Analyses of mouse breath with ion mobility spectrometry: a feasibility study

Wolfgang Vautz,1 Jürgen Nolte,1 Albrecht Bufe,2 Jörg I. Baumbach,1 and Marcus Peters2

1Department of Metabolomics, ISAS—Institute for Analytical Sciences, Dortmund; and 2Department of Experimental Pneumology, Ruhr-University Bochum, Bochum, Germany

Submitted 19 June 2009; accepted in final form 11 January 2010

Vautz W, Nolte J, Bufe A, Baumbach JJ, Peters M. Analyses of mouse breath with ion mobility spectrometry: a feasibility study. J Appl Physiol 108: 697–704, 2010. First published January 14, 2010; doi:10.1152/japplphysiol.00658.2009.—Exhaled breath can provide comprehensive information about the metabolic state of the subject. Breath analysis carried out during animal experiments promises to increase the information obtained from a particular experiment significantly. This feasibility study should demonstrate the potential of ion mobility spectrometry for animal breath analysis, even for mice. In the framework of the feasibility study, an ion mobility spectrometer coupled with a multicapillary column for rapid preseparation was used to analyze the breath of orotracheally intubated spontaneously breathing mice during anesthesia for the very first time. The sampling procedure was validated successfully. Furthermore, the breath of four mice (2 healthy control mice, 2 with allergic airway inflammation) was analyzed. Twelve peaks were identified directly by comparison with a database. Additional mass spectrometric analyses were carried out for validation and for identification of unknown signals. Significantly different patterns of metabolites were detected in healthy mice compared with asthmatic mice, thus demonstrating the feasibility of analyzing mouse breath with ion mobility spectrometry. However, further investigations including a higher animal number for validation and identification of unknown signals are needed. Nevertheless, the results of the study demonstrate that the method is capable of rapid analyses of the breath of mice, thus significantly increasing the information obtained from each particular animal experiment.

Address for reprint requests and other correspondence: W. Vautz, ISAS—Institute for Analytical Sciences, Dept. of Metabolomics, Bunsen-Kirchhoff-Straße 11, 44139 Dortmund, Germany (e-mail: vautz@isas.de).

It is without controversy that exhaled breath is a carrier of comprehensive information about the metabolic state of the subject, regardless of species (1, 2, 17, 23–25, 27, 35, 43, 49). Ion mobility spectrometry (IMS) and related spectrometry techniques have shown promise for analyses of human breath for medication and therapy control as well as for early diagnosis (6, 8, 20, 29–32, 34, 44, 46–48). However, animals are frequently used in preclinical trials to investigate the effects of drugs or diseases to protect patients from negative side effects or to exploit the devolution and effects of diseases for the development of effective drugs or therapy (19, 33). Analysis of the breath of such animals could give information about the state of their metabolism on one hand and could enable validation of the transferability of the animal model to human metabolism by comparison of metabolic markers on the other. The ovalbumin (OVA)-induced mouse asthma model is a well-characterized disease model that has been used in a vast number of studies to investigate induction and ongoing allergic immune response (19). The majority of the published work concentrates on cellular infiltration, hyperreactivity of airways, and cytokine production. However, there are only a few reports dealing with the analysis of volatile compounds in the exhaled breath of mice, predominantly concentrating on the measurement of nitric oxide (NO) (33). Since analysis of exhaled breath from small animals is technically complex, there is no published report on the analysis of other volatile compounds besides NO to date.

Therefore, during the present feasibility study IMS coupled with rapid preseparation has been applied to analysis of the breath of mice for the very first time. The resulting information could be used to explore metabolic pathways under the influence of drugs and/or diseases, hence resulting in more rapid results for experiments.

IMS was first developed for military use, e.g., for detection of warfare agents, and for security purposes with regard to the detection of explosives or drugs of abuse (4, 13, 16). In recent years the method is increasingly in demand for biological applications (26, 28) or for process control (5, 36, 40, 42) and food quality and safety (14, 18, 37, 38). The promising results from breath analysis using IMS coupled with rapid preseparation (6, 8, 17, 20, 29–31, 32, 34, 44, 46–48) encouraged its application in different disease models. Therefore, its suitability for analysis of the breath in mice should be investigated. The operation of ion mobility spectrometers under ambient conditions (pressure, temperature) requires little technical expense, and therefore low-cost instruments may be used.

Application of IMS coupled with a multicapillary column (MCC/IMS) to animals’ breath requires adaptation of sampling procedures. This should not cause problems when analyzing the breath of mammals with dimension comparable to humans, because similar volumes (several liters) and flow rates (up to 60 l/s) can be expected from their breath. However, it is challenging in significantly smaller animals like rats and mice. The pulmonary flow of mice is only up to 1 ml/s, and the tidal volume is in the range of 100–200 μl.

Fig. 1. Slope of pulmonary flow and of tidal volume with time of an anesthetized spontaneously breathing mouse as an example.

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In a pilot study, the sampling procedure of MCC/IMS was optimized for the typical flow rates and volumes expected from the breath of mice. The sampling was validated and then applied to anesthetized and intubated mice. The MCC/IMS data were evaluated by comparison with a database of known analytes and validated by additional solid-phase microextraction (SPME)-gas chromatography-mass spectrometry (GC-MS) analyses. However, to obtain significant metabolic information, a higher number of animals must be included in the study. In the present study, we wanted to demonstrate the feasibility of comprehensive breath analysis—even of mice—with MCC/IMS.

METHODS

Ion mobility spectrometry. IMS is a sensitive, fast technique for the detection of gas-phase analytes (4, 13, 16). The MCC/IMS used for the present study was custom designed at ISAS. With the help of a β-radiation source, the available gas (synthetic air) is ionized, resulting in protonated water clusters—the so-called reactant ions—that are recognized in the spectra as reactant ion peaks (RIPs). When an analyte enters the ionization chamber, proton transfer leads to ionized analyte molecules and to an associated decrease of the reactant ions. The ions are accelerated by an external electric field toward the detector (Faraday plate), and their drift velocity can be determined by measuring the drift time of the ions. The so-called ion mobility \( K \) corresponds to the drift velocity normalized to the electric field. A further normalization to the temperature and pressure of the available gas leads to the so-called reduced ion mobility \( K_0 \), which is characteristic for the analyte and independent on the experimental conditions. Additionally, rapid preseparation by MCC was applied to the analyses of complex and humid samples like human breath. Thus the retention time of the analytes is obtained as additional information for their identification. Furthermore, signal height is a measure for the concentration of the related analyte in the sample.

Custom-designed ion mobility spectrometers with rapid gas chromatographic preseparation using multicapillary columns (MCC/IMS) have been developed and applied to human breath (6, 8, 20, 29–31, 32, 34, 44, 46–48) successfully, thus giving useful additional information about the metabolic state of the subject.

With the experimental setup given in Table 1, the analysis of breath was completed after 5–10 min. The data obtained from such an analysis (≈1,000,000 data points—signal intensity vs. ion mobility and retention time) were analyzed immediately after the measurement by comparison with an analyte database and a pattern database, respectively.

From measurements of the tidal volume and the pulmonary flow of one of the mice of the study population (see Fig. 1), the maximum flow rate during exhalation of \( \approx 1,000 \) μl/s restricts the sample flow dramatically to \( \approx 100–200 \) μl/s. Furthermore, the observed breath frequency of 1.5–2.5 Hz requires a fast flow sensor and switches to enable the exclusive sampling of exhaled breath without contamination/dilution by room air.

With this information about the mice’s respiratory conditions, sampling was optimized for mouse breath. With the help of a bypass, a sample flow of 5 ml/min was drawn from the main stream with a flexible-tube pump only during exhalation. The differentiation between exhalation and inhalation was controlled by a sensitive flow sensor (see Table 1). The threshold for sampling was set to 6 ml/min (100 μl/s). Because this threshold is higher than the sample flow, dilution of the sample with room air can be avoided. The sample flow flushed the sample loop for 300 s but only if this threshold was exceeded, thus safeguarding a fourfold exchange of the volume of the sample loop with the breath sample. The result was a total sampling
duration of ~10 min. After the sampling period, the volume of the sample loop was introduced via a six-way valve into the preseparation column by a carrier gas flow of 150 ml/min.

The signals detected at the Faraday plate were amplified and converted to digital signals with a usbADC3 analog-to-digital converter. A notebook computer recorded the data with the ISAS software qIMS. Evaluation of the obtained three-dimensional data set (signal height vs. ion mobility and retention time) was carried out with the software BB_IMSanalyse (custom designed at ISAS) and included statistical treatment of the data such as denoising, compensation of the RIP to enable the identification of small signals close to the high RIP signal, and alignment of the retention time on behalf of known signals (3, 9–12, 41). All hardware and software were custom designed at ISAS.

To control memory effects and contamination of the instrument and furthermore to observe the potential influence of room air, a room air sample was analyzed before each breath analysis and an instrument blank was analyzed before each room air and each breath sample.

This method enables the rapid, sensitive, and selective detection and quantification of many volatile or semivolatile gas-phase compounds. However, detection is restricted to those substances that can be ionized with the ionization method applied. Therefore, substances like NO, for example, cannot be detected.

Animals. Female 5- to 6-wk-old BALB/c mice (Charles River, Sulzfeld, Germany) were used in these experiments. Mice were acclimated to the animal facility for 2 wk before the experiments. The vivarium was temperature controlled and maintained under a 12:12-h light-dark cycle. Food (Standard-Pellets for rat and mice, Altromin, Lage, Germany) and water were provided ad libitum. All animal experiments were approved by the appropriate governmental authority (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Recklinghausen, Germany).

Sensitization and airway challenge. Two mice were sensitized intraperitoneally by injecting 20 μg of OVA (grade V, Sigma-Aldrich, St. Louis, MO) emulsified in 2.2 mg of aluminum hydroxide (Imjec-

Fig. 3. Isoflurane signals in the 3-dimensional (3D) multicapillary column (MCC)-ion mobility spectrometer (IMS) chromatogram of mouse breath analyses (middle and bottom) that are not observed in room air (top). With each chromatogram, the single spectra at the cross-lines are displayed at bottom and the single chromatograms of both peaks on right. Top: room air for a mouse 15 min after inhalation of isoflurane in the laboratory. Middle: breath of mouse 15 min after inhalation of isoflurane; clear monomer and dimer ions signals are detected. Bottom: breath of mouse 24 h after inhalation of isoflurane; the monomer ion is still detectable.

Fig. 4. MCC/IMS chromatogram of analyses of breath of a mouse sensitized and challenged with ovalbumin. Indicated signals are compared with blank and room air measurements in Fig. 5. Center: 3D MCC/IMS chromatogram. Right: single chromatograms indicated by the cross lines. Bottom: mobility spectra indicated by the cross lines.
tAlum, Pierce, Rockford, IL) in a total volume of 200 μl on days 1 and 14. On days 28 and 38 mice underwent airway challenge by exposure to OVA aerosol for 30 min. OVA aerosol was generated with a PARI-Boy aerosol generator from a 1% OVA solution, resulting in deposition of ~5 μg of OVA in the lungs of mice (31a). This protocol leads to strong eosinophilic airway inflammation and hyperreactivity as shown previously (22). Two control mice were sham sensitized with aluminum hydroxide alone and challenged with OVA.

Connection of mice to IMS. Mice were investigated on days 39, 40, and 41 after onset of the experiment. For the measurements the mice were anesthetized by intraperitoneal injection of 65 μg/g ketamine (Ratiopharm, Ulm, Germany) and 13 μg/g xylazine (Bayer, Leverkusen, Germany). After relaxation of muscles mice were orotracheally intubated with a 1-mm-diameter endotracheal tube as described elsewhere (15). Subsequently the tube was connected to a pneumotachometer of a plethysmographic box type 871 (Hugo Sachs Electronics, March-Hugstetten, Germany) as shown in Fig. 2.

Study population and procedure of breath analysis. The breaths of four different mice—two control mice and two mice with allergic airway inflammation—were investigated two times each with the experimental setup described above. Each measurement included a blank sample to control contamination of the instrument, a room air analysis in which the sample was drawn through the complete sampling system without the mouse, and finally a breath analysis.

Validation of sampling procedure. Before analyzing the breath of mice obtained from the above-described experimental setup, it was necessary to validate the sampling procedure. It must be guaranteed that the sample represents exhaled breath only and not room air or inhaled air. Furthermore, it must be ensured that the volume of the sample loop is sufficiently filled with a real sample. Therefore, a mouse was exposed to an isoflurane atmosphere in an adjacent laboratory for few seconds and was removed again before being anesthetized. Fifteen minutes after this procedure, the mouse was anesthetized as described above, intubated, and connected to the sampling unit (see Fig. 2). Before that, the pure analyte was detected by MCC/IMS in the laboratory. The positions at which the monomer and the dimer ion peak of isoflurane were found are indicated by the cross-lines in Fig. 3.

The MCC/IMS analysis of the mouse’s breath from this experiment was compared with room air samples before and after the breath analysis. Isoflurane obtains well-known negative ion signals in the IMS spectra that were identified by a calibration carried out earlier. Those signals were observed in the breath sample only and not in the room air analyses (see Fig. 3). A monomer and a dimer ion signal could be observed 15 min after inhalation of the substance. Even after 24 h a clear but smaller signal from the monomer still remained in the spectra.

The presence of the isoflurane signals in breath and their absence in the room air analyses demonstrate clearly that the exclusive sampling of exhaled mouse breath is ensured as well as sufficient flushing of the sample loop.

SPME-GC-MS. A SPME fiber was exposed for 300 s vertically to the mouse’s exhalation flow. In this case both inhaled and exhaled air were sampled on the fiber. Afterwards, the analytes adsorbed on the fiber were analyzed by thermal desorption (TD)-GC-MS for identification of unknown analytes (48).

The sample was thermally desorbed and injected splitless at 250°C into an Agilent Technologies 6890N GC system connected with an Agilent Technologies 5973 mass-selective detector (MSD; Gerstel, Mülheim, Germany). The initial oven temperature of 35°C was maintained for 2 min, increased by 7°C/min to 250°C, and held for 7 min. A HP-5MS capillary column (60 m × 0.25 mm × 0.25-μm film...
thickness; Wicom, Heppenheim, Germany) was used for compound separation with helium as carrier gas at a constant flow rate of 1.0 ml/min. Electron ionization mode was used with 70 eV, and a mass range of m/z 33–450 was detected.

For comparison, the proposed analytes were measured with MCC/IMS. All analytes (puriss. p.a.) used for reference measurements were obtained from Sigma-Aldrich.

RESULTS

Figure 4 shows the IMS chromatogram of the breath of one of the mice affected with allergic airway inflammation as an example. Three peaks are indicated to demonstrate the evaluation procedure and the significance of the particular signals. All three peaks could be identified by comparison with the analyte database developed at ISAS as nonanal, decanal, and decamethylcyclopentasiloxane (DMCPS). Nonanal and decanal are known as common metabolites in human breath as well, while DMCPS is suspected to originate from plastic parts of the sampling system. It can be assumed that the mouse inhales the substance emitted from the plastics and—because not all of it can be adsorbed in the lung—exhales it again. The presence of decanal and nonanal to a lower extent in room air than in the breath samples can be explained by the presence of breathing personnel in the laboratory.

To validate these assumptions, a comparison of the three selected peaks as detected in the instrument blank, in room air, and in the breath of the mouse was carried out with the evaluation software BB_IMSanalyse developed at ISAS. The results are displayed in Fig. 5. From the blank measurement it can be concluded that the instrument was free from contamination: there is only a very small signal of nonanal. The reference measurement of room air shows a DMCPS peak significantly higher than in the breath of the mouse and inversely higher signals of nonanal and decanal in the breath analysis than in room air. It can be concluded that DMCPS derives from the sampling system and is partially absorbed by the mouse’s lung, while decanal and nonanal are definitely metabolites from the mouse.

With this approach, all particular signals found by the breath analysis were investigated. In total 28 specific signals could be detected as indicated in Fig. 6; 12 of these could be identified by comparison with the ISAS analyte database, and the others are indicated with the synonyms P1–P16. The database itself was validated by GC-MS data. For the identification of unknowns, additional SPME-GC-MS analyses were carried out on the breath of three of the investigated mice. However, the detected concentrations were too low to enable a distinct identification, and as a consequence the sampling on SPME needs thorough improvement.

To demonstrate the difference between the breath of healthy mice and mice affected with allergic airway inflammation the

![Fig. 6. MCC/IMS chromatogram of a mouse affected with allergic airway inflammation. All signal areas detected and used for further comparison are indicated. The 12 peaks identified by comparison with the reference database are indicated with the name of the analyte and the unknowns with the synonyms P1–P16. The graph is displayed as specified in Fig. 4.](image-url)
signal height for all detected signals was determined and compared between the two groups. For this purpose, the mean values of signal height in the breath of each healthy mouse and of each asthmatic mouse for all detected peaks normalized to the mean of the particular peak are shown in Fig. 7. This demonstrates that the deviation from mouse to mouse is broadly varying depending on the metabolite between >20% (e.g., P5, P7) and <5% (e.g., ammonia). Furthermore, for a few peaks it can be seen that the concentrations are different between healthy and asthmatic mice (e.g., benzaldehyde, hex-

Fig. 7. Comparison of the mean signal intensity of all detected peaks for each particular healthy and asthmatic mouse (4 investigated mice, 2 healthy, 2 asthmatic). Values are normalized to the mean signal intensity of each particular peak. a.u., Arbitrary units.

Fig. 8. Mean signal intensity of the peaks with significant difference between healthy and asthmatic mice. Values are normalized to the mean signal intensity of each particular peak.
DISCUSSION

Here we demonstrate for the first time that it is possible to analyze the molecular composition of breath from spontaneously breathing mice sensitized and challenged with OVA. Before the breath of mice obtained from the experimental setup described above was analyzed, the sampling procedure was validated successfully. With this experimental setup, the breath of four different mice—two control mice and two mice with allergic airway inflammation—was investigated. Interestingly, we found differences in the concentration of volatile compounds in the exhaled air in mice from the different treated groups. However, there are also some compounds such as P6 that give a similar signal, revealing that it is not a general phenomenon that all compounds are detectable in breath of mice with allergic airway inflammation at lower levels that could possibly be due to mucus deposition in the airways. Indeed, the signals of P3, P5, and P8 were always higher in the breath of the healthy mice. Therefore, those signals are preliminarily in doubt as being representative for the characterization of allergic airway inflammation in the breath of mice and should be further characterized.

Although our experiments demonstrate clearly that it is possible to detect differences between exhaled air from the healthy and the inflamed lung, it is self-evident that from eight particular analyses of the breath of two healthy mice and two mice with allergic airway inflammation no general conclusions on a possible differentiation of the groups based on the detected peaks can be drawn. The study population needs to be increased to validate the significance of the peaks for the differentiation of healthy and asthmatic mice. Furthermore, the possibility must be kept in mind that a single peak will not be sufficient to characterize the mice but rather a pattern of several peaks that will further increase the number of required breath analyses. This will be the objective of further studies together with the characterization of unknown analytes by GC-MS to obtain a comprehensive view of compounds exhaled by the inflamed lung. This is an indispensable step in exploring whether those peaks derive from metabolic processes or from, for example, medication or anesthesia.

Conclusions and outlook. An ion mobility spectrometer equipped with a multicapillary column for rapid preseparation (MCC/IMS) was successfully applied for the first time to the analysis of the breath of anesthetized but spontaneously breathing healthy mice and mice with allergic airway inflammation. The sampling procedure was briefly optimized for this purpose. However, it can be expected that further optimization, especially with regard to minimization of the tube volumes between mouse and the MCC/IMS and to the sample flow rates, will increase the quality of the results.

The analytes in the breath of the mice that could be identified are also found quite frequently in human breath. Other, not yet identified signals were not included in the ISAS database for human metabolites, and therefore no comment can be made as to whether they are relevant. The SPME-GC-MS measurements could not give further information on the identity of those peaks, because the preconcentration duration was too short with regard to the low concentrations and to the detection limits of GC/IMS. In the future, the preconcentration step must be optimized to solve this problem.

Appropriate sampling of exclusively exhaled breath was validated by analysis of the breath of a mouse marked with a well-known analyte. From the twofold analysis of the breath of two healthy and two asthmatic mice the MCC/IMS signals of three presently unidentified analytes are preliminarily in doubt as being representative for a differentiation of healthy status and allergic airway inflammation in the breath of mice. Indeed, the number of particular breath analyses and investigated subjects is by far too low to allow general conclusions. Nonetheless, this present feasibility study could demonstrate the potential of IMS as a suitable tool to obtain additional relevant information from each particular animal experiment. Once the relevant pattern of metabolites is identified, this could lead to a reduction of the number of experiments and thus to an abatement of the suffering of the subjects.

ACKNOWLEDGMENTS

The dedicated work of Luzia Seifert at ISAS—Institute for Analytical Sciences, Dortmund, was essential for the investigations reported here.

W. Vautz developed the sampling and was involved in planning and realization of the experiments and in the evaluation of the MCC/IMS data. J. Nolte carried out the GC-MS analysis and gave the interpretation of the data obtained. A. But and J. I. Baumbach initiated the cooperation and gave advice for the realization. A. But from the medical point of view and J. I. Baumbach from the IMS point of view. M. Peters provided the mice, prepared them for the experiments, and was involved in planning and realization of the experiments. All authors helped to draft the manuscript and read and approved the final version.

GRANTS

The financial support of the Bundesministerium für Bildung und Forschung and the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen is gratefully acknowledged. The project was founded partly by the project BAMDOD (Breath-gas analysis for molecular-oriented detection of minimal diseases) of the European Union (LSHC-CT-2005-019031) and the high-tech strategy funds of the Federal Republic of Germany (Project Metabo-lit-01SF0716).

DISCLOSURES

The authors declare that they have no competing interests.

REFERENCES

Innovative Methodology

IMS ANALYSES OF MOUSE BREATH