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GC–MS analysis of breath odor compounds in liver patients

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ABSTRACT

Background: Liver diseases can cause a sweet, musty aroma of the breath, called fetor hepaticus. Even in a stage of cirrhosis, the disease can be asymptomatic for many years. Breath analysis might be helpful to detect occult liver pathology.
Study objective: This study examined whether specific breath odor compounds can be found in liver patients, suffering from cirrhosis, which might be useful for diagnosis.
Materials and methods: Fifty-two liver patients and 50 healthy volunteers were enrolled. Alveolar air was analyzed by gas chromatography–mass spectrometry. Using discriminant analysis a model for liver disease was built.
Results: Dimethyl sulfide, acetone, 2-butanone and 2-pentanone were increased in breath of liver patients, while indole and dimethyl selenide were decreased. Sensitivity and specificity of the model were respectively 100% and 70%.
Conclusions: Fetor hepaticus is caused by dimethyl sulfide and to a lower extent by ketones in alveolar air. Breath analysis by GC–MS makes it possible to discriminate patients with breath malodor related to hepatic pathologies.

1. Introduction

Halitosis has a significant socio-economic impact and may reveal disease. It was neglected until recently by scientists and clinicians and is hardly covered in the medical curricula [1].

The vast majority of pathologies causing halitosis lies within the oropharynx (tongue coating, gingivitis, periodontitis, and tonsillitis) and the sulfur containing gases (hydrogen sulfide, methyl mercaptan, and dimethyl sulfide) play a predominant role [2,3]. In 10–15% of the patients, however, breath malodor has an extra-oral cause [4,5]. Examples are foreign bodies in the nose, purulent sinusitis, regurgitation esophagitis and other local factors. Systemic diseases or pathologies distant from the oropharynx, are sometimes revealed by bad smelling metabolites carried by the bloodstream to the lungs. Exhalation of the volatiles that are organoleptically perceived causes halitosis. According to the literature, these extra-oral causes are sometimes associated with a typical odor as a result of specific volatile organic compounds (VOCs) in breath (Table 1) [5–7]. In clinical practice, diabetes mellitus has been associated with the sweet smell of acetone and kidney failure results in a fishy odor. These observations suggest that VOCs in exhaled breath could provide, in a non-invasive way, valuable information about the subjects' pathophysiological condition [6–9].

Liver disease is an important extra-oral cause of bad breath. Patients with various degrees of hepatocellular failure and portosystemic shunting of blood may acquire a sweet, musty or slightly fecal aroma of the breath, termed fetor hepaticus, which has been mainly attributed to sulfur compounds [10]. If the metabolizing function of the liver fails, the concentration of the metabolites, normally processed in the liver, will increase and they will enter again the systemic circulation. Part of them will then be exhaled.

Most patients who complain about breath malodor consult a periodontologist, house doctor or dentist. It is important that clinicians can discriminate liver patients from those with oral malodor. Chronic liver disease, even in a stage of cirrhosis, can be asymptomatic for many years. In this study, gas chromatography–mass spectrometry (GC–MS) was used to examine whether specific odor compounds can be found in breath of liver patients. This could then further be used for differential diagnosis.

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Table 1
Intra- and extra-oral causes of halitosis and their related compounds

Cause	Specific compounds
Oral malodor	Hydrogen sulfide, methyl mercaptan, dimethyl sulfide and dimethyl disulfide
Diabetes mellitus—weight reduction	Acetone, other ketones
Uremia—kidney failure	Dimethylamine, trimethylamine, ammonia
Liver diseases	Dimethyl sulfide, ethanethiol, C2–C5 aliphatic acids (acetic acid, proprionic acid), butyric acid, isobutyric acid, and isovaleric acid
Lung carcinoma	Acetone, 2-butanone, *n*-propanol, aniline, and *o*-toluidine
Upper respiratory/oropharyngeal carcinoma	C2–C8 normal and branched organic acids
Trimethylaminuria	Trimethylamine
Food: garlic/onions	Allyl methyl sulfide
Other potential compounds	Indole, skatole, cadaverine, putrescine, carbon disulfide, and dimethyl selenide

Refs. [5–7].

2. Subjects, materials and methods

2.1. Subject selection

Fifty-two patients (19 females) with liver cirrhosis of various degrees and etiologies (alcohol, medication, hepatitis, primary sclerosing cholangitis, sarcoidosis, primary and biliary cirrhosis) and 50 age-matched healthy volunteers (29 females) were enrolled. All subjects signed informed consent and the research was approved by the Clinical Trials Committee of the University Hospital Leuven. The healthy volunteers were thoroughly questioned on their medical history. All confirmed they were not suffering from any known disease or were not receiving any medical treatment. Nine of them were smokers. Patients with cirrhosis previously confirmed at the hospital by various biochemical and radiological investigations and liver biopsy were selected. 12 of them were smokers. Their MELD-score (Model for End stage Liver Disease), which expresses the degree of liver impairment, ranged from 7 to 40. 35 of them took at least one of the following medications: lactulose, spironolactone, antibiotics, furosemide and propanol. Samples were taken at least 30 min after consumption of any food or beverages, before lunch and at least 2 h after tooth brushing. Volunteers were asked to refrain from eating garlic and onions or any spicy food, 24 h before measurement. They also refrained from drinking alcohol and coffee and to use a mouth rinse 24 h prior to the gas sampling. Fasting was not imposed to avoid the appearance of elevated concentrations of the ketones acetone, 2-pentanone and 2-butanone [11]. Fasting would also have been an impediment for the practical use of this approach. Patients were excluded if they had a history of surgical shunt or transjugular intrahepatic portosystemic shunt (TIPSS), severe chronic obstructive disease or asthma, sedatives or narcotics within the 48 h prior to enrollment, a neurological disorder, Wilson's disease or diabetes mellitus requiring treatment with insuline.

2.2. Sample collection

Sample collection of alveolar air occurred as previously described using a commercial device (Bio-VOC® sampler, Markes International Limited, Rhondda Cynon Taff, UK) [12,13]. Briefly, the following procedure was used. After 60 min rest, the subjects performed a single slow vital capacity breath, into an inert, non-emitting Teflon®-bulb, which has an open end so that the first part of the breath passes through the sampler and only the last portion (150 ml) is trapped. Alveolar air was transferred immediately from the sampler to a sorbent tube to capture all VOCs. This procedure was repeated three times.

The Bio-VOC® sampler was also used to take a sample of room air (same procedure). Two layer sorbent tubes containing 200 mg TenaxTA and 200 mg Unicarb (carbonized molecular sieve) (Markes International Limited) were used. The sorbent tubes were preconditioned with constant flow (90 ml/min) of nitrogen (purity 6.0, a nitrogen purifier: Alltech Associates, Lokeren, Belgium, was used to further increase the purity) using the following temperature program: 1 h at 100 °C, 1 h at 200 °C, 1 h at 300 °C and 30 min at 335 °C. They were then sealed by both Swagelok fitting and PFTE ferrules and stored at 4 °C.

2.3. VOC extraction and analysis

Analysis of samples was performed by GC–MS combined with thermal desorption as previously described [12,13]. VOCs were desorbed and concentrated in a thermal desorber (Unity®, Markes International Limited) at 250 °C onto a −10 °C cold trap for 6 min (helium flow 50 ml/min). The cold trap, packed with the same sorbents as the sorbent tubes, was then heated rapidly to 250 °C and VOCs were transferred to a gas chromatograph (HP6890N, Agilent Technologies, Diegem, Belgium). Column (capillary column, HP5MS, 30 m × 0.25 mm × 0.25 μm film thicknesses, Agilent Technologies) temperatures were ramped as follows: −40 °C for 1 min, 4 °C/min to 180 °C, 0.10 min hold and 30 °C/min to 300 °C, 0.25 min hold. Liquid nitrogen was used as cryogen. Column head pressure of helium carrier gas was set to 10 psi. Purity of helium was at least 6.0 and a helium purifier (Alltech Associates) was used to further increase the purity. Identification of VOCs occurred in a mass spectrometer (HP5973, Agilent Technologies). Mass range was applied from 30 to 350 amu.

2.4. Data management

The presence of all compounds, which have already been associated with halitosis (Table 1), was examined in all breath and environmental samples as previously described [12,13]. Therefore, for each compound an extracted ion chromatogram of the ions, specific for that compound, was made using the Chemstation software (Agilent Technologies). For double-checking, the observed SCAN spectrum was compared with the spectrum in the NIST98 library.

2.5. Quantification

For each compound, detected in at least one breath sample, a calibration curve was made. This procedure has been previously described by Van den Velde et al. [13]. Based on the calibration curve, the concentration of each compound was automatically calculated in both breath and environmental samples. If the compound was also present in the environment, the environmental concentration was subtracted from the concentration in the breath samples [14].

2.6. Statistical analysis

For each compound, a Mann–Whitney *U*-test was performed to detect significant differences between healthy volunteers and liver patients. To correct for multiple testing a Bonferroni correction was included. Forward stepwise discriminant analysis was used to build a model for liver disease. Therefore, volunteers and patients were

Table 2
Descriptive statistics of the detected compounds (in nmol/mol or ppbv)

	Liver median	Lower Q	Upper Q	Healthy median	Lower Q	Upper Q	Sign
Acetone	765.13	465.81	1739.16	212.25	144.56	325.92	+
2-Pentanone	1.56	0.98	2.69	0.38	0.25	0.61	+
2-Butanone	3.11	1.61	6.86	0.38	0.06	1.42	+
Dimethyl sulfide	29.02	10.44	61.52	13.79	7.51	23.24	+
Dimethyl disulfide	0.00	0.00	0.03	0.00	0.00	0.06	
Dimethyl trisulfide	0.00	0.00	0.00	0.00	0.00	0.00	
Carbon disulfide	0.01	−0.60	0.13	−0.03	−0.04	0.03	
Allyl methyl sulfide	0.09	0.00	0.26	0.08	0.04	0.19	
Indole	0.00	0.00	0.05	0.18	0.06	0.32	+
Skatole	0.00	0.00	0.00	0.00	0.00	0.00	
Dimethyl selenide	0.08	0.00	0.15	0.56	0.38	0.70	+
1-Propanol	7.80	1.90	9.50	8.10	2.65	10.76	

Lower Q: lower quartile. Upper Q: upper quartile. Sign: compounds that are significantly different between liver patients and healthy volunteers are indicated with +.

randomly assigned to a training set (40 healthy volunteers, 40 liver patients) to build the model and a prediction set (10 healthy volunteers, 12 liver patients) to test the model. Correlations between the compounds and between the compounds and the other parameters (MELD, medication, age) were determined using the Spearman correlation coefficient.

3. Results

Accuracy and reproducibility of the method were previously described [12,13]. In alveolar breath of the test subjects, 12 breath odor compounds were detected (Table 2). Acetone, 2-butanone, 2-pentanone and dimethyl sulfide were significantly higher in the breath of liver patients. Indole and dimethyl selenide on the other hand were significantly lower ($p < 0.001$) in the latter group. Significant correlations were found between acetone and 2-pentanone ($R = 0.64$, $p < 0.001$), indole and skatole ($R = 0.42$, $p = 0.002$) and between dimethyl disulfide and dimethyl trisulfide ($R = 0.51$, $p = 0.001$).

Discriminant analysis identified four independent compounds that discriminate best between healthy volunteers and liver patients. With these compounds, two classification functions were built, one for healthy volunteers and one for liver patients (Table 3). 75 of the 80 subjects from the training group and 19 of the 22 test subjects were classified correctly. Fig. 1 demonstrates the posterior probabilities (in Bayesian statistics, this is the probability that a hypothesis is true after the data have been analyzed) for all cases. Sensitivity and specificity of the model were respectively 100% and 70% (using the results of the test subjects). Table 4 shows the concentrations of the compounds of the model for the three test subjects that were classified incorrectly.

Table 3
Classification functions

Compounds	Healthy	Liver
Dimethyl selenide	12.45	1.60
2-Pentanone	0.013	0.66
Dimethyl sulfide	0.00074	0.013
Indole	2.69	0.15
Constant	−4.89	−1.88

Healthy: coefficients of variables of classification function for healthy volunteers. Liver: coefficients of variables of classification function for liver patients.

Table 4
Healthy volunteers incorrectly classified by the model

	Gender	Age	Indole	2-Pent	DMSel	DMS
1	F	47	0	1.39	0.1	12.05
2	M	21	0.2	1.02	0.16	4.57
3	F	35	0.025	0.69	0.07	18.73

F: female, M: male. Compound concentrations in nmol/mol or ppbv.

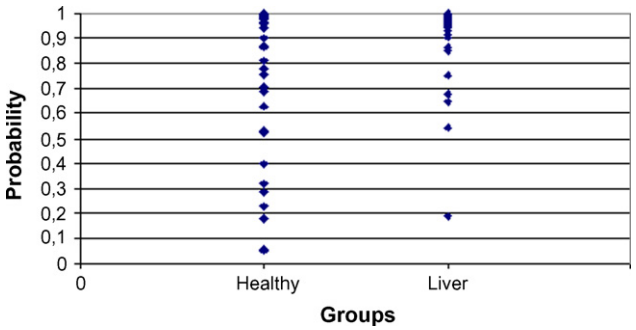

Fig. 1. Probability: probability that a case belongs to the group; if the probability <0.5, the case was classified incorrectly.

For 2-pentanone, a significant correlation was found with the MELD-score ($R = 0.53$, $p < 0.001$).

No correlations were found between the compounds from the model and medication intake or age of the healthy subjects. The same applies between men and women or between smokers and non-smokers.

4. Discussion

Liver diseases are one of the prominent extra-oral causes of bad breath. Even in a stage of cirrhosis, the disease can be asymptomatic for many years. It is important that one can discriminate these patients among the many suffering from breath malodor mostly of oropharyngeal or other origins. The present study examined whether specific breath odor compounds can be found in breath of liver cirrhosis patients, which can be used for differential diagnosis.

Dimethyl sulfide, acetone, 2-pentanone and 2-butanone were significantly higher in alveolar air of liver patients. These compounds are most likely to explain the bad smell of the breath. Dimethyl sulfide was previously identified as an important metabolite for fetor hepaticus [10]. Sulfur containing compounds are generated by incomplete metabolism of sulfur containing amino acids in the transamination pathway. Normally, concentrations in blood and alveolar breath are low [7]. Impairment of liver function increases the level of these compounds, which have a characteristic smell, like the odor of rotten cabbage [15]. The characteristic sulfur odor could only be detected by the human nose (sniffing the breath by the physician) at an alveolar dimethyl sulfide concentration of more than 30 nmol/mol or ppbv (observed in 25 of the 52 liver cirrhosis patients), which is consistent with previous investigations [10].

Next to dimethyl sulfide, other sulfur compounds like hydrogen sulfide and methyl mercaptan have also been suggested as possible mediators. However, in vitro experiments have shown that the free –SH group of methyl mercaptan immediately reacts with blood, resulting in an irreversible binding and oxidation. In this way, transport of methyl mercaptan from blood into alveolar air is not easy. The same holds for hydrogen sulfide. On the other hand, dimethyl sulfide is a neutral, stable molecule that can be transported from blood into alveolar air and be expired [16]. The metabolism of sulfur compounds has also been elucidated by Suarez et al. in the colon of rats. In contrast to dimethyl sulfide, hydrogen sulfide and methyl mercaptan were rapidly metabolized by the colonic mucosa and liver [17].

The increased concentrations of the ketones acetone, 2-pentanone and 2-butanone are believed to be the result of hepatic insuline resistance, a common disorder in patients with hepatic steatosis and end stage liver disease. Insuline resistance leads to an increase of triglycerides and free fatty acids and ketones are formed during lipolysis [18]. Moreover, in a study with rats, inhibition of CYP2E1, one of the cytochrome P450 enzymes in the liver, caused a remarkable increase of these compounds. Thus, the disturbed liver metabolism might also explain the increased levels [19].

Ketones produce a sweet smell, similar to the fragrance of rotten apples [15]. It is important to realize that this sweet odor is much more difficult to detect by sniffing the breath than the sulfur odor. Indeed, the odor index (=the ratio between the vapor pressure and the 100% recognition threshold) of acetone (720), 2-pentanone (2000) and 2-butanone (3800) is much lower than the one of dimethyl sulfide (2,760,000). This means that dimethyl sulfide is much more odorous, explaining why it is probably the most relevant for the characteristic fetor hepaticus.

On the other hand, indole and dimethyl selenide were both significantly lower in breath of liver patients. Dimethyl selenide is an excretion product of the essential micronutrient selenium. Excessive intake leads to a garlic-like breath [20]. In patients with chronic liver disease, however, selenium levels are decreased [21,22]. This explains the low levels of dimethyl selenide in breath of these patients.

Indole is derived from the catabolism of tryptophan. In patients with liver failure there is evidence that the degradation of this aromatic amino acid by the liver is impaired leading to higher levels of free tryptophan [23]. As a consequence the metabolites of this compound like indole are also increased in plasma of these patients. The reason for the decreased breath concentration is not clear. Indole binds to albumin in blood. The lack of albumin and its lower binding capacity for endogenous and exogenous compounds in liver patients are potential explanations [24].

Discriminant analysis identified four compounds that discriminate best between healthy volunteers and liver patients. Sensitivity and specificity of the model were respectively 100% and 70%. These results indicate that breath analysis is a powerful tool to detect liver disease. For 2-pentanone, a significant correlation was found with the MELD score, which gives an idea about the severity of liver disease. This means that 2-pentanone not only has diagnostic value but can also give information about the degree of liver pathology.

All three healthy volunteers who were classified incorrectly were smokers. It is possible that smoking interferes with the results and skews the predictive ability of the model. 2-Pentanone, for example, is one of the cigarette ingredients and this might explain the rather high concentration of this compound in the incorrectly classified volunteers. It is not clear whether smoking is also responsible for the rather low levels of indole and dimethyl selenide.

In this study, alveolar air was used. In two previous studies performed with the same GC–MS system and sampling method, large differences were found between alveolar air and mouth air [12,13]. If mouth air, dead space air and alveolar air are mixed, a dilution takes place. This dilution factor cannot be ignored because the dilution factor unavoidably varies, e.g. when the test subject is breathing deeply or shallowly. Concentrations of metabolic compounds can be several times higher in alveolar air than in dead space air. Moreover, some compounds like dimethyl sulfide and indole can also be produced in the oral cavity. Dimethyl sulfide is formed by anaerobic bacterial breakdown of sulfur containing amino acids such as from the tongue coating or impacted food remnants, and can thus cause oral malodor. The same holds for indole, which is produced by bacterial breakdown of tryptophan.

Food intake could have had some impact on the detected concentrations of the compounds. However, because this method might be used in clinical practice for diagnosis, we chose for a general view of the breath composition of non-fasting healthy volunteers and liver patients and did not ask the volunteers to fast during 24 h. Fasting causes elevated concentrations of the ketones acetone, 2-pentanone and 2-butanone. Acetone concentrations after fasting (4.1 μmol/mol or ppmv) were even very close to the range of diabetic breath (1.7–3.7 ppmv) in a previous study [11]. We did however ask the volunteers to refrain from eating garlic, onions or spicy food and drinking alcohol or coffee 24 h before measurements. Breath samples were taken at least 30 min after consumption of any food or beverages and before lunch.

Within the limits of this experimental set-up we conclude that fetor hepaticus is mainly caused by dimethyl sulfide and to a lower extent by the ketones acetone, 2-butanone and 2-pentanone. Breath analysis by GC–MS makes it possible to discriminate patients with breath malodor related to hepatic pathologies.

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