Published in final edited form as:

Expert Opin Ther Pat. 2016 December ; 26(12): 1393–1398. doi:10.1080/13543776.2016.1217995.

Stable isotope biomarker breath tests for human metabolic and infectious diseases: a review of recent patent literature

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Abstract

Introduction—Stable isotope breath tests can rapidly and quantitatively report metabolic phenotypes and disease in both humans and microbes *in situ*. The labelled compound is administered and acted upon by human or microbial metabolism, producing a labelled gas that is detected in exhaled breath.

Areas Covered—This review details the unique advantages (and disadvantages) of phenotypic stable isotope based breath tests. A review of recent US patent applications and prosecutions since 2010 is conducted. Finally, current clinical trials, product pipelines and approved products are discussed.

Expert Opinion—Stable isotope breath tests offer new approaches for rapid and minimally invasive detection and study of metabolic phenotypes, both human and microbial. The patent literature has developed considerably in the last 6 years, with over 30 patent applications made. Rates of issuance remain high, although rejections citing 35 U.S.C. §101(subject matter eligibility), §102 (novelty), §103 (obviousness) and §112 (description, enablement and best mode) have occurred. The prior art is significantly greater for human metabolism than microbial, and may drive differing rates of future issuance. These biomarker and diagnostic tools can enable optimization of drug doses, diagnosis of metabolic disease and its progression, and detection of infectious disease and optimize its treatment.

Keywords

Stable Isotope; Breath test; infection; metabolic disorders

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Declaration of interest

G Timmins is cofounder and current Chief Science Advisor for Avisa Pharma, a clinical stage company that has licensed a number patents (of which Timmins is inventor or co-inventor) on ¹³CO₂ breath test detection of infectious diseases from the technology transfer arm of UNM, STC.UNM. Timmins has license revenue and stock interest managed through STC.UNM. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

1. What is a Stable Isotope Breath Test?

One of the classical ways to study human metabolism is to administer a non-radioactive, stable isotope labeled tracer, and follow its transformation into products by GC-MS or LC-MS of plasma samples. In breath tests however, the stable isotope labeled tracer is transformed into a labeled gaseous or highly volatile product that is excreted through the lungs, and so can be conveniently sampled in exhaled breath, and detected by a range of spectroscopic techniques. The first commercial product was the *Helicobacter pylori* breath test, in which a ^{13}C -urea in a drink is converted by *H. pylori* urease into $^{13}\text{CO}_2$ that is detected in breath by infrared or mass or laser spectroscopy. The field has grown considerably in the last few years, and this review will focus upon patent and other developments since 2010.

2. Why can Phenotypic Biomarkers be as Important as Genotypic Markers?

a) Genotype does not always predict phenotype

Knowledge of an organism's genotype can enable optimal therapeutic treatment. For example, knowing that a person's genotype predisposes them to metabolize a drug rapidly or slowly can enable optimal drug dosing. Likewise, knowing that a pathogenic microorganism's genotype predisposes it to be sensitive or resistant to a proposed antibiotic regimen can enable optimal antimicrobial therapy. However, this holds true only when the linkage of genotype precisely and entirely defines the patient's or microbe's phenotype, but frequently there are non-genetic factors that confound the genotype to phenotype linkage. For humans, although genotype may help predict their drug metabolism phenotype by showing polymorphisms in their cytochrome P450 system, these enzymes can be further induced and/or inhibited in complex manners by concurrently taking other drugs,¹ supplements,^{2,3} or even foods⁴ resulting in genotype-phenotype discordance. Therefore direct determination of drug metabolic phenotype would lead to improved therapy. Likewise in pathogenic microbes, the link between genotype and drug resistance phenotype is of variable strength: in some cases the linkage is unambiguous and allows treatment decisions to be reliably made (*e.g.* tuberculosis diagnosis with rifampin resistance by PCR in Cepheid's Xpert[®] MTB/RIF). However, the linkage between genotype by sequencing and phenotypic pyrazinamide resistance is much more ambiguous, and so the resistance of many of these genotypes is not suitable to guide use of this drug.⁵ Furthermore other resistance mechanisms occur in microorganisms related to persistence such as stochastic expression of activating enzymes⁶ (although this may be epigenetically controlled) or growth in biofilm modes⁷ that cannot be determined by genotype.

b) Many important diseases are inherently phenotypic

The most pressing metabolic diseases, such as obesity, diabetes, pre-diabetes or liver diseases, have to be diagnosed on the basis of phenotype. For example, insulin resistance is diagnosed on the basis of the metabolic phenotype in patients treated with a glucose tolerance test, or gastroparesis (slow stomach emptying) is diagnosed on signs and symptoms and confirmed with scintigraphy after a radiolabeled solid meal.⁸ Similarly, many important aspects of infectious disease are phenotypic, such as biofilm growth, persistence,

or the production of key toxins. For all these diseases, although there can be genetic predispositions, they must be diagnosed by determining the patient's or microbe's phenotype, not genotype.

3. Why Use Stable Isotope Based Breath Tests?

Exhaled breath contains a wide variety of volatile compounds, from excreted gasses such as CO₂ NO or CO, through to a wide range of organic molecules that were first analyzed and described by Pauling *et al.*⁹ This field has significantly expanded, with its own dedicated journal, *The Journal of Breath Research* becoming established in 2007. The study of physiology or disease through analyzing these chemical “fingerprints”, termed the volatome, shows great promise, with tests such as exhaled NO levels entering into clinical practice guidelines.¹⁰ These tests are non-invasive, and do not require the administration of a tracer compound, which makes regulatory approval much more straightforward. However, there are certain circumstances when the administration of a stable isotope labeled tracer can provide important information that is not reflected in the unlabeled volatome. Generally, these measurements are reported as changes in stable isotope ratios, for example changes in the ratio of ¹³CO₂ to ¹²CO₂ in exhaled breath that are measured in per mille (‰) in reference to a standard as δ¹³CO₂, where:¹¹

$$\delta^{13}\text{CO}_2 = 1000 \times \left[\left(\frac{{}^{13}\text{CO}_2/{}^{12}\text{CO}_2 \text{ Sample}}{{}^{13}\text{CO}_2/{}^{12}\text{CO}_2 \text{ Standard}} \right) - 1 \right]$$

This is done because in some cases, there are no detectable changes in the unlabeled volatome that can be used in detection. For example, in the diagnosis of gastroparesis the use of a ¹³C-labeled meal allows detection of gastric emptying, as this is immediately followed by its absorption and metabolism and so an increase in δ¹³CO₂ in exhaled breath:¹² whereas no overall change would be detected in the absolute amount of exhaled CO₂ in the unlabeled volatome. Likewise, changes in the absolute amount of exhaled CO₂ cannot be used to study oral glucose tolerance or drug metabolism, but use of ¹³C-labeled glucose or drug allows detection via changes in δ¹³CO₂ in exhaled breath.

In other cases, there may be multiple pathways to a particular product, with only one pathway providing diagnostic specificity. For example, HCN is present in the normal human volatome,¹³ and elevated levels of HCN in breath have been associated with *Pseudomonas aeruginosa* lung infection.¹⁴ There are other sources of exhaled HCN, such as smoking or from breakdown of absorbed cyanogenic foods, which are independent of *P. aeruginosa* metabolism and so would confound measurements made using absolute amounts of HCN in exhaled breath. Since *P. aeruginosa* synthesizes HCN from glycine¹⁵ by cyanide synthase¹⁶, administration of ¹³C- labeled glycine and measurement of changes in exhaled δ¹³C of exhaled HCN could be used to specifically determine the pseudomonal HCN formation.

Likewise, it is often important to obtain information regarding a specific site or tissue, rather than the whole body as is measured by the unlabeled volatome. For example, if measurements of bacterial urease activity in the lung are desired, without contributions from urease producers in the gastrointestinal (GI) tract, inhaled ¹³C-urea delivers the tracer

directly to the lung. Then rapid metabolism *in situ* in the lung generates a rapid signal of $^{13}\text{CO}_2$ in breath before urea is systemically absorbed, transported to the GI tract, metabolized to $^{13}\text{CO}_2$ which is then transported to the lungs and exhaled- termed the 'kinetic window'.^{17,18} Therefore, there are good reasons why stable isotope tracer breath tests are developed, despite the extra expense and regulatory burdens involved in making and administering a labeled compound to humans.

4. Human Metabolic, Drug Metabolism and other Breath Test Patent Applications

The USPTO website was searched for stable isotope breath test patent applications since 2010, then the public pair website was searched for information regarding the prosecution of these US patent applications, leading to the information in Table 1 regarding eighteen applications. Of these patents, many deal with determination of overall energy balance, carbohydrate metabolism or health, while others focus upon the determination of drug metabolic phenotype in the important enzymes cytochromes P450 2C19 and P450 2D6. Others deal with enabling easier diagnosis of gastroparesis or in diagnosis of a range of hepatic disease. In addition to human metabolism patent applications, there were other uses described for stable isotopes as markers or taggants. In Melker *et al.* a system for ensuring medication adherence is described using stable isotopes to label a dosage form (such as a tablet): upon tablet dissolution and drug absorption an increase in stable isotope labeled gas formed from the taggant is measured in breath.¹⁹ In Keidan, confirmation of the intravascular placement, for example of an intravenous (i.v.) line for a cytotoxic agent, is enabled by administering ^{13}C -labeled bicarbonate that is excreted as $^{13}\text{CO}_2$ in the breath: this avoids incorrect i.v. placement and patient harm, such as through extravasation.²⁰

In the prosecution of these patents, final rejections have only been issued for three of these applications, while two non-final rejections are pending and seven have issued. The dominant basis for rejection has typically been obviousness under 35 U.S.C. §103, as has been observed with other stable isotope patent application rejections,²¹ although 35 U.S.C. §101 and §102 have also been cited. However, human metabolic studies using stable isotopes have been reported since the 1950's,²² and so a considerable body of prior art exists, especially in diabetes and related metabolic syndromes.

However, one potential counter-argument against §103 is found in the rejections of Gabriel,²³ regarding detection of cancers using oxygen isotope ratios. Multiple final and non-final rejections noted "The predictability of breath biomarker analysis for predicting cancer is unknown in the prior art. Indeed, Smith et al. teach that "[b]reath analysis is a relatively new area of research and much more needs to be done." (p. 255, 1st col., 2nd para.). They further indicate that while this research is valuable, there is no guarantee that diagnostic biomarkers will be found, especially not the specific compounds (instead of complex fingerprints of many compounds) that would be the pinnacle of accomplishment in this field." This analysis might support arguments as to the unpredictable efficacy of breath test detection (which is a complex function of many variables many of which cannot be experimentally varied) combined with the difficulty in determining specific compounds as

opposed to ‘fingerprints’. Such arguments might prove persuasive in enabling continued issuance in this area.

5. Microbial Metabolic Breath Test Patent Applications

The USPTO website was searched as described previously, but for microbial metabolism, as opposed to human, with eleven patent applications detailed in Table 2. These cover the use of a wide range of metabolic transformations, predominantly using stable isotope labeled substrates, for the detection of microbial metabolism within humans, for improved diagnosis and treatment of microbial disease. For example, conversion of doubly ^{15}N -labeled isoniazid to the rare analyte $^{15}\text{N}_2$ by mycobacterial KatG is described by Timmins *et al.*²⁴ and also in a companion publication, to allow rapid diagnosis of isoniazid susceptible tuberculosis.²⁵ Since microbial metabolic processes are often different from those of humans, there is considerable potential for specific determination of their metabolism *in situ*, despite extensive host metabolism. In a hybrid approach, Assadi-Porter *et al.* describe how changes in endogenous exhaled gas isotope ratios that occur during infection, as human metabolism alters, can be used to diagnose the onset of sepsis, this approach does not require use a tracer.²⁶ Perkett describes how fractionation of breath- measuring isotope ratios early and late during a breath- can determine the site of infection, to discriminate between tracheal colonization or deep lung infection and improve therapy.²⁷ Finally, Rigas *et al.* describe a breath test for *H. pylori* based upon unlabeled urea, and detecting increases in breath ammonia, mirroring prior breath tests that have used isotope detection.^{28,29}

In the prosecution of these eleven patents, final rejections have only been issued for one, while two non-final rejections are pending and five have issued. Again, as for the human metabolic breath tests, the basis for rejection has usually been obviousness under 35 U.S.C. §103, although 35 U.S.C. §101 and §102 have also been cited. As lead inventor in many of these patents, the author’s opinion is that issuance proceeded smoothly and uneventfully, presumably due to a much smaller body of prior art than is the case for human metabolism. There are also more uncontrollable variables than for human metabolism tests, that make successful reduction to practice highly unpredictable.¹⁷ The clinical utility of many of these breath tests remains to be developed, depending upon the specificity of the bacterial metabolism probed. The inhaled urea breath test possibly detects a very wide range of lung pathogens, and would not be a stand-alone test, although the association of urease positivity with most drug resistant ESKAPE pathogens,³⁰ might make it useful in pneumonia diagnostic algorithms. In contrast, there appears greater specificity for the use of inhaled labeled isoniazid for drug-sensitive *M. tuberculosis*, although it may still be best applied as part of an overall tuberculosis diagnostic and treatment approach.

6. Current Trials, Product Pipeline and Approved Products

The US Clinicaltrials.gov website was searched for open or yet to enroll clinical trials using the terms isotope and breath, and the results presented in Table 3. Ongoing trials are testing the capabilities of metabolic breath tests to: optimally diagnose gastroparesis through the use of an improved labelled lipid instead of octanoate (a fatty acid); to diagnose a variety of liver diseases using ^{13}C -labeled palmitate or methacetin; and to study excess caloric load.

Additionally, there have been new publications on cytochrome P450 breath tests for 2C19,^{31,32} and 2D6,^{33,34} that continue to demonstrate their promise to accurately diagnose complex drug metabolic phenotypes and drug–drug interactions. Also ongoing are trials to use longitudinal changes in breath $^{13}\text{CO}_2$ without requiring a tracer to detect sepsis in adult trauma and intensive care unit contexts.

In the product pipeline, Exalenz Bioscience continues to develop and commercialize a range of liver disease breath test diagnostics that could represent significant advances in areas of great unmet clinical need. These tests focus upon acute liver failure, chronic liver disease, clinically significant portal hypertension, hepatocellular cancer and non-alcoholic steatohepatitis. Avisa Pharma is developing an inhaled ^{13}C -urea breath test for use in the detection of a range of lung pathogens in diseases such as tuberculosis and pneumonia. Regarding approved products, the FDA recently approved the gastric emptying breath test developed by Advanced Breath Diagnostics, in 2015 and this now joins the two other approved stable isotope breath test products for the detection of *H. pylori*, BreathID Hp[®] (marketed by Exalenz) and BreathTek[®] marketed by Otsuka America Pharmaceuticals Inc.

7. Expert Opinion

The field of stable isotope breath test diagnostics continues to show significant potential for continued expansion, both from the development of the intellectual property reviewed here and also from ongoing discovery in this area. The recent FDA approval of the gastric emptying breath test, for use in the diagnosis of gastroparesis- a disease that can only be diagnosed by phenotype- confirms the author's opinion that this approach can be of much broader clinical use than just the diagnosis of *H. pylori*. The significant body of issued patents and completed and ongoing clinical trials, both historical and recent (covered in this Expert Opinion) cover a wide range of human metabolic disease, cancer and infectious disease which have significant unmet diagnostic and/or treatment monitoring needs.

There are many potential future potential areas of development. In the case of human metabolism, these include tests for both common and rare metabolic disease. An area that should receive attention is analogous to the many genotype based biomarkers and companion diagnostics for targeted cancer therapies: the development of companion diagnostics to support reimbursement and drive the utilization of new therapeutic options. In many cases, these tests take a relatively long time (several hours), and so can be inconvenient to administer in ambulatory settings. However, the development of low cost and intelligent $^{13}\text{CO}_2$ sensors with suitable prompting interfaces could enable many of these metabolic tests (such versions of the glucose tolerance test, or gastric emptying) to be conveniently performed in patients homes, with result automatically checked and uploaded into electronic patient records.

Another area of significant need is the development of both broad based and highly specific tests for a range of important microbial pathogens and their phenotypes. For example, the author and collaborators are developing a range of phenotype based drug resistance/ sensitivity tests for tuberculosis where the genotype to phenotype linkage is weak or absent, such as for pyrazinamide and the new drug Delamanid (DelybaTM) developed by Otsuka.

Outside of TB, there is a huge need for the improved usage and stewardship of antibiotics to counter the severe threat that extensive antibiotic resistance poses. Breath tests that could differentiate between pneumonias caused by viruses as opposed to bacteria, or caused by bacteria likely to be resistant (such as the ESKAPE pathogens) could prove powerful adjuncts in a range of pneumonia treatment algorithms and enable optimal stewardship by preventing unnecessary antibiotic use, or restricting use to narrow spectrum agents. Additionally, studies should be performed to determine if some of these markers (*e.g.* urease) are rapidly decreased with optimal therapy earlier than clinical signs and symptoms, so that they could guide antibiotic usage, and perhaps provide new end points for ending therapy earlier.

Also, it is worth noting that because of the high concentration of CO₂ in exhaled breath, and the significant natural abundance of ¹³C (1.1%) it requires both a significant dose of ¹³C-labelled tracer and its metabolism to ¹³CO₂ for detection. The use of tracer isotopes that are rare, or of tracers that are metabolized to gasses that are of low abundance in exhaled breath, could greatly increase the sensitivity of the approach. Therefore the development of analytical techniques such as GC-IRMS in highly portable and rugged formats would enable point of care detection of a wide range of analytes and could even support isotope and tracer multiplexing so that several different tests could be run simultaneously. In the extreme case, broad and differential patterns of labelling could enable additional information and/or specificity to be obtained from broad based volatome studies.

Finally, it is worth noting that many of these kinds of tests can be used in animals to enable more comprehensive studies of metabolism in a wide range of experimental models of both physiology and pathophysiology (elegantly reviewed by McCue and Welch)³⁵, but also in the veterinary diagnosis of disease.

Acknowledgments

Funding G Timmins is currently funded by an NIH grant, AI117224.

Reference annotations

* Of interest

** Of considerable interest

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Article highlights

- Stable isotope breath tests can diagnose a range of metabolic disease, pathophysiology and infectious disease, and can act as biomarkers of treatment response.
- These tests directly measure phenotype, and provide information that can either complement, or is not available from, genotypic information.
- There is a significant and growing body of patent applications in this area, still with a relatively high rate of issuance.
- Several clinical trials of stable isotope breath tests are both ongoing and planned, and a new test for use in the diagnosis of gastroparesis has recently been approved.
- Significant commercial activity in this area bodes well for the development and approval of a wide range of diagnostic and biomarker tests to improve patient outcomes.

Table 1**Status of Recent Metabolic Breath Test US Patent Applications**

Application	Status and Rejection Basis
Methods of determining energy balance... ³⁶	No office action *
Sniffing smartphone ³⁷	Issued
Method for measuring carbohydrate metabolism ³⁸	No office action
Medication adherence monitoring system ¹⁹	Non-final rejection §103
Methods for diagnosis, prognosis... ³⁹	No office action
Gastric emptying breath tests ⁴⁰	Non-final rejection §103
Providing evidence whether an intravascular... ²⁰	No office action
Single-point gastric emptying breath tests ⁴¹	Response to restriction filed
Breath test device and method ⁴²	Issued
Detection of rate changes in systematic... ⁴³	Issued
Superior analyzer for raman spectra... ⁴⁴	Issued
Triple isotope method and analyzer... ⁴⁵	Issued
Stable isotopic biomarker measurement... ²³	Final Rejection §112
Methods to evaluate cytochrome P450 2C19... ⁴⁶	Restriction required
Method for determining the liver performance... ⁴⁷	Final rejection §101, 103
Fluid bed meal containing a marker.. ⁴⁸	Issued
Method and composition to evaluate cytochrome... ⁴⁹	Final rejection §102, 103
Breath test device and method ⁵⁰	Issued

* USPTO last accessed 2/19/2016

Table 2**Status of Recent Microbial Breath Test US Patent Applications**

Application	Status
Diagnostic agent and diagnostic method... ⁵¹	Final rejection §103
Methods for diagnosing bacterial infections ⁵²	No office action
Compositions and kits for diagnosing infections ⁵³	Non-final rejection §103
Methods of using isoniazid for the diagnosis... ²⁴	Restriction required
Rapid test for detection of infection... ⁵⁴	Issued
Determination of location of bacterial load ²⁷	No office action
Identification of disease characteristics using... ²⁶	Issued
Detection of <i>H. pylori</i> utilizing unlabeled urea ⁵⁵	Amended after Non-final rejection §101, 102, 103
Diagnosis of infection in the lungs of patients ⁵⁶	Issued
Methods of diagnosing and treating small... ⁵⁷	Issued
Non-invasive rapid diagnostic test for... ⁵⁸	Issued

Table 3

Ongoing Clinical Trials and Approved Products

Study/Product Name	Status
Prediction Value of the BreathID ¹³ C-Methacetin Breath Test... ⁵⁹	Recruiting
¹³ C-Trioctanoate breath test as a measurement of gastric... ⁶⁰	Recruiting
Palmitate breath test to assess fatty acid oxidation in... ⁶¹	Recruiting
Changes in ¹³ CO ₂ / ¹² CO ₂ delta value in exhaled breath... ⁶²	Recruiting
Changes in ¹³ CO ₂ / ¹² CO ₂ delta value in exhaled breath... ⁶³	Recruiting
The Breathe Light study ⁶⁴	Recruiting
OBT measurement to differentiate between presence and... ⁶⁵	Not yet recruiting
Prediction value of the BreathID ¹³ C-methacetin breath test... ⁵⁹	Recruiting
Assessing portal hypertension with methacetin breath test ⁶⁶	Recruiting
Gastric Emptying Breath Test (Advanced Breath Diagnostics)	US Approved
BreathID Hp® (Exalenz)	US Approved
BreathTek® Urea Breath Test for <i>H. Pylori</i>	US Approved