The Importance of Alcohol Testing by Gas Chromatography *vs* the Cordebard Classical Method Modified in the Medico Legal Investigation

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Considering the growing number of requests from the criminal investigations authorities addressed to the institutions of legal medicine, testing of blood alcohol concentration both in the living person and in the corpse, we believe that a presentation of the two methods which are used in our country, is a topic of interest at present. The purpose of this article is to provide the reader with the technical details on how blodd alcohol concentration is realised by means of the gas chromatographic method and the classical one, (Cordebard modified by D. Banciu and I. Droc) respectively. Another purpose of this article is to also show, in a comparative way, the elements that make the gas chromatographic method superior to the former one.

Keywords: blood alcohol concentration, Gas Chromatography, Cordebard classical method modified by D. Banciu and I. Droc.

Determining the value of alcohol in the living people and corpses is a routine scientific activity in forensic practice. Recently, there has been an increase in the number of these determinations, especially in the process of finding evidence of offenses committed by drivers, or even in rarer cases [1-6].

In our country, following the Decision no. 5 of 15 June 2018 issued by the Superior Council of Forensic Medicine, the gas chromatographic method has been implemented as an official method for the determination of blood alcohol concentration in living people and corpses [7-17]. However, until 01.01.2019, both methods - the gas chromatographic method and the classical one, Cordebard modified by D. Banciu and I. Droc [5] will be used, whereas from this date on, only the first method of determination will be employed in Forensic practice [6-10].

Experimental part

Quantitative determination of ethyl alcohol in biological samples by headspace gas-cromatography with flame ionization detector

In the following subchapter, we will describe the principle that we based our work on and the way in which we proceeded for identifying and determining ethylic alcohol and other volatile organic substances, by using gas chromatography with flame ionization detector, with the Headspace method. The method will use tert-butanol as an internal standard.

The principle of the method

Volatile compounds are analysed within biological fluids (blood, urine, vitreous humour, cerebrospinal fluid) with a gas chromatograph using the internal standard. The vial containing the biological fluid and the internal standard is hermetically sealed with a lid provided with a teflon liner and placed in a device called the headspace unit where it is thermostated at a programmed temperature. The volatile components in the sample are evaporated partially until balanced is reached, thus forming a gas mixture which has a constant composition over time in the atmosphere above the sample and reflecting its composition. The *headspace*, through a series of programmed activities, takes up a determined volume of gas above the sample and transfers it under the action of the carrier gas into the gas chromatograph injector and hence passes into the column.

Along the chromatographic column, the sample components are separated by specific interactions with the stationary phase deposited on the walls. At the exit of the column, the sample components come out in turn and enter the flame ionization detector. In the detector, the sample components, together with the carrier gas, are passed through a hydrogen flame where they are burned and thus electrically charged particles result. The detector's response to the passing of a component is a very weak electrical current, the intensity of which is proportional to the concentration of that particular component in the sample.

The detector signal is graphically recorded over time and thus a chromatogram is obtained, to identify the alcohol and determine it quantitatively.

Equipment and materials

The equipments and materials involved in the research we mention:

-Gas chromatograph of general usage, which is equipped with one or two flame ionisation detectors (FID) and a computer on which the software for data acquisition and processing is installed.

-One or two chromatographic capillary columns dedicated to analysing volatile organic compounds in the biological samples (ethyl alcohol, methyl alcohol, acetone, acetaldehyde, isopropanol).

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-A Headspace unit used for analysing the samples by the vapor space method, equipped with an autosampler.

-Crimper type stapler (for fixing and sealing caps on sample bottles).

-For the homogenisation of blood samples, an ultrasound homogenizer or a vortex type homogenizer may be used.

-Analytical balance

-Automatic pipettes with a capacity of 100 ± 1000μL, 10-100 μL and 1000-5000μL

-Peaks of automatic pipettes (1000μL, 5000 μL)

-20 mL vials (sample vials) which are used for preparation of samples.

The following reactives were used:

-Distilled water,

-Certified standards of aqueous alcohol in various concentrations.

-Certified standards of whole blood alcohol in various blood concentrations.

-tert-Butanol purity over 99.5%

-ethyl alcohol

-ammonium sulphate p.a.

The chromatographic system consists of a gas chromatograph and a head space unit. The Parameters for gas chromatograph used are: Oven temperature (isotherm); splitting rate; gas pressure at the head of the column (constant); gas carrier; make-up; hydrogen flow rate for FID; air flow for FID; temperature on FID detectors; capillary columns.

The parameters set for head space are: oven temperature; loop temperature; transfer line temperature; GC cycle time; Vial Equilibration time; pressurization time and the injection time.

Solutions prepared in the laboratory

The internal standard (tert-butanol) concentration may range from 0.15 to 0.40‰. As example, in order to obtain an internal standard solution of 0.15‰ tert-butanol, weigh the analytical balance into a 25 mL volumetric flask containing approximately 8-10 mL of deionized water (t-butanol density 0.775 g/mL) 0.15 g of tert-butanol, are added 10 mL of water and homogenized. The solution thus obtained is introduced quantitatively into a 1000 mL volumetric flask containing about 700 mL of solution containing 1 mole of ammonium sulfate. Make up and homogenize.

Calibration of the method

It is an essential operation in the validation process of a method of analysis and it is meant to find a linear mathematical relationship between the concentration of an analyte and the analytical sistem response. The mathematical relationship given by the equation of the line: y (peak area) = a + bx (x-analyte concentration) is used to calculate the analyte concentration in the samples to be analyzed. Calibration is performed by recording the analytical signal for a minimum of seven standard solutions (certified) of alcohol, in aqueous solution or in whole blood. For the construction of the calibration line, standard certified solutions of alcohol in water or in whole blood are used.

The calibration sequence is as follows:

- sample blank (example: 250 μL ultrapure water with 1750 μL internal standard),

- standard solutions, in increasing order of concentrations,

- sample blank (example: 250μL ultrapure water with 1750 μL internal standard).

The calibration curve is built and automatically integrated. A correlation coefficient of at least r = 0.999 is acceptable.

After performing the calibration curve, check the calibration curve, with certified standards of ethanol in whole blood. The result of the calibration is printed and archived.

Sample preparation for analysis

The steps for the sample preparation are the following:

-Headspace vials are numbered or marked to identify samples.

-Place 1750 μL of internal standard solution into the test vials and add 250 μL sample to this solution.

-Seal the vial with a metal cap.

-In the same way, blank samples and control samples are prepared.

-Seal the bottle with a metal cap.

-In the same way, blank samples and control samples are prepared.

-Place the prepared flasks in the headspace autosampler in the specified order.

-Chromatograms are printed and archived.

Results and discussions

Acceptance criteria

In accordance with a large number of published operating standards, the following acceptance criteria for the determination of ethyl alcohol in biological samples are proposed for controls and respectively for the samples to be analysed.

For controls, the confidentiality rates of the main values mentioned in the quality certificates of each control will be accepted. If the results obtained on the control samples do not fall within the acceptance limits, their analysis is restored. If the non-compliant results are repeated, the calibration curve is restored.

For samples to be analyzed, **i**n the case of two-column equipments, the arithmetic mean shall be calculated and the result given by 2 decimal places. In the case of equipments with one column the arithmetic mean of the values of the two injections of the same sample shall be calculated and the result shall be two decimal places. A maximum difference between the results of the 2 chromatographic columns of ± 0.05g‰ is acceptable.

In case of the *blood collected from people*, if the alcohol concentration is greater than 0.15 g‰, will be reported as such in the analysis bulletins. Concentrations of alcohol less than or equal to 0.15 g‰, shall be reported in the bulletin at a lower (or equal) value of 0.15‰ - the result is considered to be negative. In the case in which the *blood is collected from the corpse*, an alcohol concentration greater than 0.20 g‰, will be reported as such in the analysis bulletins. Concentrations of alcohol of less than or equal to 0.20 g‰ shall be reported in the bulletin as follows: l*ess than (or equal to) 0.20‰ - the result is considered to be negative.*

Quantitative determination of alcohol in biological products

Isolation of the biological product

For the distillation of *blood*, 5 mL accurately measured blood, are brought into a 1000 mL wide neck distillation flask, than 45 mL of 2.5% succinic acid solution are added. The flask need to be added to a Vigreux distillation column, continued with a descending refrigerant, fitted with a flattening. The distillate is collected in a 25 mL flask, dropping 2-3 drops of distilled water into which the alonge end is inserted. After that the distilling starts (warming the flask on an asbestos screen) and are collected 25 mL of distillate.

In the case of *urine*, we need to place exactly 5 mL of urine in a distillation flask and continue the proceses, as in the case of the blood sample.

For *organs*, a quantity is thawed with purified sand and passed to the distillation flask, repeated several times with distilled water. After this, succinic acid is added and the process continues, similar to the blood one.

To be careful, iIn cases where there is less than 5 mL, take all existing material (1, 2, 3, 4 mL), make up to 50 mL with succinic acid and collect a distillate Niolum 5 times the amount of material put into work. (Example: 3 mL sample + 27 mL succinic acid, distilled = 15 mL).

Cordebard nitrochromic method modified by D. Banciu and I. Droc

Compared to the Nicloux method [5, 6], it has the advantage that oxidation is made stoichiometrically in a well-determined time and is more specific for ethyl alcohol.

The principle of the method consists in isolation of the ethanol by distillation, than oxidized at room temperature to acetic acid by a nitro-chromic mixture. The excess of the dichromate is iodometrically recovered.

$$3CH_3CH_2OH + 2K_2Cr_2O_7 + 16HNO_3 = 3CH_3COOH + 2Cr_2(SO_4)_3 + 4KNO_3 + 4Cr(NO_3)_3 + 11H_2O$$

$$K_2Cr_2O_7 + 6KI + 14HNO_3 = 2Cr(NO_3)_3 + 8KNO_3 + 3I_2 + 7H_2O$$

$$I_2 + 2Na_2S_2O_3 = 2NaI = Na_2S_4O_6$$

The reactives involved in the processes are: nitric acid (ρ = 1.4 - 1.42g/cm^3), devoid of reducing substances (Merck); Potassium iodide solution 2% (freshly prepared) and aqueous solution of potassium dichromate N/6,9 (0.144 N). Exactly 7.1065 g of potassium dichromate is required, previously sprayed and dried at 125 - 150°C to constant weight, than is brought to a 1000 mL volumetric flask, dissolved in distilled water and then is filled to the mark. It needs to be kept in the dark in bottles with a stopper. The sodium thiosulfate aqueous solution N/23 (0.0435 N) is used, being weighted 11g of sodium thiosulphate with five water molecules ($Na_2S_2O_3$-$5H_2O$), dissolved in distilled water, than needed to be added 0,10 g of anhydrous sodium carbonate in a 1000 mL volumetric flask and filled up to the mark. The solution is left for at least 5-6 days in the dark. It is needed to be checked daily. *Establishing the corection factor* of the thiosulphate solution is carried out titrating it with respect to the potassium dichromate solution (0.144 N). In this case the steps of preparation are: 5 mL of distilled water are poured in a iodometric flask, than 3 mL of potassium dichromate solution exactly measured with a microbiuret is added, after that 4 mL of nitric acid and then 20 mL of 2% potassium iodide solution are added. Top the vial and titrate with the tiosulfate N/23 solution after 3 min.

The correction factor is calculated accordingly:

$$F = 10/n,$$

where: 10 = mL potassium dichromate ($K_2Cr_2O_7$) N/23, corresponding to the 3 mL solution of potassium dichromate N/6.9 in working order. n = mL of thiosulfate consumed at titration. Typically 3 L of mother liquor is prepared.

Dosing of the sample: 5 mL of distillate is brought into a iodometric flask for titration, than 3 mL of potassium dichromate (N/6,9) is added with 1/100 microtiter, then 4 mL of nitric acid is poured. The vial needs to be closed and leave the solution for 15 min, time necessary for oxidation of ethyl alcohol. The oxidation time should be rigorously respected (an extension of it allows the oxidation of acetone, ether and other volatile compounds). Than we need to add 20 mL of 2% potassium iodide, close the vial, after 2-3 min titrate the iodine released from the potassium dichromate extract with the sodium tiosulfate solution N/23.

Calculation of the amount of alcohol expressed in grams, contained in 1000 mL of blood, is given by the formula:

$$C_{g‰} = (10 - n \text{ xF})/2,$$

where: 10 mL of $K_2Cr_2O_7$ (N/23) solution to which corresponds the 3 mL of potassium dichromate solution taken to work, capable of oxidizing 5 mg of ethyl alcohol; N = mL sodium thiosulphate N/23 used for titration, multiplied by the correction factor of the thiosulfate solution.

Conclusions

Based on the presented information we can draw the following conclusions:

-The gas chromatographic method is more accurate and precise than the classical method, as it does not interfere with the presence of other alcohols. In the classical method, for example, one cannot distinguish between methyl and ethyl alcohol.

-Alcohol levels in the penal limit are more rigorously established due to lower error than the classical method.

-The amount of biological material (blood) required for analysis is much lower (about 100 times) in the gas chromatographic method than in the classical one.

-Human error is much lower in the gas chromatographic method. In the classical method, the result depends on the visual evaluation of the operator, which gives a degree of subjectivity, and cannot be found in the gas chromatographic method.

-In the gas chromatographic method, the reports for each analysis are kept in the device memory. In the classical method, the results are written in a register, and cannot be checked retroactively.

-The gas chromatographic method is instead more expensive than the classical one, as the disposables needed to make use of this method involve higher costs.

-The gas chromatographic method, considering by all the above-mentioned elements, has undoubtedly a superior value for evidence collection than the Cordebard method modified by D. Banciu and I. Droc.

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Manuscript received: 16.02.2018