BRIEF ARTICLE

^{13}C-methacetin breath test reproducibility study reveals persistent CYP1A2 stimulation on repeat examinations

Anna Kasicka-Jonderko, Anna Nita, Krzysztof Jonderko, Magdalena Kamińska, Barbara Błońska-Fajfrowska

Anna Kasicka-Jonderko, Anna Nita, Krzysztof Jonderko, Magdalena Kamińska, Barbara Błońska-Fajfrowska, Department of Basic Biomedical Science, School of Pharmacy, Medical University of Silesia, PL-41-205 Sosnowiec, Poland
Correspondence to: Dr. Anna Kasicka-Jonderko, Department of Basic Biomedical Science, School of Pharmacy, Medical University of Silesia, Kasztanowa street 3, PL-41-205 Sosnowiec, Poland. akj@sum.edu.pl
Telephone: +48-32-2699830 Fax: +48-32-2699833

Abstract

AIM: To find the most reproducible quantitative parameter of a standard ^{13}C-methacetin breath test (^{13}C-MBT).

METHODS: Twenty healthy volunteers (10 female, 10 male) underwent the ^{13}C-MBT after intake of 75 mg ^{13}C-methacetin p.o. on three occasions. Short- and medium-term reproducibility was assessed with paired examinations taken at an interval of 2 and 18 d (medians), respectively.

RESULTS: The reproducibility of the 1-h cumulative ^{13}C recovery (AUC_{0-60}), characterized by a coefficient of variation of 10%, appeared to be considerably better than the reproducibility of the maximum momentary ^{13}C recovery or the time of reaching it. Remarkably, as opposed to the short gap between consecutive examinations, the capacity of the liver to handle ^{13}C-methacetin increased slightly but statistically significantly when a repeat dose was administered after two to three weeks. Regarding the AUC_{0-60}, the magnitude of this fixed bias amounted to 7.5%. Neither the time gap between the repeat examinations nor the gender of the subjects affected the ^{13}C-MBT reproducibility.

CONCLUSION: ^{13}C-MBT is most reproducibly quantified by the cumulative ^{13}C recovery, but the exactitude thereof may be modestly affected by persistent stimulation of CYP1A2 on repeat examinations.

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INTRODUCTION

There is no surprise that non-invasive diagnostic procedures in medicine attract much attention from either side participating in the search of any cause of malfunctioning of the organism-patients would definitely prefer methods sparing them the necessity of encroaching the body integrity, like for example a liver biopsy, whereas physicians too would prefer to avoid inflicting pain on their patients. A brilliant idea of per oral administration of ^{13}C-enriched substrates in order to get information on the metabolic efficiency or functional mass of the liver is a

practical answer to the requirement outlined above. Continuous research work has brought about considerable progress and nowadays it is possible to assess by means of ^{13}C breath tests the microsomal, the cytosolic, and the mitochondrial function of the liver[1-4]. A look at pertinent literature shows that during the past decade, from among the compounds applied for microsomal ^{13}C breath tests, ^{13}C-methacetin has steadily been making its way to be recognized as the most frequently used substrate. A number of features support its usefulness as a functional liver probe: a fast metabolism to acetaminophen and ^{13}CO$_2$ by cytochrome P450 1A2 (CYP 1A2), safety at low doses applied for a breath test, and a low cost[1-4]. Accordingly very promising results on its diagnostic usefulness were obtained in patients with chronic hepatitis C virus infection[5,6], primary biliary cirrhosis[7,8], non-alcoholic steatohepatitis[9], and various stages of liver cirrhosis[10-14], including those awaiting a liver transplantation[15].

A vital asset of any measurement or diagnostic method used in medical practice is an ability to provide reproducible results. Quite surprisingly a search of data on the reproducibility of the ^{13}C-methacetin breath test (^{13}C-MBT) revealed this item as being almost a completely blank research area. We decided therefore to search in this prospective study for a quantitative parameter which would offer the best reproducibility for a standard ^{13}C-MBT.

MATERIALS AND METHODS

Subjects

Twenty healthy non-obese subjects, 10 female and 10 male were invited to enter the study; their mean age was 25 years (range 21-31 years), and their average body mass index was 22.38 ± 0.64 kg/m^2. During a screening interview the participants declared themselves as being in full health according to the World Health Organisation criteria[16]. Among the participants three (1 male and 2 female) were habitual cigarette smokers. All the subjects categorically denied a systematic use of significant amounts of alcoholic beverages. None of the volunteers did take any medication except for oral hormonal contraception which was used by 4 female. Exclusion criteria to attend the study comprised a history of surgery affecting the digestive tract anatomy, with the exception of appendectomy, current use of any drugs which might affect gastrointestinal motility, and pregnancy. In every subject the normal structure and size of the liver was confirmed by means of an ultrasonographic examination performed by a qualified investigator.

The study was conducted in accordance with the Helsinki Declaration, and every volunteer gave written consent to participate after getting information as to the aim, protocol and methodology of the project. During the introductory interview the subjects were instructed not to eat any food with a naturally increased ^{13}C content, such as products made of maize, cane sugar, pineapple, or kiwi fruit for 48 h preceding the examination[17-19].

Experimental protocol

Every subject underwent three examination sessions, held on separate days. In half of the volunteers the two first sessions were taken 2-4 d apart, and the third one was pursued 2-3 wk later. In the other half of the subjects the schedule was inversed, i.e. the first two examination measurements were taken 2-3 wk apart, followed by a third one 2-4 d after the second one. The assignment of the order of intervals separating the sessions (short-long or long-short) was randomized. Accordingly, the assessment of the short-term reproducibility involved 20 pairs of examinations separated by a median 2 d break (range 2-4 d), whereas the medium-term reproducibility was evaluated on 20 pairs of the most distant examinations, i.e. taken at a median interval of 18 d (range 17-23 d).

The volunteers came to the laboratory in the morning, after a 12-h overnight fast and abstaining from cigarette smoking (if applicable). In the female volunteers the examinations were taken always within the same phase of their menstrual cycle. After a 15-min rest in a sitting position, necessary for stabilization of the metabolism, a basal sample of the exhaled air was collected. At the time point designated "0" the subjects took 75 mg ^{13}C-methacetin (code INC590P, Euriso-Top SA, Saint-Aubin, France; according to the certificate of analysis, the manufacturer guarantees ≥ 99% isotopic ^{13}C atom enrichment determined by proton NMR) orally dissolved in 200 mL unsweetened black tea. Samples of expiratory air for ^{13}CO$_2$ measurement were collected at 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 40, 50, 60, 75, 90, 105, 120, 150 and 180 min[20]. The procedure of collecting the breath air was standardized: the subjects took in breath and held it for 10 s, then steadily blew the air into a special aluminium covered plastic bag of 1.1 L capacity (Fischer Analysen Instrumente GmbH, Leipzig, Germany) equipped with a mouthpiece and an unidirectional valve[21,22]. The bag was closed with a plastic cork immediately at the end of the exhalation and stored for analysis. The subjects remained seated quietly and fasted throughout the whole period of the collection of the samples.

Measurement of ^{13}CO$_2$ and derivation of the breath test parameters

Concentrations of ^{13}CO$_2$ in the probes of the exhaled air were measured with the use of a non-dispersive isotope-selective infrared spectrometry apparatus (IRIS, manufactured by Wagner Analysen Technik Vertriebs GmbH, Hamburg, Germany; a model equipped with 16 ports for simultaneous mounting of bags with air samples was used). Following the procedures described previously[20,23,24], curves of the momentary and cumulative recovery of ^{13}C in the exhaled air relative to the administered oral dose of ^{13}C-methacetin were constructed within the time domain of 0 to 180 min. Subsequently the following parameters were derived: D$_{max}$ - the maximum momentary ^{13}C recovery in expiratory air, T$_{max}$ - the time elapsing from the intake of the substrate until the occurrence of the D$_{max}$, and AUC$_i$ - the cumulative ^{13}C recovery within
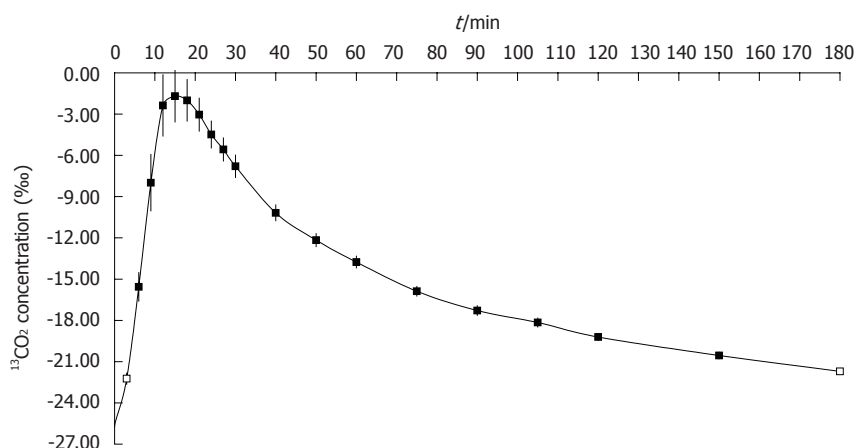
Figure 1 Time course of the ^{13}CO$_2$ content within the total pool of exhaled CO$_2$ after peroral administration of 75 mg ^{13}C-methacetin in 20 healthy volunteers who each underwent three breath tests on separate days. Filled rectangles represent the time points at which the rise in ^{13}CO$_2$ concentration was statistically significantly different from the baseline measurement, open rectangles stand for values not statistically different from the baseline. The values shown are mean ± SE.

Table 1 Reproducibility of the ^{13}C-methacetin breath test

| | D_{max} | | T_{max} | | AUC_{0-60} | |
	S_term	M_term	S_term	M_term	S_term	M_term
CVp	16.23%	16.46%	30.57%	32.49%	10.00%	10.00%
RC	15.55% dose/h	16.80% dose/h	16.86 min	16.73 min	5.84% dose	5.86% dose
Bias proportional	N	N	N	Y	N	N
Bias fixed	N	Y	N	N	N	Y
Delta$_{0.05}$	3.80% dose/h	3.51% dose/h	4.0 min	3.8 min	1.43% dose	1.26% dose

T_{max}: Time to reach the maximum momentary ^{13}C elimination; D_{max}: Maximum momentary ^{13}C elimination; AUC_{0-60}: 60-min cumulative ^{13}C elimination in expiratory air, respectively; S_term: Short term reproducibility assessment on the basis of 20 pairs of ^{13}C-methacetin breath tests separated by a median 2-d break; M_term: Medium-term reproducibility evaluation involved 20 pairs of examinations accomplished at a median interval of 18 d. CV$_P$: Coefficient of variation for paired examinations; RC: Repeatability coefficient. Delta$_{0.05}$: The least difference detectable at $P = 0.05$ level, two-tailed in the case of 20 paired examinations.

a time span i, which was calculated by integration of the respective part of the momentary ^{13}C recovery curve.

Statistical analysis

The data were subjected to the Bland and Altman statistic for calculation of repeatability coefficients and check for a proportional or fixed bias[25,26]. In addition the reproducibility of breath test parameters was expressed in terms of the coefficient of variation for paired examinations, CV$_p$[27,28]. Sets of absolute values of individual differences in paired measurements were compared with the use of the paired or unpaired *t* test (where appropriate) in order to check if factors such as the time gap between the examinations, or the gender of the subjects affected the reproducibility. Other statistical methods comprised the use of a repeated measures analysis of variance (R_ANOVA) followed by a *post hoc* check on the significance of differences between means, which was accomplished with the Tukey's honest significant difference test[29]. Statistical significance was set at the $P < 0.05$ level, two-tailed. The results are presented as mean ± SE. All statistical analyses were performed with the use of Statistical 6.0 software[30].

RESULTS

Similar basal concentrations of ^{13}CO$_2$ in the expiratory air were observed on the three study days, amounting to -25.79‰ ± 0.25‰, -25.44‰ ± 0.21‰ and -25.59‰ ± 0.22‰. After oral intake of the ^{13}C-methacetin solution an expected biphasic course of ^{13}CO$_2$ content within the exhaled air was observed, characterized by a rapid rise followed by a less steep decline. R_ANOVA indicated that when referred to the basal situation, the rise in breath ^{13}CO$_2$ was statistically significant between the 6th and the 150th minute (Figure 1).

Basic reproducibility assessment

As shown in Table 1, the T_{max} exhibited rather an unsatisfactory reproducibility, whereas the D_{max} displayed a fair reproducibility, supposedly sufficient from the point of view of diagnostic applications of the breath test. The reproducibility of the cumulative ^{13}C recovery relative to the administered substrate dose was strongly dependent on the time span included for the calculation of this parameter, and improved rapidly with inclusion of the subsequent measurement points (Figure 2). The AUC_{0-60} attained a CV$_p$ of 10%-identical in both the case of the
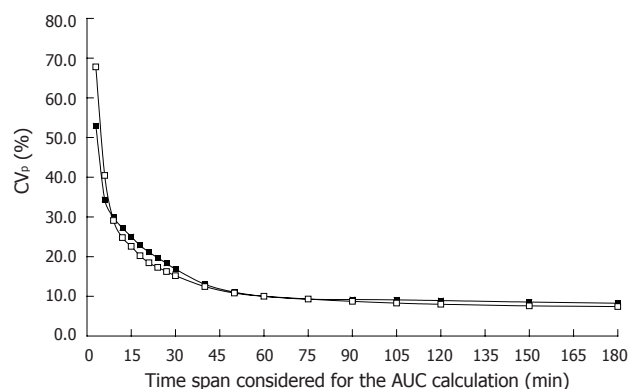
Figure 2 Relationship of the reproducibility of the cumulative ^{13}C recovery, expressed in terms of the coefficient of variation for paired examinations (CV$_P$), on the time span taken for the calculation of the area under the curve. The short-term reproducibility (open rectangles) assessment involved performance of 20 pairs of ^{13}C-methacetin breath tests separated by a median 2-d break, whereas 20 pairs of examinations accomplished at a median interval of 18 d were considered for the evaluation of the medium-term reproducibility (filled rectangles). AUC: Area under the curve.

short- or the medium-term reproducibility. The inclusion of data from beyond the 60 min did not bring about much refinement of the reproducibility of the AUC.

No statistically significant difference between the short- and medium-term reproducibility of the breath test parameters was found. Moreover, none of the parameters considered had a different short- or medium-term reproducibility in men and women.

Bland and Altman statistics of reproducibility

Bland and Altman plots pertaining to representative parameters of the ^{13}C-MBT are provided in Figure 3. Bland and Altman statistics revealed no proportional bias in either D$_{max}$ or AUC$_{0-60}$ (Table 1). The same applied to the short-term reproducibility of T$_{max}$, whereas analysis of its medium-term reproducibility showed that the slope of the linear regression of the between-day differences on the corresponding means of the paired measurements was statistically significantly different from zero (Table 1).

The Bland and Altman statistic disclosed a fixed bias in the case of the medium-term reproducibility of either the D$_{max}$ or the AUC$_{0-60}$. It means that the mean differences between the paired measurements taken a median of 18 d apart differed statistically significantly from zero. A closer look at the pertinent differences indicated that the ability of the liver to handle ^{13}C-methacetin increased when a repeat dose was administered after two to three weeks (D$_{max}$: 34.60% ± 2.75% dose/h on the first administration *vs* 39.08% ± 2.77% dose/h on the second administration, $P = 0.015$; AUC$_{0-60}$: 20.80% ± 0.99% dose on the first administration *vs* 22.27% ± 0.98% dose on the second administration, $P = 0.017$). No such effect was observed if two doses of ^{13}C-methacetin were administered in close sequence [D$_{max}$: 34.26% ± 2.41% dose/h on the first administration *vs* 34.91% ± 2.68% dose/h on the second administration, not significant (NS); AUC$_{0-60}$: 21.16% ± 0.96% dose on the first administra-

tion *vs* 21.03% ± 1.07% dose on the second administration, NS]. A graphical representation of the phenomenon observed is provided in Figure 4.

DISCUSSION

Without any doubt provision of reproducible measurement results is a feature of paramount importance, expected to be assured by methods designed for research and/or clinical applications in medicine[28,31-33]. Breath tests performed with the use of stable isotopes, such as ^{13}C, cannot be considered an exception in this respect, especially because during the past decade their clinical use has been constantly growing[24,34,35]. An increase in the number of relevant scientific papers indicates that, from among the tests dedicated to assess the activity of cytochrome P450, known as the microsomal breath tests, the ^{13}C-MBT has lately taken the lead. Accordingly, Yaron Ilan in his very recent review[36] provides evidence-based arguments that the ^{13}C-MBT is a powerful tool to aid hepatologists in bedside decision making.

While searching the literature we came across only one study which was aimed at the evaluation of the reproducibility of the ^{13}C-MBT. Petrolati *et al*[15] performed repeat measurements separated by an interval of 12 wk in 10 healthy volunteers. This scarcity of data encouraged us to undertake a systematic, prospective study on the reproducibility of the quantitative measures of a standard ^{13}C-MBT. Much effort was given in order to assure comparable conditions while performing the ^{13}C-MBT. Accordingly, all the examinations were started at the same time in the morning, a 15-min rest always preceded the collection of the basal samples of expiratory air, the procedure of taking the breath samples was standardized, and comfortable surroundings were provided to the volunteers so that they could stay relaxed while maintaining a sitting position throughout the examination. Moreover, the female participants were always examined in the same phase of the menstrual cycle. While designing the study protocol we decided to adopt the currently accepted mode of administration of ^{13}C-methacetin, namely a fixed oral dose of 75 mg[5,6,8,13,15,20,37,38]. It should be noted, however, that formerly other body mass adjusted dosage regimens were applied, encompassing 5 mg/kg in the pioneer work by Krumbiegel *et al*[39], then 2 mg/kg[7,11,12,40], as well as 1 mg/kg[10,41], and finally Iikura *et al*[42] applied in infants 0.5 mg/kg ^{13}C-methacetin.

Taking into account the investigative nature of our research, we generously took as many as 19 samples of breath air throughout 3 h. This approach appears to be precedential in nature, because for routine clinical use just a few measurement points are usually considered[15,42]. Nine samples were collected in the study by Zipprich *et al*[13] (at 10, 20, 30, 40, 50, 60, 80, 100 and 120 min after application of the substrate), and twelve samples of the expiratory air-every 15 min for 3 h of observation – were taken by Ciccocioppo *et al*[41]. A team of German researchers from Bochum collected thirteen samples-at 5, 10, 15, 20,
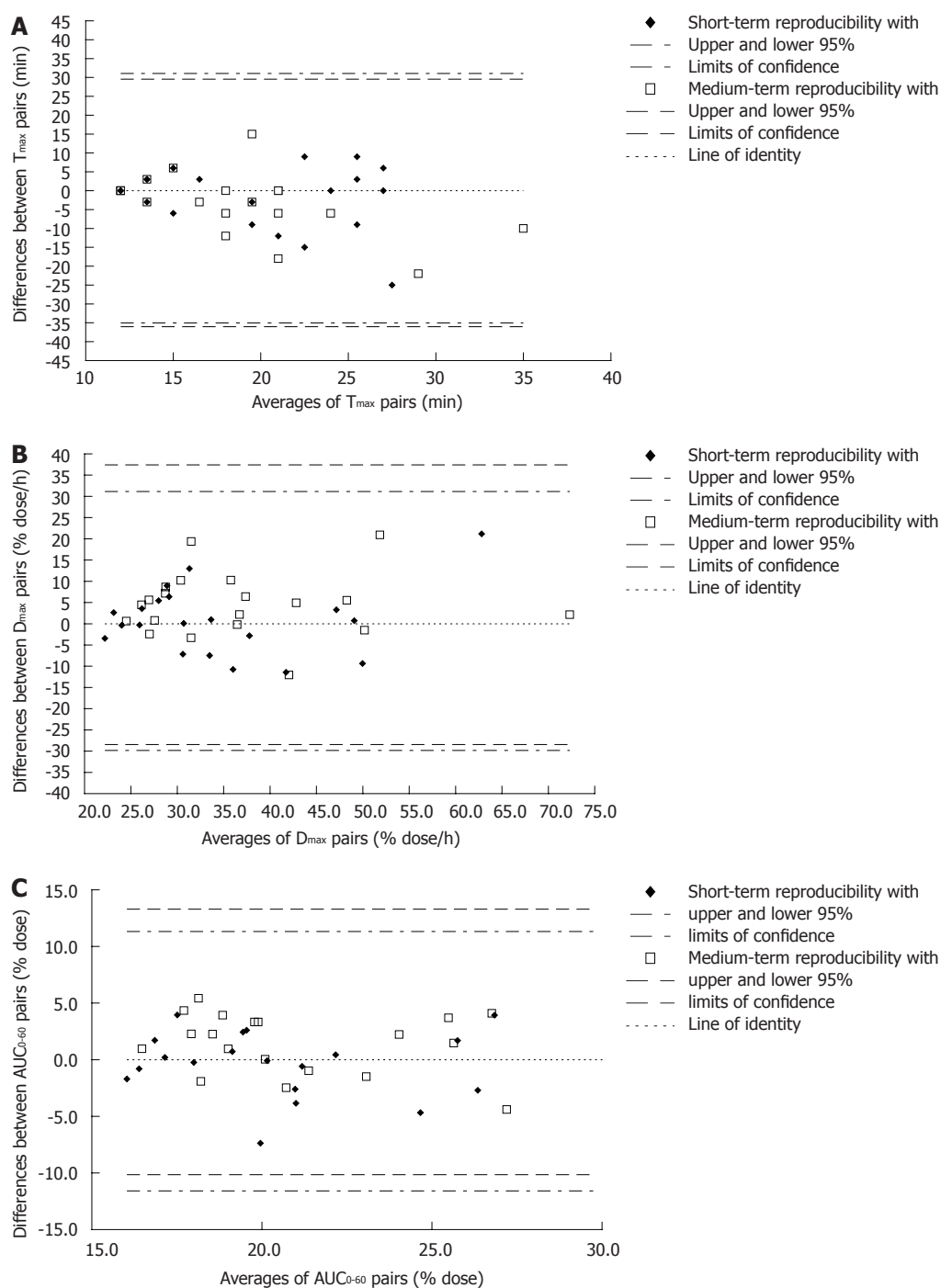
Figure 3 Bland and Altman statistics (plot of differences between pairs *vs* their means) of the short- (filled diamonds) and medium-term (open rectangles) reproducibility of the ^{13}C-methacetin breath test. T_{max}: Time to reach the maximum momentary ^{13}C elimination (panel A); D_{max}: Maximum momentary ^{13}C elimination (panel B); AUC_{0-60}: 60-min cumulative ^{13}C elimination in expiratory air (panel C). On each panel the respective borders of the 95% confidence intervals are plotted.

30, 40, 50, 60, 80, 100, 120, 150 and 180 min after administration of ^{13}C-methacetin[12]. One should mention that our frequent sampling of breath air (an aliquot was taken every 3 min during the first half hour) is similar to the new approach recently proposed by Lalazar *et al*[6,38] who introduced a "continuous" ^{13}C-MBT wherein samples of breath air are taken every 2-3 min and analysed with a laser-based device.

Our intention was to determine the length of the T_{max} with the greatest exactitude possible. Nevertheless, with the CV_p between 30.6% and 32.5%, the reproducibility of this parameter appeared to be a bit disappointing. Apparently the T_{max} may therefore not be the most useful parameter for the purposes of clinical decision making. On the other hand, we established on the basis of the within-subject study protocol that the repeatability of the T_{max} was sufficient to discern a difference in its length as small as 4 min ($P = 0.05$ level, two-tailed, $n = 20$).
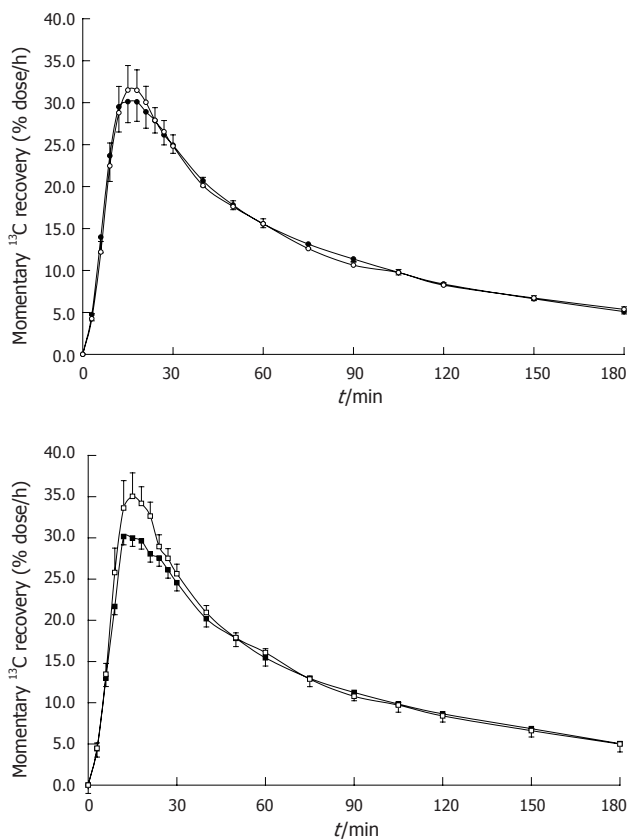
Figure 4 Corresponding curves of the momentary ^{13}C recovery in breath air after peroral administration of 75 mg ^{13}C-methacetin in 20 healthy volunteers if two examinations were taken at a median of 2 d apart (panel A) or if the examinations were separated by a median 18 d gap (panel B). Open symbols, the first administration, filled symbols, the second administration, the values shown are mean ± SE. See the text for the results of the statistical comparison of the matching D_{max} and AUC_{0-60} values.

D_{max} (CV_p of 16%) showed better reproducibility, whereas the most reliable with regard to its repeatability appeared to be cumulative ^{13}C recovery in expiratory air. At this point it is important to emphasize that the reproducibility of the cumulative ^{13}C recovery was dependent on the time span considered for its calculation. Hence the CV_p improved rapidly with inclusion of the subsequent measurement points, achieving a reasonable value of 10% for the period comprising the first 60 min following the administration of the substrate. Consideration of later measurement points offered only a small further improvement in the reproducibility of the AUC. Our finding provides an argument in support of limiting the time for sample collection to 1 h when performing the ^{13}C-MBT-which is a standpoint coherent with the procedure proposed lately by the research group headed by Yaron Ilan from Israel[6,38].

Of great importance is the finding that in the case of each of the considered parameters, characterizing quantitatively the result of the ^{13}C-MBT, the reproducibility depended neither on the time gap between the repeat examinations nor on the gender of the subjects. Thus a convincing proof of reliability of this test has been obtained. Quite unexpectedly a thorough analysis of reproducibility, involving the application of the Bland and Altman statistic, disclosed the existence of a fixed bias in the case of the medium-term reproducibility of the D_{max} and the AUC_{60}. It was found that those two parameters increased statistically significantly if the repeat examinations were separated by a two- to three-week gap, which was not the case when the results of examinations taken at a close sequence were compared. A similar trend can, however, be discerned when looking at the results obtained by Petrolati *et al*[15] in their attempt to evaluate the reproducibility of the ^{13}C-MBT. They found that the 45-min cumulative ^{13}C recovery (AUC_{0-45}) in breath air after peroral intake of 75 mg ^{13}C-methacetin amounted to 17.5% ± 2.8% dose and to 18.8% ± 4.3% dose on the first and the second occasion, respectively. Although the two means did not differ statistically significantly ($P = 0.30$), it is evident that the AUC_{0-45} determined on the second occasion increased by 7.4%. Quite a similar magnitude of increment was determined in our study-the AUC_{0-60} rose by 7.5% on the repeat measurement taken after two to three weeks ($P = 0.017$), whereas it remained unchanged with an average difference of -0.6% (NS) between measurements performed a median of 2 d apart. A delayed and persisting stimulation of a minor, but statistically significant, magnitude of the CYP1A2 capacity to metabolize methacetin would therefore be inferred. It remains unclear if the phenomenon disclosed would have a clinically relevant impact. It is rather obvious that not all patients with liver disease require performance of a series of ^{13}C-MBTs, which most likely may pertain to those awaiting liver transplantations[15]. At present one cannot predict whether a stimulation of CYP1A2 observed on repeated ^{13}C-MBT in healthy volunteers will occur also in patients with major impairment of the liver functional reserve. Nevertheless the error in estimating the cumulative ^{13}C recovery in breath air caused by a stimulation of CYP1A2 by ^{13}C-methacetin seems to be quite small, not exceeding 8% according to our results, as well as those obtained by Petrolati *et al*[15].

Summing up, the study renders evidence that the ^{13}C-MBT provides satisfactorily reproducible results. Remarkably, the cumulative ^{13}C recovery appear to be the most reproducible quantitative index of the test, the computation of which over the first hour following administration of the substrate offers a reasonable 10% coefficient of variation, regardless of the time span separating the repeat measurements. One should be aware that the exactitude of this parameter may be modestly affected by a persistent stimulation of CYP1A2 on repeat examinations.

COMMENTS

Background
Great progress has been made during the past two decades with respect to the diagnostic use of stable isotopes in hepatology - it is now possible to assess by means of ^{13}C breath tests the microsomal, the cytosolic, or the mitochondrial function of the liver. From the point of view of a patient those tests are particularly attractive as in many instances they may result in avoidance of the inconvenience of a repeat liver biopsy.

Research frontiers
A crucial asset of any measurement or diagnostic method used in medical practice is provision of reproducible results. Stable isotope breath tests cannot be exempted from this scrutiny. Quite surprisingly, the reproducibility of one of the most popular among the ^{13}C breath tests applied in hepatology, the ^{13}C-methacetin breath test (^{13}C-MBT) has not been sufficiently examined before.

Innovations and breakthroughs
This prospective study is the first one dedicated to thorough evaluation of the repeatability of the ^{13}C-MBT. According to the study results, cumulative ^{13}C recovery is the most reproducible quantitative index of the ^{13}C-MBT, the computation of which over the first hour following administration of the substrate offers a 10% coefficient of variation, regardless of the time span separating the repeat measurements. An additional finding is that in the case of repeat examinations the exactitude of this parameter may be modestly affected by a persistent stimulation of CYP1A2 responsible for a fixed bias which amounted to 7.5%.

Applications
Promising results on the usefulness of the ^{13}C-MBT to evaluate reliably and accurately the metabolic liver functional reserve were documented in patients with various stages of liver cirrhosis, including those awaiting a liver transplantation, as well as those with chronic hepatitis C virus infection, primary biliary cirrhosis, or non-alcoholic steatohepatitis.

Terminology
The breath tests dedicated to get information on the metabolic efficiency of the liver are based on an elegant but simple idea of monitoring the elimination of $^{13}CO_2$ in expiratory air after per oral administration of ^{13}C-enriched substrates. Depending on the chemical structure of such substrates, the microsomal, the cytosolic, or the mitochondrial function of the liver may be evaluated noninvasively.

Peer review
The study was conducted carefully and the paper is well written.

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