Review paper

$^{13}CO_2$ breath tests in non-invasive hepatological diagnosis

Joanna Musialik[1], Krzysztof Jonderko[1], Anna Kasicka-Jonderko[1], Magdalena Buschhaus[2]

[1]Department of Basic Biomedical Science, School of Pharmacy in Sosnowiec, Medical University of Silesia, Katowice, Poland
[2]Centre for Therapeutic Discovery, Medical Research Council Technology, London, UK

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Address for correspondence: Prof. Krzysztof Jonderko MD, PhD, Department of Basic Biomedical Science, Medical University of Silesia, 3 Kasztanowa St, 41-205 Sosnowiec, Poland, phone: +48 32 269 98 30, fax: +48 32 269 98 33, e-mail: kjonderko@sum.edu.pl

Abstract

In liver diagnostics, a simple, non-invasive test with high sensitivity and specificity is permanently being sought in order to assess the degree of liver damage. In addition to liver biopsy, algorithms using blood parameters or elastometry are used in clinical practice. However, these methods do not provide information about the true liver reserve, so the liver breath test seem to be a promising diagnostic tool. The basis of this test depends on the ability of particular hepatocyte enzyme systems to metabolise a tested substance labelled with a stable carbon isotope. The kinetics of $^{13}CO_2$ elimination with expiratory air then permits quantitative assessment of the functional liver reserve and the degree of organ damage. In this paper the most commonly used tests, grouped according to the main metabolic pathways, are described. The usefulness of liver breath tests in specific clinical situations, both as a diagnostic and prognostic tool, is presented.

Introduction

By the severity of liver disease we mean both the advancement of dynamic processes, such as inflammatory activity, and the progression of fibrosis including liver cirrhosis. This information allows for appropriate therapeutic decisions to be made and helps plan for further procedures. The gold standard in this assessment remains, despite the development of non-invasive methods, histopathological examination of a liver biopsy specimen, which provides relatively accurate information on inflammatory activity and fibrosis. This invasive examination is difficult to be considered ideal, as it poses risk of complications to the patient, thus making it unsuitable for frequent administration, and more so due to the possibility of sample error – too small size of a sample or taken too superficially under the liver capsule. Finally, a liver biopsy may sometimes not be possible due to medical reasons, or simply because of patient fear. These factors have led to the rapid development of non-invasive methods, of which the most popular has become elastography (Fibroscan, Echosens, France), in which, with the use of ultrasound waves, the degree of liver "stiffness" is measured and the amount of deposited connective tissue is assessed, taking into account the fact that with progression of fibrosis the velocity of the wave propagation increases. Almost simultaneously, a number of laboratory diagnostic algorithms have been formed, based on different combinations of parameters determined in the peripheral blood, both biochemical and serological. The simplest of these, as well as the most sensitive, include the APRI index [1] established on the relation between activity of aspartate aminotransferase (AST) and platelet count, the FORNS index [2] based on the dependence between patient's age, cholesterol level, activity of gamma glutamyl transpeptidase, and platelet count, and FibroIndex [3], which uses the activity of AST, gamma globulin concentration, and platelet count, all of these having found their place in daily practice. These algorithms confirm the likelihood of liver cirrhosis with high probability; however, further differentiation of the degree of fibrosis requires extensive diagnostic methods or expensive commercial tests such as Fibrotest (Biopredictive, France), or liver biopsy. All of the mentioned methods, however, do not provide information about the actual capacity of the organ and its functional reserve.

Dynamic tests accessing liver function

The main functions of the liver beyond the synthesis of biologically important substances, such as coagulation factors and albumin, are cleansing, metabolism, and excretion of endo- and xenobiotics. Evaluation of the synthetic liver function is relatively simple through routine blood tests. Evaluation of the detoxification function is difficult and requires specialised diagnostic methods because it depends both on the amount of active hepatocytes and on hepatic blood flow. The specificity of liver function requires assessment of both the ability to transport organic anions, which translates into bilirubin and bile acids, and the rate of metabolism of exogenous substances. There has been ongoing research into simpler, non-invasive, and safer methods for the patient for assessing functional organ reserve. One of the better known, but now rather historical methods, is a test with bromosulfophthalein (BSP), which, despite turning out to be useful in detecting even clinically silent liver damage, requires an intravenous dye injection followed by several blood samples to comply with the colorimetric assessment. A second simpler test utilises indocyanine green (ICG), requiring only a single blood sample. It is, however, less sensitive in detecting mild liver dysfunction but is still used occasionally today [4, 5].

Breath tests

An ideal test to assess hepatic reserve should use a non-toxic substance, metabolised in the liver, administered orally, and easy to evaluate in non-invasively collected material: urine, saliva, or breath. Close to this ideal are breath tests utilising a carbon ^{13}C labelled substance that is metabolised in the liver to carbon dioxide and exhaled by the patient along with the expiratory air. The measure of the recovered marker and the time of its appearance in the exhaled air allows for the evaluation of liver metabolic activity. Despite many metabolic pathways in the liver, assessment of one pathway is sufficient to measure hepatic functional reserve [5]. These tests use different metabolic pathways for xenobiotics and therefore are divided into three groups: microsomal, mitochondrial, and cytosolic – evaluating the efficiency of enzymatic systems located in different areas of the cell. Table I shows the distribution of breath tests and the substances utilised.

Microsomal tests

These tests evaluate the efficiency of a microsomal enzyme complex associated with the effective metabolism of drugs and toxic substances. It is located on the smooth endoplasmic reticulum, with cytochrome P450 being the most important group among the enzymes that form it. Cytochrome P450 is a heterogeneous group of proteins, differing in both structure and location. The function of these enzymes is involvement in the processes of oxidation, in which oxygen formed during metabolism combines with reduced heme iron. The metabolised substance combines with cytochrome in the region adjacent to the iron. During the process of oxidation, the iron is oxidised and one molecular oxygen atom is incorporated in the xenobiotic while the other is incorporated in the resulting water.

The ability of the liver to remove xenobiotics depends on two factors: liver perfusion and the extraction factor (*E*), which is the ratio of the difference in concentration of a given substance in the blood entering and leaving the liver and the concentration of the substance in the blood flow. Based on this factor, some assays are restricted by blood flow through the liver (when the *E* factor is higher than 0.7) or provide information about the metabolic liver reserve (when the *E* factor is lower than 0.3). Due to the detoxification functions, the microsomal activity can be a good indicator of liver function, and in addition the activity of cytochrome P450 is reduced in the course of viral liver diseases, due to the reduction in expression of genes encoding these enzymes under the influence of secreted interferons, transforming growth factor (TGF), or other cytokines in response to hepatitis C virus (HCV) infection, for instance. Animal studies have also shown that the function of this cytochrome may be dependent on fibrosis in the liver with sinusoid capillarisation [6].

Within the group of microsomal tests, invasive tests feature where a labelled substance is administered intravenously (antipyrine, caffeine, lidocaine), or non-invasive where the substance is administered orally (aminopyrine, diazepam, methacetin, and phenacetin). For obvious reasons, non-invasive tests are replacing invasive ones because, beyond the disadvantage associated with administration of the substance, adverse side effects have also occurred. Non-invasive tests in which

Table I. Division of liver breath tests

Test group	Metabolised compound
Microsomal	Aminopyrine, phenacetin, methacetin, erythromycin, diazepam, caffeine
Mitochondrial	α-Ketoisocaproic acid, methionine, octanoic acid
Cytosolic	Galactose, phenylalanine, tyrosine, fructose, alanine, ornithine

the test substance is labelled with carbon ^{13}C are based on the same principle – one of the carbon atoms of the examined substance is replaced with the stable isotope ^{13}C, which will eventually be detected in expiratory air [4, 5, 7]. There are two distinct subgroups in this group of tests: (a) tests limited by enzyme activity (aminopyrine and caffeine) and (b) tests limited by the liver blood flow (methacetin and phenacetin).

An example of a test from the first group is the aminopyrine breath test (ABT), which for many years was one of the most frequently performed tests to evaluate the function of cytochrome P450. After oral administration of the substrate – labelled dimethylaminoantipyrine – it is completely absorbed from the intestine, and then metabolised in the liver during the two-step N-demethylation by the cytochrome P450 system. Finally, bicarbonate is formed, which in part is excreted in breath. In addition, due to the low extraction ratio (E = 0.2), aminopyrine metabolism is not dependent on changes or abnormalities (presence of vascular shunts) in the liver blood flow [7]. The work of Giannini *et al*. [8] demonstrated the usefulness of ABT in differentiating the severity of chronic hepatitis C (CHC). The individual test parameters allowed for isolation of patients with post-inflammatory cirrhosis and differentiation of patients with low ($F \leq 2$ by METAVIR) and advanced ($F > 2$ by METAVIR) fibrosis. However, a 2-hour cumulative recovery of ^{13}C in expiratory air proved to be useful for the assessment of necrotic-inflammatory activity.

A subsequent test within this group is a test utilising caffeine (CBT, caffeine breath test), which has many of the features of an ideal test: low extraction ratio, moderate cost of the substrate, and, especially, a single sample requirement, received 1 or 2 h after administration, permitting the caffeine test to enter into daily practice alongside routine blood tests [6]. In a recently published study, the test allowed the assessment of the progression of non-alcoholic fatty liver disease (NAFLD). In this study, significant differences were noted in the subsequent groups of patients with NAFLD: the group with steatosis inflammation, cirrhosis, as well as simple steatosis [9, 10].

An example of a test from the second group is the methacetin breath test (MBT). Methacetin (N-(4-methoxyphenyl)acetamide), a derivative of phenacetin, is rapidly metabolised by D-methylation in the liver oxidative system, leading to the formation of acetaminophen and carbon dioxide [6]. Additional factors including, age, sex, smoking, or the use of other drugs has less influence on the methacetin metabolism, in contrast to aminopyrine [11–14]. In addition, this test has very good reproducibility [15], and the only limitation is the high rate of extraction, which may be of importance in patients with severe portal hypertension or in the presence of vascular shunts.

This test proved to be highly sensitive (89%) in recognition of liver cirrhosis of different aetiology, and in patients with CHC [16–18]. The MBT also showed the highest sensitivity and specificity in comparison to other non-invasive indicators (Fibrotest, APRI, De Ritis' ratio) with regards to predicting liver cirrhosis [19]. It has also allowed for indication of inflammatory activity in the livers of patients with CHC and normal transaminase levels [20]. However, in a dual study using MBT and phenylalanine breath test, only MBT permitted differentiation of patients at various stages of liver cirrhosis induced by HCV infection [21]. The MBT also proved to be a sensitive, non-invasive test to monitor return of normal liver function after liver transplantation and to monitor improvement in liver function in acute liver inefficiency, indicating improvement a few days earlier than specified by standard biochemical tests [22, 23]. The case study by Hydzik *et al*. [24] demonstrated the usefulness of the test in predicting the outcome of a patient with liver failure post consumption of *Amanita phalloides*, showing only a slight percentage (0.09%) of $^{13}CO_2$ uptake on the fourth day of the disease, and then 0.02% the next day, whereas a normal result is an average of 12%. Recently it was reported that the results from within the first 30 min of the ^{13}C-MBT test reliably reflect the disease advancement stage in primary biliary cirrhosis [25].

Another microsomal test is the erythromycin test, although it did not find wider application because of the lack of correlation between the test results and biochemical parameters or severity of liver disease. Nonetheless, its usefulness has been demonstrated in predicting the neurotoxicity of tacrolimus, an immunosuppressive drug used in the treatment of vascularised organ transplantation and acute liver transplant dysfunction or its insufficiency [7].

Mitochondrial tests

Mitochondrial function in the liver cell is related to the metabolism of carbohydrates, proteins, fats, and xenobiotics, as well as being a major source of cellular energy. Mitochondrial liver dysfunction induces manifestation, or progression, of chronic liver disease by inducing microsteatosis. This occurs in NAFLD or pregnancy, Reye's syndrome, or in the case of toxic effects of certain drugs like amiodarone and tetracycline, or antiviral drugs [26]. Mitochondrial damage in these instances is due to impairment of trans-membrane electron transport and/or synthesis of ATP [26].

One of the primary mitochondrial tests uses α-ketoisocaproic acid (KICA). After oral administration this

acid undergoes almost exclusive decarboxylation in liver mitochondria following inhibition of its second metabolic pathway, which is transamination into leucine. Therefore, in order to block the latter pathway, the patient receives KICA together with leucine. Recent studies have shown that the KICA breath test (KICA-BT) is characterised by very good reproducibility in the short and medium term [27].

The usefulness of KICA-BT was demonstrated by examining KICA metabolism in healthy volunteers who were administered with ethanol or acetylsalicylic acid. Alcohol significantly reduced the decarboxylation of KICA and delayed the appearance of the marker in exhaled air, whereas acetylsalicylic acid increased the decarboxylation of KICA by inducing transformation of NAD+ to NADH [26, 28]. The usefulness of KICA in the analysis of the potential toxicity of drugs subjected to mitochondrial changes was established. The recognised toxicity of tacrolimus was considered due to inhibition of KICA decarboxylation and disturbance of the energetic processes in the liver, causing acute (lasting up to 5 h) suppression of the energy production in hepatocytes [8]. Recently it was reported that the metabolism of ^{13}C-KICA augments during the intake of combined oral contraceptives containing ethinyl estradiol, reflecting enhanced liver mitochondrial metabolic activity [29]. Despite several articles demonstrating the usefulness of the KICA-BT test in the evaluation of alcoholic liver disease, this test was not implemented as a routine clinical diagnostic, because alcohol probably also affects other determinants of KICA changes such as decarboxylase phosphorylation, the availability of coenzyme A, and intramitochondrial calcium levels [26]. An interesting double study by Palmieri *et al*. [30] demonstrated the usefulness of KICA-BT for evaluation of liver function in patients with liver cirrhosis and hepatocellular carcinoma (HCC), demonstrating decreased values in KICA-BT, without significant changes in MBT results. Also, further deterioration in KICA-BT results detected in patients previously treated with ablation using electromagnetic waves of radio frequency (RFA, radiofrequency ablation) was an early predictor of HCC recurrence.

The methionine breath test (MetBT) is another mitochondrial test in which the utilised amino acid is processed via two metabolic pathways. In a healthy liver, methionine undergoes transsulphuration into homocysteine, involving adenosyltransferase. In the failure of the first pathway, methionine is metabolised by transamination into α-keto-β-methiolbutyrate, which is further subjected to changes in the carboxylic acids cycle with the release of labelled $^{13}CO_2$ [26]. This test has proven useful in assessing acute toxic liver steatosis after administration of valproic acid [31] and in patients with liver steatosis or cirrhosis of various aetiologies, wherein the amount of $^{13}CO_2$ released per hour in the latter group was inversely proportional to the severity of the disease in Child-Turtcotte-Pugh score (CTP) [32]. Another study showed the usefulness of MetBT in assessing the viability of liver grafts within the first days after transplantation [26].

Cytosolic tests

The aromatic amino acid metabolism takes place in the cytosol of hepatocytes, and its concentration in the cytoplasm is directly dependent on the function of the liver cell. The best-known and most used test in this group is the breath test with labelled phenylalanine (PheBT). This amino acid is metabolised primarily by irreversible hydroxylation to tyrosine involving phenylalanine hydroxylase. Hydroxylation of phenylalanine and the latter tyrosine transamination have limited enzymatic capacity, so analysing the presence of the end product ($^{13}CO_2$) in the expiratory air correlates well with the function of the liver enzymes, and since both of these metabolic pathways are only found in the liver, they are sensitive indicators of liver dysfunction [33]. Enzymes tested in cytosolic assays with phenylalanine or tyrosine (TyrBT, tyrosine breath test) are not subjected to the potential induction caused by simultaneously administered medications that may interfere in the classical microsomal assays. Furthermore, there are no adverse reactions to the amino acids used in the test, as described in the literature. On the contrary, aminopyrine, for example, has a potential risk of inducing agranulocytosis, and hence has not been approved in the United States [33]. However, the disadvantage of PheBT is the involvement of an alternative metabolic pathway in acute liver failure, and the resulting metabolites, being neurotransmitters, are likely to exacerbate hepatic encephalopathy [33]. Also, the reproducibility of the ^{13}C-PheBT appears to be remarkably worse than in the case of either ^{13}C-MBT or ^{13}C-KICA-BT [34].

This test, however, confirmed its usefulness by discriminating between groups of patients with compensated and decompensated cirrhosis, as well as between patients with oesophageal varices of varying degrees of severity [35, 36]. Another study showed that the Phe-BT result was an independent survival predictor in patients with hepatic impairment [37].

A subsequent cytosolic test evaluates galactokinase efficiency. The galactose breath test (GBT) has a lower value if liver disease is associated with simultaneous alcohol consumption, and this pertains also to concomitant diabetes. This is caused by diluting out of labelled glucose, arising from the conversion of galactose, in bodily fluids, which leads to a reduction in $^{13}CO_2$ con-

Table II. Clinical usefulness of selected liver breath tests

Test group	Substrate	Clinical usefulness
Microsomal	Aminopyrine	Staging of chronic hepatitis C
	Methacetin	Staging of liver cirrhosis, acute liver failure, assessment of fibrosis in chronic hepatitis C or primary biliary cirrhosis, evaluation of graft functioning after liver transplantation
	Erythromycin	Assessment of drug toxicity
Mitochondrial	α-Ketoisocaproic acid	Assessment of drug toxicity, staging of alcoholic or non-alcoholic fatty liver disease, relapse of hepatocellular carcinoma
	Methionine	Assessment of drug toxicity, staging of non-alcoholic fatty liver disease, staging of liver cirrhosis
Cytosolic	Phenylalanine/tyrosine	Diagnosis of liver cirrhosis, survival prognosis in liver cirrhosis patients
	Galactose	Staging of chronic hepatitis C or primary biliary cirrhosis

centration [33]. However, in patients with primary biliary liver cirrhosis, the test confirmed its high specificity and sensitivity for the evaluation of liver function, as well as in the early stages of the disease, further allowing patients to be grouped according to the severity of fibrosis, which may be useful for non-invasive diagnosis of disease progression in this rare disease entity [38].

Conclusions

Although breath tests have been available for many years, they have not taken a stable position among the non-invasive diagnostic methods of liver damage. It may seem peculiar, given the results and statistics presented in this review. Table II summarises the types of breath tests and their clinical utility. However, in recent years there has been a renewed interest in this group of studies. This is most likely due to the general trend among physicians to replace invasive tests (poorly reproducible and involving higher risks to the patient) with non-invasive tests that can be repeated frequently and therefore can be used for continuous monitoring of disease progression. Among these are breath tests – a simple and safe way to monitor not only damage to the liver tissue due to fibrosis, but also its metabolic reserve. No single serological test, or even expensive diagnostic kits, would give such a precise insight into the metabolic efficiency of the liver. Often we are faced with the dilemma of whether it is worth commencing a treatment or it would be best to wait. This is particularly the case with CHC, where the effectiveness of currently available treatment in Poland is just 50%, and in more advanced cases it is even further reduced. Breath tests could therefore help us in making therapeutic decisions, especially in difficult patient cases – with advanced disease or after a failed treatment. In Poland it is possible to perform these tests in several centres and the cost of the study is much lower than elastography (Fibroscan) or Fibrotest. Consequently, the decision as to whether these tests will become routine hepatological diagnostics depends solely on our knowledge and belief.

Conflict of interest

The authors declare no conflict of interest.

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