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THE EVALUATION OF THE REPEATABILITY OF THE ¹³C-KETOISOCAPROATE BREATH TEST FOR ASSESSING HEPATIC MITOCHONDRIAL FUNCTION.

ABSTRACT

The ¹³C-ketoisocaproate (¹³C-KICA) breath test (BT) has been recently proposed as a non-invasive test for assessing hepatic mitochondrial function. Results of the ¹³C-KICA BT can be expressed as different parameters. However, the best parameter for expressing the ¹³C-KICA BT result is uncertain which hinders use of the BT in routine clinical practice. We have investigated the repeatability of different parameters of ¹³C-KICA BT. Thirteen healthy adult subjects (5 men and 8 women) underwent a ¹³C-KICA BT on two occasions separated by a gap of approximately 30 days. There were no significant differences between the repeated measurements for all the parameters of the ¹³C-KICA BT over 30 days. Furthermore, the Bland Altman statistics showed no fixed or proportional bias for any of the parameters of the ¹³C-KICA BT. The cumulative ¹³C-dose enrichment over 60 min had the lowest within subject variability of 12% compared to all other parameters of the ¹³C-KICA BT. The cumulative ¹³C-dose enrichment over 60 min could be a very useful parameter for the ¹³C-KICA BT to detect impaired hepatic mitochondrial function in patients with chronic liver diseases.

Keywords: ¹³C-ketoisocaproate breath test, hepatic mitochondrial function, non-alcoholic fatty liver disease, cumulative ¹³C-dose enrichment over 60 min, repeatability, Bland Altman statistics, within subject variability.

1. Introduction

The ^{13}C -ketoisocaproate (^{13}C -KICA) breath test (BT) has been recently proposed as a non-invasive test for assessing changes in hepatic mitochondrial function in people with non-alcoholic fatty liver disease (NAFLD) [1, 2].

Studies comparing results following oral and intravenous administration of ^{13}C -KICA in healthy subjects provide robust evidence that the ^{13}C -KICA BT (following the oral administration of ^{13}C -KICA) measures hepatic and not muscle decarboxylation of ^{13}C -KICA and thus is highly specific for testing hepatic mitochondrial function [3, 4]. The results of the ^{13}C -KICA BT can be expressed as different parameters such as ^{13}C -dose enrichment in exhaled breath, percent ^{13}C -dose recovered in exhaled breath, or as cumulative percent ^{13}C -dose recovered in exhaled breath (cPDR) over a defined period of time. Previous studies have shown that the time of peak exhalation of $^{13}\text{CO}_2$ after the oral administration of ^{13}C -KICA was between 50 to 60 min [4, 5] and therefore the cPDR over 60 min has been used to express the results of the ^{13}C -KICA BT [2, 6]. However, there is uncertainty about what is the optimum outcome parameter and the time span over which to conduct the ^{13}C -KICA BT. To date, only a few studies have tried to determine the best parameter for expressing the ^{13}C -KICA BT [4, 5, 7]. Results from these studies have suggested that the cPDR over 2 hr offered the best repeatability while only one study showed no improvements in repeatability of cPDR measured beyond 60 min. Therefore, there is a need for more studies to validate the repeatability of ^{13}C -KICA BT when expressed as cPDR over the first 60 min, since ^{13}C -KICA BT measurements up to 60 min best reflect hepatic, rather than muscle mitochondrial function. Furthermore, the effect of other factors on the repeatability of the ^{13}C -KICA BT such as the time frame between measurements in subjects and the sex of the subjects

is uncertain [7, 8]. We have investigated the optimum measurement of ^{13}C -KICA BT that shows best repeatability over 60 min. The specific aim of the study was to determine which parameter of the ^{13}C -KICA BT showed the best repeatability for its potential use in assessment of hepatic mitochondrial function in clinical practice.

2 Materials and Methods

This study was carried out according to Good Clinical Practice and the Declaration of Helsinki. The research protocol was approved by the Southampton and South West Hampshire local research ethics committee (15/SC/0619) and written informed consent was obtained from all subjects prior to their participation.

2.1 Subjects

Thirteen healthy subjects, (5 men and 8 women, mean (\pm SD) age of 44 ± 15.4 years and mean BMI (\pm SD) of 24.3 ± 4.4 kg/m²) underwent the ¹³C-KICA BT protocol on two occasions with a gap of approximately 30 days between each test. The inclusion criteria for recruitment of healthy volunteers were: men and women, age >18 years, no medical history of chronic liver disease, not taking any prescribed medications, and alcohol consumption ≤ 14 units/week for women and ≤ 21 units/week for men. The exclusion criteria included alcohol consumption (>14 units/week for women and >21 units/week for men) and surgery affecting the digestive tract anatomy, with the exception of appendectomy. The presence of liver fat or liver fibrosis was ascertained by use of transient elastography (FibroScan, Echosens, Paris, France) performed by a trained clinician (ES). The Controlled Attenuation Parameter (CAP) (db/M) measurement was assessed and a threshold of CAP score of > 248dB/M was applied to diagnose the presence of liver fat [9]. The technical background for CAP has been previously described in detail [10] and the results are expressed in dB/m. The liver stiffness measure (kPa) was assessed as a proxy measure of liver fibrosis and the details of the technical description and examination procedure have been described previously [11]. Results are expressed as the median value in kilopascals (kPa) [12].

Anthropometric measurements such as Body Mass Index (BMI) was recorded along

with biochemical measurements such as alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin and total protein in fasting serum using commercially available kits according to the manufacturers' instructions.

2.2 ¹³C-ketoisocaproate breath test (¹³C-KICA BT) protocol

The ¹³C-KICA BT was undertaken twice in healthy subjects (separated by approximately 30 days) in order to assess the repeatability of the test. Subjects refrained from alcohol for 24 hr and fasted overnight for at least 12 hr prior to each test. All subjects were at rest for the duration of the study and remained fasted throughout. On the morning of the study, to standardize CO₂ production, subjects were asked to lie down on a bed and carbon dioxide produced (VCO₂) at rest was measured by indirect calorimetry (GEM Nutrition, UK) for 25 minutes prior to the start of the ¹³C-KICA BT protocol. Paired breath samples for baseline measurement of isotopic abundance were collected from each subject exhaling directly into 12 ml Exetainer breath tubes (LABCO Ltd, High Wycombe, UK) via straws. Each subject then ingested a solution containing 1 mg/kg body weight of 2-keto-[1-¹³C]-isocaproic acid (99% -¹³C; Cambridge Isotopes, USA) along with 20 mg/kg body weight of L-leucine (Sigma) in 200 ml of water. Further paired breath samples were collected every 10 min for 60 min. The measurement of ¹³C abundance in each breath sample was carried out by Continuous Flow Isotope Ratio Mass Spectrometry (CF-IRMS, ABCA System, SERCON, Crewe, UK). The ¹³C-dose enrichment (atom % excess) on breath as ¹³CO₂ was calculated from the ¹³C-abundance measurements according to Equation 1:

$$^{13}\text{C-dose enrichment on breath (atom \% excess)} = \frac{\text{delta over baseline (DOB)}}{100/1000} \times R_{\text{PDB}} \times 1000 \quad (1)$$

Where DOB is defined as the difference between the basal ^{13}C abundance of breath CO_2 before administration of ^{13}C -ketoisocaproate ($\delta_{\text{unenriched}}$) and the ^{13}C -abundance of breath CO_2 at a time point after administration (δ_{enriched}), relative to Vienna Pee Dee Belemnite (PDB).

$$\text{DOB} = \delta_{\text{enriched}} - \delta_{\text{unenriched}}$$

and $R_{\text{PDB}} = 0.0112372$ which is defined as the $^{13}\text{C}/^{12}\text{C}$ isotopic ratio of Vienna Pee Dee Belemnite.

The cumulative ^{13}C -dose enrichment on breath over n time points up to time t (atom % excess) was calculated using the trapezoidal method [13] according to Equation (2).

$$\begin{aligned} \text{Cumulative } ^{13}\text{C}\text{-dose enrichment on breath over time t (atom \% excess)} = & \text{Breath } ^{13}\text{C}\text{-} \\ & \text{dose enrichment}_{(n)} + \text{Breath } ^{13}\text{C}\text{-dose enrichment}_{(n-1)} / 2 \times \text{change in time t (min)} + \\ & \text{cumulative breath } ^{13}\text{C}\text{-dose enrichment}_{(n-1)}. \end{aligned} \quad (2)$$

The percent ^{13}C -dose recovered on breath per min (PDR/min) and the cumulative percent ^{13}C -dose recovered on breath over 60mins (cPDR over 60 min) were expressed as a percentage of the administered ^{13}C -KICA dose and were calculated using the ^{13}C -dose enrichment (atoms % excess) according to Equation (3):

$$\text{PDR/min (\%)} = \text{Atom \% excess} \times \text{VCO}_2 \text{ (mmol/min)} \times 100 / \text{Dose (mmol)} \quad (3)$$

where the volume of CO₂ output (VCO₂ in mmol/min) is measured by indirect calorimetry.

$$\text{Dose (mmol)} = {}^{13}\text{C-KICA substrate administered (mmol)} \times n \times P/100 \quad (4)$$

where P= ¹³C isotopic purity (%) of ¹³C-substrate and n = number of ¹³C atoms per molecule of ¹³C-KICA substrate.

The cPDR over the n time points up to time t (%) was calculated using the trapezoidal method [13] according to Equation (5):

$$\text{cPDR over time t (\%)} = [\text{PDR}_{(n)} + \text{PDR}_{(n-1)}]/2 \times \text{change in time (min)} + \text{cPDR}_{(n-1)} \quad (5)$$

2.3 Statistical analysis

Data are presented as means \pm SDs if normally distributed. A linear-mixed effects model analysis for repeated measures was undertaken in order to explore the effect of sex and time on the parameters of the ^{13}C -KICA BT. The linear mixed effects model was constructed with each parameter of the ^{13}C -KICA BT with sex, visit and sex-by-visit interaction as fixed effects. Visit was defined as the two occasions when the ^{13}C -KICA BT was undertaken in healthy subjects (separated by approximately 30 days) in order to assess the repeatability of the test. A repeated co-variance structure of scaled identity was used in the linear mixed model analysis. The estimates of fixed effects on each parameter of the ^{13}C -KICA BT were calculated using restricted maximum likelihood. The primary tests of interest in the linear mixed effects model analysis were the significance of sex, visit and sex-by-visit interaction on each parameter of the ^{13}C -KICA BT.

The repeatability of the ^{13}C -KICA BT was assessed using the method of Bland and Altman to check for the presence or absence of any proportional or fixed bias in each parameter of ^{13}C -KICA BT and where 95% of the differences or limits of agreement (LOA) lie between $\pm 1.96 \times \text{SD}$ of the mean difference. The coefficient of repeatability (COR) which is the value below or equal to which the absolute differences between two measurements of the ^{13}C -KICA BT would lie with 0.95 probability was calculated [14] according to Equation (6):

$$\text{z-score} \times \text{SD of differences} \times 2^{1/2} \quad (6)$$

where the z-score = 1.96 (i.e., the z-score for 95% confidence with a two-tailed $p < 0.05$).

The repeatability of the ^{13}C -KICA BT parameters was also expressed as the within subject coefficient of variation (COV) for paired examinations [15].

The minimum detectable change (MDC) which is the minimal amount of change outside of the measurement error that reflects a significant change in each ^{13}C -KICA BT parameter within a subject rather than due to random variation was calculated [16] according to Equation (7):

$$\text{z-score} \times \text{Standard error of the Mean (SEM)} \times 2^{1/2} \quad (7)$$

where the z-score = 1.96 (i.e., the z-score for 95% confidence with a two-tailed $p < 0.05$).

Data were analysed using Statistical Package for the Social Sciences (SPSS) Version 21.0 (IBM, New York, USA). Comparison of continuous variables between groups was performed with Students t-tests for normally distributed data. Univariate associations between anthropometric, biochemical and other measures of liver fat and fibrosis and the ^{13}C -KICA BT were investigated using either the Spearman's rank correlation for non-normally distributed data or Pearson's correlation for normally distributed data.

3 Results

The clinical and anthropometric measurements of 13 healthy subjects (5 men and 8 women) on the first visit are shown in table 1. Men had significantly higher body weight and higher plasma concentrations of ALP and total protein compared to women (all $p < 0.05$), while there were no significant differences in BMI, ALT, bilirubin, albumin, liver stiffness, CAP scores and the ^{13}C -KICA BT result between men and women. Furthermore, univariate associations between the ^{13}C -KICA BT and the clinical measurements in healthy subjects were investigated and showed that there were no significant correlations between the results of the ^{13}C -KICA BT and any of the clinical measurements: ALT ($r = -0.32$, $p = 0.3$), ALP ($r = -0.1$, $p = 0.75$), bilirubin ($r = 0.04$, $p = 0.9$), albumin ($r = 0.00$, $p = 1.0$), Total protein ($r = -0.14$, $p = 0.64$), liver stiffness score ($r = 0.04$, $p = 0.9$) and CAP scores ($r = 0.17$, $p = 0.6$).

The time course of the recovery of the ^{13}C -label on the breath in 13 healthy subjects after the oral administration of ^{13}C -KICA on 2 occasions (visits 1 and 2) and expressed as the percent ^{13}C -dose recovered per min on breath is shown in figure 1. Each visit showed a rapid appearance of ^{13}C -label on breath which peaked within 30 min and declining slowly thereafter. There was no significant difference in the PDR at 60 min (visit 1 $0.33 \pm 0.06\%$ vs visit 2 $0.31 \pm 0.05\%$; $p = 0.3$). Furthermore, there were no significant differences between the repeated measurements of the ^{13}C -KICA BT expressed as cumulative percent ^{13}C -dose recovered over 60 min between men and women ($17.5 \pm 2.8\%$ versus $19.71 \pm 4.5\%$; $p = 0.2$) although there was a trend towards an increased ^{13}C -KICA oxidation in women compared to men.

The results of the linear mixed effects model analysis for repeated measures for parameters of the ^{13}C -KICA BT are shown in table 2. This analysis showed that across all the parameters of the ^{13}C -KICA BT (cumulative ^{13}C -enrichment over 60 min, percent ^{13}C -dose recovered at 60 min and cumulative percent ^{13}C dose recovered over 60 min), there was no significant effect of sex and time, nor was there any interaction between sex and time, on the repeated measures for parameters of ^{13}C -KICA BT.

The Bland and Altman plots for the parameters of the ^{13}C -KICA BT expressed as the percent ^{13}C -dose recovered per min and cumulative percent ^{13}C dose recovered over 60 min are shown in figure 2. The plots showed that twelve out of thirteen data points fell within the 95% limits of agreement (LOA) for the percent ^{13}C -dose recovered per min. However, for the cumulative percent ^{13}C -dose recovered over 60 min (cPDR over 60mins), all 13 data points were within the 95% LOA. Furthermore, in table 2, the Bland Altman statistic showed no fixed or proportional bias for any of the parameters for expressing the ^{13}C -KICA BT.

Data on the repeatability of the parameters of the ^{13}C -KICA BT such as the within-subject coefficient of variation (COV), coefficient of repeatability (COR) and minimum detectable change (MDC) are shown in table 2. The cumulative ^{13}C -dose enrichment over 60 min had the lowest within subject COV of 12% with a COR of 0.4 atoms % excess and MDC of 0.11 atoms % excess when compared to the other parameters of the ^{13}C -KICA BT.

4 Discussion

The results of this study show that the ^{13}C -KICA BT over 60 min has excellent repeatability over the period of approximately 4 weeks that separated the two ^{13}C -KICA BTs. Our data suggest that the cumulative ^{13}C -dose enrichment on breath over 60 min was the most repeatable parameter for assessing hepatic mitochondrial function. By using only a 30 day separation between the two BTs, the repeatability of the BT measurements was unlikely to be influenced by physiological changes affecting hepatic mitochondrial function in each subject undergoing repeat testing.

In this study, we have determined the most repeatable parameter of the ^{13}C -KICA BT for assessing hepatic mitochondrial function. As suggested in a previous study [17], one of the most important requirements for all ^{13}C -liver function BT is to provide consistent and repeatable results. In addition, the choice of the most convenient parameter for the ^{13}C -KICA BT will involve achieving a balance between minimising the variability of the parameter within individuals, increasing the ability of the ^{13}C -KICA BT to reliably detect impaired hepatic mitochondrial function and the practicality of completing the ^{13}C -KICA BT with minimal subject effort. Our data show that the cumulative ^{13}C -dose enrichment over 60 min had the lowest within subject variability compared to other parameters of the ^{13}C -KICA BT. The low within subject variability of the cumulative ^{13}C -dose enrichment in breath over 60 min without measuring the VCO_2 could allow a more useful application of the ^{13}C -KICA BT for assessing hepatic mitochondrial function. This is because having access to indirect calorimetry to measure the VCO_2 and its associated costs may be limited in the clinical setting and therefore could have a significant impact upon the translation of the ^{13}C -KICA breath test from a research setting into clinical practice. However, there is still a need to determine whether the

omission of CO₂ production from calculating the results of the ¹³C-KICA BT will alter the diagnostic power to detect impaired hepatic mitochondrial function in subjects (especially if there are marked differences in CO₂ production between individuals) compared to cPDR over 60 min, which is normalised for CO₂ production.

Previous studies [5, 18] have shown the time course of the ¹³CO₂ excretion profile with an early peak between 30-40 min followed by a slow decline as confirmed by the current observations. Our study showed that there was no significant relationship between the clinical measurements of liver function and hepatic mitochondrial function assessed by the ¹³C-KICA BT in healthy subjects. In addition, our data showed no significant differences in the results of the ¹³C-KICA BT between men and women or with repeated measurements separated by 4 weeks. Although no significant differences in the results of the ¹³C-KICA BT were observed between men and women, the hepatic mitochondrial metabolism of ¹³C-KICA after the oral administration tended to be higher in women than men. Our finding was also consistent with that observed in earlier studies [8, 18, 19] indicating a possible effect of sex hormones on hepatic mitochondrial function in women. However, a recent study has shown that differences in the kinetic parameters of the ¹³C-KICA BT between men and women was nullified when the results of the ¹³C-KICA BT were adjusted for body composition [4]. Therefore, the effect of sex on the kinetic parameters of ¹³C-KICA BT before and after adjusting for body composition requires further investigation in order to establish normal ranges for the ¹³C-KICA BT in men and women.

The assessment of hepatic mitochondrial function can be performed by using other ¹³C-liver function breath tests that utilise the hepatic metabolism of other ¹³C-metabolic

probes such as ^{13}C -glycine and ^{13}C -methionine. However in this study, we chose ^{13}C - α -ketoisocaproate due to its less complicated and specific metabolic pathway in the hepatic inner mitochondrial membrane [20] compared to that of ^{13}C -glycine and ^{13}C -methionine [21, 22]. Furthermore, the high metabolic demand for glycine and methionine under different pathological states or metabolic states makes using these alternative ^{13}C -metabolic probes unsuitable for assessing hepatic mitochondrial function directly.

Our study had some limitations that should be considered. First, we studied only a relatively small number of men and women; thus, the study lacked power to identify any small differences that might exist between men and women. Secondly, we did not extend the test beyond 60 min and so cannot comment on the repeatability of the test beyond this time point. That said, we aimed to assess hepatic rather than muscle mitochondrial function and therefore chose to terminate the test at 60 minutes. Thirdly, we studied healthy subjects and it is not clear to what extent the findings may relate to those with liver injury or disease.

5 Conclusions

In our study, we have shown that the ^{13}C -KICA BT over 60 min is highly repeatable over 30 days in healthy subjects. It may be possible to simplify the ^{13}C -KICA BT by expressing results as cumulative ^{13}C -dose enrichment on breath over time avoiding the need to estimate the VCO_2 in individuals, which could make the test better suited to clinical practice. However, the effect of not estimating the VCO_2 on the performance of the test to detect impaired hepatic mitochondrial function in patients with chronic liver diseases will need to be determined.

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Disclosure statement

No potential conflict of interest has been reported by the authors.

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TABLE 1 Characteristics of the study population and anthropometric and clinical parameters measured at the screening visit (Visit 1).

Abbreviations: - ALT- Alanine aminotransferase, ALP-Alkaline phosphatase, CAP – Controlled attenuation parameter, ^{13}C -KICA - ^{13}C -ketoisocaproate, cPDR - cumulative percent ^{13}C -dose recovered on breath. All values are presented as mean \pm SD. * - significant differences at $p < 0.05$.

FIGURE 1 Kinetic curves representing the time course of the % ^{13}C -KICA dose recovered on breath (PDR) per min during the ^{13}C -KICA breath test in 13 healthy subjects on two occasions (visits 1 and visit 2) 30 days apart. All values are presented as mean \pm SEM.

FIGURE 2 Plot of differences between pairs versus their means of (A) % ^{13}C -KICA dose recovered on breath at 60 mins and (B) cumulative % ^{13}C -dose recovered (cPDR) on breath over 60mins for the ^{13}C -KICA breath test.

The dashed lines denote borders of the 95% confidence intervals plotted as 95% limits of agreement (LOA) for the lower boundary (LB) and upper boundary (UB) while the solid lines denote the mean differences between the repeated measurements of the parameters of ^{13}C -KICA breath test.

TABLE 2 Comparison of different parameters of the ^{13}C -KICA breath test (^{13}C -KICA BT) by linear mixed model analysis with repeated measures and measures of repeatability of different parameters of the ^{13}C -KICA BT. This table shows a linear mixed effects model with estimates and p-values of fixed effects of sex, visit and sex x visit interaction on the parameters of ^{13}C -KICA BT.

Abbreviations: standard deviation (SD), limit of agreement (LOA), confidence interval (CI), lower boundary (LB), upper boundary (UB), coefficient of variation (COV), coefficient of repeatability (COR) and minimum detectable change (MDC).

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