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Determination of Endogenous Ethanol in Blood and Breath by Gas Chromatography-Mass Spectrometry

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JONES, A. W., G. MÅRDH AND E. ÄNGGÅRD. *Determination of endogenous ethanol in blood and breath by gas chromatography-mass spectrometry.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 267–272, 1983.—We describe methods for the determination of endogenous ethanol in biological specimens from healthy abstaining subjects. The analytical methods were headspace gas chromatography (GC) for plasma samples and gas chromatography-mass spectometry (GC/MS) with deuterium labelled species $^2H_3$-ethanol and $^2H_5$-ethanol as internal standards for breath analysis. Ethanol in rebreathed air was about 10% higher than in directly analysed end-expired alveolar air. Known volumes of rebreathed air were passed through a liquid-N_2 freeze trap and the volatile constituents of breath were concentrated prior to analysis by GC or GC/MS. Besides endogenous ethanol, peaks were seen on the chromatograms for methanol, acetone and acetaldehyde as well as several as yet unidentified substances. The endogenous alcohols ethanol and methanol were confirmed from their mass chromatograms and the GC/MS profile also indicated the presence of endogenous propan-1-ol. The concentration of endogenous ethanol in plasma showed wide inter-subject variations ranging from below detection limits to 1.6 μg/ml (34.8 μmol/l) and with mean±SD of 0.39±0.45 μg/ml (8.5±9.8 μmol/l). We aim to characterise further the role of endogenous ethanol with the main focus on dynamic aspects such as the rate of formation and turnover.

Analysis Blood Breath Endogenous ethanol Gas chromatography-mass spectrometry

ALTHOUGH several investigators have confirmed the presence of ethanol in samples of blood and breath from healthy abstaining subjects [6, 22, 23], the biological significance of the concentrations found is still unsettled [5,15]. The source of endogenous ethanol is usually ascribed to microbial fermentation of ingested sugars in the gut but normal metabolic pathways have also been implicated [2, 14, 20].

We were interested in endogenous ethanol, particularly in the possibility of studying its dynamic aspects such as the rate of formation and turnover. This paper presents details of the analytical methods that we have used to determine endogenous ethanol in blood and breath and some preliminary results of the concentrations present. The occurrence of endogenous ethanol in biological fluids from healthy abstaining subjects was unequivocally established by the use of gas chromatography-mass spectrometry for analysis and with deuterium labelled analogues of ethanol as internal standards.

METHOD

Subjects

The test subjects were healthy men recruited from our laboratory staff. Their ages ranged from 21–38 years and all of them had abstained from alcoholic beverages for at least 24 hr before the tests.

Sampling of Blood and Breath

Samples of blood (10 ml) were drawn from an antecubital vein after the subjects had fasted overnight. The bloods were collected in vacutainers (Becton-Dickinson Ltd., USA) pretreated with sodium fluoride to give a final concentration in blood of 10 mg/ml. Within 30 min of taking the blood samples, they were centrifuged at 1400 g in a Vortex table centrifuge for 15 min to obtain plasma.

The samples of breath were taken after subjects rebreathed the initial exhalation 5 times. Each subject attached a nose clip, took a moderately deep inhalation and exhaled into a 4 liter Tedlar® bag made of polyvinyl fluoride film (Dupont Corporation, USA). The bag and breath-inlet tube (1 cm i.d.) were kept warm inside a heating jacket, maintained at 40°C, to prevent condensation of water vapor and the subsequent loss of water-soluble constituents of breath. The initial exhalation was rebreathed from the bag and this rebreathing maneuver was repeated until 5 cycles were complete. The final exhalation was trapped in the heated bag. The temperature of the expired breath was measured with a thermistor device as described previously [11].

Immediately after the rebreathing procedure was completed, a measured volume of the breath was drawn from the heated bag and led through a glass U-tube packed with glass spheres (3 mm diameter). The U-tube was pre-cooled in liquid nitrogen (−196°C) and was flushed continuously with nitrogen until just before being attached to the supply of breath. All connections from the heated bag to the inlet of the U-tube were warmed to about 50°C by electrically heated tape. The rebreathed air was aspirated through the U-tube at the rate of 1.5 liter/min for about 30–60 sec. The exact volume of breath passing through the U-tube was monitored

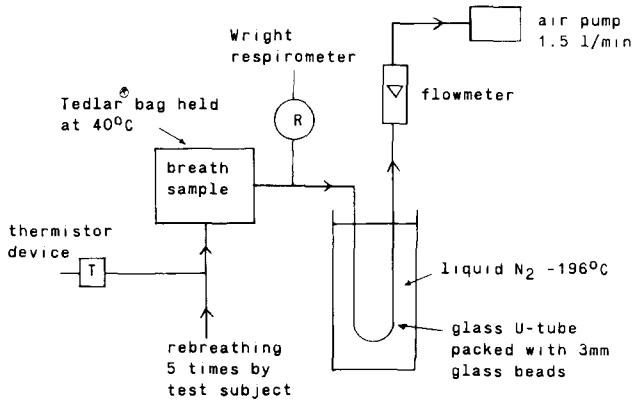

FIG. 1. Schematic diagram of the equipment used to sample breath and to freeze-trap a measured volume for gas chromatography analysis.

with a Wright respirometer (British Oxygen Co., U.K.). The arrangement of the apparatus for freeze-trapping the volatile constituents of breath is shown schematically in Fig. 1.

To analyze reference standard substances, we added aqueous solutions of the agents to a U-tube before it was flushed with nitrogen and immersed in the freeze trap. The dilutions of the standards were chosen so that the total volume of liquid added was approximately equal to the water content of the expired breath sample. Expired breath leaves the mouth saturated with water vapor at about 34.5°C [11].

Analysis of Blood and Breath Samples

To analyze the endogenous volatiles in blood or plasma, we employed headspace gas chromatography after saturating the biological specimen with sodium chloride or potassium carbonate to enhance the vapor phase concentrations of the volatile agents [19]. Blood or plasma (1 ml) was transferred into a 22 ml glass bottle containing 1 g of the salt. The bottles were made air tight with rubber septa and pressure sealed with aluminum caps. The bottles were positioned in an aluminum block heater with tight fitting holes completely covering the sides of the glass vessels. The samples were equilibrated at 60±1.0°C for 30 min. Preliminary trials showed that this was enough time to get complete equilibrium of the volatile agents between the gas and liquid. Samples of the headspace vapor (1 ml) were removed with a warmed gas-tight syringe (S.G.E. Ltd., Victoria, Australia). The syringe was filled with 1 ml of room air which was injected into the headspace above the liquid. The syringe-plunger was then moved up and down to the 1 ml graduation mark 4 times to obtain an equilibrated sample. A 1 ml aliquot of the headspace gas was removed and injected into the gas chromatograph.

The condensed breath samples were transferred from the liquid-N_2 trap to the gas chromatrographic column according to the method described by Dannecker *et al.* [4].

Preparation of the Nitrite Ester Derivatives of Methanol and Ethanol

For GC analysis, we used the nitrite ester derivatives of ethanol and methanol [7,13]. These volatile esters of nitrous acid and primary alcohols were generated directly in the headspace flasks with methyl nitrite (b.pt. −12°C) and ethyl nitrite (b.pt. 17°C). The biological sample (1 ml) was mixed with 0.2 ml $NaNO_2$ (2% w/v in water) and the flask was made air tight and immersed in an ice-water bath at 5°C. A 0.2 ml aliquot of H_2SO_4 (8% v/v in water) was injected through the septum and the contents of the flask were thoroughly mixed and kept at 5°C for 10 min. A sample of the headspace gas (1 ml) was removed for analysis by gas chromatography. The nitrite esters are readily synthesised according to the following reaction:

$$C_2H_5OH + 2NO_2 \rightleftarrows C_2H_5ONO + HNO_3$$

The advantage of making this derivative of ethanol is that 5°C equilibrium suppresses the volatility of the other endogenous substances, such as acetone, giving clean chromatograms. Moreover, the higher molecular weight for the nitrite esters make them potentially more useful for mass fragmentographic analysis.

Gas Chromatographic Analysis

The quantitative analysis of ethanol and its separation from other low-molecular weight volatiles was done with a Pye 104 gas chromatograph equipped with flame ionization detector. For direct headspace analysis Porapak Q (80–100 mesh) (Waters Associates, USA) was packed into a glass column (2 m × 2 mm i.d.) kept at 120°C with nitrogen (20 ml/min) as the carrier gas. The air and hydrogen flow rates to the detector were adjusted to achieve highest sensitivity with optimal signal to noise ratios. For assay of nitrite esters, Tenax GC (30–60 mesh) supplied by Supelco Ltd., USA was packed into a 2 m × 2 mm i.d. silanized glass column kept at 60°C with nitrogen (20 ml/min) as carrier gas. The condensed breath samples were analysed on the Porapak Q column held initially at 40°C for 2 min, during transfer of the sample to the column, and then heated as quickly as possible to 90°C and finally programmed from 90–150°C at 5°C per min.

The detector responses were fed to a Hewlett Packard reporting integrator Model 3390 A. The detection limit for ethanol with these techniques, to give a response exceeding twice the noise width, was about 50 ng/ml plasma (1.1 μmol/l).

Gas Chromatography-Mass Spectrometry

An LKB 2091 gas chromatograph-mass spectrometer was used to analyze freeze-trapped breath samples and equilibrated headspace gas. The gas chromatograph was a modified version of the Pye 104 instrument. The mass spectrometer was equipped with a multiple ion detector and a Finnigan INCOS data system was used to collect and process the results. The chromatographic operating conditions were the same as described above. The flash heater of the mass spectrometer was at 230°C, ion source 270°C and the trap current was set at 50 μA. The energy of electrons was 70 eV for repetitive scanning and 22.5 eV for selected ion monitoring. In the assay of condensed samples of breath, repetitive scanning mode was used, m/z 20–200 at 3 sec/scan. The resulting spectra were stored on a magnetic disc and were manipulated in various ways including peak matching in a computer generated library of spectra, and preselected mass fragments were displayed as mass chromatograms.

TABLE 1

MASS SPECTRA OF STANDARD REFERENCE SUBSTANCES*

Reference substance	MW	Mass-fragments (% intensity) base peak	molecular ion	m/z
Methanol	32	31 (100)	32 (88)	29 (15)
$^2H_3$-Methanol C^2H_3OH	35	33 (100)	35 (80)	30 (10)
Acetaldehyde	44	44 (100)	44 (100)	29 (75)
Ethanol	46	31 (100)	46 (10)	45 (50)
$^2H_3$-Ethanol $C^2H_3CH_2OH$	49	31 (100)	49 (15)	48 (60)
$^2H_5$-Ethanol $C^2H_3C^2H_2OH$	51	33 (100)	51 (20)	49 (32)
Acetone	58	43 (100)	58 (40)	44 (5)
Propan-1-ol	60	31 (100)	60 (3)	59 (8)
Methyl nitrite	61	30 (100)	61 (8)	29 (12)
Ethyl nitrite	75	30 (100)	75 (0)	60 (40)

*Principal mass fragments (m/z) from electron-impact (70 eV) mass spectra of low-molecular weight alcohols, acetone, acetaldehyde and the nitrite ester derivatives of methanol and ethanol. MW=molecular weight, m/z=ratio of mass to charge of the ion fragment.

Chemicals and Solvents

Standard solutions of the reference substances were made-up in water that was double distilled from alkaline permanganate. The chemicals and solvents were of analytical grade purity and were obtained through local suppliers (Kebo Grave Ltd., Stockholm). The deuterium labelled alcohols, $^2H_3$-methanol (C^2H_3OH) and $^2H_6$-ethanol ($C^2H_3C^2H_2O^2H$), were brought from Merck Ltd., Darmstadt, Germany and $^2H_3$-ethanol ($C^2H_3CH_2OH$) from Stohler Isotope Chemicals Ltd., USA. When $^2H_6$-ethanol is mixed with water it becomes converted into $^2H_5$-ethanol, $C^2H_3C^2H_2OH$.

RESULTS

Mass Spectra of Reference Materials

Table 1 shows the principal mass fragments obtained from the electron-impact (70 eV) spectra of headspace gas samples in equilibrium with dilute solutions of the substances in water. The base peak, molecular ion and the second most intense ion are given. These fragmentation patterns were used to identify the endogenous components seen on the chromatograms from freeze-trapped samples of breath. The low intensity of the molecular ions of nitrite ester derivatives of methanol and ethanol limits their usefulness for quantitative analysis by selected ion monitoring under the present operating conditions. The spectra of the primary alcohols methanol, ethanol and propan-1-ol all have m/z 31 as their base peak. This mass fragment seems to be characteristic for primary aliphatic alcohols and corresponds to the molecular species CH_2OH^+ [17]. The deuterium labelled ethanol analogues are useful as internal standards for mass fragmentographic analysis and can help to elucidate the fragmentation pattern of a substance on electron impact.

Increases in Expired Ethanol After Rebreathing

Table 2 compares the concentration of ethanol in end-expired alveolar air and in breath from the same subject after rebreathing 5 times. Both samples were taken for analyses within 2–3 min. The concentrations of ethanol and the temperatures of expired breath increase with the number of cycles of rebreathing. With between 3–5 complete rebreathed cycles, the concentrations of ethanol rises by about 10% on average. These results speak in favor of using rebreathing air for studies on the excretion of endogenous volatiles in breath [12].

Concentration of Endogenous Ethanol in Plasma

Headspace analysis of plasma samples from healthy abstaining subjects gave three definite peaks on the chromatogram. From retention times, these peaks were identified as acetone, ethanol or methanol. The relative intensities of the peaks varied widely among different subjects with the acetone response always being the most pronounced. The endogenous concentration of ethanol was quantitated and ranged from only trace amounts (below detection limits) to 1.6 μg/ml (34.8 μmol/l). The distribution of values was markedly skewed, 0.39±0.454 μg/ml (mean±SD, n=17). The median endogenous ethanol concentration in these subjects was 0.14 μg/ml (3.0 μmol/l). The same range of values was seen when the nitrite ester derivative of ethanol were analysed.

Gas Chromatography-Profile Analysis

Figure 2 shows typical chromatograms from analysis of the constituents of breath after freeze-trapping a sample in liquid nitrogen. The peaks labelled 1–5 were identified by comparison with retention times of reference standards. In line with the findings from direct headspace analysis of plasma the relative concentrations of endogenous volatiles in breath were acetone > ethanol > methanol > acetaldehyde. Peak 5 was a pentadiene, possibly 2-methyl-1,3-butadiene (isoprene), but this may have arisen as an artifact from the rubber tubes used in the sampling system. In the chromatographic profile of expired breath, several other endogenous species were evident and attempts are being made to charac-

TABLE 2

INCREASES IN EXPIRED-ETHANOL AFTER SUBJECTS REBREATHED*

Number of rebreathing cycles	Temperature of breath	Percent increase in expired-ethanol	
		Observed	Temp. corrected
1	34.9 ± 0.19	5.3 ± 0.90	4.2 ± 1.17
2	34.9 ± 0.18	11.2 ± 0.62	7.7 ± 0.53
3	35.0 ± 0.20	13.7 ± 1.22	10.4 ± 1.20
4	35.2 ± 0.20	14.8 ± 0.92	10.0 ± 0.60
5	35.2 ± 0.15	16.9 ± 1.36	11.6 ± 0.96

*The results after rebreathing were compared with control breaths after subjects took a deep inspiration and expiration (end-expired alveolar air). The mean temperature of control breath was 34.5 ± 0.16°C (±SE n=30). The blood/breath ratio of ethanol for samples of rebreathed air was 2000:1. The temperature-corrected values were derived from ethanol's air/blood temperature coefficient of solubility based on experiments in-vitro [8]. The figures presented are means ± SE (n=6).

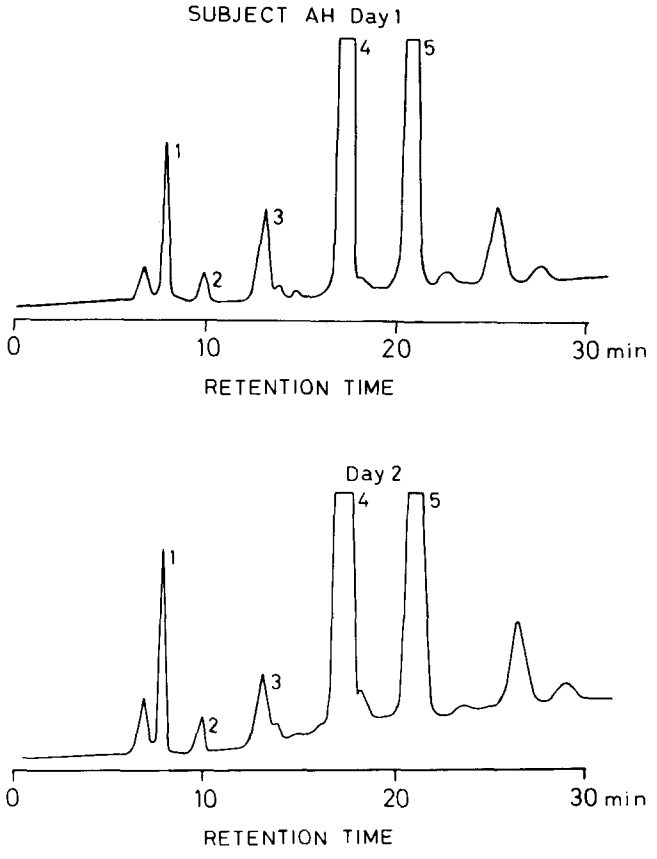

FIG. 2. Gas chromatograms derived from the analysis of freeze-trapped condensates of breath from a healthy abstaining subject tested on two consecutive days. The numbered peaks are (1) methanol, (2) acetaldehyde, (3) ethanol, (4) acetone (5) tentatively identified as 2-methyl-1,3-butadiene (isoprene).

terise them. Figure 2 illustrates excellent day to day reproducibility for the breath profile of this subject.

Mass Chromatograms of Breath Condensates

Considerably stronger evidence for the presence of a particular endogenous species is furnished from the mass spectrum of the substance. Figure 3 displays a computer generated mass fragmentogram of a gas chromatographically analysed freeze-trapped sample of breath from a healthy subject. As well as similar retention times to standard reference substances, the same mass fragments in approximately the same relative abundance are seen. The run made on the breath condensate (lower part of Fig. 3) was at a much higher sensitivity than the standard sample. In spite of a higher background noise in tracings of the biological sample, the mass fragments agree well with the standards. The presence of endogenous acetaldehyde could not be confirmed in this breath condensate. The fragment at m/z 31 appears at 3 different retention times and these are well matched with methanol, ethanol and propan-1-ol, respectively. The ethanol fragmentation pattern m/z 31 (base peak) m/z 45 and m/z 46 (parent ion) was corroborated by the use of deuterium labelled internal standards.

DISCUSSION

The occurrence of small amounts of ethanol in the blood and tissue from normal human subjects has interested scientists for more than a century [9]. Most of the earlier experimental studies of this topic were hampered by technical difficulties and the methods of analysis used to quantitate ethanol lacked sufficient specificity.

Nevertheless, in more recent work, employing gas chromatographic methods of analysis, increasing evidence has emerged to show that endogenous ethanol does exist, but the concentrations seen have large inter-individual variations [6, 16, 22]. Our results, as yet on a relatively small material, show a markedly skewed distribution of values ranging from below detection limits (50 ng/ml, 1.1 μM) to 1600 ng/ml (34.8 μM) with a median value of 140 ng/ml (3.04 μM). The reason for the wide inter-individual variation in healthy abstaining subjects is hard to explain but the overall range of values

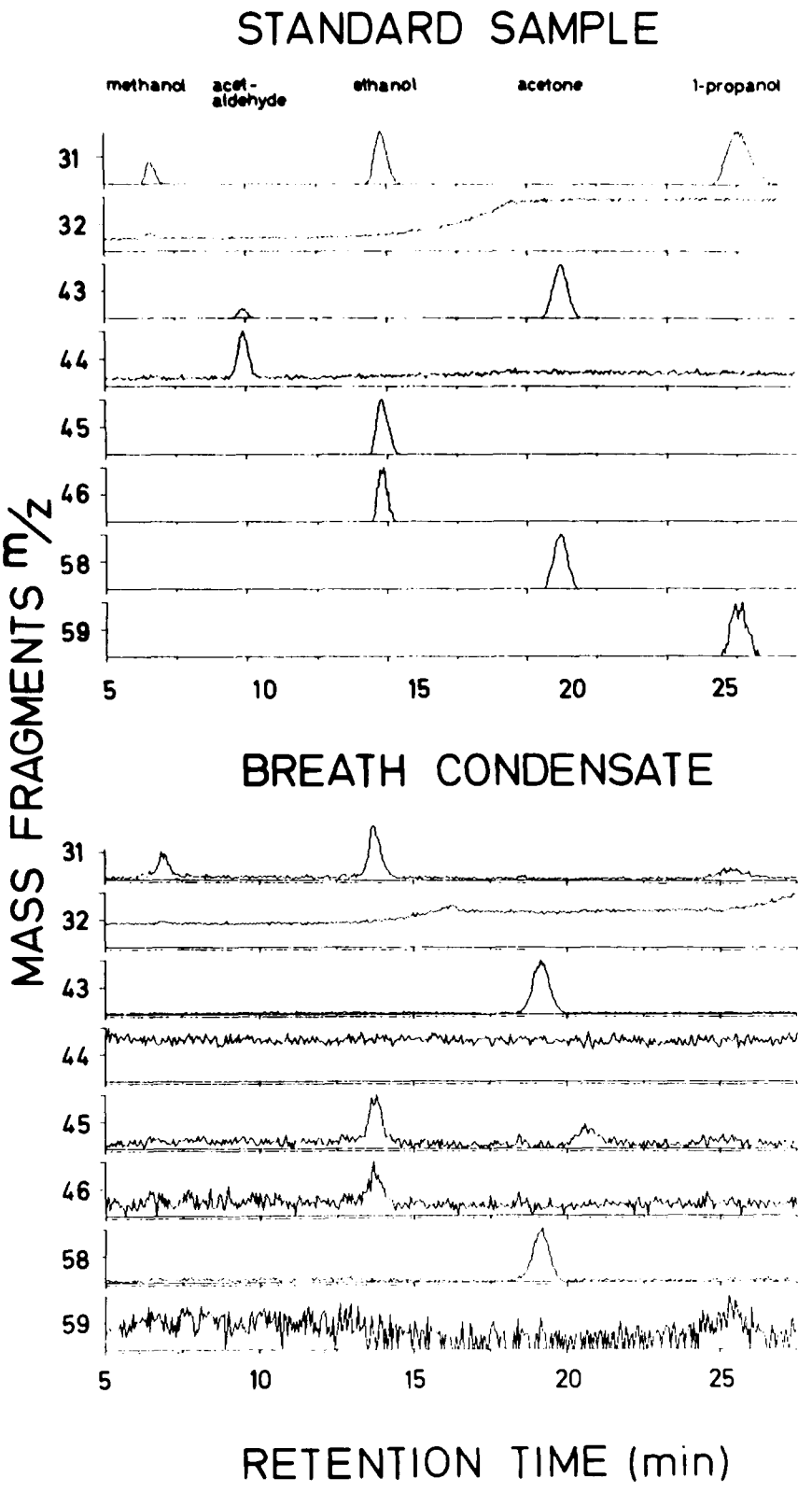

FIG. 3. Mass chromatograms resulting from a freeze-trapped condensate of breath from a healthy abstaining subject. The upper part of the figure shows mass chromatograms from a 5 component mixture of reference substances. The lower part of the figure shows the breath condensate.

shows good agreement with a recent study of endogenous ethanol with a sensitive headspace gas chromatography method of analysis [22].

Because the concentration of endogenous ethanol in blood samples is often near the limits of detection by conventional gas chromatography, we focused attention on the use of expired breath as a biological specimen for the assay and a mass spectrometer as the detector. The concentration of gases and volatile agents in breath reflects their concentration in the pulmonary arterial blood [10]. Breath analysis can be considered as being similar to headspace gas analysis but with equilibrium occurring between the volatile constituents of blood and alveolar air in the lungs at about 37°C. If important physiological factors, such as the temperature of breath and the vital capacities of test subjects are controlled, then the analysis of breath will provide a reliable estimate of the coexisting concentration of ethanol in blood [10,11].

By means of a suitable enrichment step, such as a cryogenic trap [3,4] or a porous polymer adsorbent material [1], the volatile endogenous substances expired in breath are easily concentrated. The profiles of trace volatile substances in human respiratory gas gives results that are directly proportional to the levels in arterial blood and therefore reflect the concentrations reaching the brain. Our GC (Fig. 2) and GC-MS (Fig. 3) profiles of endogenous substances in breath are being repeated in a larger group of subjects.

The possibility that endogenous ethanol is formed by decarboxylation of pyruvic acid with acetaldehyde as the intermediate has long been known [21]. Treonine, deoxyribose phosphate and β-alanine have been proposed as non-microbial precursor of endogenous ethanol via acetaldehyde [5]. Endogenously formed ethanol yields acetaldehyde as oxidation product and this substance is often implicated in the adverse biochemical and pharmacological effects of ethanol [18]. The condensation of acetaldehyde with various biogenic amines or formation of acetaldehyde-adducts with certain amino-acids and proteins may have untoward effects in predisposed individuals [18].

The rates of formation and turnover of a biologically active substance is more valuable information than its steady-state level [15]. The turnover of endogenous ethanol has apparently never been experimentally approached. It seems likely that there may be an uneven distribution in the vascular system with sharp arterial-venous differences and that the actual concentration present in a tissue may be blood-flow limited.

The use of GC/MS methods of analysis with deuterium labelled species as internal standards and positive identification of the principal mass fragments m/z 31, m/z 45 and m/z 46 gives unequivocal evidence for the existence of endogenous ethanol in blood and breath from normal human subjects. But the origin and biological significance of the endogenous ethanol remains to be established.

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