Journal of Chromatography A, 1254 (2012) 87–97

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Allergic asthma exhaled breath metabolome: A challenge for comprehensive two-dimensional gas chromatography

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ARTICLE INFO

Article history:
Received 12 March 2012
Received in revised form 5 July 2012
Accepted 9 July 2012
Available online 16 July 2012

Keywords:
Allergic asthma
Exhaled breath
Volatile metabolites
Headspace-solid phase microextraction
Comprehensive two-dimensional gas chromatography–time of flight mass spectrometry

ABSTRACT

Allergic asthma represents an important public health issue, most common in the paediatric population, characterized by airway inflammation that may lead to changes in volatiles secreted via the lungs. Thus, exhaled breath has potential to be a matrix with relevant metabolomic information to characterize this disease. Progress in biochemistry, health sciences and related areas depends on instrumental advances, and a high throughput and sensitive equipment such as comprehensive two-dimensional gas chromatography–time of flight mass spectrometry (GC × GC–ToFMS) was considered. GC × GC–ToFMS application in the analysis of the exhaled breath of 32 children with allergic asthma, from which 10 had also allergic rhinitis, and 27 control children allowed the identification of several hundreds of compounds belonging to different chemical families. Multivariate analysis, using Partial Least Squares-Discriminant Analysis in tandem with Monte Carlo Cross Validation was performed to assess the predictive power and to help the interpretation of recovered compounds possibly linked to oxidative stress, inflammation processes or other cellular processes that may characterize asthma. The results suggest that the model is robust, considering the high classification rate, sensitivity, and specificity. A pattern of six compounds belonging to the alkanes characterized the asthmatic population: nonane, 2,2,4,6,6-pentamethylheptane, decane, 3,6-dimethyldecane, dodecane, and tetradecane. To explore future clinical applications, and considering the future role of molecular-based methodologies, a compound set was established to rapid access of information from exhaled breath, reducing the time of data processing, and thus, becoming more expedite method for the clinical purposes.

1. Introduction

Asthma is a complex inflammatory disorder characterized by allergic inflammation, smooth muscle contraction, bronchial hyperresponsiveness, hypertrophy and hyperplasia of smooth muscle, hypersecretion of bronchial mucus, activation of mast cells, eosinophils, lymphocytes, epithelial cells, macrophages, disruption of the bronchial epithelium and production of free radicals with variable symptoms (e.g. cough, dyspnoea, wheezing, chest pain) [1]. Allergic asthma is the most common form of asthma and is increasing considerably, in developed countries such that it is now one of the commonest chronic disorders in the world, and is also associated with high direct and indirect health costs, especially related with diagnosis and treatment.

In recent years, non-invasive techniques that may be useful for the assessment of airway inflammation have been found in the analysis of exhaled breath. Inflammation plays a critical role in many physiological changes of the body including inflammatory lung diseases like asthma. Inflammation is accompanied by oxidative stress and subsequently lipid peroxidation and during this process polyunsaturated fatty acids are converted into volatiles that are secreted via the lungs. Hundreds of different volatiles are present in human breath, and their relative concentrations may alter via the disease [2]. Exhaled breath has been studied using one-dimensional gas chromatographic (1D-GC) process in lung diseases, such as asthma [2,3], cystic fibrosis [4] and lung cancer [5,6]. Although such approach often provides satisfying analytical results an in-depth chromatogram analysis frequently indicates that some peaks are the result of two or more co-eluting compounds. Comprehensive two dimensional gas chromatography (GC × GC) employs two orthogonal mechanisms to separate the constituents of the sample within a single analysis, based on the application of two GC columns coated with different stationary phases, which increases peak capacity as a result of the product of the peak capacity of the two dimensions. For example, a non-polar/polar phase combination (NP/P), connected in series through a modulator interface achieves this goal. For instance, using a cryogenic modulator, the interface samples small (several seconds) portions of the first

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0021-9673/$ – see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.chroma.2012.07.023

Table 1
Characteristics of the studied population: allergic asthma and control children.

	Allergic asthma (n = 32)	Control (n = 27)
Age in years (range/median)	4–16/9	3–6/5
Gender (male/female)	18/14	15/12
Pathology		
Allergic asthma (AA)	22 (69%)	–
Allergic asthma + allergic rhinitis (AA + AR)	10 (31%)	–
Allergens[a]		
Dust mite	18 (56%)	–
Dust mite + gramineae	5 (16%)	–
Gramineae	4 (13%)	–
Dust mite + cat fur + gramineae	2 (6%)	–
Dust mite + cat fur	1 (3%)	–
Gramineae + cat fur	1 (3%)	–
Dust mite + cockroach	1 (3%)	–

Therapy

Corticosteroid	Leukotriene receptor antagonist	Bronchodilator	Anti-histamine	Nasal corticosteroid	Allergic asthma (n = 32)	Control (n = 27)
x	x	x	–	–	1 (3%)	–
x	–	x	–	–	9 (28%)	–
x	x	–	–	–	5 (16%)	–
–	x	–	x	x	2 (6%)	–
–	–	x	x	–	5 (16%)	–
–	x	x	–	–	1 (3%)	–
–	–	–	x	x	2 (6%)	–
–	x	–	x	–	1 (3%)	–
No therapy		–			6 (19%)	

[a] Results obtained by prick-tests.

dimension (^{1}D) eluate by cryofocusing, and re-injects them into the second column (^{2}D). Each ^{1}D peak is modulated several times, largely preservating the ^{1}D separation. Co-eluting compounds from ^{1}D undergo additional separation on ^{2}D [7]. Sensitivity and limits of detection are improved due to focusing of the peak in the modulator and separation of analytes from chemical background [8] compared to 1D-GC. ToFMS (time-of-flight mass spectrometry) brings several advantages such as full mass spectra acquisition at trace level sensitivity and mass spectral continuity, which allows for deconvolution of spectra of co-eluted peaks [9]. To the best of our knowledge, GC × GC–ToFMS methodology has never been reported before to study allergic asthma exhaled breath volatile composition. However, GC × GC–ToFMS has been used with multibed sorption trap for exploring human exhaled breath volatile composition [10], and searching potential biomarkers for active smoking [11], and combined with automated needle trap for breath analysis of patients undergoing cardiac surgery [12]. These studies revealed the potential of this technique in breath analysis. Thus, this study aims to obtain a deeper knowledge of allergic asthma based on exhaled breath analysis using a previously developed HS-SPME extraction technique, as well as several other exhaled breath sampling parameters [3], combined with GC × GC–ToFMS system. The first step was to check the separation potential of GC × GC–ToFMS and sensitivity issues, important parameters in exhaled breath analysis, a complex matrix with several compounds in the micromolar to nanomolar range [13]. Secondly, Partial Least Squares-Discriminant Analysis (PLS-DA) and Monte Carlo Cross Validation (MCCV) were performed to assess both the predictive power and classification models robustness. Moreover PLS-DA regression vectors were used to help understand metabolic variations important to class discrimination.

2. Experimental

2.1. Standards and materials

Several reagents were used to perform this study: linear alkanes (C_8–C_{20}) in hexane (99.5%, Fluka, Madrid, Spain), linear alkenes (C_8–C_{20}) (98%, Sigma–Aldrich, Madrid, Spain), aldehydes: hexanal (98%, Sigma–Aldrich, Madrid, Spain), (*E*)-2-nonenal (95%, Acros Organics, Geel Belgium), decanal (98%, Sigma–Aldrich, Madrid, Spain), ketones: 3-heptanone (97%, Sigma–Aldrich, Madrid, Spain), 5-methyl-3-heptanone (94%, Sigma–Aldrich, Madrid, Spain), 3-octanone (98%, Sigma–Aldrich, Madrid, Spain), absolute ethanol was supplied by Panreac (99.5%, analytical grade, Barcelone, Spain). Ultra pure water was obtained from a Milli-Q system from Millipore (Milford, MA, USA).

For the sensitivity studies, a stock solution of each standard (1 g/L) was prepared in absolute ethanol and made up to volume, and from this a solution of 100 mg/L was set up. A working solution was prepared to yield different concentrations and to reproduce a two-phase system (headspace and coating fibre), as in breath analysis, 5 μL was added to a 120 mL SPME flask and sealed with an aluminium crimp cap with a vial was capped with a PTFE septum (Chromacol, Hertfordshire, UK), and concentrations ranged from 20 to 200 × 10^3 pg/L.

The SPME holder for manual sampling and fibre were purchased from Supelco (Aldrich, Bellefonte, PA, USA). The SPME device included a fused silica fibre coating partially cross-linked with 50/30 μm divinylbenzene-carboxen-poly(dimethylsiloxane) (DVB/CAR/PDMS). Prior to use, the SPME fibre was conditioned at 270 °C for 60 min in the GC injector, according to the manufacturer's recommendations. Then, the fibre was daily conditioned for 10 min at 250 °C.

2.2. Samples

A group of 32 children with allergic asthma, from which 10 had allergic asthma and allergic rhinitis, and 27 healthy control children volunteered for this study (n = 59). The characteristics of the patients and controls are presented in Table 1. A *naive* patient was also included in this study. This patient was a 9 years old female child that had never taken an asthma drug and was diagnosed by physicians with allergic asthma based on symptoms history and skin prick tests were performed being positive for dust mites. After the first consult, this child was prescribed a combination