Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 798 (2003) 179–186
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Highly sensitive gas chromatographic determination of ethanol in human urine samples

Michael Zilly*, Peter Langmann, Ulrike Lenker, Verena Satzinger, Diana Schirmer, Hartwig Klinker

Department of Internal Medicine, Division of Infectious Diseases and Hepatology, Medizinische Poliklinik, University of Wuerzburg, Josef-Schneider Strasse 2, D-97080 Wuerzburg, Germany

Received 10 April 2003; received in revised form 9 September 2003; accepted 11 September 2003

Abstract

In order to evaluate recent alcohol consumption, a very sensitive and specific gas chromatographic method for ethanol determination in human urine samples was developed. The non-invasive method was performed without any pretreatment and carried out on a Stabilwax capillary column, 30 m × 0.53 mm × 1.0 μm film thickness. Helium was used as carrier gas with a constant inlet pressure of 27.72 kPa (0.277 bar) and a flame ionization detector (FID). Quantification was performed with the use of acetonitrile as an internal standard (IS). The calibration curve was linear throughout the concentration range from 0.5 to 500 mg/l. The calculated intra- and inter-day coefficients of variation were below 8%. A clear chromatographic separation of ethanol from methanol, acetone, 1-propanol and 2-propanol was achieved.

Keywords: Ethanol

1. Introduction

Alcohol use and abuse plays an important role in clinical medicine. Ethanol is a main progression factor of chronic viral hepatitis to liver cirrhosis. In patient guidance, decision making in concern of organ transplantation, compliance or starting special therapy options in a clinical setting, a main interest is to categorize abstinence from social drinking or heavy drinking habits without invasive techniques [1,2]. Recent studies showed no significant endogenous ethanol production in urine from healthy patients [3–6]. As ethanol levels in plasma or serum show a continuous drop, low levels of alcohol can potentially be missed, performing a detection in plasma by routine laboratory measurement only, while urine samples remain positive even after hours depending on individual rehydration volumes with non-alcoholic beverages and time intervals concerning acts of urination. Here, we describe the validation of a non-invasive and highly sensitive flame ionization detector (FID) gas chromatographic (GC) detection method for ethanol in urine which can detect even very small amounts of alcohol in urine. This FID method shows an easy and economic performance for laboratories who do not have a feasibility to use headspace technique. Urine samples can directly be injected without specific preanalytical procedures. Main concern of this method was to evaluate low or even very low urine levels in a non-invasive way, to allow an easy differentiation between full abstinence and alcohol consumption. Patients were recruited from the outpatient care unit specialized in hepatology and infectious diseases.

2. Experimental

2.1. Chemicals and reagents

Ethanol, acetonitrile, acetone, methanol, 1-propanol and 2-propanol, all gradient grade, were obtained from Merck (Darmstadt, Germany). Distilled water came from Delta Select (Pfullingen, Germany). Blank, alcohol free urine was obtained from healthy abstinent volunteers.

2.2. Chromatographic equipment and conditions

Ethanol analysis was carried out on an Agilent Technologies 6890 N gas chromatograph equipped with a flame

* Corresponding author. Fax: +49-931-20136485.
E-mail address: zilly_m@klinik.uni-wuerzburg.de (M. Zilly).

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2003.09.037

ionization detector and an autosampler (Fa. Agilent, serie 7683, Waldbronn, Germany). An Agilent chemstation (Fa. Agilent) was used for peak identification and integration. Chromatography was performed with a Stabilwax capillary column, 30 m × 0.53 mm × 1.0 μm film thickness (Restek Corp. Europe, Bad Soden, Germany) protected by a guard column (Hydroguard FS, 1 m × 0.53 mm i.d.). The injection port of the chromatograph was installed with a glass liner, partly filled with silanized glass wool (SERVA Feinbiochemica, Heidelberg, Germany) to prevent contamination of the analytical column with non-volatile material from urine. The GC conditions were: column temperature, 40–200 °C (2 min hold at 40 °C, 10 °C/min from 40 to 100 °C and 20 °C/min from 100 to 200 °C); injection temperature, 200 °C; detection temperature, 300 °C; helium flow rate, 5.7 ml/min. The injection mode was split (split ratio, 25:1; split flow, 142.2 ml/min; total flow, 151.1 ml/min) and the injection volume was 2 μl. For GC quantification, the peak area of each compound was used.

2.3. Standard preparation

The initial stock solution of ethanol (1 mg/ml) and acetonitrile (internal standard (IS)) (195 μg/ml) were prepared by dissolving the compounds in distilled water. The ethanol stock solution was appropriately diluted with water for the preparation of working solution at concentrations of 0.5–500 mg/l.

These solutions are stable for at least 3 months at 4 °C. Urine calibration samples were prepared with an ethanol concentration of 2.72, 27.2 and 136 mg/l, respectively. The appropriate amount of the working solutions and the internal standard (20 μl) were added to blank urine to achieve the mentioned range of calibration concentrations. The retention time was 5.4 min for the internal standard and 4.3 min for ethanol, respectively.

2.4. Sample preparation

First, 20 μl of IS was pipetted into separate snap-cap microcentrifuge vials containing 200 μl of human urine. Each tube was then vortex-mixed and centrifuged for 5 min at 15,000 U/min. The supernatant from each tube was transferred to individual GC vials with glass microinserts and placed in an automated sample injector. Two microliters of the supernatant was injected into the gas chromatograph.

2.5. Preparation of quality control samples

A quality control (QC) stock solution was prepared by dissolving 1 mg ethanol in 1 ml distilled water. Subsequent QC stock samples were prepared at three concentration levels 2.72, 27.21, and 136 mg/l by serial dilution with blank, alcohol free urine. On each validation day, working QC samples were prepared freshly at each level by the same procedure as the sample preparation described earlier.

2.6. Specificity and selectivity

In order to evaluate any interference of endogenous compounds with the analytical method, an analysis of spiked blank urine samples as well as for spiked distilled water probes was performed. Exogenous interference, i.e. by different medication or food intake was ruled out by the analysis of $n = 200$ patient samples collected from our laboratory. Testing the methodological selectivity for ethanol a specific differentiation from methanol, acetone, 1-propanol and 2-propanol was evaluated using spiked blank urine samples.

2.7. Limit of detection

The limit of detection (LOD) for ethanol in urine was defined by the lowest detectable concentration yielding a signal-to-noise ratio of three, indicating a significant difference of spiked and blank urine samples of three individuals as determined by the two-tailed, paired Student's *t*-test.

2.8. Limit of quantification

For the concentration to be accepted as the lower limit of quantification (LOQ), the measure of accuracy (percent deviation from the nominal concentration) and precision (relative standard deviation) are to be less than 8%. All samples were assayed four times. The LOQ was 0.25 mg/l (5.425 μmol/l). The upper limit of quantification (ULQ) was arbitrarily set at 500 mg/l (10,850 μmol/l). In case of ethanol amounts above the ULQ, a dilution series with distilled water (urine: distilled water starting at 1:1) was prepared for these urine probes.

2.9. Accuracy, precision and linearity

Accuracy was calculated as the relative error of the nominal concentration. Precision was expressed in terms of relative standard deviation and obtained by analysis of variance (ANOVA) for each test concentration using the analytical run as the grouping variable.

Intra-day accuracy and precision of the method were determined by measuring three replicate QC samples at three different urinary ethanol concentrations (ethanol concentrations: 2.72, 27.21 and 136 mg/l; Table 1).

To obtain the inter-day accuracy and precision three samples of each concentration were analyzed at seven different days as described earlier (Table 2). Daily standard curves were evaluated by duplicate analysis of nine spiked urine samples for ethanol in the range of 0.5–500 mg/l.

A linear weighted least-squares regression analysis (1/concentration squared) to plot the observed peak

Table 1
Stability of urine samples: intra-day and day-by-day accuracy (RE%) and precision (CV%) for the analysis of ethanol in urine samples is shown for a 24 h period at room temperature, for 7 days at 4 °C and 60 days at −20 °C and for a period of four freeze–thaw cycles over 7 days ($n = 4$ samples for each)

	Deployed ethanol concentration		
	2.72 mg/l	27.21 mg/l	136 mg/l
Intra-day analysis			
Mean	2.76	27.33	136.21
S.D.	0.02	0.064	0.54
CV%	0.84	0.24	0.39
RE%	−1.59	−0.43	−0.15
Twenty-four hours at room temperature			
Mean	2.78	27.59	144.27
S.D.	0.08	0.58	1.72
CV%	2.94	2.11	1.19
RE%	−2.21	−1.40	−6.08
Seven days at 4 °C			
Mean	4.11	28.25	155.55
S.D.	0.04	0.06	0.35
CV%	0.85	0.22	0.22
RE%	−51.23	−3.84	−14.37
Sixty days at −20 °C			
Mean	3.68	27.76	139.81
S.D.	0.02	0.35	5.67
CV%	0.57	1.26	4.05
RE%	−35.42	−2.03	−2.80
Four freeze–thaw cycles			
Mean	3.03	28.15	140.77
S.D.	0.04	0.75	0.93
CV%	1.30	2.65	0.66
RE%	−11.57	−3.45	−3.51

area/internal standard ratio of ethanol was performed. The linearity of five calibration curves was tested with the *F*-test for lack of fit, using a weight factor of (1/concentration).

2.10. Analysis of patient samples

Urine samples were obtained from patients at our outpatient department for hepatology and infectious diseases suffering from chronic hepatitis. All patients were recommended to avoid any amount of alcohol due to their liver disease. Spontaneous urine samples of $n = 85$ patients were obtained by a standardized procedure for midstream urine. Urine was immediately stored at −20 °C until further analysis. The presented method with acetonitrile as IS seems not to be suitable for all toxicological purposes as possible acetonitrile poisonings are observed.

2.11. Calculation and data analysis

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 11.0.dt. (SPSS, Chicago, IL, USA).

Table 2
Inter-day accuracy (RE%) and precision (CV%) for the analysis of ethanol in urine samples over 10 different days (A–J; $n = 3$ samples for each)

	Deployed ethanol concentration		
	2.72 mg/l	27.21 mg/l	136 mg/l
Detected ethanol concentration (mg/l)			
A			
Mean	2.45	26.68	132.28
S.D.	0.19	0.37	2.63
CV%	7.79	1.40	1.99
RE%	9.92	1.95	2.74
B			
Mean	2.69	26.99	136.34
S.D.	0.03	0.08	0.72
CV%	0.98	0.30	0.53
RE%	1.10	0.80	−0.25
C			
Mean	2.62	27.47	136.79
S.D.	0.02	0.04	0.32
CV%	0.58	0.15	0.23
RE%	3.80	−0.96	−0.58
D			
Mean	2.55	26.94	133.87
S.D.	0.01	0.11	0.40
CV%	0.39	0.41	0.30
RE%	6.25	0.98	1.57
E			
Mean	2.62	26.42	133.14
S.D.	0.04	0.10	0.74
CV%	1.38	0.37	0.56
RE%	3.68	2.90	2.11
F			
Mean	2.60	26.87	135.51
S.D.	0.01	0.12	1.19
CV%	0.22	0.46	0.88
RE%	4.53	1.25	0.36
G			
Mean	2.52	26.73	134.45
S.D.	0.02	0.17	1.13
CV%	0.82	0.62	0.84
RE%	7.23	1.76	1.14
H			
Mean	2.41	26.64	136.36
S.D.	0.01	0.08	0.34
CV%	0.41	0.29	0.25
RE%	11.40	2.11	−0.26
I			
Mean	2.47	27.23	140.84
S.D.	0.01	0.10	0.55
CV%	0.57	0.38	0.39
RE%	9.19	−0.07	−3.56
J			
Mean	2.47	26.56	138.99
S.D.	0.03	0.08	0.46
CV%	1.02	0.30	0.33
RE%	9.07	2.39	−2.20

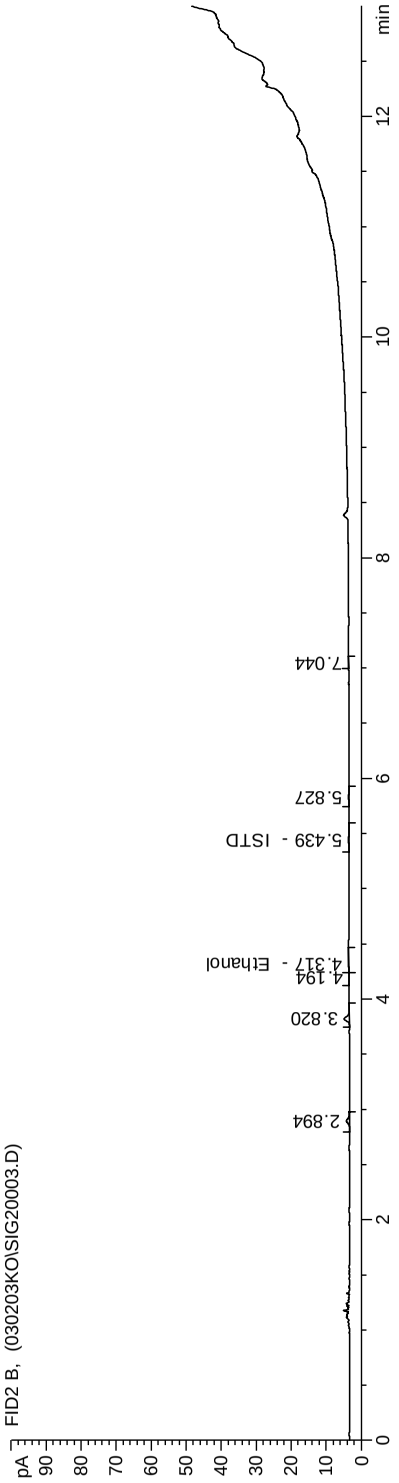

Fig. 1. Chromatogram of a blank urine sample.

3. Results and discussion

3.1. Chromatography and detection

Urine analysis for the presence of ethanol or illicit substances was described previously with several (headspace) GC methods [7–9]. Our FID GC detection method can easily be used for direct injection of urine samples without any pretreatment. An equal baseline and very good sensitivity of our assay was reached. For protection of the column from being contaminated with non-volatile material, a glass wool filled glass liner has proved suitable and satisfactory. More than 100 urine sample injections can be performed without deteriorated performance of the glass liner. The use of the internal standard acetonitrile was simple and made our assay reliable. Peak shape, separation from contaminating urine compounds or metabolites as well as the separation from other endogenous alcohols and acetone were optimal by using a Stabilwax capillary column.

3.2. Specificity and selectivity

Drug-free urine samples obtained from healthy individuals were devoid of interference near the retention time of ethanol and the internal standard, respectively. Fig. 1 shows the chromatogram of blank urine.

The analysis of urine samples containing ethanol showed interference neither with the preparation procedure nor with the analytical method or any concomitant drugs used by the patients (Fig. 2).

3.3. Limit of quantification and detection

The limit of detection for ethanol in urine was determined at 0.1 mg/l. The lower limit of quantification was reached at a concentration of 0.25 mg/l (Fig. 3). The upper limit of quantification was arbitrarily set at 500 mg/l. For our purpose to differentiate between alcohol abstinence and the consumption of alcohol by moderate drinking, this limit was satisfactory. If the detected amount for ethanol in a sample was above 500 mg/l, controlled dilution series were performed till the sample amount was below the ULQ and results were accordingly recalculated.

In literature, the description of a rare “autobrewery syndrome” with accumulation of very low endogenous ethanol blood concentrations can possibly lead to very low urinary ethanol levels, but these findings are uncommon, the detected blood levels ranged from 0 mg/l to a maximum of

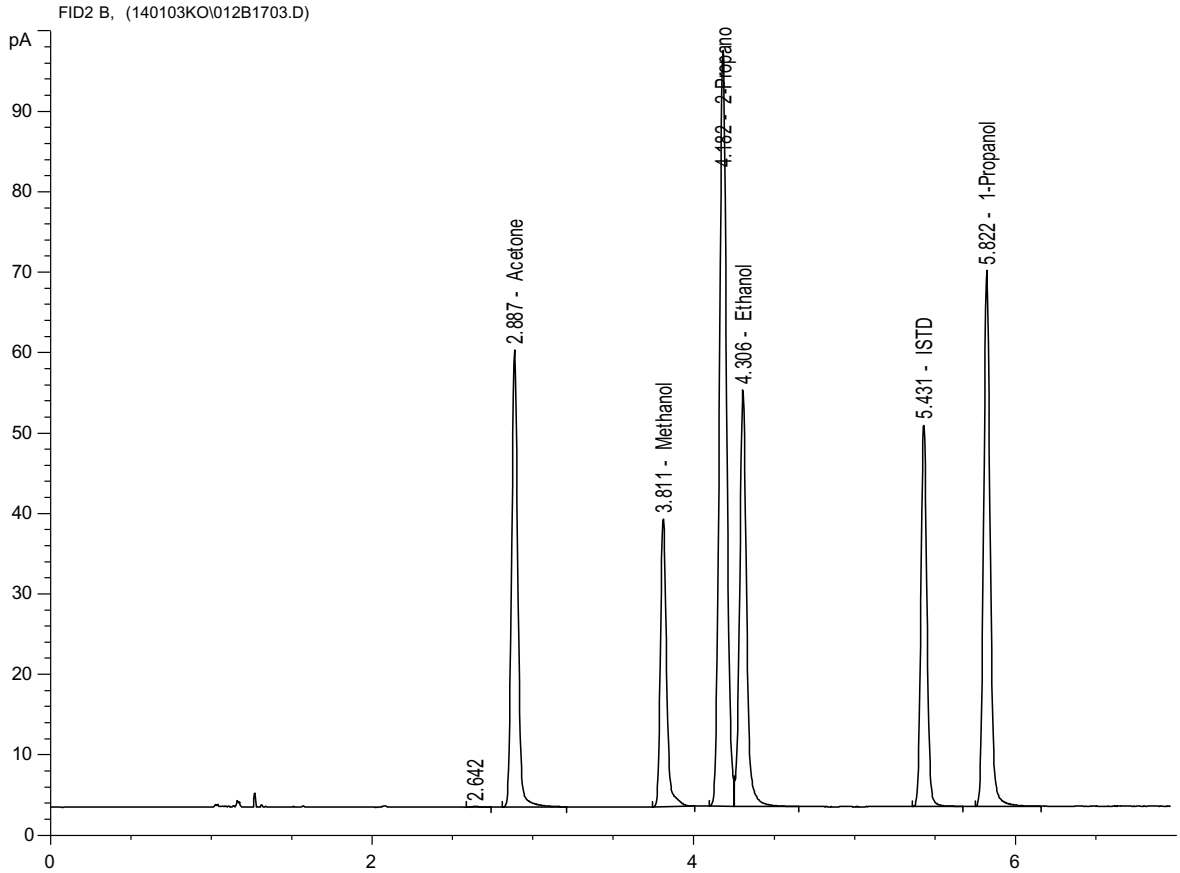

Fig. 2. Chromatographic separation of ethanol and other alcohols and ketones. Concentrations: acetone, 24.69 mg/l; methanol, 24.68 mg/l; 2-propanol, 19.5 mg/l; ethanol, 25.0 mg/l; 1-propanol, 25.0 mg/l.

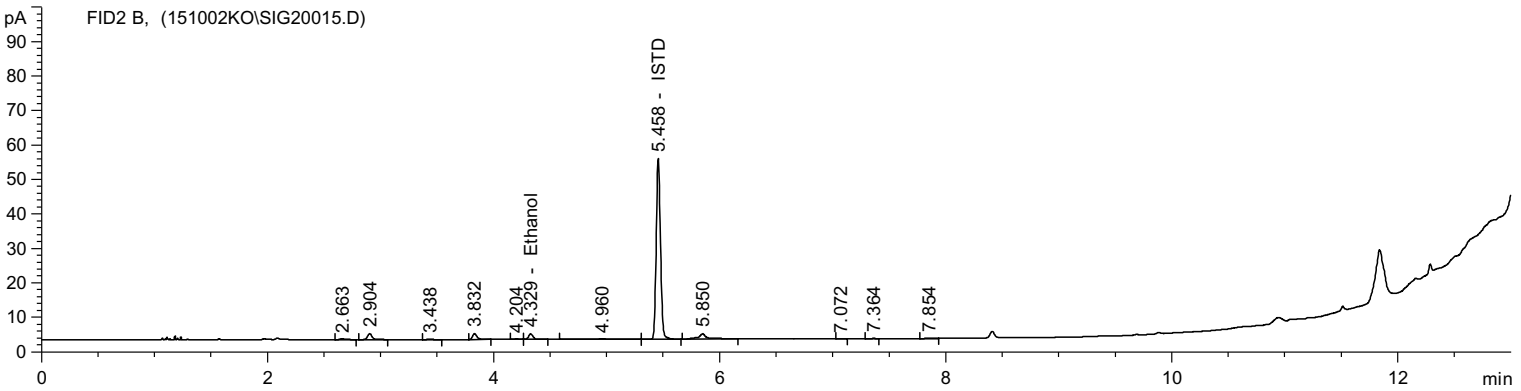

Fig. 3. Chromatogram of a blank urine, spiked with ethanol (0.259 mg/l) and IS (CH_3CN) (LOQ = 0.25 mg/l).

0.8 mg/l in a single venous blood specimen. Those authors stated, that these concentrations are far too low to have any forensic or medical significance, what perhaps needs to be discussed anew [6]. As ethanol is filtered readily in the kidneys and is not completely reabsorbed concentrations of ethanol in blood and urine should range at comparable levels, with a trend to higher levels in urine. Those small amounts of up to 0.8 mg/l ethanol detected in venous blood produced by an "autobrewery syndrome" may even become nearly undetectable in a spontaneous urine sample performing a single urine analysis in respect of possible dilution over time. Experiments by Jones [10] showed, that the amount of ethanol excreted unchanged in urine was only about 0.7–1.5% of the amount of alcohol ingested by moderate drinking. Anyway, taking into account the exact urine volume and the total amount of alcohol consumed by a person, a recalculation of the corresponding amount of ethanol in blood should still be at a comparable level. Advantages of using the urinary ethanol detection method described here are found in its sensitivity and non-invasiveness. Besides that, plasma levels can already return to normal levels while urinary ethanol remains elevated for hours—till the bladder is voided.

3.4. Accuracy, precision, linearity and recovery of the assay

The intra-day accuracy in between a 24 h period at room temperature, day-by-day over 7 days at 4 °C and 60 days at −20 °C including four freeze–thaw cycles over 7 days is shown in Table 1. Precision of the method was determined in six analytical runs including three different concentrations. Precision (CV%) ranged from 0.22 to 4.05% (Table 1). In contrast to the aqueous preparation, urine samples showed a higher variability for low urine ethanol concentrations with an increase of the ethanol concentration over the time. The results of intra-day validation as well as inter-day accuracy and precision (up to 60 days follow-up) of ethanol are shown in Table 1.

Using the ratios of the observed peak areas for ethanol and the internal standard in nine spiked samples analyzed in duplicate, the standard curves showed a correlation coefficient of 0.999 (range: 0.25–500 mg/l) as determined by least-square analysis. All calibration curves proved to be linear in the respective range listed earlier in the *F*-test for lack of fit.

Stability studies show increasing amounts of ethanol in urine probes after being stored for 3–4 months at −20 °C after being thawed for repetitive analysis. This is supposed to be due to secondary infection with microbes, i.e. such as yeasts, which can produce detectable amounts of alcohol in vitro [4,5].

3.5. Analysis of patient samples

Urine samples were taken from patients with chronic hepatitis of our outpatient department for hepatology and

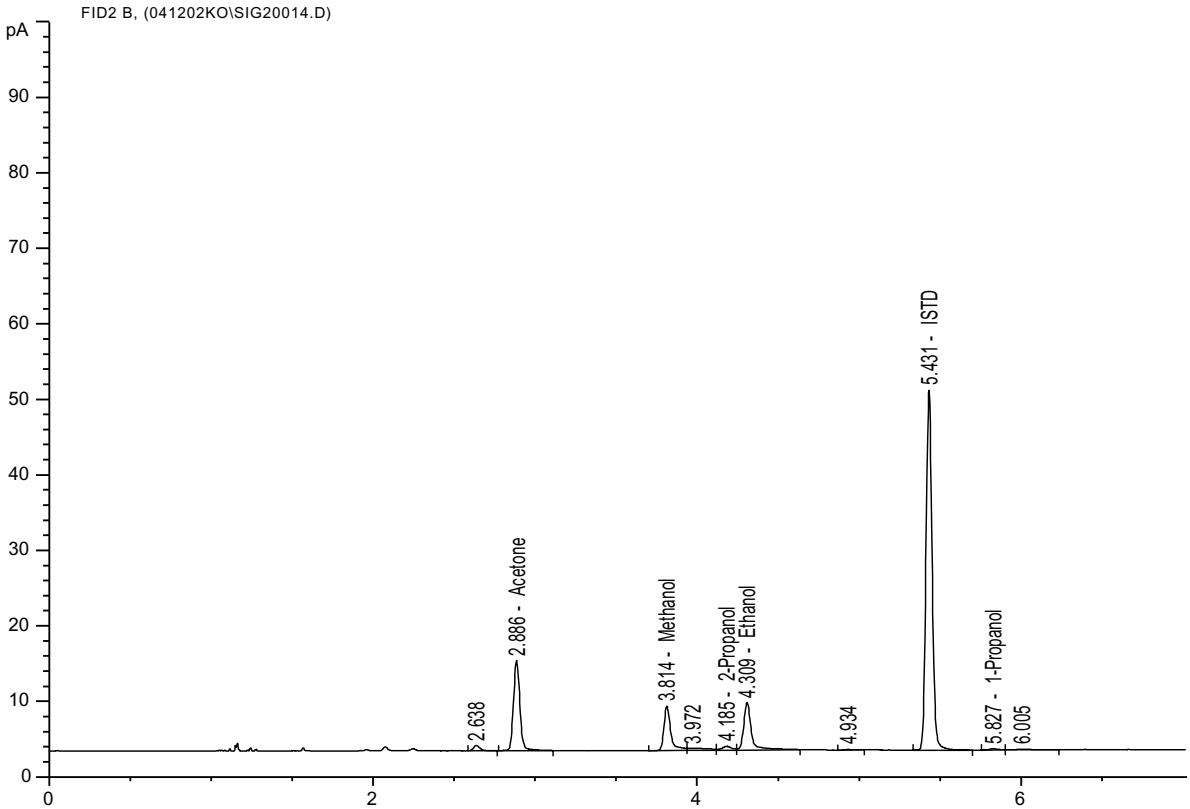

Fig. 4. Chromatogram of a blank human urine sample spiked with internal standard and a low ethanol urine concentration (2.17 μg ethanol/ml urine (RT 4.309)).

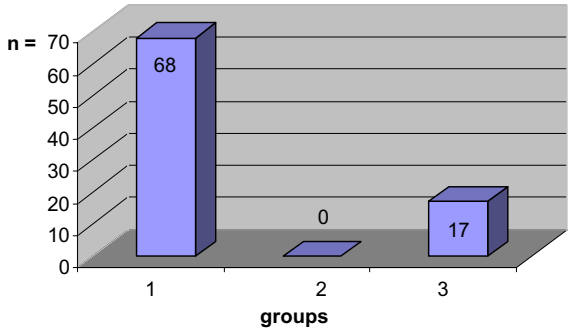

Fig. 5. Urinary ethanol levels in $n = 85$ outpatients: group 1, $n = 68$ without any detection; group 2, $n = 0$ with a detection ranging from LOD to LOQ; group 3, $n = 17$ with a detection of above LOQ of 0.25 mg/l ethanol in a spontaneous urine probe (range: 0.67–2855 mg/l; median: 69.4 mg/l; mean ± S.D.: 401.7 ± 789.75 mg/l).

infectious diseases. Urine concentrations of ethanol in our examples were between 2 and 2855 mg/l urine. The presented results, shown in Fig. 4, demonstrate the applicability of the assay for monitoring alcohol-abuse by urine ethanol detection in hepatitis-infected patients. In Fig. 5, the distribution of $n = 85$ patient samples concerning their ethanol concentration in urine is displayed.

4. Conclusion

We present a validated, reliable and convenient assay for an extremely sensitive determination of ethanol in urine. The described GC assay can readily be used in a standard hospital laboratory without any prior urinary sample preparation steps by direct injection. In our hands, the described procedure was most suitable. Calibration curves for ethanol ranging from 0.25 to 500 mg/l are appropriate for our purposes to differentiate between (absolute) abstinence and (low) alcohol consumption and therefore proofed suitable for assessment of patient's compliance by a non-invasive test. Also alcohol rehabilitation programs could benefit from knowing even such small amounts of alcohol in urine in patients' guidance and for decision of further treatment options. For detection of higher amounts of ethanol in urine, i.e. in patients with ethanol intoxication, the detection range can easily be enlarged by dilution series. Besides that, in these cases plasma alcohol levels should still be clearly enhanced. In comparison to formerly described detection methods using headspace GC [7], our method is more sensitive and as easy to perform but shows an even better cost effectiveness. Our method also was at a comparable sensitivity with a better peak shape and baseline separation compared to [11]. The described method can be carried out on nearly every GC system without the need of headspace technique or mass spectrometry. The practicability of this highly specific and economic non-invasive detection method for further evaluation of ethanol intake is demonstrated by the analysis of 85 urine levels of patients regularly seen in our unit. This GC method can be used for monitoring alcohol intake in patients not suffering from a urinary tract infection. Chromatographic separation of ethanol as well as for other alcohols and ketones was also possible (acetone, methanol, 1-propanol, 2-propanol).

References

[1] F. Musshoff, T. Daldrup, J. Chromatogr. B: Biomed. Sci. Appl. 713 (1998) 245.
[2] P.C. Sharpe, Ann. Clin. Biochem. 38 (2001) 652.
[3] J.J. Saady, A. Poklis, H.P. Dalton, J. Forensic Sci. 38 (1993) 1467.
[4] A. Helander, O. Beck, A.W. Jones, J. Forensic Sci. 40 (1995) 95.
[5] A. Jones, L. Hylen, E. Svensson, A. Helander, J. Anal. Toxicol. 23 (1999) 333.
[6] B.K. Logan, A.W. Jones, Med. Sci. Law 40 (2000) 206.
[7] C.L. Correa, R.C. Pedroso, J. Chromatogr. B: Biomed. Sci. Appl. 704 (1997) 365.
[8] M.C. Yu, B.K. Tang, R.K. Ross, Cancer Epidemiol. Biomarkers Prev. 4 (1995) 849.
[9] J.A. Iffland, Med. Sci. Law 42 (2002) 207.
[10] A.W. Jones, Forensic Sci. Int. 45 (1990) 217.
[11] A. Tangerman, Clin. Chem. 43 (1997) 1003.