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Interpretation of Non-Invasive Breath Tests Using ^{13}C-labeled Substrates – A Preliminary Report With ^{13}C-Methacetin

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Abstract
Non-invasive breath tests can serve as valuable diagnostic tools in medicine as they can determine particular enzymatic and metabolic functions in vivo. However, methodological pitfalls have limited the actual clinical application of those tests till today. A major challenge of non-invasive breath tests has remained the provision of individually reliable test results. To overcome these limitations, a better understanding of breath kinetics during non-invasive breaths tests is essential. This analysis compares the breath recovery of a ^{13}C-methacetin breath test with the actual serum kinetics of the substrate. It is shown, that breath and serum kinetics of the same test are significantly different over a period of 60 minutes. The recovery of the tracer $^{13}CO_2$ in breath seems to be significantly delayed due to intermediate storage in the bicarbonate pool. This has to be taken into account for the application of non-invasive breath test protocols. Otherwise, breath tests might display bicarbonate kinetics despite the metabolic capacity of the particular target enzyme.
Key words: liver function, liver function test, ^{13}C-breath test, methacetin, cytochrome P450 1A2, LiMAx test
Abbreviations: NBT, non-invasive breath tests; DOB, delta over baseline; HPLC, high performance liquid chromatography

Introduction

Non-invasive breath tests (NBT) with ^{13}C-labeled substrates have been applied for the assessment of specific enzymatic/ metabolic functions and the diagnosis of particular diseases [1, 2]. NBTs are based on in vivo metabolism of certain ^{13}C-labeled substrates into a product and ^{13}C-labeled carbon dioxide by a specific target enzyme. The interpretation of the test results assumes that the appearance and recovery of $^{13}CO_2$ represents the concurrent in vivo metabolism of the substrate (Fig. 1).

Expired $^{13}CO_2$ can be detected by mass spectrometry [3], non-dispersive isotope selective infrared spectroscopy [4] or other methods [5]. Breath sampling can be performed in bags or tubes [6], or by direct online analysis [7]. Thus NBTs can determine in vivo metabolism without repeated blood sampling, which makes it more acceptable and comfortable for both physicians and patients. However, $^{13}CO_2$ is not directly exhaled from the target enzyme, but needs to be transported from the investigated organ as bicarbonate ($H\,^{12}CO_3^-$ / $H\,^{13}CO_3^-$) into the lung [8]. Methodological studies reported the kinetics of $^{13}CO_2$ excretion already in the 1970-80ies [9-13]. It is known that emerging bicarbonate has a relatively long halftime of approx. 60 minutes [14] and that ultimately only 70% of the emerging $^{13}CO_2$ is excreted [15]. This could significantly interfere with NBT results [8]. However, these data did neither influence the design of later breath test protocols nor the algorithms of NBT interpretation. Different ways for calculation of test readouts have been described in literature: Some authors used single time points (Fig. 2; # 1-4) - whether at chosen arbitrary points in time like 15, 30 or 60 minutes (Fig. 2; # 2-4) [16] or maximal abundance (Fig. 2; # 1) [7]. Other authors applied area-under-curve analysis (Fig. 2; # 5) [17, 18].

However, it remains somehow undefined which way actually provides the most valid and reliable test readout. The aim of this analysis was to explore the correlation between substrate and $^{13}CO_2$ kinetics during the intravenous ^{13}C-methacetin breath test to improve the analytic algorithms.

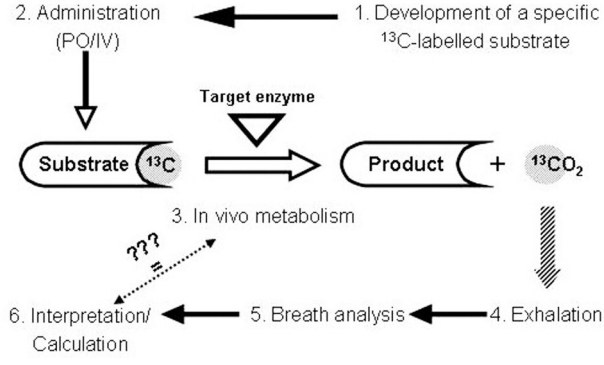

Fig. 1. General principle of non-invasive breath tests using ^{13}C-labeled substrates. The close connection between breath test interpretation and in vivo metabolism is a essential precondition for the validity of a test.

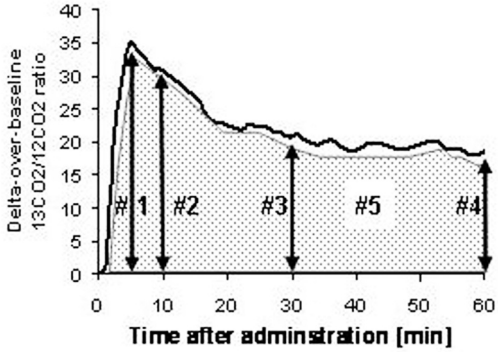

Fig. 2. Algorithms of test interpretation in non-invasive breath tests for calculation of the enzymatic capacity. It is shown an exemplary plot of breath recovery ($^{13}CO_2/^{12}CO_2$ ratio) after administration of 2 mg/kg ^{13}C-labeled methacetin. #1- Maximum of delta-over-baseline (DOB); #2- DOB at 15 minutes; #3- DOB at 30 minutes; #4- DOB at 60 minutes; #5- Cumulative recovery by calculation of area under DOB curve.

Methods

The experimental study was performed in healthy volunteers after approval by the faculties ethics review board. The persons were assessed by a specific breath test using ^{13}C-methacetin as substrate for the hepatic cytochrome P450 1A2 system and thereby blood samples were drawn to determine the substrate kinetics. The methodology was based on the previously reported LiMAx test of Stockmann et al. [7]. The substrate was administered into a peripheral vein as a bolus in a dose of 2 and 4mg/kg body weight.

Breath Sampling and Analysis

An online protocol of breath analysis was applied, to enable a high sampling rate to enable kinetic analysis of breath recovery. Breath samples were automatically drawn and analyzed with a frequency of as approximately 1/min by a modified nondispersive isotope-selective infrared spectroscopy based device (FANci2-db16, Fischer Analyseninstrumente, Leipzig, Germany). Exhaled breath was collected by a special two-way face mask. Mean baseline $^{13}CO_2/^{12}CO_2$ ratio was recorded ten minutes before injection for the calculation of delta-over-baseline (DOB) $^{13}CO_2/^{12}CO_2$ ratio values. The presented $^{13}CO_2/^{12}CO_2$ ratio is standardized by the Pee Dee Belemnite standard [12]. For each test, a total of 46 breath samples were automatically analyzed.

Blood Sampling and Analysis

Bloods samples were drawn from a peripheral vein before injection of the substrate, and after 30 seconds, 1, 2, 5, 10, 20, 30 and 60 minutes. Samples were taken in a standardized way. Firstly, 5 mL of blood were sampled and discarded. Secondly, a sample of 5 mL was taken in a serum tube for analysis. Finally, the catheter was flushed by 10 mL of 0.9% sodium chloride solution. Serum probes were centrifuged with 3,000 rpm for 4 minutes and the serum aliquot was taken into a separate tube. Probes were analyzed for the concentration of methacetin by high performance liquid chromatography (HPLC). The analysis was performed by a specialized pharmacologist, who was blinded from the breath test results. For sample preparation 50 µL serum were mixed with 100µL of a acetonitrile methanol solution (1 : 1) and centrifuged 14,000 rpm for 8 minutes. Finally, 10µL of each sample was applied to the analyzer. A commercial HPLC-Test-Kit for measurement of levetiracetam in serum (Chromsystems GmbH, Munich, Germany) was used for analysis. The Kit-conditions were modified for estimation of methacetin. Chromatography was performed with a LC-6B system (Shimadzu, Duisburg, Germany) at a flow rate of 1.5 mL/min, with UV-detection at 260 nm. The sensitivity was 0.5µg/mL with proven test linearity up to a concentration of 100 µg/mL. The mean inter-assay variability for methacetin was 6.8%.

Results

The pilot experiment was performed in a 34-year old male healthy volunteer without any history of hepatic or extra-hepatic disease. His healthy condition was confirmed by routine clinical biochemistry including a standard pattern of parameters (Aspartat-aminotransferase, alanine-aminotransferase, bilirubin, albumine, creatinine, urea, blood count, prothrombin time) and a

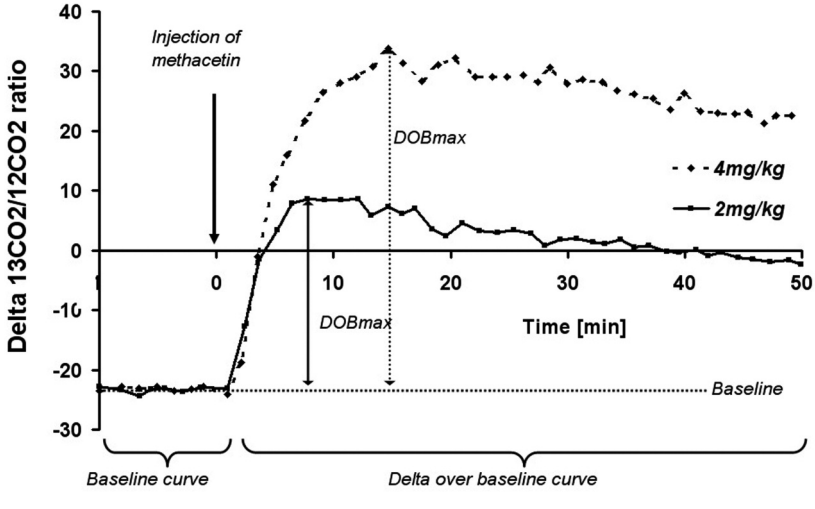

Fig. 3. Breath recovery curve of $^{13}CO_2/^{12}CO_2$ ratio from the ^{13}C-methacetin breath test. ^{13}C-methacetin was applied intravenously in a dosage of 2 and 4 mg/kg and breath recovery was analyzed for in total 60 minutes.

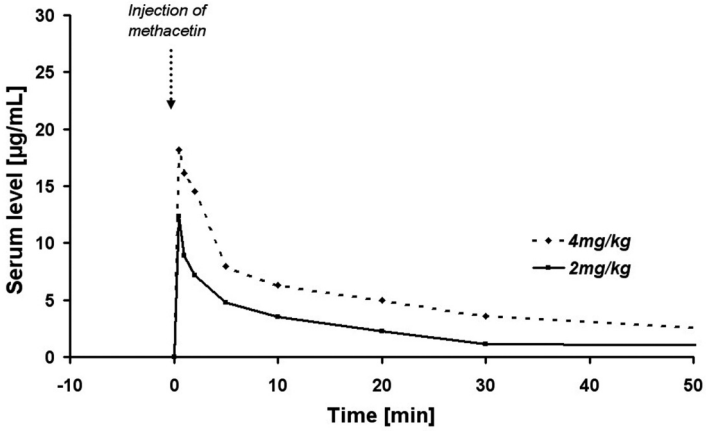

Fig. 4. Serum kinetics of ^{13}C-methacetin from the ^{13}C-methacetin breath test ^{13}C-methacetin was applied intravenously in a dosage of 2 and 4 mg/kg and blood serum samples were drawn during breath analysis.

standard history taking and clinical examination. The tests were performed in a resting position on two consecutive days.

A baseline $^{13}CO_2/^{12}CO_2$ ratio of -23.1 ± 0.3 was measured before injection. The intravenous ^{13}C-methacetin injection lead a rapid increase of DOB, leading to the maximum of DOB (DOBmax) already within 7 minutes for a dose of 2 mg/kg and 15 minutes for a dose of 4 mg/kg (Fig. 3). The $^{13}CO_2/^{12}CO_2$ ratios increased up to + 8.7 (2 mg/kg), and + 33.8 (4 mg/kg) leading to DOBmax values of 31.7 (2 mg/kg), and 57.1 (4 mg/kg), respectively. Consequently, the DOB values continuously decreased slowly, leading to $^{13}CO_2/^{12}CO_2$ ratios after 60min of -2.4 (DOB60min = 20.7 [2 mg/kg]) and 22.6 (DOB60min = 45.9 [4 mg/kg]) (Fig. 3).

By definition, the maximum of serum concentration of ^{13}C-methacetin was reached directly after intravenous injection (first sample after 30 seconds). A maximum of 12.3 µg/mL was determined after injection of 2 mg/kg, and maximum of 18.2 µg/mL after 4 mg/kg, respectively. The concentration rapidly decreased during intracorporeal distribution within few minutes, declining down to 4.8 µg/mL (2 mg/kg) and 8.0 µg/mL (4 mg/kg) within 5 minutes. Thereafter, the concentration further decreased by hepatic metabolism to 1.0 µg/mL (2mg/kg) and 2.1 µg/mL (4mg/kg) at 60 minutes after injection (Fig. 4).

Discussion

Any protocol of breath analysis for dynamic breath test should aim to display the actual metabolism at its best. The literature has reported the successful differentiation between diseased and non-diseased groups by NBTs using ^{13}C-labeled substrates [1, 2]. However, this is only a pre-condition for the successful implementation into clinical diagnostics. Individually reliable test results that prove superior prognostic power in comparison to preexisting diagnostic tests are required [19] and the different algorithms require further standardization for clinical application. If $^{13}CO_2$ is not expired directly but retained inside the body during the active metabolism, this has to be taken into account for the methodology of breath sampling and the correct interpretation of test results. These preliminary results confirm the significant difference between serum kinetics of methacetin and the kinetics of $^{13}CO_2$ in expired breath. Intravenous injection of ^{13}C-methacetin leads to a very early maximum of DOB values within less than 10 minutes, while the substrate levels have already decreased significantly from its maxima directly after injection. This could be interpreted that the physiological metabolism of ^{13}C-labeled methacetin is extremely fast at the administered dosages. Moreover the $^{13}CO_2$ excretion and thus breath recovery appears to be significantly delayed in comparison to the continuously rapid decrease of the substrate serum levels. The prolonged pulmonary excretion of $^{13}CO_2$ over one hour strongly confirms that the quickly produced $^{13}CO_2$ is not completely expired, but a certain magnitude is stored as bicarbonate inside different body compartments. As the ^{13}C-methacetin breath test was meant to analyze cytochrome capacity and not individual bicarbonate kinetics, this phenomenon needs to be considered more thoroughly. As a consequence, protocols that determine test readouts from single time point breath samples could be significantly influenced by individual bicarbonate kinetics. In contrast, the online assessment analyzes a large number of breath samples – without any sampling bags or tubes – and thus could also determine the individual bicarbonate kinetics. As a result, the maximum of $^{13}CO_2$ excretion can be accurately determined at an early point after injection and might be more closely connected to the fast in vivo methacetin metabolism (Fig. 1). Nevertheless, these effects need to be further investigated and confirmed in larger numbers of healthy volunteers and liver diseased patients. In conclusion, accurate test results from NBTs could only be obtained, when other influencing factors such as the physiological serum kinetics of the substrate and the bicarbonate kinetics are taken into account in the development of suitable test protocols.

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