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ION MOBILITY SPECTROMETRY IN THE DIAGNOSIS
OF SARCOIDOSIS: RESULTS OF A FEASIBILITY STUDY

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A feasibility study with ion mobility spectrometry (IMS) was started to find characteristic peaks of volatile organic compounds in exhaled air of 10 mL sampling volume, which might be relevant for the diagnosis of sarcoidosis. Therefore, breath samples of 9 patients with sarcoidosis and suspicion of sarcoidosis because of mediastinal lymph node enlargement were investigated. The 5 patients with confirmed sarcoidosis showed a highly congruent distribution of metabolites in exhaled air which was different in main component analyses from patients with unspecific mediastinal lymph node enlargement. These results are a first step in breath analysis by IMS in patients with sarcoidosis. The IMS as a new method in breath analysis and the first results of the investigations are presented and discussed in detail.

Key words: exhaled breath, ion mobility spectrometry, sarcoidosis

INTRODUCTION

Analysis of exhaled breath gains more attention in the diagnosis of pulmonary diseases and the assessment of their activity. The recognition of relevant and determinating substances is of main importance. Therefore, an overview about the composition of human exhaled breath is necessary, to come to a detailed analysis of specific substances proposed as biomarkers. In comparison to other analytical methods, ion mobility spectrometry offers rather good conditions to get this overview fast. Furthermore, it provides a detailed separation of volatile organic substances detected especially in very low concentrations as relevant for human breath analysis.
More than 70000 ion mobility spectrometry (IMS) devices are used worldwide for the detection of chemical agents, drugs and explosives, for example in the German Reichstag or at many airports worldwide. But, there is only little knowledge about this gas analysis method, especially concerning applications for medical questions.

IMS is based on suitable ionisation of gaseous metabolites, followed by a separation of either positive or negative ions formed in a drift tube at ambient pressure and often at ambient temperature. Swarms of ions are separated with short impulses (about 10-100 µs) in drift tubes with a length of only a few centimetres (Fig. 1). A very small electric current (nA to pA) generated at a Faraday-plate forms a spectrum of the running time of the ions. As the drift tube uses ambient pressure in contrast to mass spectrometry, which needs high vacuum, the drift time is determined not only by the ion mass, but also by the number of collisions with neutral molecules.

The time the ions need for passing a certain distance is conversely proportional to the mobility of the ions. Under distinct conditions, the ion mobility allows the identification of analytes. Generally, ion mobility spectrometry is not a method for identification of unknown conjunctions in a gas, but in contrast to all procedures so far and under special conditions it realizes very low detection limits (ng/L to pg/L, ppmv to pptv-range) without any pre-concentration. Of special attraction is the combination of IMS with suitable gas chromatographic columns, so that a separation of mixtures is available within a few minutes. This enlarges the field of possible applications to rather complex mixtures as occurring in human breath.

As many other spectroscopic or mass spectrometric procedures can only be managed by comparably high financial costs and with qualified staff, it is desirable to have cost saving systems for special analytical problems, e.g., for the monitoring or fast screening of certain and in many cases selected analytes. Running a procedure at ambient air and pressure is often a criterion for the use of IMS. Furthermore, comparable short times for analysis – one spectrum lasts less than 50 ms, a complete breath analysis takes less than 500 s – mean an important advantage of this method. Further information and detailed descriptions of
different ion mobility spectrometers, different forms of ionisation and data interpretation procedures can be found in literature (1-3).

First data about clinical application of IMS exist for breath analysis in lung cancer and airway infections (4-10). Interstitial lung disease and mediastinal lymph node enlargement are challenging diagnostically and therapeutically. It would be desirable to have a diagnostic method with high selectivity, which allows the separation of different forms of interstitial lung disease as well as diseases with mediastinal lymph node enlargement. As a first step, breath analysis with IMS has been undertaken in a disease that can be diagnosed with high certainty, as sarcoidosis (11).

In the present feasibility study, exhaled breath from patients with sarcoidosis and suspicion of sarcoidosis was analyzed in order to find common and discriminating volatile organic compounds. The objective was the characterization of exhaled breath by IMS, but not the identification of the underlying analytes and their concentration. The latter would have gone beyond the initial question at this time and needs further investigations.

MATERIAL AND METHODS

In July 2006, breath analysis by IMS was tested in patients with known sarcoidosis and those with suspicion of sarcoidosis because of mediastinal lymph node enlargement. The study was approved by the institutional Ethics Committee for Human Research.

Ion Mobility Spectrometer

For breath analysis, an ion mobility spectrometer developed by the ISAS - Institute for Analytical Sciences Dortmund was used. In this spectrometer, a 550 MBq $^{63}$Ni β-radiation source was applied for the ionization of the carrier gas air. It is connected with a polar multi-capillary-column (MCC, OV-5, Sibertech Ltd., Novosibirsk, Russia) used as pre-separation unit.

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<th>Table 1. Multi-capillary-column (MCC) parameters.</th>
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<td><strong>PARAMETER</strong></td>
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<td>Ionisation source</td>
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<td>Electric field strength</td>
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<td>Length of drift region</td>
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In this MCC, the analytes in exhaled breath were sent through 1000 parallel capillaries, each with an inner diameter of 40 µm and a film thickness of 200 nm. The total diameter of the separation column is 3 mm. This allows the realization of carrier gas velocities from 300 mL/min, which are optimal for effective operation of the IMS. The relevant MCC-parameters are summarized in Table 1.

**Sampling**

The sampling was realized by charging the MCC with the volume of a sample loop (10 mL). This guarantees a constant sample volume for analysis and the adherence to analogue test conditions.

The test starts with the exhalation through a mouth piece connected with a Teflon bulb. At the end of exhalation an electric 6-way-valve is switched and 10 mL of gas in the sample loop are given to the MCC. The first step of analysis is a chromatographic separation of compounds occurring within the breath in the MCC by chromatographic effects known. Having passed the MCC the pre-separated analytes enter the ionization chamber of the IMS in an ideal case one after another (Fig. 2). Here follows the ionization of carrier gas molecules by the $^{63}\text{Ni}$ β-radiation source and fast ion-molecule reactions to form ionized molecules of the analytes by different types of collisions including charge transfer reactions. A further separation of the ions formed takes place in the electric field within a drift tube. The detection of ionized analytes is realized on a Faraday-plate at the end of the drift region of the IMS.

During the sampling period, exhaled breath flows through the sampling loop. A miniaturized suction pump (type G6/02-850163, ASF Thomas, Wülfrath) was connected to the outlet of the loop, to realize a homogenous breath samples flow of different testing persons. The suction rate is 350 mL/min. The suction pump guarantees a sufficient sample volume also in patients with difficulties to exhale properly. The flow rates of drift- and carrier gas (both synthetic air) are hold constant using two mass flow regulators (Typ PR4000, MKS Instruments München, Germany). To clean the system, the sample loop is flushed with synthetic air (purity 99.999 %) after each sampling procedure. For the determination of blind value, samples of room air were analyzed before analyzing exhaled air. The difference between IMS-chromatograms of ambient room air and exhaled air allows the recognition of contaminations in the IMS and avoids its spreading.

**RESULTS**

Breath analysis with IMS was taken in 9 patients: 5 patients had sarcoidosis and in 4 patients sarcoidosis could be excluded histologically.
Details about the patients

Patient 1: 48 years old female with histologically confirmed sarcoidosis in radiological stage III. Respiratory failure at rest or exertion was excluded. Arterial hypertension was treated with a β-blocker.

Patient 2: 30 years old male with acute Loefgren Syndrome and mediastinal lymph node enlargement (diameter 3.5 cm). Under steroid treatment (80 mg prednisone) regression of lymph node enlargement. No lung functional impairment during exercise testing up to 125 watts.

Patient 3: 50 years old male with suspicion of sarcoidosis. Radiologically sarcoidosis stage II with mediastinal lymph node enlargement and small pulmonary nodules. BAL with lymphocytic alveolitis (27%) and a T4/T8 ratio of 5. Confirmation of sarcoidosis in lung biopsies. Extrapulmonary sarcoidosis with hepatic involvement (increased liver enzymes) and abdominal lymph node enlargement.

Patient 4: 60 years old male with mediastinal lymph node enlargement, COPD, coronary heart disease, diabetes with renal insufficiency, and hyperuricaemia. Lung function with combined restrictive and obstructive dysfunction. Respiratory insufficiency with a PaO₂ of 57 mmHg and a reduced diffusion capacity (TLCO) of 75%. Histology of mediastinal lymph node biopsies showed unspecific reactive changes.

Patient 5: 67 years old female with posttuberculous calcifications in the right upper lobe and an obstructive stenosis of the right bronchial tree with middle lobe syndrome and dyslectasis of the upper lobe. Enlargement of mediastinal lymph nodes. HR-CT consistent with sarcoidosis stage II. Impaired lung function with obstructive ventilation and reduced TLCO (63%). Lymph node biopsies with epitheloid granulomas.

Fig. 3. Typical IMS Chromatogram as a heatmap. Inlet: single spectrum on the retention time of 3 s (dashed line in the heatmap) - 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 heatmap and the corresponding single spectrum - the individual mobilities are shown in addition.
Patient 6: 52 years old patients with lower limb swelling, sustained cough, and mediastinal lymph node enlargement. Lung function not impaired. BAL with lymphocytic alveolitis and CD4/CD8 ratio of 8.3. Lymph node biopsies showing granulomas, epitheloid cells, and giant cells.

Patient 7: 64 years old patient. For 20 years working in a coal mine. Acute viral airway infection. No lung functional impairment. In a CT scan enlarged mediastinal lymph nodes. Reactive inflammatory changes of the lymph nodes. Normalization of lymph node size in follow-up.

Patient 8: 65 years old male with enlarged pretracheal lymph nodes (1.6 cm) in a CT scan. No complaints. Normal lung function. Histologically anthracotic fibrosis and sinus histiocytosis.

Patient 9: 65 years old patient with productive cough and weight loss of 2 kg in 3 months. Smoker with 60 pack/years. Obstructive lung function with FEV1%VC of 65%. Normal lung volumes. Enlarged medistinal and hilar lymph nodes in a CT scan. Histologically anthracotic lymph nodes.

Evaluation of IMS-Chromatograms

The IMS Chromatograms of the patients were compared with single spectra and IMS Chromatograms of healthy persons, as shown exemplarily in Fig. 3. This represents a typical IMS Chromatogram and a single spectrum of exhaled breath with signals of humidity, ammonia, acetone, and air from a healthy person. Little variations of drift and retention time can result from changing conditions during measurements, e.g., the temperature and pressure, which directly influence ion mobility. That is why the mobility scale is used in data bank supported analyses. Actually, the drift time was used as the real parameter in order to present measurements directly.

A visual analysis of IMS chromatograms of the first 4 patients showed additional peaks at positions 19.47 ms / 40.1 s, 20.14 ms / 47.8 s and 21.91 / 47.9 s (Fig. 4) in exhaled breath of Patient 1 compared with healthy persons. These signals could also be seen in Patient 2. One of these signals was also detected in
Patient 3. The IMS-chromatogram of Patient 3 showed additional broad peaks at retention times between 10-70 s and drift times of 18 ms as well as retention times between 50-100 s and corresponding drift times of 20 ms.

Patient 4, in whom sarcoidosis could be excluded, showed none of the peaks that had been seen in patients with sarcoidosis (Fig. 5). Signals with retention times lower than 30 s were not considered because of methodological reasons.

The evaluation of chromatograms of 5 additional patients showed that the initial consideration of three discriminating signals was not sufficient for visual interpretation and definite diagnosis, because of variable peak compositions. Therefore, from all 9 chromatograms 13 peaks were chosen for discrimination. The position of these 13 peaks is marked with a cross and shown exemplarily in Fig. 6.

With regard to the signal height, a classification into 5 classes was made: -1 - no signal, 0 - in noise, 1 - minimal signal, 2 - distinct signal, 3 - high signal. The IMS Chromatogram of a patient with sarcoidosis shown in Fig. 6 exhibits clearly 6 signals from the 13 signals under consideration. These 6 signals are characterized by drift time (ms)/retention time (s) as follows: 42/19, 50/22, 70/23, 109/25, 145/22, and 238/30. Peaks at 42/219 and 70/23 were classified with 3, the peak at 109/25 with 2 and the signals at 50/22, 145/22 and 238/30 with 1. Peak
43/22 is noisy, whereas the signals at 50/22, 128/21, 150/21, 173/20, 196/22, and 144/24 were absent (therefore characterized with -1). The occurrence or absence of these signals was characterized for all 9 patients in the same manner. With these data based on peak position and height, a principal component analysis was made using (Unscrambler®), Version 9.6, Camo, Woodbridge, USA). This allowed a separation of the patient groups with and without sarcoidosis.

Fig. 7 shows the scores of 3 principal components which are close together in the case of patients with sarcoidosis, whereas patients without sarcoidosis show different and variable positions with a higher scatter, because of other influences. Investigations with a greater number of cases will probably allow to distinguish between the two groups and also to define the analytes most relevant in the loadings of the principal component analysis carried out.

**DISCUSSION**

For the diagnosis and differentiation of pulmonary disorders, the development of non-invasive and highly discriminating diagnostic tools is desirable. Breath analysis by analysis of breath condensate has not yet proven its diagnostic
potential. Furthermore, the potential of different methods of breath analysis has to be regarded on the background of many different questions. One question is whether breath analysis allows diagnosing a distinct disease or whether disease activity or prognosis can be estimated. To assess the diagnostic value of new techniques in breath analysis it is useful to test them in healthy persons and in diseases which can be proven or excluded with high certainty.

In contrast to the determination of single analytes or makers, which first have to be found, selected and tested in regard of sensitivity and specificity, IMS allows a complete visualization and separation of volatile organic compounds in exhaled breath rather fast.

A typical single spectrum and an IMS-chromatogram of a healthy person show the main components in air (indicated as RIP = reactant ion peak with a drift time at 17 ms), humidity and ammonia (drift time at 16 ms), ethanol (drift time at 17.5 ms) and acetone (drift time at 18.5 ms - see Fig. 3). At the inlet it can be seen that these components have the same retention time, i.e., they need the same time for passage through the multicapillary columns, but can be separated by a different drift time, i.e., different passage times in the electric field. At a detection limit of retention times above 10 s and drift times above 17 ms there are no further signals.

Fig. 7. Results of a principal component analysis of IMS Chromatograms of breath of 9 patients with suspicion of sarcoidosis including the signals marked exemplarily in Figure 6. The group of patients with confirmed sarcoidosis is marked by red symbols, the group of patients with non-confirmed suspicion is marked by green symbols; the numbers are the ID of the patients.
For patients with pulmonary disorders additional signals and pathologic changes of the spectrograms have to be expected. Although the signals with different peak positions and peak heights do not give evidence about the underlying analyte, it can be assumed that identical disorders have similar peaks resp. peak distributions, which allows recognition of distinct diseases by typical peak patterns.

Up to now, IMS has been applied in patients with airway infections and lung cancer (10, 12, 13). It could be shown that patients with lung cancer had highly specific peak clusters that are distinctly different from those in healthy persons. In patients with airway infections, peaks disappeared after antibiotic treatment, so that they had to be referred to either metabolic processes induced by bacteria or the bacteria themselves. In vitro head space analyses over bacterial cultures showed typical spectra for different bacteria (6), which could be found in IMS Chromatograms of patients with airway infections (10).

These first results show that ion mobility spectrometry provides an analysis of exhaled breath with more than 1 million data points and is suitable for gaining a topographic visualization of volatile organic breath compounds, thus enabling the detection and recognition of analytes which are relevant in underlying metabolic processes. IMS offers the advantage that a pre-concentration of exhaled breath can be avoided. As data acquisition is possible within 15 min, IMS offers ideal conditions for practicable clinical tests.

For an initial clinical validation of IMS it was regarded as useful to take breath analysis in patients who are suspected to have disorders that can be diagnosed with high certainty, such as sarcoidosis. There are only few data about breath analysis in patients with sarcoidosis, which concern only single components of breath, such as NO, ethane, H$_2$O$_2$ and 8-isoprostane (14-19). These investigations have shown that NO has no diagnostic value in sarcoidosis, whereas H$_2$O$_2$ and 8-isoprostane, as markers of oxidative stress, allow an estimation of disease activity, but do not give any diagnostic or differential diagnostic evidence, as they are also found elevated in tuberculosis. Ethane, as marker of lipid oxygenation, is relevant for disease prognosis, not only in sarcoidosis but also in interstitial lung diseases (14). So, there is no analyte or combination of different analytes in exhaled breath that makes a diagnosis of sarcoidosis possible.

The first step in breath analysis of patients with sarcoidosis was the identification of cluster of peaks in IMS chromatograms which are typical and diagnostic for sarcoidosis. The second step, a chemical identification of the volatile organic substances by mass spectrometry was not yet done in this study, because it would have exceeded the primary intention of peak cluster recognition. After having identified the volatile organic substances it would be possible to conclude from the peak positions the underlying metabolites and to avoid additional mass spectrometry.

The present study in patients with sarcoidosis and excluded sarcoidosis was taken as a feasibility study. The main interest was in comparing the chromatograms of both groups. For this purpose, spectrograms of the first 4
patients examined were interpreted visually. Such visual evaluation of the first 2 patients showed a complete correspondence in 3 peaks with the IMS Chromatograms. This is conspicuous because both patients have a different clinical presentation of sarcoidosis. One patient had sarcoidosis stage III without disease activity, whereas the other one showed acute sarcoidosis (Löfgren-Syndrom). The correspondence of three peaks was striking, but could not be regarded as specific for sarcoidosis until a bigger number of patients with sarcoidosis will have been tested. A correlation with disease activity, however, is unlikely, but cannot be fully excluded. As Patient 4 had no corresponding peaks compared with the first three patients, a consideration of sarcoidosis could be excluded by visual interpretation of the IMS Chromatogram.

Difficulties of visual interpretation are underlined by Patient 3, who only showed one peak which was identical in the position with those in Patient 1 and Patient 2. For interpretation of IMS Chromatograms - this is evident in Patient 3 - physical phenomena have to be taken into consideration. The detection of 2 rather large and broad peaks at a drift time of 18 ms over a retention time from 10-60 s, can be a reason for missing peaks at a retention time of 403 s and a drift time at 23 ms. This may be caused by the fact that ion formation in spectrometers is a matter of concurrence, which means that charges are transferred from the reactant ion peak (RIP) to the respective analytes.

In the case of highly different affinity of protons, many ions are formed for an analyte with a high proton affinity, whereas for an analyte with a low affinity there do not remain enough reactive ions, so that it is impossible to produce a peak formation corresponding to this analyte. Measurements with different MCCs, to have a better pre-separation of the analytes with different retention times, or the use of IMS with a negative electric field are necessary to get a better differentiation of different positive and negative ions, which are hidden in this big broad peak. There is also a need for additional methodological work. Increasing the resolution of the IMS chromatograms can be helpful as well. Furthermore, the actual study shows that with an increasing number of tested patients the peak distribution, which means both peak positions and intensities, shows a variability that cannot be discriminated by visual interpretation. The reason is that the occurrence of VOC is influenced not only by the primary disease itself but also by concomitant disorders and metabolic processes as well as nutrition. Therefore, the interpretation of IMS Chromatograms of a greater population is no longer possible by visual interpretation, but affords a statistical approach for the registration and analysis of peaks, comparable with the composition of peak clusters in patients with bronchial carcinoma by electronic nose (20, 21) or the breath analysis of VOC with GC/MS (22, 23).

A small population in this study was similarly evaluated by a main component analysis, by which a separation of patients with sarcoidosis from patients with non-specific mediastinal lymph node enlargement was possible. Two groups could be found. As expected, the points representing sarcoidosis
were closer together, whereas patients without sarcoidosis had different points with greater distances to one another.

At this moment, the results cannot be transferred with certainty to all patients with sarcoidosis or mediastinal lymph node enlargement. But the peak clusters found in these different groups and the separation by main component analysis can be regarded as a first step toward a classification of exhaled breath by IMS in sarcoidosis. Further studies with greater numbers of patients are necessary. The additional testing of exhaled breath by mass spectrometry may allow to relate peaks to corresponding and chemically identified VOC and to get insights into underlying metabolic processes.

In conclusion, ion mobility spectrometry is suitable for the examination of volatile organic compounds in exhaled breath and offers the chance to get more quantitative and qualitative information about metabolic processes occurring. The first aim in the diagnostic approach is detection of typical clusters of peaks, characterizing special pulmonary diseases, so that these patterns can be applied in the primary diagnostic testing. Secondly, the identification of underlying metabolic processes is of interest. Additional mass spectrometric investigations are necessary for relating peaks and specific VOC. The data of this feasibility study in sarcoidosis have to be confirmed in further studies with a higher number of patients, with the intention to develop IMS as a diagnostic tool in sarcoidosis.

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