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A GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC STUDY OF PROFILES OF VOLATILE METABOLITES IN HEPATIC ENCEPHALOPATHY

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SUMMARY

Volatile organic substances present in blood plasma and cerebrospinal fluids of certain control groups of human subjects and cirrhotic patients some of whom were suffering from hepatic encephalopathy were quantitatively analysed and identified. A rapid, reproducible, direct injection capillary column gas chromatographic method was developed for the concentration and detection of such volatiles at mg/l and lower concentrations. Of at least forty volatiles detected, twenty-one were identified. The mean concentration of one of these, 3-methylbutanal, was found to be significantly elevated ($p < 0.01$) in chronic encephalopathics (2.37 ± 0.79 mg/l, $n = 18$), when compared to the controls (0.30 ± 0.08 mg/l, $n = 20$). Furthermore, the concentration of this component increased with the clinically diagnosed severity of the encephalopathic state. The presence of 3-methylbutanal is related to leucine, a branched-chain amino acid linked with hepatic encephalopathy.

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INTRODUCTION

Hepatic encephalopathy is a complex neuropsychiatric syndrome, probably multifactorial in origin. It is generally believed that the major factor in this disease involves toxic nitrogenous substances formed in the intestine by bacterial action on proteins [1]. However, the nature of these toxic substances is unknown and is the subject of much speculation [2, 3]. Originally, it was felt that increased blood ammonia levels in encephalopathy were responsible for the resulting toxicity [4] but hyperammoniaemia is not always associated with the incidence of coma [5]. Abnormalities of amino acid metabolism [6, 7], particularly the variable elevation of the concentrations of aromatic amino acids such as phenylalanine, tryptophan and methionine and recently the consistent depression in the levels of the branched-chain amino acids (BCAA) valine, leucine and isoleucine [8—11] have been noted. This decrease in levels of the BCAA's has been attributed so far to the effect of high levels of circulating insulin in the blood resulting in an increase of muscle BCAA uptake [12].

Since hepatic encephalopathy has been linked to deranged protein metabolism and it is unlikely that the amino acids are, in themselves, toxic, it becomes necessary to consider the possibility that one or more of their metabolites may be toxic. There is some prior evidence to suggest that the metabolism of amino acids produces volatile substances such as low-molecular-weight aldehydes, ketones and alcohols [13]. Some of these components may be related to the resulting toxicity in hepatic encephalopathy. The study of the volatile components in the body fluids of subjects with hepatic encephalopathy would therefore appear to be of interest.

The study of the volatile components in body fluids, in general, is a relatively new field [14—18] with potential as a novel means of studying disease. Various methods exist for the study of low-molecular-weight substances of biological origin. These methods apply either solvent extraction, headspace analysis or direct injection techniques, and are either very time consuming or non-quantitative. Goldberg and Sandler [19] have developed a simple, rapid and quantitative method for the analysis of body fluid volatiles, which in slightly modified form, has been used in this study. With such a method it should be possible to produce metabolic profiles to distinguish between the diseased and normal state or act as indicators of certain diseases. This study shows the application of the method to hepatic encephalopathy as one such disease.

EXPERIMENTAL

Analysis for volatiles

A gas chromatographic (GC) method of analysis for body fluid volatiles [19] developed in our laboratory, was based on the direct injection of 100 μl of sample, removal of water in a condenser at 0°C and subsequent sample trapping and concentration on a small, cooled precolumn packed with Tenax GC.

The present method is a modification of the above with the Tenax GC precolumn being replaced with a 1 m × 0.5 mm I.D. stainless-steel capillary tube immersed in liquid nitrogen. Recoveries of low-molecular-weight volatiles were

improved over the previous method. After trapping for 5 min the sample is injected on to the column by means of a microvolume switching valve and heating of the trap to 100°C.

The volatiles are separated by GC using wall-coated, open-tubular columns and a flame ionization detector. The column was a 100 m × 0.5 mm I.D. nickel tube prepared according to the method of Bertsch et al. [20]. It was coated with Ucon 50 HB 5100 and had an efficiency of 100,000 theoretical plates, based on the peak for 2-pentanone ($k = 5$). The carrier gas (nitrogen) flow-rate (1.5 ml/min) was pressure controlled at 153 kPa. The column was held at 50°C for 10 min, then programmed at 2°C/min, to 150°C and held at 150°C to the end of the analysis. Make-up nitrogen was added at the detector base to give a total nitrogen flow-rate of 25 ml/min. Hydrogen and air flow-rates were set at 25 ml/min and 300 ml/min, respectively.

Quantitative analysis of volatiles

The concentrations of the volatiles in the sample were determined using an internal standard, *o*-xylene, added to produce a concentration of 2.6 mg/l (0.5 ml *o*-xylene standard, 132 mg/l, added to 2 ml sample). Calibration standards of 1 mg/l of various volatiles were analysed and calibration coefficients were calculated. An Autolab System 1 (Spectra Physics, Technical Marketing Assoc., Toronto, Canada) computing integrator with the calculation accessory was used to measure the peak areas and perform the calculations, giving the concentrations of the volatiles in mg/l.

Volatile identification

Components were identified by analysis on two columns of differing polarities (OV-17 and Ucon 50 HB 5100). Further confirmation of the component identification was achieved by connecting the concentrating system to a Du-Pont 21-492 mass spectrometer. The column used in this portion of the study was a glass open-tubular column 50 m × 0.5 mm I.D. coated with OV-17 (All-tech, Arlington Heights, IL, U.S.A.). The operating conditions were otherwise maintained as described above.

Sampling

Blood samples were drawn into heparinized green stopper B-D vacutainers, cooled in ice and transferred into ice-chilled stoppered bottles. Samples not analysed immediately were frozen until needed. Skin disinfection prior to blood sampling was performed with soap and water rather than 2-propanol to eliminate possible contamination of the sample from the latter source.

Cerebrospinal fluid (CSF) samples were obtained by lumbar puncture. The CSF samples were taken only when necessary for other tests and therefore very few samples were analysed.

Subjects and samples

Samples of blood were collected from human subjects clinically classified as to their state of health into one of two major groups, (1) non-encephalopathic controls (20 subjects) and (2) encephalopathic (18 subjects). The control group was divided into four subgroups as follows: six normals under no dietary

control, five subjects undergoing a colonoscopy examination who were sampled after a fast, four non-dietary controlled cirrhotics and five post portacaval shunt patients on protein-reduced maintenance diets. All the post shunt patients had experienced previous episodes of encephalopathy. The encephalopathic group was divided into two subgroups, one consisting of thirteen patients in grades 1 and 2 (mild) hepatic encephalopathy, and the other consisting of five patients in grades 3 and 4 (severe) hepatic encephalopathy. Blood samples, taken from these subjects on a number of different occasions while they were in the same clinical state, were analysed.

Three CSF samples from one patient in grade 4 hepatic coma were taken on different days and analysed by the same procedure. The results were compared with those from samples taken from two different non-encephalopathic control subjects.

Amino acid analysis

Plasma samples from ten individuals in the study were deproteinized with equal volumes of 15% sulphosalicylic acid, containing 200 μmol/l norleucine as internal standard. The free neutral amino acid concentrations were determined on 300 μl of plasma using a Beckman Spinco amino acid analyser Model 116/119.

RESULTS

The method developed for the analysis of the volatile components was found to be reproducible to within ± 0.06 mg/l at the 95% confidence level when standard solutions were used. When the same blood plasma sample was analysed on three consecutive days the reproducibility was within ± 0.07 mg/l with 95% confidence [21]. An analysis of variance showed no significant differences between standard solution or blood plasma sample replications.

A quantitative comparison of the chromatographic profiles of the volatiles showed that there were distinct differences between non-encephalopathic (Fig. 1a) and encephalopathic subjects (Fig. 1b). Subsequent quantitative analysis confirmed this. The methods used for identification of components and their plasma and CSF concentrations in the control and encephalopathic subjects are given in Table I.

Two components, 3-methylbutanal and furfural, exhibited significant concentration differences between groups. In blood samples from encephalopathics the former was present at about eight times the concentration in the control groups and the latter at a concentration of about 50% of that in the control group.

In CSF the average concentration of 3-methylbutanal in the fluid of the subject in grade 4 hepatic encephalopathy (25.13 mg/l) was found to be almost twenty times higher than that for the control subjects (1.28 mg/l). Furfural was not detected in any of the CSF samples.

When the results from patients in grades 1 and 2 hepatic encephalopathy were compared with those for grades 3 and 4, it was found that the concentration level of 3-methylbutanal increased with the severity of the encephalopathic state. The mean concentration of 3-methylbutanal in mild encephalo-

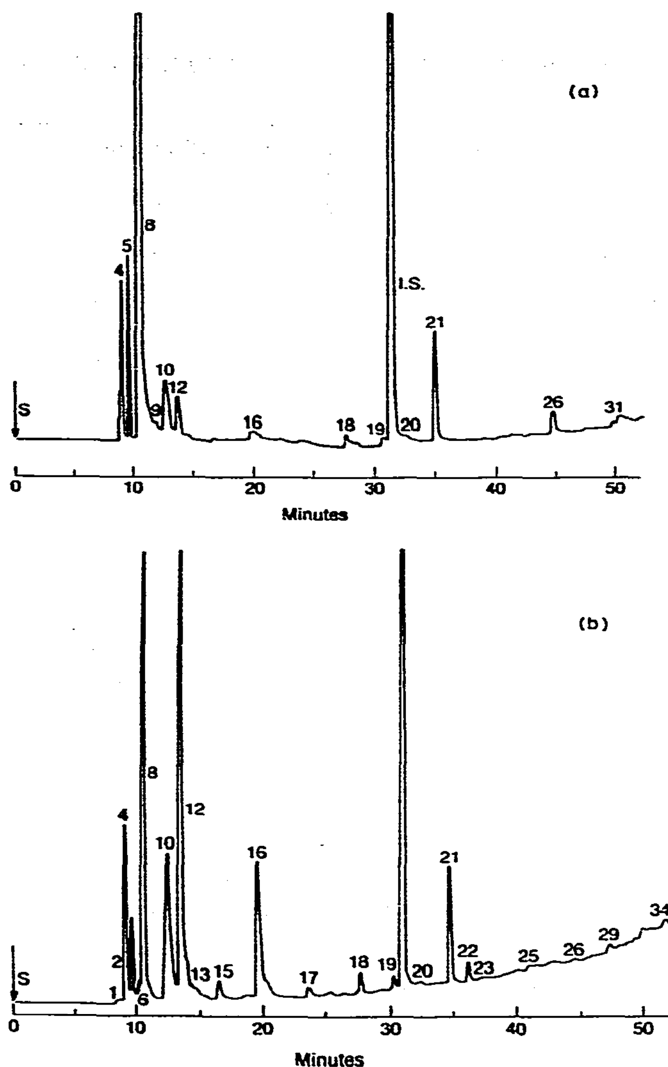


Fig. 1. Chromatograms of plasma volatile metabolites in (a) normal and (b) encephalopathic subject. Conditions: 100 m × 0.25 mm I.D. nickel column coated with Ucon 50 HB 5100; nitrogen carrier gas at a flow-rate of 1.5 ml/min; 50°C for 10 min then programmed to 150°C at 2°C/min. Peaks are identified in Table I.

pathics, grades 1 and 2 (1.30 ± 0.32 mg/l, n = 13) was significantly less than ($p < 0.025$) the mean for grades 3 and 4 encephalopathic subjects (5.13 ± 2.46 mg/l, n = 5). The results are presented graphically in Fig. 2.

Plasma amino acid analysis showed that the ratio of BCAA to aromatic amino acids was 3 for normal subjects and between 0.58 and 1.93 for cirrhotic subjects whether encephalopathic or not. Subjects in grade 4 encephalopathy had high plasma BCAA levels, yet others [22, 23] have claimed that increasing the plasma BCAA level would be beneficial in the management of hepatic encephalopathy.

TABLE I

CONCENTRATIONS (mg/l ± S.E.M.) OF IDENTIFIED VOLATILE COMPONENTS IN PLASMA AND CSF

Peak No.	Component	Method of identification*			Control group (mg/l)		Encephalopathic group (mg/l)	
					Plasma ($n = 20$)	CSF ($n = 2$)	Plasma ($n = 18$)	CSF ($n = 3$)
4	Ethanal	U	O	M	0.76 ± 0.15	2.26	0.75 ± 12	1.19
6	Propanal	U			0.04 ± 0.01	0.01	0.04 ± 0.01	0.04
8	Propanone	U	O	M	9.66 ± 2.55	1.84	17.44 ± 6.59	157.61
10	Butanone + 2-	U	O	M	2.32 ± 0.83	0.24	1.33 ± 0.25	1.60
	methylbutanal**			M				
11	Ethanal +	U	O		3.11 ± 1.93	ND	0.13 ± 0.03	ND
	2-propanol**	U	O	M				
12	3-Methylbutanal	U	O	M	0.30 ± 0.08	1.28	2.37 ± 0.79***	25.13
13	2-Butanal			M	0.05 ± 0.01	0.02	0.05 ± 0.02	1.15
14	2-Pentanone +	U	O		0.05 ± 0.02	ND	0.46 ± 0.37	0.04
	3-Pentanone**	U						
16	3-Methyl-1-							
	butanal	U			0.20 ± 0.06	0.05	0.22 ± 0.06	0.13
18	1-Hexanal			M	0.07 ± 0.01	0.01	0.16 ± 0.07	2.46
19	3-Heptanone	U			0.09 ± 0.02	ND	0.06 ± 0.01	0.01
20	2-Heptanone	U			0.05 ± 0.01	0.02	0.08 ± 0.02	0.08
21	Furfural			M	0.53 ± 0.06	ND	0.28 ± 0.04***	ND
23	Methylpyrrole			M	0.03 ± 0.01	ND	0.07 ± 0.02	0.06
25	4-Heptanone +			M	0.07 ± 0.03	0.01	0.04 ± 0.01	0.04
	2-octanone**	U	O					
28	Benzaldehyde			M	ND	ND	0.06 ± 0.03	0.24

*U = Identified by chromatography on Ucon 50 HB 5100 column; O = identified by chromatography on OV-17 column; M = identified by combined gas chromatography—mass spectrometry.

**Components not resolved on Ucon column.

***Significant difference between plasma concentrations ($p < 0.01$).

DISCUSSION

Low-molecular-weight volatile substances in body fluids are present due to the metabolism of higher-molecular-weight components such as amino acids, fatty acids and carbohydrates. Changes in the concentration of the volatiles parallel changes in the concentrations of the high-molecular-weight component. The volatiles may therefore be used as an indirect measurement of the metabolism of amino acids, fatty acids and sugars. Furthermore, the volatiles give the total of the metabolism of related substances. Treatment of hepatic encephalopathy consists of protein withdrawal and elimination or modification of the colonic bacteria.

It has been shown [24] that in the bacterial degradation of certain amino acids, volatile substances may be produced. In subjects suffering from hepatic encephalopathy, the bacterial formation of volatiles from amino acids must be

considered. Therefore, it is the metabolic pathways of bacteria which must be used in explaining the presence of those volatile components directly related to hepatic encephalopathy.

Two components, 3-methylbutanal and furfural show significant differences in plasma concentrations when encephalopathic and non-encephalopathic subjects are compared. Only the former is present in the CSF.

Thus, on the basis that CSF is the transfer agent to the brain for such substances, only 3-methylbutanal could have a neurological effect in hepatic encephalopathy. Pilotti et al. [25] have shown that 3-methylbutanal is toxic in the sense that it inhibits cell multiplication in vitro at concentration levels comparable to those found in the blood plasma of the encephalopathic subjects in this study and below the levels found in CSF samples of such a subject. Rats injected with sufficient 3-methylbutanal to give a blood concentration of this substance comparable to patients with hepatic encephalopathy showed EEG patterns similar to encephalopathics [26].

Fig. 2 shows the concentration of 3-methylbutanal for all the subject groups. Two points of interest are the relatively high levels of 3-methylbutanal in the normal group as compared to the colonoscopy subjects and the low levels of this component in the shunt group as compared to the encephalopathics. The high level in the normals would indicate that 3-methylbutanal is of dietary origin, either endogenous or exogenous, since the colon group was sampled after a fast. Furthermore, the fact that high levels of 3-methylbutanal in the normals are not associated with encephalopathy shows that other components such as ammonia, fatty acids, amino acids or mercaptans [2, 3] must be involved. A person susceptible to encephalopathy is apparently sensitive to toxic substances not removed due to the absence of the normal liver function. The low levels in the shunt patients seem to bear this out. These patients, with improper liver function, become encephalopathic when their protein intake is high. Together with the onset of encephalopathy, the plasma 3-methylbutanal increases. The formation of this aldehyde is thus related to the metabolism of ingested protein, in particular the BCAA's.

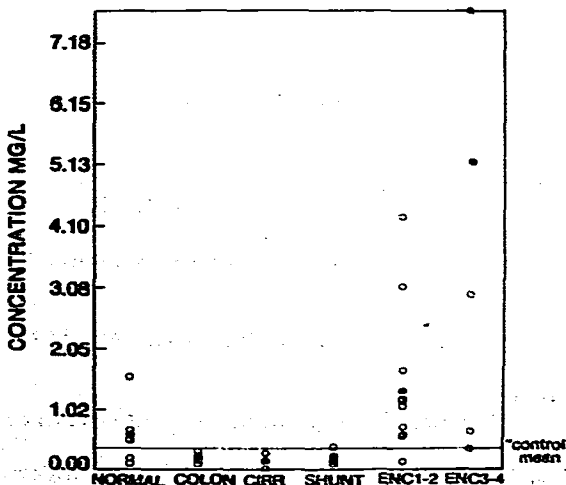
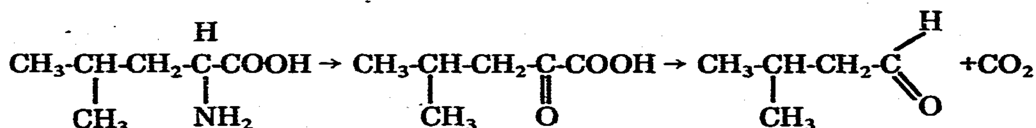


Fig. 2. 3-Methylbutanal concentrations in blood plasma in all groups in individual subjects. Group description as in text. (o) Subject mean; (•) group mean ± S.E.M.

It is proposed that 3-methylbutanal is formed by the catabolism of leucine as follows:



The literature supports the possible formation of this compound from leucine. Yabumato and Jenning [27] have proposed the formation of 3-methylbutanal from leucine, 2-methylbutanal from isoleucine and methylpropanal from valine. That is, all three BCAA's have been considered capable of producing volatile branched-chain aldehydes. Yu and Spencer [28] have demonstrated the production of [^{14}C] 3-methylbutanal from [^{14}C] leucine using an enzyme extract from tomato. Non-enzymatically, these volatiles may be formed from their corresponding amino acids by the Strecker degradation [29], which has been postulated in several biological systems. Recently [30] it has been shown that 3-methylbutanal is formed by isolated bacteria grown on agar supplemented with yeast extract.

CONCLUSION

A quantitative, reproducible method for the analysis of body fluid volatiles has been developed. This has been applied to the study of hepatic encephalopathy.

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