Critical Factors in Selecting an Internal Standard for Accurate Determination of Blood Alcohols in Post Mortem Samples

Sky Countryman, Kory Kelly, and Christopher Fernandez
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA

Though use of n-Propanol as an internal standard for GC blood alcohol analysis is common, it can be difficult to resolve when analyzing post mortem samples. Further, n-Propanol can also be formed in the body's natural decay process. A solution to this is to use other analytes as an internal standard. However, neither the Restek® nor the J&W® BAC column pairs were able to suitably resolve t-Butanol. They were able to resolve 2-Butanol, but this resulted in double the total analysis time. The Zebron™ ZB-BAC1 & ZB-BAC2 columns were able to fully resolve both t-Butanol and 2-Butanol from other target analytes, while maintaining rapid analysis times of less than 2 minutes.

Introduction

Last year, nearly 35 % of all traffic fatalities were alcohol related[1], reinforcing the need for accurate methods of determining blood alcohol content (BAC). Gas chromatography has become the standard analytical testing device for BAC in both confirmatory testing as well as post mortem investigations.

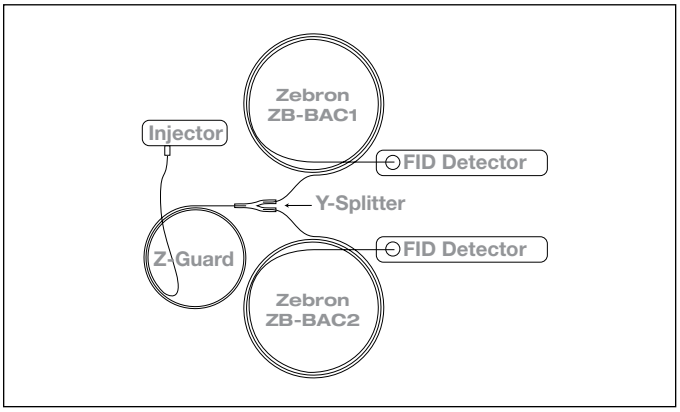
To improve the accuracy of this test, many labs are moving towards a dual column approach with the use of an internal standard **(Figure 1)**. The use of n-Propanol as an internal standard is common, but can be problematic when analyzing post mortem samples because it can also be formed in the body's natural decay process[2]. The objective of this work was to provide a method for analyzing post mortem samples using a different internal standard, while still retaining fast analysis with good accuracy and precision.

The determination of BAC is done to help either confirm the use/abuse of alcohol or to help identify the contributing factors in the cause of death for post mortem samples. Many analysis protocols today are favoring the use of dual column headspace GC methods to confirm the presence of ethanol in a blood sample. Due to the potential for other volatile components to be present in a patient's sample, either from metabolic processes or by contamination of common solvents, there are other compounds that are also included in analysis, including methanol, acetaldehyde, isopropanol, and acetone.

To account for systematic error associated with the measurement of BAC, it is common practice to use an internal standard. The most commonly referenced internal standard in the literature is n-Propanol. However, in post mortem samples, bacterial metabolism can result in the presence of n-Propanol, which could interfere with quantitation and lead to artificially lower calculated values of ethanol concentration.

The purpose of this work was to evaluate two alternative internal standards for BAC analysis, 2-Butanol and t-Butanol, which could be used in place of n-Propanol for normal analytical testing as well as post mortem samples. In order to compare quantitation values, n-Propanol was also included for reference. Three different sets of columns were evaluated for this analysis: the Restek Rtx®-BAC1 and Rtx®-BAC2 columns from Restek®, the J&W® DB-ALC1 and DB-ALC2 columns, and the Phenomenex Zebron ZB-BAC1 and ZB-BAC2 columns.

Figure 1.
Dual GC column configuration for blood alcohol analysis.



Method

A calibration curve **(Figure 3)** was prepared with analytes at concentrations of 0.025, 0.050, 0.100, 0.200, and 0.400 % in water (total volume) inside a 20 mL headspace vial. Note that the concentration of internal standards in each sample was always at 0.100 %. Analysis of each sample was conducted on an Agilent® 6890 gas chromatograph equipped with an Overbrook Scientific® Inc. (HT-200) autosampler and two new capillary columns. Column dimensions are listed with each chromatogram **(Figure 2)**. The columns were installed such that they would lead from the same injection port and guard column and split off into two separate flame ionization detectors. All parameters for the GC method are listed with the chromatograms. Linearity, LOD, LOQ, and reproducibility were evaluated for Zebron BAC1 and 2 columns **(Tables 1-2)**.

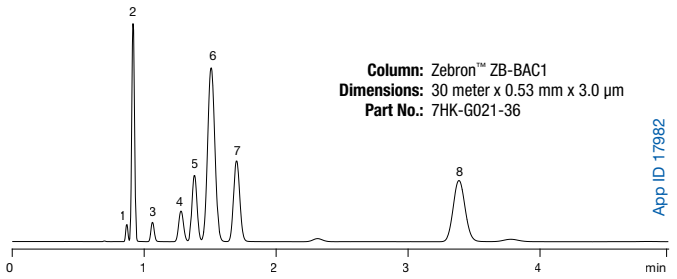
Results and Discussion

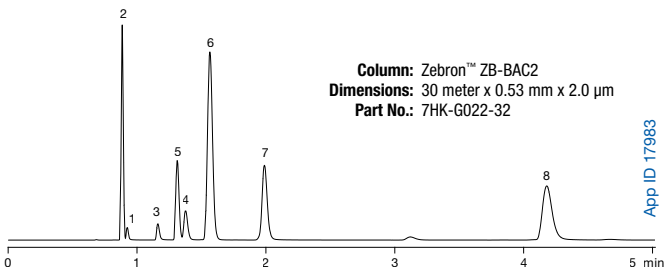
The Restek Rtx-BAC and the J&W DB-ALC columns showed co-elution between acetone and t-Butanol **(Figure 2)**. This co-elution with the internal standard would interfere with quantitation by the column pair, making the analysis inaccurate. Since the analysis was performed at 40 °C, it was impractical to optimize the GC oven conditions to achieve separation. If dual column analysis was required, neither column set would be suitable for use with t-Butanol as an internal standard.

Both the Restek and the J&W column pairs were able to separate all target analytes from 2-Butanol. However, the analysis time significantly increased due to the late elution of this internal standard. While this does provide a solution for post mortem samples, it doubles time necessary for analysis on both columns. This increased analysis time can negatively impact lab productivity if this standard were used for routine BAC testing in place of n-Propanol.

Next, we evaluated the Zebron ZB-BAC1 and ZB-BAC2, a new set of columns designed for BAC analysis. The Zebron pair was able to fully resolve t-Butanol from other target analytes on both columns in under 2 minutes. The use of t-Butanol actually shortened the total analysis time for the method by approximately 9 %. The

Figure 2A.
Blood Alcohols Using Zebron ZB-BAC Columns



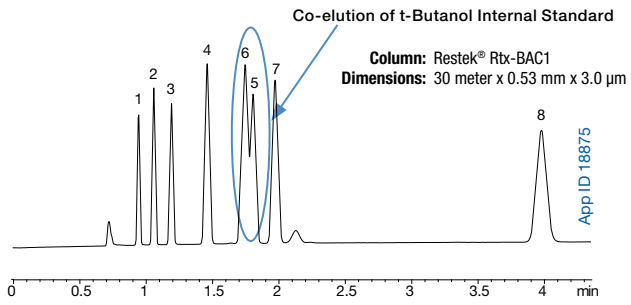


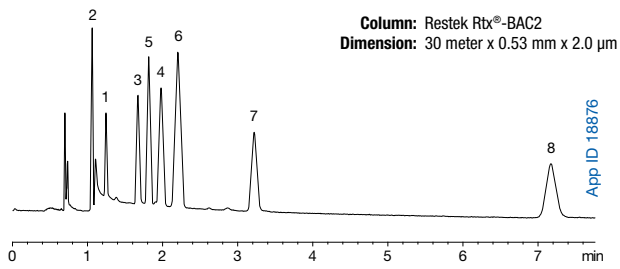
Injection: Split 0.8:1 @ 150 °C, 1 mL
Carrier Gas: Helium @ 80 cm/sec (constant flow)
Oven Program: 40 °C Isothermal
Detector: FID @ 250 °C

Sample: Analytes 0.025 % and internal standards 0.100 % in water
1. Methanol
2. Acetaldehyde
3. Ethanol
4. Isopropanol
5. Acetone
6. t-Butanol (IS)
7. n-Propanol (IS)
8. 2-Butanol (IS)

* Note additional separation of target analyte ethanol from neighboring peaks

Figure 2B.
Blood Alcohols Using Restek Rtx-BAC Columns

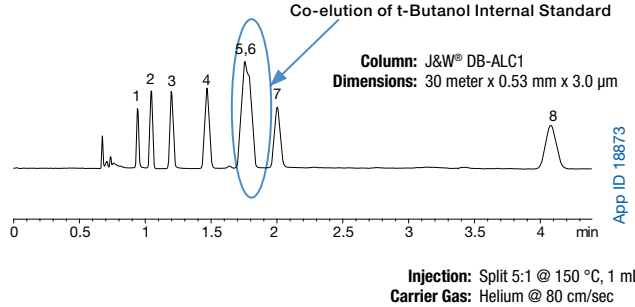


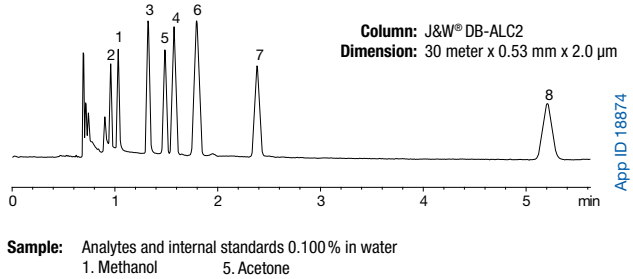


Injection: Split 5:1 @ 150 °C, 1 mL
Carrier Gas: Helium @ 80 cm/sec (constant flow)
Oven Program: 40 °C Isothermal
Detector: FID @ 220 °C

Sample: Analytes and internal standards 0.100 % in water
1. Methanol
2. Acetaldehyde
3. Ethanol
4. Isopropanol
5. Acetone
6. t-Butanol (IS)
7. n-Propanol (IS)
8. 2-Butanol (IS)

Figure 2C.
Blood Alcohols Using Agilent J&W DB-ALC Columns





Injection: Split 5:1 @ 150 °C, 1 mL
Carrier Gas: Helium @ 80 cm/sec (constant flow)
Oven Program: 40 °C Isothermal
Detector: FID @ 220 °C

Sample: Analytes and internal standards 0.100 % in water
1. Methanol
2. Acetaldehyde
3. Ethanol
4. Isopropanol
5. Acetone
6. t-Butanol (IS)
7. n-Propanol (IS)
8. 2-Butanol (IS)

internal standard 2-Butanol was also separated on both columns with a run time under five minutes. Labs would have the option to use either internal standard, for routine testing and maintain fast analysis times.

A further benefit of the Zebron™ column pair was the increased resolution and high reproducibility of ethanol from other close eluting components improving analytical precision. Using Zebron columns, the calibration curve for all compounds was found to be linear with correlation coefficients (R^2) within a range of 0.9980 – 0.9998 **(Figure 3)**. The agreement in quantitative results between each of the three possible internal standards was very good, indicating that either t-Butanol or 2-Butanol would be suitable alternatives to n-Propanol for method quantitation. Method LOD and LOQ were determined to be less than 0.0003 % and 0.001 % respectively for all compounds **(Table 1)**. These levels are significantly below the required detection limits of the method indicating that sensitivity requirements can easily be met.

Conclusion

The use of n-Propanol as an internal standard can be problematic when analyzing blood alcohol content in post mortem samples via GC. To help improve method accuracy and eliminate issues associated with n-Propanol, we performed dual column GC analyses utilizing two alternative internal standards (t-Butanol and 2-Butanol) on three different BAC GC column pairs. Our findings revealed that the Phenomenex Zebron ZB-BAC1 and -2 columns are the optimal solution for fast blood alcohol analysis of post mortem samples.

Neither the Restek® nor the Agilent® J&W® BAC column pairs suitably resolved t-Butanol from other target analytes. Both column pairs were able to fully resolve 2-Butanol. However, complete resolution of 2-Butanol required the method analysis time for both columns to double.

In contrast, the Phenomenex Zebron ZB-BAC1 & and ZB-BAC2 columns were able to fully resolve both t-Butanol and 2-Butanol from other target analytes, while maintaining rapid analysis times. Quantitative analysis on the Zebron BAC pair showed equivalent performance between all three internal standards (n-Propanol, t-Butanol, and 2-Butanol). The LOD & and LOQ for this column set were far below the control limits required by current testing regulations.

Table 1.
Estimated Limits of Detection (LOD) and Quantitation (LOQ) on Zebron ZB-BAC1 and ZB-BAC2 phases. The LODs and LOQs are also displayed in units of ppm in addition to % blood alcohol content.

Compound	Zebron ZB-BAC1		Zebron ZB-BAC2	
	LOD	**LOQ**	**LOD**	**LOQ**
Methanol	0.0003 % (2.9 ppm)	0.001 % (9.7 ppm)	0.0003 % (3.0 ppm)	0.001 % (10.1 ppm)
Acetaldehyde	0.00002 % (0.2 ppm)	0.00008 % (0.8 ppm)	0.00002 % (0.2 ppm)	0.00006 % (0.6 ppm)
Ethanol	0.0003 % (2.6 ppm)	0.0009 % (8.8 ppm)	0.0002 % (2.2 ppm)	0.0007 % (7.4 ppm)
Isopropanol	0.0002 % (1.8 ppm)	0.0006 % (5.8 ppm)	0.0001 % (1.4 ppm)	0.0005 % (4.6 ppm)
Acetone	0.00008 % (0.8 ppm)	0.0003 % (2.6 ppm)	0.00005 % (0.5 ppm)	0.0002 % (1.7 ppm)

Figure 3.
Calibration curves on (A) Zebron ZB-BAC1 and (B) Zebron ZB-BAC2 phases. Each analyte is designated by the following: Methanol (-◆-), Ethanol (-▲-), Isopropanol (-○-), Acetone (-✶-), and Acetaldehyde (-■-). Insets show a magnified view to decipher the methanol and ethanol calibration curves.

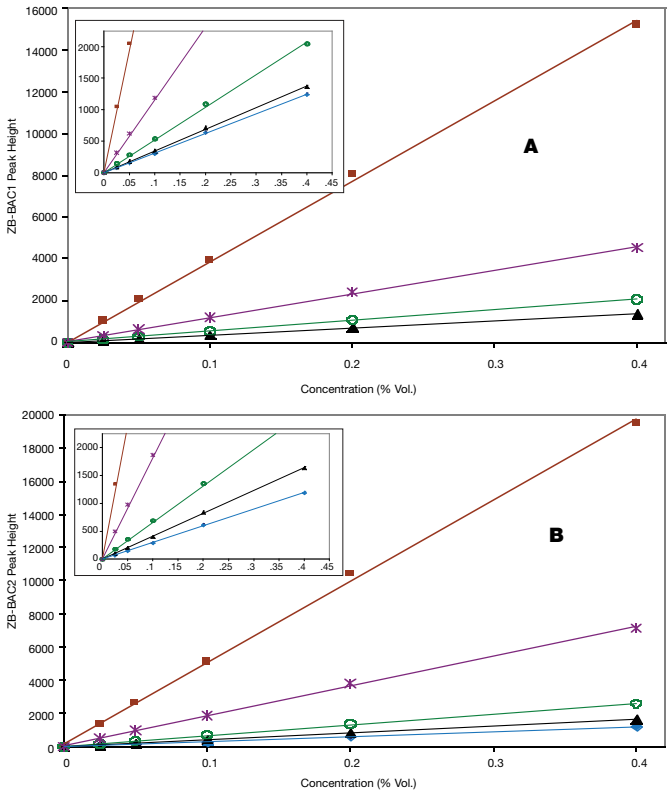


Table 2.
High reproducibility of Zebron ZB-BAC1 and ZB-BAC2 for blood alcohols. Relative RSD values for replicate injections (n=5) were taken at the lowest concentration in the calibration curve of 0.025 %.

Zebron ZB-BAC1

Compound	Peak Area % RSD (Absolute)	Peak Area % RSD (Relative to t-Butanol)	Peak Area % RSD (Relative to n-Propanol)	Peak Area % RSD (Relative to 2-Butanol)
Methanol	3.1	2.8	2.7	2.6
Acetaldehyde	2.1	1.6	1.5	1.4
Ethanol	3.4	2.8	2.7	2.6
Isopropanol	3.2	2.6	2.5	2.4
Acetone	2.2	1.6	1.5	1.4

Zebron ZB-BAC2

Methanol	2.1	1.6	1.4	1.3
Acetaldehyde	3.1	2.7	2.5	2.4
Ethanol	3.2	2.7	2.5	2.4
Isopropanol	2.2	1.6	1.4	1.3
Acetone	3.2	2.7	2.5	2.4

APPLICATIONS

Australia
t: 02-9428-6444
f: 02-9428-6445
auinfo@phenomenex.com

Austria
t: 01-319-1301
f: 01-319-1300
anfrage@phenomenex.com

Belgium
t: +31 (0)30-2418700
f: +31 (0)30-2383749
beinfo@phenomenex.com

Canada
t: (800) 543-3681
f: (310) 328-7768
info@phenomenex.com

Denmark
t: 4824 8048
f: 4810 6265
nordicinfo@phenomenex.com

Finland
t: 09-4789 0063
f: +45 4810 6265
nordicinfo@phenomenex.com

France
t: 01 30 09 21 10
f: 01 30 09 21 11
franceinfo@phenomenex.com

Germany
t: 06021-58830-0
f: 06021-58830-11
anfrage@phenomenex.com

Ireland
t: 01 247 5405
f: +44 1625-501796
eireinfo@phenomenex.com

Italy
t: 051 6327511
f: 051 6327555
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

Mexico
t: (55) 5018 3791
f: (310) 328-7768
tecnicomx@phenomenex.com

Netherlands
t: 030-2418700
f: 030-2383749
nlinfo@phenomenex.com

New Zealand
t: 09-4780951
f: 09-4780952
nzinfo@phenomenex.com

Norway
t: 81 00 20 05
f: +45 4810 6265
nordicinfo@phenomenex.com

Puerto Rico
t: (800) 541-HPLC
f: (310) 328-7768
info@phenomenex.com

United Kingdom
t: 01625-501367
f: 01625-501796
ukinfo@phenomenex.com

All other countries:
Corporate Office USA
t: (310) 212-0555
f: (310) 328-7768
info@phenomenex.com

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References

1. National Highway Traffic Safety Administration J.E. *Monitoring.*
2. Nanikawa, R.; Ameno, K.; Hashimoto, Y.; Hamada, K. *Medicolegal Studies on Alcohol Detected in Dead Bodies – Alcohol Levels in Skeletal Muscle*. Forensic Sci. Int., 20, 133-140.

Ordering Information

Zebron ZB-BAC1 GC Columns			
ID(mm)	**df(µm)**	**Temp. Limits °C**	**Part No.**
30-Meter			
0.32	1.80	-20 to 260/280 °C	7HM-G021-31
0.53	3.00	-20 to 260/280 °C	7HK-G021-36

Zebron ZB-BAC2 GC Columns			
ID(mm)	**df(µm)**	**Temp. Limits °C**	**Part No.**
30-Meter			
0.32	1.20	-20 to 260/280 °C	7HM-G022-25
0.53	2.00	-20 to 260/280 °C	7HK-G022-32

If Zebron GC Columns do not provide you with equivalent separations as compared to any other GC column of the same phase and dimensions, return the column with comparative data within 45 days for a FULL REFUND.

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