Non-$^{13}$CO$_2$ targeted breath tests: a feasibility study

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Abstract

Breath tests allow a non-invasive and fast diagnostic of different specific enzymes’ phenotypic functionality. Over the last decade several $^{13}$C-breath tests were successfully tested, with the $^{13}$C-urea breath test being approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA). The use of other targets than labeled $^{13}$CO$_2$ in exhaled breath offers additional possibilities. High sensitivity analytical technologies, such as proton-transfer reaction time-of-flight mass spectrometry, enable the detection of different volatile targets in the low ppb (parts per billion) range in real-time.

In the current study volunteers received 0.8 mg deuterated 2-propanol, which was converted to d$_3$-acetone (m/z 62.08) by alcohol dehydrogenase. D$_3$-acetone (m/z 62.08) appeared in exhaled breath concentrations up to 30 ppb (at maximum). Parallel consumption of ethanol seems to reduce the activity of the enzyme, resulting in approximately 15–30% reduction of the produced d$_3$-acetone. Phenotypic determination of enzyme activities is important, since the functionality of enzymes is influenced by factors such as age, sex, life-style, diet, organ function, metabolism, etc, which cannot be entirely accounted for by genetic factors.

Keywords: breath tests, VOCs, personalized medicine

1. Introduction

Breath analysis provides a picture of different metabolic processes in a non-invasive way. It has the potential to become a powerful tool for determining enzyme activities, organ functionality and transport processes, since it could be applied as a screening method in hospitals or even in medical practices. As an analytical tool it could be used to determine appropriate drug dosages and medicinal efficacy and play a role in screening the progress of diseases. Thus, breath analysis is especially applicable to personalized medicine.

Currently, breath tests investigated in the clinical diagnostics use stable isotopic labeled substrates, mostly $^{13}$C-labeled compounds. The only $^{13}$C-based breath test approved by the Food and Drug Administration (FDA) and applied in clinical routine is the $^{13}$C-urea breath test [1] which detects Helicobacter pylori, the cause of most duodenal and stomach ulcers by chronic inflammation of the inner lining of the stomach.

Other $^{13}$C-based breath tests applied for determination of enzyme activities are the $^{13}$C-dextromethorphan and the $^{13}$C-pantoprazol breath test [2, 3]. In both tests $^{13}$CO$_2$ is released in exhaled breath after metabolism of the precursors.
During these tests exhaled air samples are collected before and after administration of the precursor and the so called DOB-value (delta over baseline [%]) is determined as difference of measured $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratio in specified time points by non-dispersive infrared spectroscopy (or isotope ratio mass spectrometry). These tests provide a quick, non-invasive procedure with high patient compliance and could potentially be produced at low-cost once $^{13}\text{C}$-labelled substrates are produced in high throughput.

The dose of precursor to be ingested for a $^{13}\text{CO}_2$-based breath test is determined by a simple calculation: it should lead to a difference in delta-over-baseline of about 1 delta unit, which corresponds to a difference in concentration of approximately 500 ppb. Such a difference is easily measurable but is at the border of the natural fluctuation, since changes in breathing patterns and other natural variations may lead to a change in delta of about 0.5 delta units.

Compounds other than $^{13}\text{CO}_2$, which do not usually present in exhaled breath, could be used with reduced precursor amount. Since such target compounds can be measured on the ppt-level (e.g. at 100 ppt), the dose of a precursor compound for a breath test could be less than 0.1 mg.

Today, detection of the produced targets at the reduced level of 100 ppt is possible due to state-of-the art analytical systems such as gas chromatography with mass-spectrometric detection (GCMS), ion mobility spectrometry (IMS), laser spectrometry, or proton-transfer reaction (time-of-flight) mass spectrometry (PTR-MS and PTR-TOF-MS). The latter direct mass spectrometric techniques enable monitoring of breath volatiles even in real-time, as shown in previous studies focusing on concentration changes e.g. isoprene and acetone during different physiological states such as during sleep or exercise [4, 5].

To elucidate biological processes isotopic labeling of compounds is helpful when the expected target volatile is already part of the breath constituents under normal conditions. Beside the above mentioned $^{13}\text{C}$ labeling other stable isotopes such as deuterium (D) or $^{15}\text{N}$ can be also applied.

It should be noted that labeling with $^{13}\text{C}$ is limited by the fact that VOCs appearing normally in the exhaled breath also contains 1.1% naturally occurring $^{13}\text{C}$, which will increase the amount of the produced labeled target compound. Another approach is to use a substrate in which a methyl group is replaced by a fully deuterated methyl group, so that the two VOCs differ by 3 amu instead of 1 amu. Thus, the labeled VOC does not interfere with any other compound including a few hydrated groups, and it is possible to reduce substantially the amount of the substrate needed for a unique identification. Winkler et al [6] used deuterated ethanol for examination of ethanol metabolism by the enzyme alcohol dehydrogenase (ADH). The administered amount of $d_3$-ethanol was 0.5 µg/kg, which means approx. 400 mg dose for a volunteer with 85 kg body weight.

In the present study we selected 2-propanol for demonstrating the capability of monitoring the enzyme functionality of alcohol dehydrogenase with a lower amount of administered substrate. 2-Propanol is widely used in chemical industry, e.g. as solvent in production of hair and skin cosmetic products, disinfectants and as anti-freeze agent in fuel systems. 2-propanol is of low toxicity by any route. The LD50 values for several animal species after oral administration varied between 4475 and 7990 mg/kg body weight [7]. It can cause irritation of the respiratory system, eyes and mucous membranes. Higher exposure levels affect the central nervous system and may lead to nausea, hypertension and dizziness.

The major metabolic pathway of 2-propanol is oxidation by liver alcohol dehydrogenase (ADH) to acetone, which is eliminated mainly via urine and exhaled air from the body [8, 9]. Acetone may also be further metabolized to acetate, formate and carbon dioxide [10]. ADH comprises a family of enzymes grouped into several classes. They catalyse the oxidation of primary and secondary alcohols to aldehydes and ketones, respectively, and also can catalyse the reverse reaction.

Class I ADH occurs in the liver but is also observed to a lesser extent in the gastrointestinal tract, kidneys, and lungs [11, 12]. ADH belonging to class II is detected only in the liver, whereas class III was found in all examined tissues. Class IV isoenzyme of ADH is expressed preferably in the upper part of digestive tract [13].

In the reported experiments we used deuterated 2-propanol, $d_3$-2-propanol in order to eliminate the interference with any other VOCs including a few hydrated groups. The applied dose of 0.8 mg demonstrates the functionality of breath tests with a 50–100 fold reduction of the substrate compared to often applied drug doses (e.g. ~50 mg).

2. Materials and methods

2.1. Test protocol

Two volunteers (co-authors, non-smokers) were recruited for the study. Volunteers arrived in the morning after at least 12 h of fasting. Two types of tests were carried out on two separate days for each person. The first test started with the administration of $1 \mu l \ d_3$-isopropanol (0.8 mg) dissolved in ca. 150 ml of tap water. Breath analysis was started before drinking the test solution and continued for 30 min, then continued with 15–30 min intervals during the next 2 h, and finally hourly for approximately 6 h.

A second test was performed aimed at inhibition of the activity of alcohol dehydrogenase starting with drinking 50 ml of hard liquor (vodka, alc. content 40%) identically after at least 12 h of fasting. 15 min later a baseline breath measurement was carried out and the test solution containing the $1 \mu l \ d_3$-isopropanol in tap water was administered. The acquisition of spectra was performed following the timeline of the first test. Room air was checked before and during the tests to control for environmental contamination.

2.2. Real-time breath analysis using PTR-TOF MS

For the current study a high-sensitivity PTR-TOF MS (PTR-TOF 8000, Ionicon Analytik Gesellschaft mbH, Innsbruck, Austria) was used. The principles of operation are described extensively elsewhere [14, 15]. For on-line breath sampling the PTR-TOF MS was combined with a
The BET sampler (buffered end-tidal sampler, Ionicon Analytik Gesellschaft mbH [16]).

The usual pressure and temperature in the drift tube of the PTR-TOF MS was 2.35 mbar and 80 °C, respectively. The drift voltage was 635 V, and the resulting E/N 66.1 V cm−1. The TOF was working at 3.5 * 10⁻⁷ mbar pressure. The temperature of the BET system was kept constant at 60 °C during sampling. A gas flow of 20 mL min⁻¹ was applied, carrying exhaled air from the BET (40 ml sample volume) into the PRT-TOF MS through a 60 °C heated line. Sucking the sample from the BET at low flow rate eliminates mixing the end-tidal breath with room air. During sampling candidates breathed into the BET system continuously by inhaling the air through the nose and exhaling through the mouth. While exhaling acetone at m/z 59 and the protonated water dimer signal (H₃O⁺·H₂O) at m/z 37 were monitored to determine the end-tidal fraction.

2.3. Data analysis

The PTR-TOF MS produces a full scan every 40 μs. 100 000 of these spectra are integrated to increase the signal-to-noise ratio and reduce the amount of data. Consequently, one integrated spectrum every 4 s was recorded using TofDaq Recorder (vers. 1.2.93, TOFWERK AG, Thun, Switzerland). Data evaluation procedure including mass calibration, ion extraction is described elsewhere [17]. For normalization of the signal with respect to the precursor ion H₃O⁺ m/z 21 (¹⁸O isotope) was used.

The distribution of calibration curve parameters (for calibration of acetone, acetaldehyde and ethanol) was obtained through bootstrapping simulation [18]. To determine detection limits we used the defining relations described in a previous work [18]. The reported critical values are: detection decision (LC, false positive risk < 5%) and detection limit (LOD, false negative risk < 5%).

2.4. Calibrations

Test gases for the compounds acetone, acetaldehyde, ethanol were prepared in zero air (hydrocarbon impurities < 10 ppb) with 90% relative humidity at 20 °C using a test gas generator (GASLAB, Breitfuss Messtechnik GmbH, Harpstedt, Germany).

| Compound   | CAS     | Ions [m/z] | LC [ppb] | LOD [ppb] | R²   | SF [ncps/ppb] |
|------------|---------|------------|----------|-----------|------|---------------|
| acetaldehyde | 75-07-0 | 45.03      | 13.71    | 27.43     | 0.99 | 144.7         |
| ethanol    | 64-17-5 | 47.04      | 23.6     | 54.2      | 0.94 | 2.68          |
| acetone    | 67-64-1 | 59.05      | 0.1      | 0.2       | 0.99 | 161.0         |

**Figure 1.** Exhaled breath signals at m/z 62 before (green) and 20 min after administration of d₃-isopropanol (red).
Germany). All chemicals were purchased from Sigma-Aldrich (Vienna, Austria). In the gas generator aqueous solutions of the substances (0.02 mol% for acetone, 0.65 mol% for ethanol and 0.32 mol% for acetaldehyde) were introduced into a vaporizer with determined flow rate and evaporated at 100 °C. Vapours were diluted with zero air by means of integrated mass flow controllers producing gas standards of the selected volatiles within the range of 1 and 1000 ppb for acetone and acetaldehyde and between 100 ppb and 50 ppm for ethanol. The saturation of the signal was reached above 30 ppm for ethanol, thus a non-linear function was applied for calculation of the concentration. The linear range was up to 10 ppm. Beside the ion C\textsubscript{2}H\textsubscript{6}OH\textsuperscript{+} at m/z 47.04 we observed also the hydrated form H\textsubscript{2}O C\textsubscript{2}H\textsubscript{6}OH\textsuperscript{+} at m/z 65.06 for ethanol. For acetaldehyde the ion H\textsubscript{2}O C\textsubscript{2}H\textsubscript{5}O\textsuperscript{+} at m/z 63.04 was observed in addition to the ion C \textsubscript{2}H\textsubscript{5}O\textsuperscript{+} at m/z 45.03. The calibration parameters are summarized in Table 1.

3. Results

During the applied breath test the production of acetone is expected in exhaled air at comparatively high levels (around 400–1000 ppb). Isotopic labelling of 2-propanol was necessary to monitor small concentration changes in the low ppb-range as a consequence of the limited precursor dose of 1 µl administered by the volunteers. Therefore, d\textsubscript{3}-isopropanol was used to be converted to d\textsubscript{3}-acetone forming a signal at m/z 62, where no interfering peaks arise. Figure 1 shows the PTR-TOF MS signals of exhaled breath before (baseline) and 20 min after drinking the precursor representing the changes in m/z 62 intensity, which occurs after drinking the d\textsubscript{3}-isopropanol exclusively.

Signals of d\textsubscript{3}-acetone differed between the two volunteers during the breath test (marked with open circles in figures 2(a) and (b)). At the beginning of the test m/z 62.08 exhibits a concentration level of around 0 ppb. Immediately after drinking the test solution a fast and strong increase in the d\textsubscript{3}-acetone concentration can be observed for 2–3 s exceeding 25 ppb, followed by an immediate decrease almost to the starting level (approx. 2 ppb).

The first concentration peak could be due to an immediate metabolism of d\textsubscript{3}-isopropanol by alcohol dehydrogenase located in the mucosa of the mouth [19, 20]. Supporting this assumption, Erstgard et al observed an increased acetone concentration in saliva of a female volunteer after 2-propanol exposure [8]. After the sharp and narrow peak at the beginning the d\textsubscript{3}-acetone level starts to increase and reaches its maximum at around 20 min after precursor administration. Then the concentration decreases quickly over the next 10 min and moderately during the following 6 h, ending in a concentration of around 1 ppb, which still higher than the baseline level.

Considering that 100% of the labelled d\textsubscript{3}-isopropanol is metabolized to d\textsubscript{3}-acetone by ADH enzymes (1.12E-5 mol) around 6.2% of the produced d\textsubscript{3}-acetone (7.90E-07 mol) was exhaled in the first 7.4 h (duration of the measurement).

Since the enzyme activities can be influenced by factors such as age, sex, environmental effects, medication, etc, volunteers performed a second test by drinking 50 ml of vodka prior to the administration of the precursor (as described above) to examine whether the parallel consume of ethanol influences the metabolism of 2-propanol by ADH.

As it can be seen in figure 2(a)–(b) the maximum d\textsubscript{3}-acetone levels were reduced in this second test after drinking of hard liquor in both volunteers. Volunteer 1 repeated test 1 and test 2 three times at three different days for estimation of the produced d\textsubscript{3}-acetone concentration. Mean concentration of formed d\textsubscript{3}-acetone was 41.8 ± 10.2 ppb (mean ± SD) in the test with d\textsubscript{3}-isopropanol in tap water without swallowing the hard liquor and 28.5 ± 2.5 ppb (mean ± SD) after drinking vodka. Although the number of measurements is limited, the results show that a tendency for influence on the enzyme activity by the additional substrate, ethanol, can be suggested. The high standard deviation of 10.2 ppb found in test 1 might point out, that different factors such as the consumed food, physical activity, or other daily changing parameters can affect the test results.
Accepting that 100% of the labelled d₃-isopropanol is metabolized to d₃-acetone by ADH enzymes (1.12E-5 mol) around 7% (7.1% ± 1.9% considering the three repetitions) of the produced d₃-acetone (~8.94E-07 mol) was exhaled in the first 4.2 h measurement duration in the test without drinking vodka, while around 6% after drinking the hard liquor; ~7.52E-07 mol (5.9% ± 0.2% regarding the three reps).

These first experiments showed differences in production of d₃-acetone for the two tested volunteers, while the general trend of the d₃-acetone concentration during the tests remained similar in test 2. However, the assumption that this variation lays in differences found in alcohol consumption habits, metabolism rate and individual tolerances or e.g. in daily fluctuations, should be examined in a higher number of subjects.

3.1. Changes in acetone concentration

Figure 3 shows changes in unlabelled acetone m/z 59 concentrations during the tests; these changes seem to be affected by
the consumed hard liquor but not by the administered labelled precursor. Different studies suggest and report the formation of acetone after ethanol consumption [6, 21], which can be observed in figure 3 during both tests.

In test 1 (without vodka) acetone concentration is stable at around 650 ppb for volunteer 1 and 350 ppb for volunteer 2 during the first hour, followed by an increase over the next 4 h of fasting. In the second test an increasing profile with a maximum of 1400 ppb at approx. 100 min after drinking of vodka (85 min after administration of d₃-isopropanol marked with blue dashed line) can be observed for volunteer 1. In case of volunteer 2 the maximum exhaled acetone concentration was measured at approx. 1 h after drinking vodka (thus, 45 min after administration of the precursor).

The changing ethanol concentration in exhaled air was also monitored in parallel. As it can be seen in figure 4, their concentration as measured by m/z 47 ion reached maxima (12 ppm for volunteer 1 and around 50 ppm for volunteer 2) approximately 1 h after vodka consumption, followed by a rapid drop over 40–60 min. It should also be mentioned that
around 50 ppm the signal is fully saturated, thus above this level the quantification is not possible. After 180 min exhaled breath ethanol values for both volunteers drop to 1–2 ppm.

Beside m/z 47.04 we observed a considerable change in the signal of m/z 65.06 (displayed on figure 5). Based on calibration results and its similar trend to ethanol, this ion can be assigned to hydrated ethanol, H₂O·C₂H₆OH⁺.

It should be mentioned that Winkler et al [6] assumed the formation of other protonated isotopes of deuterated acetone, namely C₃D₆O-H⁺. However, the exact mass of m/z 65.06 and the high concentration argue in favor of the formation of H₂O·C₂H₆OH⁺. In our opinion the formation of C₃D₆O-H⁺ is unlikely, since deuterated methyl groups usually do not exchange deuterium.

Acetaldehyde, the expected metabolic product of ethanol was monitored at m/z 45.03 (figure 6). For volunteer 1 (with lower exhaled ethanol levels) the maximal breath concentration of around 360 ppb could be measured 1 h after vodka consumption, while for volunteer 2, ~460 ppb could be detected after 30 min following alcohol ingestion (figure 7). However, because no time delay could be observed in acetaldehyde signal formation and while at the same time vodka contains high acetaldehyde levels (according to our headspace investigations around 25 ppm), the increased acetaldehyde levels might arise mostly from the hard liquor. In contrast, the vodka consumed in the experiments contained acetone in the low ppb-range and its influence is therefore limited to the exhaled acetone concentrations.

4. Conclusions

Targeting molecules other than ¹³CO₂ might open up additional possibilities, if the expected target metabolite appears in exhaled air only after administration of the substrate and the analytical device is sensitive enough for its detection in the low ppb range.

High sensitivity analytical technologies, such as proton-transfer reaction time-of-flight mass spectrometry enable a sensitive detection of different volatile targets. Since sample preparation is not required, a direct on-line and real-time monitoring of targets in exhaled breath is possible. Our previous experiments with ¹³C-breath tests showed that carrying out the tests according to the same protocol is of utmost importance to achieve comparable results.

In a breath test we used deuterated 2-propanol for monitoring the activity of alcohol dehydrogenase. The use of isotopic labeling was necessary in order to recognize the converted product, d₃-acetone (m/z 62.08) in the low ppb range beside the normal acetone breath level.

The applied precursor dose was 0.8 mg, leading to a detectable signal of around 30 ppb d₃-acetone (at the peak of response) in exhaled breath.

The activity of ADH was influenced by consumption of hard liquor in parallel to deuterated 2-propanol. This second feasibility test leads to a reduction of the produced d₃-acetone concentration for both volunteers. However, reproducibility of these tests and influence of other factors such as body weight, age, and drinking habits, etc have to be confirmed through additional investigations in a larger sample.

Moreover, time profiles of acetone and acetaldehyde productions could be monitored in real-time. We observed a rise in acetone levels exceeding the level of 1 ppm within 1 h of the administration of vodka. The concentration of ion m/z 45.03 increased almost 100 times compared to the test without vodka, which corresponds to acetaldehyde (C₂H₅O⁺) with high probability.

We observed that high ethanol levels in breath yielded higher acetone and acetaldehyde signals for volunteer 2 in
comparison to volunteer 1. We assume that the detected acet-aldehyde level is linked together with the comparatively high level of acetalddehyde in vodka, while a part of the detected acetone concentration arises from metabolism of ethanol contained in the hard liquor.

As the monitoring of physiological processes will increase current knowledge of metabolic pathways, it will help to explain the possible origin of volatiles appearing in exhaled air. Monitoring targets in known metabolic processes beside $^{13}$CO$_2$ in exhaled air could be used for predicting various enzymatic processes with reduction of the applied dose.

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