

## Atypical Kinetics of Valproic Acid Glucuronidation *In vitro* and *In vivo* in Humans

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### Abstract

Atypical kinetics has been observed *in vitro* for both cytochrome P450 and UDP-glucuronosyltransferase catalyzed enzymatic reactions. The *in vitro* and *in vivo* kinetics of valproic acid- glucuronide (VPA-glucuronide) formation was investigated in humans.

VPA-glucuronide formation kinetics was investigated *in vitro* using human cryopreserved hepatocytes (pool of 10). Estimates of  $V_{max}^{app}$  ( $39.5 \pm 3.3$  pmol/min/ $10^6$  cells),  $S_{50}^{app}$  ( $224 \pm 34$   $\mu$ M) and  $n$  ( $2.34 \pm 0.28$ ) was obtained by fitting VPA-glucuronide formation rate vs VPA incubation concentration data to the Hill equation. *In vitro* Eadie-Hofstee plots were "hooked" and characteristic of atypical sigmoidal/autoactivation kinetics.

*In vivo* Eadie-Hofstee plots of VPA-glucuronide formation rate (calculated from urinary excretion rate data for VPA-glucuronide) were constructed using data from four human subjects given a 1000 mg VPA oral dose [1]. The positive slope of linear regression lines for these plots was consistent with *in vivo* atypical sigmoidal/autoactivation kinetics.

In summary, these data build upon our previous observations in sheep and provide the first demonstration that VPA-glucuronide formation exhibits atypical kinetics *in vitro* in human hepatocytes. Available *in vivo* data is consistent with *in vitro* results suggesting that VPA-glucuronidation exhibits atypical sigmoidal/autoactivation kinetics *in vivo* in humans.

**Keywords:** Atypical kinetics; Sigmoidal/autoactivation kinetics; *In vitro* - *in vivo* extrapolation; Enzyme kinetics; Glucuronidation

### Introduction

Valproic acid (2-propylpentanoic acid, VPA) is a broad spectrum anticonvulsant with a unique branched-chain fatty acid structure [2]. Previously, we observed evidence of both *in vitro* and *in vivo* autoactivation kinetics of VPA-glucuronide formation in sheep [3]. Approximately, 70-80% of the administered VPA dose is metabolized to VPA-glucuronide such that manifestation of *in vivo* autoactivation kinetics was evident in both the appearance of VPA-glucuronide in urine and the decline of unbound VPA in plasma. In humans, VPA-glucuronide is a major metabolite of VPA accounting for 10-70% of the administered dose [4]. The UDP-glucuronosyltransferases (UGT) capable of metabolizing VPA in humans include UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT 1A10 and UGT2B7 [5,6]. Interestingly, glucuronidation reactions involving UGT1A6 and UGT2B7 have shown evidence of autoactivation kinetics with substrates other than VPA [7,8]. Reports of enhanced rates of glucuronidation in humans at high doses of VPA (1000 mg) have been previously described in literature [9]. These enhanced rates could not be accounted for entirely by increases in unbound VPA concentrations that occurred with increasing dose and are consistent with autoactivation kinetic behavior. Thus, well-designed *in vitro* enzyme kinetics studies of VPA glucuronidation spanning a clinically relevant VPA concentration range may show evidence of *in vitro* autoactivation kinetics of VPA glucuronidation. In addition, careful examination of the clinical literature on VPA in humans may reveal evidence of *in vivo* atypical sigmoidal/autoactivation kinetics consistent with our observations in sheep. The objective of the current study is to characterize the kinetics of VPA-glucuronide formation *in vitro* using human hepatocytes.

In addition, *in vivo* data on VPA-glucuronidation in humans will be examined for evidence of atypical sigmoidal/autoactivation kinetics.

### Methods

#### *In vitro* valproic acid glucuronidation kinetics

The formation kinetics of VPA-glucuronide were examined in cryopreserved human hepatocytes (pool of 10) (CellzDirect Inc., Durham, NC) under the following incubation conditions: VPA (6.4 to 800  $\mu$ M) and  $0.5 \times 10^6$  cells/ml human liver hepatocytes in Dulbecco's Modified Eagle Medium fortified with glucose. The incubations were performed in triplicate on three separate occasions at each VPA concentration in an incubator at 37°C, 95% humidity and 5% CO<sub>2</sub>. The total incubation volume was 100  $\mu$ L. Incubations were terminated at 60 minutes by the addition of 200  $\mu$ L of ice-cold acetonitrile containing the internal standard (diclofenac at 0.1  $\mu$ M final concentration; Sigma-Aldrich Co., St. Louis, MO) and 3% formic acid. The resulting samples were centrifuged at 3000 g for 10 minutes at 4°C and 10  $\mu$ L of the supernatant was injected into a liquid chromatography coupled with a tandem mass spectrometer (LC-MS/MS) for quantitation of VPA-glucuronide.

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VPA-glucuronide was quantitated using a modification of a LC-MS/MS method previously described by Wong et al. [3]. The instrument consisted of an Agilent 1200 liquid chromatography system (Agilent Technologies Inc. Santa Clara, CA) coupled with an Applied Biosystems ABI-4000 triple quadrupole mass spectrometer (Applied Biosystems Foster City, CA). Samples were injected by a CTC Analytics HTS PAL autosampler (Leap Technology Inc., Carrboro NC) onto Thermo Scientific Hypersil gold C<sub>18</sub> column (50 mm × 1.9 mm i.d.; Thermo Electron Corporation, San Jose, CA). The mobile phase, consisting of (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid, was delivered at a flow rate of 0.4 mL/min, using the following gradient program: 5% B for 0.4 min, linear gradient to 90% B between 0.4 and 3 min, held at 90 % B until 3.5 min, then re-equilibrated to initial conditions after 3.5 min with the total run time was 5.5 min. The mass spectrometer was operated in the negative ion mode with multiple reaction monitoring (VPA-glucuronide: 319.0 > 143.0, internal standard: 293.9 > 250.0) at a collision energy of 28 ev and 17 ev. The dwell time for both channels was set at 100 ms. The calibration curve was prepared by spiking authentic standards to blank hepatocyte samples after the incubations were quenched. The peak-area ratio of VPA-glucuronide to internal standard was linear ( $r^2 > 0.99$ ) over a concentration range of VPA-glucuronide from 0.5 nM to 2500 nM. The coefficient of variation was less than 15% at all of the concentrations.

The rate of VPA-glucuronide formation vs. VPA concentration data from the human hepatocyte experiments were fitted to the Hill (Equation 1) and the Michaelis-Menten equation (Equation 2) using GraphPad Prism V4.02 (GraphPad Software Inc., San Diego, CA).

$$V = \frac{V_{\max}^{app} \times C^n}{S_{50}^{appn} + C^n} \quad (1)$$

$$V = \frac{V_{\max}^{app} \times C}{K_m^{app} + C} \quad (2)$$

Where  $V$  is the rate of VPA glucuronidation,  $V_{\max}^{app}$  is the apparent maximum rate of VPA glucuronidation,  $C$  is the VPA incubation concentration, and  $S_{50}^{app}$  or  $K_m^{app}$  is the apparent substrate concentration where the rate of VPA-glucuronide formation is 50% of  $V_{\max}^{app}$ , and  $n$  is the Hill coefficient.

Since clearance for compounds exhibiting sigmoidal/autoactivation kinetics are concentration dependent, calculation of maximal clearance due to autoactivation ( $CL_{\max}$ ) was performed using Equation 3 [10].

$$CL_{\max} = \frac{V_{\max}^{app}}{S_{50}^{app}} \times \frac{(n-1)}{n(n-1)^{1/n}} \quad (3)$$

### ***In vivo* Eadie-Hofstee Plots for VPA glucuronidation in humans**

*In vivo* Eadie Hofstee Plots were generated using data extracted from Bialer et al. [1]. Figures of unbound VPA serum concentration-time profiles and plots of amount of VPA-glucuronide remaining to be excreted in urine vs. time profiles were digitized using UN-SCAN-IT v6 (Silk Scientific Corporation, Orem, Utah). Urinary excretion rate of VPA-glucuronide ( $V$ ) and unbound VPA serum concentrations at the midpoint of the urine collection interval ( $C_{\text{mid}}^u$ ) were calculated and used to generate *in vivo* Eadie-Hofstee plots ( $V$  versus  $V/C_{\text{mid}}^u$ ). Data

is presented for four human subjects where  $\geq 3$  or more data points were available for plotting. For the remaining two subjects, only one or two data points were available for plotting *in vivo* Eadie-Hofstee plots due to less frequent urine collection during the post-dose period where VPA unbound concentrations could be measured; and therefore were excluded from the analysis. Linear regression of *in vivo* Eadie-Hofstee plots was performed using Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA).

## **Results**

### ***In vitro* valproic acid glucuronidation kinetics**

Figure 1 shows plots of VPA-glucuronide formation rate vs. VPA incubation concentration and the corresponding Eadie-Hofstee plots from a representative human hepatocyte experiment fitted to the Hill (Figure 1A and 1B) or Michaelis-Menten equation (Figure 1C and 1D). A summary of the associated kinetic parameter estimates are presented in Table 1. The Eadie-Hofstee plot exhibits the “curved” shape that is characteristic of sigmoidal/autoactivation kinetics. It is better described by the fitted line generated using the Hill equation (Figure 1B) rather than the classical Michaelis-Menten equation (Figure 1D).

Based upon plots shown in Figure 1, *in vitro* VPA glucuronidation in human hepatocytes exhibits atypical sigmoidal/autoactivation kinetics under the VPA concentration range tested. Since clearance for compounds exhibiting sigmoidal/autoactivation kinetics are concentration dependent, calculation of maximal clearance due to autoactivation was performed using Equation 3. The estimated  $CL_{\max}$  describing VPA-Glucuronidation was 15 mL/hr/kg.

### ***In vivo* Eadie-Hofstee Plots for VPA glucuronidation in humans**

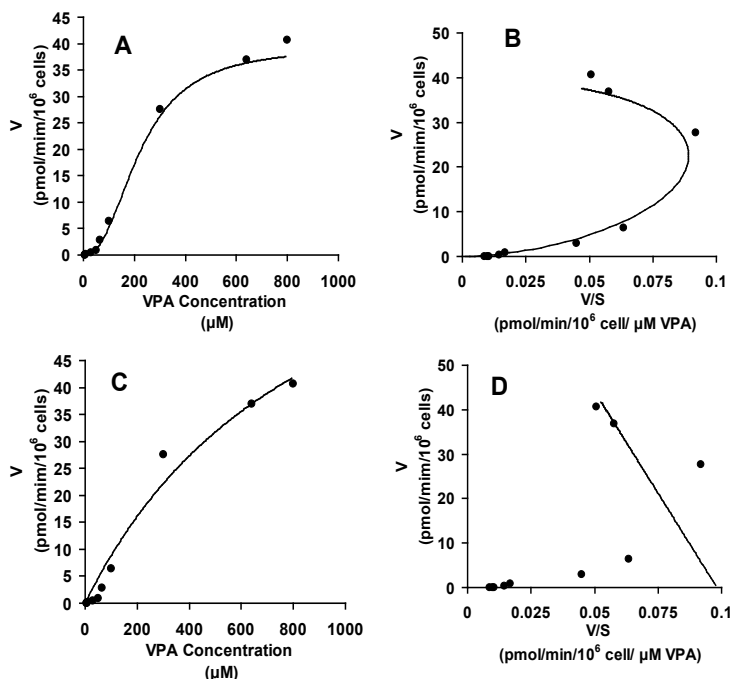
*In vivo* Eadie-Hofstee Plots for VPA glucuronidation are presented in Figure 2. The X-axis for *in vivo* Eadie-Hofstee Plots ( $V/C_{\text{mid}}^u$ ) represents the average unbound intrinsic clearance for VPA-glucuronidation over specific urine collection intervals. Since VPA glucuronidation did not appear to be saturated based upon the *in vivo* Eadie-Hofstee Plots, data from human subjects were not fit to either the Michaelis-Menten or Hill equation. Instead, data from *in vivo* Eadie-Hofstee Plots were fitted to a linear regression line and the associated equations are presented in Figure 2. The slope of the regression line was used to discriminate whether VPA-glucuronide formation *in vivo* exhibited kinetics that were consistent with either classical Michaelis-Menten kinetics or atypical sigmoidal/autoactivation kinetics since the slope of the Eadie-Hofstee Plots are uniquely negative and positive, respectively, at lower non-saturating substrate concentrations (Figure 3). For all four human subjects presented, the slope of the linear regression line was positive (3.92 to 6.03), consistent with sigmoidal/autoactivation kinetics.

## **Discussion**

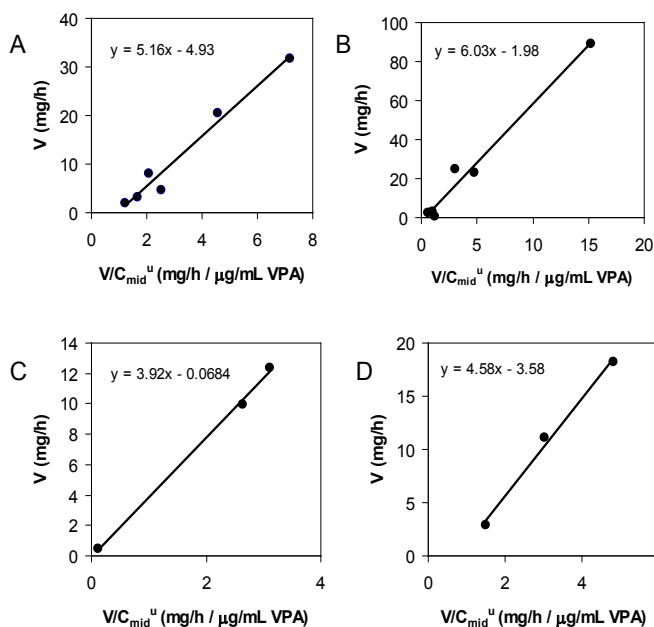
Aside from one report of atypical sigmoidal/autoactivation kinetics of VPA glucuronidation *in vitro* in sheep [3], this phenomenon has yet

	$V_{\max}^{app}$ (pmol/min/10 <sup>6</sup> cells)	$S_{50}^{app}$ or $K_m^{app}$ ( $\mu$ M)	$n$
Hill	39.5 ± 3.3	224 ± 34	2.34 ± 0.28
Michaelis-Menten	89.8 ± 28.0	915 ± 472	-

**Table 1:** Summary of kinetic parameters describing VPA-glucuronide formation kinetics in human hepatocytes.



**Figure 1:** Plot of VPA-glucuronide formation rate vs VPA incubation concentration and a Eadie-Hofstee plot from a representative human hepatocyte (pool of 10) experiment fitted to the Hill (A and B) or Michaelis-Menten equation (C and D). V is defined as the rate of valproic acid-glucuronide formation and S is defined as substrate (VPA) concentration in the figure.



**Figure 2:** (A-D) *In vivo* Eadie-Hofstee plots for VPA glucuronidation for four individuals following administration of a single 1000 mg oral dose of sodium valproate (V = urinary excretion rate of VPA-glucuronide; C<sub>mid</sub><sup>u</sup> = unbound VPA plasma concentration at the midpoint of the urine collection interval). Regression lines and associated equations are presented.

to be reported for VPA glucuronidation *in vitro* in humans or other species. Previous investigations characterizing the enzyme kinetics of VPA glucuronidation were not designed with autoactivation kinetics in mind and therefore, this phenomenon may have been missed. In an earlier manuscript [3], we reported our investigation into the

translation of *in vitro* sigmoidal/autoactivation kinetics to the *in vivo* situation. Our efforts focused on the *in vitro* and *in vivo* kinetics of VPA glucuronidation in sheep. The unique *in vivo* disposition of valproic acid in sheep made it an ideal animal model to conduct our investigation. VPA is a low clearance compound that is primarily

eliminated by the liver such that the clearance of unbound VPA is essentially its intrinsic metabolic clearance. In sheep, glucuronidation is the main route of VPA elimination (70 to 80%), and the urinary excretion of VPA-glucuronide is formation rate limited. Finally, VPA is a very safe compound, thus enabling the administration of high doses that result in a broad range of *in vivo* concentrations. Based upon these characteristics, evidence of *in vivo* sigmoidal/autoactivation kinetics manifested itself in both the unbound VPA plasma concentration-time profile, and in the urinary excretion of VPA-glucuronide following administration of a 100 mg/kg intravenous dose. Our observations of *in vivo* sigmoidal/autoactivation kinetics were further substantiated by *in vitro* experiments demonstrating autoactivation kinetics of VPA-glucuronide formation using sheep liver microsomes [3] and sheep hepatocytes (unpublished data).

Since VPA shows minimal microsomal binding [3], *in vitro* incubation concentrations correspond to unbound concentrations *in vivo*. Human subjects in a clinical study described by Bailer et al. [1] received a single 1000 mg oral dose of valproic acid which resulted in unbound levels ranging from approximately 2.5 to 63  $\mu\text{M}$  (0.36 to 9.0  $\mu\text{g/mL}$ ). VPA concentration ranges investigated in previous *in vitro* studies were substantially higher (500  $\mu\text{M}$  to 15 mM; [5] and 100  $\mu\text{M}$  to 20 mM; [6]. In the current study, we performed an enzyme kinetics experiment using pooled cryopreserved hepatocytes over a concentration range that not only covered the observed unbound VPA concentration range but extended  $\sim 13$  fold higher (5 to 800  $\mu\text{M}$  or 0.72 to 115  $\mu\text{g/mL}$ ). The extension of the upper incubation concentration to 800  $\mu\text{M}$  was based upon our observations that the kinetics of VPA-glucuronide formation was not fully characterized *in vivo* (Figure 2). It must be noted that the 800  $\mu\text{M}$  incubation concentration is still closer to the lower end of VPA concentrations used in previous *in vitro* investigations.

Our *in vitro* enzyme kinetics study using human hepatocytes clearly show that the *in vitro* formation kinetics of VPA-glucuronide is atypical in nature over a more clinically relevant concentration range (Figure 1). The Eadie-Hofstee Plot exhibits the characteristic “curved” shape that is associated with atypical sigmoidal/autoactivation kinetics and provides evidence that this phenomenon occurs *in vitro* in humans. Finally, the estimated  $\text{CL}_{\text{max}}$  (15 mL/hr/kg) based upon kinetic parameters from the hepatocyte study is in line with reported estimates of VPA-glucuronide formation clearance observed in humans (12-22 mL/hr/kg) [11].

Enhanced rates of *in vivo* glucuronidation in humans at high doses of VPA have previously been described in the literature. Granneman et al. [9] reported that an increase in VPA dose from 250 mg to 1000 mg resulted in a 13.6 fold increase in the dose normalized urinary excretion rate of VPA-glucuronide. Increases in VPA unbound fraction (i.e. 10% unbound at 250 mg versus 40% unbound at 1000 mg) could at most account for a four-fold increase in glucuronidation, leaving much of the change unexplained. A second report by Anderson et al. [12] showed dose-dependent increases in the intrinsic formation clearance of VPA-glucuronide in healthy volunteers treated with consecutive 4 day regimens of VPA at daily oral doses of 250, 500, and 1000 mg. Data were also presented showing increases in VPA-glucuronide formation clearance with increasing dose for patients undergoing chronic VPA therapy. As multiple-dose regimens were involved, possible inductive effects of VPA may have contributed to the observations of Anderson

et al. [12]. Nevertheless, human data presented in both reports described above are consistent with our previous observations of *in vivo* autoactivation kinetics of VPA glucuronidation in sheep [3].

Observations of *in vivo* sigmoidal/autoactivation kinetics reported in literature are rare. Compounds exhibiting sigmoidal/autoactivation kinetics *in vitro*, often do not achieve the range of concentrations required to characterize the phenomenon *in vivo*. Alternatively, the design of *in vivo* studies to characterize autoactivation kinetics may not be feasible for many of these compounds due to the complexity of their disposition in an *in vivo* system. Our ability to observe and characterize the *in vivo* autoactivation kinetics of VPA glucuronidation in sheep previously was in part due to the *in vivo* tolerability of VPA at high doses [3]. Intravenous doses of 100 mg/kg administered to sheep provided a large concentration range of unbound drug in plasma, thus allowing for reliable estimation of *in vivo* enzyme kinetic parameters from modeling of unbound plasma concentration-time profiles [3]. Similar characterization in humans is not feasible since doses normally administered are far less than those employed in our previous studies in sheep. In addition, although a major pathway in humans (i.e. 10-70%; [4], glucuronidation does not consistently contribute to the elimination of VPA to the same extent as in sheep (i.e. 70-80%; [3]. Therefore, our approach of characterizing sigmoidal/autoactivation kinetics of VPA glucuronidation using unbound VPA plasma-concentration time profiles in sheep is not possible in humans since pathways other than glucuronidation can play a major role in VPA elimination from plasma.

An alternative approach to detect *in vivo* sigmoidal/autoactivation kinetics of VPA glucuronidation is through the use of diagnostic *in vivo* Eadie-Hofstee Plots [3]. Figure 3 illustrates the distinct shapes of Eadie-Hofstee Plots resulting from classical Michaelis-Menten kinetics and atypical sigmoidal/autoactivation kinetics. In our previous manuscript [3], we presented *in vivo* Eadie-Hofstee Plots generated from VPA-glucuronide urinary excretion data and unbound VPA plasma concentrations from five sheep dosed at 100 mg/kg iv. All *in vivo* Eadie-Hofstee plots presented had the characteristic “curved” shape associated with autoactivation kinetics. Figure 4 is an *in vivo* Eadie-Hofstee Plot from a single animal dosed at 10 mg/kg iv, a dose closer in range to that used clinically in humans. At this lower dose, only the bottom portion of the characteristic “curved” Eadie-Hofstee plot is evident due to a more limited range of concentrations achieved *in vivo*. At low non-saturating substrate concentrations, the slope of the

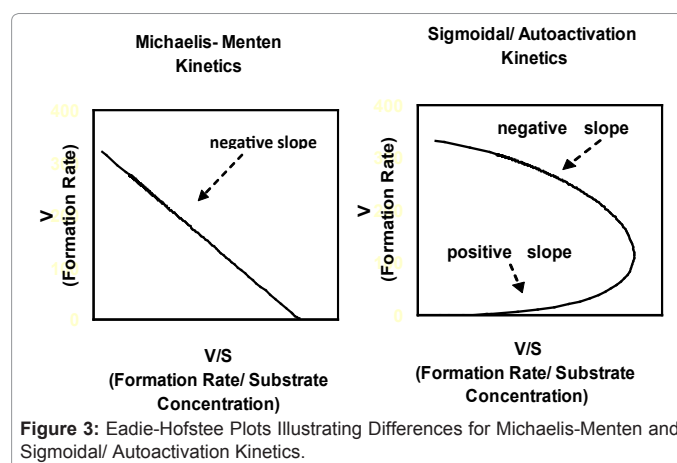
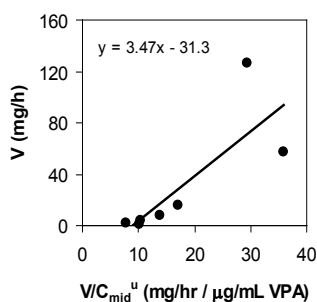


Figure 3: Eadie-Hofstee Plots Illustrating Differences for Michaelis-Menten and Sigmoidal/ Autoactivation Kinetics.





**Figure 4:** Representative in vivo Eadie-Hofstee Plot for individual sheep dosed at 10 mg/kg iv (V = urinary excretion rate of VPA-glucuronide;  $C_{mid}^u$  = unbound VPA concentration in plasma at the midpoint of the urine collection interval). Regression line and associated equation is presented. Data extracted from Wong et al. [13].

the Eadie-Hofstee Plot is positive due to increasing unbound intrinsic clearance with increasing unbound VPA concentrations (Figure 3 and Figure 1B). The linear regression line through the data from sheep in figure 4 has a positive slope consistent with what would be expected for atypical sigmoidal/autoactivation kinetics. Eadie-Hofstee Plots from four human subjects given a single oral dose of 1000 mg VPA show a similar positive slope reflective of atypical *in vivo* kinetics in humans (Figure 2).

In summary, we have shown that the *in vitro* kinetics of VPA in human hepatocytes is atypical in nature under clinically relevant concentrations. In addition, based upon our analysis of existing data, the *in vivo* kinetics of VPA glucuronidation in humans are consistent with atypical sigmoidal/autoactivation kinetics.

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