Ion mobility spectrometry for the detection of volatile organic compounds in exhaled breath of lung cancer patients – Results of a pilot study.

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Abstract:

Background:
Analysis of exhaled breath, especially of volatile organic compounds (VOCs), is of increasing interest in the diagnosis of lung cancer. Compared to other methods of breath analysis, ion mobility spectrometry (IMS) offers a ten-fold higher detection rate of VOCs. By coupling the ion mobility spectrometer with a multi-capillary column as a pre-separation unit, IMS offers the advantage of an immediate two-fold separation of VOCs with visualisation in a three-dimensional chromatogram. The total analysis time is about 500 s compared to gas chromatography/mass spectrometry (GC/MS) of about 1 h. Therefore it seemed reasonable to test IMS in breath analysis.

Methods:
In this pilot study 32 patients with lung cancer were subjected to a breath analysis by IMS. Their IMS-chromatograms were compared with those of 54 healthy controls. An IMS that was built for special clinical application was used to identify characteristic peaks of VOCs, which might be relevant for the diagnosis of lung cancer in exhaled air of 10 mL volume.

Results:
By a combination of 23 peak regions within the IMS-Chromatogram, patients with lung cancer, including a patient with carcinoma in situ, were classified and differentiated from healthy persons with an error rate of 0.

Conclusion:
Breath analysis by IMS can detect a discriminating combination of VOCs in lung cancer patients. By pattern recognition without the need for chemical analysis of the underlying VOCs, IMS has the potential to facilitate lung cancer diagnosis.

Key words:
breath analysis, exhaled breath, lung cancer, ion mobility spectrometry, volatile organic compounds

Abbreviations:
ATS = American Thoracic Society; COPD = chronic obstructive pulmonary disease; ERS = European Respiratory Society; GC = gas chromatography; IMS = ion mobility spectrometry; MCC = multi-capillary-column; MS = mass spectrometry; nA = nano-Ampere; pA = pico-Ampere; VOC = volatile organic compounds
Introduction:

There is a wish to be able to diagnose pulmonary disorders and assess their activity by analysis of breath. Different methods such as exhaled breath condensate, [1] the electric nose, [2-4] and the measurement of volatile organic compounds by gas chromatography/mass spectrometry [5-9] have been focused on, especially in COPD, asthma, lung cancer and interstitial lung disease. But as yet the ideal method has not been found.

The success of any method depends on the recognition of discriminating substances that can be used as biomarkers. This however needs an overview of the composition of VOCs in the exhaled breath, which then permits the selection of discriminating compounds.

Thus Horvath et al., [1] who summarised the general requirements for breath analysis in the recommendations of ATS/ERS, concluded that the ideal test should offer a "breathogram".

This breathogram can be offered by Ion Mobility Spectrometry (IMS). [10-16] In contrast to other analytical methods IMS enables detection and separation of all VOCs in exhaled breath and their visualisation in a three-dimensional so-called IMS-chromatogram. The diagnostic approach to breath analysis with IMS is based on the measurement of a disease-specific VOC peak combination, allowing diagnosis by pattern recognition without the need for chemical identification of the underlying VOCs.

These methodological advantages give rise to the question of whether IMS is superior to other methods of breath analysis such as gas chromatographic/mass spectrometric (GC/MS-) analysis of VOCs, [5-9] or the electronic nose. [2-4]

Generally, IMS is based on ionisation of gaseous metabolites, which are separated with short impulses (about 10 – 100 µs) in drift tubes with lengths of only a few centimetres (Fig. 1) at ambient pressure. The very small electric current (nA to pA) generated at a Faraday plate forms the spectrum of the running time of the ions. The combination of IMS with gas chromatographic columns guarantees a pre-separation of gaseous metabolites before entering the drift tube.

Unfortunately, IMS is not a method for identification of unknown compounds in a gas. However, the main advantages of IMS are its ability to detect very low concentrations of compounds (ng/L to pg/L, ppm, to ppt,-range) without any pre-concentration, and the short time for analysis; a spectrum takes less than 50 ms, a complete breath analysis less than 500 s. Because of the relative simplicity of the technique, and encouraged by our first results in the detection of airway infections [17] and sarcoidosis, [18, 19] IMS was used in this feasibility and pilot study to evaluate its potential for the non-invasive diagnosis of lung cancer.

Material and methods:

From 1/7/2004 to 30/11/2004 and following IRB approval from the institution, breath analysis with IMS was performed in healthy employees of the ISAS – Institute for Analytical Sciences – and in voluntary patients of the Hemer Lung Hospital who had a cytological or histological diagnosis of lung cancer. In this pilot study, which was designed to generate preliminary data and a training set of VOC profiles, no distinctions, e.g. regarding smoking history or COPD, were made. Furthermore patients with lung cancer were not differentiated by stage or histological type of tumour. All participants had given their written informed consent for participation in this study.
Ion mobility spectrometer:

For breath analysis an ion mobility spectrometer (Fig. 2) developed by the ISAS was used. In this spectrometer a 550 MBq $^{63}$Ni β-radiation source was applied for the ionisation of the carrier gas (air). It was connected to a polar multi-capillary-column (MCC, type OV-5, Sibertech Ltd., Novosibirsk, Russia) used as the pre-separation unit. In this MCC the analytes of exhaled breath were sent through 1,000 parallel capillaries, each with an inner diameter of 40 µm and a film thickness of 200 nm. The total diameter of the separation column was 3 mm. The relevant MCC parameters are listed in Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$^{63}$Ni-IMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionisation source</td>
<td>$^{63}$Ni (510 MBq)</td>
</tr>
<tr>
<td>Electric field strength</td>
<td>326 V/cm</td>
</tr>
<tr>
<td>Length of drift region</td>
<td>12 cm</td>
</tr>
<tr>
<td>Diameter of drift region</td>
<td>15 mm</td>
</tr>
<tr>
<td>Length of ionisation chamber</td>
<td>15 mm</td>
</tr>
<tr>
<td>Shutter opening time</td>
<td>10 μs – 1 ms</td>
</tr>
<tr>
<td>Shutter impulse time</td>
<td>20, 100 ms</td>
</tr>
<tr>
<td>Drift gas</td>
<td>synthetic air (20.5% O$_2$ (4.5), 79.5% N$_2$ (5.0))</td>
</tr>
<tr>
<td>Drift gas flow</td>
<td>100 … 300 mL/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>24 °C (room temperature)</td>
</tr>
<tr>
<td>Pressure</td>
<td>101 kPa (ambient pressure)</td>
</tr>
<tr>
<td>MCC</td>
<td>OV-5, polar</td>
</tr>
<tr>
<td>Column temperature</td>
<td>30 °C</td>
</tr>
</tbody>
</table>

Table 1: Characteristics of ion mobility spectrometer

Sampling Gas Collection:

The study subjects were asked to exhale through a mouthpiece connected to a Teflon bulb. Exhaled breath passed through an unheated sampling loop. A miniaturised suction pump (type G6/02-850163, ASF Thomas, Wülfrath, suction rate 350 mL/min) was connected to the outlet of the loop, to realise a homogeneous breath sample flow. At the end of exhalation, thus providing a mainly alveolar sample, an electric 6-way-valve was switched and 10 mL of gas in the sample loop were directed to the MCC for chromatographic separation of breath compounds. Having passed through the MCC the pre-separated analytes entered the ionisation chamber of the IMS (Fig. 3).

In here the carrier gas molecules were ionised by the $^{63}$Ni β-radiation source and fast ion-molecule reactions, forming ionised molecules of the analytes by different types of collisions, including charge transfer reactions. A further separation of the ions formed took place in the electrical field within the drift tube.

The ionised analytes were detected on a Faraday-plate at the end of the drift region. For the determination of blind values (i.e. environmental VOCs), samples of room air were considered before analysing exhaled breath to check for interfering measurement characteristics. Further data interpretation was based on the difference between the chromatograms. Fig. 4 represents a typical IMS-chromatogram and a single spectrum of exhaled breath from a healthy person.
Analysis of IMS-chromatograms:
The raw IMS data were first treated by a baseline correction so that intensity values varied around zero in areas of pure noise. Then the time-axes were transformed to adjust for different instrumental and environmental factors, such as length of the drift tube or ambient pressure. Next, the single measurements were subjected to a peak localisation procedure. The set of peak positions found in the whole sample of measurements was then analysed at once in a cluster procedure. The resulting clusters were the basis for a definition of typical peak areas (Fig. 5) of the analysed data set. Derived from these peak areas, new peak variables were defined as the mean intensity in the areas, which was calculated by going back to the original single measurements [detailed description in 23]. In this way, different numbers of variables were tested for their ability to form a differentiating cluster step by step. For each set of variables a possible validation set was built. Smoking history, tumour cell type and stage of cancer were not influential in development of the model.

Statistical methods:
The data were processed by different statistical methods. All steps of the analysis were performed with the software package R.[20] First, the LOWESS method was used to apply a baseline correction to the data as it offers the desired amount of robustness. For the task of peak localisation, a merging regions algorithm was used to distinguish between different peak areas.[21] For the clustering of peak positions the results of Ward’s method were used as starting values for the k-means method to obtain a stable, but well-adjusted clustering. Before the final application of a linear discriminant analysis for separation of the two groups of lung cancer patients and controls, a multiple t-test procedure was used to identify differentially expressed variables and thus reduce the complexity of the discrimination task. [22, 23] To evaluate the classification results, the error rate was estimated by leave-one-out cross-validation. A single observation from the original sample was used as the validation data, while the remaining observations served as training data for development of the model. The leave-one-out procedure incorporated the multiple testing procedure as well as the discriminant analysis. The resulting set of variables included in the model remained stable, while the coefficients of the discriminant rule were of course slightly changed. Afterwards the established rule was used to check the correctness of the resulting classification. The number of wrong assignments gives a reliable estimation of the actual error rate.[23] By executing a stepwise selection based on the standardised discriminant coefficients and the estimated error rate, the model was further optimised.

Results:
Altogether, 32 patients (24 men, 8 women) with histologically proven lung cancer were studied before the initiation of therapy. Mean age was $65.1 \pm 9.6$ years, body weight $74.9 \pm 13.7$ kg and height $169.4 \pm 8.3$ cm. Seven patients had a small cell lung carcinoma (SCLC), 24 patients a non-small-cell lung carcinoma (NSCLC), including 1 patient with a carcinoma in situ, 1 patient had a mixed tumour with NSCLC- and SCLC and 5 patients had an undifferentiated carcinoma. The tumour stage ranged from Tis to T4N3M1 (stage 0: 1 patient, stage 2: 3 patients, stage 3: 9 patients, stage 4: 17 patients). Seventeen patients were ex-smokers, 6 patients had never smoked and 7 patients still smoked. Fifty-four healthy persons (39 men, 15 women – 12 smokers, 42 non-smokers) without cancer served as controls. Mean age was $46 \pm 12$ years, body weight $81 \pm 16$ kg, height $181 \pm 9$ cm.
By applying the peak pattern analysis as described (see: Material and methods) a set of 23 variables was differentially expressed between the classes to a multiple test level of 0.001%. Based on these variables a linear discriminant analysis was conducted. By applying the leave-one-out method to 32 patients and 54 healthy controls, all patients with lung cancer, including the patient with carcinoma in situ, and all healthy persons were classified correctly (Fig. 6). This yielded a 100% negative and positive predictive value, respectively. The error rate estimated by the leave-one-out method was 0. The separation of both groups was independent from the smoking status (Fig. 7). In the lung cancer and the control group nonsmokers as well as smokers were present. No clustering occurred on the discriminant value scale for smokers or nonsmokers, neither in the left part related to lung cancer nor in the right part related to the control group.

Discussion:

Our data show that IMS can produce a discriminating combination of VOC peaks in lung cancer patients. The expression of a constellation of 23 VOC peak areas allowed the classification of a person as a tumour patient or a healthy person with an accuracy of 100%. The error rate estimated by the leave-one-out method was 0%.

The leave-one-out method was used for cross validation; a single observation from the original samples was used as the validation data, and the remaining observations served as the training data. In addition, the number of VOC-peak areas was reduced in a stepwise selection to reduce model complexity and optimise the error rate. By considering 23 VOC peak areas and applying the leave-one-out method to 32 patients and 54 controls, all were classified correctly. This means that in all cases the leave-one-out patient was classified correctly when the model was built on the base of 23 VOC peak areas, while the prediction error was > 0% when more VOC peak areas were included in the model. The next step will be to validate the model developed by the 23 VOC peaks and trained with data from 32 patients and 54 controls in larger populations using blinded data.

These data demonstrate that breath analysis by IMS has the potential to facilitate lung cancer recognition with high accuracy, as it was reported for GC-/MS-analysis [5, 8], electric nose,[2-4] coloric-array-systems [24] or even dogs specially trained for the detection of lung cancer patients [25] reaching sensitivities and specificities between 71.4% and 99%.

The correct classification even of the patient with a carcinoma in situ by IMS may indicate that the VOC composition is not influenced by the tumour volume and stage itself, but that it likely represents an expression of metabolic processes influenced by the tumour. Data from Deng et al.,[26] who found increased concentrations of hexanal and heptanal in exhaled breath as well as in blood from lung cancer patients, support this assumption. For a definite answer additional studies are necessary in patients with carcinoma in situ and in patients before and after complete tumour resection.

First examinations by Gordon et al. of the value of VOCs in the detection of lung cancer showed nearly 300 different VOC peaks in GC-/MS-profiles of exhaled breath from patients with lung cancer.[5] Capture of VOCs by sorbent traps, followed by solid phase micro-extraction or thermal desorption and gas chromatographic/mass spectrometric determination,
as described by Phillips et al. and other investigators, have remained the relevant methods in breath analysis. These methods use the cluster formation of VOCs for the diagnosis of lung cancer. However, the determination of special discriminating clusters is limited using the electric nose or GC-/MS-analysis. No method of VOC-analysis has yet been able to determine a consistent tumour-specific VOC pattern. Furthermore the comparison of subgroups (smokers vs. lung cancer, COPD vs. lung cancer, healthy persons vs. COPD) was based on different VOCs, such as acetone, methyl-ethyl-ketone, n-propanol, pentane, 2-methylpentane, 2,4-dimethylpentane, different alkanes and methylated alkanes, methylpentane and isoprene or alcohols and different disulphides. Interestingly, even after tumour resection Poli et al. found a decrease of VOCs (isoprene and decane) that had not been tumour-specific in the primary diagnostic approach.

In contrast to these methods, the methodological properties of IMS, which provides a complete breathogram with more than 1 million data points and a ten-fold lower detection limit of VOCs, appear superior, especially because the distinct separation of all VOCs by retention time and mobility (drift time) and the determination of their concentration creates a three-dimensional topography of VOCs in exhaled breath. This unique VOC classification explains the higher probability of finding a disease-specific and discriminating VOC peak combination that allows diagnosis by pattern or cluster recognition without the necessity of a chemical analysis of the underlying VOCs.

The central question of the present study, that is, whether IMS can detect a specific combination of VOC markers in exhaled breath of patients with lung cancer that differentiates them from healthy persons with high accuracy, is answered by the data shown. Other studies either compared lung cancer patients with healthy persons, or healthy persons with lung cancer patients, patients with asthma, PAH and COPD, as well as smokers and non-smokers. This makes the studies and their results difficult to compare. Since the most significant question concerns the existence of a malignancy, our IMS study was designed only to determine whether lung cancer patients show a VOC-pattern that separates them from controls, regardless of concomitant, especially pulmonary disorders. Smoking status was not influential on the discrimination of both groups, as might have been suspected at first. This result supports the data of Philips et al. who showed that smoking, histology and TNM had no influence on the discrimination by VOCs.

The IMS study has certain limitations. It included and compared lung cancer patients with healthy controls, but did not address a further prospective classification of lung cancer patients and healthy controls based on the pre-selected combination of VOCs. This difference in study design may explain the higher accuracy compared to former studies on breath analysis. The lung cancer patients were not matched to patients without lung cancer, and we did not include a further control group with other lung disease. So the discriminating cluster of VOCs is not necessarily a tumour-specific one. Furthermore most of the patients had an advanced tumour stage.

Further studies are necessary for the discrimination of different kinds of lung disease and different stages of lung cancer. These studies need blinding with respect to diagnosis to minimise bias. Finally proper selection of the control group and the classification of subgroups (smoking status, COPD, medication) are necessary.
However, our pilot study was primarily focused on the possibility and the advantages of ion mobility spectrometry as an analytical method. We intended to show that IMS, as a new method in breath analysis, can produce discriminating patterns of VOCs. These positive results could not be expected in advance.

The IMS-pattern does not yield direct information about the underlying analytes. However, the original intention in IMS diagnostics is the separation of patient groups by discriminating pattern recognition. This is consistent with Gordon et al.,[5] who regarded knowledge about chemical identity as unnecessary for peak classification.

However, in a second step that is comparable to other methods of breath analysis, determination of the VOCs is possible by additional mass spectrometric analysis. The stepwise relation of analysed VOCs to a defined peak position in a chromatogram opens the way to a three-dimensional „IMS-cartography of exhaled breath“. After this process has been finished the peak position allows correlation to the original VOC. If it is possible to find disease-specific and diagnostic analytes or combinations of analytes, exhaled breath might exclusively be tested for these VOCs. Additionally the identification of these VOCs may give further information about tumour biology and underlying metabolic processes.

In conclusion, breath analysis by IMS in lung cancer patients and healthy controls provided a classification of both groups with an accuracy of 100%, using a combination of 23 discriminating peak regions.

The immediate two-fold separation of VOCs that allows their visualisation in a three-dimensional chromatogram represents an important advantage of IMS over other methods. As the IMS procedure is based on pattern recognition, mass spectrometric analysis is not obligatory.

Data acquisition times of only 15 minutes offer good pre-conditions for the clinical use of IMS in breath analysis, especially if future computerised peak distribution analysis facilitates and accelerates further evaluation.

For a reliable diagnosis and separation of lung cancer from other disorders further measurements and an evaluation of the discriminating pattern in a larger group of patients are necessary.
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Competing interests:
The authors have no conflicts of interest to disclose.

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Legends:

Fig. 1:
Working principle of an ion mobility spectrometer – left: ionisation and reaction region to form ions of the analytes; right: drift chamber to separate the ions formed. The gas inlet is the entrance point of the carrier gas air with all molecules including the metabolites – the drift gas is a pure gas flowing towards the ions drifting to avoid entrance of non-ion at the ion shutter – the gas outlet is the outlet of the carrier and the drift gas – the aperture grid protects the Faraday plate – the drift rings stabilise the electric field within the drift region.

Fig. 2:
Ion mobility spectrometer at the Hemer Lung Clinic including the mouthpiece, the MCC/MS, the temperature control unit for the MCC and the bottles with synthetic air.

Fig. 3:
Sampling starting with carrier gas inlet (1, from the mouthpiece) using a six-port-valve (2) to introduce 10 mL of breath into the Multi-Capillary column (3, MCC) for pre-separation, followed by entrance of pre-separated breath compounds into the ionisation region of the IMS (4) and outlet (5).

Fig. 4:
Typical IMS-Chromatogram as heat map – inlet: single spectrum on the retention time of 3 s (dashed line in the heat map) – the signals related to acetone (blue quadrangle), the reactant ion peak (RIP) and ethanol (green quadrangle), humidity and ammonia (black quadrangle) are marked in the heat map and the corresponding single spectrum – the individual mobilities are shown in addition.

Fig. 5:
Differential expressed variable areas (typical peak areas).

Fig. 6:
Result of linear discriminant analysis (x = patient with carcinoma in situ).

Fig. 7:
Influence of the smoker status on the discriminant values as shown in Figure 6.
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