Highly sensitive gas chromatographic analysis of ethanol in whole blood, serum, urine, and fecal supernatants by the direct injection method

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A highly sensitive, reproducible, and rapid gas chromatographic method for ethanol determination in various biological specimens (human whole blood, serum, urine, and fecal supernatants) was developed. The method involves direct injection of the biological specimen into the gas chromatograph, without any pretreatment. Contamination of the gas chromatographic column with nonvolatile material was prevented by the use of a glass liner in the injector. This liner, which acted as a precolumn, was partly filled with small glass beads. Injection was performed in between the glass beads. More than 50 injections of the various biological specimens could be done before the liner had to be replaced by a new one. This injection technique between glass beads allows direct injection of large sample volumes up to 10 μL without disturbing the gas chromatographic separation. Injection of these large sample volumes made the method very sensitive. The detection limit for ethanol amounted to 0.1 mg/L (2 μmol/L) when using an injection volume of 5 μL. Attention has also been paid to simultaneously monitoring ethanol, methanol, acetaldehyde, and acetone in blood and urine of control subjects.

INDEXING TERMS: alcohol • acetalddehyde • acetone • methanol • propanol • butanol.

Numerous methods have been described for the determination of ethanol in whole blood, serum, and urine, the most popular being gas chromatography (GC), chemical assays, and enzymatic assays [1–3]. GC is the most precise and reliable method for alcohol determination in blood and other biological fluids, and has become the gold standard in forensic toxicology. However, in clinical chemistry, GC has often been disregarded as a technique full of difficulties and requiring specifically trained personnel.

Concerning GC, many methods are available in the literature [2, 3]. Methods requiring solvent extraction or distillation are time and sample consuming and should be considered obsolete. The two major techniques used nowadays are headspace sampling and direct specimen injection. These two techniques can also be fully automated. The headspace technique is quite laborious, requires larger volumes of the biological specimen than does direct injection, and is less sensitive than the direct injection technique. Moreover, the headspace technique might be subject to serious analytical errors due to variations in partitioning of ethanol between the gas and liquid phases, depending on the liquid matrix used [4, 5]. Direct injection [3, 5–7] obviates all the sample type discrepancies observed with headspace analysis. The only drawback of direct injection is its possible polluting effect on the injection port, on the precolumn, on the column, and on the injection syringe. Many investigators have advocated preparation of protein-free filtrates of the biological specimen or dilution before analysis [3].

The objective of the present paper was to develop a sensitive, reliable, easy-to-use, and rapid procedure for the determination of ethanol in whole blood, serum, urine, and fecal supernatants by using the direct injection GC technique.

Materials and Methods

REAGENTS
Ethanol, methanol, acetone, n-propanol, isopropanol, n-butanol, and isobutanol, all analytical grade (>97% purity), were obtained from Merck (Darmstadt, Germany).
L-Lactic acid and periodic acid came from Sigma (St. Louis, MO). The column packing material, 10% SP-1200/1% H₃PO₄ on 80/100 Chromosorb W AW, came from Supelco (Bellefonte, PA). The glass wool (dimethylchlorosilane treated) was from Chrompack (Middelburg, The Netherlands) and the small glass beads with a diameter of 1 mm from Tamson (Zoetermeer, The Netherlands).

PREPARATION OF BIOLOGICAL SPECIMENS
Whole blood, urine, and fecal material were obtained from healthy volunteers who were recruited from the laboratory personnel. In the experiments with whole blood, heparinized whole blood was used. Fecal samples were homogenized with a blender and ultracentrifuged for 2 h at 4 °C and 30 000g. The supernatant (fecal water) was carefully removed and stored at −20 °C until analysis. For a more convenient procedure, one might dilute the feces 3 to 5 times with distilled water. After vortex-mixing, 1 mL of the homogeneous suspension was transferred into a conical polypropylene micro sample tube (Eppendorf, Hamburg, Germany; 1 mL) and centrifuged for 1 min at 10 000×g in an Eppendorf centrifuge. The clear supernatants, 50-μL Hamilton syringe (Model 1705, Chrompack) with a removable needle (needle gauge 22G), penetrating the glass beads by at least 1.5 cm. Injection inside the glass beads of the liner resulted in a sharp single peak for acetaldehyde.

Ethanol, methanol, and acetone did not separate at a column temperature of 120 °C. However, in ethanol-intoxicated patients the methanol and acetone concentrations are usually <1% of that of ethanol and therefore do not interfere with the ethanol determination in these patients. When concerned with simultaneously monitoring ethanol, methanol, acetaldelyde, and acetone, a lower column temperature of 60 °C must be used, resulting in an

GAS CHROMATOGRAPHY
The gas chromatograph used was a Chrompack Model CP 9001, equipped with a flame ionization detector, and a CP-9010 automatic liquid sampler (Chrompack). Data handling was done with the Maestro chromatography data system (Chrompack). The injection port of the chromatograph was installed with a hand-made glass liner (length 8 cm, o.d. 6 mm, i.d. 3 mm) (Fig. 1). This liner, which acted as a pre-column to prevent contamination of the gas chromatographic column with nonvolatile material from blood, urine, and fecal supernatants, was stoppered with a dimethylchlorosilane-treated glass wool plug and partly filled with small glass beads with a diameter of 1 mm. Injection of whole blood, serum, urine, and fecal supernatants was performed by means of a 50-μL Hamilton syringe (Model 1705, Chrompack) with a removable needle (needle gauge 22G), penetrating the glass beads by at least 1.5 cm. Injection by <1.5 cm beneath the surface of the glass beads mostly resulted in a broad tailing peak for ethanol. The plunger of the syringe had a Teflon tip to provide an inert leak-tight seal.

For routine analyses, 2-μL injections were performed. For more sensitive determinations, injection volumes up to 10–20 μL might be used. The liner was replaced within seconds by a new one after some 50 2-μL injections or 10 10-μL injections of whole blood, serum, urine, or fecal supernatants. By then, the glass beads in the vicinity of the injection area had a brown-black color, due to contamination with nonvolatile material.

The conditions were as follows: Column: 2 m x 2 mm i.d., glass, packed with 10% SP 1200/1% H₃PO₄ on 80/100 Chromosorb W AW. Column temperature: 120 °C or 60 °C; injection port temperature: 200 °C; detector temperature: 180 °C. Detector output attenuation: 2. Carrier gas: N₂, 20 mL/min; H₂, 30 mL/min; air, 300 mL/min. Freshly packed columns were conditioned overnight at 190 °C with a flow of nitrogen carrier gas, before being connected to the detector. A few 1-μL injections of 10% formic acid were made to clear the column of unknown impurities. When using a new liner, two 2-μL injections of distilled water were made to clear the new glass beads inside the liner of some unknown impurities that might disturb the gas chromatographic separation. The time to replace the liner, stabilize the system, and to decontaminate the new liner took ~3 min.

Acetaldelyde and ethanol separated at a column temperature of 120 °C. Acetaldelyde was generated inside the gas chromatographic liner by oxidation of lactic acid with periodic acid [8]. For this, the syringe was filled with 0.3 μL of 150 g/L periodic acid and 1 μL of a lactic acid solution (25 mmol/L). Injection inside the glass beads of the liner resulted in a sharp single peak for acetaldelyde. Ethanol, methanol, and acetone did not separate at a column temperature of 120 °C. However, in ethanol-intoxicated patients the methanol and acetone concentrations are usually <1% of that of ethanol and therefore do not interfere with the ethanol determination in these patients. When concerned with simultaneously monitoring ethanol, methanol, acetaldelyde, and acetone, a lower column temperature of 60 °C must be used, resulting in an
almost baseline separation between all the mentioned volatiles.

CALIBRATION AND RECOVERY STUDIES
An aqueous stock calibrator of ethanol was prepared with a concentration of 500 g/L. This solution was stored at 4 °C. To 1-mL samples of water, whole blood, serum, urine, and fecal supernatants were added 1, 2, 4, 6, 8, and 10 μL of this stock calibrator, resulting in solutions of 0.5, 1, 2, 3, 4, and 5 g/L, respectively. The most diluted calibrator (0.5 g/L) was further diluted to afford solutions with ethanol concentrations ranging from 0.25 to 10 mg/L. The aqueous calibrators were used for daily calibration.

The intraassay reproducibility was determined for three calibration solutions (0.01, 0.5, and 5 g/L) in water, whole blood, serum, urine, and fecal supernatant, by analyzing each sample six times on the same day. The interassay reproducibility was determined by analyzing the same samples on six different days during a 3-month period. In between, the samples were stored at −20 °C.

Results
GAS CHROMATOGRAPHIC SEPARATION
Figure 2a–e shows gas chromatograms of five calibrator solutions of ethanol (1, 2, 3, 4, and 5 g/L) in whole blood. Injection within glass beads gave a sharp peak for ethanol with a retention time of 0.43 min (oven: 120 °C). The same sharp peaks were obtained for injections with water, serum, urine, and fecal supernatants, supplemented with ethanol. Some broadening of the ethanol peak was sometimes observed after 50–100 injections of the biological specimen, due to contamination of the glass beads with nonvolatile material. Injection of large sample volumes (10 μL or more) also sometimes resulted in peak broadening. Peak broadening resulted in lower peak heights but the peak area was not influenced by peak broadening. Because peak area was used for daily calibration, peak broadening did not influence the outcome of the analysis. Nevertheless, the liner was routinely replaced by a new one after 502-μL injections of biological specimen. Injection within the glass beads must be performed at a distance of at least 1.5 cm beneath the surface of the glass beads inside the liner. Injection at a distance of <1.5 cm beneath the surface gave a broad tailing peak for ethanol (Fig. 2f). Injection in the gas phase of an empty liner without glass beads often gave broad or double peaks for ethanol.

Many reported methods on analysis of ethanol are concerned with simultaneously monitoring acetaldehyde, methanol, or acetone concentrations [5, 9–11]. The peak of acetaldehyde (retention time: 0.36 min) was clearly separated from that of ethanol (retention time: 0.43 min) at a column temperature of 120 °C. Ethanol, methanol, and acetone coincided at 120 °C. An almost baseline separation between acetaldehyde, methanol, acetone, and ethanol was obtained at a column temperature of 60 °C (Fig. 3). Some forensic important congeners [12] of ethanol (isopropanol, n-propanol, isobutanol, n-butanol) are also included in Fig. 3 and showed baseline separation at 60 °C. No carryover problems were seen for ethanol, nor for any of the other volatiles studied.

CALIBRATION, RECOVERY, AND PRECISION
Daily calibration was performed with the aqueous ethanol calibration solutions (concentration: 1–5 g/L). A quite good linear correlation was obtained between peak area and concentration (Fig. 4). No significant differences were observed between the calibration line of ethanol in water and those of ethanol in whole blood, serum, urine, and fecal supernatant. The biological matrix did not influence the gas chromatographic analysis. Although not shown, the calibration curves were also linear in the low concentration range (0.0001–0.5 g/L).

The percentage recovery of ethanol from water, whole blood, serum, urine, and fecal supernatant was excellent, with low intra- and interassay CVs (Table 1). The interas-
say reproducibility was determined by analyzing the same samples on six different days during a 3-month period. In between, the samples were stored at −20 °C.

The detection limit, corresponding to a peak area equal to four times the background noise, was 0.25 mg/L, when using 2-μL injections and a column temperature of 60 °C. This limit could be lowered to 0.10 mg/L by injecting larger amounts (5 μL) of biological sample. Injection between glass beads allows injection of at least 5–10 μL of biological sample without resulting in peak broadening or other disturbances. Injection of larger volumes sometimes resulted in peak broadening without disturbing peak area. For a given concentration, a linear correlation was obtained between the volume injected and the peak area, at least for injections up to 10 μL (y = 24.293x + 1035, r = 0.9999, Fig. 5). Above this volume, the response was not linear anymore. When applying these large volumes, the liner should be replaced by a new one after some 10 injections. Although not studied in detail, the detection limits for acetaldehyde, methanol, and acetone lie in the same low range as that for ethanol. Injection of 1 μL of an aqueous calibrator solution (Fig. 3) gave comparable peak areas for all the volatiles studied.

Injection of 5 μL of a blank urine sample (Fig. 6a), serum sample (not shown), or blank whole-blood sample (Fig. 6d) from a healthy volunteer who had no alcoholic drinks during the last 3 days before the sampling gave no detectable ethanol peaks (<0.1 mg/L). This was repeated for two other volunteers with the same results. No interferences were observed with other constituents from whole blood, serum, urine, or fecal supernatant, allowing detection of very small amounts of ethanol. Fig. 6b and 6e show the spectra after addition of 5 mg/L ethanol to the corresponding blanks (see Fig. 6a and 6d). Fig. 6c shows the spectrum of the first morning urine of the same volunteer who drank one glass of wine the evening before. The ethanol concentration in this sample amounted to 3.4 mg/L. The ethanol concentration in the serum of a normal social drinker amounted to 1.2 mg/L (Fig. 6f). The control urine, whole-blood, and serum samples studied here all contained small amounts of acetone (0.3–2.0 mg/L). In the control samples containing no ethanol, methanol was not detected either (<0.2 mg/L). Methanol was present in urine and blood samples of volunteers who had several alcoholic drinks and consequently high ethanol concentrations. The methanol concentration in a urine sample containing 0.8 g/L ethanol amounted to 5.2 mg/L and in a serum sample containing 0.8 g/L ethanol to 3.7 mg/L. The acetone concentrations in these two samples amounted to 5.2 and 1.3 mg/L, respectively. The first two small peaks in the spectra of Fig. 6 were also present after injection of 5 μL of distilled water and were designated as injection peaks. The latter had the same retention time as acetaldehyde. The area of this injection peak corresponded to 0.5–0.8 mg/L acetaldehyde. When measuring acetaldehyde, the concentration has to be corrected for this amount. Moreover, one should use a new liner because after ~10 injections a
Table 1. Mean percentage recovery of ethanol from water, whole blood, serum, urine, and fecal supernatant, and the intraassay and interassay variation, as measured for three different concentrations.

<table>
<thead>
<tr>
<th>Added amount of ethanol, g/L</th>
<th>0.01</th>
<th>0.5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Recovery from water*</td>
<td>102 ± 10</td>
<td>98 ± 4</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>CV, %</td>
<td>10.2</td>
<td>3.8</td>
<td>3.1</td>
</tr>
<tr>
<td>% Recovery from whole blood</td>
<td>101 ± 10</td>
<td>96 ± 5</td>
<td>98 ± 4</td>
</tr>
<tr>
<td>CV, %</td>
<td>9.7</td>
<td>5.7</td>
<td>3.7</td>
</tr>
<tr>
<td>% Recovery from serum</td>
<td>101 ± 10</td>
<td>94 ± 7</td>
<td>94 ± 1</td>
</tr>
<tr>
<td>CV, %</td>
<td>10.1</td>
<td>7.4</td>
<td>0.8</td>
</tr>
<tr>
<td>% Recovery from urine</td>
<td>102 ± 6</td>
<td>98 ± 5</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>CV, %</td>
<td>6.3</td>
<td>5.5</td>
<td>6.4</td>
</tr>
<tr>
<td>% Recovery from fecal supernatant</td>
<td>106 ± 7</td>
<td>99 ± 6</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>CV, %</td>
<td>6.3</td>
<td>5.7</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Intraassay

% Recovery from water*: 102 ± 10, 98 ± 4, 95 ± 3
CV, %: 10.2, 3.8, 3.1

% Recovery from whole blood: 101 ± 10, 96 ± 5, 98 ± 4
CV, %: 9.7, 5.7, 3.7

% Recovery from serum: 101 ± 10, 94 ± 7, 94 ± 1
CV, %: 10.1, 7.4, 0.8

% Recovery from urine: 102 ± 6, 98 ± 5, 100 ± 6
CV, %: 6.3, 5.5, 6.4

% Recovery from fecal supernatant: 106 ± 7, 99 ± 6, 100 ± 3
CV, %: 6.3, 5.7, 2.7

Interassay

% Recovery from water*: 95 ± 8, 98 ± 4, 98 ± 4
CV, %: 9.0, 4.1, 4.2

% Recovery from whole blood: 108 ± 8, 101 ± 6, 98 ± 3
CV, %: 7.1, 6.1, 2.8

% Recovery from serum: 108 ± 4, 109 ± 8, 97 ± 4
CV, %: 5.3, 7.4, 4.0

% Recovery from urine: 101 ± 9, 105 ± 3, 99 ± 1
CV, %: 8.5, 3.0, 1.5

% Recovery from fecal supernatant: 101 ± 7, 98 ± 4, 102 ± 3
CV, %: 7.0, 4.0, 3.0

*Mean ± SD (n = 6).

Injection in the gas phase might therefore result in a slower evaporation of the sample and, as a consequence, peak broadening. The gas chromatograph used (Chrompack CP 9001) is ideal, because this chromato-

contaminated liner resulted in two liner peaks with the same retention time as acetaldehyde and isopropanol.

Discussion

With the technique described in the present study, whole blood, serum, urine, and fecal supernatants can be analyzed by GC for the presence of ethanol without any pretreatment. Direct injection inside a glass liner filled with glass beads, which acted as a precolumn, protects the GC column against serious contamination with nonvolatile material. This injection technique was also recently applied for the determination of fecal short-chain fatty acids [13]. The glass beads inside the liner ensure that injection of the sample always takes place against hot glass, providing an immediate evaporation of the sample. Injection against hot glass appears to be very important for obtaining sharp peaks. Broad peaks for ethanol were often seen after injection in the gas phase of an empty liner. This might be explained by a temperature difference. The glass beads have the same temperature as the injector (200 °C), whereas the temperature in the gas phase of an empty liner is surely lower, mainly because of cooling by the N₂ carrier gas stream through the liner.

Fig. 5. The peak area response of ethanol vs injection volume. Injected sample: whole blood with an ethanol concentration of 1 g/L; column temperature: 120 °C.

Fig. 6. Gas chromatograms of a blank urine sample from a healthy volunteer (a), the same urine sample supplemented with 5 mg/L ethanol (b), a morning urine sample of the same volunteer after consumption of one glass of wine the evening before (c), a blank whole-blood sample from a healthy volunteer (d), the same blood sample supplemented with 5 mg/L ethanol (e), and a serum sample from a healthy volunteer (social drinker) (f).

Peak 1, acetaldehyde; peak 2, isopropanol; peak 3, acetone; peak 4, ethanol. Column temperature: 60 °C; injection volume: 5.0 μL; recorder output: 4 mV.
graph is provided with an injection port containing the required liner. The same gas chromatographic column has been in use now for >3 years. No deterioration of the column has been observed after >10,000 injections of blood, serum, urine, and fecal supernatants. Detection of low physiological concentrations of ethanol, methanol, and acetone should be performed at a column temperature of 60 °C. The column should be replaced by a new one when separation between these volatiles becomes insufficient.

INTERNAL CALIBRATOR
Most authors applying the direct injection technique do use an internal calibrator [6, 11, 14, 15], also because injection of small sample volumes (0.5 μL or less) is subjected to unacceptable large sample errors. However, my technique uses larger volumes (1 μL or more), eliminating these large sample errors and consequently the need for an internal calibrator, as was apparent from the very small intra- and interassay variations. An aqueous external calibrator of ethanol meets all the requirements. The calibration graphs for ethanol in water did not differ from those in whole blood, serum, urine, and fecal supernatant. Two calibrations during the day, one at the beginning and one at the end, were sufficient. The intra- and interassay CVs in peak area, when injecting 1 μL of an external ethanol calibrator of 1 g/L, were <5%. Nevertheless, when one is uncomfortable with the approach of external calibration, one might easily use one of the higher alcohols (n-propanol, isobutanol, n-butanol, see Fig. 3) as internal calibrator.

SENSITIVITY
The effects of alcohol intoxication lie in the concentration range 0.2–6 g/L [31]. In this study, a column temperature of 120 °C was used for this concentration range. No separation was obtained between ethanol, methanol, and acetone at 120 °C. However, the concentrations of methanol and acetone in alcohol-intoxicated patients are usually <1% that of ethanol [16, 17] and therefore do not interfere with the ethanol determination. Similar low values for methanol and acetone (~1–5 mg/L) were found in this study for a blood and urine sample containing 0.8 g/L ethanol. Conventional gas chromatographic methods for ethanol determination lack sensitivity at <10 mg/L (0.2 mmol/L) [6, 7, 18, 19] and are not suited for the determination of normal physiological concentrations of ethanol in the blood or urine of control subjects because these concentrations lie below this limit. GC-mass spectrometry (MS) methods have been applied to measure such normal concentrations [9, 15, 18]. However, MS is a technique much more sophisticated than GC and requires highly trained personnel. The present GC method has a detection limit (0.1 mg/L) similar to GC-MS. Such a low limit was obtained by injecting large sample volumes (5 μL or more) and using a column temperature of 60 °C. The presence of interfering substances, particularly methanol and acetone, has been a concern in the forensic measurement of ethanol in blood and urine. Almost baseline separation between ethanol, methanol, and acetone was obtained at 60 °C. This is necessary because normal physiological concentrations of acetone lie in the same low range as those of ethanol [9] and would interfere with ethanol at a column temperature of 120 °C. A mean urine ethanol concentration of 1.4 mg/L was found for healthy social drinkers [18]. I did not detect any alcohol (<0.1 mg/L) or any methanol (<0.2 mg/L) in the urine of three healthy volunteers when they abstained from alcohol for at least 3 days. Literature values of normal physiological methanol concentrations in blood and urine range from undetectable (<0.6 mg/L) to 3.8 mg/L [20–22].

QUANTIFICATION
Quantification on the basis of peak area was excellent. Quantification on the basis of peak heights is less desirable, because peaks may become somewhat broadened, especially when injection was performed inside a liner already contaminated with nonvolatile material from previous injections or when applying large sample volumes (10 μL or more). This broadening lowered peak heights but had no influence on peak area.

INJECTION SYRINGE
Plugging of the syringe appeared to be a serious problem during direct injection of whole blood or serum, especially when using the 10-μL Hamilton syringes with a needle gauge of 26S (Hamilton code 701). In my experience, immediately washing the syringe as recommended by some authors [6, 11] did not solve the problem, nor did the use of Hamilton syringe cleaning wires. The use of a cleaning wire even worsened the plugging. Plugging could be overcome by the use of a 25-μL or 50-μL gas-tight syringe with a Teflon plunger tip and a removable needle with a needle gauge of 22S (Hamilton code 702 and 703, respectively). Immediately after each injection, the plunger was removed and the syringe was washed by filling it from above with a 9 g/L saline solution by means of a second syringe. The plunger was then reinstalled and pressed down, thereby cleaning the needle from protein or other nonvolatile deposits.

DIRECT INJECTION VS HEADSPACE TECHNIQUE
Direct injection and headspace GC are the two most often used GC techniques for measuring ethanol in biological specimens. The headspace technique is quite laborious and is subject to various analytical errors, mostly due to sample-type discrepancies [5]. The type of biological specimen influences the partitioning of ethanol between liquid and headspace vapor, as does the type of salt added as a salting-out agent. Moreover, headspace techniques require larger volumes of biological specimen than direct
injection and have higher detection limits. In my hands, the headspace technique was inferior to direct injection.

STORAGE

The ethanol concentrations in water, blood, serum, urine, and fecal supernatant appeared to be stable for at least 3 months at −20 °C. No significant changes in ethanol concentrations were observed during storage. Similar data were found in literature for storage of blood. During a 2-week period, no significant changes were observed in the blood concentration of ethanol, whether stored at room temperature, under refrigeration, or in the freezer [14, 23].

In conclusion, the direct injection method as presented here is a highly sensitive, rapid, and reliable gas chromatographic procedure for measuring ethanol in various biological specimens. The direct injection method between glass beads may be a step forward in measuring all kinds of volatile substances in biological material. Once running, the method is easy to perform and does not require highly and specifically trained personnel, making this gas chromatographic method also suited to the field of clinical chemistry.

I thank all the healthy volunteers participating in this study.

References