Effects of Valproic Acid on Organic Acid Metabolism in Children: A Metabolic Profiling Study

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Young children are at increased risk for valproic acid (VPA) hepatotoxicity. Urinary organic acid profiles, as a surrogate of mitochondrial function, were obtained in children 1.9 to 17.3 years of age (n = 52) who were undergoing treatment with VPA for seizure disorders. Age-matched patients receiving treatment with carbamazepine (CBZ; n = 50) and healthy children not undergoing treatment (n = 22) served as controls. Age-related changes in organic acid profiles were observed in all three groups. Although the untreated and CBZ control groups were indistinguishable from each other with respect to the principal-component analysis (PCA) score plots of the subjects, a distinct boundary was apparent between the VPA and each of the control groups. Interindividual variability was observed in the VPA-induced alterations in endogenous pathways corresponding to branched-chain amino acid metabolism and oxidative stress. The data suggest that more detailed metabolomic analysis may provide novel insights into biological mechanisms and predictive biomarkers for children at highest risk for serious toxicity.

Valproic acid (VPA), a widely prescribed antiepileptic drug, is associated with a severe idiosyncratic hepatotoxicity characterized by microvesicular steatosis and necrosis. Although it is rare, this toxicity can be fatal, particularly in children <2 years of age, those with developmental delays or metabolic disorders (especially disorders of mitochondrial function), and those concurrently receiving enzyme-inducing medication.¹–³ Although the exact mechanism of VPA-induced hepatotoxicity has not been definitively identified, it is hypothesized that this effect is mediated through interference with mitochondrial β-oxidation. Competitive inhibition of β-oxidation enzymes⁴,⁵ and depletion of carnitine,⁶ coenzyme A,⁴,⁵,⁷ and glutathione⁸ stores during VPA metabolism may impair lipid metabolism, resulting in steatosis. In addition, oxidative stress⁹,¹⁰ may contribute to the toxic effects of VPA.

Valproate is a substrate for branched-chain amino acid metabolism,¹ utilizing the same enzymes and cofactors that are needed for mitochondrial lipid metabolism. It is thought that, because of its small size, VPA diffuses passively across the mitochondrial outer membrane, independent of the carnitine shuttle.⁴,⁵ Once inside the mitochondrial matrix, VPA is converted to valproyl-CoA, a substrate for dehydrogenation by 2-methyl branched-chain acyl-CoA dehydrogenase, forming 2-ene-VPA-CoA.⁵ The latter is converted into 3-OH-VPA-CoA by the β-oxidation enzyme enoyl-CoA hydratase and then into 3-keto-VPA-CoA by an unidentified membrane-bound NAD⁺-dependent dehydrogenase.⁵ Approximately 40% of the 3-keto-VPA-CoA is cleaved into propionyl-CoA and pentanoyl-CoA by an unidentified thiolase, with the remaining 60% probably being hydrolyzed to 3-keto-VPA.¹¹

Several studies have been conducted to investigate the effects of VPA on cellular metabolism, using supratherapeutic doses in rodents.¹²–¹⁴ Although the results of these studies uniformly demonstrate alterations in metabolic end points, no unifying mechanism of hepatotoxicity has been presented. In one study using ¹³C-labeled glucose, VPA was reported to cause a...
of bacterial contamination of the urine sample. A regression analysis was performed between sample age and individual organic acid concentrations. Seven organic acids were identified as having significant nonzero correlations with sample age ($P < 0.05$): 2-ketoisocaproic, succinic, methylsuccinic, acetonitrile, isocitric, methylicitric, and 4-hydroxyphenylpyruvic acids. None of these correlations retained significance after the application of a Bonferroni-corrected $\beta$ level of $0.0007$ ($\alpha = 0.05/n = 70$). Removal of the data for the organic acids that were most strongly correlated with sample age did not appreciably alter the principal-component model (described in more detail below), suggesting that the storage of the samples pending analysis contributed little variance to the data set.

Prior to assessing VPA’s effects on organic acid profiles, the underlying effects of subject age in the data set were determined. Normalization of the data set was shown to introduce some age correlation structure. All organic acid levels are reported as mmol acid/mol creatinine in order to account for variations in urine dilution. However, this normalization can be problematic in pediatric samples, in which the creatinine excretion is expected to increase with age. Creatinine level showed a significant correlation to subject age ($r = 0.383$; $r^2 = 0.145$; $P = 0.0061$). The excretion rate of creatinine did not differ significantly between the carbamazepine (CBZ) and VPA groups, indicating that these drugs do not alter creatinine excretion, as has also been shown previously.

A principal-component model was generated, and the principal components were then regressed against age. The first principal component (PC1) accounted for 31.2% of the variance and explained in vitro studies using isolated rat liver mitochondria that demonstrated that pyruvate uptake across the mitochondrial membrane is severely inhibited by VPA and its metabolites, along with diminished rates of ATP synthesis fueled by pyruvate. Efforts to understand the mechanisms through which VPA alters cellular metabolism have implications not only for hepatotoxicity but also for weight gain, a common side effect of VPA therapy thought to be related to increased availability of long-chain fatty acids.

Attempts to identify the specific factor(s) underlying the increased risk for VPA-induced hepatotoxicity in young children have focused on the pathways of VPA biotransformation, with particular interest in those that differ substantially between children and adults in order to gain insights into the mechanisms leading to preferential toxicity in susceptible children. A common, but not universal, finding is a role for 2-n-propyl-4-pentenoic acid (4-ene-VPA), a potentially hepatotoxic metabolite formed by $\omega-1$ oxidation of VPA (see Supplementary Figure S1 online). Although it has been suggested that 4-ene-VPA formation is increased in younger children, age-related differences have not been reproducible. However, thiol conjugates of VPA metabolites potentially reflect the burden of reactive metabolites formed from VPA and have been reported to decline with increasing age in childhood. In a comprehensive analysis of VPA metabolites in 91 children receiving VPA mono- and polytherapy, we were unable to replicate any age-related changes in urinary VPA metabolite concentrations when developmental changes in urinary creatinine concentration were taken into consideration (K.E. Price et al., unpublished data). Consequently, the current investigation pursues an alternative hypothesis, namely, that developmental differences in mitochondrial function exist, and that introduction of a medium-chain fatty acid load (e.g., VPA) may cause age-dependent perturbations in mitochondrial function, as measured by urinary organic acid profiles.

RESULTS

Subjects

This study involved 127 children 1.7–17.6 years of age. The demographic parameters of the study sample are presented in Table 1.

Effects of sample and subject age on organic acid profiles

The concentrations of urinary organic acids determined in this study were comparable to values reported in Swiss, Turkish, and American pediatric populations. However, because these data were generated from a set of residual urine samples, the effect of sample age (storage time) on organic acid profiles was assessed. Findings in the literature indicate that levels of lactic, 2-hydroxyglutaric, 2-ketoglutaric, succinic, 3-hydroxypropionic, and hippuric acids are likely to change as a result

Table 1 Demographic information for the study cohort

| Characteristic | CBZ    | VPA    | No medication |
|---------------|--------|--------|---------------|
| Gender        |        |        |               |
| Male          | 34     | 34     | 11            |
| Female        | 16     | 18     | 11            |
| Age (years)   | 10.4 ± 4.1 | 10.2 ± 3.9 | 11.1 ± 4.9 |
|               | (1.7–17.6) | (1.9–17.3) | (3.0–17.3)   |
| Weight (kg)   | 40.7 ± 21.3 | 33.8 ± 15.8 | 41.4 ± 17.8 |
|               | (11.2–99.1) | (7.6–86.4) | (13.9–67.9) |
| Race          |        |        |               |
| AA            | 1      | 4      | 4             |
| C             | 4.2    | 5.1    | 18            |
| H             | 4      | 4      | 4             |
| AA/C          | 3      | 4      |               |
| Race          |        |        |               |
| AA            | 4      | 4      |               |
| C             | 1      | 1      |               |
| Oth           | 1      | 1      |               |
| Dosage (mg/kg/day) | 16.6 ± 10.1 | 25.9 ± 18.7 | N/A          |
|               | (3.1–45.8) | (7.6–138.9) |               |

AA, African American; AA/C, African American/Caucasian; AS, Asian; C, Caucasian; CBZ, carbamazepine; H, Hispanic; N/A, not applicable; Oth, Other; VPA, valproic acid.
Effect of drug administration on organic acid profiles

In the principal-component analysis (PCA) model described above, the first three components explained 44.4% of the variation in the data set, with PC1 explaining 31.2% of the variance. The scores plot is presented in Figure 2a. Control and CBZ samples overlapped completely and were mostly confined to a cluster along PC2. There was a distinct boundary between the control/CBZ and VPA groups, with the VPA subjects showing interindividual variability in the extent to which individual samples deviated from the control/CBZ grouping; an age-related trend was identified for PC1.

Because VPA is an eight-carbon medium-chain fatty acid, we considered the possibility that the observed difference in organic acid profiles was slower than the age-related increase in creatinine level. When PC1 was regressed with subject age, the trends were parallel for the VPA and control groups (Figure 1). These results indicate that age-related changes in urinary organic acid profiles were preserved in all three groups and further imply that this relationship was not affected by drug administration.

**Figure 1** Age trends identified by principal-component analysis (PCA). Subject age was regressed against first principal component (PC1) scores separately for each group: control (red squares), carbamazepine (green triangle), and valproic acid (blue circle). The lines of best fit for each group are colored to match the color of the symbol for that group. The similarity in the slopes of the regressed lines indicates that age-related changes in organic acid profiles were similar in each group and independent of drug treatment.

**Figure 2** Principal-component analysis (PCA) scores plot. (a) PCA was performed on the data correlation matrix to uncover the major sources of variation in the data set. The first principal component (PC1) accounts for 31.2% of the variance and is primarily defined by 2-hydroxyglutaric, 3-hydroxyisobutyric, 3-hydroxyisovaleric, and glutaric acids. The second principal component (PC2) is primarily defined by an inverse relationship between uracil and 4-hydroxyphenylpyruvic, 2-ketoisovaleric, acetoacetic, 2-hydroxybutyric, 2-keto-3-methylvaleric, and 2-ketoisocaproic acids. The control (open squares) and CBZ (closed triangles) groups were superimposable, whereas a distinct boundary was observed between the control/CBZ and VPA (closed circles) groups, with the VPA subjects showing interindividual variability in the extent to which data for individual samples deviate from those for the control/CBZ grouping. To address the possibility that the observed changes attributed to VPA were due to organic acids that may have been derived from VPA metabolism, organic acids exceeding specified correlation thresholds with any VPA metabolite were successively excluded from the PCA analysis. (b) The original score plot containing the full data set. The threshold was set at $R^2 = 0.5$ and was sequentially reduced to thresholds of (c) $R^2 = 0.4$ and (d) $R^2 = 0.3$. The distribution of values was then compared with those for the full data set. The data are labeled by group (open squares, control; closed triangle, CBZ; closed circle, VPA). CBZ, carbamazepine; VPA, valproic acid.
acid profiles between VPA and the other two groups was due to the formation of “endogenous” organic acids derived from the metabolism of VPA or the further metabolism of its primary metabolites. The PCA was repeated after excluding data for organic acids that correlated with VPA metabolites to an extent greater than a threshold value of \( r^2 = 0.5 \) (eight organic acids; Figure 2b), \( r^2 = 0.4 \) (15 organic acids; Figure 2c), or \( r^2 = 0.3 \) (24 organic acids; Figure 2d). The removal of data pertaining to these organic acids did not result in any demonstrable change in the plots relative to Figure 2a, and the distinct boundary between VPA and control/CBZ samples persisted in all the plots. That is, intercorrelations among the VPA metabolites and organic acids had little bearing on the calculation of the initial VPA PCA model, lending credibility to the model’s robustness and the observed trends.

The PCA model could not distinguish between healthy control subjects and those receiving CBZ. Therefore, the organic acids identified as significant on the PC2 axis per this model relate solely to VPA-induced changes in metabolism. PC2 is defined primarily by an inverse relationship between concentrations of uracil and 4-hydroxypseudouracil, 2-ketoisovaleric, acetocetic, 2-hydroxybutyric, 2-keto-3-methylvaleric, and 2-ketoisocaproic acids. These organic acids provide insights into the metabolic pathways that are uniquely perturbed by VPA. 2-Keto-3-methylvaleric and 2-ketoisovaleric acids are intermediates in branched-chain amino acid metabolism, which is known to be altered by VPA; concentrations of these acids are also elevated in lactic acidosis and ketoacidosis. 2-Hydroxybutyric acid, as a byproduct of glutathione synthesis, is a marker of oxidative stress, which is also a known effect of VPA. 2-Keto-3-methylvaleric acid, which is generated by L-leucine metabolism, is found in very high levels in maple syrup urine disease but has not been reported to be altered by VPA therapy.

Other, more subtle, trends were noted in the PC scores plot. When the first three principal components were regressed against demographic variables, PC1 showed a highly significant \( P < 0.0001 \) correlation with age, group, body weight, and dose. A table of \( P \) values for PC1 regressed to various demographic variables is shown in Table 2, representing regression for the data as a whole and broken into data for individual groups. PC2 was significantly correlated with age, group, race, and body weight (Table 3). PC3 was significantly correlated with group, and the presence of concurrent medications was classified (for VPA subjects) as monotherapy, noninducer medications, and enzyme-inducing medications (Table 4).

### Differential effects of VPA and CBZ on organic acid profiles

The CBZ group served as a control for the potential effect of seizure disorders on organic acid profiles. Comparison of the two groups by means of PCA identified a significant effect of VPA on urinary organic acid profiles in children but did not show any effect of CBZ. Subsequently, the data were modeled using linear discriminant analysis, and a unique metabolic effect from both VPA and CBZ was noted. A significant two-canonical-variable model was calculated from the data, with canonical coefficient 1 (CC1) accounting for 66.9% of the data variation and CC2 accounting for the remaining 33.1%. The misclassification rate was 2.4%, with 3 of the 124 samples misclassified—one CBZ sample was misclassified as a VPA sample, and two samples (one CBZ and one VPA) were classified as samples of healthy controls. The differentiation between group mean values was highly significant.

### Table 2 P values for the regression of principal component 1 against various demographic variables, using both the entire data set and the data set broken into three groups (CBZ, VPA, and control)

| Variable | All | CBZ | Control | VPA |
|----------|-----|-----|---------|-----|
| Group    | <0.0001 | —   | —       | —   |
| Age      | <0.0001 | <0.0001 | 0.0005 | <0.0001 |
| Gender   | 0.7373  | 0.9514 | 0.5308  | 0.8603 |
| Race     | 0.6576  | 0.9027 | 0.5130  | 0.1453 |
| Weight   | <0.0001 | 0.0003 | 0.0002  | <0.0001 |
| Dose     | <0.0001 | 0.1121 | —       | 0.0019 |
| Concurrent medications | —   | —   | 0.5339  | —   |

Principal component 1 represents the linear combination of factors that accounts for the largest amount of variability in the data, and each succeeding component (principal component 2, principal component 3) has the highest variance possible under the constraint that it is uncorrelated with the preceding components. Principal component 1 accounted for 31.2% of the variability in the current data set.

### Table 3 P values for the regression of principal component 2 against various demographic variables, formatted as described in Table 2

| Variable | All | CBZ | Control | VPA |
|----------|-----|-----|---------|-----|
| Group    | <0.0001 | —   | —       | —   |
| Age      | 0.0002  | <0.0001 | 0.0039 | 0.0125 |
| Gender   | 0.8431  | 0.2905 | 0.4777  | 0.6490 |
| Race     | 0.0380  | 0.2142 | 0.3559  | 0.1964 |
| Weight   | 0.0069  | 0.0003 | 0.0040  | 0.0113 |
| Dose     | 0.3045  | 0.2674 | —       | 0.6031 |
| Concurrent medications | —   | —   | —       | 0.6926 |

### Table 4 P values for the regression of principal component 3 against various demographic variables, formatted as described in Table 2

| Variable | All | CBZ | Control | VPA |
|----------|-----|-----|---------|-----|
| Group    | 0.0239 | —   | —       | —   |
| Age      | 0.7877  | 0.5544 | 0.2582 | 0.8455 |
| Gender   | 0.3792  | 0.0640 | 0.2715  | 0.9151 |
| Race     | 0.6321  | 0.4677 | 0.6304  | 0.9294 |
| Weight   | 0.9032  | 0.4108 | 0.6008  | 0.5823 |
| Dose     | 0.7792  | 0.5114 | —       | 0.5942 |
| Concurrent medications | —   | —   | —       | 0.0057 |

Significant correlations are shown in bold. CBZ, carbamazepine; VPA, valproic acid.
CBZ group also showed some significant nonzero age-related effects on mitochondrial function. The two groups and are therefore potential organic acid biomarkers (results were compared. Eight organic acids were identified as in each of the groups (VPA, CBZ, and healthy control), and the CBZ and VPA groups. We regressed organic acid levels to age in patients treated with VPA and CBZ. Age-dependent perturbation of organic acid profiles in patients treated with VPA and CBZ

Differential metabolic perturbation by age was noted in the in children are probably associated with increased metabolic demands. Given that impairment of mitochondrial β-oxidation has been implicated in VPA hepatotoxicity, and developmental changes in mitochondrial function may be implied by the age-dependent changes in normal ranges for urinary organic acid concentrations used in pediatric settings to diagnosis inborn errors of metabolism, we hypothesized that the developmental context in which VPA is administered may be an important determinant for age-related differences in the risk for serious forms of toxicity. The data presented in this study represent an initial exploration of this alternative approach.

Few, if any, studies have used comprehensive profiling of urinary organic acids or other biomarkers to assess alterations in mitochondrial metabolism by VPA on a population basis in children. The data presented in this study provide a unique opportunity to examine the variability in endogenous metabolic consequences of VPA treatment in children and to formulate hypotheses for further prospective studies. Data models revealed that a systemic metabolic change occurs in response to VPA treatment in children who show no evidence of overt hepatic damage, although significant interindividual variability in metabolic responses to VPA was observed. A final key finding was an age-dependent metabolic response to VPA, thereby giving a new perspective to the unexplained VPA toxicity in young significant, as gauged by the Wilks’ lambda test ($P < 0.0001$). The canonical plot is shown in Figure 3. Note that in this model the CBZ and control samples were distinguished, as compared with the PCA model presented above. This finding suggests that CBZ perturbs the metabolism through a mechanism distinct from that of VPA. Given that PCA did not detect this difference, the perturbation from CBZ is probably more subtle.

On examination of the canonical plot, the CC1 separated the VPA samples from the control and CBZ samples. CC1 is primarily defined as a contrast axis between pimelic, 2-hydroxyglutaric, 4-hydroxyphenylpyruvic, and succinic acids on the one hand and glycolic, azelaic, and 3-methylglutaric acids on the other. Five of these (pimelic, 2-hydroxyglutaric, succinic, azelaic, and 3-methylglutaric acids) are dicarboxylic acids known to be excreted in larger quantities under conditions of oxidative stress and impaired fatty acid oxidation. CC2 separated the CBZ samples from the VPA and control groups (with some separation of the VPA and control samples also noted). CC2 is primarily defined by a contrast between citric, 3-hydroxyisobutyric, and glutaric acids on the one hand and aconitic and pimelic acids on the other. Increased excretion of dicarboxylic acids implies increased oxidative stress, and intermediates in the Krebs cycle also appear to be altered.

**Age-dependent perturbation of organic acid profiles in patients treated with VPA and CBZ**

Differential metabolic perturbation by age was noted in the CBZ and VPA groups. We regressed organic acid levels to age in each of the groups (VPA, CBZ, and healthy control), and the results were compared. Eight organic acids were identified as having significant nonzero age-related trends in the VPA group alone: pyruvic ($R = −0.49$), ethylmalonic ($R = −0.51$), 5-hydroxyhexanoic ($R = −0.31$), suberic ($R = −0.37$), sebacic ($R = −0.38$), and hippuric ($R = −0.33$) acids, as well as isovalerylglucose ($R = −0.30$). These did not show significant trends in the other two groups and are therefore potential organic acid biomarkers of age-dependent VPA effects on mitochondrial function. The CBZ group also showed some significant nonzero age-related trends in 3-hydroxyisobutyric ($R = −0.32$), 4-hydroxybutyric acids, potential markers of oxidative stress.

**DISCUSSION**

The analyses presented in this report represent a new approach to the characterization of age-related differences in VPAs effects on a biological system. In the past, attention has been focused on a terminal olefin metabolite of VPA, 4-ene-VPA, because of its structural similarity to known hepatotoxins such as 4-pentenoic acid. Kondo et al. reported that 4-ene-VPA formation is increased in younger children and declines with increasing age, but these findings of age-related differences have not been replicated by others. Evidence for increased bioactivation of VPA in younger children has been presented by Gopaul et al. on the basis of increased urinary concentrations of N-acetylcysteine conjugates of (E)-2,4-diene VPA, reflecting detoxication of reactive VPA metabolites by glutathione conjugation.

In urinary metabolite studies, it is common practice to apply a correction factor, using urinary creatinine concentrations to adjust for the effects of hydration status on observed drug and metabolite concentrations. However, creatinine production increases with age between birth and adolescence with the acquisition of muscle mass and is affected by gestational age in newborns and anorexia in adolescents (summarized in ref. 33). Therefore, in a comprehensive analysis of VPA metabolite profiles in 91 children receiving VPA as either monotherapy or polytherapy, we were unable to replicate any age-related changes in urinary VPA metabolite concentrations when developmental changes in renal creatinine concentration were taken into consideration (K.E. Price et al., unpublished data). We therefore hypothesized that changes in body composition that are characteristic of growth and development in children are probably associated with increased metabolic demands. Given that impairment of mitochondrial β-oxidation has been implicated in VPA hepatotoxicity, and developmental changes in mitochondrial function may be implied by the age-dependent changes in normal ranges for urinary organic acid concentrations used in pediatric settings to diagnosis inborn errors of metabolism, we hypothesized that the developmental context in which VPA is administered may be an important determinant for age-related differences in the risk for serious forms of toxicity. The data presented in this study represent an initial exploration of this alternative approach.

Few, if any, studies have used comprehensive profiling of urinary organic acids or other biomarkers to assess alterations in mitochondrial metabolism by VPA on a population basis in children. The data presented in this study provide a unique opportunity to examine the variability in endogenous metabolic consequences of VPA treatment in children and to formulate hypotheses for further prospective studies. Data models revealed that a systemic metabolic change occurs in response to VPA treatment in children who show no evidence of overt hepatic damage, although significant interindividual variability in metabolic responses to VPA was observed. A final key finding was an age-dependent metabolic response to VPA, thereby giving a new perspective to the unexplained VPA toxicity in young children.
children and potentially spurring future investigation. Because we carried out retrospective analysis of residual urine samples, interpretation is limited to the identification and description of potentially interesting metabolic changes and issues related to pediatric metabolic profiling, subject to validation in future prospective investigations.

Age trends are a significant issue in the metabolic profiling of pediatric populations. As discussed above, creatinine excretion changes with age but is commonly used to correct for fluctuations in urine concentration between samples. Therefore, correction for urinary creatinine concentration typically would introduce an age trend into the data (if one were not already present). Furthermore, those involved in the medical treatment of young children are well aware that the range of normal values for individual organic acids changes with increasing postnatal age, despite these values routinely being reported relative to urinary creatinine level. The challenge in studies of this type is to differentiate changes attributable to the intervention from those arising from the background “noise” generated by increasing age, particularly if the factor of interest is itself age-dependent. An important finding in this study is that organic acid profiles changed with time, and this change was independent of drug administration. To the extent that individual organic acids can serve as surrogate biochemical markers of mitochondrial dysfunction, the data raise the possibility of a mitochondrial “phenotype” and suggest that this phenotype changes with age. A major challenge will be to refine the assessment of mitochondrial phenotypes to determine the phenotype (i.e., at a younger age) that is most susceptible to toxicity, thereby providing testable hypotheses for the mechanisms of toxicity.

VPA differentially affected the pattern of organic acid excretion. This was evident from the varying distance of points representing individual VPA patients from points representing central/CBZ patients is the PCA scores plot. The metabolic changes reflected by the data are drug specific, but not disease specific, given that the data for the CBZ group (the disease control group) did not differ significantly from those for the healthy controls. Furthermore, an age-dependent metabolic response to VPA was observed. Both PCA and discriminant analysis showed deviation of the VPA metabolic profiles relative to the control and CBZ groups. Clustering revealed groupings of VPA subjects according to age and identified two subsets of young subjects within the VPA group. Finally, it is notable that there is considerable interindividual variability in the extent to which patient data deviate from those of the control/CBZ cluster; some patients in the VPA group had values indistinguishable from those of the control and CBZ groups, whereas others had values that deviated considerably relative to control values. Taken together, these findings suggest that when VPA is administered in children, it produces a perturbation of a dynamic system with a medium-chain fatty acid load and that the consequences in terms of mitochondrial function may change significantly throughout childhood. In this context, it would not be unexpected to find interindividual variability in the ability to respond to this perturbation across a treated population with, perhaps, more limited ability to adapt at discrete ages/developmental stages.

A major limitation of this study is its design. The goal of the original investigation was to characterize population variability in the patterns of VPA biotransformation and the effect of age on those profiles. This report represents a change in perspective from what children’s developing systems do to the drug to what the drug does to children’s developing systems, and it includes the necessary secondary analysis of the samples to address the change in perspective. All the subjects in this study were deemed healthy by standard laboratory measures, and it was therefore not possible to address specifically the relationship between organic acid profiles and hepatotoxicity. Also, the study was designed to collect urine under steady-state conditions after patients had been stabilized on their doses of VPA and CBZ. Therefore, it was not possible to assess the extent to which organic acid profiles changed after VPA treatment. Nevertheless, the findings provide valuable insights and testable hypotheses for future studies.

An additional limitation of the study is the scope of analytes used to assess metabolic effects of VPA. Urinary organic acid profiles are routinely available in tertiary-care pediatric settings and were used in this investigation as surrogate measures of metabolic function and, potentially, mitochondrial function. Expanding the repertoire of analytes to interrogate the response of a broader complement of biological pathways to VPA administration, analogous to the metabolomic approaches that have been applied in both animal and human studies to identify endogenous profiles predictive of acetaminophen-induced hepatotoxicity, has the potential to elucidate biological mechanisms and identify sensitive and specific biomarkers for toxicity, especially those predictive of children at highest risk for serious toxicity.

Overall, this study indicates that human pediatric metabolic profiling of individual response to VPA provides a new approach to investigating the mechanistic basis of age-related susceptibility to VPA hepatotoxicity. Clearly, prospective investigation is needed to identify more precisely the factors contributing to variability in metabolic response to VPA. Longitudinal studies of better design are needed for more efficient identification of individual toxicity risk by examining metabolic response to VPA over time.

METHODS

Subjects. A retrospective analysis was conducted on residual urine samples of subjects from two separate studies. The first was a study of children receiving CBZ (n = 50) or VPA (n = 52) for routine management of seizure disorders. These subjects were recruited at Children’s Mercy Hospital and Clinics (Kansas City, MO), Kosair Children’s Hospital (Louisville, KY), and Primary Children’s Medical Center (Salt Lake City, UT) and were age and gender matched. In the original study, children of both genders were eligible if they were between 1 and 16 years of age, stably maintained on VPA or CBZ for at least 2 weeks (either as monotherapy or in conjunction with other antiepileptic agents as determined by their primary treating physician), and without clinical evidence of hepatic or renal dysfunction. Exclusion criteria included (i) any clinical contraindication preventing collection of a urine sample (≥5 ml) during routine health visits and (ii) noncompliance with VPA or CBZ therapy during the 24 h prior to sample collection. Urine samples were collected over one complete steady-state dosing interval of VPA or CBZ. On the day prior to a scheduled clinic visit, the last daily dose of VPA was to be administered after the participants had emptied
their bladders. All the urine produced overnight (generally 8–12 h) was subsequently collected in containers (provided in advance to the participants) until the next (morning) dose of medication was taken. After collection, the urine containers were either brought to the clinic by the participants/guardians or recovered from the homes by study personnel.

A comparison group of healthy children receiving no medications and with no underlying medical conditions (n = 22), who were participating in two phenotyping studies investigating the ontology of CYP2D6 at Children’s Mercy Hospitals and Clinics, were selected so as to span the age distribution of the CBZ and VPA subjects. In children aged 2 to 5 years, an overnight collection (as described for the VPA/CBZ study) was used, whereas a 4-h collection interval was used for children >5 years. All the original studies were approved by the institutional review boards at each participating institution; the use of residual samples for the current study was approved by the Children’s Mercy Hospital Pediatric Institutional Review Board and ratified by the institutional review boards at the partner institutions. Written informed consent from parents/guardians was obtained, as well as assent from the patients, if applicable, prior to participation in the study. This study was designated as protocol 10606 within the Eunice Kennedy Shriver National Institute of Child Health and Human Development Network of Pediatric Pharmacology Research Units and was registered as study NCT00224952 at ClinicalTrials.gov.

**Analysis of organic acids.** Organic acid analysis was conducted by the Biochemical Genetics Laboratory at Children’s Mercy Hospitals and Clinics in accordance with the standard operating procedure established in the laboratory and based on methods described by Tanaka et al. and Sweetman et al. Samples were stored at a temperature of –20°C or below until gas chromatography/mass spectrometry analysis. Prior to analysis, the creatinine concentration was determined for each urine sample, using the Jaffe method. A volume of urine containing 1 µmol creatinine was then diluted to 2 ml with water. Tropic and ketocaproic acid were added as internal standards. The solutions were alkalinized with hydroxylamine and NaOH and heated at 60°C for 30 min. The reaction was stopped with cooling, and the solution was acidified with 6 N HCl. Organic acids were extracted with 2 × 2 ml volumes of ethyl acetate and dried at 30°C under nitrogen. Derivatization was performed using 150 µl N,O-bis(trimethylsilyl)trifluoroacetamide:pyridine (2:1 v:v). The mixture was heated at 65°C for 30 min. All analyses were performed by means of gas chromatography/mass spectrometry, using an Agilent Technologies (Santa Clara, CA) 5890 Series II GC coupled to an HP 5972 MSD. A 30-meter Phenomenex Zebron ZB-1 (100% dimethylpolysiloxane) capillary column of 0.25-mm internal diameter was used, with stationary film thickness of 0.25µm. The injection volume was 1 µl. The initial temperature of the column oven was 60°C; this was increased to 280°C; this was increased to 280°C. Helium carrier gas was maintained at a flow rate of 1 ml/min. Detection was performed using a mass selective detector with mass range of 50–550 amu.

**Supplementary Table S1** online. The 20 excluded acids showed no detectable levels in any of the urine samples and therefore contributed no variation to the data set. In addition, results for some analytes were reported as 0 mmol/mol creatinine in several subjects. To facilitate logarithmic transformation and statistical analysis, undetectable concentrations were arbitrarily assigned a value 10 times smaller than the minimum reported value for those analytes. One subject in the VPA group (a 4-year-old boy) was classified as an outlier, with levels of 36 acids outside the upper quartile plus 1.5 times the interquartile range, which is up to 15 times outside the next nearest value. The data for this subject were excluded from the model.

**Statistical analyses.** Of the 91 organic acids reported for each sample, 71 were included in the statistical analyses (see Supplementary Table S1 online). The 20 excluded acids showed no detectable levels in any of the urine samples and therefore contributed no variation to the data set. In addition, results for some analytes were reported as 0 mmol/mol creatinine in several subjects. To facilitate logarithmic transformation and statistical analysis, undetectable concentrations were arbitrarily assigned a value 10 times smaller than the minimum reported value for those analytes. One subject in the VPA group (a 4-year-old boy) was classified as an outlier, with levels of 36 acids outside the upper quartile plus 1.5 times the interquartile range, which is up to 15 times outside the next nearest value. The data for this subject were excluded from the model.

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**CONFLICT OF INTEREST**

The authors declared no conflict of interest.
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