUALITY CONTROL

To assess the accuracy and correctness of linearity, it was necessary to prepare three quality controls of concentrations 0.3; 2.5 and 4 g / l. calculated concentrations (C_{cal}) and their corresponding relative errors (RE) are listed in Table 2.

Table 2. Results of quality control

CQ (g/l)	C_{cal} 1	C_{cal} 2	C_{cal} 3	Average	RE %
0,3	0,297	0,295	0,271	0,288	4,046
2,5	2,091	2,330	2,353	2,258	9,682
4	3,693	3,972	3,908	3,858	3,553

On the basis of the Table 2, the relative errors (RE) calculated for the average of three injections was 9.68% as the maximum value which is below 10%. Therefore the results found in the study of the linearity are acceptable.

3.3 EVALUATION OF THE LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

The limit of detection (LOD) and the limit of quantification (LOQ) are determined according to the procedure described by Accreditation Program Laboratory Analysis [11].

The limit of detection (LOD) and the limit of quantification (LOQ) were obtained experimentally, after the analysis of 20 test materials related to blank solution (plasma + internal standard).The LOD and LOQ were calculated to be 0.06 g/mL and 0.1 g/mL, respectively.

3.4 REPEATABILITY AND REPRODUCIBILITY

The reproducibility and repeatability of the method have been evaluated by calibration curve method (residual standard deviation σ), by injecting the six replicate injections of standard concentration (2 g/L) in three different days using the same concentration for solutions under the same experimental conditions [12]. In this case practical concentration of solutions was appropriate to theoretical concentration. The results of system repeatability and reproducibility are depicted in Table 3.

Table 3. Results from the study of repeatability and reproducibility

day	**Day 1 (repetability)**	**Day 2**	**Day 3**
calculated concentrations g/L	1,986 1,847 1,918 2,037 1,915 1,839	1,993 1,972 1,886 1,953 1,993 2,212	2,098 1,872 1,707 1,849 1,766 1,995
σ_L(repeatability)	0.077	--	
CV_L(repeatability)	4.032%		
σ_R (reproducibility)	0.114		
CV_R(reproducibility	4.932%		
Bias %	3.25		
Recovery percent (TR%)	96.75		

The difference between results was insignificant and the value for the coefficient of variation (CV), for all levels was less than 10%. On the basis of the results obtained, we observed a good repeatability and a good reproducibility

3.5 PRECISION AND ACCURACY

The accuracy of the method was calculated in terms of the percent bias (Bias %) and recovery percent (RP), based on the results found in the study of the reproducibility (theoretical concentration 2 g/L). Low values of bias (3.25%) and high value of the recovery percent (96.75%) indicated a good precision and accuracy of the proposed method.

3.6 STABILITY

In order to determinate the stability of ethanol in plasma. The evolution of ethanol in plasma was studied for a concentration of 2g / L in three different storage temperatures (-20 ° C, + 4 ° C and 23 ° C).Table 4.

Table 4. Results from the study of the stability

day	**T=-20 °c**	**T= +4 °c**	**T = 23 °C**
1	2,053	2,091	2,148
4	2,006	2,074	2,096
12	1,879	1,828	1,987
14	1 ,886	1,876	1,9629
18	1,805	1,773	1,759
20	1,691	1,747	0,102

On the basis of the calculated concentration obtained, the curve stability was plotted. The study of the stability has allowed us to choose the optimal routing and sample storage conditions, preferably at 4 ° C and the maximum retention period (one week). (fig5).

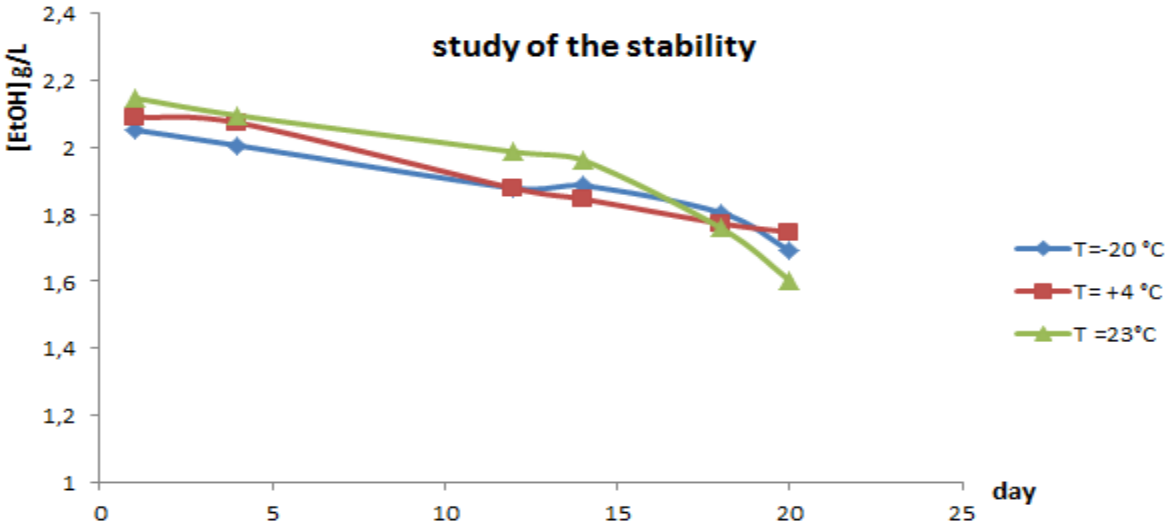

Fig. 5. Curve stability study of ethanol 2g / L

4 CONCLUSION

A novel method has been validated for the quantitation of ethanol by HS-GC–Fid. The set of criteria for method validation have shown that the performance achieved is in accordance with the specifications in advance.

In this respect this method was shown to possess all the characteristics of a solid analytical method. The method was shown to possess excellent sensitivity, selectivity, repeatability, robustness, linearity, and ease of use. This technique can be used as a method for the determination of ethanol in the plasma in the Laboratory of Toxicology and Pharmacology Poison Control and Pharmacovigilance Centre of Morocco (CAPM)

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