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Ethyl glucuronide in vitreous humor and blood postmortem specimens: analysis by liquid chromatography-electrospray tandem mass spectrometry and interpreting results of neo-formation of ethanol

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Abstract

Introduction. The determination of ethyl glucuronide (EtG), a stable and sensitive marker that is specific to alcohol intake, finds many applications both in the forensic toxicology and clinical fields.

Aim. The aim of the study is to examine the possibility of using a cadaveric biological matrix, vitreous humor (VH), to determine EtG as a marker of recent ethanol use.

Methods. The blood, taken from the femoral vein, and the VH were obtained from 63 autopsy cases. Analysis of the EtG was performed using an LC/MS/MS system. Analyses of the ethanol and putrefaction biomarkers, such as acetaldehyde and n-propanol, were performed using the HS-GC/FID technique in both the matrices.

Results. In 17 cases, both ethanol and EtG were absent in both matrices.Nineteen cases presented ethanol in blood from 0.05 to 0.30 g/L, EtG-Blood concentration from 0.02 to 3.27 mg/L, and EtG-VH concentration from 0.01 mg/L to 2.88 mg/L. Thirteen cases presented ethanol in blood > 0.05 g/L but EtG concentration in blood and VH lower than 0.01 mg/L, are part of these 8 samples presented acetic aldehyde and n- propanol in blood or VH, means identification of putrefaction indicators. Fourteen cases presented ethanol in blood > 0.46 and EtG concentration in blood and VH higher than 0.01 mg/L.

Conclusions. The determination of EtG in biological material is important in those cases where the intake of ethanol appears doubtful, as it allows us to exclude the possibility of any post-mortem formation of ethanol.

Key words

- ethyl glucuronide
- ethanol
- VH
- peripheral blood

INTRODUCTION

In the western world the consumption of ethanol is a social and economic problem of great importance. According to statistics, 20-30% of the costs relating to health care are to be attributed to alcohol abuse [1].

In particular, in Italy the habit of consuming alcoholic beverages affects almost 13 500 people, aged 11 and over.

If the percentage of consumers, according to their consumption habits, for example with meals or between meals, is considered there are marked differences in gender. Almost one in two men also consumes alcohol between meals (46.20%), whilst women with the same habit are approximately one in four (26.8%). The differences in gender are almost negligible in the age group ranging from 18-24 years [2].

The identification and quantification of biomarker, specific for the consumption and/or abuse of alcohol, is of relevance for forensic purposes since it offers the possibility of distinguishing between the intake of alcohol in matrices such as blood and serum (in the short term), urine (in the medium term) and hair and meconium (in the long term) [3-5].

Ethyl glucuronide (EtG), being a direct metabolite of

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ethanol, could be used as highly specific direct marker for assessing the intake of alcohol in the medium term. It was identified for the first time in 1952 by Kamil, in the urine of rabbits, as triacetyl-methyl-ester [6].

EtG is a non-volatile polar, relatively stable molecule. It derives from the conjugation of ethanol with glucuronic acid activated via mediation of UDP-glucuronyl transferase (UGT) in the endoplasmic reticulum of the hepatocytes.

Only 0.02-0.06% of the ethanol ingested is transformed into EtG.

The kinetics of EtG has been examined. This study showed that the comsumption of a quantity of ethanol, capable of determining a level of alcohol of 0.50 g/Kg, is sufficient to result in the formation of EtG, that can be detected in the blood after just four hours from ingestion, and remains for up to 14 hours [7].

In the field of toxicology and forensics, EtG is of particular interest due to its possible applications [8-10].

For example, it may be used to differentiate between ante-mortem alcohol intake and post-mortem formation due to putrefactive processes. In postmortem ethanol analysis, the most commonly used sample matrix is whole blood. It is known that blood is susceptible to possible post-mortem changes. Indeed, a certain quantity of ethanol may be generated by post-mortem fermentative phenomena, initiated by several microorganisms, including *Escherichia coli*, starting from glucidic substrates in the blood, thus biasing analytical findings [11, 12]. To overcome this limitation, the use of an alternative/complementary matrix to the blood is of great relevance for forensic purposes [13].

Vitreous humor (VH), has a liquid character and resists putrefaction, which make it an attractive material for quantitative analysis in cases where blood is not available or deteriorated.

VH is less affected by these changes and should, therefore, provide a more reliable estimation of ante-mortem drug concentrations [14].

AIM

The aim of the study is to examine the possibility of using a cadaveric biological matrix, VH, to determine EtG as a marker of recent alcoholic use to supplement blood alcohol concentration measurements [15].

The use of this alternative matrix, already successfully experimented in studies on the detection of ethanol, can allow to obtain important data on the ante-mortem alcohol intake [16, 17].

MATERIALS AND METHODS

Reagents

The following reagents were used for the analysis: β-d-ethyl glucuronide and β-d ethyl glucuronide d-5 supplied by Cerilliant®, both at a concentration of 100 µg/mL and with a high degree of purity, acetonitrile ultra resi-analyzed® (J.T.Baker®), methanol (Sigma-Aldrich®), water (J.T.Baker®), formic acid 5M (Agilent Technologies, Milan, Italy). Tert-butanol (Sigma-Aldrich®). The aqueous solution was filtered on 0.45 µm PTFE filters (SUN.Sri, Duluth, GA,USA).

Samples

For this study samples were analysed from 63 autopsies (53 male subjects and 10 female) performed at the Institute of Forensic Medicine at the University of Brescia, from 2003-2011. In all cases, blood was taken from the femoral vein and VH from one or both eyeballs. Blood and VH were collected in medico-legal autopsies with a syringe and a needle, within 48 hours after the discovery of the deceased.

The "blank" samples for preparing the standard calibration curves on blood were supplied by the Spedali Civili di Brescia Hospital's transfusion service and tested negative for ethanol and EtG.

The "blank" samples for preparing the standard calibration curves on VH were obtained from deceased patients that underwent to legal autopsy and tested negative for ethanol and EtG.

Following collection, blood specimen preserved with potassium floride, and VH samples, were stored at -20 °C until analysis.

Analytical method – Ethanol, acetaldehyde, n-propanol analysis

Blood and VH samples were subjected to quantitative determination of ethanol and identification of putrefaction indicators such as acetaldehyde and n-propanol using headspace method [18-22].

Analyses were performed using Agilent Technologies® 6850 Series CG System coupled with a flame ionization detector (FID) equipped with a Agilent J &W DB-LC1 capillary column (30 m x 0.32 mm *i.d.*, 1.8 µm film thickness).

The injection was performed in split ratio mode 20:1. Before injection samples were heated at 60 °C for 20 minutes.

The analytes were identified by comparison of the retention times with those of standard solutions. A satisfactory separation required 6 min.

Ethanol concentration was performed using tert-butanol as the internal standard in linear calibration with 0.05-0.10-0.20-0.50-1.00-2.00-5.00 g/L aqueous ethanol solution. The lower limit of quantification (LLOQ) was 0.05g/L [23, 24].

Statistical issues

Dosages of ethanol blood, ethanol-VH, EtG-Blood, EtG-VH, were means of 63 samples tested in duplicate.

The comparison of ethanol blood and EtG-VH concentration and comparison of ratio ethanol blood/EtG-Blood and ethanol blood/EtG-VH when applicable, Scatter diagram regression were used ($P < 0.05$ was considered as statistically significant).

Analytical method – Ethyl glucuronide analysis

An HPLC 1260 System equipped with an infinity system model 1260 injector and paired with an Agilent Technologies 6460 Triple Quad LC/MS triple quadrupole mass spectrometer was used for the quantitative dosage of EtG.

Chromatographic separation of EtG and internal standard Ethyl glucuronide – d5 was achieved using INERTSIL ODS-3 100 × 3 mm, 3 µm coupled with a

security guard column. The flow rate was 0.3 mL/min with mobile phase of 0.01% Formic Acid in water (A) and acetonitrile (B). The column was equilibrate with 2% B for 3 min, A linear gradient to 95% B was applied over 12 min. The total run time was 15 min per sample [25-27].

An Agilent (Waldbronn, Germany) 6460 triple quadrupole mass spectrometer equipped with a Jet Stream-Electrospray ion source operating in negative-ion mode was used as detection system. The optimal source parameter were: source temperature: 300 °C, drying gas flow: 12 L/min, drying gas temp: 350 °C, nebulizer pressure: 40 psi, capillary voltage: 3.500 V and nozzle voltage: 0V.

The transitions were optimized and were selected as follows: m/z 221.1, 85.3 and 221.1, 75.2 for Ethyl glucuronide and 226.1, 85.1 and 226.1, 75.0 for Ethyl glucuronide-d5 (EtG-D5).

For all transition, dwell time was set at 20 ms.

EtG VALIDATION FOR BLOOD AND VH SAMPLES

Linearity of calibration

To validate the quantitative analytical method for EtG in the blood and VH samples, 4 calibration curves were prepared for both matrices by adding 80 µL of blood/VH "blank" to 20 µL of pure EtG standard in a methanol solution at known and scalar concentrations, to obtain the following calibration points: 0.00-0.01-0.02-0.05-0.1-0.2-0.5-1.00 mg/L.

20 µL of deuterated internal standard (EtG-d5) was added to each calibration point, at concentration of 0.50 ng/µL (0.10 mg/L), freshly prepared from the stock solution before each analysis and stored at -20 °C.

The blood and VH samples were deproteinized by adding 250 µl of acetonitrile and 250 µL of methanol, respectively, mixed and centrifuged at 13000 rpm for 10 minutes. The supernatants were transferred to a conical test tube and were dried under a stream of nitrogen [15]. The dried extracts were then reconstituted in 500 µL of ultra-filtered water containing formic acid at 0.01%, transferred to vials with screw tops and analysed using LC/ESI-MS/MS.

The linearity of the two methods was verified by preparing 4 calibration curves for each matrix; the results obtained are represented by regression lines with the least-squares method.

Selectivity

Selectivity was tested by analysing 10 samples of "blank blood" and 10 samples of "blank VH", supplemented with the internal standard (EtG-D5) at a concentration of 0.1 mg/L, to verify the absence of interfering substances that could co-elute with the interested analytes.

Precision and accuracy

To evaluate the intraday and inter-day precision of this method, 6 replicates of "blank blood" and "blank VH" samples, spiked with different concentration of EtG standard (0.01 mg/L, 0.05 mg/L, 0.10 mg/L e 0.20 mg/L) were analysed on 4 different days.

The intraday and inter-day precision was estimated by calculating the coefficient of variation percentage (C.V.%).

The accuracy for both matrices was calculated from the percentage of deviation of the mean of the results from the corresponding nominal value.

Long term stability

The stability of the analytes was assessed using real samples of blood and VH that resulted positive for EtG upon first analysis. These were stored in the dark at -20 °C and analysed again after 15 days and 30 days of storage.

Matrix effect

The matrix effect was determined by comparing the responses obtained from the analysis of 6 replicates of aliquots of blank matrix of blood and VH, fortified at a concentration of 0.05-0.10-0.20 mg/L, with those obtained from the analysis of similar aliquots of water, fortified at the same concentrations.

Examined samples

To evaluate the concentration of EtG in the blood and VH samples, 100 µL of each samples, brought to room temperature, were spiked with 20 µL (0.10 mg/L) of deuterated internal standard (EtG-d5), freshly prepared from the stock solution and stored at -20 °C until use.

The blood and VH samples were deproteinized by adding 250 µL of acetonitrile and 250 µL of methanol respectively mixed and centrifuged at 13000 rpm for 10 minutes. The supernatants, once transferred to a conical test tube, were dried under a stream of nitrogen.

The residues were then reconstituted with 500 µL of ultra-filtered water containing formic acid at 0.01%, transferred to vials with screw tops and analysed using LC/ESI-MS/MS.

RESULTS

To achieve the aim of the present study, that is the possible use of VH sample as a marker of recent alcoholic intake, it was necessary to verify that the applied LC/ ESI-MS/MS method could be specific, sensitive, precise and accurate for determining EtG in the biological samples.

First, the linearity response for blood and VH samples was studied. The method showed a linear response between 0.00 and 1.00 mg/L for both matrices (R2 blood = 0.998, R2 VH = 0.995) *(Table 1)*.

The selectivity of the developed method was evaluated analyzing blank samples. The absence of interfering substance that could co-elute with the interested analytes and thus could bias the quantification results, confirmed that the method was highly specific for both matrices.

Then the EtG stability in both matrices was evaluated on 10 positive blood and VH samples over a period of 4 weeks. Samples were kept in the dark at -20 °C and subjected to analysis after 30 days. The average variation detected between the first (day 1) and second analysis (day 30) was of 8%.

Table 1
Blood and vitreous humor calibration results

	Blood			Vitreous humor		
Date	**Slope**	**Intercept**	**R^2**	**Slope**	**Intercept**	**R^2**
70711	0.746	0.035	0.999	0.808	0.201	0.989
190711	0.703	0.018	0.999	0.710	0.366	1.000
030811	0.746	0.012	0.996	0.702	0.239	0.999
260911	0.783	0.037	0.998	0.747	0.316	0.993
Average	0.745	0.025	0.998	0.742	0.281	0.995
DS	0.033	0.012	0.001	0.048	0.074	0.005

Table 2
Accuracy and precision data. Evaluation in blood and vitreous humor

Matrix	**Nominal conc. (mg/L)**	**Mean calculate (mg/L)**	**Accuracy (bias% n = 24)**	**Intraday precision (CV% n = 6)**	**Interday precision (CV% n = 24)**
Blood	0.01	0.012	22.00	14.60	15.25
	0.05	0.059	17.30	7.90	8.72
	0.10	0.115	14.50	6.00	7.62
	0.20	0.219	9.30	6.30	5.99
Vitreous humor	0.01	0.011	15.00	14.30	15.13
	0.05	0.058	16.00	6.40	6.33
	0.10	0.109	9.00	5.20	6.11
	0.20	0.204	2.00	3.00	4.23

The sensitivity of the tests was confirmed by assessment of the LLOQ (S/N ratio of 10) and of the LOD (S/N ratio of 3) for EtG both in blood and in VH (LOQ blood and VH 0.01mg/L, LOD blood and VH 0.005 mg/L.)

Evaluation of the precision of the analytical method allowed us to verify that the coefficient of variation, calculated for each concentration analysed, was less than 12% of all the concentrations examined.

The accuracy, determined on the same concentrations of the analyte used to estimate precision, on the basis of the data obtained provided a percentage error within ± 15% (*Table 2*).

To investigate possible EtG matrix effects, including ionization or suppression, the signal responses from standards prepared in water and those prepared in matrix extract (blood end VH) were compared.

The concentration was higher in blood. The matrix effect at a concentration of 0.10 mg/L, proved to be less than 15% for both matrices (*Table 3*).

After validation of the analytical method, 63 autopsy samples of blood and VH were subjected to analysis to detect EtG; the results were subsequently compared with the quantities of ethanol in both matrices to obtain further information on the intake of alcohol.

Furthermore, two important putrefaction markers were evaluated in the cadaver blood, acetic aldehyde and n-propanol, produced by post-mortem fermentation processes, with the aim of discovering whether the source of the ethanol in the blood was of a putrefactive nature. *Table 4* shows the analytical results of the 63 autopsy cases that were examined.

Table 3
EtG matrix effect: mean concentration at 0.050- 0.100 -0.200 mg/L for aqueous standards, blood and vitreous humor

EtG matrix effect	**Mean concentration mg/L (n = 6)**	**Accuracy % (n = 6)**
0.050 mg/L Prepared standard		
Aqueous	0.048	- 4.00
Blood	0.058	16.00
Vitreous humor	0.057	14.00
0.100 mg/L prepared standard		
Aqueous	0.105	5.00
Blood	0.112	12.00
Vitreous humor	0.108	8.00
0.200 mg/L prepared standard		
Aqueous	0.203	1.50
Blood	0.218	9.00
Vitreous humor	0.206	3.00

Seventeen cases (1, 2, 4, 6, 22, 23, 24, 32, 37, 39, 44, 46, 48, 51, 54, 57, 60) on 63 presented ethanol in blood = 0.00 g/L or ≤ 0.05 g/L (LLOQ), blood and and EtG-VH concentration were ≤ LLOQ (0,01 mg/L) ± 15%.

Nineteen cases presented ethanol in blood from 0.05 to 0.30 g/L, EtG-Blood concentration from 0.02 to 3.27 mg/L, median 0.18 mg/L and mean 0.83 mg/L. The same

Table 4
Samples results: ethanol and EtG concentration in blood and vitreous humor (VH); qualitative results of n-propanol and acetic aldehyde in blood and vitreous humor (VH)

SAMPLE	Ethanol (g/L)		EtG (mg/L)		n-propanol		Acetic aldehyde		Sex	Cause of death
	Blood	v.h.	Blood	v.h.	Blood	v.h.	Blood	v.h.		
1	0.00	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	cardiac death
2	0.00	0.00	0.01	0.00	Assent	Assent	Assent	Assent	m	drug intoxication
3	0.93	0.81	2.59	0.42	Assent	Assent	Assent	Assent	m	haemorrhage
4	0.00	0.00	0,00	0.00	Assent	Assent	Assent	Assent	m	polytrauma
5	0.10	0.06	0.07	0.02	Assent	Assent	Assent	Assent	m	cardiac death
6	0.00	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	cardiac death
7	0.20	0.05	0.75	0.49	Assent	Assent	Assent	Assent	m	polytrauma
8	0.59	0.34	0.45	0.22	Assent	Assent	Assent	Assent	m	suffucation
9	0.27	0.18	0.00	0.00	Assent	Assent	Present	Present	m	coronary heart disease
10	1.45	0.00	3.51	1.60	Assent	Assent	Assent	Assent	m	drug intoxication
11	0.30	0.12	3.24	1.67	Assent	Assent	Assent	Assent	m	drug intoxication
12	1.86	0.00	0.01	0.04	Assent	Assent	Assent	Assent	m	thorax trauma
13	0.18	0.10	2.27	2.13	Assent	Assent	Assent	Assent	m	cardiac death
14	1.82	0.00	2.22	2.54	Assent	Assent	Assent	Assent	f	cardiac death
15	1.25	0.00	0.00	0.00	Assent	Assent	Present	Assent	m	drug intoxication
16	0.28	0.16	0.02	0.01	Present	Present	Assent	Present	f	cerebral trauma
17	0.05	0.05	0.68	0.28	Assent	Assent	Assent	Assent	m	drug intoxication
18	0.05	0.05	0.14	0.10	Assent	Assent	Assent	Assent	m	haemorrhage
19	0.05	0.05	0.12	0.01	Assent	Assent	Assent	Assent	m	suffucation
20	0.49	0.00	0.00	0.00	Assent	Assent	Present	Assent	m	cardiac death
21	0.31	0.10	0.01	0.12	Assent	Assent	Assent	Assent	m	thrombosis
22	0.00	0.00	0.01	0.01	Assent	Assent	Assent	Assent	m	drug intoxication
23	0.00	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	cardiac death
24	0.00	0.00	0.01	0.01	Assent	Assent	Assent	Assent	m	drug intoxication
25	1.71	0.32	0.61	0.30	Assent	Assent	Assent	Assent	m	coronary heart disease
26	0.10	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	drug intoxication
27	0.20	0.20	0.25	0.12	Assent	Assent	Assent	Assent	m	drug intoxication
28	0.56	0.00	0.01	0.00	Assent	Assent	Present	Present	f	cerebral trauma
29	0.12	0.00	0.07	0.04	Assent	Assent	Assent	Assent	m	drug intoxication
30	0.22	0.00	0.00	0.01	Assent	Assent	Assent	Assent	m	polytrauma
31	0.39	0.20	0.00	0.02	Assent	Assent	Present	Present	f	suffucation
32	0.00	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	thrombosis
33	0.15	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	drug intoxication
34	0.37	0.05	0.00	0.00	Assent	Assent	Present	Assent	m	drug intoxication
35	0.55	0.29	0.07	0.02	Assent	Assent	Assent	Assent	f	cerebral trauma
36	1.99	0.23	18.3	12.53	Assent	Assent	Assent	Assent	m	drug intoxication
37	0.00	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	suffucation
38	0.20	0.11	3.27	0.18	Assent	Assent	Assent	Assent	m	polytrauma
39	0.00	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	coronary heart disease
40	0.20	0.05	2.12	2.67	Assent	Assent	Assent	Assent	m	drug intoxication
41	0.20	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	cardiac death
42	0.55	0.57	2.73	0.50	Assent	Assent	Assent	Assent	m	drug intoxication
43	2.32	0.32	20.05	5.86	Assent	Assent	Assent	Assent	m	drug intoxication

(continues)

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SAMPLE	Ethanol (g/L)		EtG (mg/L)		n-propanol		Acetic aldehyde		Sex	Cause of death
	Blood	v.h.	Blood	v.h.	Blood	v.h.	Blood	v.h.		
44	0.00	0.00	0.00	0.00	Assent	Assent	Assent	Assent	f	haemorrhage
45	1.56	0.32	5.03	2.99	Assent	Assent	Assent	Assent	f	suffucation
46	0.00	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	polytrauma
47	0.05	0.05	0.18	0.38	Assent	Assent	Assent	Assent	m	drug intoxication
48	0.00	0.00	0.01	0.00	Assent	Assent	Assent	Assent	m	suffucation
49	0.15	0.10	0.06	0.02	Assent	Assent	Assent	Assent	f	haemorrhage
50	0.46	0.36	11.59	4.24	Assent	Assent	Assent	Assent	f	drug intoxication
51	0.00	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	drug intoxication
52	0.29	0.05	0.02	0.00	Present	Assent	Assent	Assent	m	haemorrhage
53	0.10	0.31	0.43	0.50	Assent	Assent	Assent	Assent	m	haemorrhage
54	0.00	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	cardiac death
55	0.05	0.05	0.92	2.88	Assent	Assent	Assent	Assent	f	drug intoxication
56	0.05	0.05	1.74	0.12	Assent	Assent	Assent	Assent	m	drug intoxication
57	0.00	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	drug intoxication
58	1.18	1.29	11.98	6.90	Assent	Assent	Assent	Assent	m	drug intoxication
59	0.06	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	haemorrhage
60	0.00	0.00	0.00	0.00	Assent	Assent	Assent	Assent	m	suffucation
61	0.27	0.10	0.03	0.52	Assent	Assent	Assent	Assent	m	cardiac death
62	0.09	0.05	0.16	0.06	Assent	Assent	Assent	Assent	m	hanging
63	1.21	0.99	3.58	0.43	Assent	Assent	Assent	Assent	m	cardiac death

samples presented EtG-VH concentration from 0.01 mg/L to 2.88 mg/L, median 0.22 mg/L, and mean 0.65 mg/L, while in the same samples acetic aldehyde and n-propanol were absent, indicating that the prior intake of alcoholic beverages is likely (cases: 5, 7, 11, 13, 16, 17, 18, 19, 27, 29, 38, 40, 47, 49, 53, 55, 56, 61, 62).

Thirteen cases presented ethanol in blood > 0.05 g/L but EtG concentration in blood or/and VH lower than 0.01 mg/L (cases: 9, 15, 20, 21, 26, 28, 30, 31, 33, 34, 41, 52, 59).

Twelve cases presented ethanol in blood > 0.06 g/L but ethanol VH concentration 0.00 g/L (cases: 10, 12, 14, 5, 20, 26, 8, 29, 30, 33, 42, 59). In 9, 15, 16, 20, 28, 31, 34 and 52 samples acetic aldehyde and n- propanol were present in blood or VH, means identification of putrefaction indicators (*Table 5*).

Fourteen cases presented ethanol in blood > 0.46 and EtG concentration in blood and VH higher than 0.01 mg/L (cases: 3, 8, 10, 12, 14, 25, 35, 36, 42, 43, 45, 50, 58, 63).

Figure 1 shows correlation between ethanol in blood and EtG-VH in 63 cases.

The correlation coefficient (r) was 0.585 with $P < 0.0001$ and 95% confidence interval for r = 0.395 to 0.727.

Figure 2 shows the correlation between ratio ethanol in blood /EtG-Blood and ethanol in blood /EtG-VH in 34 comparable samples.

The correlation coefficient (r) was 0.798 with $P < 0.0001$ and 95% confidence interval for r = 0.629 to 0.895.

DISCUSSION

This allows us to assume a probable neo-formation of ethanol from a cadaveric source, supported by the presence of acetaldehyde or n-propanol as a indicators of fermentation (samples 9, 15, 16, 20, 28, 31, 34 and 52).

Samples 20 and 28 showed ethanol in blood concentration were 0.49 and 0.56 respectively, but EtG blood concentration was ≤ LLOQ ± 15%: this fact probably indicates a post-mortem ethanol neo-formation.

Cases 10, 26, 30, 33, 41 and 59 were positive for ethanol in blood (> 0.06 g/L), but EtG in blood and VH concentration ≤ LLOQ ± 15%. Acetaldehyde and n- propanol were absent.

Cases 15, 20, 28 presented ethanol in blood of 1.25 g/L, 0.49 g/L and 0.56 g/L respectively, but negative for EtG in both matrices, as well as showing the presence of acetaldehyde in the blood.

Analyzing the results of correlation between ethanol in blood and EtG-VH the data had a linear distribution on the graphic (*Figure 1*), except for the samples whit EtG-VH > 1.00 mg/L when we can note the dispersion. *Figure 2* shows minor dispersion of the data.

Study of relationship between EtG-VH and EtG-Blood showed that more than 90% of the samples feature matching. Indeed the cases 2, 28, 48 and 52 had EtG only in blood but not in VH. This can be explained by the LLOQ EtG -Blood concentration. The cases 30 and 31 had EtG only in VH but not in blood.

Table 5
Nineteen cases presented ethanol in blood from 0.05 to 0.30 g/L, EtG-Blood concentration from 0.02 to 3.27 mg/L and EtG-VH concentration from 0.01 mg/L to 2.88 mg/L, 13 cases presented ethanol in blood > 0.05 g/L but EtG concentration in blood and VH lower than 0.01 mg/L. Samples 9, 15, 16, 20, 28, 31, 34 and 52 acetic aldehyde and n- propanol were present in blood or VH, means identification of putrefaction indicators

Sample	Ethanol (g/L)		EtG (mg/L)		n-propanol		Acetic aldehyde	
	Blood	v. h	Blood	v. h	Blood	v. h	Blood	v. h
5	0.10	0.06	0.07	0.02	Assent	Assent	Assent	Assent
7	0.20	0.05	0.75	0.49	Assent	Assent	Assent	Assent
9	0.27	0.18	0.00	0.00	Assent	Assent	**Present**	**Present**
11	0.30	0.12	3.24	1.67	Assent	Assent	Assent	Assent
13	0.18	0.10	2.27	2.13	Assent	Assent	Assent.	Assent.
15	1.25	0.00	0.00	0.00	Assent	Assent	**Present**	Assent
16	0.28	0.16	0.02	0.01	**Present**	**Present**	Assent	**Present**
17	0.05	0.05	0.68	0.28	Assent	Assent	Assent	Assent
18	0.05	0.05	0.14	0.10	Assent	Assent	Assent	Assent
19	0.05	0.05	0.12	0.01	Assent	Assent	Assent	Assent.
20	0.49	0.00	0.00	0.00	Assent	Assent	**Present**	Assent
21	0.31	0.10	0.01	0.12	Assent	Assent	Assent	Assent
26	0.10	0.00	0.00	0.00	Assent	Assent	Assent	Assent
27	0.20	0.20	0.25	0.12	Assent	Assent	Assent	Assent
28	0.56	0.00	0.01	0.00	Assent	Assent	**Present**	**Present**
29	0.12	0.00	0.07	0.04	Assent	Assent	Assent	Assent
30	0.22	0.00	0.00	0.01	Assent	Assent	Assent	Assent
31	0.39	0.20	0.00	0.02	Assent	Assent	**Present**	**Present**
33	0.15	0.00	0.00	0.00	Assent	Assent	Assent	Assent
34	0.37	0.05	0.00	0.00	Assent	Assent	**Present**	Assent
38	0.20	0.11	3.27	0.18	Assent	Assent	Assent	Assent
40	0.20	0.05	2.12	2.67	Assent	Assent	Assent	Assent
41	0.20	0.00	0.00	0.00	Assent	Assent	Assent	Assent
47	0.05	0.05	0.18	0.38	Assent	Assent	Assent	Assent
49	0.15	0.10	0.06	0.02	Assent	Assent	Assent	Assent
52	0.29	0.05	0.02	0.00	**Present**	Assent	Assent	Assent
53	0.10	0.31	0.43	0.50	Assent	Assent	Assent	Assent
55	0.05	0.05	0.92	2.88	Assent	Assent	Assent	Assent
56	0.05	0.05	1.74	0.12	Assent	Assent	Assent	Assent
59	0.06	0.00	0.00	0.00	Assent	Assent	Assent	Assent
61	0.27	0.10	0.03	0.52	Assent	Assent	Assent	Assent
62	0.09	0.05	0.16	0.06	Assent	Assent	Assent	Assent

Knowing the kinetics of ethanol and its direct metabolite, it is possible to assume in these cases that there was an intake of alcohol within three hours prior to death.

This could be explained by the fact, clearly expressed in scientific literature, that the microsomal enzyme responsible for the ethanol glucuronidation reaction exists as a super-family of enzymes. The polymorphic variants in the genes that encode UTG may have a significant impact on the ability of man to synthesise EtG and may contribute to explaining the inter-individual difference in the level of EtG after consuming alcohol [28].

CONCLUSIONS

Introduction of the determination of EtG into the analytical protocol of a chemical-toxicological laboratory is of significant interest not only on an exclusively clinical level (rehabilitation programs for alcoholic patients) but also, and above all, on the penal and administrative aspects of forensic-toxicological investigations. In fact, the identification of EtG in biological samples is an extremely useful analytical-diagnostic indicator in highlighting the prior intake of alcohol as it is a medium term marker of such intake.

In the interest of forensics and toxicology, in cases

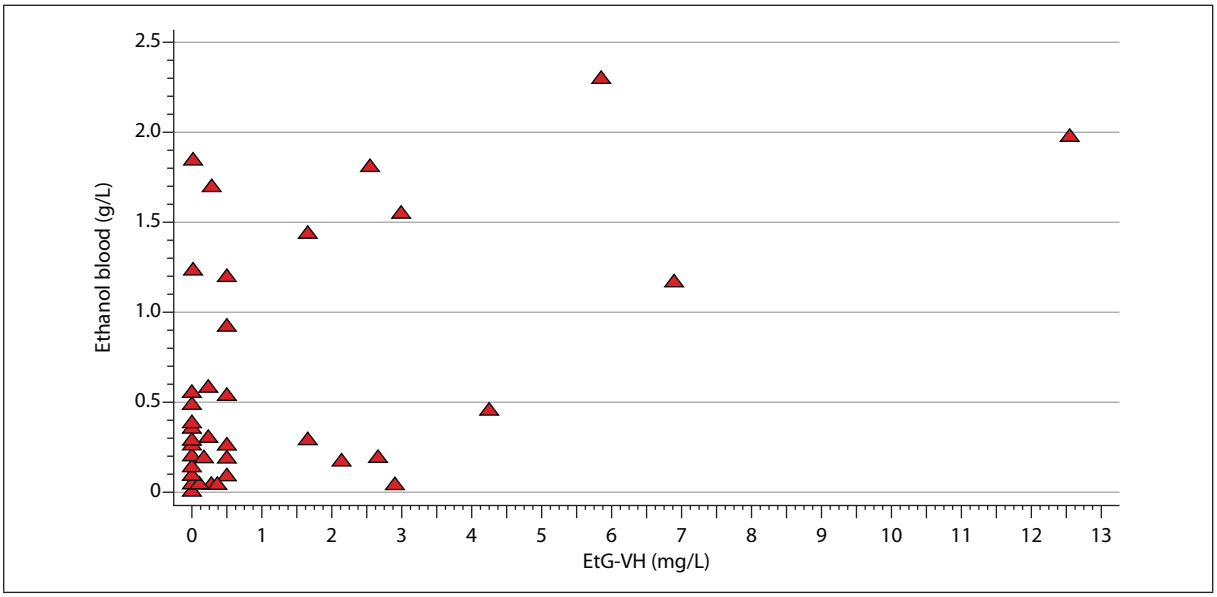

Figure 1
Samples results: comparison of distribution of the ethanol in blood (g/L) and EtG-VH (mg/L) concentration in 63 cases.

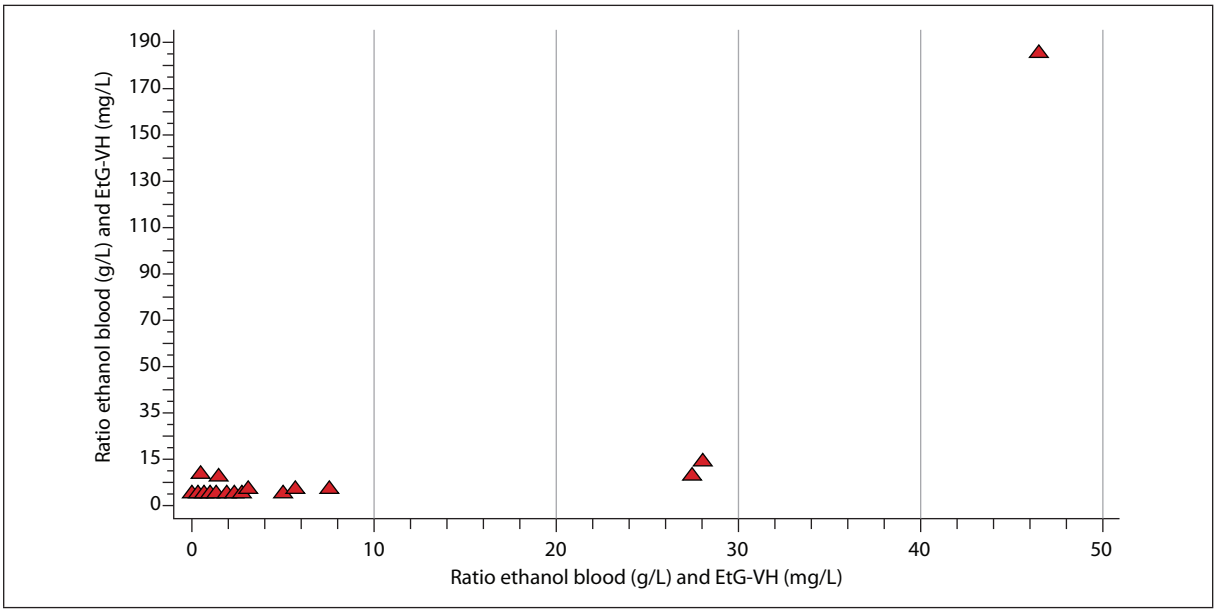

Figure 2
Samples results: comparison of ethanol in blood (g/L) and EtG-VH (mg/L) ratio with ethanol in blood (g/L) and EtG-blood (mg/L) in 34 cases.

where the intake of ethyl alcohol cannot be clearly traced solely by correspondence with the EtG datum detected in the biological fluids being examined, it is important to evaluate not only ethanol but also other parameters that could provide further indications on the source of the alcohol found, such as n-propanol and acetaldehyde, which in high concentrations can be a good indication of suspected putrefaction [29, 30].

In conclusion, our study has clearly confirmed the possibility of using VH to determine EtG following the intake of ethyl alcohol. In particular, it can be said that the EtG detected in this matrix, in real cases taken from corpses at different degrees of preservation, proved to be an excellent parameter for differentiating between ante-mortem intake of ethanol and post-mortem formation due to putrefactive phenomena.

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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