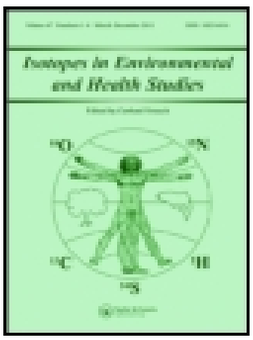

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Scrutiny of ^{13}C-phenylalanine breath test reproducibility

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ABSTRACT

We evaluated the reproducibility of the ^{13}C-phenylalanine breath test (^{13}C-PheBT). On three separate days, 21 healthy volunteers (11 F and 10 M) underwent ^{13}C-PheBT with 100 mg L-[1-^{13}C]phenylalanine taken orally. Short-term reproducibility was evaluated with paired examinations taken 3 days apart; paired examinations separated by 23 days (median) served for the medium-term reproducibility assessment. Expiratory air was sampled at 19 points throughout 3 h. Determined limited reproducibility of the ^{13}C-PheBT must be taken into consideration while interpreting the results of this diagnostic tool. The results of this study imply the following conclusions: (i) From among the three parameters examined, the cumulative ^{13}C recovery area under the curve (AUC) offers much better reproducibility than the maximum momentary ^{13}C recovery in the expiratory air (D_{max}) or the time to reach the maximum momentary ^{13}C recovery (T_{max}) (ii) Collection of the breath air samples for 2 h results in a much better reproducibility of AUC, than for 1 h only; (iii) Reproducibility of ^{13}C-PheBT is affected neither by the duration of the time gap between repeated tests nor by gender; (iv) Comparison with data obtained formerly reveals that reproducibility of the ^{13}C-PheBT is worse than either that of of the ^{13}C-methacetin (^{13}C-MBT) or the ^{13}C-alpha-ketoisocaproic acic (^{13}C-KICA-BT) breath tests. This finding will have to be taken into consideration while interpreting the results of this diagnostic tool.

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Breath test; ^{13}C-phenylalanine; carbon-13; isotope application in diagnostic medicine; liver function; men; reproducibility

1. Introduction

Measurement of functional reserve of the liver (FRL) is needed in clinical circumstances requiring assessment and monitoring of the advancement of dynamic processes, such as inflammatory activity, and the progression of fibrosis leading to liver cirrhosis [1,2]. The obtained information allows then for appropriate therapeutic decisions to be made and helps plan further procedures, such as liver transplantation [3–5]. Also, post-transplantation survey necessitates a proper assessment of the FRL [5].

In the past, intravenously administered dyes were used for evaluation of the FRL, namely bromosulfophthalein or indocyanine green. The former one appeared to be quite cumbersome because it required drawing of several blood samples. The latter,

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although based on a single blood sample only, turned out, however, to be less sensitive in detecting mild liver dysfunction [6].

Breath tests utilizing carbon ^{13}C-labelled substances constitute a modern tool for the measurement of the FRL. These tests fulfil the crucial requirements of a good, non-invasive diagnostic method: a non-toxic substance is administered orally, whereas the final metabolite – $^{13}CO_2$ – is measured in expiratory air samples [7,8]. Rather strikingly, despite being available for over 30 years now, the ^{13}C liver breath tests (^{13}C-LBTs) have not been accepted as a routine diagnostic tool yet.

Recently, the pages of *Digestive Diseases & Sciences* were the place of an interesting polemic which was initiated by the state-of-the-art paper by Afolabi et al. [9], wherein the authors insisted on the necessity of subjecting the ^{13}C-LBTs to scrutiny of a three-stage model of clinical evaluation. We shared this viewpoint to some extent, expressing our opinion that still a great deal of work would have to be done regarding standardization of the ^{13}C-LBTs [10]. On the other hand, a light at the end of the tunnel seems to be visible already now, because multicentre phase III trials aimed at the implementation of ^{13}C-LBTs in clinical practice appear to be under way [11].

The cytosol of hepatocytes is the place of the aromatic amino acid metabolism. Phenylalanine is processed there primarily by irreversible hydroxylation to tyrosine involving phenylalanine hydroxylase [12]. Tyrosine is next converted by tyrosine aminotransferase into hydroxyphenylpyruvic acid. Because these metabolic pathways are only found in the liver, they are sensitive indicators of liver dysfunction. Hydroxyphenylpyruvate is further metabolized, through dioxygenation, to homogentisic acid, ultimately bringing about CO_2 [12]. Hence, the ^{13}C-phenylalanine breath test (^{13}C-PheBT) appeared to be useful for discrimination between patients with compensated and decompensated cirrhosis, as well as between patients with oesophageal varices of varying degrees of severity [1,2,4]. It was also reported that the ^{13}C-PheBT result was an independent survival predictor in patients with hepatic impairment [3,5].

Recently, in a Letter to the Editor of the *Digestive Diseases and Sciences*, we presented the preliminary results of examinations on the reproducibility of the ^{13}C-PheBT [10]. Quite strikingly, the reproducibility of the ^{13}C-PheBT seemed to be worse than in the case of either the ^{13}C-methacetin breath test (^{13}C-MBT) or the ^{13}C-alpha-ketoisokaproic acid breath test (^{13}C-KICA-BT). We decided therefore to pursue an extended study focused on the ^{13}C-PheBT reproducibility and factors which may affect it. The obtained results are reported and discussed in this paper.

2. Methods

2.1. Subjects

Twenty-one healthy unpaid volunteers (11 women and 10 men, mean age 24.4 ± 0.6 years, body mass index amounted to 22.33 ± 0.63 kg/m^2) were recruited. Inclusion criteria comprised a non-smoking status, negative statement of systematic use of a significant amount of alcoholic beverages, and in women denial of use of hormonal contraception. Every participant declared herself/himself as being in full health according to the World Health Organization criteria during a medical interview. Exclusion criteria comprised a history of surgery affecting the digestive tract anatomy (except for

appendectomy), current use of any drugs, and pregnancy. Normal structure and size of the liver were confirmed in every subject by means of an ultrasonographic examination performed by a qualified investigator (AKJ). Approval for conduct of the study was obtained from the Bioethics Committee of the Medical University of Silesia. The study was conducted in accordance with the Helsinki Declaration, and every volunteer gave a written consent to participate after getting information as to the aim, protocol, and methodology of the project. The subjects were instructed not to eat any food of naturally increased ^{13}C content, such as products made of maize, cane sugar, pineapple, and kiwi fruit for 48 h preceding the examination [13].

2.2. Protocol

Every subject agreed to undergo three examination sessions, held on separate days. Two schedules of examinations were assigned in random order to the volunteers: (A) the first two breath test were separated by 2–4 days, and the third was taken 3–4 weeks later, or (B) the first breath test was followed by a break of 2–4 weeks, after which the second and the third tests were accomplished with a 2–4 days gap between them. Accordingly, the short-term reproducibility was assessed on the set of 21 paired examinations taken at a median gap of 3 days (interquartile range, i.q.: 2.75–4 days; mean: 3.2 day), whereas a set of 21 pairs of the most distant examinations, accomplished at a median 23-day break (i.q. 21–28 days; mean 25.1 days), served for the evaluation of the medium-term reproducibility.

The volunteers came to the laboratory in the morning, after a 12-h overnight fast. Initially, they rested for 15 min in a sitting position, and thereafter two basal samples of the exhaled air were collected. A standardized mode of collecting the samples was adopted: the subjects took in breath and held it for 10 s, then steadily blew the air through a straw of 3 mm inner diameter into a 12-ml glass vial (Exetainer®, Labco Ltd, UK) which was tightly closed with a cap immediately at the end of the exhalation [14].

At the time point designated '0', the subjects took orally 100 mg L-[1-^{13}C]phenylalanine (^{13}C-Phe; code CLM-762-SP; ≥99.1 % enrichment in ^{13}C certified by the manufacturer Cambridge Isotope Laboratories, Inc., USA). The aqueous solution of the substrate was prepared instantly with the use of an ultrasonic homogenizer UP50H (Hielscher, Germany), equipped with a 3-mm sonotrode H3 (30 kHz; 50 W) [15]. After intake of the solution, the volunteers drank 50 ml plain water to rinse any rest of the substrate from the mouth and the oesophagus down to the stomach. Samples of expiratory air were then collected at 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 40, 50, 60, 75, 90, 105, 120, 150, and 180 min after time '0'.

During one examination session in seven subjects, the collection period was extended, and additional samples were obtained at 240, 360, 540, 720, 1440, and 2880 min. During those sessions, the subjects remained fasted for the first 6 h and were then served a light 320 kcal lunch, neutral as to the ^{13}C content. It was composed of a sandwich consisting of a 50-g wheat white bread roll smeared with 20 g butter and garnished with two slices (35 g) of lean cooked pork ham (13.0 g protein, 18.0 g fat, 24.2 g carbohydrates) and 200 ml of unsweetened black tea [16]. The subjects remained seated comfortably in the lab until lapse of 9 h. The last three expiratory air samples were collected by the volunteers themselves at home. The subjects were committed to take then at least a 15-min rest in a sitting position before blowing into the vials.

2.3. $^{13}CO_2$ measurement 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 the isotope ratio mass spectrometry (ABCA, Automated Breath ^{13}C Analyser, SerCon Ltd, UK). Before and after every series of samples, a quality control procedure was performed which involved a run of the measurement on a standard gas (5 % CO_2 within N_2) of a certified $^{13}CO_2$ content of −31.33 ‰ after every 10 breath air samples.

Curves of the momentary and cumulative recovery of ^{13}C in the exhaled air, relative to the administered oral dose of L-[1-^{13}C]Phe, were constructed within the time domain of 0–180 min. Subsequently, the following parameters were obtained: D_{max} – the maximum momentary ^{13}C recovery in the expiratory air, T_{max} – the time elapsing from the intake of the substrate until the occurrence of the D_{max}, as well as AUC_i – the cumulative ^{13}C recovery calculated by integration of the momentary ^{13}C recovery curve within the time domain until a time point *i*.

2.4. Reproducibility data on other ^{13}C-LBTs

Data necessary to compare the reproducibility of the ^{13}C-PheBT with other ^{13}C-LBTs were obtained from previously published studies devoted to reproducibility of the ^{13}C-MBT [17] and ^{13}C-KICA-BT [14], respectively.

2.5. Statistical analysis

The considered reproducibility indices comprised the coefficients of variation for paired examinations (CV_p) [18,19], Bland and Altman repeatability coefficients, as well as a check for a proportional or fixed bias [20,21].

Sets of moduli (=absolute values) of individual differences in paired measurements were compared with the use of the paired or unpaired *t*-test (where appropriate) to check if factors such as the time distance between the examinations, or the gender of the subjects affected the reproducibility.

Moreover, with the purpose of comparison among three ^{13}C-LBTs, moduli of individual percentage between-day differences in paired measurements were subjected to analysis of variance (ANOVA) followed by a *post hoc* check on the significance of differences between means, performed with the Tukey's honest significant difference (HSD) test [22,23].

Statistical significance was set at the $p < .05$ level, two-tailed. Results are presented as mean ± SE values.

3. Results

3.1. Duration of the rise of breath $^{13}CO_2$ after intake of the substrate

Examinations in seven subjects, in whom sample collection was extended to 48 h, demonstrated that already after 5 h after application of 100 mg of ^{13}C-Phe, the concentration of $^{13}CO_2$ in breath air returned to the basal level (Figure 1).

In all 21 subjects, measurement results covering a 3-h time span were available, permitting thus a more detailed analysis. After averaging for every subject, the measurements

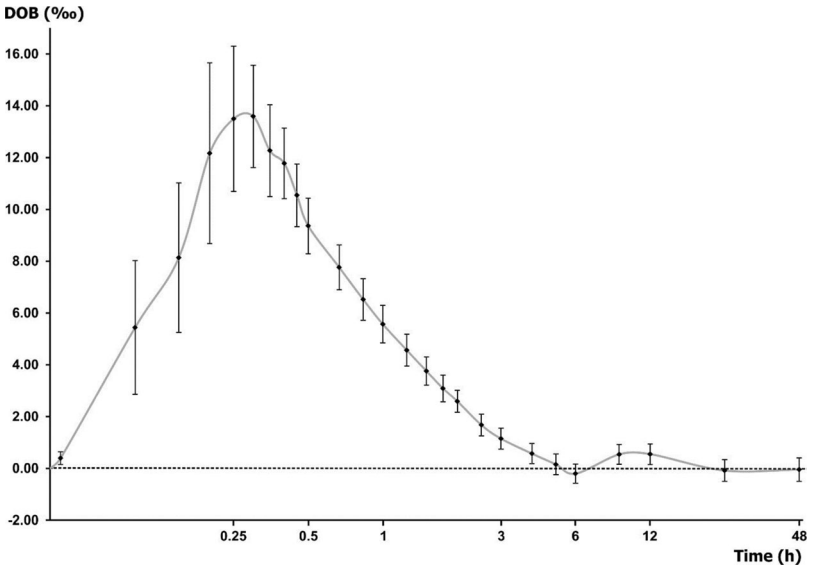

Figure 1. Time course of the rise in $^{13}CO_2$ abundance in expiratory air – DOB – observed in seven healthy volunteers in whom the collection period of breath samples after per oral intake of 100 mg L-[1-^{13}C]Phe was extended to 48 h; for the sake of clarity, the abscissa scale was subjected to a logarithmic transformation. DOB: delta over baseline.

taken on three examination sessions, the resulting set of data was subjected to R_ANOVA, which disclosed that, when referred to the basal situation, the rise in breath $^{13}CO_2$ was statistically significant between 6 and 120 min (Figure 2).

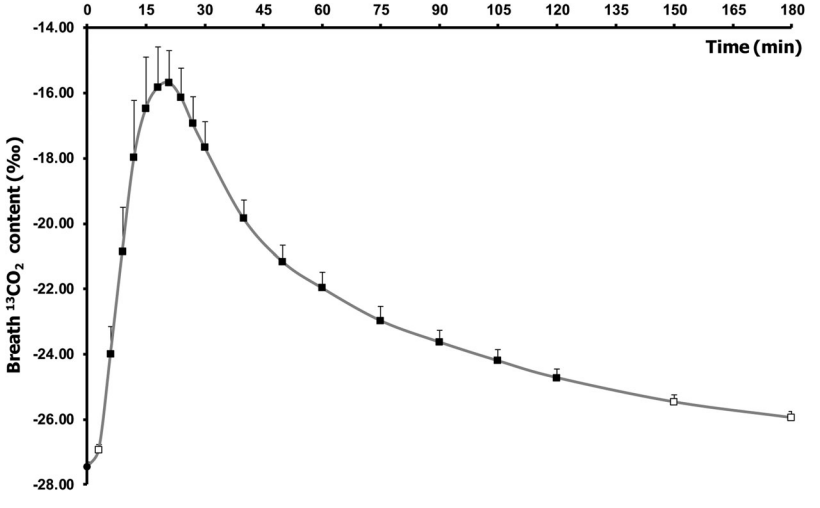

Figure 2. Time course of the $^{13}CO_2$ content within the total pool of the exhaled CO_2 after per oral intake of 100 mg L-[1-^{13}C]Phe observed in 21 healthy volunteers of whom everyone underwent three breath tests on separate days. Filled rectangles represent the time points at which the rise in $^{13}CO_2$ was statistically significantly different from the baseline measurement, and open rectangles stand for values not statistically different from the baseline.

3.2. Reproducibility of the ^{13}C-PheBT

The data on reproducibility of the quantitative parameters of the ^{13}C-PheBT is given in Table 1. The best reproducibility was that of the AUC, while T_{max} reproducibility was the worst, and D_{max} reproducibility was ranked in between of them. Except for a borderline significance ($p = .0496$) of the fixed bias in the case of short-term reproducibility of T_{max}, in no other instance did the Bland and Altman statistics reveal any fixed or proportional bias, which means that the mean differences between paired measurements did not differ statistically significantly from zero, and the slope of the linear regression of those differences on the corresponding means of the paired measurements was not statistically significantly different from zero (respective Bland and Altman plots are depicted in Figure 3).

It can be inferred from Table 1 that consideration of a longer interval for computation of the AUC improved the reproducibility of this parameter. Indeed, this supposition is confirmed by a graphic presentation of the relationship of the CV_p of the AUC on the length of time span considered for its computation; an improvement of reproducibility was observed until 120 min, whereas inclusion of data beyond the second hour did not bring about any further refinement thereof (Figure 4). This graph also provides evidence for very similar short- and medium-term reproducibility of the AUC, if calculated on data comprising at least 30-min sample collection span.

An in-depth analysis revealed next that there was no statistically significant difference between the short- and medium-term reproducibility in the case of any of the breath test parameters assembled in Table 1. Moreover, gender did not affect the reproducibility of the ^{13}C-PheBT parameters.

3.3. Comparison of reproducibility among three liver function breath tests (LFBTs)

ANOVA disclosed statistically significant effects of the kind of LFBTs on the reproducibility of their quantitative parameters (Table 2). *Post hoc* comparisons revealed that the reproducibility of AUC_{60} and AUC_{120} was statistically significantly better in the case of ^{13}C-MBT than with ^{13}C-PheBT. Also, the reproducibility of T_{max} and AUC_{120} was superior

Table 1. Reproducibility of the ^{13}C-PheBT.

	D_{max}		T_{max}		AUC_{0-60}		AUC_{0-120}	
	S_term	M_term	S_term	M_term	S_term	M_term	S_term	M_term
CV_p	26.26 %	25.59 %	30.89 %	30.20 %	21.78 %	23.11 %	17.30 %	19.19 %
RC	9.12 % dose/h	8.89 % dose/h	16.0 min	15.4 min	4.18 % dose	4.49 % dose	5.00 % dose	5.76 % dose
Bias proportional	N	N	Y	N	N	N	N	N
Bias fixed	N	N	N	N	N	N	N	N
$Delta_{0.05}$	2.10 % dose/h	2.07 % dose/h	3.4 min	3.6 min	0.98 % dose	1.01 % dose	1.19 % dose	1.37 % dose

D_{max}: maximum momentary ^{13}C elimination; T_{max}: time to reach the maximum momentary ^{13}C elimination; AUC_{0-60} and AUC_{0-120}: 60-min and 120-min cumulative ^{13}C elimination in expiratory air, respectively; S_term: short-term reproducibility assessment on the basis of 21 pairs of ^{13}C-PheBTs separated by a median 3-day break; M_term: medium-term reproducibility evaluation involved 21 pairs of examinations accomplished at a median interval of 23 days; RC: repeatability coefficient; $Delta_{0.05}$: the least difference detectable at $p = .05$ level, two-tailed in the case of 21 paired examinations.

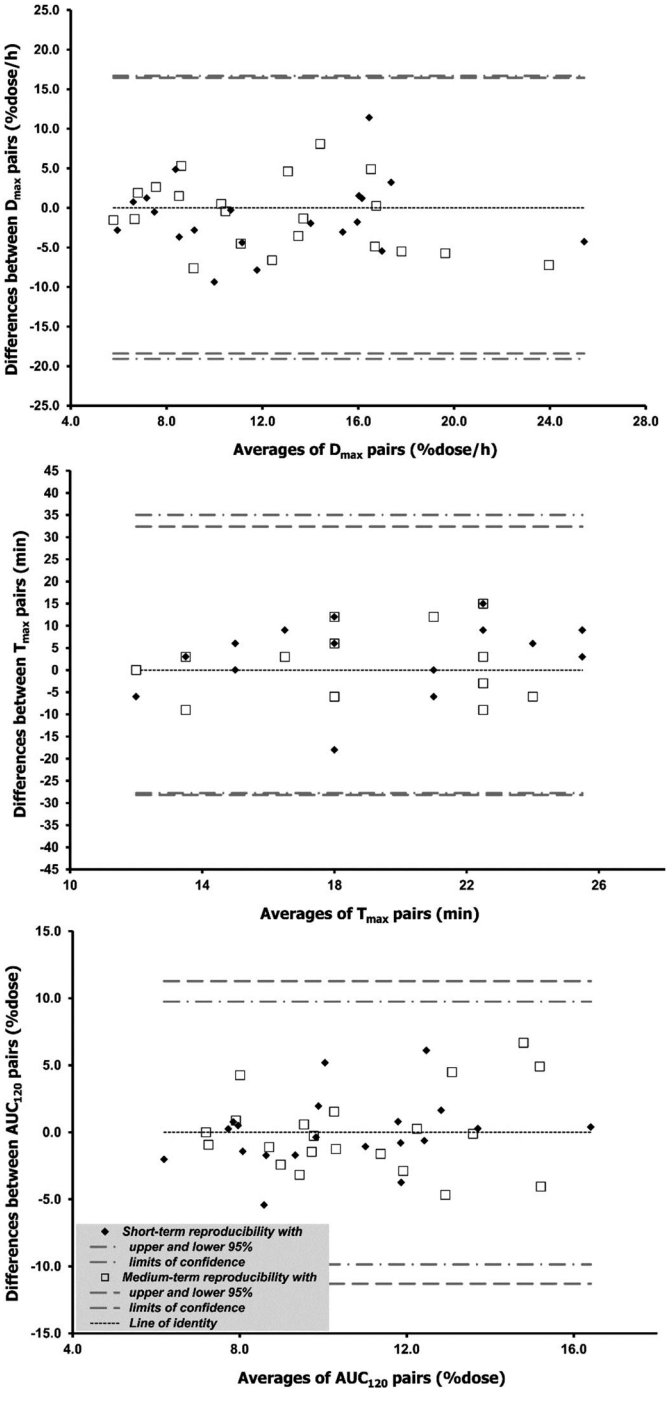

Figure 3. Bland and Altman statistics (plot of differences between pairs vs. their means) of the short- (filled diamonds) and medium-term (open rectangles) reproducibility of the ^{13}C-PheBT. D_{max}: maximum momentary ^{13}C elimination (upper panel); T_{max}: time to reach the maximum momentary ^{13}C elimination (middle panel; due to occurrence of some identical values of T_{max}, the total number of points depicted in this panel is less than 21 for a given category); AUC_{120}: 2-h cumulative ^{13}C elimination in expiratory air (bottom panel). On each panel, the respective borders of the 95 % confidence intervals are plotted – cf. the legend in the bottom panel.

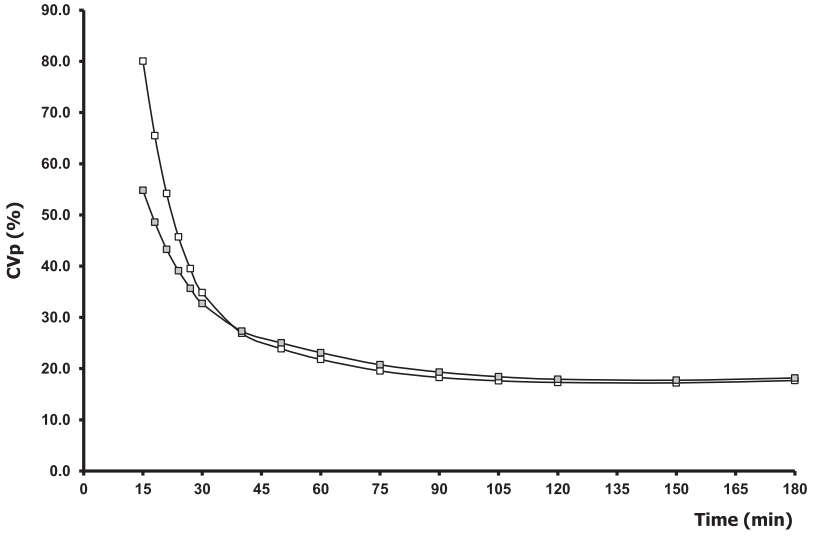

Figure 4. Relationship of the reproducibility of the cumulative ^{13}C recovery in expiratory air after per oral intake of 100 mg L-[1-^{13}C]Phe, expressed in terms of the CV_p, on the time span considered for computation of the area under the curve (AUC). The short-term reproducibility assessment involved the performance of 21 pairs of ^{13}C-PheBT separated by a median 3-day break, whereas 21 pairs of examinations accomplished at a median interval of 23 days were taken for evaluation of the medium-term reproducibility.

Table 2. Comparison of reproducibility of three liver function breath tests performed by means of ANOVA on moduli of individual percentage between-day differences.

			^{13}C-LBT	
Breath test parameter	ANOVA result	^{13}C-PheBT	^{13}C-MBT	^{13}C-KICA-BT
T_{max}	$F_{2,52}$=4.047 p = .023	40.1 ± *4.6*	26.5 ± *4.0*[a]	23.7 ± *3.5*[b]
D_{max}	$F_{2,52}$ = 3.003 NS (p = *0.058*)	30.8 ± *3.7*	21.5 ± *3.4*	16.5 ± *2.5*[c]
AUC_{60}	$F_{2,52}$ = 5.252 p = .0084	23.2 ± *3.3*	9.5 ± *1.0*[d]	15.7 ± *2.0*
AUC_{120}	$F_{2,52}$ = 6.396 p =0.0033	20.0 ± *2.9*	10.1 ± *1.1*[e]	9.6 ± *1.2*[f]

Note: Data pertaining to ^{13}C-MBT and ^{13}C-KICA-BT reproducibility were obtained from [17] and [14], respectively.
T_{max} = time to reach the maximum momentary ^{13}C elimination (D_{max}); AUC_{60} and AUC_{120} = cumulative 1- and 2-h ^{13}C elimination in expiratory air; NS = not significant.
Results of the *post hoc* Tukey's HSD examining differences between the means: [a]p = .064 (NS), [b]p = .039, [c]p = .059 (NS), [d]p = .006, [e]p = .0084, and [f]p = .013 in every case vs. the ^{13}C-PheBT

with ^{13}C-KICA-BT than with ^{13}C-PheBT. For none of the parameters was the reproducibility of the ^{13}C-MBT any different from that of the ^{13}C-KICA-BT (Table 2).

4. Discussion

Good reproducibility of a diagnostic test determines the reliable results and thus allows for its use in clinical practice. So far, however, no systematic studies were conducted regarding the repeatability of the ^{13}C-PheBT. The authors of existing scientific reports have focused their attention mainly on demonstrating the usefulness of ^{13}C-PheBTs in patients with liver disease.

For instance, Tugtekin et al. [24] performing the assay of the ^{13}C-PheBT demonstrated differences between oral administration of the marker and its administration by the

intravenous route. The study involved 12 healthy volunteers and 10 patients with liver cirrhosis. During the study, in which ^{13}C-Phe was administered orally, the volunteers were given 100 ml of an aqueous solution containing a marker at 2 mg/kg body mass, and samples were taken at 10, 20, 30, 45, and 60 min. Two days later, during a second trial, the volunteers were given intravenously 20 ml of a solution containing 2 mg/kg ^{13}C-Phe. Expiratory air samples were taken at 18 points during the 4 h after injection of the marker. Significant differences between patients with liver cirrhosis and healthy volunteers were evident in the first 10 min after intravenous administration. A per oral approach yielded comparable separation between 30 and 60 min. The authors point out that most probably a variable gastrointestinal resorption kinetics after oral application had caused this difference [24].

Freeman et al. [25] also conducted a study wherein ^{13}C-Phe was administered orally and intravenously. They studied the functional liver function after partial hepatectomy in 10 healthy liver donors. ^{13}C-Phe was administered intravenously at 2, 4, and 7 days after the operation while orally after 3, 5, 8, 14, 28, and 56 days, respectively. The total duration of the per oral test (3.33 mg/kg body mass) was 90 min during which time nine breath samples were taken. In contrast, when the substrate was administered intravenously (20 ml solution containing 2 mg/kg ^{13}C-Phe), samples were collected within 30 min. After hepatectomy, a significant diminution in the oxidation of the orally administered ^{13}C-Phe (a decrease of the order of 65–90 %) was observed, which was maintained up to 56 days after surgery. Noteworthy, Freeman et al. also observed a delay in the production of $^{13}CO_2$ when the substrate was taken orally, as compared to its intravenous administration in the same individual. The authors cited suggest that orally administered amino acids may not be well absorbed and/or metabolized in some subjects for weeks after partial hepatectomy, whereas intravenously delivered substrates are much better oxidized by the regenerating liver [25].

Several features and methodological approaches distinguish this study on the ^{13}C-PheBT reproducibility. First, a representative group of 21 healthy volunteers was involved. Because ^{13}C-Phe as a very light powder would not easily dissolve in water and would rather tend to form flocculent snippets floating on its surface, we meticulously worked with an ultrasonic homogenizer to solubilize the substrate. Moreover, after the subjects drank the aqueous ^{13}C-Phe solution, an additional aliquot of pure water was given to them to rinse any rest of the substrate from the mouth and the oesophagus down to the stomach. Ultimately, an unprecedented frequency of breath air sampling was deliberately adopted because we wanted to get an insight, as exact as possible, on the time course of the $^{13}CO_2$ concentration curves.

The finding that the best reproducibility characterized the AUC, whereas T_{max} and D_{max} did not perform so well, is quite easy to explain. The latter two parameters are derived from the curve of momentary ^{13}C recovery in the expiratory air, which may be prone to be affected by short-time fluctuations of metabolism. On the contrary, from a mathematical point of view, AUC reflects an integral of the momentary ^{13}C recovery in the expiratory air, and as such conveys information of the total metabolism of the ^{13}C-labelled substrate within the duration of a $^{13}CO_2$ breath test.

With all this effort, either the short- or the medium-term reproducibility of the ^{13}C-PheBT was found inferior when compared to the other two breath tests, which we scrutinized before: the ^{13}C-MBT [17] and the ^{13}C-KICA-BT [14]. One should recall here that the

latter tests examine liver metabolic activities other than ^{13}C-PheBT – these are the microsomal function in the case of ^{13}C-MBT and the mitochondrial capacity in the case of ^{13}C-KICA-BT. A potential advantage of the ^{13}C-PheBT over microsomal breath tests consists of the fact that the latter may be affected by enzymatic induction due to drugs (cimetidine, erythromycin, rifampicin, phenobarbital) [12] or a diminished ability of the liver to handle a ^{13}C-substrate exerted by cigarette smoking [26] or combined oral contraceptives containing ethinylestradiol [27]. The mentioned effects may interfere with the results of ^{13}C-MBT.

It seems that variable and unpredictable absorption of ^{13}C-Phe, as outlined already by Tugtekin et al. [24] and Freeman et al. [25] may account for the phenomenon disclosed in the current study. From a practical point of view, a moderate reproducibility of the ^{13}C-PheBT may cause not only a considerable intra-individual variability of its quantitative results but also quite a vast 'normal range' interval. Indeed, a closer look at the pertinent literature discloses that the latter problem was already encountered by other authors. The well-documented studies by Festi et al. [28] and Lara Baruque et al. [29] involved an unusually large size of control groups, composed of healthy volunteers, which in the first of the cited works amounted to 40, and in the second to as many as 48.

5. Conclusions

The results of this study imply the following conclusions:

(i) From among the three parameters examined, AUC offers much better reproducibility than D_{max} or T_{max};

(ii) Collection of the breath air samples for 2 h results in a much better reproducibility of AUC, than for 1 h only;

(iii) Reproducibility of ^{13}C-PheBT is affected neither by the duration of the time gap between repeated tests nor by gender;

(iv) Comparison with data obtained formerly reveals that reproducibility of the ^{13}C-PheBT is worse than either the ^{13}C-MBT or ^{13}C-KICA. This finding will have to be taken into consideration while interpreting the results of this diagnostic tool.

Disclosure statement

No potential conflict of interest was reported by the authors.

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