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Breath biomarkers for detection of human liver diseases: preliminary study

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Chronic liver disease is initially occult, has multiple aetiologies, involves complex diagnostic questions, and requires follow-up because progression is likely. Blood tests and biopsies are generally used, but have disadvantages. We have developed a new test for liver disease based on abnormal concentrations of metabolic products detected in exhaled breath. This test can be used, in conjunction with other clinically accepted diagnostic protocols, to detect and classify chronic liver diseases. Samples of breath collected from spontaneously breathing human subjects (86 patients presenting with 13 liver diseases and 109 subjects with normal liver function) were concentrated cryogenically and analysed by wide-bore capillary gas chromatography using various detectors. The concentrations of various molecules in exhaled breath were examined for potential use as biomarkers of liver function. Subjects with chronic liver diseases could be differentiated from those with normal liver function by comparing levels of breath carbonyl sulphide, carbon disulphide and isoprene; these differences were confirmed and correlated by comparing the levels with standard clinical blood markers of liver damage. The presence of chronic liver failure can thus be detected with sensitivity and specificity by quantifying sulphur-containing compounds arising from the abnormal metabolism associated with liver disease. The breath test we have developed appears to distinguish between hepatocellular and biliary tract aetiologies, and allows staging for severity. This approach may provide the clinician with a simple, non-invasive technique for use in the screening of large populations and follow-up for patients with chronic liver disease.

Keywords: breath carbonyl sulphide, carbon disulphide, dimethyl sulphide, isoprene, liver diseases, hepatocellular diseases, diseases of the bile duct, staging disease.

Introduction

The concept that blood, urine and other body fluids and tissues can be sampled and analysed to yield clinical information for the diagnosis of disease states or to monitor therapy is the foundation of modern clinical diagnosis and medical practice. The use of breath as a collectable sample has not received similar clinical use because the low concentrations (p.p.b.) of the analyte required concentration of the molecules of interest for chemical analysis. Recent advances in on-line concentration and capillary gas chromatography have suggested that the use of exhaled breath to study disease processes should now be re-examined.

The collection of exhaled breath has major advantages since it is non-invasive and represents minimal risk to the personnel collecting the samples. The composition of breath is a reflection of the identities and concentrations of the molecules

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present in the circulating blood, their volatilities, lipid solubilities and rates of diffusion across the alveolar membrane. With few exceptions (oxygen and carbon dioxide), the rate of excretion of molecules into breath is governed by the rate of perfusion of the blood through the pulmonary vasculature (cardiac output) and the minute ventilation. Diffusion across the alveolar membrane is governed by changes in free energy. The potential use of breath tests in medicine was reviewed by Phillips (1992). Currently the only clinical breath tests used routinely are based on the quantification of hydrogen (Perman 1991) and ^{13}C- or ^{14}C-labelled carbon dioxide (Phillips 1992). These tests are based on the administration of diagnostic agents that are metabolized to hydrogen or carbon dioxide. However, it has been demonstrated by many researchers that exhaled breath also contains a number of other endogenous species present at trace levels that are produced during normal or abnormal physiological function.

In their classic paper, Chen *et al.* (1970) identified methyl mercaptan, ethyl mercaptan and dimethyl sulphide in the breath of cirrhotic patients in hepatic coma, and proposed that these sulphur-containing molecules might arise from the incomplete metabolism of methionine. They confirmed their hypothesis by feeding methionine to normal and cirrhotic patients and found that levels of these sulphur-containing compounds increased in the breath of all the subjects, particularly those with impaired liver function. Subsequently, Kaji *et al.* (1978), Tangerman *et al.* (1983, 1985) and Hisamura (1979), using improved gas chromatographic methods, demonstrated that the levels of all of these sulphur-containing molecules are elevated in the breath of patients with cirrhosis compared with subjects with normal liver function. More recently, Phillips and his collaborators (Phillips and Greenberg 1987a, b, 1991, 1992b, Phillips *et al.* 1993) have reported the identification of several breath biomarkers, including sulphur-containing compounds, in the exhaled breath of normal and diseased human subjects.

Previous studies performed in our laboratories focused on the analysis of breath ethane to monitor the oxygen free radical-mediated lipid peroxidation that occurs in acute and chronic settings (for review see Risby and Sehnert 1999). The goal of the present study was to determine whether other exhaled breath biomarkers could be used as sensitive indicators of liver function or as markers to distinguish between liver diseases with different pathologies. If successful, routine breath analysis could provide a non-invasive means for the early diagnosis of liver disease or to monitor the efficacy of therapy.

Materials and methods

Human studies

This study was approved by the Johns Hopkins Joint Committee on Clinical Investigation. Samples were collected from consenting adults (50 males, 36 females) who attended a liver clinic at the Johns Hopkins Hospital and from normal adult volunteers (65 males, 44 females) with similar age and race distributions.

Blood collection

A blood sample, drawn via venipuncture into a vacutainer tube, was collected from each study subject. Breath and blood samples were collected at the same visit.

Breath collection

Breath samples were collected by means of a two-way non-rebreathing valve (NRV) (Hans Rudolf, Kansas City, Missouri, USA) connected by respiratory tubing to a gas-tight collection bag (22.4 l, Calibrated Instruments, Inc., Ardsley, New York, USA). Two biological filters and a disposable mouthpiece were used in-line on the mouthport of the NRV. This breath collection system, which has essentially no backpressure, has been used to collect breath from adults and neonates (Arterbery *et al.* 1994, Miller *et al.* 1997, Schwarz *et al.* 1997). Breath was collected after 1 min of normal breathing. To reduce the potential influence of variations in total and regional alveolar ventilation, which that may tend to alter the concentration of volatile compounds in the breath, breath samples were collected from a seated subject using a predetermined ventilation pattern. During breath collection, the study subject was asked to breathe with a frequency of approximately 12 breaths/min, and to attempt to maintain a tidal volume that approximated their basal respiratory requirement. Total breath collection over 1 min has been found to produce a representative sample, correcting for any differences in individual breaths, and is more reproducible than the collection of end-tidal breath samples (Risby and Sehnert 1999).

Room air collection

Room air was sampled with a glass syringe at the time of breath collection and stored in gas-tight collection bags (3 l, Calibrated Instruments Inc.). These samples were used to determine the background concentrations of the analyte molecules.

Storage of gaseous samples

Our previous research has shown that breath and room air samples are stable in gas collection bags for at least 48 h, although all samples were analysed within 6–8 h of collection.

Analysis of carbon dioxide in exhaled breath

Aliquots (20 ml) from the collected samples of exhaled breath were analysed for carbon dioxide using the LB-3 CO2 (Beckman Instruments, Fullerton, California, USA). This instrument was calibrated daily using a certified gas standard of carbon dioxide. The concentration of carbon dioxide in the collected breath sample was used in the normalization of breath data and to check the quality of the collected breath sample. Any sample of breath that contained less than 15 Torr of CO2 was not used in any data analysis.

Concentration of room air and exhaled breath samples

Gaseous samples were concentrated by cryogenic adsorption onto a polymeric adsorbent as follows. A stainless steel wide-bore capillary tube (20 cm, 1.65 mm outer diameter; 1.19 mm inner diameter) packed with 2,6-diphenyl-*p*-phenylene oxide (60–80 mesh) (Tenax TA, Alltech Associates, Deerfield, Illinois, USA) was connected to a six-port stainless steel gas sampling valve (1.59 mm inlet) (Valco Instruments Co. Inc., Houston, Texas, USA) in place of the standard gas sampling loop. The length of the adsorbent packing was 10 cm and the packing was retained on either side with plugs of silanized glass wool. The collection tube was submerged in an ethanol/liquid nitrogen slush bath (−121°C) for 6 min to allow the collection tube and its contents to equilibrate. After equilibration, 60 ml of collected gas was drawn through the collection tube using a gas-tight syringe. Cryogenic temperatures increase the adsorption of low boiling point gases onto the surface of the organic adsorbent. After the gas had been sampled, the slush bath was replaced with a specially designed heating block maintained at 160°C to thermally desorb the collected breath molecules. We have determined that the collection tube and its contents reached 160°C within 30 s. The thermally desorbed breath concentrate was injected immediately onto the gas chromatographic column by rotation of the gas-sampling valve. The valve was rotated back to its fill position after 45 s and the collection tube was flushed with ultrapure nitrogen to clean it prior to analysis of the next sample.

Volatile analyte molecules, such as methane and ethane, are quantitatively adsorbed onto the surface of the polymeric adsorbent at −121°C without trapping the major components of breath (nitrogen or oxygen). The break-through volumes using the collection tube and the polymeric adsorbent at −121°C have been investigated. All of the molecules identified to date in breath were collected with approximately 100% efficiency using sampling volumes up to 200 ml. Similarly, thermal desorption at 160°C has been found to quantitatively desorb the molecules of interest without significant tailing of the solute peaks.

The concentration system has been validated by comparing peak areas for the analyte obtained with regular gas sampling loops and more concentrated gas samples to the peak areas for the same analyte molecules contained in diluted gas samples using the collection tube, cryogenic concentration and thermal desorption.

Capillary gas chromatographic analysis of samples of room air, and exhaled breath

A modification of the method developed in our laboratory (Arterbery *et al.* 1994) was used to analyse the concentrated gas samples. The concentrated gas samples were initially analysed using a wide-bore (60 m, 0.53 mm inner diameter) fused silica capillary column wall coated with dimethyl silicone (7 μm) using a non-selective flame ionization detector (Varian 3700, Walnut Creek, California, USA). Separation was performed using the following temperature programme: hold at 25°C for 10 min, heat from 25 to 200°C at a rate of 5°C/min, and hold at 200°C for 5 min. Subsequently, the concentrated gas samples were analysed using a wide-bore (30 m, 0.53 mm inner diameter) fused silica capillary column wall coated with dimethyl silicone (7 μm) with a sulphur-selective flame photometric detector (Shimadzu 9A, Shimadzu Corp., Columbia, Maryland, USA) using the following temperature programme: hold at 25°C for 5 min, heat from 25 to 200°C at a rate of 5°C/min, and hold at 200°C for 5 min. Linear gas (helium) velocities of 25 cm s^{-1} at 25°C were used for both separation protocols. The analytical methods were calibrated before daily use by injection of standard gas mixtures of sulphur-containing compounds or C1–C6 straight chain hydrocarbons using the regular gas sampling loops with different volumes. We have demonstrated that the limit of quantification using 30 ml of exhaled breath is < 0.1 p.p.b. for the compounds of interest.

Identification of all the compounds was based on retention volumes and electron impact mass spectral data (MD 800, Fisons Instruments, Beverly, Massachusetts, USA). Identical gas chromatographic conditions and columns were used for the confirmational studies by gas chromatography-electron impact mass spectroscopy. Identification was confirmed by authentic standards.

Since it is difficult to ensure complete timed collection of exhaled breath, the concentrations of the biomarkers were expressed in units of pmol l^{-1}, corrected to an alveolar concentration of 40 Torr of carbon dioxide, and the breath samples were corrected for background levels of the analyte molecules in room air. Only ethane was found in room air at measurable levels.

Assay for breath compounds

The coefficient of variation for the determination of levels of breath compounds in replicate samples was found to be 3% ($n = 100$).

Liver function and other clinical tests

The blood samples collected from each study subject were submitted to the following analyses: serum urea nitrogen, serum total bilirubin, serum alanine aminotransferase, serum aspartate aminotransferase, serum alkaline phosphatase, serum albumin, differential white blood cell count, and total serum cholesterol. These tests were performed in the clinical chemistry laboratories of the Johns Hopkins Hospital using standard assays.

Grading of patient status was performed by clinical assessment of how close the patient was to dying of liver failure if a transplant was not performed. This assessment included the degree of abnormality in blood tests relevant to liver function, liver biopsy, clinical assessment of the degree of physiological and nutritional impairment, and the frequency and severity of potentially fatal complications such as episodes of variceal bleeding, spontaneous bacterial peritonitis and severe encephalopathy. This assessment was similar to the 'points' system now used by United Network for Organ Sharing (UNOS) for grading patients by degree of severity of liver disease (Bylaws, UNOS, Richmond, Virginia, USA, 1999).

Data analysis

All data were entered into a spreadsheet (Excel, Microsoft Corporation, Richmond, Washington, USA) and data analyses were performed using the statistical package STATA (STATA Corporation, College Station, Texas, USA). Clinical chemistries and exhaled breath data were entered as continuous variables. Gender, age, race, disease state and staging of liver disease were entered as categorical variables. Analysis of variance, Student's *t*-test, comparisons of means (Bonferroni and Scheffe tests), linear regression, multiple regression, logistic regression analyses and non-parametric tests (Wilcoxon and Kruskal–Wallis) were used to examine the data.

Results

Table 1 shows the demographics of the study population. Seventy-five per cent of the study subjects with liver disease presented with alcoholic or cryptogenic cirrhosis, hepatitis C or sclerosing cholangitis, while the remaining 25% presented with one of an additional eight liver diseases. This disease distribution is typical of the patient population that attends this clinic. Liver function tests and related

Table 1. Demographics of study population.

	Number
Gender	
Male	115
Female	80
Liver status or liver disease	
Alcoholic cirrhosis	17
α_1-Antitrypsin deficiency	1
Autoimmune cirrhosis	6
Biliary obstruction	1
Cryptogenic cirrhosis	13
Cystic fibrosis	1
Fulminant hepatitis	1
Hepatitis B	4
Hepatitis C	21
Normal	109
Primary biliary cirrhosis	6
Sclerosing cholangitis	12
Steatohepatitis	3

blood chemistries (serum urea nitrogen, serum total bilirubin, serum alanine aminotransferase, serum aspartate aminotransferase, serum alkaline phosphatase, serum albumin and total serum cholesterol) and a clinical assessment of the stage of liver disease (none, early, mid or late stage) were obtained for each study subject at the time of study

Exhaled breath samples were collected at the same visit as when the blood samples were drawn. Figure 1 shows an example of a capillary gas chromatographic separation of the major constituents found in the exhaled breath collected from one of the study subjects with liver disease. This separation was monitored using a flame ionization detector that responds non-selectively to any compound that contains a carbon–hydrogen bond. The major components in exhaled breath

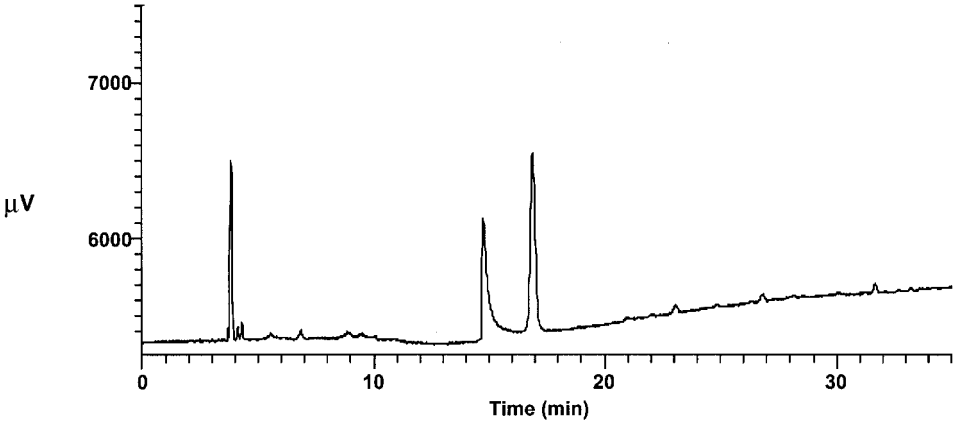

Figure 1. Capillary gas chromatogram of a sample (30 ml) of human breath collected from a study subject with liver disease. The column effluent was monitored with a flame ionization detector. The identifications and retention times of the peaks are as follows: methane, 3.85 min; ethane, 4.14 min; ethane, 4.31 min; dimethyl ketone, 14.76 min; 2-methyl-1,3-butadiene (isoprene), 16.90 min. This breath profile was typical for both normal and diseased subjects.

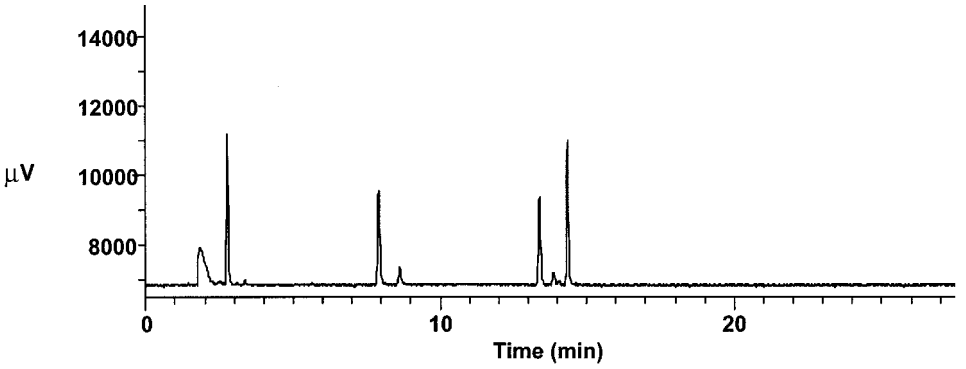

Figure 2. Capillary gas chromatogram of a sample (60 ml) of human breath collected from the same study subject with liver disease as in figure 1. The column effluent was monitored with a flame photometric detector. The identifications and retention times of the peaks are as follows: carbonyl sulphide, 2.77 min; methyl sulphide, 7.93 min; carbon disulphide, 8.64 min; three congeners of allyl methyl sulphide, 13.41, 13.87 and 14.37 min. In normal subjects breath sulphur compounds were absent unless they had consumed garlic.

in all study subjects were methane, ethene, ethane, dimethyl ketone and isoprene. This breath profile is typical of all the breath samples collected during the study. Figure 2 shows the separation of the same breath sample using a sulphur-selective detector (flame photometric detector); the major compounds observed were carbonyl sulphide, dimethyl sulphide, dimethyl disulphide, carbon disulphide and the three congeners of allyl methyl sulphide. The sulphur compounds observed in figure 2 are not detectable or are below the detection limits for the flame ionization detector. Note that the two gas chromatograms cannot be superimposed since chromatographic conditions and column lengths are different.

The distributions of the clinical chemistry and breath data were found to be non-normally distributed for the study subjects with liver diseases; therefore, statistical analyses of the data could only be performed using non-parametric tests (Wilcoxon and Kruskal–Wallis). However, if the raw data is transformed logarithmically, the resulting data are normally distributed and parametric tests such as the *t*-test, analysis of variance, multiple comparison tests (Bonferroni and Scheffe tests), linear regression, multiple regression and logistic regression can be used to investigate the transformed data.

Initially the differences between the means of the clinical chemistry data for the study subjects with liver disease and the means of the clinical chemistry data for the study subjects with normal liver function were examined using non-parametric tests on the raw data and parametric tests on the log-transformed data. The results of the parametric tests (*t*-tests) are shown in table 2, together with the means and standard deviations for the raw data. As expected the means of the clinical chemistry data, with the exception of serum urea nitrogen (not shown), for study subjects with liver diseases were significantly different from those for study subjects with normal liver function.

When the various molecules detected in exhaled breath were examined, the mean concentrations of carbonyl sulphide, carbon disulphide and isoprene in the breath of subjects with liver diseases were found to be significantly different from the corresponding means in the breath of normal subjects. No significant differences were observed between the concentrations of ethane, dimethyl sulphide or

Table 2. Summary of the results of comparisons (t-test based on the log-transformed data) for various measurements (means ± SD) between study subjects with normal liver function and study subjects with abnormal liver function, hepatocellular injury or injury to the bile duct.

Test	Normal	Liver disease	Hepatocellular injury	Bile duct injury
Serum				
Alanine aminotransferase ($IU\,l^{-1}$)	21 ± 14	93 ± 164**	96 ± 185**	85 ± 65**
Albumin ($g\,dl^{-1}$)	4.6 ± 0.3	3.6 ± 2.4**	3.7 ± 2.6**	3.3 ± 0.9**
Alkaline phosphatase ($IU\,l^{-1}$)	71 ± 20	220 ± 181**	168 ± 94**	391 ± 9275**
Aspartate aminotransferase ($IU\,l^{-1}$)	22 ± 6	120 ± 198**	126 ± 224**	102 ± 60**
Total bilirubin ($mg\,dl^{-1}$)	0.8 ± 0.4	5.7 ± 8.6**	4.9 ± 7.4**	8.2 ± 11.6**
Total cholesterol ($mg\,dl^{-1}$)	186 ± 40	164 ± 70**	151 ± 50**	NS
Breath				
Carbon disulphide ($pmol\,l^{-1}$)	966 ± 1063	1473 ± 541**	1883 ± 6125**	121 ± 283**
Carbonyl sulphide ($pmol\,l^{-1}$)	3778 ± 7660	7136 ± 3358**	9163 ± 3535**	445 ± 604**
Isoprene ($pmol\,l^{-1}$)	5975 ± 2264	10903 ± 6378**	11556 ± 6687**	NS

** p value < 0.002; NS, not statistically significant.

acetone (data not shown) found in the breath of these two study groups. These results are summarized in table 2.

Three additional sulphur-containing molecules were identified in the breath of some of the study subjects. These molecules were the congeners of allyl methyl sulphide and were present in the breath of study subjects who had eaten foods containing garlic within the last 24 h. These molecules are also shown in figure 2 (retention times 13.41, 13.87 and 14.37 min). The rates of clearance (metabolism) of allyl methyl sulphides from the breath were found to be subject dependent but not liver disease dependent (data not shown).

Since our study population did not have a sufficient number of subjects diagnosed with specific liver diseases to allow statistical analyses to be performed by disease state, our study subjects with liver diseases were stratified into two populations: study subjects with hepatocellular injury (alcoholic cirrhosis, (1-antitrypsin deficiency, autoimmune cirrhosis, cryptogenic cirrhosis, fulminant hepatitis, hepatitis B or C, and steatohepatitis) ($n = 66$) and study subjects with diseases of the bile duct (cystic fibrosis, primary biliary cirrhosis, sclerosing cholangitis and biliary obstruction) ($n = 20$). This distinction may be confounded in the late stages of liver diseases by the development of extensive liver cirrhosis. The differences between the means of the clinical and breath data for these two study populations were compared individually with normal subjects ($n = 109$). The results of these comparisons (t-tests using log-transformed data) are shown in table 2. As expected, significant differences were seen in the means of the clinical chemistry data for study subjects with hepatocellular injury and those with diseases of the bile duct versus subjects with normal liver function.

The mean levels of carbonyl sulphide and carbon disulphide in the breath of study subjects with hepatocellular injury were significantly elevated compared with the mean level found in the breath of normal subjects. The difference between the mean levels of isoprene in these two study groups was also significant. The sensitivity of breath carbonyl sulphide to detect hepatocellular injury was 48%, with a specificity of 88%. It is interesting to note that the mean concentrations of carbonyl sulphide and carbon disulphide in the breath of subjects with diseases of the bile duct were significantly lower than those with normal liver function (table

Table 3. Values of p from the analysis of variance (based on the log-transformed data) of various measurements of hepatocellular and bile duct injury as a function of severity of liver disease.

Test	Hepatocellular injury	Bile duct injury
Serum		
Alanine aminotransferase ($IU\,l^{-1}$)	< 0.00001	< 0.00001
Albumin ($g\,dl^{-1}$)	< 0.00001	< 0.00001
Alkaline phosphatase ($IU\,l^{-1}$)	< 0.00001	< 0.00001
Aspartate aminotransferase ($IU\,l^{-1}$)	< 0.00001	< 0.00001
Total bilirubin ($mg\,dl^{-1}$)	< 0.00001	< 0.00001
Total cholesterol ($mg\,dl^{-1}$)	< 0.00001	< 0.00001
Urea nitrogen ($mg\,dl^{-1}$)	0.026	0.0001
Breath		
Carbon disulphide ($pmol\,l^{-1}$)	0.20	0.14
Carbonyl sulphide ($pmol\,l^{-1}$)	< 0.00001	< 0.00001
Dimethyl sulphide ($pmol\,l^{-1}$)	0.01	0.4
Ethane ($pmol\,l^{-1}$)	0.03	0.9
Isoprene ($pmol\,l^{-1}$)	0.07	0.2

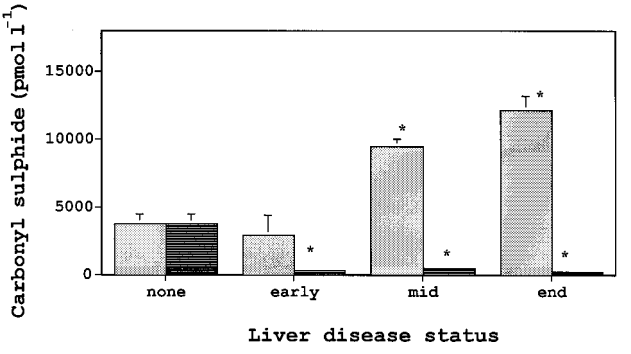

Figure 3. Breath carbonyl sulphide examined as a function of severity of liver disease for hepatocellular and bile duct injury using analysis of variance and comparisons of means. Results are expressed as mean + SE of the raw data. Hepatocellular injury, light bars; bile duct injury, dark bars. * denotes statistically significant difference from appropriate control.

3), while the mean concentrations of breath carbonyl sulphide and carbon disulphide in the subjects with hepatocellular injury were significantly higher than in those with normal liver function. There were no significant differences between the mean concentrations of breath isoprene in study subjects with diseases of the bile duct compared with normal subjects.

The clinical chemistry and breath data for normal subjects and for those with hepatocellular injury and those with diseases of the bile duct were examined separately as a function of the severity of liver disease using analysis of variance (ANOVA) and comparisons of means. Table 3 shows the summary of the analysis of variance for the various measurements as a function of the severity of liver disease (staging). With the exception of serum urea nitrogen, the serum chemistries showed significant increases as liver disease progressed (data not shown). The means of the serum chemistries increased (cholesterol decreased) as the liver disease became more severe (data not shown). Only breath carbonyl sulphide was found to be correlated with severity of disease.

The data for breath carbonyl sulphide (mean and standard error of raw data) as a function of liver disease staging are shown in figure 3 (see also table 3). Breath

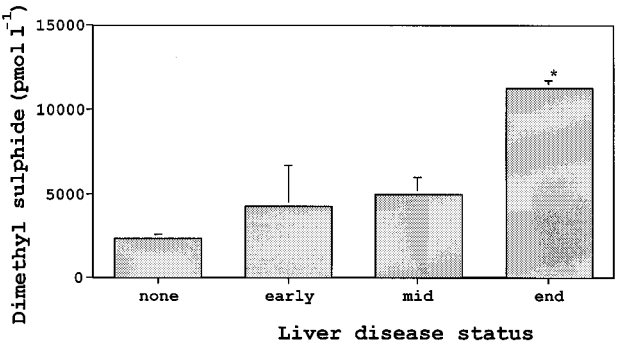

Figure 4. Breath dimethyl sulphide examined as a function of severity of liver disease in hepatocellular injury using ANOVA and comparisons of means. Results are expressed as mean + SE of the raw data.

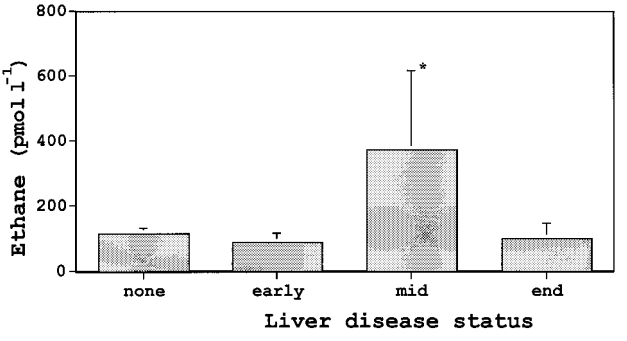

Figure 5. Breath ethane examined as a function of severity of liver disease in hepatocellular injury using ANOVA and comparisons of means. Results are expressed as mean + SE of the raw data.

concentrations of carbonyl sulphide increased with staging for the study subjects with hepatocellular injury and decreased as a function of staging in patients with diseases of the bile duct. Increases with the severity of the disease were also observed for dimethyl sulphide in the study group with hepatocellular injury (table 3 and figure 4), whereas no statistically significant differences were observed for the stage of liver disease in the study group that had diseases of the bile duct (data not shown). Levels of breath ethane were the only other breath biomarker that showed any statistical significance as a function of staging in these study subjects. There was a significant increase in breath ethane for study subjects with hepatocellular injury (table 3 and figure 5) at mid-stage disease. Interestingly, no correlation between breath carbon disulphide and staging was observed in hepatocellular or bile duct injury.

No changes in breath sulphur-containing compounds have been found in subjects with other diseases, including cardiac diseases, renal disease and hyperlipidaemias (unpublished studies, data not shown). Additionally, none of the results showed significant differences on the basis of gender.

Discussion

Fetor hepaticus, described as a characteristic sweet, musty odour of the breath, has been attributed since the 1920s to the abnormal excretion of mercaptans (alkanethiols) and dimethyl sulphide. The production of volatile sulphur compounds as a result of impaired liver function has been studied from a number of perspectives; figure 6 shows a schematic summary of the pathways involved. Although the degradation of methionine by liver mitochondria was first observed by Canellakis *et al.* (1953a, b), the complete transamination pathway in the liver has yet to be defined (Scislowski and Pickard 1994). The production of methanethiol and other volatile sulphur compounds has been shown to require the presence of either 2-oxoglutaric acid and pyruvate or glyoxylic acid (Scislowski and Pickard 1994). The branched chain 2-oxyacid dehydrogenase complex probably controls the transaminative flux of these compounds. Degradation of methionine can also occur in the gut by the action of bacterial methionine γ-lyase (Johnston *et al.* 1981), although it has been proposed that the gut is not the source of methanethiol and dimethyl sulphide in liver disease (McClain *et al.* 1980). The production of methanethiol depresses the synthesis of urea (Derr and Zieve 1982), with the result that circulating levels of ammonia, biogenic amines and short-chain fatty acids are increased, thereby setting up the scenario for hepatic encephalo-

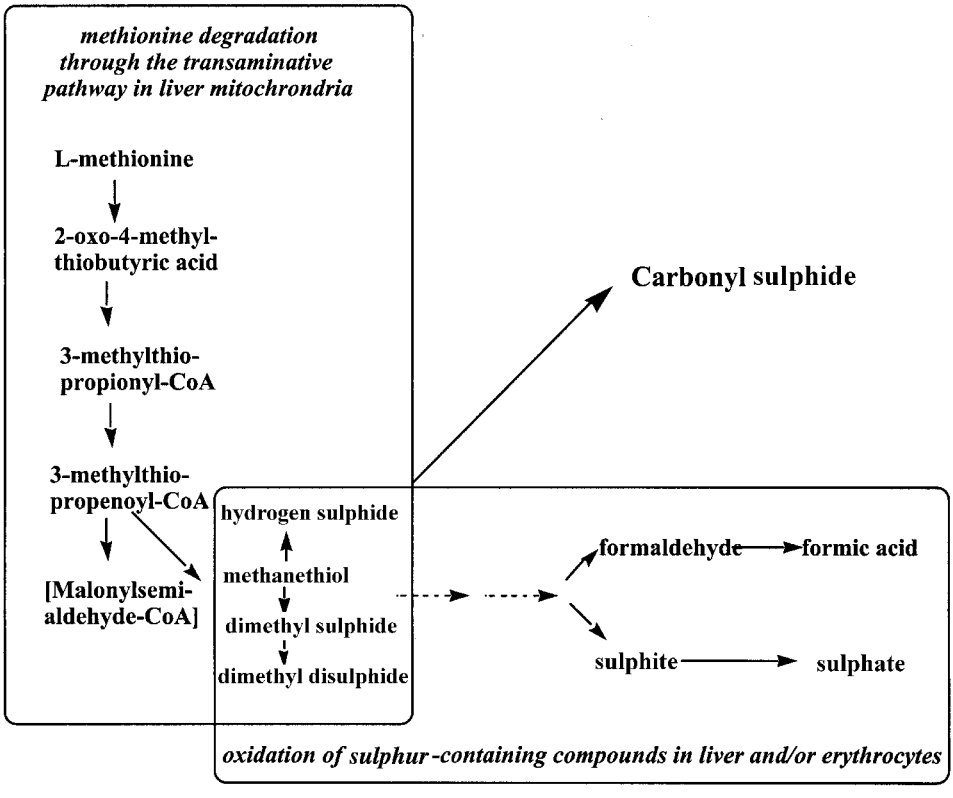

Figure 6. Schematic summary of the published pathways for the formation of volatile sulphur-containing compounds from methionine and their oxidation to inorganic sulphur-containing compounds.

pathy. Methanethiol blocks cytochrome C oxidase and the enzymes involved in protection from peroxidative damage (Finkelstein and Benevenga 1986). Animals with compromised livers as a result of a toxic insult, prolonged ischaemia or partial hepatectomy are demonstrably more sensitive to the depressive effects of methanethiol on the central nervous system (Vahlkamp *et al.* 1979, Zieve *et al.* 1985b), thereby suggesting that the liver is the major organ responsible for the oxidation of methanethiol (Zieve *et al.* 1985a). It has been demonstrated that methanethiol can be oxidized by erythrocytes to formic acid and sulphite or sulphate (Blom and Tangerman 1988). Similar oxidative pathways for the removal of volatile sulphur compounds are probably involved in both liver and erythrocytes.

If the complete scheme shown in figure 6 is considered, it appears that the production of volatile sulphur-containing compounds from methionine and their removal by oxidation are in a steady state, with low concentrations of circulating sulphur-containing compounds present in human subjects with normal, healthy livers. However, since impairment of liver function increases the level of reduced sulphur-containing compounds, liver disease must affect hepatic oxidation more than it affects transamination production. This observation suggests that cytochrome C oxidase, which is present in hepatocytes, may be involved in the oxidation of sulphur-containing compounds. Therefore it is reasonable to hypothesize that as the extent of hepatic damage progresses, the levels of sulphur-containing compounds will increase, the extent of urea synthesis will decrease and the likelihood of hepatic encephalopathy will increase. However, in our studies methanethiol was not detected in the breath of any of the study subjects. Since these results were in conflict with published data (Chen *et al.* 1970, Kaji *et al.* 1978, Hismura 1979, Tangerman *et al.* 1985), we investigated whether methanethiol in human breath was stable. A known concentration of methanethiol was added to a sample of human breath and the sample was analysed over time. The signal observed initially for methanethiol was less than anticipated and decreased rapidly with time. It is well known that methanethiol is extremely labile and difficult to analyse by gas chromatography. Additionally, methanethiol is rapidly oxidized to methyl sulphide and methyl disulphide. Studies are in progress that are aimed at defining whether this loss in signal for methanethiol is the result of oxidation that occurs in the gas-tight collection bag or during the cryogenic concentration and thermal desorption steps.

The physiological origins for carbon disulphide and carbonyl sulphide are currently unknown. Carbon disulphide has been identified in the breath of human subjects who are receiving disulfiram [bis(diethylthiocarbamoyl)disulphide] as aversion therapy for chronic alcoholism (Paulson *et al.* 1977, Rychtarik *et al.* 1983, Faiman *et al.* 1984, Phillips *et al* 1986, Phillips and Greenberg 1992a); none of the study subjects was receiving disulfiram therapy. However, the levels of breath carbon disulphide and the levels of carbonyl sulphide could be correlated in these samples of breath (data not shown). Carbonyl sulphide has been detected as a result of oxidative metabolism of carbon disulphide in isolated rat hepatocytes and liver microsomes (Dalvi *et al.* 1975, Chengelis and Neal 1987). This oxidation has been shown to be produced by the mixed-function oxidase enzyme system. Carbonyl sulphide has also been identified in the breath of rats exposed to carbon disulphide (Dalvi and Neal 1978). It is possible that the origin of breath carbonyl sulphide is the oxidative metabolism of carbon disulphide. There was also a correlation between the levels of exhaled breath methyl sulphide and the levels

of breath carbonyl sulphide (data not shown). This correlation suggests that carbonyl sulphide could also be produced during the incomplete metabolism of sulphur-containing essential systems, including methionine.

Our data suggest that the precursors of carbonyl sulphide are excreted in bile, and gut bacterial action is responsible for carbonyl sulphide production. This hypothesis is based on observation that levels of breath carbonyl sulphide decrease as a function of disease status in study subjects with diseases of the bile duct. The observation that the levels of dimethyl sulphide were not comparably reduced with the severity of the biliary duct dysfunction suggests that the production of carbonyl sulphide and dimethyl sulphide do not share a complete common pathway. It is possible that dimethyl sulphide (and/or methanethiol) may be oxidized by gut bacterial action to carbonyl sulphide, and bile duct dysfunction reduces this pathway.

The statistically significant increases in the levels of breath ethane in study subjects with hepatocellular injury judged to have mid-stage liver disease supports the proposition that hepatocellular injury may involve oxygen free radical-mediated inflammation. This inflammation decreases as the liver becomes more cirrhotic in end-stage disease. Increased ethane may reflect the contribution from study subjects who presented with hepatitis B or C, which are inflammatory diseases. Additional studies are planned to investigate the involvement of oxygen free radicals in the pathogenesis of hepatocellular injury and to determine the biological origin of carbonyl sulphide. In these preliminary studies, no attempts were made to control for diet or for evidence of dental disease. These variables will also be addressed in future studies.

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