ETHANOL (ETHYL ALCOHOL) in SERUM and/or in PLASMA by UV – CODE Z05810

BIOCHEMISTRY AND METABOLISM

The ethyl alcohol is an aliphatic alcohol of PM=46.07.

Usually it is assumed orally and quickly absorbed from stomach, small intestine and colon. The time necessary in order to complete the different process of absorption is from 2 to 6 hours, it varies with different factors such as the presence of food and other liquids, the time employed for the ingestion of the drink, the biological variability among individuals.

The alcohol, for its solubility in water and the low molecular weight, as soon as absorbed is quickly distributed in tissues and fluids of the organism, exceeding also the encephalic barrier and the placental one. The course of the alcohol concentration in some biological liquids has been determined experimentally from several authors, also recently (Fig. 1). It has been demonstrated that the maximum plasmatic concentration is achieved after approximately 20 minutes from the assumption as well as salive and expired air, while in urines the maximum concentration is achieved after approximately two hours from the assumption.

![Fig. 1. Ethyl alcohol: concentration in the biological liquids](image)

After the absorption, the Ethanol is mostly metabolized (90-98%) for oxidative way, to a speed directly proportional to the corporeal weight constant in the time. The first reaction of oxidation occurs in the liver, for share of the enzyme Alcohol It dehydrogenates with NAD like coenzyme and Hydrogen accepter with Acetaldehyde production; this is subsequently oxidized to Acetic acid, due to the enzyme Aldehyde Dehydrogenates. The kinetic of elimination does not have an exponential course, like the major part of the exogenous substances, but is pseudolinear; after the completion of the phase of absorption and distribution, when the concentration value is maximum C0, the relation between whole blood concentration Ct and time t, it is given from the equation: Ct = C0 - kt, with k between 10 and 25 mg/dL/h (Fig.2).

![Fig.2. Urines and expired air represent also the main ways of elimination of the alcohol and its products of oxidation. The kinetic of elimination, altogether regulated from the equation of Michaelis Menten, assumes however a pseudolinear course after the completion of the phase of absorption and distribution.](image)
The excretion of alcohol not modified, usually, interests 2% of the assumed amount and mostly happens through kidneys and lungs, even if small amounts are found also in saliva and other organic liquids; it can go up until 10% in case of massive ingestion.

The urine concentration is little bit higher than the hemactic one, the alveolar one is approximately 0.05%.

The concentration in plasma is approximately 10% more than the entire blood

**ACUTE POISONING**

The excessive assumption of alcohol provokes, in the short terms, clinical situations of varied gravity that could call on the presence of the doctor of the First Aid, both for the direct effects of the drunkenness and for the indirect ones (car accidents).

The doctor could apply to the Laboratory for different aims such as:

- to assess one possible cause of coma, in patient without trauma cranial;
- to assess the cause of coma in patient with trauma cranial (for ex. as a result of car accidents);
- to diagnose drunkenness in patient with doubtful symptomatology.

It has been shown there is a relationship between Blood Alcohol Concentration- BAC and Drunkenness

A blood level of 0.5% or more is commonly fatal. Levels of even less than 0.1% can cause intoxication, with unconsciousness often occurring at 0.3–0.4%. The amount of ethanol in the body is typically quantified by blood alcohol content (BAC), the milligrams of ethanol per 100 milliliters of blood. The table at right summarizes the symptoms of ethanol consumption. Small doses of ethanol generally produce euphoria and relaxation; people experiencing these symptoms tend to become talkative and less inhibited, and may exhibit poor judgment. At higher dosages (BAC > 100 mg/dl), ethanol acts as a central nervous system depressant, producing at progressively higher dosages, impaired sensory and motor function, slowed cognition, stupefaction, unconsciousness, and possible death.

In America, about half of the deaths in car accidents occur in alcohol-related crashes. There is no completely safe level of alcohol for driving; the risk of a fatal car accident rises with the level of alcohol in the driver's blood. However, most drunk driving laws governing the acceptable levels in the blood while driving or operating heavy machinery set typical upper limits of blood alcohol content (BAC) between 0.05% to 0.08%.

We only remember that the Italian Legislation does not consider crime the alcohol consumption, excepting when drinker has to drive (August 2003 Law 1, n. 214 - Art. 186). The suspicion of such crime authorizes the organs of highway patrol to carry out the assessment of “psycho-physical alteration” due to the alcohol; assessment that must be carried out with instruments and procedures determined from regulations (DPR 16 December to you 1992, n. 495 - Art. 379).

The procedures of the examinations of the presence of alcohol in the blood are dictated from the article n°186 of the Rule of the Road.

In Italy the level of whole blood concentration for the state of drunkenness has been fixed to 0.5 g/l.

**CHEMICAL ANALYSIS BY HPLC**

The present method determines the concentrations of the Ethanol in serum and/or in plasma derivatized it and reading it in HPLC $\lambda = 385 \text{ nm}$.

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This product fulfills all the requirements of Directive 98/79/EC on in vitro diagnostic medical devices (IVD). The declaration of conformity is available upon request.

| Release N° 003 | Ethanol in serum by UV | March 2010 |
TECHNICAL FEATURES

Principle of the Method:
The sample is diluted and incubated at 40 °C for 30 minutes. After cooling, the deproteinization solution is added and, at the surnatant, is added the derivatization solution. The solution is incubated at 70 °C for 15 minutes. After cooling the solution is diluted and directly injected into HPLC system.

Recovery : 100%

Sensitivity : < 0,02 g/l

Dynamic Range of the Method : 0,02 - 3,16 g/l

Reference Values:

Ref. Art. n° 186 of the Rule of the Road: Guide in state of drunkenness

0,5 g/l < Ethanol < 0,8 g/l:
pecuniary penalty from 500,00 to 2,000,00 euro and arrest till 1 month – additional administrative pecuniary penalty with driving licence suspension from 3 to 6 months;

0,8 g/l < Ethanol < 1,5 g/l:
pecuniary penalty from 800,00 to 3,200,00 euro and arrest till 3 months - additional administrative pecuniary penalty with driving licence suspension from 6 months to 1 year;

Ethanol > 1,5 g/l:
pecuniary penalty from 1,500,00 to 6,000,00 euro and arrest till 6 months - additional administrative pecuniary penalty with driving licence suspension from 1 to 2 years.

Components of the kit:
All the reagents are ready-to-use and stable 3 years at 2 – 8 °C.

Reagent A – Buffer Solution N° 1, 1 x 20 ml
Reagent B – Reagent B, 1 x 5 ml
Reagent C – Buffer Solution N° 2, 1 x 10 ml
Reagent D – Deproteinization Solution, 1 x 10 ml
Reagent E – Derivatization Solution, 1 x 10 ml
Reagent F – Test Sol./Chemical Standard, 1 x 10 ml See Warnings
Reagent G – Diluting Solution, 1 x 100 ml
Reagent M – Mobile Phase, 3 x 500 ml

Minimum Instrumental equipment required:
Isocratic HPLC System with loop of 50 µl or 100 µl. Spectrophotometric Detector λ=385 nm Chromatograms Recorder

Optional Equipment:
Autosampler
Operational Computer

Whole Blood Collection Procedure:
DON’T USE alcoholic disinfectants. Collect 3 ml of whole blood in a test tube with anticoagulant K3EDTA+NaF. Centrifuge at 4000 rpm for 5 minutes. Separate plasma and store at –20 °C. Stable 4 weeks. The test tubes must to be stored well closed.
**PREANALYTICAL PROCEDURE**

**Dilution of Test Solution 3,16 g/l**

Pipette in a glass tube with teflon cap:
- 800 µl of H$_2$O HPLC grade
- 200 µl of Reagent F – Test Solution

Pipette in a glass tube with teflon cap:
- 200 µl of Reagent A – Buffer Solution N° 1
- 50 µl of Reagent B
- 50 µl of diluted Test Sol.

**Vortex for 10 sec.**

- Incubate at 40 °C for 30 minutes

**Cooling**

Add:
- 100 µl of Reagent C – Buffer Sol. N° 2
- 100 µl of Reagent D – Deproteinization Sol.
- 100 µl of Reagent E – Derivatization Sol.

**Vortex for 10 sec.**

- Incubate at 70 °C for 15 minutes

**Cooling at vortex for 10 sec.**

Transfer 100 µl of derivatized in a vial and add 900 µl of Reagent G – Diluting Solution.

**Vortex for 10 sec.**

**INJECTION**:

- Inject 50 µl of this solution in the chromatographic system.

Verify that the Test Solution has retention time similar to fig. 3. If the Test is all right you can start with the analytical procedure; if not, check the functionality of the analytical system.
ANALYTICAL PROCEDURE

STEP 1: Dilution of Samples, Calibration Chemical Standard and Controls

<table>
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<th></th>
<th>Samples</th>
<th>Chemical Standard</th>
<th>Controls</th>
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<tr>
<td>Water HPLC grade</td>
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<td>800 µl</td>
<td>800 µl</td>
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<tr>
<td>Samples</td>
<td>200 µl</td>
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<tr>
<td>Reagent F – Chemical Standard</td>
<td>200 µl</td>
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<td></td>
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<tr>
<td>Controls</td>
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<td>200 µl</td>
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</table>

**IMPORTANT**: It is important to use standard solution just prepared.

STEP 2: Preparation of Samples, Calibration Chemical Standard and Controls

Pipette in a glass tube with teflon cap:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Samples</th>
<th>Standard</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A – Buffer Sol. N° 1</td>
<td>200 µl</td>
<td>200 µl</td>
<td>200 µl</td>
<td>200 µl</td>
</tr>
<tr>
<td>Reagent B</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>Water HPLC grade</td>
<td>50 µl</td>
<td></td>
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</tr>
<tr>
<td>Samples</td>
<td>50 µl</td>
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<td></td>
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<tr>
<td>Controls</td>
<td></td>
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<td></td>
<td>50 µl</td>
</tr>
</tbody>
</table>

**Vortex for 10 sec.**

STEP 3: Incubate at 40 °C for 30 minutes

**Cooling at Room Temperature** and **Vortex for 10 sec.**

STEP 4: Deproteinization Procedure

Add:

- 100 µl of **Reagent C** – Buffer Sol. N° 2
- 100 µl of **Reagent D** – Deproteinization Sol.

**Vortex for 10 sec. and centrifuge at 5000 rpm for 5 minutes**

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STEP 5: Transfer 400 µl of surnatant in glass tubes with teflon cap and add:

- 100 µl of Reagent E – Derivatization Sol.

STEP 6: Incubate at 70 °C for 15 minutes

*Cooling at Room Temperature* and *Vortex for 10 sec.*

STEP 7: Diluting Procedure

Pipette:

- 100 µl of derivatized sample.

*N.B.: at this step, the sample is stable 3 days at 2-8 °C*

INJECTION:

- Inject 50 µl of this solution in the chromatographic system.
**ETHANOL in SERUM and/or in PLASMA - Warnings**

**REAGENT F : CHEMICAL STANDARD / TEST SOLUTION**

| Ethanol | 3,16 g/l |

**SPECTROPHOTOMETRIC DETECTOR PARAMETERS**

| λ          | 385 nm |
| GAIN       | 0.001 AUFS |
| INTEGRATION TIME | 10 sec. |

**HPLC COLUMN PROTECTION**

To save the analytical column Reverse Phase GENESIS 4.6 x 150 mm, 4 μ, the use of Metasaver Precolumn Filter 0.5 um (1 x 10 pcs.) cod. ZA6005 is obligatory.

**HPLC COLUMN CONDITIONING**

Install a new analytical C₁₈ Reversed-Phase column (GENESIS 4.6 mm x 150 mm, 4 μ). Disconnect the detector and flux 30 ml of solution and subsequently 30 ml of HPLC water, set flow at 1.2 ml / min. Don’t recycle the washing solutions. Filter the mobile phase with a vacuum system and a suitable filter of 0.22 μ. Condition the column with the mobile phase at a flow of 1.2 ml / min. and discharge the first 30 ml. Condition furtherly the column for 30 min. also at recycling phase. Finally inject the Chemical Standard and verify the quality of the HPLC run. **It is possible to make analysis at recycling phase.** If Room Temperature is > 20° C is better to preserve the Mobile Phase at 2 – 8° C among runs.

**COLUMN CLEANING**

Disconnect the detector. Flux 30 ml of H₂O and discharge. Flux a solution made of H₂O: Methanol or Acetonitrile ( 20 : 80 v/v ) for 30 min and discharge. When you re-use the column, flux 30 ml of H₂O before a new conditioning with the mobile phase.

**HPLC PARAMETERS**

| LOOP       | 50 µl or 100 µl |
| FLOW       | 1.2 ml/min. |
| PRESSURE   | About 80 bar |

**INTEGRATOR HP – 3394 / 3395 / 3396 PARAMETERS**

| ATTENUATION | 7 or 8 |

**OPERATIONAL COMPUTER PARAMETERS**

**IN CONFORMITY WITH THE SPECIFICATION OF OPERATIONAL COMPUTER SOFTWARE**

**ACCESSORIES AND CONSUMABLES**

<table>
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<tr>
<th>CODE</th>
<th>DESCRIPTION</th>
<th>PACKAGING</th>
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</thead>
<tbody>
<tr>
<td>Z05820</td>
<td>Chemical Standard for Ethanol in serum and/or in plasma</td>
<td>2 x 5 ml</td>
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<tr>
<td>ZFM15960E</td>
<td>Genesis C 18 (150 x 4.6mm -4 um) Analytical Column</td>
<td>1 PK</td>
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<tr>
<td>ZA6005</td>
<td>Metasaver Precolumn Filter 0.5 um</td>
<td>1 x 10 PK</td>
</tr>
<tr>
<td>S29057U</td>
<td>Standard glass vials of 2 ml with screw cap</td>
<td>1 x 100 PK</td>
</tr>
</tbody>
</table>
ETHANOL in SERUM and/or in PLASMA by UV
(Reference Chromatograms)

**Fig. 3 : Test Solution**
R.T. 10.330  Ethanol  3.16 g/l

**Fig. 4 : Plasma sample of a sober subject**
R.T. 10.330  Ethanol  3.16 g/l
ETHANOL in SERUM and/or in PLASMA by UV
(Reference Chromatograms)

**Fig. 5:** Plasma sample of a medium drinker
R.T. 10.135  Ethanol  0.79 g/l

**Fig. 6:** Plasma sample of an hard drinker
R.T. 10.143  Ethanol  3.16 g/l