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Computer-Aided Headspace Gas Chromatography Applied to Blood-Alcohol Analysis: Importance of Online Process Control

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ABSTRACT: This paper describes the analysis of ethanol in blood specimens from suspect drunk drivers and the associated quality assurance procedures currently used in Sweden for legal purposes. Aliquots of whole blood from two separate Vacutainer® tubes are diluted with 1-propanol as internal standard before analysis by headspace gas chromatography (HS-GC) with three different stationary phases: Carbopak B, Carbopak C, and 15% Carbowax 20 M. The actual HS-GC analysis, the integration of chromatographic peaks, the collection and processing of results, as well as the quality control tests involve the use of computer-aided techniques. The standard deviation of analysis (y) increased with concentration of ethanol in the blood specimen (x), and above 0.50 mg/g the regression equation was $y = 0.0033 + 0.0153x$. The prosecution blood-alcohol concentration (BAC) is the mean of three separate determinations made by different laboratory technicians working independently with different sets of equipment. A deduction is made from the mean analytical result to compensate for random and systematic errors inherent in the method. At BACs of 0.5 and 1.5 mg/g, which are the statutory limits in Sweden, the allowances currently made are 0.06 and 0.09 mg/g, respectively. Accordingly, the reduced prosecution BAC is less than the actual BAC with a statistical confidence of 99.9%.

KEYWORDS: toxicology, alcohol, chromatographic analysis, computers, blood, gas chromatography, headspace technique, computer-aided, quality control, legal purposes

Statutory limits of blood-alcohol concentration (BAC) as evidence of impairment of driving were introduced in Sweden in 1941 [*1*]. The present law stipulates two different BAC limits; 0.5 and 1.5 mg/g.[2] A distinction is made between a more serious offense called drunken driving (1.5 mg/g) and impaired driving (0.5 mg/g). Conviction for drunken driving carries a mandatory prison sentence for two to eight weeks and withdrawal of the driving license for one to two years. These severe penalties, even for a first offense, make it essential that the chemical-technical methods used to determine BAC are accurate, precise, and that the variations that occur in day-to-day work are carefully controlled.

About 22 000 drunk drivers are apprehended in Sweden each year and all blood specimens are sent to the same central laboratory for quantitative determination of ethanol. The result used for prosecution must not exceed the true value with at least 99.9% confidence. This statistical safeguard is ensured by making a deduction from the mean of 3 single deter-

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[2]0.50 mg/g = 0.53 mg/mL = 0.053 g% w/v; specific gravity of whole blood is taken to be 1.06.

minations. The day-to-day precision of blood-alcohol measurements must be carefully monitored to ensure that the size and structure of the analytical variations remain within acceptable tolerance limits. This requires a rigorous program of quality control and good laboratory practice generally. The control charts should be generated online to ensure that the process is "under control" before analytical reports are returned to the police authorities.

This paper describes a computer-aided analytical system based on HS-GC for determination of ethanol in blood specimens from drunken drivers. A novel feature of our method is the use of online quality control charts. This provides close scrutiny of analytical errors, both random and systematic, and ensures that they remain stable over time. Because the standard deviation of an analysis increases with the concentration of ethanol in the blood specimen, a bigger allowance is necessary at higher BACs. In this way, the same level of confidence (99.9%) is guaranteed for all suspects. The primary laboratory results are plotted as control charts after transformation of the data into normalized ranges. This ensures a normal distribution of errors so that the whole spectrum of BAC measurements can be displayed on the same control diagram.

Materials and Methods

Blood Specimens

In Sweden all blood specimens from suspect drunk drivers are analyzed at the National Laboratory of Forensic Chemistry. Blood specimens (2 by 10 mL) are taken from a cubital vein into Vacutainer® tubes (Mediplast AB, Stockholm) containing a mixture of sodium fluoride (100 mg) as preservative and ethylenediaminetetraacetate (EDTA) (25 mg) as the anticoagulant. The specimens of blood are then labelled with the name of the suspect, the time and date of sampling, and the name of the person, normally a licensed nurse, who took the specimens. The tubes of whole blood and whenever possible one or two specimens of urine are sent to our laboratory by express mail together with a police report. This contains details about the suspect such as name, date of birth, address, type of motor vehicle as well as various particulars about the traffic incident, that is, traffic accident, moving traffic offense, or routine control.

The specimens of blood and urine arrive 12 to 24 h after sampling and each package is opened one at a time in a specially allocated room with two laboratory workers present at all times. Information written on the blood specimen tubes is compared with the police protocol and any inconsistencies are carefully noted. The same registration number is then given to both tubes of blood, any accompanying specimens of urine, and the police report. The blood specimens are stored in a refrigerator at 4°C pending analysis and the police protocols are locked away in an office.

Preparation of Blood for Analysis

Before analysis, the blood specimens are allowed to mix thoroughly in a rotamix device for about 10 min. Exactly 0.1-mL aliquots are removed and diluted eleven fold with an aqueous solution of 1-propanol (0.08 mg/mL) as internal standard using a fixed volume diluter-dispenser (Eppendorf Ltd., FRG). The blood is then dispensed directly into 22-mL vials and made airtight with silicon rubber stoppers and crimped-on aluminum caps. The registration number of the blood specimen is written on the vials to allow a check on correct position number during dilution and later when setting up the headspace gas chromatography (HS-GC) analysis. This dilution equipment is reserved for blood from living subjects and the transfer tubing is washed with internal standard between consecutive specimens to avoid carryover effects.

Aqueous alcohol standards, 0.50, 1.00, and 1.50 mg/mL (Merck Ltd., Damstadt, FRG), are supplied in flame-sealed borosilicate glass ampoules. These standards are used to generate calibration factors for HS-GC and are analyzed every tenth blood specimen as control of within-run and between-run accuracy. Standards are diluted with 1-propanol in exactly the same way as blood specimens with the same diluter-dispenser. A triplicate determination is made on each blood by three different laboratory technicians working independently with different sets of equipment. The aliquots analyzed are taken from two separate Vacutainer tubes as an extra precaution against sample mix-up. Aqueous alcohol standards prepared ourselves by volumetric dilution of absolute ethanol show excellent agreement with those supplied by Merck. Figure 1 presents a flowchart of the analytical scheme currently used for blood-alcohol analysis.

Headspace Gas Chromatography

The instrument park for blood-alcohol analysis consists of 3 Sigma 2000 gas chromatographs, 3 HS-100 automatic headspace samplers, 3 LIC-100 computer Integrators, and 1 professional computer model 7500. The GC equipment and the computer were obtained from Perkin-Elmer Corp., Norwalk, Connecticut. The headspace sampler allows the analysis of 100 vials in each run. Before starting the analysis, each operator enters the registration

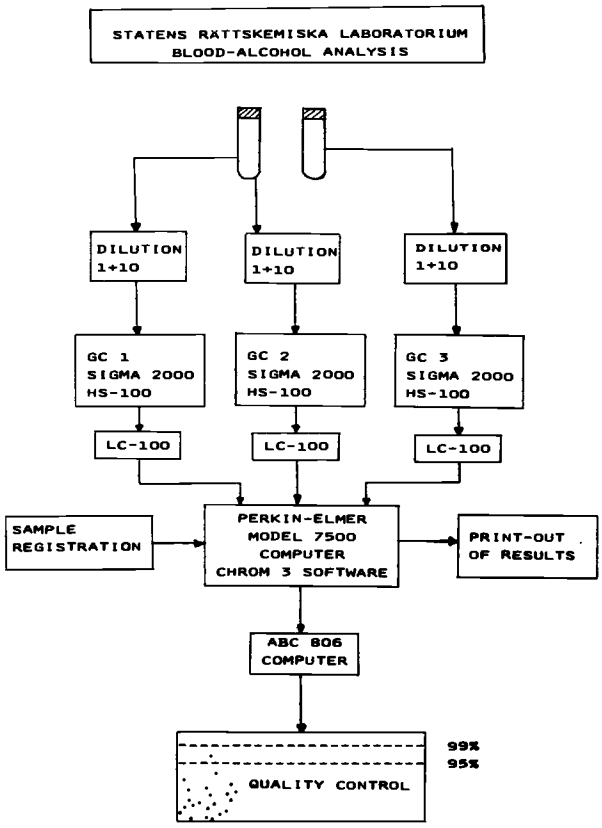

FIG. 1—*Flow diagram illustrating the various steps involved in the analysis of ethanol in blood for legal purposes with online process control of results.*

number of the blood specimen into the computer and this corresponds to a certain position number in the actual series. The regression relationship (peak height ratio versus concentration of ethanol) is calculated based on a 3-point calibration with triplicate determinations for each of the standards 0.50, 1.00, and 1.50 mg/mL.

The evaluation of results and preparation of reports are done with computer programs written by Martin Arnold (Perkin-Elmer Corp., Norwalk, Connecticut). When the series of blood specimens are analyzed, the registration numbers and positions in the run are compared for the three GC instruments. If these match, the average BAC, standard deviation, and range of the triple determination are calculated. An analytical report is printed on specially designed paper stickers. If the precision falls within the predetermined tolerance limits, the stickers are attached directly to the police protocols. Finally, the registration number of the blood specimen printed on the sticker is compared with the number on the police protocol and if these check, the papers are undersigned by the chief analyst and returned to the police. As an extra safeguard to ensure specimen identity, the suspect's name and the date and time of blood sampling according to the police protocol are compared with a laboratory list with the same information but taken from the tubes of blood.

Chromatographic Operating Conditions

Table 1 summarizes the operating conditions used for HS-GC. All three instruments are connected to the same computer-controlled operating system and the conditions are generally similar to those described in the literature for blood-alcohol analysis by HS-GC [*2–9*].

Quality Control Charts

During the printout of analytical reports under the control of the Perkin Elmer computer, a separate desktop computer (ABC 806 Luxor data AB, Sweden) performs an independent statistical analysis and generates quality control charts online. One chart depicts random analytical errors (precision) derived from the range and standard deviation (SD) of each triplicate determination. A second chart monitors the accuracy of the assay by plotting residuals. These are defined as the differences together with their sign between the concentration of alcohol measured in the standard solutions every tenth blood and the target values. The

TABLE 1—*Operating conditions used for analysis of ethanol in blood specimens from suspected drinking drivers by headspace gas chromatography with flame ionization detector and Sigma 2000 GC and HS-100 sampler from Perkin-Elmer.*

	Stationary Phase		
Chromatographic Parameter	Carbopak C, 80–100 0.2% Carbowax 1500	Carbopak B, 60–80 5% Carbowax 20M	15% Carbowax 20M Chromosorb W
Glass column	2 m by 3 mm id	2 m by 3 mm id	2 m by 3 mm id
Oven temp.	100	100	120
Run time	2.3 min	2.3 min	2.3 min
Detector temp.	150°C	150°C	170°C
N_2 flow rate	20 mL/min	20 mL/min	20 mL/min
Equilib. time	18 min	18 min	18 min
Equilib. temp.	40°C	40°C	40°C
Transfer temp.	70°C	70°C	70°C
Pressuriz. time	0.5 min	0.5 min	0.5 min
Inject time	0.08 min	0.08 min	0.08 min
Withdrawal time	0.2 min	0.2 min	0.2 min
Cycle time	2.8 min	2.8 min	2.8 min

magnitude of the residuals also gives a check on linearity of the detector response because 0.5, 1.0, and 1.5 mg/mL are included in each series after every tenth blood.

Statistical Background

The present system of analytical quality control has its roots in a 1956 Swedish Government report about reliability of blood-alcohol analysis for legal purposes [*10*]. However, in 1956 no consideration was given to the change in precision of analysis as a function of BAC. It was felt that because the average BAC among Swedish drunk drivers was about 1.5 mg/g, three times more than the statutory limit, a constant subtraction term would suffice even though it was recognized that this gives a greater margin of security to those with BAC near the lower statutory limit. Nowadays the increase in SD with concentration of ethanol is taken into account when calculating the allowance for random error. The functional relationship between SD and BAC was determined from 4247 consecutive determinations made when the method was first introduced.

Instead of the conventional precision control charts, which usually depict the range or SD plotted against sample number in the series, we used the technique of plotting normalized (studentized) range [*11*]. A single control chart therefore serves to display all blood specimens regardless of the BAC. On a conventional control chart, the specimens that deviate most would be those at high BAC implying less efficient control of precision at low BAC. The normalized range (Nr) is calculated by dividing the observed BAC range of each triple determination by the SD of the mean BAC estimated from the regression relationship relating SD and concentration of alcohol for a large population of blood analyzed by the same method ($N = 4247$). This SD was constant until a BAC of 0.50 mg/g was reached but thereafter increased according to the equation $y = 0.0033 + 0.0153x$.

The mean of a triplicate determination has a standard error of $SD/\sqrt{3}$ and the 99.9% confidence limit in a one-tailed test is given by $SD/\sqrt{3} \times 3.09$. The allowances for random analytical variations are therefore 0.0196 mg/g at 0.5 mg/g BAC and 0.047 mg/g at 1.5 mg/g.

$$\text{Normalized range (Nr)} = \frac{\text{BAC range (max} - \text{min)}}{\text{SD}}$$

where

$$y\ (\text{SD}) = 0.0033 + 0.0153x\ (\text{BAC}) \text{ for specimens above 0.5 mg/g}$$
$$\text{SD} = 0.011 \text{ for samples less than 0.5 mg/g BAC}$$
$$\text{SE} = \text{SD}/\sqrt{3}$$
$$\text{99.9\% confidence limit} = \text{SE} \times 3.09 \text{ (one-tailed test)}$$

Results

Specificity for Measuring Ethanol

Table 2 gives the retention times of ethanol and other low-molecular weight volatiles with three different stationary phases used for blood-alcohol analysis. Note that 2-butanone and ethanol have the same retention times with 15% Carbowax 20M as the column packing. Otherwise, ethanol is well resolved from potential interfering substances and the total analysis time is only about 3 min.

Relationship Between Precision and Blood-Ethanol Concentration

Figure 2 shows the relationship between precision of analysis defined as SD of a single determination and the mean concentration of alcohol in the blood. Above 0.5 mg/g, the SD

TABLE 2—*Relative retention times with three different GC stationary phases for ethanol compared with other low-molecular weight volatile agents that might be encountered in forensic blood specimens. The retention times are relative to 1-propanol internal standard.*

Substance	Relative Retention Time, min		
	Carbopak C, 80–100 0.2% Carbowax 1500	Carbopak B, 60–80 5% Carbowax 20M	15% Carbowax 20M Chromosorb W
Methanol	0.33	0.35	0.68
Acetone	0.63	0.46	0.57
Ethanol	0.47	0.52	0.71
2-Propanol	0.76	0.71	0.68
2-Butanone	1.52	0.80	0.71
1-Propanol	1.00 (1.54)	1.00 (1.85)	1.00 (1.83)[a]

[a]Absolute retention time, min.

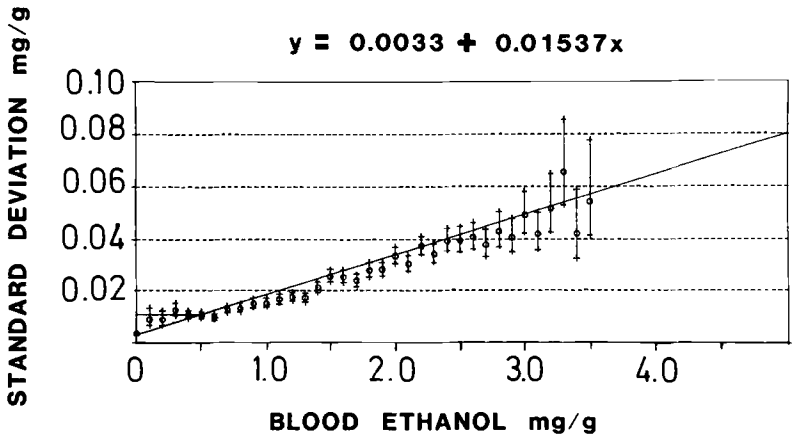

FIG. 2—*Relationship between precision of blood-alcohol analysis and concentration of ethanol. The regression equation was calculated by the maximum likelihood method for specimens with BAC above 0.5 mg/g.*

clearly increases as BAC increases. The regression relationship was calculated by the method of maximum likelihood to compensate for the increase in variance as BAC increases [*12*]. The regression equation above 0.5-mg/g BAC was $y = 0.0033 + 0.0153x$.

Control Chart for Within Laboratory Precision

Figure 3 shows a typical control chart for monitoring within laboratory precision. This kind of chart includes a new feature, namely, the use of normalized range, sometimes called studentized range. The tolerance limits are set at a normalized range of 3.31 for 95% of specimens and 4.12 for 99% (see Ref *11*, Table A-10). This implies that a maximum of 5 and 1% of specimens should fall outside these limits of 3.31 and 4.12, respectively. The average SD for each series of blood is calculated and used for long-term control of drift in the precision of the method.

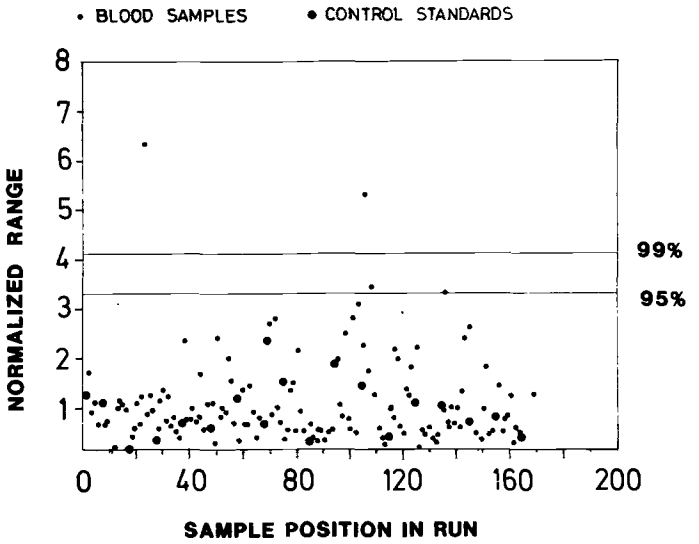

FIG. 3—*Control chart to monitor within-laboratory precision of blood-alcohol analysis. Normalized range is plotted on the vertical axis and sample number in the series on the horizontal axis.*

Control Chart for Systematic Errors

Figure 4 shows a typical control chart used to monitor systematic errors in the method. A perfectly accurate method would show all residuals equal to zero. The use of three ethanol standards (0.50, 1.00, and 1.5 mg/mL) gives a check on linearity of the method. The mean residual and its deviation from zero are calculated for each series of blood analyzed. Based on 417 residual values obtained during 28 consecutive days, the mean ± SD was 0.007 ± 0.00375 mg/g. No tendency was seen for the residual to change with the concentration of alcohol. The allowance (deduction) for systematic error is therefore 0.007 + (3.09 × 0.00375) = 0.0172 mg/g. If the mean residual in a series exceeds this critical value all the blood specimens are reanalyzed.

Precision of Analysis During Long-Term Routine Use

Figure 5 shows the relationship between SD of a single determination and BAC for 15 288 consecutive blood specimens after the method was well established in routine use. Extreme values were excluded before this linear regression relationship was calculated. The SD seems to remain constant until a BAC of 0.75 mg/g is reached, but thereafter, a linear increase with BAC is obvious. The correlation coefficient was 0.97 ± 0.058 ($p < 0.001$) and the regression equation above 0.75 mg/g was y (SD) = $-0.0016 + 0.0104x$ (BAC). The magnitude of the regression coefficient (0.0104) implies that the SD of analysis increases by about 0.01 mg/g for each 1.0-mg/g increase in BAC. The difference between this result and the slope of the regression line in Fig. 2 can be explained by eliminating extreme value data and the improvement in precision that comes from long-term routine use of the method.

Deductions Made for Analytical Errors

Figure 6 (upper part) shows the average weekly BAC among individuals apprehended in Sweden for driving under the influence. Over twelve months, the weekly BAC was remark-

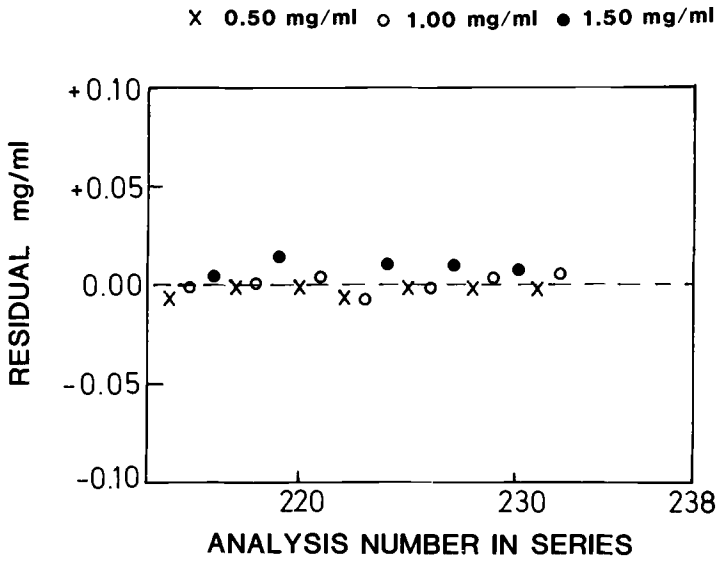

FIG. 4—*Control chart depicting systematic errors or accuracy of blood-alcohol analysis. The residuals are the differences between the known target concentration of the standard (0.50, 1.00, or 1.50 mg/mL) and the mean results obtained by gas chromatographic analysis on three different instruments.*

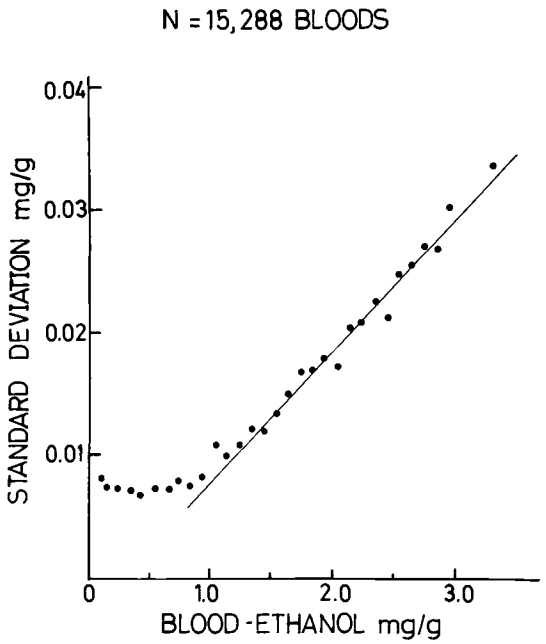

FIG. 5—*Relationship between precision of blood-alcohol analysis and concentration of ethanol for 15 288 specimens analyzed during 1986. Outlying values with normalized range above ten were excluded. The regression equation at BAC above 0.75 mg/g was* $y = -0.0016 + 0.0104x$. *The mean SD for all blood within intervals of 0.1-mg/g BAC is plotted.*

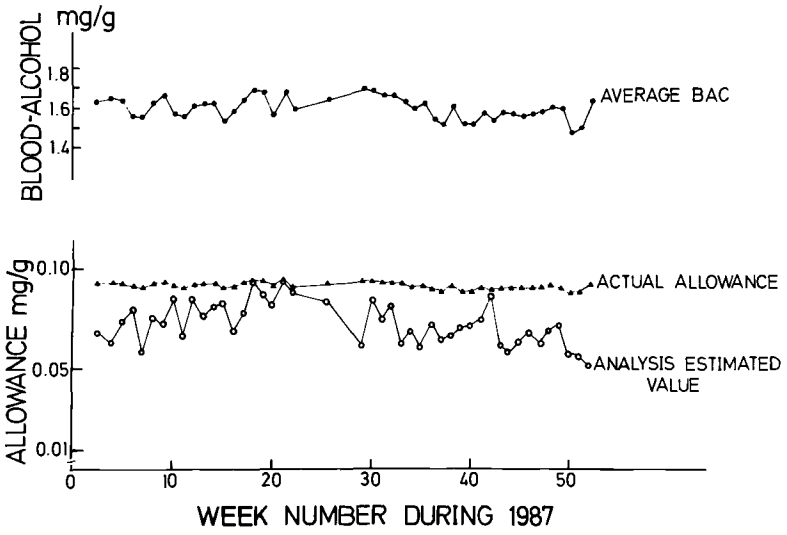

FIG. 6—*Weekly record of the average BAC of Swedish DWI offenders* (upper part) *and the concentration-dependent allowance made for analytical variations* (middle). *The analysis estimated value* (lower part) *is derived from the actual random and systematic errors for each weekly period.*

ably constant at about 1.66 mg/g. The magnitude of the deductions used to derive the prosecution BAC are also shown in Fig. 6 (middle). This allowance is simply a function of the mean BAC provided that the quality control requirements are met. In the lower part of Fig. 6, the deductions were calculated with reference to the actual average SD (random error) and residual (systematic error) for each weekly period. These analysis-estimated values fluctuate over the year but are always less than the allowances actually made.

Interlaboratory Collaborative Study

Table 3 shows the results obtained from a collaborative study of blood-alcohol analysis conducted at five Scandinavian laboratories. The study involved three blood specimens taken from suspected drunk drivers. Aliquots of blood were sent coded to the participating laboratories. The precision reflects within-run and between-run components of variance and the coefficient of variation (CV) was less than 2% for the BAC range studied.

TABLE 3—*Interlaboratory collaborative study of forensic blood-alcohol analysis in the Nordic countries. Headspace gas chromatography was used for analysis and the results represent mean ± SD for three to four replicate determinations made on each of two consecutive days.*

	Ethanol Concentration in Blood, Mean ± SD, mg/g		
Country	Specimen 1	Specimen 2	Specimen 3
Denmark	2.99 ± 0.015	2.23 ± 0.008	1.38 ± 0.00
Finland	3.01 ± 0.079	2.24 ± 0.048	1.37 ± 0.034
Iceland	3.02 ± 0.023	2.24 ± 0.011	1.38 ± 0.012
Norway	3.08 ± 0.067	2.32 ± 0.072	1.41 ± 0.024
Sweden	3.00 ± 0.025	2.24 ± 0.014	1.39 ± 0.014

Discussion

Widmark's classic chemical method of blood-alcohol analysis was published in 1922 [*13*] and this became widely used for legal purposes in Sweden and most of Europe. In the early 1950s, enzymatic methods were developed for blood-alcohol analysis [*14*] because these offered a better specificity for analyzing ethanol compared with wet-chemical oxidation procedures. The risk of interference was especially important when autopsy material was analyzed. The alcohol dehydrogenase (ADH) method was used in Sweden from the mid-1950s until 1985 when the present HS-GC method was introduced.

HS-GC has emerged as the method of choice for analysis of ethanol as well as other low molecular weight volatiles in body fluids for research and medicolegal purposes. The present method is similar to many well-established HS-GC techniques described in the literature [*2–6, 9*]. Special precautions must be taken to eliminate the risk of mix-up of specimens and to ensure a high degree of quality control of the day-to-day analytical results. To this end, aliquots of blood are removed from two different Vacutainer tubes from each suspected driver. Furthermore, three laboratory assistants each make a single determination working independently with different sets of equipment.

The notion of making an allowance to adjust the mean result of analysis for errors in the method is a long-established tradition in Sweden. This is especially relevant when per se statutes are enforced and the BAC result cannot be rebutted. The amount subtracted from the mean depends in part on the number of replicate determinations, the inherent variability of the assay, and the degree of probability required that the reduced value will not exceed the true BAC. If the SD of the method of analysis is unknown, confidence limits should be set according to student's t-distribution for the appropriate degrees of freedom, being one less than the number of replicate determinations. But for a method of analysis in routine use, the SD is normally well defined for a sufficiently large material so that the normal distribution can be used. However, a prerequisite for use of the normal distribution with infinite degrees of freedom is that the random errors in each series are under statistical control.

Besides day-to-day control of within-laboratory precision, an important aspect of proficiency testing is to make regular interlaboratory collaborative studies. This entails sending the same blood specimens to all participating laboratories. When different countries are involved, these interlaboratory tests cannot be done under blind conditions, that is, the control blood is not sent mixed together with daily specimens. Table 3 shows that within-laboratory precision is acceptable with a CV of less than 2%. The mean BAC reported by the various laboratories also agreed well.

The present deduction made from the mean of a triplicate determination of BAC before reports are sent to the police is comprised of three elements: (1) a component for random analytical variations, the magnitude of which depends on the underlying BAC; (2) a component for systematic deviations (accuracy); and (3) an additional allowance to guard against possible long-term drift in the analytical procedures. This latter value is set at 0.024 mg/g. Thus a driving while intoxicated (DWI) suspect with measured mean BAC of 0.5 and 1.5 mg/g gets an allowance of 0.06 and 0.09 mg/g, respectively. In practice, this ensures that an individual with a true BAC just below one of the critical legal limits still runs a 1 in 1000 risk that the reduced value is too high and therefore above the statutory limit. Our strategy of including Component 3 above in the amount deducted considerably lowers the likelihood of unjust treatment owing to analytical errors.

Before control charts are approved or rejected by the chief chemist, all outlying values must be carefully scrutinized. This is necessary to find if there are assignable causes which might account for the extreme values. If less than 5% of blood specimens in a series fall outside the critical 3.31 limit, this can be tolerated because it implies an improvement in the analytical precision. Indeed, this suggests that a smaller deduction from the mean can be made to maintain 99.9% confidence in the prosecution BAC. But to change the subtraction term based on results from a limited series of blood specimens is not advisable. The SD is

likely to increase again later as illustrated in Fig. 6. During the summer months when more experienced laboratory staff take vacation, the SD seems to fluctuate more and tends to increase. This trend is also seen when less experienced technicians are made responsible for one of the replicate assays of each triplicate determination.

If more than 5% of results fall outside the critical normalized range of 3.31, this should serve as a warning signal that the system is in danger of becoming "out of statistical control." If more than 1% of blood have normalized range above 4.12, this signifies the action limit and the whole series should be reanalyzed if assignable causes cannot be found. But before this is done, the chromatograms and the tubes of blood should be carefully examined. The principal reason for outliers stems from the presence of 2-butanone as well as ethanol in the blood specimens. Some drinking drivers in Sweden drink denatured alcohol [*15*]. Table 2 shows that this ketone has the same retention time as ethanol with 15% Carbowax 20M as stationary phase. This gives an abnormally large BAC range for the triplicate determination. Partial coagulation of blood in one or both of the Vacutainer tubes is another cause of outliers. The concentration of ethanol in serum is about 12% greater than in whole blood [*16*].

Problems in getting both Vacutainer tubes filled with blood can introduce a time delay between the consecutive specimens, and BAC might therefore change through metabolism. There are instances when dilution of one of the tubes of blood has occurred during sampling such as when victims of a traffic accident are given an intravenous infusion of fluids at the roadside or when they arrive at hospital. If the infusion coincides with taking blood for alcohol analysis, an unintentional dilution of one or both of the specimens is likely. It is obviously important to distinguish true analytical variations from other sources of error which are outside the control of the laboratory. Reanalysis of only the outlying blood specimens in the series regardless of the number is not good practice. This would mean that blood in the same series, but which happens to fall within the tolerance limits, needs to get a larger allowance to ensure 99.9% confidence in the prosecution value.

The precision of blood-alcohol analysis by HS-GC with automated sampling techniques has a CV of less than 1% and this probably represents a lower limit in routine applications. With our method, the CVs for different GC instruments, two tubes of blood, and different operators are included in this figure. The remaining random error can be tolerated and adjusted as described above by reducing the mean result so that the final prosecution value is less than the true BAC with high statistical confidence such as 99.9% in a one-tailed test as used for legal purpose in Sweden.

Acknowledgment

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