

Rapid Identification of Dihydropyrimidine Dehydrogenase Deficiency by Using a Novel 2-¹³C-Uracil Breath Test

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ABSTRACT

Purpose: Dihydropyrimidine dehydrogenase (DPD)-deficient cancer patients have been shown to develop severe toxicity after administration of 5-fluorouracil. Routine determination of DPD activity is limited by time-consuming and labor-intensive methods. The purpose of this study was to develop a simple and rapid 2-¹³C-uracil breath test, which could be applied in most clinical settings to detect DPD-deficient cancer patients.

Experimental Design: Fifty-eight individuals (50 “normal,” 7 partially, and 1 profoundly DPD-deficient) ingested an aqueous solution of 2-¹³C-uracil (6 mg/kg). ¹³CO₂ levels were determined in exhaled breath at various time intervals up to 180 min using IR spectroscopy (UBiT-IR₃₀₀). DPD enzyme activity and *DPYD* genotype were determined by radioassay and denaturing high-performance liquid chromatography, respectively.

Results: The mean (±SE) C_{max} , T_{max} , δ over baseline values at 50 min (DOB₅₀) and cumulative percentage of ¹³C dose recovered (PDR) for normal, partially, and profoundly DPD-deficient individuals were 186.4 ± 3.9, 117.1 ± 9.8, and 3.6 DOB; 52 ± 2, 100 ± 18.4, and 120 min; 174.1 ± 4.6, 89.6 ± 11.6, and 0.9 DOB₅₀; and 53.8 ± 1.0, 36.9 ± 2.4, and <1 PDR, respectively. The differences between the normal and DPD-deficient individuals were highly significant (all *P*s < 0.001).

Conclusions: We demonstrated statistically significant differences in the 2-¹³C-uracil breath test indices

(C_{max} , T_{max} , DOB₅₀, and PDR) among healthy and DPD-deficient individuals. These data suggest that a single time-point determination (50 min) could rapidly identify DPD-deficient individuals with a less costly and time-consuming method that is applicable for most hospitals or physicians' offices.

INTRODUCTION

Dihydropyrimidine dehydrogenase (DPD) deficiency is an autosomal codominantly inherited pharmacogenetic syndrome with a variable phenotype that ranges from partial to complete loss of DPD enzyme activity (1, 2). The clinical impact of DPD deficiency has been dramatically demonstrated by studies showing that 43–60% of the patients with severe toxicity (including death) after administration of standard doses of 5-fluorouracil (5-FU) are partially or profoundly DPD deficient (3, 4). Population studies have shown the prevalence of partial and profound DPD deficiency to be 3–5% and 0.1% in the general population, respectively (5–7).

Several methods including high-performance liquid chromatography, mass spectrometry, thin layer chromatography, and denaturing high-performance liquid chromatography (DHPLC) have been developed to identify DPD deficiency in cancer patients (8–11). Unfortunately, these methods remain too complex and time consuming for routine clinical use and are unavailable in most treatment facilities. The availability of a method to accurately determine exhaled ¹³C in breath offers a novel approach for the detection of DPD deficiency through the administration of 2-¹³C-uracil. Recent studies have successfully used a similar approach to diagnose *Helicobacter pylori* infection in a Food and Drug Administration-approved ¹³C-urea breath test and to examine metabolic abnormalities in carbohydrate, lipid, and amino acid metabolism (12–16).

In the present study, we describe the development of a rapid, simple, and noninvasive 2-¹³C-uracil breath test (UraBT), which can be performed in most cancer-treatment facilities or physicians' offices. After oral administration of 2-¹³C-uracil, ¹³CO₂ in exhaled breath was measured and indices [C_{max} , T_{max} , δ over baseline values at 50 min (DOB₅₀), and cumulative percentage of ¹³C dose recovered in breath (PDR)] were assessed for their ability to discriminate between healthy and DPD-deficient individuals.

MATERIALS AND METHODS

Principle of the UraBT. The principle of the UraBT is based on metabolism of 2-¹³C-uracil by the enzymes of the pyrimidine-catabolic pathway to produce ¹³CO₂ (see below, Eq. A). In DPD-deficient individuals, reduced 2-¹³C-uracil catabolism would be expected to result in decreased exhaled ¹³CO₂ levels.

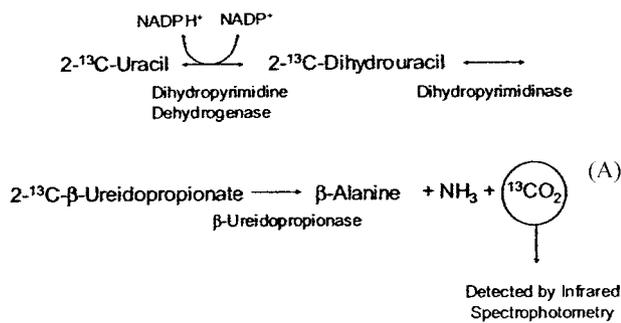
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$^{13}\text{CO}_2$ and $^{12}\text{CO}_2$ in exhaled breath samples is measured by IR spectrometry using the UBiT-IR₃₀₀ (Meretek Diagnostics, Lafayette, CO). The amount of $^{13}\text{CO}_2$ present in breath samples is expressed as a δ over baseline ratio that represents a change in the $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratio of breath samples collected before and after 2- ^{13}C -uracil ingestion (see Eq. B; Ref. 17).

$$\text{DOB} = \left[\frac{^{13}\text{CO}_2}{^{12}\text{CO}_2} \right]_{\text{post-dose sample}} - \left[\frac{^{13}\text{CO}_2}{^{12}\text{CO}_2} \right]_{\text{pre-dose sample}} \quad (\text{B})$$

The amount of 2- ^{13}C -uracil metabolized and released into the breath as $^{13}\text{CO}_2$ was determined for each time point using the equation described by Amarri *et al.* (18). These results were expressed as PDR. The UBiT-IR₃₀₀ instrument used to calculate the $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratio is discussed in detail elsewhere (17).

Study Design. Informed consent was obtained from each volunteer who participated in this institutional review board-approved study at the University of Alabama at Birmingham. Exclusion criteria for healthy individuals included respiratory or metabolic disorders.

Preliminary studies in four healthy individuals [one man and three women; mean age, 32 years (range: 22–48 years)] and three partially DPD-deficient individuals (one man and two women, ages 27, 23, and 31, respectively) evaluated the dose of 2- ^{13}C -uracil and time course that could provide maximal separation of breath patterns between these groups. Doses of 2- ^{13}C -uracil included fixed doses of 100, 200, or 300 mg as well as doses adjusted to body weight (1, 3, 6, or 12 mg/kg) examined from 0 to 180 min after ingestion.

After dose optimization and the determination of the time frame to be examined, subsequent studies used a 6 mg/kg dose of 2- ^{13}C -uracil evaluated over 180 min. The UraBT indices (C_{max} , T_{max} , DOB₅₀, and PDR) were determined in 50 healthy individuals [19 men and 32 women; mean age, 30 years (range, 19–70 years)] and seven partially DPD deficient individuals [six men and one woman; mean age, 35 years (range, 23–59 years)] and one profoundly DPD-deficient individual (one woman; age, 58 years). Two of the partially DPD-deficient individuals who participated in the initial dose escalation studies also participated in this phase of the study. In addition, all individuals participating in this study were phenotypically characterized by a DPD radioassay and genotypically characterized for known sequence variations associated with DPD deficiency by DHPLC with confirmation by sequence analysis as described below.

Uracil Breath Test. Following an overnight fast, volunteers started the protocol at approximately 8 a.m. All volunteers

were weighed and baseline breath samples were collected in 1.2-liter aluminum-lined bags (Otsuka Pharmaceuticals, Tokushima, Japan). Volunteers then ingested an aqueous solution (over a period of 15 s) containing 6 mg/kg bodyweight of 2- ^{13}C -uracil (99.9%; Cambridge Isotope Laboratories Inc., Andover, MA). This was followed by the collection of 21 breath samples over 180 min (obtained every 5 min for 30 min and then every 10 min thereafter). The $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratio of each breath sample was determined by IR spectroscopy using the UBiT-IR₃₀₀ instrument and data analysis performed as described above.

DPD Radioassay. Sixty ml of blood was drawn from a volunteer's peripheral vein at approximately 12 p.m. on the same day as their UraBT to limit variation resulting from the circadian rhythm in DPD enzyme activity (19). DPD activity in peripheral blood mononuclear cells (PBMC) was determined using a radioassay described previously (8). Individuals with PBMC DPD activity ≤ 0.18 nmol/min/mg protein were considered to be DPD deficient (5).

DHPLC Analysis of the DPYD Gene. DHPLC analysis was used to genotype the coding region of the *DPYD* gene of "normal" and DPD-deficient individuals as described previously (11). All *DPYD* sequence variants identified by DHPLC were confirmed by DNA sequencing using a dideoxynucleotide chain termination method (Big Dye Kit; Applied Biosystems, Foster City, CA) and capillary electrophoresis on an ABI 310 Automated DNA Sequencer (Applied Biosystems).

Discrimination of Normal and DPD-Deficient Individuals. Statistical comparisons between the 50 healthy and eight DPD-deficient individuals (seven partially and one profoundly deficient) for each of the UraBT indices (C_{max} , T_{max} , DOB₅₀, and PDR) were made using the two-sample *t* test and the signed rank Wilcoxon test. The performance of the UraBT as a potential diagnostic test was evaluated through statistical classification procedures using linear discriminant functions (a mathematical rule for categorizing subjects as DPD deficient or healthy based on their breath patterns). The accuracy of the resulting classification was assessed through the observed false-positive rate (FPR = proportion of healthy individuals incorrectly categorized as DPD deficient) and the observed false-negative rate (FNR = proportion of DPD-deficient individuals incorrectly categorized as healthy). The sensitivity and specificity of the UraBT were defined as the percentage of healthy and DPD-deficient individuals that were correctly classified [(1-FPR) \times 100% and (1-FNR) \times 100%, respectively]. Additionally, the performance of this classification scheme was further evaluated using cross-validation.

Assay Variability. Interassay variability was examined using repeated measures ANOVA to find any significant differences between breath test profiles within seven individuals (four healthy and three partially DPD deficient) who repeated the UraBT from 1 to 8 months after their initial examination. Additionally, equivalence between the individuals' different UraBT profiles was examined using a bioequivalence test as described by Phillips (20) and Diletti *et al.* (21). Intra-assay variability was examined among four healthy individuals (five repetitions), analyzed using repeated measures ANOVA, and summarized by coefficient of variation.

Assessment of Breath Collection Bag Integrity with Time. The integrity of the breath collection bags was assessed after storage at room temperature for up to 210 days. One hundred and thirty-two samples from 24 volunteers underwent duplicate analysis 90 ($n = 30$), 120 ($n = 32$), 150 ($n = 20$), 180 ($n = 25$), or 210 ($n = 25$) days after their initial examination. Integrity was analyzed using repeated measures ANOVA and summarized by coefficient of variation.

Statistical Analysis. All statistical summaries and analyses as described above were produced in SAS version 8.2, using procedures such as MEANS, GLM, MIXED, NLIN, NPARIWAY, and DISCRIM.

RESULTS

Preliminary UraBT Studies. Preliminary studies demonstrated that the administration of 2-¹³C-uracil dose adjusted to kilogram (body weight) generated less variable ¹³CO₂ breath patterns and indices (C_{max} , T_{max} , DOB_{50} , and PDR) than single-fixed doses of 100, 200, or 300 mg. The variability in breath patterns between the largest (107 kg) and smallest (50 kg) volunteers (volunteers A and D, respectively) when a fixed 300-mg dose of 2-¹³C-uracil is administered is shown in Fig. 1A. The reduction in variability after standardization of the 2-¹³C-uracil dose to 6 mg/kg is shown in Fig. 1B. Similar results were also observed in the breath patterns from the other volunteers who received both fixed and weight-adjusted doses (data not shown). Preliminary studies also demonstrated that the optimal dose of 2-¹³C-uracil that was needed to achieve maximal separation between the breath patterns of healthy and partially DPD-deficient volunteers was 6 mg/kg (data not shown). Time course studies from 0 to 180 min demonstrated that the elimination phase of ¹³CO₂ breath patterns from normal and DPD-deficient individuals could be examined within 180 min (data not shown).

Discrimination of Normal and DPD-Deficient Volunteers Using the UraBT. The ¹³CO₂ breath patterns and PDR (mean \pm SE) from normal, partially, and profoundly DPD-deficient volunteers are shown in Figs. 2 and 3, respectively. Highly significant differences in the UraBT indices (C_{max} , T_{max} , DOB_{50} , and PDR) were observed between "normal" and DPD-deficient individuals (all $P_s < 0.001$; Table 1).

Multiple linear discriminant functions were computed to determine which UraBT indices best classified DPD-deficient individuals. The discriminant function fitted on the DOB_{50} was demonstrated to be the optimal classification rule over all other time points. Subjects having a $DOB_{50} < 128.9$ were classified as DPD deficient, and those with a $DOB_{50} \geq 128.9$ were classified as normal. Using these criteria, the UraBT demonstrated an observed 100% sensitivity (with all of the DPD-deficient individuals correctly identified as DPD deficient) and 96% specificity (with 48 of 50 individuals correctly classified as normal).

Inter- and Intra-Assay Variability. Assessment of the interassay variability of the UraBT demonstrated the results were reproducible with no significant differences observed between original breath patterns and those obtained from a second UraBT repeated several months later. Bioequivalence was sig-

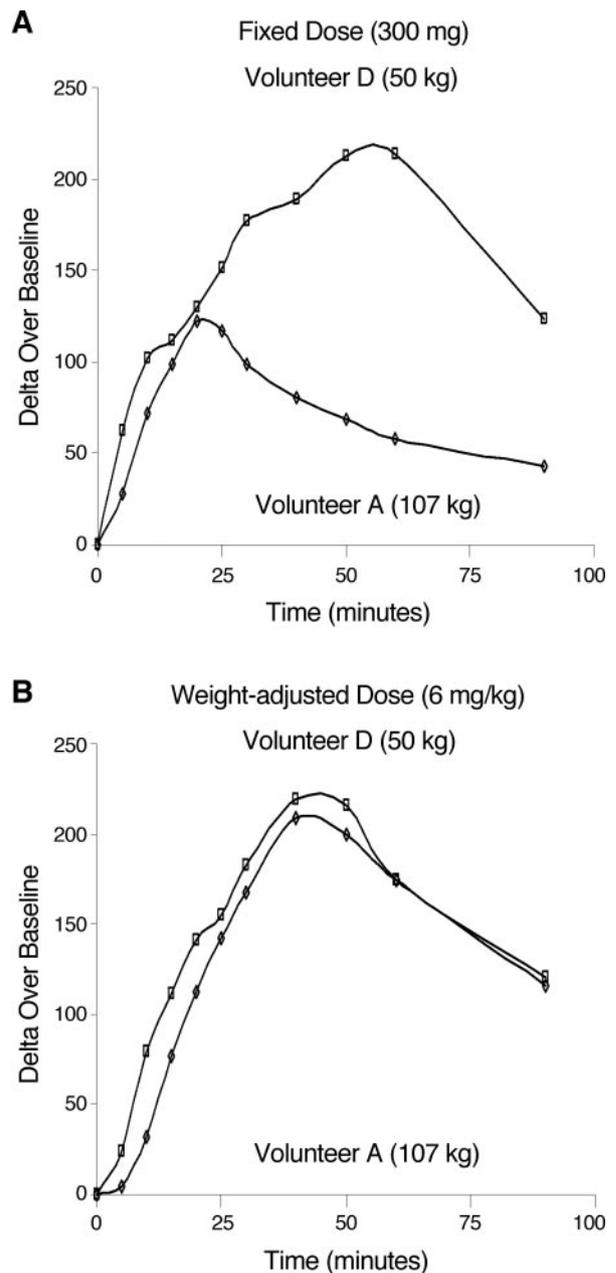


Fig. 1 Examination of variability of ¹³CO₂ breath patterns after administration of a fixed dose (300 mg) and a weight-adjusted dose (6 mg/kg) of 2-¹³C-uracil. Shown are the ¹³CO₂ breath patterns from two healthy volunteers [volunteers A (\diamond) and D (\square)] after administration of a fixed 300-mg dose (A) and a weight-adjusted (6 mg/kg) dose (B) of 2-¹³C-uracil. Less variability was observed in breath patterns when the dose of 2-¹³C-uracil was standardized to weight (6 mg/kg). In the fixed dose studies (A), the dose adjusted to weight for largest (107 kg) individual (volunteer A) corresponded to 2.8 mg/kg, whereas the dose adjusted to weight for smallest (50 kg) individual (volunteer D) corresponded to 6 mg/kg.

nificantly demonstrated at a 5% level of significance. All intra-assay coefficient of variations were $< 5\%$.

Integrity of Breath Collection Bags. ¹³CO₂ content in breath collection bags stored at room temperature was assessed

90, 120, 150, 180, or 210 days after their initial examination. No significant differences were observed between the $^{13}\text{CO}_2$ content of sample bags before and after storage. $^{13}\text{CO}_2$ levels of breath samples before and after storage were highly correlated ($R^2 > 99\%$; Fig. 4)

DPD Enzyme Activity by Radioassay. The DPD enzyme activity of all individuals enrolled in this study was determined. The mean (\pm SE) PBMC DPD activity of the 50 normal individuals was 0.30 ± 0.01 nmol/min/mg protein (range, 0.19 to 0.44). The mean PBMC DPD activity of the seven partially deficient individuals was 0.10 ± 0.02 nmol/min/mg protein (range, 0.03 to 0.17). The PBMC DPD activity of the profoundly deficient individual was undetectable.

DPYD Genotype. No sequence variants previously associated with DPD deficiency were identified in the *DPYD* gene of the “normal” individuals (data not shown). Table 2 summarizes the sequence variations identified in the partial and profoundly DPD-deficient individuals (D-1 through D-8). D-1, D-2, D-3, and D-4 demonstrated a heterozygous *DPYD**2A (IVS14 + 1G>A) genotype and a partially DPD-deficient phenotype. D-3 also demonstrated an additional heterozygous sequence variant in exon 19 (2329G>T, A777S). D-6 demonstrated a homozygous *DPYD**2A (IVS14 + 1G>A) genotype and a profoundly DPD-deficient phenotype (no detectable DPD en-

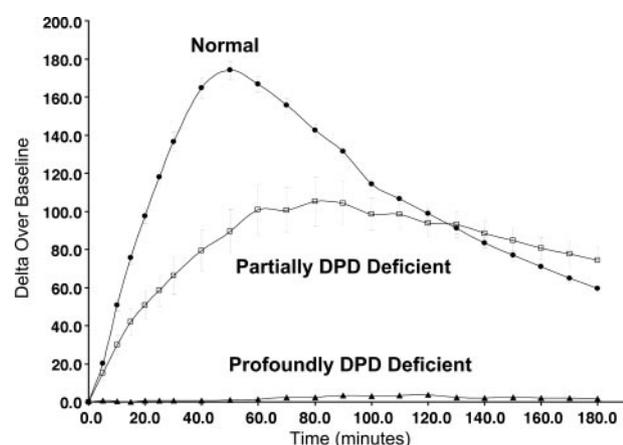


Fig. 2 $^{13}\text{CO}_2$ breath patterns from 50 normal and 8 DPD-deficient volunteers. Normal (●), partially (□), and profoundly (▲) DPD-deficient individuals ingested a 6 mg/kg oral solution of 2- ^{13}C -uracil. Breath samples were collected for 180 min after ingestion and the amount of ^{13}C label in breath (expressed as δ over baseline) was determined for each time point (mean \pm SE).

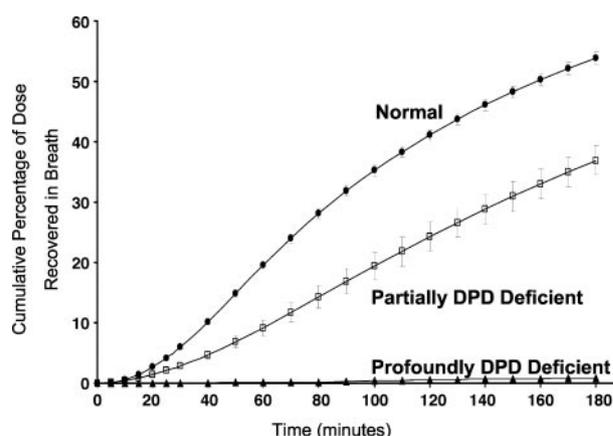


Fig. 3 Percentage of ^{13}C dose recovered from the breath of 50 normal and 8 DPD-deficient volunteers. Normal (●), partially (□), and profoundly (▲) DPD-deficient individuals ingested a 6 mg/kg oral solution of 2- ^{13}C -uracil. Breath samples were collected for 180 min after ingestion, and the percentage of ^{13}C dose recovered in breath was determined for each time point (mean \pm SE).

zyme activity). D-7 demonstrated three heterozygous sequence variations [*DPYD**13 (1679T>G, I560S), *DPYD**9A (85 T>C, C29R) and 496 A>G, M166V] and a partially DPD-deficient phenotype. D-5 and D-8 had no known sequence variants previously associated with DPD deficiency within the coding region of the *DPYD* gene, although a partially DPD-deficient phenotype was demonstrated.

DISCUSSION

In the United States, adverse drug reactions have been shown to account for over 100,000 fatalities/year and remain the fourth leading cause of death after heart disease, cancer, and stroke (22). It is estimated that 31–34% of the 2 million patients who receive 5-FU/year exhibit severe toxicity (including death) and that approximately 50% of these patients with adverse drug reactions secondary to 5-FU have reduced DPD activity or no DPD activity (3, 23–25). Although the frequency and lethality of DPD deficiency distinguishes this syndrome from other pharmacogenetic disorders (*i.e.*, thiopurine methyltransferase deficiency), the clinical diagnosis of DPD deficiency remains difficult because the appearance of life-threatening toxicity is typically the first symptom of this pharmacogenetic syndrome. Despite over 10 years of research, clinically feasible assays to

Table 1 Uracil breath test indices

Volunteers ^a	C_{\max} (DOB) ^b	T_{\max} (minutes) ^b	DOB ₅₀ (DOB) ^b	Percentage dose recovered ^b
Normal ($n = 50$)	186.4 ± 3.9	52.0 ± 2.0	174.1 ± 4.6	53.8 ± 1.0
Partially DPD ^c deficient ($n = 7$)	117.1 ± 9.8	100 ± 18.4	89.6 ± 11.6	36.9 ± 2.4
Profoundly DPD deficient ($n = 1$)	3.6	120	0.9	<1

^a Fifty normal and eight (seven partially and one profoundly) DPD-deficient individuals performed the 2- ^{13}C -uracil breath test as described in “Materials and Methods.”

^b Highly significant differences in 2- ^{13}C -uracil breath test indices were observed between normal and DPD-deficient individuals (all P s < 0.001). The data shown are expressed as a mean \pm SE.

^c DOB₅₀, δ over baseline 50 min following 2- ^{13}C -uracil ingestion; DOB, δ over baseline; DPD, dihydropyrimidine dehydrogenase.

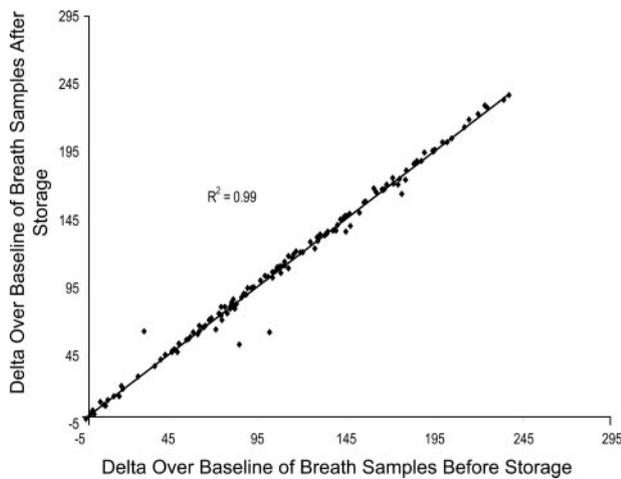


Fig. 4 Integrity of breath collection bags. One hundred thirty-two breath samples underwent duplicate analysis after storage at room temperature for 90 ($n = 30$), 120 ($n = 32$), 150 ($n = 20$), 180 ($n = 25$), or 210 ($n = 25$) days after their initial examination to establish the ability of breath collection bags to retain $^{13}\text{CO}_2$.

identify DPD-deficient individuals are not yet available in cancer treatment facilities.

In the current study, a facile, noninvasive UraBT was developed and optimized to discriminate between healthy, partially, or profoundly DPD-deficient individuals. This approach exploits the difference in $2\text{-}^{13}\text{C}$ -uracil catabolism between normal and DPD-deficient individuals. Previous pharmacokinetic studies from our laboratory in a profoundly DPD-deficient patient demonstrated reduced 5-FU catabolism with prolonged elimination half-life compared with the “normal” population (26). Additional studies by our laboratory using eniluracil (a potent DPD inhibitor) showed similar results (27). Taken collectively, these data suggested that the reduced catabolism of $2\text{-}^{13}\text{C}$ -uracil in DPD-deficient individuals should result in a subsequent decline in $^{13}\text{CO}_2$ levels in breath.

Preliminary studies of the UraBT demonstrated that an

administered dose of 6 mg/kg $2\text{-}^{13}\text{C}$ -uracil generated less variable breath indices (C_{max} , T_{max}) than single-fixed doses of 100, 200, or 300 mg (Fig. 1) and that the elimination phase of $^{13}\text{CO}_2$ in normal and DPD-deficient individuals could be examined within 180 min. Administration of 6 mg/kg of $2\text{-}^{13}\text{C}$ -uracil to normal, partially, and profoundly DPD-deficient individuals showed a significantly reduced C_{max} , DOB_{50} , and PDR and a significantly increased T_{max} in deficient individual compared with normal individuals (Figs. 2 and 3; Table 1). The maximal $^{13}\text{CO}_2$ δ over baseline differences in breath pattern indices were observed at 50 min comparing normal and DPD-deficient individuals ($P < 0.001$). Applying a cutoff value of 128.9 DOB_{50} min, all eight DPD-deficient individuals were correctly identified (100% sensitivity). In addition, 48 of 50 normal individuals were also correctly identified (96% specificity). These data suggest that one time point (50 min) may be used to discriminate between normal and DPD-deficient patients; however, larger population studies are required to clearly establish the optimal time point where δ over baseline cutoff values would provide maximal separation.

To determine whether exhaled breath samples could be stored and shipped to distant sites for analysis, the integrity of the breath collection bags were examined for changes in $^{13}\text{CO}_2$ levels after storage at room temperature for up to 210 days. As shown in Fig. 4, no significant changes were observed in the $^{13}\text{CO}_2$ levels. Taken collectively, the ability to use one time point to discriminate between normal and DPD-deficient individuals and the stability of the breath samples suggests that the UraBT offers the first potentially useful diagnostic assay for the identification of DPD-deficient individuals.

Interestingly, there was no strong correlation between PBMC DPD activity and any of the UraBT indices. This is likely because determining DPD enzyme activity from a single tissue (PBMC) cannot be directly compared with the systemic metabolism of $2\text{-}^{13}\text{C}$ -uracil by multiple organs (*i.e.*, liver, colon, lung, brain, spleen, and PBMC), which are known to have various levels of DPD (28, 29).

All DPD-deficient individuals examined in this study

Table 2 Sequence variants identified in the coding region of the *DPYD* gene in DPD deficient volunteers

Location of <i>DPYD</i> sequence variant	D-1 ^a	D-2	D-3	D-4	D-5	D-6	D-7 ^a	D-8
Exon 2							<i>DPYD</i> *9A 85T>C C29R	
Exon 6							496A>G M166V	
Exon 13							<i>DPYD</i> *13 1679T>G 1560S	
Exon 14	<i>DPYD</i> *2A IVS14+IG>A	<i>DPYD</i> *2A IVS14+IG>A	<i>DPYD</i> *2A IVS14+IG>A	<i>DPYD</i> *2A IVS14+IG>A		<i>DPYD</i> *2A IVS14+IG>A homozygous		
Exon 19			2329G>T A777S					
DPD enzyme activity (nmol/min/mg)	0.07	0.17	0.06	0.14	0.08	Undetectable	0.03	0.17

^aData from Johnson et al. (1).

were genotyped to identify the molecular basis of their DPD deficiency (Table 2). Individuals D-1 through D-4 (with a partially DPD-deficient phenotype) demonstrated a heterozygous *DPYD**2A genotype, whereas D-6 (with a profoundly DPD-deficient phenotype) demonstrated a homozygous *DPYD**2A genotype. D-3 demonstrated a heterozygous sequence variation in exon 19 (2329G>T, A777S), which has been observed previously in a partially DPD-deficient patient (30). D-7 (with a partially DPD-deficient phenotype) demonstrated a heterozygous *DPYD**13, *DPYD**9A, and M166V genotype. Both *DPYD**2A and *DPYD**13 have been confirmed by our laboratory and others to result in DPD deficiency, whereas *DPYD**9A and M166V have been observed in individuals with normal activity (31–33). Interestingly, D-5 and D-8 were identified as DPD deficient (using both the radioassay and UraBT); however, no known sequence variants were identified in the coding region of the *DPYD* gene. The molecular basis for DPD deficiency in these individuals remains unknown and may be attributable to sequence variations occurring in an untranslated region of the *DPYD* gene (including the promoter, 5′ untranslated region, 3′ untranslated region, or intronic regions).

Previous studies have suggested that approximately 50% of cancer patients with severe 5-FU toxicity are DPD deficient; however, the etiology of 5-FU toxicity in the remaining patients is unclear. Increased 5-FU toxicity has also been linked to deficiencies in the dihydropyrimidinase and β-ureidopropionase enzymes and alterations in the thymidylate synthase promoter (34–37). Future studies will examine whether similar methodologies may be used to identify patients with these other pyrimidine-catabolic disorders.

In summary, this study demonstrates that the UraBT can rapidly discriminate between normal, partially, and profoundly DPD-deficient individuals (within 50 min) and offers a useful screening method that can be applied in most clinical settings (e.g., hospitals and physicians' offices) to identify DPD-deficient individuals before 5-FU chemotherapy. Furthermore, this novel approach may be used to collect samples at remote locations with subsequent analysis at a centralized reference laboratory.

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