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Secondary electrospray ionization coupled to high-resolution mass spectrometry reveals tryptophan pathway metabolites in exhaled human breath

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Many studies and personalized medicine in general require frequent measurements and/or rapid results of biomarker levels. Here we show that 20 low volatility metabolites of the tryptophan pathway can be detected in exhaled human breath. This real-time and non-invasive method offers an attractive alternative to blood analysis.

Amino acids play a central role in human biochemistry as the building blocks of proteins. Furthermore, they also play a role as neurotransmitters and are the starting materials for the biosynthesis of several vital compounds.[1] Tryptophan (Trp or W) is an aromatic, non-polar proteinogenic essential amino acid with an indole ring as a side chain. The catabolism of tryptophan in humans results in the formation of kynurenine derivatives as key intermediates.[2] They can then undergo deamination or further catabolism resulting in oxidation to acetyl-CoA. By this pathway, kynurenine serves also as an intermediate for the synthesis of the co-factors NAD^+ and $NADP^+$.[2] Besides this, tryptophan also acts as a precursor for the synthesis of the neurotransmitters melatonin and serotonin.[2] Disorders in tryptophan metabolism result in diseases such as vitamin B6 responsive xanthurenic aciduria, hydroxy-kynureninuria, tryptophanuria and Hartnup disease.[3]

Secondary electrospray ionization (SESI) coupled to high-resolution mass spectrometry (HRMS) has been shown to be a powerful technique for breath analysis and biomarker identification.[4] Over the last few years, it has been applied to several metabolic studies, including differentiation of obstructive sleep apnea patients from their ''breathprints''.[5] As a breath analysis technique, SESI-HRMS is capable of detecting hundreds of compounds that enter the breath from the blood *via* the lung.[6] Usually thought to be restricted to volatile or semi-volatile compounds, SESI-HRMS nowadays can detect low volatility compounds as well, thanks to the great sensitivity of specially designed SESI sources and state-of-the-art MS instrumentation.

We hypothesized that this extreme sensitivity may reveal compounds that are metabolically linked to tryptophan. Therefore, the aim of this work was to detect Trp metabolites in real time and non-invasively in exhaled breath (as opposed to conventional blood tests, which are time-consuming).

The chemical nature of tryptophan and its metabolites, including the aromaticity and low polarity, may provide just enough volatility for these compounds to be detected in breath by means of SESI-HRMS. From a data set from 12 different subjects, we tried to extract 37 metabolites related with human tryptophan metabolism, as protonated ions $[M + H]^+$ and within a window of ± 2 ppm, from on-line analyses of exhaled breath. 20 out of 37 metabolites (54%, Table 1) were clearly detected in every subject, which shows the potential of SESI-HRMS to assess tryptophan metabolism. It should be highlighted that the mass range of detected metabolites reached up to 265 u (formyl-*N*-acetyl-5-methoxykynurenamine), which is beyond the mass range limit ($\sim$150–200 u) of competing breath analysis techniques such as proton transfer reaction (PTR)[7] or selected ion flow tube (SIFT)[8] mass spectrometry.

Even though the elemental composition of the metabolites detected is reliable, since it is based on HRMS, it is always possible that isobaric compounds may be interfering. Therefore, in order to further strengthen identification, one of the compounds showing sufficiently intensity (5-methoxyindoleacetate, Fig. 1A and B) was selected for on-line tandem mass spectrometry. Results (Fig. 1C) showed agreement between spectra obtained from breath and from a standard[9] for the three main fragments of 5-methoxyindoleacetate (Fig. 1D), which confirms the identity of this compound. Several other peaks are visible in the breath tandem mass spectrum (Fig. 1C), which are not related to the compound studied. These peaks are fragments from other, isobaric compounds that were co-isolated in the first low-resolution stage of the mass spectrometer, as can be seen in Fig. 1A. Additional confirmation of the compound identities was obtained for two metabolites by comparing ultra-high performance liquid chromatography (UHPLC) retention times from standards and exhaled breath condensate. As can be seen in

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Table 1 Tryptophan pathway metabolites detected in breath by SESI-HRMS

Name[a]	Formula	$[M + H]^+$
Anthranilate	$C_7H_7NO_2$	138.0550
Tryptamine	$C_{10}H_{12}N_2$	161.1073
4,8-Dihydroxyquinoline 4,6-Dihydroxyquinoline	$C_9H_7NO_2$	162.0550
3-Methyldioxyindole	$C_9H_9NO_2$	164.0706
Indole-3-acetate 5-Hydroxyindoleacetaldehyde	$C_{10}H_9NO_2$	176.0706
3-Hydroxykynurenamine 5-Hydroxykynurenamine	$C_9H_{12}N_2O_2$	181.0972
5-Methoxyindoleacetate	$C_{11}H_{11}NO_3$	206.0812
4-(2-Aminophenyl)-2,4-dioxobutanoate	$C_{10}H_9NO_4$	208.0604
L-Kynurenine	$C_{10}H_{12}N_2O_3$	209.0921
N-Acetylserotonin	$C_{12}H_{14}N_2O_2$	219.1128
5-Hydroxy-L-tryptophan	$C_{11}H_{12}N_2O_3$	221.0921
4-(2-Amino-3-hydroxyphenyl)-2,4-dioxobutanoate	$C_{10}H_9NO_5$	224.0554
3-Hydroxy-L-kynurenine 5-Hydroxykynurenine	$C_{10}H_{12}N_2O_4$	225.0870
N-Formylkynurenine	$C_{11}H_{12}N_2O_4$	237.0870
6-Hydroxymelatonin	$C_{13}H_{16}N_2O_3$	249.1234
Formyl-*N*-acetyl-5-methoxykynurenamine	$C_{13}H_{16}N_2O_4$	265.1183

[a] Compounds in boldface: identity confirmed by tandem HRMS or UHPLC.

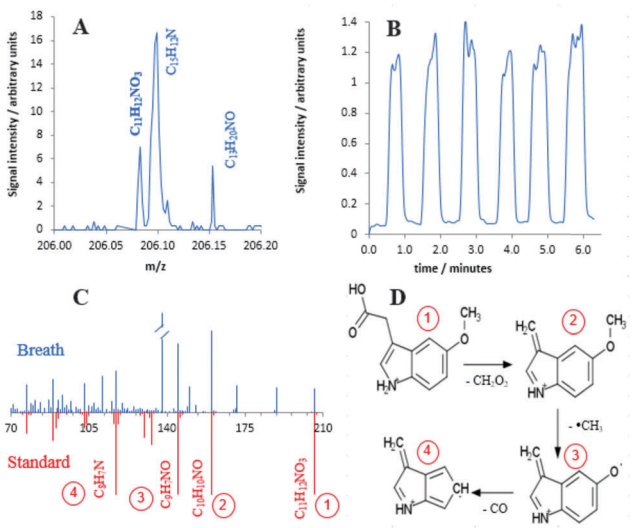

Fig. 1 High-resolution mass spectrum of 5-methoxyindoleacetate (m/z = 206.0812) and neighboring isobaric peaks (A). Times traces of 5-methoxyindolacetate from six consecutive exhalations (B). Comparison of tandem MS spectra of 5-methoxyindolacetate from breath and a standard (C). Tandem MS fragmentation pathway of 5-methoxyindolacetate (D).

Fig. 2, excellent matches were found for the two compounds studied, indole-3-acetate and 3-hydroxykynurenine. No signal was found in UHPLC for any other metabolites, most probably because of lack of sensitivity in comparison to SESI-HRMS and/or poor recoveries in the exhaled breath condensate collection process.

A look into the tryptophan metabolic pathway (Fig. 3) revealed that the 20 metabolites detected are closely related, being preferentially located in the same branches. Breath concentrations

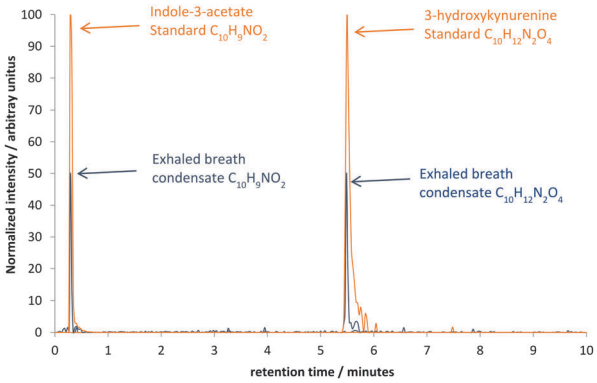

Fig. 2 UHPLC-HRMS extracted ion chromatograms for indole-3-acetate and 3-hydroxykynurenine standards (red) and from exhaled breath condensate (blue).

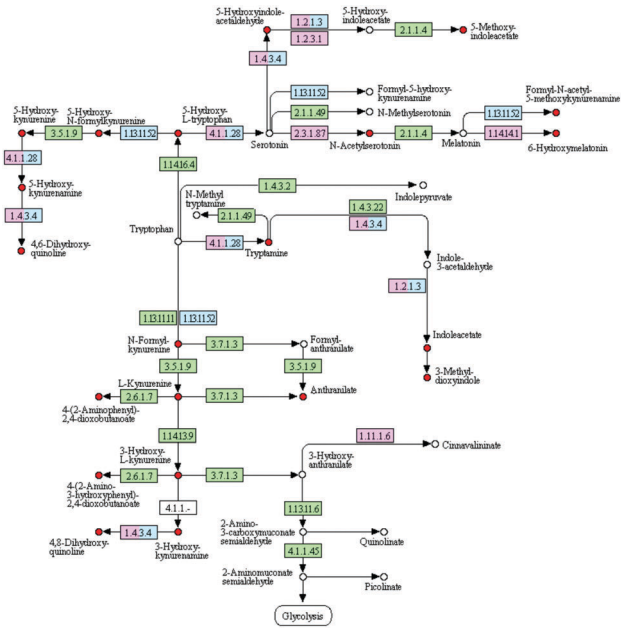

Fig. 3 Tryptophan metabolites detected in breath by means of SESI-HRMS (red). Pink, pathways related to a disease. Blue, reactions related to a drug. Green, other reactions known in *Homo sapiens*. Adapted from KEGG.[12]

of endogenous compounds are described by the Farhi equation[10] whose major factors are systemic and lining fluid concentrations and volatility, both of which are compound-dependent. Therefore, the detection in breath of only some of the tryptophan pathway branches may be the result of these having higher concentrations, higher volatilities (more plausible based on the chemical nature of the metabolites detected), or a combination of these two factors.

Of special interest is the detection of two metabolites in the melatonin branch (*i.e.* *N*-acetylserotonin and 6-hydroxymelatonin), which are known to be involved in the entrainment of the circadian rhythms and also have an antioxidant and neuroprotective role.[11] Real-time follow-up of these two compounds may be compelling for chronobiologists.

In conclusion, it has been shown that tryptophan metabolism can be assessed by SESI-HRMS real-time breath analysis

of volatile metabolites, covering in this way up to ~50% of the whole pathway. This opens a new way of diagnosing, and even more important, follow-up in a fast, real-time and non-invasive way, tryptophan metabolic disorders whose prognosis depends on maintaining a strict daily diet (*e.g.*, Hartnup disease). Furthermore, the detection of two metabolites related to melatonin suggests a way of assessing circadian rhythms, a field which demands frequent, or ideally on-line measurements, as demonstrated here.[13]

Numerous other applications in personalized medicine and real-time medical monitoring that require immediate results can be envisaged. Further studies should be conducted to address whether this approach can be extended to other amino acid pathways, especially those containing relatively volatile metabolites, or even further to other human metabolic pathways. It should be noted that tryptophan metabolites were detected but an absolute quantification was not carried out. Absolute quantification in SESI-HRMS is difficult to achieve; this being one of the main current limitations of the technique that needs to be addressed in the future. Nevertheless, we are confident that SESI-HRMS will be useful as a non-invasive, real-time tool for metabolic and disease monitoring.

Experimental parameters were as follows: for real-time breath analysis, 12 subjects (8 males and 5 females; age: 30 ± 6 years) with no clinical signs of lung disease and non-smokers, were asked to breathe through a heated tube (90 °C) into the inlet of a home-built SESI source that was directly coupled to a high-resolution mass spectrometer (TripleTOF 5600+, AB Sciex, Concord, ON, Canada). Six replicated measurements took less than 10 minutes. Additional experimental parameters and protocols have been described elsewhere.[14a] For tandem HRMS analysis, product ion scan experiments were run as previously described.[14b] The local ethics committee approved the study (KEK-ZH-Nr. 2014-0076) and all participants gave their written informed consent. Exhaled breath condensate samples were collected using a homemade device following the recommendations suggested by the ATS/ERS task force. For analysis, they were injected into an ACQUITY UPLC system (Waters, MA, USA) and separated in a C18 ACQUITY column (2.1 mm × 100 mm, 1.7 μm, Waters, MA, USA). A 10 minute gradient (0.4 mL min^{-1}) was set from 95/5% to 10/90% of a water/acetonitrile mixture acidified with 0.1% formic acid. The eluent from the column was introduced into a LTQ Orbitrap (Thermo Fisher Scientific, MA, USA) mass spectrometer working at a resolution of 30 000 at *m*/*z* 400.

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