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BRIEF REPORT

QUANTIFICATION BIAS IN BLOOD ALCOHOL DETERMINATION BY HEADSPACE GAS CHROMATOGRAPHY

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ABSTRACT: The quantification of blood alcohol concentration is routinely performed in many forensic laboratories by gas chromatography. In this short report, the influence of the matrix miss-match between samples and calibrators (e.g. the use of aqueous standards to quantify blood samples) and the actual efficiencies of different internal standards used to normalise the ethanol signal were considered. Test samples in blood and water were prepared from a certified reference standard of ethanol. The samples were analysed by gas chromatography using n-propanol and t-butanol as internal standards. The collected data provided evidences that a sub-optimal setting of the considered variables can be responsible for a quantification bias up to 15%. These issues are of practical relevance in the medico-legal sector and should be considered during the process of method development.

KEY WORDS: blood alcohol concentration**,** calibrator, internal standard, gas chromatography, bias.

INTRODUCTION:

Human blood samples are the primary type of evidence received for medico-legal testing of alcohol concentration by gas chromatography. Whole blood is the sample of choice, since plasma typically entails an overestimation[1]. For the purpose of forensic testing, different thresholds of alcohol concentration are set before a person is charged with a fine or crime. The consequent need to produce medico-legal reports underlines the importance of the careful development and validation of the analytical methods applied. The literature on this argument is vast and many possible influencing factors are known[2, 3]. Method development should start from tuning of the most important parameters; in this context we feel that further attention should be paid to the choice of the internal standard and calibrators. The quantification of unknown samples is achieved by interpolation of a calibration curve. For this reason, reference materials at known concentrations of ethanol are widely available on the marketplace[4]. The majority of these materials are aqueous solutions produced according to ISO Guide 34 and satisfy the regulatory needs posed by ISO 17025. However, the opportunity to use aqueous calibrators to quantify

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blood samples should be carefully evaluated, even if an internal standard is used to correct for the differences among samples and calibrators[5, 6]. The actual efficacy of the internal standard needs to be experimentally verified. A "good" internal standard is chemically similar to ethanol without any chromatographic overlap with the target substance or other volatiles that could be present in the samples (e.g., acetone, methanol or acetaldehyde). *n*-propanol is commonly considered the best suited substance for this purpose, even if *t*-butanol is also used[5,6]. The samples can also be diluted with water up to practically correct for the matrix mismatch (usually a 1:10 dilution is sufficient)[7]. However, this procedure inevitably increases the limit of quantification with evident constraints in dealing with the "zero tolerance" threshold posed for young drivers or commercial vehicle operators.

MATERIALS AND METHODS:

In this report we present some data retrieved from the development of the analytical procedure adopted at our laboratory. The method was arranged starting from the protocol suggested by the producer of the available equipment (i.e. a PerkinElmer Clarus 500 gas chromatographer equipped with a Turbomatrix40 headspace autosampler). The original parameters were: sample equilibration: 30 min 70 °C, vial pressurisation: 15 psi for 3 min, transfer line temperature: 110 °C, injection: 200 °C, oven: 40 °C isothermal, column: Elite-BAC1 (PerkinElmer Cod. N9316579), carrier gas: N_2.

n-propanol and *t*-butanol were both tested as internal standard. Samples were mixed with ammonium sulphate in order to increase the partition coefficient of alcohol in the gas phase and amplify the analytical response[8]. A fixed volume of sample (1 ml) was added to 0.9 ml of 1 M ammonium sulphate and to 0.1 ml of 8 g/L *t*-butanol or *n*-propanol (internal standard).

Two type of test samples were prepared: an intermediate series of sample at 0.30, 0.75, 1.50, 3.75, 7.50 g/L of ethanol in water were produced independently from a mother solution 15 g/L. All the samples (including the mother solution) were further diluted 1:5 in water and blood (previously checked for the absence of ethanol). At the end of the process, 2 independent series at 0.06, 0.15, 0.30, 0.75, 1.50 and 3 g/L in blood and water were obtained. This dilution scheme ensured that each concentration was independent, and the matrix composition uniform within each series. The second type of samples consists of 4 solutions at the nominal concentrations of 0.5 and 2 g/L in water and blood. These samples were prepared by adding 100 and 400 µl of a 0.5 g/L ethanol standard to a final volume of 1 ml of water or blood.

RESULTS:

The first type of test samples described in the "materials and method" session (i.e. 2 independent series at 0.06, 0.15, 0.30, 0.75, 1.50 and 3 g/L of ethanol in blood and water) were prepared and analysed independently 10 times. For each repetition, blood and water samples were analysed within the same analytical session. For each concentration, it was expected that the average analytical response (i.e. the ethanol/internal standard area ratio) of the blood and water samples would be similar due to normalisation with the internal standard. However, a progressive response difference was observed (Table 1). The disparity becomes clearly significant among samples normalised by *t*-butanol, while it is not supported by a statistical evidence when *n*-propanol is used. In order to quantify the possible bias, test samples at the nominal concentrations of 0.5 and 2 g/L in water and blood were used (i.e. the second type of test samples described in the "Materials and methods" section). The 2 couples of samples were produced and analysed independently 10 times using a quantitative method calibrated by commercial certified reference materials based on water solutions. As reported in Table 2, the average error ranges from 2% at 0.5 g/L with *n*-propanol up to 16% at 2 g/L with *t*-butanol. The error is indeed negligible, if the analytical samples are matrix matched with the calibrators (i.e. water samples quantified against water calibrators).

DISCUSSION:

The presented data show how a matrix difference between samples and calibrators and/or the actual efficacy of the internal standard can bias the outcome of the alcohol test by gas-chromatography. These factors should be carefully considered during method development. Our data point out the superiority of *n-propanol* for BAC determination. However, a few additional aspects should be considered, especially if *t*-butanol is selected as internal standard. As the error seems to increase with the amount of ethanol, the use of a fixed correction factor becomes difficult. On the other hand, the practice of making up "in-house" standards by spiking known concentrations of ethanol into "blank" blood samples can lead to regulatory problems associated with the medico-legal sector. The whole process should be carefully controlled and documented, even though it would be unrealistic for a medical laboratory to have enough resources to produce certified reference materials. A possible solution could be the use of commercial blood standards that are industrially manufactured by spiking ethanol into a blood matrix. However, the availability of such materials is still limited and the assigned concentration is usually an average value derived from a number of independent certified laboratories (usually these standards don't comply with the ISO guide 34 requirements).

CONCLUSIONS:

In conclusion, this report offers some helpful thoughts on the calibration of the analytical methods used to assess blood alcohol concentration for medico-legal purposes. These considerations may seem trivial, but a recent comment by Schug[9] shows that these analytical issues are still highly
relevant. In our practice, the guidelines of the Italian Forensic Toxicologist Group don't report any specific indication about the internal standard and the calibrators used for alcohol tests[10] and the only way to control the risk of possible bias is to asses experimentally the real efficacy of such measures adopted to minimize the matrix mismatch and to participate periodically to suitable proficiency tests on blood samples.

Table 1. Analytical response of water and blood samples

t-BUTANOL						
	Blood samples		**Water samples**			
Conc. g/L	Av. Anal. Response (a)	St.Dev	Av. Anal. Response (b)	St.Dev	**Difference (a-b)**	**p-value**
0.06	0.0096	0.0009	0.0130	0.0016	**-0.0034**	**3.E-02**
0.15	0.0301	0.0031	0.0323	0.0036	**-0.0021**	**5.E-01**
0.30	0.0746	0.0020	0.0712	0.0038	**0.0034**	**2.E-01**
0.75	0.2281	0.0022	0.1994	0.0034	**0.0286**	**3.E-04**
1.50	0.4736	0.0121	0.4073	0.0065	**0.0663**	**1.E-03**
3.00	0.9754	0.0407	0.8224	0.0148	**0.1530**	**4.E-03**
***n*-PROPANOL**						
	Blood samples		**Water samples**			
Conc. g/L	Av. Anal. Response (a)	St.Dev	Av. Anal. Response (b)	St.Dev	**Difference (a-b)**	**p-value**
0.06	0.0197	0.0012	0.0288	0.0006	**-0.0091**	**1.E-02**
0.15	0.0613	0.0034	0.0718	0.0017	**-0.0105**	**6.E-02**
0.30	0.1557	0.0136	0.1669	0.0159	**-0.0112**	**5.E-01**
0.75	0.4880	0.0287	0.4788	0.0245	**0.0093**	**8.E-01**
1.50	0.9824	0.0599	0.9699	0.0424	**0.0124**	**8.E-01**
3.00	2.0218	0.0969	1.9658	0.0551	**0.0560**	**6.E-01**

Average analytical response (ethanol/internal standard area ratio) of blood and water samples at different concentrations (0.06-3.00 g/L of ethanol) analysed using *n*-propanol or *t*-butanol as internal standards (n=10).

Table 2. Ethanol quantification in water and blood samples

Internal Standard *t*-butanol			
Cerilliant® calibrators (water)			
Nominal Conc. g/L	Av. Quant. Water samples	Av. Quant. Blood samples	Error on blood samples %
0.50	0.50	0.56	12%
2.00	2.01	2.32	16%
Internal standard *n*-propanol			
Cerilliant® calibrators (water)			
Nominal Conc. g/L	**Av. Quant. Water samples**	**Av. Quant. Blood samples**	**Error on blood samples %**
0.50	0.50	0.51	2%
2.00	2.01	2.11	5%

Average alcohol quantification of 0.5 and 2 g/L ethanol spikes in blood and water analysed using *n*-propanol or *t*-butanol as internal standard and Cerilliant® reference materials (water solutions) as calibrators (n = 10).

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