A New Method for the Determination of Ethanol in the Blood and Urine by Pulse Heating

Taizo Nagano, Tatsunori Takayasu and Hitoshi Maeda
Department of Legal Medicine, Kanazawa University School of Medicine, Takara-machi 13 1, Kanazawa 920, Japan

(Received May 22, 1989 ; accepted July 18, 1989)

Abstract. We have established a new method for the determination of ethanol and other gaseous substances in even minute amounts of biological material by utilizing a Curie-point pyrolyzer (Model JHP 3, Japan Analytical Industry) connected to GC or GC-MS. No pretreatment of the biological material is needed and the procedure is based on the principle that volatile or gaseous substances can be evaporated from biological materials by pulse heating and introduced for analysis directly to a GC or GC-MS.

The blood from rats that had been administered ethanol-d6 orally, as well as blood and urine collected from healthy volunteers that had ingested alcoholic drinks and blood and tissue specimens taken from autopsied cadavers were examined for ethanol concentrations by both this new method and by the usual head space-GC method.

To test this new method, one microliter of the blood or urine was injected into a tubularly folded ferromagnetic alloy (Pyrofoilヨ) and heated pulsatively five times at 160°C (pulse heating). The vapor that evaporated from the sample then was directly introduced to the GC or GC-MS, and the time required to complete the analysis took approximately 5 min. In parallel, for comparison purposes, portions of the same samples (0.2-0.5g) were examined by means of the head space method as well. The measured values of ethanol-d6 and ethanol in the rat blood and in the human whole blood, blood plasma, and urine that were obtained by this pulse heating procedure correlated almost perfectly with those obtained by head space method. These results show the accuracy and reproducibility of this new method. Further, "pulse heating" makes it possible to determine the presence of ethanol very quickly from a very small amount of the specimen.

We have termed this new investigative method for determining volatile and gaseous substances the "pulse heating method". Informatively, nitrous oxide (N_2O, laughing gas) was identified by this pulse heating GC-MS procedure from the blood and organ specimens of a victim that had succumbed to gas asphyxia.

Key words : Toxicology, Ethyl alcohol, Mass fragmentography

Introduction

The analysis of ethanol in biological materials is a routine procedure in the medico-legal practice and it has a great significance when investigating traffic incidents. For determining the presence of ethanol, several methods are available, though they are relatively complicated and time-consuming. Procedures that use gas chromatography for the detection of ethanol and other volatile poisons in biological materials have been reported[1)-7)]. Among them, a head space method[1)-4)] is now widely utilized because of its many advantages. This method, however, also has some disadvantages that will be mentioned in the Discussion portion of this paper. Further, Ueda and Fukui8' and Ishizawa et al.[9),10)] have reported on a possible method for determining the presence of ethanol by a process of evaporation that requires only a very small amount of the specimen to be tested and uses an electric furnace-type pyrolyzer. While their methods are theoretically appropriate, they

have not been practically utilized because of the reasons that also will be mentioned in the Discussion.

Recently, a Curie-point pyrolyzer has been developed that uses ferromagnetic alloys which very quickly generate heat by electromagnetic induction and can be warmed to an accurately defined Curie temperature[11)12)]. Thus, we have made an attempt to establish a new method for determining the presence of ethanol and other volatile and gaseous substances by pulse heating, utilizing this Curie-point pyrolyzer and connecting it to a gas chromatograph (GC) apparatus or a gas chromatograph-mass spectrometer (GC-MS). In this study, we have carried out a fundamental animal experiment using ethanol-d6 and were able to determine the presence of alcohol in human blood and urine by this method, which we have termed the "pulse heating method", and have compared our results with the results achieved by the head space method.

Materials and Methods

1. Standard solution.

Deuterium-labelled ethanol-d6 (MSD Isotopes, Canada; 0.2-2.0 mg/ml) and natural ethanol (0.2-5.0 mg/ml) were added to distilled water.

2. Ethanol-d6 containing samples.

Ethanol-d6 (25 v/v% aqueous solution) was administered orally to rats (Wister strain, ca. 200 g in weight) at a dose of 1 g per kg body weight. The blood was extracted from 15 to 90 min after this administration, and stored in small glass vials at 4°C.

3. Human blood and urine samples.

Healthy adult volunteers were given alcoholic drinks: beer in the amount of 750 and 1,300 ml; and, Japanese sake in the amount of 700 and 950 ml. Blood and urine samples then were collected at about 30 min after ingestion. The whole blood, plasma, and urine were stored at 4°C until use.

Blood samples also were collected from 12 autopsy cases and examined as well.

4. Equipments and analytical conditions.

A Curie-point pyrolyzer (Japan Analytical Industry, Model JHP-3) was connected to a GC (JEOL, Model GCG-06, FID) or a GC-MS system (JEOL, Model JMS-DX303 & DA5100 Data System). Fig. 1 is a schematic diagram that shows

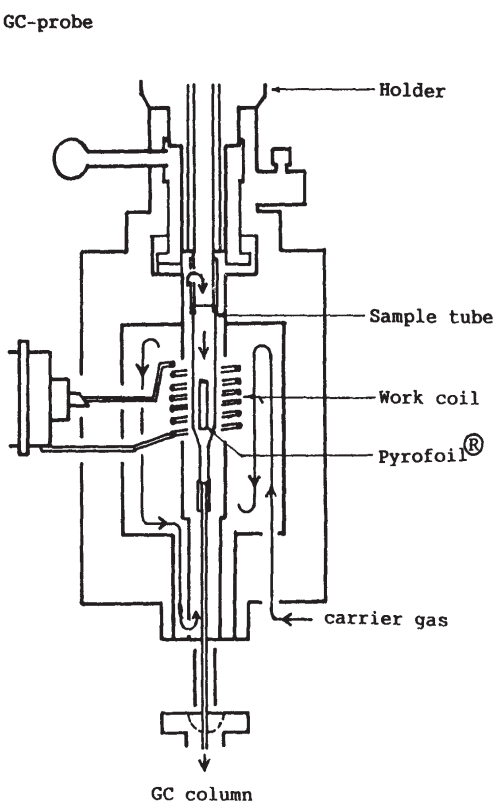

Fig. 1. Schematic illustration of GC-probe of Curie point pyrolyzer Model JHP-3.

the construction of the "GC-probe" of the pyrolyzer. Pulse heating (abbreviated as Py)-GC or -GC-MS was carried out under the following conditions:

Pyrolyzer (JHP-3)
- frequency 450 kHz, power 225W
- Pyrofoil®, of Curie-point 160°C
- needle temperature 200°C

GC (GCG-06, FID)
- column: Porapak Q (80-100), 2.6 mm × 1 m
- temperature: column oven 160°C
  - injection 180°C
  - detector 222°C
- carrier gas flow (N_2) 50 ml/min
- calculator Shimadzu Chromatopac C-R3A

GC-MS (JMS-DX303 & DA5100 Data Systems)
- GC column: Porapak Q (80-100), 2.6 mm × 1 m
- temperature: column oven 170°C
  - injection 180°C
  - separator 180°C

carrier gas flow (He)	45 ml/min
MS El (positive) mode	
accel volt	3 kV
ionization volt	70 eV
ionization current	0.3 mA
ionization chamber temperature	150°C

5. Analytical procedure.

For calibration curves, 0.5, 1.0, 2.0, 3.0, and 5.0 //I of standard solutions of ethanol-d6 and ethanol were used. Ethanol standard solutions of 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mg/ml were used to determine within-run and day-to-day precision. Each sample was divided into two portions and analyzed in parallel by both the head space method and the "pulse heating" method.

Pulse heating method : One microliter of each sample was extracted with a microsyringe and injected into a Pyrofoil® which was folded in a tubular form. The foil was immediately inserted into a "sample tube" which was fixed on a holder and set on the "GC-prove" of the pyrolyzer (Fig. 1). The sample then was heated at 160°C for 4 sec five times pulsatively at intervals of 20 sec in order to completely evaporate the volatile substances. The vapor was analyzed by the Py-GC or -GC-MS.

Head space method : According to the method of Nanikawa and Kotoku[4], samples (0.5 ml) pipetted into vials were incubated with an internal standard (0.2 mg/ml tert-butanol) at 55°C for 20-25 min. The head space gas (about 1 ml) then was analyzed by GC or GC-MS.

6. A case of determination of gaseous anesthetics in human blood and tissue specimens.

During our investigations, we also had the opportunity to analyze the amount of gaseous anesthetics present in the heart blood and the brain, and also in the heart muscle, lung, liver, and kidney by means of both methods. The above materials were obtained from a cadaver of a 9-year-old, who had died in an anesthetic accident. A small amount of each material was sealed into a vial, and frozen at –30°C immediately after being taken from the cadaver. Two days later the materials then were examined by the two methods described above.

Results

1. Mass chromatographic analysis of ethanl-d6 from animal blood by the pulse heating method.

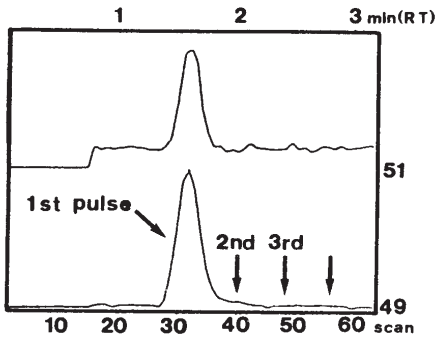

Fig 2 Mass chromatogram of ethanol d6 (d5) extracted by a computer data process from TIC Rat heart blood (1 μl) was analyzed by pulse heating at 160°C for 4 sec, five times at intervals of 20 sec No peaks appeared after the second pulse heating

Rat blood containing ethanol-d5, into which ethanol-d6 immediately changed in water, were analyzed by the pulse heating method. Mass chromatogram of ethanol-d5 (ion current profiles at m/z 49 and 51) that was extracted by a computer-data process from a series of consecutively recorded mass spectra is shown in Fig. 2. When the blood samples were examined by 5 pulse heatings, only one peak was noted at a retention time (1.6 min) of ethanol-d6 (-d5) in the mass chromatogram (Fig. 2). The El mass spectrum of the peak showed predominant ions of ethanol-d5 at m/z 33, 49 and 51. Additionally, very small ion peaks at m/z 50, 46 and 48 were observed, which were also detected in standard solutions. No other ions, including natural ethanol (m/z 31 and 45), were noted.

2. Calibration Curves.

Using 0.2-2.0 mg/ml of an ethanol-d6 solution as the standard solution, calibration curves were drawn from an estimation of the peak area and peak height of their mass-chromatograms. Each line was linear through the zero-point as shown in Fig. 3. By means of the peak area, the following equation was obtained : Y=4.40 X 106X, where Y is the peak area per 1il, and X is the concentration of ethanol-d6. Similar results were obtained when natural ethanol (0.2-5.0 mg/dl) was used instead ofethanol-d6. When 0.5, 2.0, 3.0, and 5.0ilof each standard solution was applied, the calibration curves were almost identical to that seen in the

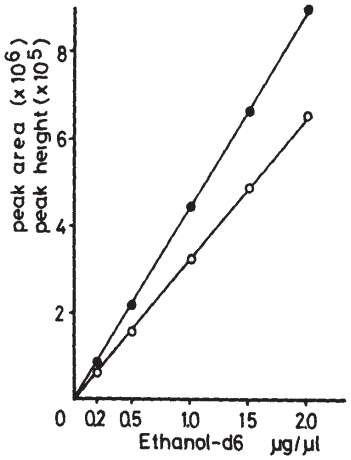

Fig 3 Calibration curves for ethanol d6 (d5) meaured at m/z 49 Standard solution in the distilled water ●—● peak area ○—○ peak height

Table 1 Measured values of ethanol d6 in the rat heart blood

sample No	Measured by	
	head space method	pulse heating method
1	0 7611 mg/ml	0 7500 mg/ml
2	0 6841	0 6921
3	0 9799	0 9667
4	0 6690	0 8269
5	0 8282	0 8924
6	0 8894	0 9990
7	0 5120	0 5227
8	1 1531	1 2860

use of 1 ìl of the standards.

For within-run precision, the coefficients of variation (CVs) ranged from 2.7 to 5.1% at 0.2-5.0 mg/ml level. For day-to-day precision, CVs ranged from 5.5 to 8.6%.

3. Measured values of ethanol d6 concentrations in the animal blood.

Ethanol-d6 (changed into -d5) concentrations in the rat heart blood measured by the pulse heating and the head space methods are given in Table 1. The measured values by both methods very closely approximated each other. The correlation between the values by both methods is shown in Fig. 4 and the regression curve was estimated per the following formula: Y=0.03 + 1.03 X (Y, the

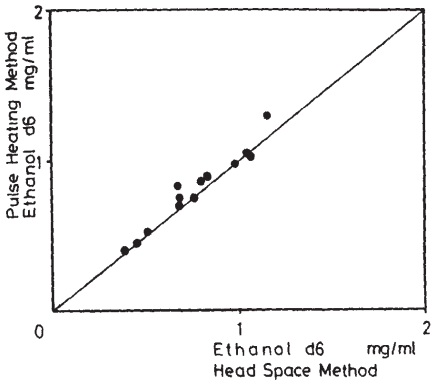

Fig 4 Comparison of ethanol d6 (d5) concentrations in the rat heart blood measured by the head space method and by the pulse heating method

Table 2 Measured values of the ethanol con centration in human blood and urine after ingestion by volunteers

sample No	Measured by	
	head space method	pulse heating method
whole blood		
1	0 475 mg/ml	0 489 mg/ml
2	0 619	0 665
3	2 474	2 485
4	2 790	2 873
plasma		
1	0 539	0 556
2	0 691	0 712
3	2 654	2 713
4	3 057	3 226
urine		
1	0 624	0 679
2	0 721	0 756
3	2 446	2 767
4	3 993	4 323

measured value by the pulse heating method; X, the measured value by the head space method) with a correlation coefficient (r) 0.965 (sample size n=15).

4. Measured values of ethanol concentration in human whole blood, plasma, and urine.

Ethanol concentrations of the whole blood, plasma, and urine collected from healthy volunteers were measured by both the pulse heating and the head space methods. The data obtained are shown in Table 2. The correlation between the

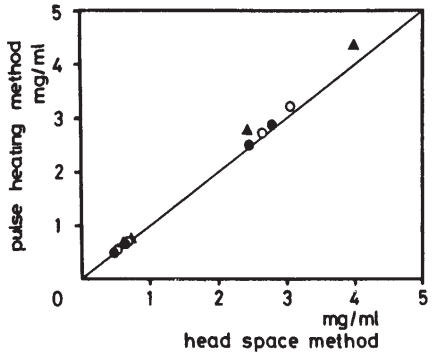

Fig 5 Comparison of ethanol concentrations in the volunteers blood and urine measured by the head space method and by the pule heating method
● whole blood ○ plasma ▲ urine

Table 3 Measured values of the blood ethanol concentration in autopsy cases

sample No	Measured by	
	head space method	pulse heating method
1	2 323 mg/ml	2 147 mg/ml
2	3 977	3 611
3	0 787	0 772
4	1 486	1 315
5	1 056	1 068
6	2 980	2 985
7	3 330	3 370
8	1 560	1 512
9	<0 001	<0 001
10	1 537	1 535
11	<0 001	<0 001
12	0 580	0 592

measured values by both methods is shown in Fig. 5 and the regression curve was estimated per the following formula: Y=0.017 + 1.03X (r=0.998, n=12).

5. Measured values of ethanol concentration in whole blood in autopsy cases.

In autopsy cases as well, the measured values of the blood ethanol concentrations by means of the pulse heating were almost identical with the head space methods (Table 3). For the correlation between both methods, the regression curve was estimated as follows : Y=0.020 + 0.952X (r=0.997, n=12).

All processes to complete the analysis required about 5 min.

6. Determination of gaseous anesthetics in the human blood and tissue specimens.

By both the pulse heating GC-MS method and the head space-GC-MS method, nitrous oxide (N2O, laughing gas) was qualitatively identified in the all tissue specimens tested.

Discussion

Because of its many advantages, the head space GC method is widely used internationally. There are many reasons for this ; the method has a high reliability and shows a fairly good reproducibility, and the head space gas can be directly applied to a GC-apparatus for measurement. However, particularly when the ethanol concentrations are very low or abnormally high or when the amount of the materials to be tested is very minute, there are also some disadvantages in utilizing this method. Contrary it is theoretically appropriate to determine ethanol and other volatile or gaseous substances which were evaporated out from the sample specimens Attempts[8)-10)] have been made to use an electric furnace type pyrolyzer to evaporate ethanol from minute samples as a method of gas content analysis. Using this approach Ueda and Fukui[8)] have tried to analyze the ethanol content by heating samples sealed in polyethylene tubes that were melted by heat. Although, as they have described, the interference from the pyrolysis products of the polyethylene containers was negligible, some small peaks appeared that resulted from polyethylene, with a longer retention time than that of the ethanol. Therefore, the next sample could not be introduced for about 22 min until all the pyrolysis product of the polyethylene had left the column. Similarly, the gas chromatographic method reported by Ishizawa et al.[9) 10)] requires more than 30 sec to reach the necessary temperature, by which time the ethanol will have completely evaporated from the specimens.

In contrast the Curie-point pyrolyzer, which we employed, possesses a valuable heating capacity that utilizes a self-heating capability of ferromagnetic alloys. In other words, by means of electromagnetic induction, hysteresis generates heat in the metal due to friction caused by the changing polarity. At the Curie-point, the metal then suddenly looses its magnetism owing to a

drastic change in the magnetic permeability. In formatively, the ferromagnetic metal, Pyrofoilㅋ, that was used in this study reaches the Curie-point within 0.2 sec, whereas the temperature in a furnace pyrolyzer rises far more slowly, taking longer than 5 sec. Further, Pyrofoil® cools rapidly due to the interruption of the electromagnetic induction. Therefore, samples injected or wrapped in a tubularly folded foil can be instantaneously and repeatedly heated to a given Curie temperature, and the heating duration can be appropriately controlled.

As shown in Fig. 2, by using a Curie-point pyrolyzer that was connected to a GC or GC-MS, it was possible to determine the ethanol content very quickly from a very small sample of the material (ca. 0.5-5㎕), whereas the head space method required ca 0.5 ml of the sample. Further, the pulse heating method could be repeated at short intervals until the ethanol-d5 was completely released from the specimens (Fig. 2). The blood samples gave only one peak (retention time, 1.6 min) in spite of 5 pulse heatings, and all the necessary processes for one analysis were completed within approximately 5 min. Informatively, the peak width of the ethanol (or -d5) was about 20 sec. For a comparison the furnace pyrolyzer method used by Ueda and Fukui[8] and by Ishizawa et al.[9),10)] gave an ethanol peak width that was about 45 sec and required approximately 22 and 25 min for one analysis, respectively.

This shows that a Py-GC (or GC-MS) by means of a Curie point pyrolyzer can be performed more rapidly than the methods that use a furnace pyrolyzer[8)~10)] and that the peaks are remarkably sharper. The Py-GC-MS analyses of ethanol-d6 (-d5) in blood samples showed no other peak than ethanol-d6. This indicates that there occurred no artifacts, such as the degradation of ethanol-d5 or pyrolysis production of interfering substances from the blood.

Further, all the calibration curves of ethanol (or -d6) obtained by repeated examinations were almost identical, and the stability of the calibration curves is reflected in the within-run and day-to-day precision.

From these data, it can be said that the pulse heating-GC or -GC-MS method has good accuracy and sufficient reproducibility for practical assays of ethanol in the blood and urine. A series of examinations to prove the reliability of this method showed that the measured values from the blood and urine (liquid samples) very closely approximated those obtained by the head space method (Figs. 4 & 5).

In the rat whole blood and in human whole blood, as well as in the plasma and urine from volunteers, the measured values obtained by the pulse heating method had a tendency to be slightly higher than the values obtained by the head space method (Tables 1& 2 and Figs. 4 & 5).

Thus, the use of a Curie point pyrolyzer is suitable for determining the ethanol content of biological materials. Employing this new method, the analysis can be very quickly performed and the materials to be tested require no pretreatment. Additionally, the quantitative accuracy and reproducibility are comparable to those obtained by the head space method.

Further, our preliminary study has shown that this method can be applied to the analysis of other volatile and gaseous substances, such as petrolic components and gaseous anesthetics, and the details of our results will be published separately.

Informatively, we had an opportunity to determine gaseous anesthetics in the heart blood and in other tissue specimens of a 9-year-old who unexpectedly died while under anesthesia. Using our pulse heating GC-MS method, laughing gas was determined in all the specimens tested. This indicate the possibility of determining the presence of volatile and gaseous drugs and poisons in all kinds of biological materials whether liquid or solid by the pulse heating method.

We are now seeking to establish a reliable and rapid screening system for determining volatile and gaseous substances in bioloigical materials.

Acknowledgement

This work has been supported in part by a Grant-in-Aid for Scientific Research (No. 60440041) from the Ministry of Education, Science, and Culture, Japan

A part of this work was presented at the 72nd Conference of the Medico-legal Society of Japan, Akita, May, 1988, at the International Congress of Forensic Science, Beijing, September, 1988, and at the 67th Annual Meeting of the Deutsche Gesellschaft fur Rechtsmedizin Kloster Banz, September, 1988.

References

1) Curry, A.S., Hurrt, G., Kent, N.R. and Powell, H.: Rapid screening of blood sample for volatile poisons by gas chromatography. Nature, 195, 603-604, 1962.

2) Bassette, R., Ozeris, S. and Whitnal, C.H.: Gas chromatographic analysis of head space gas of dilute aqueous solutious. Anal Chem, 34, 1540-1543, 1962.

3) Machata, G.: Ober die gaschromatographische Blutalkoholbestimmung. Analyse der Dampfphase. Mikrochim Acta, 6, 262-271, 1964.

4) Nanikawa, R. and Kotoku, S. : A routine method or the determination of ethanol in biological materials using gas chromatography. Jpn J Stad Alcohol, 4, 27-33, 1969 (in Japanese with English abstract).

5) Chundela, B. and Jana J.: Quantitative determination of ethanol besides other volatile substances in blood and other body liquids by gas chromatography. J Forensic Med, 7, 153-161, 1960.

6) Parker, K.D., Fontan, C.R., Yee,J.L. and Kirk, P.L.: Gas chromatographic determination of ethyl alcohol in blood for medicolegal purposes. Separation of other volatiles from blood or aqueous solution. Anal Chem, 34, 1234-1236, 1962.

7) Ueda, M. : Analytical research of acute alcoholic intoxication (Special communication). Jpn J Legal Med, 21, 261-284, 1967 (in Japanese with English abstract).

8) Ueda, M. and Fukui, Y.: A new method for the determination of ethanol in blood by pyrolysis-gas chromatography. Jpn J Stud Alcohol, 2, 100-106, 1967.

9) Ishizawa, F., Sasagawa, K., Mashiko, K.: A study on the gas chromatographic determination of ethanol in blood. Rep Natl Res Inst Police Sci, 32, 33-37, 1979 (in Japanese with English abstract).

10) Ishizawa, F. : A study on the gas chromatographic determination of ethanol in blood. Jpn J Legal Med, 34, 618-625, 1980.

ll) Simon, W., Kriemler, P., Voellmin,J.A. and Steiner, H.: Elucidation of the structure of organic compounds by thermal fragmentation. J Gas Chromatography, 5, 53-57, 1967.

12) Oguri, N. and Ohnishi, A.: Development of bioporbe Curie point pyrolyzer- GC and applications. Proc. Pittsburgh Conference, 870, 1988.