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# Acetoacetate, Acetone, and Dibenzylamine (a Contaminant in L-(+)-β-Hydroxybutyrate) Exhibit Direct Anticonvulsant Actions in Vivo

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**Summary:** *Purpose:* To investigate whether ketone bodies are directly anticonvulsant.

*Methods:* We tested the effects of acetoacetate (ACA), acetone, and both stereoisomers, D-(−)- and L-(+), of β-hydroxybutyrate (BHB) on sensory-evoked seizures in Frings audiogenic seizure-susceptible mice.

*Results:* We found that these ketone bodies, with the exception of the D-(−)-isomer of BHB, were anticonvulsant in this model. Furthermore, with gas chromatography–mass spectrometry, we confirmed that the activity of L-(+)-BHB was due to dibenzylamine, a chemical contaminant.

*Conclusions:* Our data indicate that the anticonvulsant efficacy of the ketogenic diet may be due in part to the direct actions of ACA and acetone. **Key Words:** Acetoacetic acid—Acetoacetate—3-Hydroxybutyrate—β-Hydroxybutyrate—3-Hydroxybutanoic acid—Acetone—Dibenzylamine—Ketogenic diet—Epilepsy.

The ketogenic diet (KD) is an established nonpharmacologic treatment for patients with intractable epilepsy (1,2) and was designed to mimic the biochemical changes seen upon fasting, specifically the formation of ketone bodies: β-hydroxybutyrate (BHB), acetoacetate (ACA), and to a lesser extent, acetone. Despite decades of clinical experience with the KD, the mechanisms underlying its anticonvulsant activity remain poorly understood.

Investigators have observed that ketosis is necessary but not always sufficient for seizure control with the KD. It is well known that seizure control gradually increases within the first few weeks of initiating a KD, as serum ketone levels steadily increase, but then is abruptly lost when ketosis is broken, usually through ingestion of carbohydrate (3). In addition, seizure control appears to correlate with blood BHB levels (4), although this relation is not consistent (5). Taken together, these observations suggest a direct role for ketone bodies in limiting seizure activity.

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To investigate whether ketone bodies are directly anticonvulsant, we tested the effects of ACA, acetone, and both stereoisomers, D-(−)- and L-(+), of BHB on sensory-evoked seizures in Frings audiogenic seizure-susceptible mice. We found that ACA, acetone, and the L-(+)-isomer of BHB had anticonvulsant activity in the Frings model; however, subsequent experiments established that the activity of L-(+)-BHB was due to dibenzylamine (DBA), a chemical contaminant.

## METHODS

### Anticonvulsant testing

Frings audiogenic seizure-susceptible mice were obtained from an in-house colony maintained by the Anticonvulsant Screening Program at the University of Utah. Before testing, all mice were maintained on a 12-h light/dark cycle, and food and water were available ad libitum according to the guidelines outlined in the NIH Guide for Care and Use of Laboratory Animals. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Utah.

The Frings audiogenic seizure-susceptible mouse is a model of sensory-evoked reflex seizures (6). The seizure phenotype is characterized by a sequence of wild run-

ning, loss of righting reflex, tonic flexion, and ultimately tonic extension in response to a high-intensity sound stimulus. One-month-old female Frings mice were injected intraperitoneally (i.p.) with varying doses (1–30 mmol/kg) of ACA, acetone, L-(+)-BHB, D-(−)-BHB, or the racemate, DL-BHB. Fifteen minutes after i.p. administration of the test compound, individual mice were placed into a round, plexiglas sound chamber and exposed to 110-dB, 11-KHz sound for a total of 20 s or until a full tonic extension seizure was elicited (6).

### Gas chromatography–mass spectrometry

Standards were prepared in the following manner. Stock solutions (1 mg/ml) of DBA, D-(−)-BHB, L-(+)-BHB, and the racemate, DL-BHB, were prepared by using methanol (Fisher Scientific, Pittsburgh, PA, U.S.A.) as the solvent. These samples were further diluted to 1 μg/ml and stored overnight at 2–8°C. The following day, 0.1 ml of each of these solutions was transferred to an autosampler vial equipped with a conical low-volume insert, and evaporated under $N_2$ gas. Each sample was re-dissolved in 50 μl of ethyl acetate (which had been dried with magnesium sulfate) and then reevaporated. They were again dissolved in 50 μl of ethyl acetate to which 50 μl of a derivatizing agent, perfluoroacetic anhydride, was added. Vials were then capped and heated to 70°C for 60 min. Gas chromatography–mass spectrometry (GC-MS) analysis was performed with a Hewlett Packard 5890 Series II Gas Chromatograph (equipped with an electron-capture detector, model 7673 Injector, and a HP 3396 Series II Integrator) as previously described (7).

### Chemicals

DBA ($C_{14}H_{15}N$; MW, 197.28), ethyl acetate, and perfluoroacetic anhydride were purchased through Aldrich (Milwaukee, WI, U.S.A.), Fisher Scientific (Pittsburgh, PA, U.S.A.), and Fluka (Ronkonkoma, NY, U.S.A.), respectively. Differing lots of ACA, acetone, D-(−)-BHB, L-(+)-BHB, and DL-BHB, were obtained from both Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Aldrich.

## RESULTS

After a 10 mmol/kg i.p. injection of ACA, eight of eight mice were protected against sound-induced tonic extension. Acetone also was protective in eight of eight mice administered 10 mmol/kg acetone. With 30 mmol/kg L-(+)-BHB, seven of eight mice injected were protected against sound-induced tonic extension, whereas none of eight mice given a similar dose of the D-(−)-isomer were protected 15 min after injection. Summary data obtained with ACA, acetone, and L-(+)-BHB are presented in Table 1.

At a 30 mmol/kg dose, both stereoisomers of BHB produced signs of acute toxicity characterized by mild gait abnormality, lack of exploratory activity coupled with blank stares, and watery stools. All animals recovered fully from sound-induced seizures and BHB toxicity within 2 h. Conversely, ACA and acetone doses of <20 mmol/kg were well tolerated.

**TABLE 1.** *Summary of anticonvulsant efficacy data in Frings audiogenic seizure-susceptible mice*

| Test compound | $ED_{50}$ | 95% Confidence interval |
|---|---|---|
| L-(+)-β-Hydroxybutyrate | 19.0 mmol/kg | 14.1–23.9 |
| Acetoacetate | 7.3 mmol/kg | 6.6–8.1 |
| Acetone | 3.1 mmol/kg | 1.8–4.7 |
| Dibenzylamine | 94 μmol/kg | 72.5–145 |

Surprisingly, the racemate (DL-BHB) did not protect Frings mice against sound-induced seizures, raising concerns about the effects seen with L-(+)-BHB. Additionally, the clinical relevance of L-(+)-BHB effects was raised because it is the D-(−)-isomer that is biologically produced (8). Doepner et al. (9) reported earlier that L-(+)-BHB block of transient outward $K^+$ current in myocardial mouse cells was due to DBA, which they identified with UV spectroscopy; however, no direct demonstration of this was provided. Therefore, we used GC-MS to determine whether commercial lots of the L-(+)-isomer of BHB in fact contained DBA.

In the GC-MS scan mode, the derivitized DBA resulted in a peak at 7.19 min with M/Z (mass/charge) ions of 91.10 and 202.10 as the most abundant ions. As shown in Fig. 1, using the select ion monitoring (SIM) at M/Z 202.10, the peak at 7.29 min was not present in either the DL-racemate or the D-(−)-BHB standards. However, there was evidence of DBA in the L-(+)-BHB. Using standard solutions of DBA, we estimated that there was not >0.01% DBA contamination of the D-(−)-BHB and DL-BHB compounds, but that DBA composed ~0.4% of the L-(+)-BHB. Thus 1–30 mmol of L-(+)-BHB would contain roughly 40 μmol to 1.2 mmol of DBA. Dibenzylamine also was identified in L-(+)-BHB from different lots obtained through both Aldrich and Sigma.

Given these findings, we next examined the effects of DBA in Frings audiogenic seizure-susceptible mice. Fifteen minutes after administering 200 μmol/kg DBA i.p., complete protection against sound-induced tonic extension was observed in four of four mice. Over a concentration range of 50–200 μmol/kg (four to 11 mice per dose), DBA produced a dose-dependent block of audiogenic seizures (Table 1).

## DISCUSSION

The principal finding of this study is that the ketones ACA and acetone, but not BHB, exhibited anticonvulsant efficacy in Frings audiogenic seizure-susceptible mice. Our data confirm Keith's observation (10) that

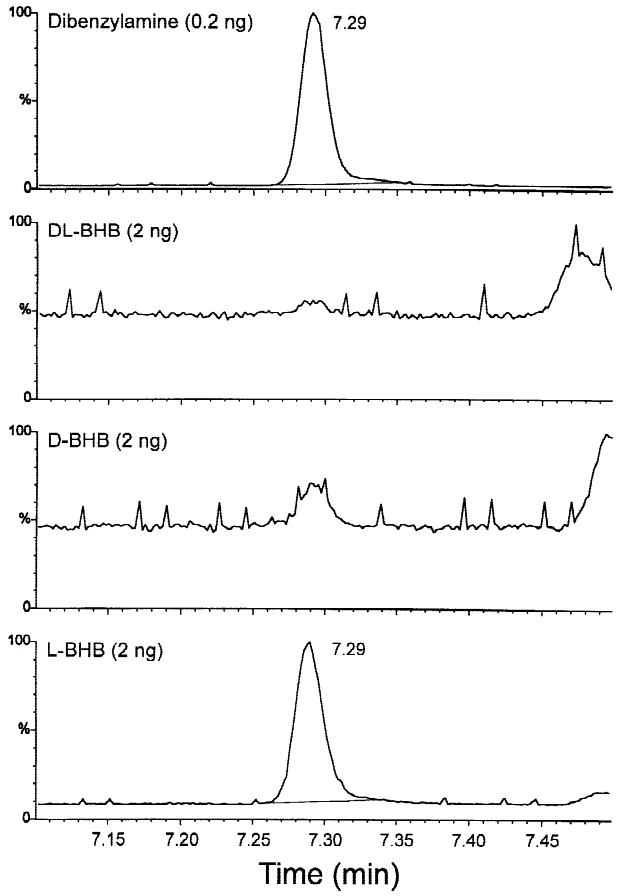


**FIG. 1.** Selected ion current chromatogram of M/Z 202.10, obtained from injecting ~0.2 ng derivatized dibenzylamine (top trace), or 2 ng of racemic β-hydorxybutyrate (BHB) (second trace), or the separate D-(−)-(third trace) and L-(+)-isomers (bottom trace) of BHB.

ACA blocked seizures induced by thujone, a convulsant constituent found in many essential oils and an antagonist of γ-aminobutyric acid $(GABA)_A$ receptors (11). We also demonstrated that the active component in L-(+)-BHB is likely to be DBA for the following reasons: (a) the racemate, DL-BHB, does not contain DBA and was inactive in the Frings model; (b) although we cannot exclude a pharmacodynamic interaction between the D- and L-enantiomers, D-(−)-BHB was not effective in blocking audiogenic seizures; and (c) Doepner et al. (9) reported a similar experience with these ketones in electrophysiologic experiments conducted in mouse myocardial cells.

Frings audiogenic seizure-susceptible mice were chosen to test for the potential anticonvulsant properties of ketones because of the promising role these mice have played in antiepileptic drug (AED) screening (6). In addition, protection against these audiogenic seizures is believed to be nondiscriminatory with respect to the differing clinical efficacies of known AEDs (i.e., agents effective against both partial and generalized seizures are all active against sound-induced seizures in Frings mice) (6).

Given the strong (but not universal) correlation between blood ketone levels and seizure control with the KD (4), it is important to determine if ketones can directly modulate neuronal excitability and synchronization. In cellular electrophysiologic experiments, BHB and ACA did not directly alter synaptic or inhibitory synaptic transmission in the hippocampus (12) and did not directly interact with $GABA_A$, ionotropic glutamate receptors, or voltage-gated sodium channels in cultured neocortical neurons (Rho et al., unpublished data).

The less prevalent ketone bodies, ACA and acetone, clearly possess anticonvulsant properties, but the precise mechanisms underlying these actions are unclear. One possibility is that cerebral acetone may contribute to the anticonvulsant actions of the KD, possibly through nonspecific general anesthetic actions or through uncoupling of gap junctions (P. Carlen, personal communication, 2001). Recently, it was observed that patients successfully treated with a KD exhibit elevated levels of acetone in the brain (13).

Acetoacetate can enter the brain via monocarboxylic transporters (MCTs) and can then be spontaneously decarboxylated to acetone. Further, brain accumulation of acetone from the periphery may be enhanced because of the high lipid solubility of this species. Elevated brain levels of BHB such as that seen in a fasting-induced ketotic state (14) also may indirectly contribute to an anticonvulsant effect, because BHB can be subsequently converted to ACA by β-hydroxybutyrate dehydrogenase. Interestingly, a ketogenic diet has recently been shown to increase MCT levels in rat brain (15).

Our experience with DBA emphasizes once again the pitfalls of ascribing specific biologic activity to chemical preparations containing active trace contaminants (9). This cautionary note may be relevant because many published studies addressing the effects of BHB in a variety of in vivo and in vitro models do not indicate which enantiomer was used. Nevertheless, although DBA possesses anticonvulsant properties (and might warrant further preclinical development), DBA also has been identified as a potent irritant and is listed in the Environmental Protection Agency inventory under the Toxic Substances Control Act (TSCA). Therefore, its future as a clinically useful anticonvulsant seems doubtful.

An additional issue merits comment: the potential biologic relevance of L-(+)-BHB. It is generally accepted that D-(−)-BHB, the stereoselective product of D-3-hydroxybutyrate dehydrogenase, is the clinically significant (i.e., physiologic) stereoisomer (8). L-(+)-BHB has not yet been directly assayed in either animals or humans, yet there is evidence that this isomer could be biologically produced, either in liver and/or brain (16–

18). Whether L-(+)-BHB is relevant to the KD is unknown.

In summary, we demonstrated that the ketone bodies ACA and acetone are directly anticonvulsant in vivo, whereas the more prominent species, BHB, is not. Interestingly, these metabolic products of BHB appear more potent as AEDs than BHB itself (Table 1). Given these findings, we propose that the anticonvulsant efficacy of the KD may be due in part to the accumulation of acetone in the brain, a hypothesis supported by previous published data (13). How acetone exerts such an action remains unclear.

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