

Supplementary Information

1. Valproic Acid

1.1 Metabolism and Antiepileptic Mechanism of Action

Valproic acid is one of the first-line antiepileptic drugs currently established in the long-term treatment of epilepsy and has shown superior efficacy in all types of idiopathic generalized epilepsies. Also it has proven useful in some psychiatric diseases. The main molecular mechanism of action of valproic acid in epilepsy therapy is not completely understood but it is believed to cause an increase in the availability of gamma-aminobutyric acid (GABA, an inhibitory neurotransmitter) or enhance GABA action or mimic GABA action.¹ VPA can also inhibit sodium and/or calcium channel function.¹ More recently, VPA has attracted much attention because of its histone deacetylase inhibitor properties which have led it to show promise as an antineoplastic agent in the treatment of several types of cancer²⁻⁵ or in “purging” latent HIV infection when administered with highly active antiretroviral therapies.⁶

The metabolism of valproic acid is mainly hepatic via glucuronide conjugation (30%-50%, VPA-glucuronide, 320.34 g/mol) and mitochondrial β -oxidation (40%).^{1, 7} The metabolites formed by oxidation include 2-ene VPA (142.19 g/mol); hydroxylation products (3-hydroxy VPA, 4-hydroxy VPA, 5-hydroxy VPA, 160.21 g/mol), approx. 10%;⁷ ketones from oxidation of hydroxy metabolites (3-keto VPA, 4-keto VPA, 159.21 g/mol); and dicarboxylic acids also from oxidation of hydroxy metabolites (propylglutaric acid, 174.19 g/mol; propylsuccinic acid, 160.17 g/mol).⁸ Another fraction of VPA is unsaturated into 3-ene VPA, 4-ene VPA (142.19 g/mol), 2,3-diene VPA, 2,4-diene VPA (140.18 g/mol), and 4-keto-2-ene VPA (157.19 g/mol).^{1, 7} In addition, valproyl glutamate (273.33 g/mol), valproyl glutamine (272.34 g/mol), and

valproyl glycine (201.27 g/mol), as well as conjugates with carnitine and coenzyme A, metabolites have been identified.⁹

1.2 Side Effects

There are several side effects associated with valproic acid intake.^{1, 10} The most common ones include nausea, vomiting, heartburn, and weight gain. Less than ten percent of patients report dose-related tremor, dermatological effects such as rash or alopecia, and neurological effects such as drowsiness, irritability and ataxia. Approximately 12% of patients report thrombocytopenia or inhibition of platelet aggregation. Rare cases of fatal hepatotoxicity, especially in children under 2 years of age, have been reported (1:30000). Teratogenicity has also been reported.

2. Materials and Methods

The chemical reagents used including acetic acid and methanol were of HPLC purity grade (Fluka, Buchs, Switzerland). Valproic acid samples were obtained in the form of Depakine® Chrono 500 tablets (Sanofi-Aventis, Paris, France). Each tablet contains 145 mg of valproic acid and 333 mg of sodium valproate in addition to saccharine and other excipients.

The standard compounds 4-hydroxy-VPA- γ -lactone¹¹ and 5-hydroxy-VPA- δ -lactone¹² were synthesized as described before. NMR Spectroscopic data for 4-hydroxy-VPA- γ -lactone was in complete agreement with the reported one.¹³ Spectroscopic data for 5-hydroxy-VPA- δ -lactone is: ¹H NMR (CDCl₃, 200 MHz): 0.94 (*t*, 3 H, *J*=7.1), 1.30-1.63 (*m*, 4 H), 1.83-1.98 (*m*, 3 H), 2.01-2.17 (*m*, 1 H), 2.39-2.55 (*m*, 1 H), 4.30 (*t*, 2 H, *J*=6.0); ¹³C NMR (CDCl₃, 50 MHz): 13.99, 20.01, 22.00, 24.58, 33.37, 39.33, 68,38, 174.81.

In Extractive Electrospray Ionization (EESI), a neutral sample (breath in this case) is delivered to the plume of a conventional electrospray formed from pure solvent. Neutral analyte molecules are charged by protonation / cationization processes when colliding with charged droplets from this ESI spray. The electrospray solution used here consisted of a methanol:water:acetic acid mixture (2:2:1) infused at 5 $\mu\text{L}/\text{min}$. The electrospray source was a commercial model operated at 3kV bias voltage; positive ion mode was used. The breath was introduced through a heated Teflon tube (80°C) at an angle of (β) 60° with respect to the electrospray needle and the distance between the MS cone and the tube was kept at 8 mm (see Figure 1). The distance between the needle and the tube was 4 mm. The electrospray needle was placed at an angle of (α) 130° with respect to the MS cone and a distance of 6 mm to the MS cone. Volunteers took a deep breath and exhaled for as long as possible (10-15s) into the heated Teflon tube, maintaining the flow at $\approx 300 \text{ cm}^3/\text{s}$, thus, both mouth and alveolar air were sampled. Six replicates of the breath measurements were done for the drug monitoring and pharmacokinetic studies. Each volunteer had his own mouth piece, and the heated tubing used to guide the breath into the ESI region was purged between volunteers to prevent memory effects. In the pharmacokinetic studies, healthy volunteers were administered a single dose of two tablets of Depakine® Chrono 500 to observe the changes in the breath fingerprint due to valproic acid ingestion. The breath fingerprint of this healthy volunteer was measured several times on different days before the ingestion of valproic acid to have a useful baseline.

A quadrupole time-of-flight mass spectrometer (QTOF Ultima, Waters Micromass, Manchester, UK) was used to perform the majority of the experiments. An LTQ Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) was used for the high mass accuracy confirmation measurements. The signal counts were normalized by the time each breath lasted. All spectra were background subtracted.

The synthetic 4-hydroxy-VPA- γ -lactone and 5-hydroxy-VPA- δ -lactone were used as standards to substantiate the identity of the VPA breath biomarker. In principle, 3-hydroxy-VPA- β -lactone could also form during the VPA metabolism, however, among three possible lactones the latter one is least stable, whereas 4-hydroxy-VPA- γ -lactone is the most favorable and stable one. The vapors of each standard were analyzed via EESI, and MS/MS was performed.

In parallel to the measurement of the VPA metabolite in exhaled breath, blood was taken from each volunteer. The protein bound VPA fraction and the free VPA were separated by ultrafiltration (Centrifree Micropartition system, Millipore Corporation, Bedford, MA 01730, USA). VPA concentrations were measured via a fluorescence depolarization assay (on a Cobas Integra 400 plus system from Roche Diagnostics GmbH, Mannheim, Germany). The necessary reagents, murine monoclonal anti-VPA buffered to pH=7.5, and with added stabilizers, preservatives, and fluorescently labeled VPA, came with the Cobas C pack for the Integra 400 plus system. A coefficient of variation of 3.3% (for serial measurements) or of 4.2% (overall) is specified by the manufacturer. If free VPA is determined on a Cobas Integra 700 system, these coefficients of variation are 1.0%, (serial) and 1.4% (overall), respectively.

3. Identification of Detected Metabolites

The synthetic 4-hydroxy-VPA- γ -lactone and 5-hydroxy-VPA- δ -lactone were used as standards to substantiate the identity of the VPA breath biomarker. In principle, 3-hydroxy-VPA- β -lactone could also form during the VPA metabolism, however, among three possible lactones the latter one is least stable, whereas 4-hydroxy-VPA- γ -lactone is the most favorable and stable one. The vapors of each standard were analyzed via EESI, and MS/MS was performed. The fragmentation pattern from the m/z 143 signal is identical for the standard vapors and the exhaled breath (Figure S1). Furthermore, the 4-OH-VPA- γ -lactone standard vapors were mixed with ammonia vapors in the ESI plume and a clear shift to m/z 160 was observed (Figure S2).

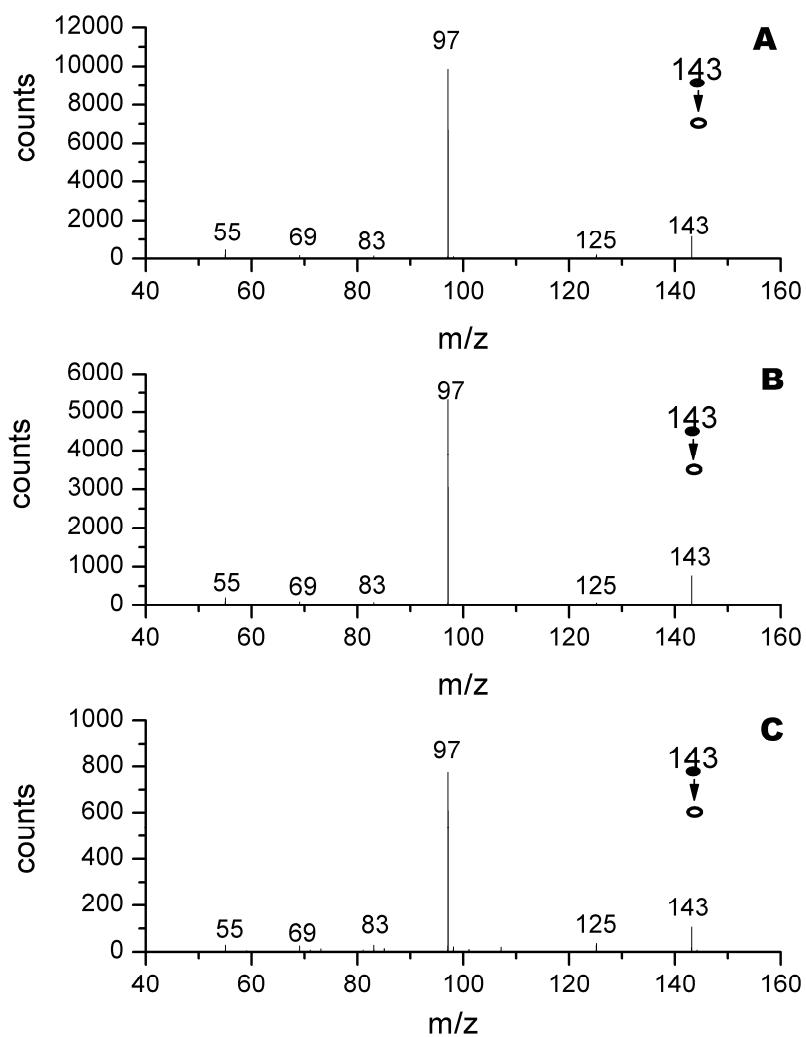


Figure S1. Collision induced dissociation fragmentation patterns of parent ion m/z 143 for (a) vapours of 4-hydroxy-VPA- γ -lactone, (b) vapours of 5-hydroxy-VPA- δ -lactone, and (c) exhaled breath of individual under VPA therapy (all analyzed via EESI).

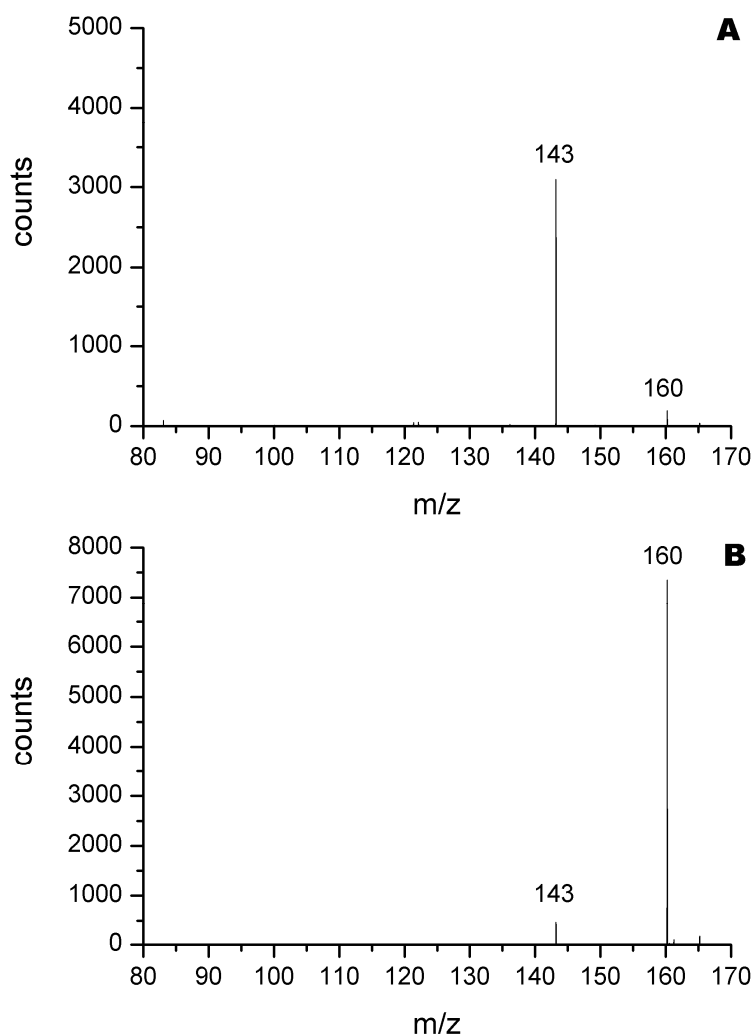


Figure S2. Spectra of 4-hydroxy-VPA- γ -lactone vapours obtained via EESI MS before (a) and after (b) being mixed with ammonia vapours. The shift in mass corresponds to NH_3 and is representative of a non-covalent ammonium complex.

The peaks at 143 m/z and 160 m/z were both subjected to MS/MS for identification purposes (see also below, Figure S4b insets). The collision energy was set between 10 and 20 units. The parent ion at 160 m/z gives major fragments at 143 m/z, 125 m/z, and 97 m/z. The parent ion at 143 m/z also gives major fragments at 125 m/z, and 97 m/z. Thus, we believe there is some in-source fragmentation of the m/z=160 ion to give 143 m/z. This was further supported by observing the ratio of 160 m/z to 143 m/z change as the Q-TOF mass spectrometer RF lens1

voltage was varied in the low range where the relative intensities of other ions of comparable m/z did not change (see Figure S3), supporting the identification of the $m/z=160$ signal as a non-covalent complex between the lactone and an ammonium ion.

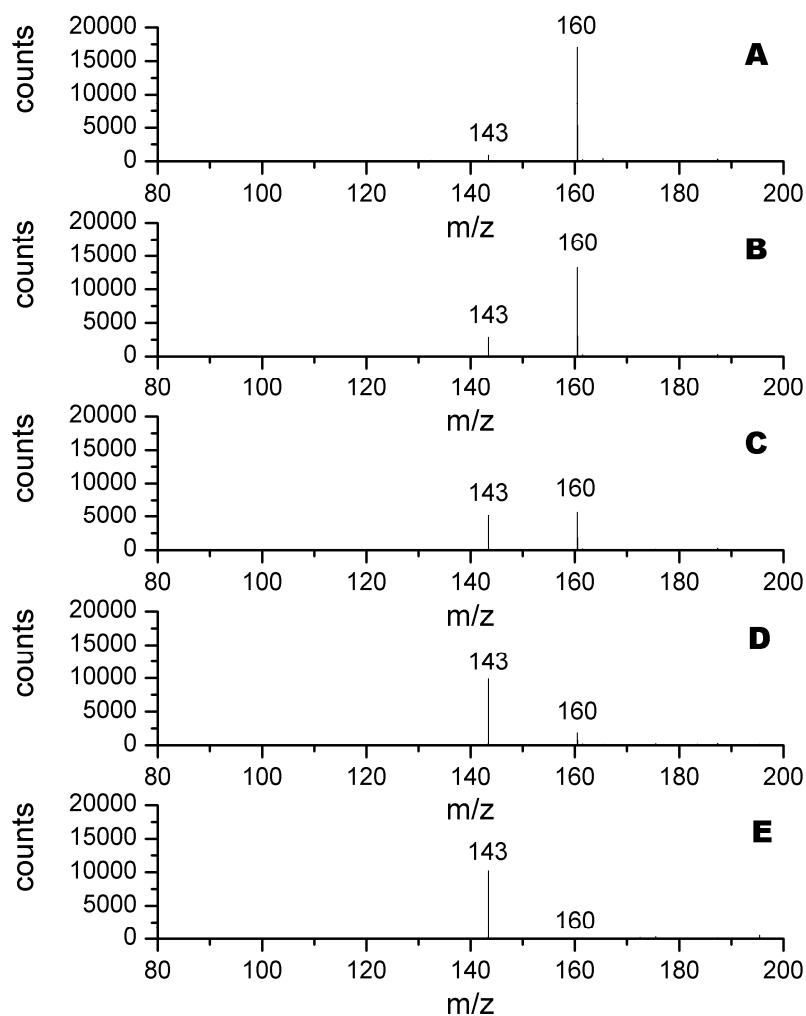


Figure S3. EESI MS of exhaled breath of individual under VPA therapy at different RF lens1 voltages: a) 35 V, b) 40 V, c) 45 V, d) 50 V, e) 55 V. Cone voltage maintained at 40 V. It is evident that an increase in the RF lens1 voltage produces fragmentation of m/z 160 to give m/z 143. The RF lens1 in our Q-TOF instrument takes the place of the skimmer. In this region, under these lower voltages, collisions with residual gas come about leading to ion cooling and droplet desolvation.

Tandem mass spectrometry measurements were also performed on an LTQ orbitrap instrument in order to get high accuracy m/z data (mass accuracy $<5\text{ppm}$) to confirm the structural information. The parent ion is at $160.1330\ m/z$ and its fragments at $143.1061\ m/z$, $125.0954\ m/z$, and $97.1005\ m/z$. Also, the accurate m/z shows that the ion at $143.1065\ m/z$, which is observed in the breath fingerprint, has virtually the same m/z as the fragment ion from $160.1330\ m/z$. We assign the compound at $m/z=160.1330\ (M+H^+)$ to a VPA metabolite with the elemental composition $C_8H_{17}O_2N$. This is further supported by the fragmentation pattern because the fragment at $143.1061\ m/z$ corresponds to a loss of NH_3 , the fragment at $125.0954\ m/z$ corresponds to a subsequent loss of H_2O , and the fragment at $97.1005\ m/z$ corresponds to a subsequent loss of CO . In addition, a fragment at $55.0534\ m/z$ was observed which could correspond to C_4H_7 . MS/MS data showing a loss of H_2O and CO also supports the lactone structure. It has been shown previously that fragmentation via collisionally induced dissociation of protonated γ -valerolactone,¹⁴ δ -valerolactone,¹⁵ and γ -butyrolactone¹⁶ results in loss of H_2O . In the case of γ -valerolactone¹⁴ and δ -valerolactone¹⁵ this is followed by a loss of CO .

4. Pharmacokinetic Studies

A healthy volunteer was administered a single dose of two tablets of Depakine® Chrono 500 to observe the changes in the breath fingerprint due to valproic acid ingestion. The breath fingerprint of this healthy volunteer was measured several times on different days before the ingestion of valproic acid to have a useful baseline. Figure S4a shows the typical breath spectral fingerprint of the healthy volunteer before ingestion of valproic acid. Figure S4b depicts the breath spectral fingerprint of the same volunteer 2hrs after the intake of valproic acid. It is clear that peaks at $143\ m/z$ and $160\ m/z$ are present in the healthy volunteer only after valproic acid ingestion.

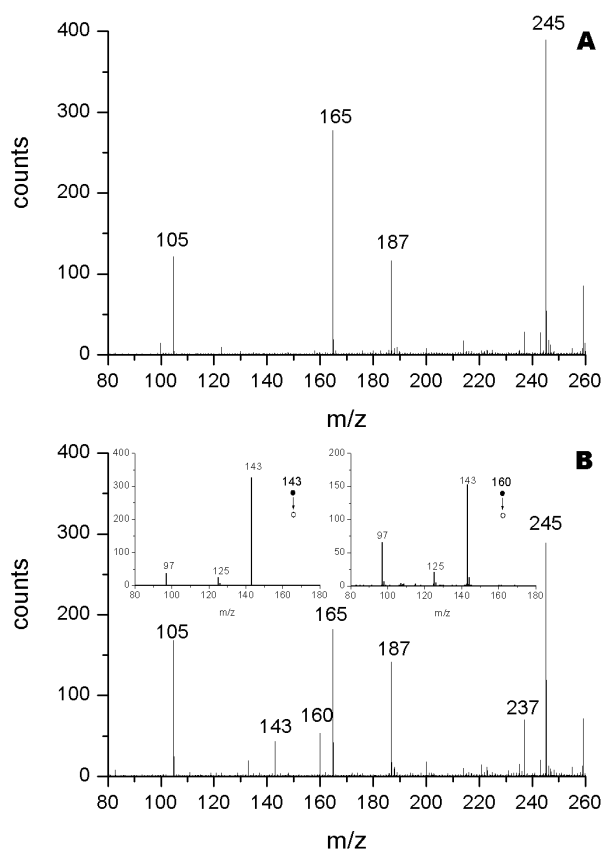


Figure S4. Breath fingerprints of a volunteer before (a) and 3.5 hrs (b) after ingestion of two tablets of Depakene CHRONO 500. It is evident that peaks at 160 m/z and 143 m/z appear in the breath only after valproic acid ingestion. The insets show CID fragmentation patterns of breath analyte at m/z=160 and m/z=143. The fragmentation patterns, along with the more exact mass determinations, indicate in-source fragmentation of the m/z =160 ion to m/z =143.

Table S1. Valproic acid half-lives obtained via exhaled breath EESI MS.

| Volunteer | Valproic Acid Half-life ($t_{1/2}$, hrs) |
|----------------|--|
| A ¹ | 10.5 |
| B ² | 9.2 |
| C ² | 7.8 |
| D ¹ | 10.2 |
| E ² | 7.0 |

¹Single dose of one Depakine® CHRONO 500 tablet.

²Single dose of two Depakine® CHRONO 500 tablets.

Finally, Table S1 and Figure S5 show the biological variation of the VPA elimination kinetics for 5 healthy volunteers who each took a single dose of VPA. Breath analysis was carried out for 3 consecutive days in each case.

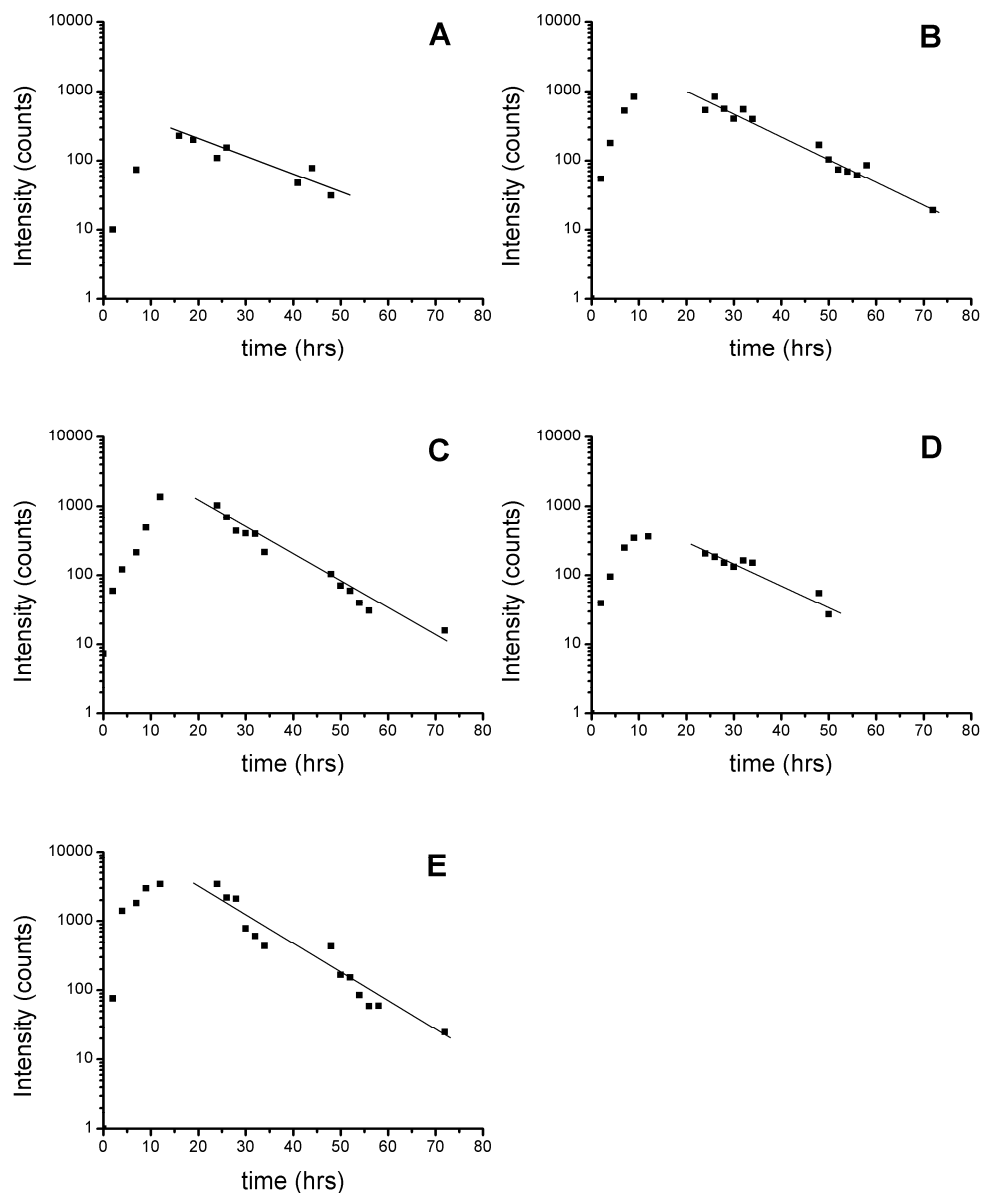


Figure S5. Temporal behavior of the VPA biomarker (m/z 143) obtained via EESI MS from exhaled breath of volunteers after taking a single dose of either one Depakine® CHRONO 500 tablet (a, d) or two tablets (b, c, e). The first-order elimination kinetic profile is evident in each volunteer.

5. Statement on Human and Other Animal Experiments

We hereby confirm that all experiments were performed in accordance with relevant guidelines and regulations. We also confirm that informed consent was obtained from all subjects who served as volunteers for the studies presented. Ethical approval was obtained from the Kantonale Ethikkommission Zürich (Ethics Committee of the State of Zurich, Switzerland) and from the Ethics Committee of East China Institute of Technology.

6. References

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