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Journal of Chromatography B, 727 (1999) 89–94

JOURNAL OF CHROMATOGRAPHY B

Ultra-sensitive method for determination of ethanol in whole blood by headspace capillary gas chromatography with cryogenic oven trapping

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Received 30 July 1998; received in revised form 28 January 1999; accepted 29 January 1999

Abstract

We have established an ultra-sensitive method for determination of ethanol in whole blood by headspace capillary gas chromatography (GC) with cryogenic oven trapping. After heating a blood sample containing ethanol and isobutyl alcohol (internal standard, IS) in a 7.0-ml vial at 55°C for 15 min, 5 ml of the headspace vapor was drawn into a glass syringe and injected into a GC port. All vapor was introduced into an Rtx-BAC2 wide-bore capillary column in the splitless mode at −60°C oven temperature to trap entire analytes, and then the oven temperature was programmed up to 240°C for GC measurements with flame ionization detection. The present method gave sharp peaks of ethanol and IS, and low background noise for whole blood samples. The mean partition into the gaseous phase for ethanol and IS was 3.06±0.733 and 8.33±2.19%, respectively. The calibration curves showed linearity in the range 0.02–5.0 μg/ml whole blood. The detection limit was estimated to be 0.01 μg/ml. The coefficients of intra-day and inter-day variation for spiked ethanol were 8.72 and 9.47%, respectively. Because of the extremely high sensitivity, we could measure low levels of endogenous ethanol in whole blood of subjects without drinking. The concentration of endogenous ethanol measured for 10 subjects under uncontrolled conditions varied from 0 to 0.377 μg/ml (mean, 0.180 μg/ml). Data on the diurnal changes of endogenous ethanol in whole blood of five subjects under strict food control are also presented; they are in accordance with the idea that endogenous blood ethanol is of enteric bacterial origin.

Keywords: Cryogenic oven trapping; Ethanol

1. Introduction

There are a number of reports dealing with analyses of ethanol by gas chromatography (GC) [1–9]. In most of these reports, conventional packed columns, which give relatively low sensitivity and poor separation, were used [1–4,8]. With wide-bore capillary columns, only a 0.1–0.5 ml volume of headspace vapor can be injected [6,9]; with medium-bore capillary columns, split injection giving less than 5% efficiency has to be used [7].

In a recent study [10] we developed a new GC method for sensitive determination of chloroform and methylene chloride in human whole blood by

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00063-8

trapping them at cryogenic oven temperature for headspace samples; this method allows us to inject large volumes of headspace vapor without any loss, resulting in much higher sensitivity. In this study, we have extended this experiment to ethanol in human whole blood and have optimized the conditions to establish the assay method.

2. Experimental

2.1. Materials

Ethanol, isobutyl alcohol, methanol and sodium sulfate were obtained from Wako (Osaka, Japan); sodium hydrosulfite from Yoneyama Yakuhin Kogyo (Osaka, Japan); the Rtx-BAC2 fused-silica wide-bore capillary column (30 m×0.53 mm I.D., film thickness 2.0 μm) from Restek (Bellefonte, PA, USA). Human whole blood was obtained from 15 healthy subjects, who volunteered to take part in this study with informed consent.

2.2. Procedure

Stock solutions of ethanol (500 μg/ml) and isobutyl alcohol [internal standard (IS), 50 μg/ml] were prepared by dissolving them in distilled water. To a 7.0-ml screw-cap vial containing 0.1 g sodium hydrosulfite and 0.5 g sodium sulfate was added 1.0 ml whole blood containing 5 μg or less of ethanol and 0.5 μg IS. The vial was rapidly sealed with a silicone-septum cap and placed on an aluminum block heater. After heating the vial at 55°C for 15 min, the 24 G needle of a glass syringe (5-ml volume) was passed through the septum. A 5-ml volume of the headspace vapor was drawn into the syringe and injected into the GC port in the splitless mode at −60°C oven temperature.

2.3. GC conditions

GC analyses were carried out on an HP 6890 series gas chromatograph equipped with flame ionization detection (FID) and with a cryogenic oven temperature device (Hewlett-Packard, Palo Alto, CA, USA). An electrically operated solenoid valve introduces liquid carbon dioxide at a rate appropriate to cool the oven to a desired temperature. The GC conditions were: column temperature, −60 to 240°C (1 min hold at −60°C, 10°C/min from −60 to 40°C, 10 min hold at 40°C, and 20°C/min from 40 to 240°C); injection temperature, 200°C; detection temperature, 240°C; and helium flow-rate, 3 ml/min. The vapor was injected in the splitless mode, and the splitter was opened 1 min after completion of the injection. For GC quantitation, the peak area of each compound was used.

2.4. Endogenous ethanol in whole blood

We collected whole blood, in the presence of EDTA–2Na, from healthy subjects (in total 15 persons) who abstained from drinking for at least 2 days. For 10 subjects, whole blood was taken at arbitrary times of the day for determination of mean ethanol level. For five subjects, diurnal changes of endogenous ethanol were examined under strict food control: three rice balls (in total 600 g) plus 180 ml of seaweed soup were taken at 09:00 and 12:00. Neither the rice balls nor the soup contained ethanol. Blood was collected at 08:30 (before breakfast), 10:30 (after breakfast), 11:30 (before lunch), 13:30 and 17:00. Ethanol in whole blood was measured immediately after sampling.

3. Results

3.1. Optimization of conditions

We tested various initial oven temperatures at −20, −30, −40, −50, and −60°C for trapping ethanol and IS vapor, as shown in Fig. 1. At −20°C, the ethanol peak was broad and overlapped an impurity peak; with a decrease of the initial oven temperature down to −60°C, the ethanol peak became sharper and more separated from the impurity peak. Thus, we adopted −60°C initial oven temperature. The IS peak was not markedly influenced by decreasing temperature in the range −20 to −60°C.

Various salts have been reported to be effective for increasing the extraction efficiency of ethanol [8]. We added 0.3, 0.5 or 1.0 g sodium sulfate to 1 ml whole blood in the presence of 5 μg ethanol. The

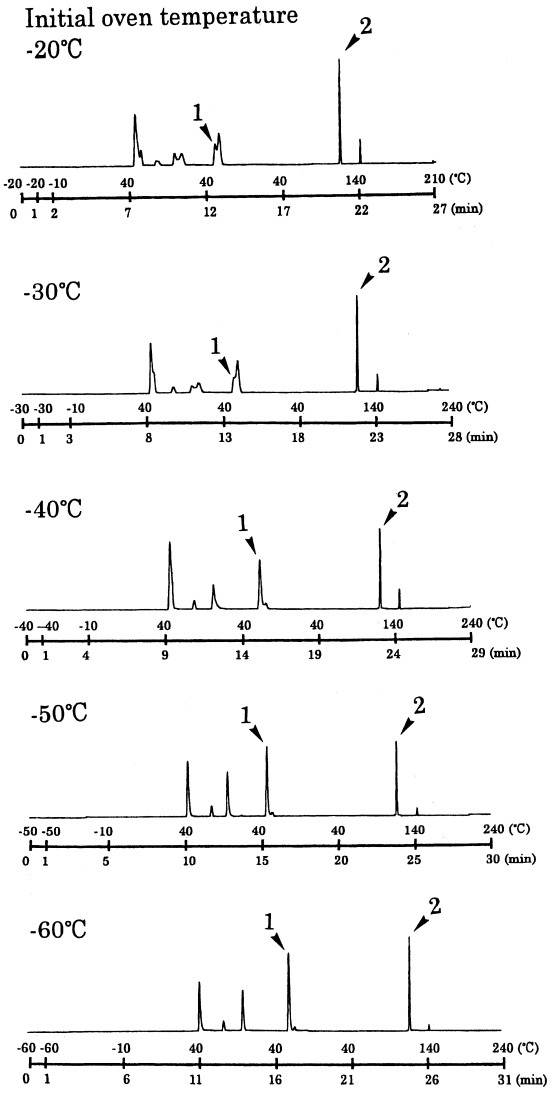

Fig. 1. Headspace capillary GC for ethanol (1) and isobutyl alcohol (IS) as a function of various initial oven temperatures. Ethanol (5 μg) and IS (0.5 μg) were added to 1 ml human whole blood for headspace extraction. The scales of the *Y*-axes are the same for each panel.

peak was highest with 0.5 g sodium sulfate; the latter was adopted in our method.

Sodium hydrosulfite, a reducing agent, has been reported to be effective for the protection of ethanol from oxidation [11]. We added 0.02, 0.05, 0.1, 0.15 or 0.2 g sodium hydrosulfite to 1 ml whole blood in the presence of 5 μg ethanol. The highest peak was obtained at 0.1 g of the reducing agent, which was adopted in our method.

We tested various capillary GC columns, such as DB-WAX, GS-Q, Rtx-Volatiles and Rtx-BAC2. The Rtx-BAC2 column gave excellent symmetrical peaks of ethanol and good separation from impurity peaks. For other columns, remarkable tailing, very short retention times and missing ethanol peaks were observed. Thus, we used the Rtx-BAC2 column in this study. According to the manufacturer's instructions for the column, the temperature range should be −20 to 240°C. In our experience, however, this column was found to be resistant to the cryogenic temperature at −60°C; it could be repeatedly used for at least 3 months with good reproducibility.

3.2. Reliability of the method

Fig. 2 shows gas chromatograms for non-extracted pure ethanol (5 μg on column) and IS (0.5 μg on column) directly injected into the GC (top panel) and for headspace extracts from 1.0 ml human whole blood in the presence (5 and 0.5 μg, respectively, in a vial) and absence of both compounds (middle and bottom panels). The retention times for ethanol and IS were 16.5 and 24.5 min, respectively. The backgrounds gave small impurity peaks; no interfering peaks appeared around the test peaks (Fig. 2, bottom panel).

The mean partition into the gaseous phase of ethanol at 5.0 μg/ml and of IS at 0.5 μg/ml was 3.06±0.733 and 8.33±2.19%, respectively (n=10 each), respectively.

The coefficients of intra-day and inter-day variations for ethanol in whole blood were 8.72 and 9.47% at 5 μg/ml whole blood (n=5 each) and 9.53 and 10.1% at 1.0 μg/ml (n=5 each).

A calibration curve for ethanol in human whole blood was drawn by plotting 11 concentrations versus 0.5 μg/ml IS. It was linear in the range 0.02–5.0 μg/ml. The equation and the r value for the curve were $y = 0.465x + 0.0206$ (r=0.999).

Fig. 3 shows gas chromatograms obtained for headspace extracts of human whole blood in the presence and absence of 0.5 μg (A) or 0.1 μg (B) spiked ethanol and 0.5 μg IS. As shown in the figure, 0.1 μg ethanol gave a peak with a signal-to-noise ratio >10. The detection limit (signal-to-noise

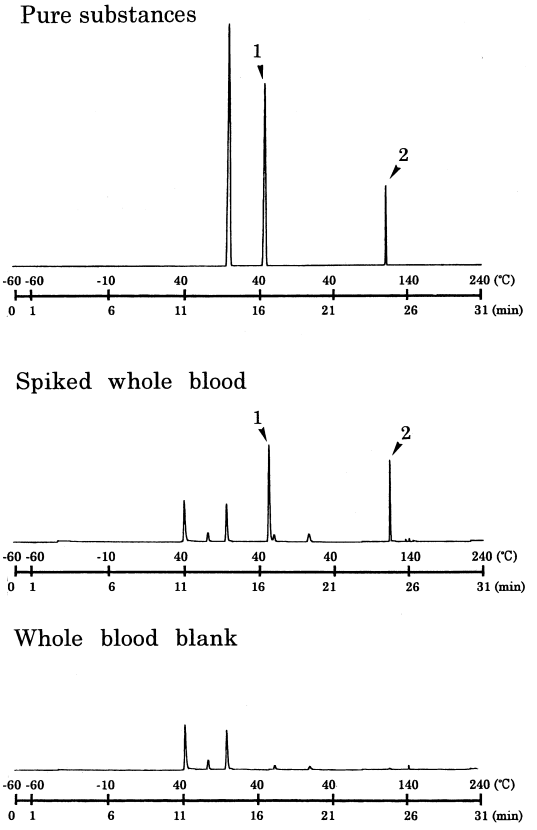

Fig. 2. Capillary GC chromatograms with cryogenic oven trapping at −60°C for pure ethanol (1) and (IS) with direct injection (top panel), for whole blood spiked with 5 μg ethanol and 0.5 μg IS in 1.0 ml (middle panel) and for whole blood in the absence of the compounds (bottom panel). The vertical scale of the top panel is 8 times lower than those of other panels.

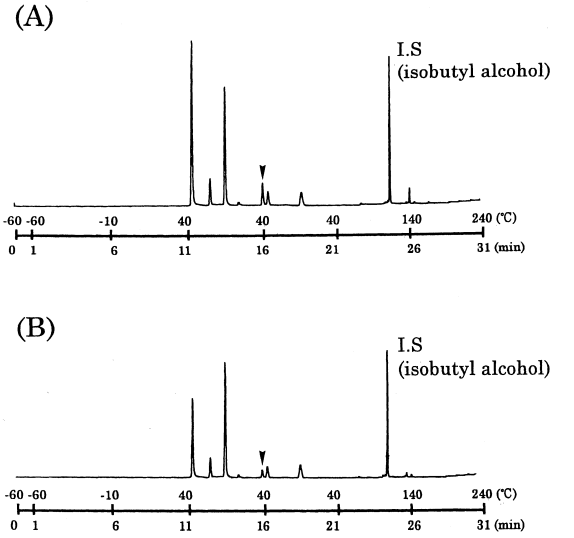

Fig. 3. Headspace capillary GC with cryogenic oven trapping for human whole blood (1.0 ml) which had been spiked with 0.5 μg ethanol (A) or 0.1 μg ethanol (B) as indicated with arrows. The amount of each IS was 0.5 μg/ml blood. The scales of the *Y*-axes are the same for both panels.

ratio 3) of ethanol in whole blood was estimated to be 0.01 μg/ml.

3.3. Endogenous ethanol present in whole blood without drinking

The concentrations of endogenous ethanol for each subject varied greatly under food-uncontrolled conditions as shown in Table 1. The mean concentration was 0.180±0.117 μg/ml (mean±SD, $n=10$).

The physical conditions of five subjects used for diurnal changes of endogenous ethanol in whole blood under strict food control are presented in Table 2. Fig. 4 shows the results of changes in blood of the above five subjects. There were large diurnal changes for each individual and large variations in the level among the five subjects. Individual 12 suffered from diarrhea, especially in the morning of the experimental day (Table 2); he showed negli-

Table 1
Concentrations of endogenous ethanol in whole blood of healthy subjects under uncontrolled conditions except for alcohol abstinence

Individual	Sex	Age	Concentration (μg/ml)
1	F	29	0.377
2	M	23	0.353
3	F	23	0.113
4	F	24	0.130
5	F	20	ND[a]
6	M	24	0.241
7	M	35	0.170
8	F	22	0.095
9	M	23	0.211
10	M	36	0.110
Mean±SD			0.180±117

[a] Below the detection limit.

Table 2
Physical conditions of the subjects used for diurnal changes of endogenous ethanol in whole blood under food control

Individual	Sex	Age	Defecation on the day	Resistance to alcohol intoxication
11	M	22	No	Strong
12	M	25	Diarrhea	Weak
13	M	23	No	Weak
14	M	27	Once (08:30)	Strong
15	M	21	No	Weak

gible levels of ethanol until 13:30. The levels increased at 17:00, except for individual 11.

3. Discussion

Recently, a microcomputer-controlled device for lowering the oven temperature to below 0°C has become available for new types of gas chromatographs. This device was originally designed for rapid cooling of an oven to reduce the time of analysis. In this study, we have used it for trapping ethanol gas inside a capillary column at cryogenic oven temperature; as much as 5 ml of gas can be injected into the column without any loss, resulting in a much higher sensitivity. This is the first report dealing with GC with cryogenic oven trapping for ethanol in biological samples.

There are a number of reports on analyses of ethanol in biological samples by GC [1–9]. Among them, Hara et al. [6] reported the highest sensitivity by headspace GC-FID with a DB-1 wide-bore capillary column; their detection limit was 8 μg/ml. Recently, O'Neal et al. [9] have reported a method of headspace GC-FID with an Rtx-BAC2 capillary column; their detection limit was 10 μg/ml. Our present method gave a detection limit of about 0.01 μg/ml, which is 1000 times lower than the above literature data.

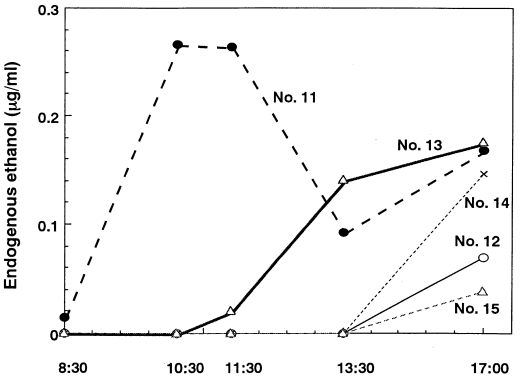

Fig. 4. Diurnal changes in endogenous ethanol in whole blood of subjects whose food intake had been under strict control. For details of foods, see text.

The ultra-high sensitivity obtained in this study is, of course, mainly due to the cryogenic oven trapping of entire analytes inside the capillary column. In addition to this new principle, the use of the Rtx-BAC2 column and sodium hydrosulfite reducing agent were very useful for increasing the sensitivity, especially when very small amounts of ethanol had to be detected.

Using the present method, we have measured endogenous ethanol concentrations in whole blood of healthy subjects under food-uncontrolled conditions, who abstained from drinking for at least 2 days (Table 1). The mean concentration obtained from 10 subjects was 0.180 μg/ml. The data for each subject are within the range reported by Lester (0–1.5 μg/ml) [12].

Diurnal changes in endogenous ethanol in the blood of five subjects were also studied under strict food control (Fig. 4). The level increased at 17:00 except for individual 11, probably because of the accumulation of intestinal contents during the day of the experiment. Individual 12, who suffered from diarrhea, and 14, who defecated in the morning (Table 2), showed negligible levels of blood ethanol until 13:30. These data suggest that fermentation of intestinal contents by bacterial flora is the main source of the endogenous blood ethanol. This is in accordance with the report that many kinds of bacteria produce ethanol [13]. Measurements of endogenous ethanol were made immediately after blood sampling, because post-mortem production of ethanol is well known [14].

In the present study, we established an ultrasensitive headspace GC-FID method for the analysis of ethanol in biological samples using cryogenic oven trapping; it is simple and requires no special GC operation, although it requires liquid carbon dioxide. Our method is, of course, not suitable for

routine analysis of ethanol in human blood after drinking, because of its extremely high sensitivity; it is useful mainly for biochemical applications on very low concentrations of ethanol in blood, tissues or cultured cells [15,16]. From the viewpoint of forensic toxicology, the present method also seems useful for measuring ethanol in very small sample volumes (microliter or milligram scale).

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