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FOCUS: EMERGING INVESTIGATORS: RESEARCH ARTICLE

Real-Time Quantitative Analysis of Valproic Acid in Exhaled Breath by Low Temperature Plasma Ionization Mass Spectrometry

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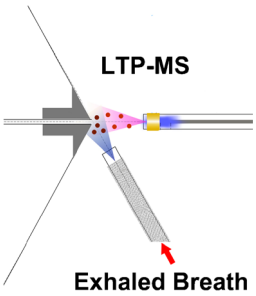

Abstract. Real-time analysis of exhaled human breath is a rapidly growing field in analytical science and has great potential for rapid and noninvasive clinical diagnosis and drug monitoring. In the present study, an LTP-MS method was developed for real-time, in-vivo and quantitative analysis of γ-valprolactone, a metabolite of valproic acid (VPA), in exhaled breath without any sample pretreatment. In particular, the effect of working conditions and geometry of the LTP source on the ions of interest, protonated molecular ion at *m/z* 143 and ammonium adduct ion at *m/z* 160, were systematically characterized. Tandem mass spectrometry (MS/MS) with collision-induced dissociation (CID) was carried out in order to identify γ-valprolactone molecular ions (*m/z* 143), and the key fragment ion (*m/z* 97) was used for quantitation. In addition, the fragmentation of ammonium adduct ions to protonated molecular ions was performed in-source to improve the signal-to-noise ratio. At optimum conditions, signal reproducibility with an RSD of 8% was achieved. The concentration of γ-valprolactone in exhaled breath was determined for the first time to be 4.83 (±0.32) ng/L by using standard addition method. Also, a calibration curve was obtained with a linear range from 0.7 to 22.5 ng/L, and the limit of detection was 0.18 ng/L for γ-valprolactone in standard gas samples. Our results show that LTP-MS is a powerful analytical platform with high sensitivity for quantitative analysis of volatile organic compounds in human breath, and can have potential applications in pharmacokinetics or for patient monitoring and treatment.

Keywords: Low temperature plasma probe, Ambient ionization, Breath analysis, Valproic acid, Valprolactone

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Introduction

Human breath contains thousands of molecules, including volatile compounds carried in the vapor phase and non-volatile compounds carried in aerosol particles. These molecules are produced by various metabolic processes in the human body, released into the blood, and passed onto the airway once the blood reaches the lungs. Everyone has a 'breathprint' that can potentially provide instantaneous information related to health [1–3]. Breath analysis has a number of advantages over traditional analytical techniques. It is a noninvasive and painless procedure. Breath sampling is easily accessible and essentially unlimitedly repeatable, which is advantageous over blood, urine, or tissue collection [4]. In addition, the matrix effects of human breath are less significant and sample preparation prior to analysis is often simpler compared with other biological fluids. Consequently, the analysis of exhaled breath is a rapidly growing research field for clinical diagnosis and monitoring purposes [5–9].

Valproic acid (VPA) is primarily used for the treatment of epilepsy. New patients will be given a blood test once or twice a month initially for determining the optimal therapeutic window. During continuous treatment, drug monitoring of VPA showed wide fluctuation in its plasma level; about 18% of patients had toxic levels while 20% of patients were below therapeutic level [10]. Thus, the patients need constant monitoring for VPA as a guide for dosage adjustment.

It has been reported that the treatment with the drug VPA led to an enhancement of 3-heptanone concentration in exhaled breath [11]. In a recent study, 3-heptanone was suggested as a potential marker for VPA in exhaled breath by PTR-MS; however, there was no correlation between 3-heptanone concentrations in breath gas and VPA blood concentrations [12].

Previous research by Gamez et al. showed a new metabolite (γ-valprolactone, or 4-hydroxy VPA-γ-lactone) in exhaled

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breath of individuals under VPA therapy through extractive electrospray ionization (EESI) mass spectrometry [13]. The mass spectra of exhaled breath showed two distinct peaks, γ-valprolactone molecular ion (*m/z* 143) and its ammonium adduct ion (*m/z* 160). The preliminary results demonstrated a linear correlation between EESI signal at *m/z* 143 and the concentration of the free VPA fraction in blood. This research showed the feasibility of monitoring VPA in exhaled breath by EESI; however, no quantitation of the VPA metabolite was reported.

The LTP source was first introduced by Harper et al. [14]. It is based on the dielectric barrier discharge by applying high alternating current voltage, and helium was employed as discharge gas. Different from other ambient plasma sources, the temperature can be as low as 30 °C so that it does not inflict thermal damage to the sample substrates, such as human skin, explosives [15, 16], etc. The LTP probe has been demonstrated as a powerful analytical tool for direct analysis of a wide variety of chemicals, especially ionizing low molecular weight compounds, more efficiently and over a relatively wide polarity range [17]. A number of diverse types of applications have been developed, including food safety [18–20], product quality control, and forensics [21, 22]. In addition, the LTP portability shows good potential for in-field chemical analysis. In previous work by Zenobi et al. [23], the active part of a capillary dielectric barrier discharge was used for on-line analysis of exhaled human breath via negative ion Q-TOF MS. Nevertheless, that work was mainly focused on fingerprinting and semi-qualitative analysis of endogenous compounds. Thus, it is clear that there is a need for quantitative analysis of γ-valprolactone in exhaled breath to improve understanding of the excreting pathways of exhaled metabolites.

The present work is aimed at developing a method for quantitative monitoring VPA metabolite in exhaled breath. For that purpose, the afterglow region of a home-made LTP source is used to ionize the γ-valprolactone in exhaled breath and analyzed via positive ion linear ion trap MS. The experimental conditions of the LTP-MS platform were systematically optimized to obtain the best signal-to-noise ratio. Under the optimized conditions, the analytical figures of merit were obtained, including the limit of detection, and γ-valprolactone in the exhaled breath was quantified in real time and in vivo, without sample preparation.

Experimental

Breath Sampling

Exhaled breath was sampled with a device composed of a disposable mouthpiece and a T-piece (Swagelok Co., Solon, OH, USA) that diverted one part of the exhaled breath to the LTP-MS interface and the other part towards a rotameter (Linde FM 4333; Union Carbide Co., Houston, TX, USA) to control breath flow rate (Figure 1). The breath directed at the LTP-MS interface passed through a PTFE tube (i.d. 3.20 mm, o.d. 6.00 mm) maintained at approximately 80 °C with heating tape (BriskHeat Co., Columbus, OH, USA). The flow rate of the breath exiting the heated tube and the rotameter was calibrated with a mass flow controller (0.01-5 LPM, 32907–69, Cole-Parmer Instrument Co., Vernon Hills, IL, USA) and compressed air. During the breath sampling procedure, the breath flow rate was controlled by instructing the volunteer to exhale (lasting typically 10–15 s) through the mouthpiece, while keeping the rotameter indicator at the same height to ensure constant exhaled breath flow rate (1–3 SLPM). Three replicates were performed per measurement. In the plots, the data of signal intensity and its error bar represent the average and the standard deviation result of the triplicates, respectively.

Preparation of Gas-Phase γ-Valprolactone Standards

Gas-phase samples were obtained from the headspace of liquid samples in sealed vials. The most concentrated sample was prepared by placing 10 mg liquid γ-valprolactone (Toronto Research Chemicals Inc., Toronto, Canada) into a sealed 10 mL sample vial and maintained at 25 °C overnight. The concentration of γ-valprolactone in the headspace of the 10 mL vial was calculated to be 450 μg/L based on the vapor pressure (0.059 mmHg at 25 °C). Diluted standards were made by mixing liquid dimethyl sulfoxide (DMSO, ACS; Mallinckrodt Baker Inc., Phillipsburg, NJ, USA, vapor pressure of 0.60 mmHg at 25 °C) and liquid γ-valprolactone to reach specific mole fractions that yield different headspace concentrations in accordance with Raoult's Law. Standards with headspace concentrations of γ-valprolactone of 112.5, 56.3, 28.1, and 14.1 μg/L were made by mixing 10.0 mg of liquid γ-valprolactone with different masses of DMSO (16.5, 38.5, 82.4, and 170.4 mg, respectively) in 10 mL sealed sample vials and maintained at 25 °C overnight.

Dilution Chamber and Quantitative Analysis Protocol

A dilution chamber (100 mL rounded bottom glass flask with a magnetic stirrer underneath, Supplementary Figure S1 in Supporting Information Section 1) was positioned to enable online mixing and dilution of the injected standard gaseous sample with exhaled breath for the quantitative analysis. The air-tight flask featured a PTFE tube to allow the exhaled breath to come in and a glass tube to direct the gas-phase mixture to the LTP-MS interface. The temperature was maintained at around 80 °C by wrapping heating tape around the tubes and the chamber. Dilutions of gas standards were made by injecting 5 μL of headspace gas from the prepared sample vials, described in the section above, directly into the dilution chamber previously filled with exhaled breath (five consecutive deep exhalations). After 5 s, the gas mixture in the flask was transported to the LTP-MS interface by active exhalation of breath from the volunteer. Blanks (injection with 5 μL gas-phase DMSO) and standard gas samples were run consecutively. A gastight syringe (50 μL, model 1705 RN SYR; Hamilton Co., Reno, NV, USA) was used for headspace standard gas sampling. The syringe was heated and flushed after each injection to prevent carryover. Three replicates were performed at each concentration.

LTP Ionization Source

The configuration of the designed ionization source is shown in Figure 1. The LTP source includes a glass tubing with i.d. and o.d. of 4.00 mm and 6.00 mm, respectively; a tungsten rod (1.59 mm diameter) acting as an internal axial electrode was centered inside the glass tubing; a copper foil (3.5 mm wide, 1.5 mm distance to glass tube exit) surrounding the outside of the glass tubing was used as the outer ring electrode. A high voltage AC power supply (PVM500; Information Unlimited, Amherst, NH, USA) was used for the generation of dielectric barrier discharge. The applied voltage and frequency of the power supply were monitored with a high-voltage probe at the powered ring electrode, and the current was measured through a resistor (1000 Ω) on the grounded axial-electrode with an oscilloscope (DSOX3034A; Agilent Technologies Co., Santa Clara, CA, USA). The flow rate of discharge gas helium (Airgas Inc., Radnor, PA, USA) was controlled with a mass flow controller (0.01-1 LPM, 32907–67, Cole-Parmer Instrument Co., Vernon Hills, IL, USA). Exhaled breath was delivered by the heated Teflon tube towards the plasma ionization source to be ionized and detected by MS.

Mass Spectrometry

The experiments were performed with a Thermo LTQ-XL linear ion-trap mass spectrometer (Thermo Scientific) in the positive ion mode. Except for removing the standard ESI house, no modification was required. Instrument parameters were set to a capillary temperature of 275 °C, a capillary voltage of +4 V, a tube lens voltage of +50 V, and a microscan count 1 with a maximum ion injection time of 100 ms. Ionized species produced transient mass spectrometric signals, which were acquired and further processed with Thermo Excalibur software (Thermo Scientific).

Results and Discussion

LTP-MS Spectra of Exhaled Breath

The exhaled breath from a volunteer under a daily dose of VPA and a typical healthy volunteer without VPA intake were analyzed in real-time by LTP - MS (Figure 2). The experimental conditions were: applied voltage, 3.0 kV; frequency, 25 kHz; helium flow rate, 0.25 SLPM; breath flow rate, 2.40 SLPM; LTP source was positioned coaxial with MS capillary; inter-electrode distance, 4 mm; distance between breath tubing and MS inlet was 11.08 mm and angle was 35°; distance between LTP and MS was 1.90 mm. The spectra show that there are two major peaks at *m/z* 143 and 160 in the mass spectrum of the volunteer under VPA treatment, which are not present in the breath mass spectrum of the healthy volunteer. These observations are consistent with those found in previous research by EESI-MS [13]. The peaks at *m/z* 143 and 160 correspond to protonated γ-valprolactone and its ammonium adduct ion, respectively. These results show that the LTP ionization source is effective for real-time breath analysis of VPA. It is also worth noting that there are many peaks present in both mass spectra shown in Figure 2, but the intensities are much lower compared with the dominant ions, which makes them hard to observe at that scale.

Optimization of LTP Source

The LTP source was characterized using the exhaled breath from the volunteer under a daily dose of VPA. The experimental parameters such as power supply voltage, electrodes distance, power supply frequency, helium flow rate, breath flow rate, and the geometry of LTP source and breath tube were experimentally optimized one-factor-at-a-time. In this study,

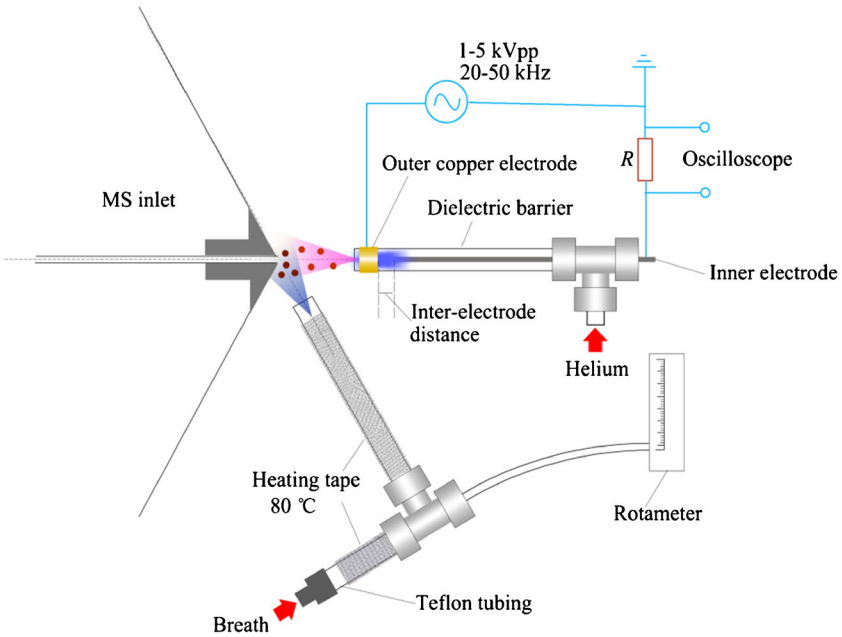

Figure 1. Schematic of experimental setup for online breath analysis by LTP-MS. Exhaled breath is introduced through a heated Teflon tube. The oscilloscope in the external circuit is used to monitor and analyze the discharge current waveform

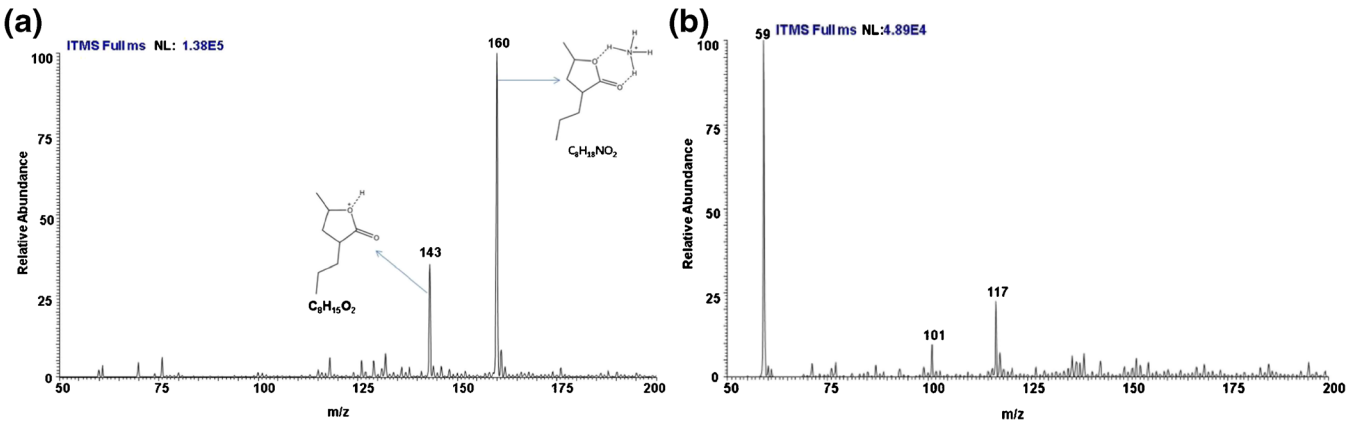

Figure 2. LTP mass spectrum of exhaled breath from: volunteer under daily dose of VPA (**a**) and volunteer without VPA intake (**b**). The protonated valprolactone (*m/z* 143) metabolite and its ammonium adduct (*m/z* 160) are evident in (**a**), but absent in (**b**)

the working conditions were selected to obtain high intensity, good signal-to-noise ratio (S/N), and low RSD values for the signal of protonated γ-valprolactone (*m/z* 143) and its ammonium adduct (*m/z* 160). The noise is taken as the signal intensity when no breath is exhaled. Note: the optimization trends of different operating parameters for *m/z* 160 were found to be very similar to *m/z* 143 (Supporting Information Section 2).

Power Supply Voltage and Inter-Electrode Distance

The effect of the applied peak-to-peak voltage on the analytical signal was found to depend on the inter-electrodes distances. Thus, a systematic study was performed where the applied voltage was varied as a function of the inter-electrode distance, which was taken as the distance between the tip of the axial electrode and the edge of the copper foil electrode farthest from the end of the glass tube. When the electrodes were just overlapped, the value was defined as the zero point, 0 mm. The negative value represents a movement of the inner axial electrode toward the outlet of glass tubing. Positive values denoted a movement of the inner electrode farther away from the end of the glass tubing. The initial conditions were: applied frequency, 28 kHz; helium flow rate, 0.25 SLPM; breath flow rate, 2.40 SLPM; the LTP source was positioned coaxial with MS capillary, the distance between breath tubing and MS inlet was 11.08 mm, and angle was 35°; distance between LTP and MS was 1.90 mm. Figure 3a shows the effect of the voltage on the signal intensity level of protonated γ-valprolactone (*m/z* 143) under different inter-electrode distances. The applied voltage is limited at the lower range by the plasma ignition-voltage threshold. An upper applied voltage of 4 kV was maintained to prevent arc formation between the powered electrode and the MS capillary inlet. When the electrodes overlap, the plasma needs a relatively low voltage to be ignited and in general the signal intensity increases with voltage. It is evident that the voltage necessary to generate the plasma is higher as the inter-electrode distance is increased, which results in a narrower voltage working range. As it can be seen in Figure 3b, the highest S/N is obtained when the two electrodes distance was 0 mm and power supply voltage was 2.53 kV; that is because the noise was very low compared with higher voltage; however, the plasma became less stable (the signal RSD% was 49.8%). When the inter-electrode distance is 8 mm, it has the highest signal intensity and good RSD value (6.60%), but also lower S/N values; when the inter-electrode distance is 4 mm, it gave the second highest intensity at 3.0 kV with good S/N and RSD value (as low as 5.48%). Moreover, it can be seen that at 4 mm distance, the signal intensity increases with voltage first, then it arrives at the maximum when voltage is 3.0 kV. If the voltage is increased, the intensity will drop dramatically. This trend can be explained by the plasma mode. Diffuse plasma can be generated at lower voltages, and the signal intensity increases with voltage, which means a higher amount of ionizing plasma species is being produced, as supported by the plasma current waveforms (Supporting Information Section 3). However, as the applied voltage exceeds 3.0 kV, the plasma will become filamentary as first evidenced by the current waveform, where characteristic multiple spikes begin to appear, and is eventually observable with the naked eye. In this mode, the discharge constricts and the plasma reagent ion population distribution changes, which will result in the signal intensity decreasing. In summary, 4 mm inter-electrode distance at 3.0 kV applied voltage was chosen as the optimum conditions for the experiments discussed below.

Geometry of LTP Source

The effects of the distance between LTP probe and MS inlet (0.63–3.80 mm, shorter distances were avoided to prevent arcing) was studied. Inter-electrode distance was chosen at 4 mm with 3.0 kV applied voltage, and other experimental conditions were the same with the initial ones. The result (Figure 3c) shows that the distance between LTP probe and MS inlet does not affect the signal intensity as dramatically as

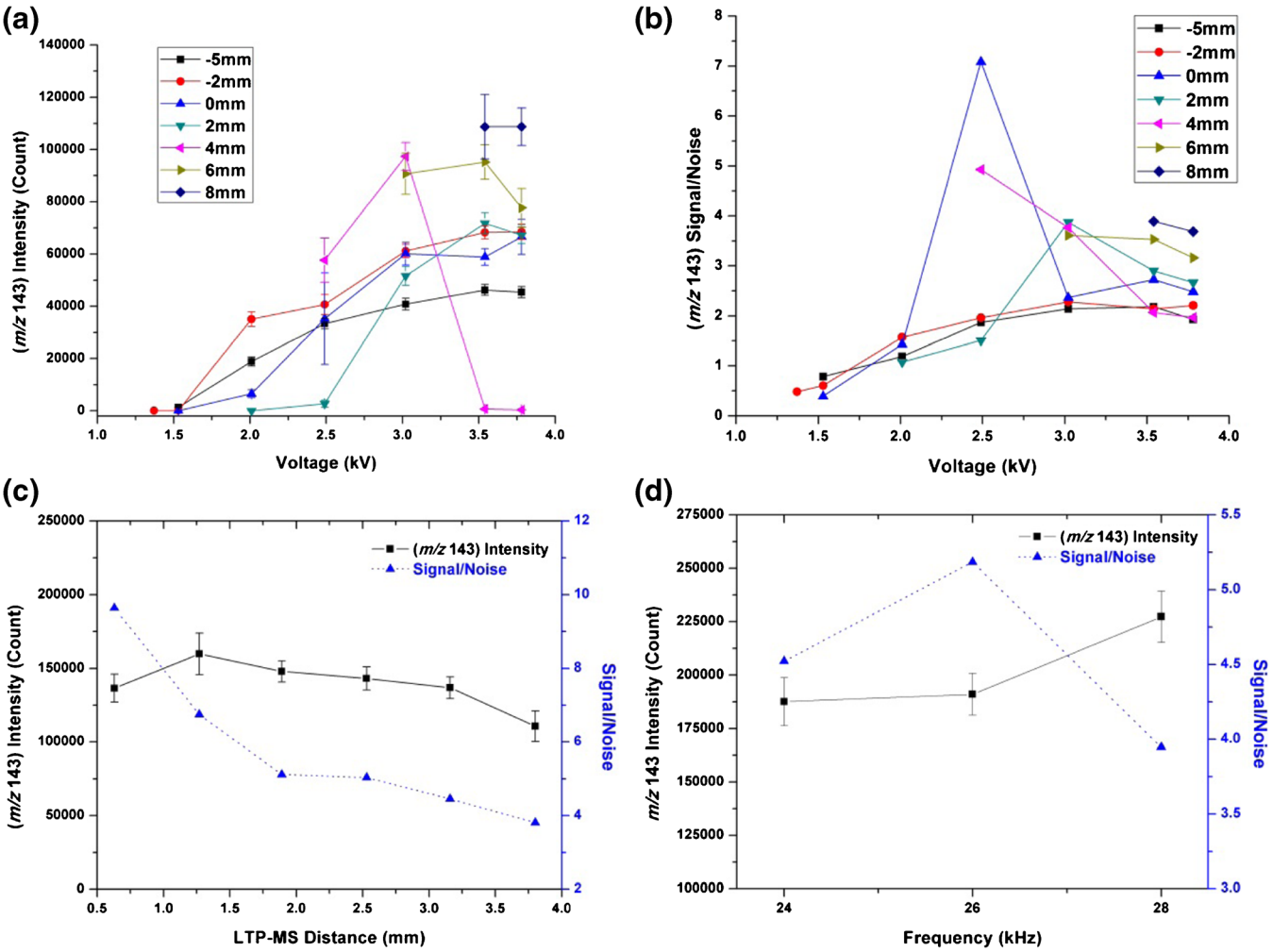

Figure 3. Effect of operating conditions on signal intensity and S/N at *m/z* 143: (**a**) signal intensity versus applied voltage for different inter-electrode distances, (**b**) S/N versus applied voltage for different inter-electrode distances, (**c**) signal intensity (black solid line) and S/N (blue dashed line) as a function of LTP to MS distance, (**d**) signal intensity (black solid line) and S/N (blue dashed line) as a function of frequency

other parameters studied. Nevertheless, the S/N is clearly influenced by the LTP probe/MS distance. It is clear from the Figure 3c that at longer distances the S/N value follows the signal trends, which indicates that the noise intensity is approximately constant at distances greater than 1.8 mm. On the other hand, the S/N increases at distances below 1.8 mm despite the decrease in signal intensity, thus showing the noise is much lower in this region. At 0.63 mm, the highest S/N was observed; thus, this distance was selected as optimum for the following experiments.

AC Frequency

In this study, the optimal conditions obtained above were applied and other parameters were not changed, except the power supply frequency was adjusted to maintain a high voltage of 3.0 kV to the copper electrode; it was found that the effective frequency range was between 28 kHz and 24 kHz. Under the same voltage, it can be seen that the signal intensity increases when the frequency increases; however, it is accompanied with higher noise. From Figure 3d, 26 kHz was chosen as the best frequency to have good intensity, RSD (9.53%), and highest S/N (this was also the same case for ions at *m/z* 160, see Supplementary Figure S3b).

Helium Flow Rate

Helium flow rate tested in this section was increased from 0.2 to 0.4 SLPM (utilized in previous study), and the breath flow rate was chosen between 2.4 SLPM and 2.95 SLPM (which was the accessible range with the rotameter setup to keep a comfortable exhalation). Other conditions were: applied voltage, 3.0 kV; frequency, 26 kHz; the LTP source was positioned coaxial with MS capillary; inter-electrode distance, 4 mm; distance between breath tube and MS inlet, 11.08 mm; angle between breath tube and LTP source, 35°; distance between LTP and MS, 0.63 mm. As clearly shown in Figure 4a, when the helium flow rate was set to be 0.2 SLPM, if the breath flow

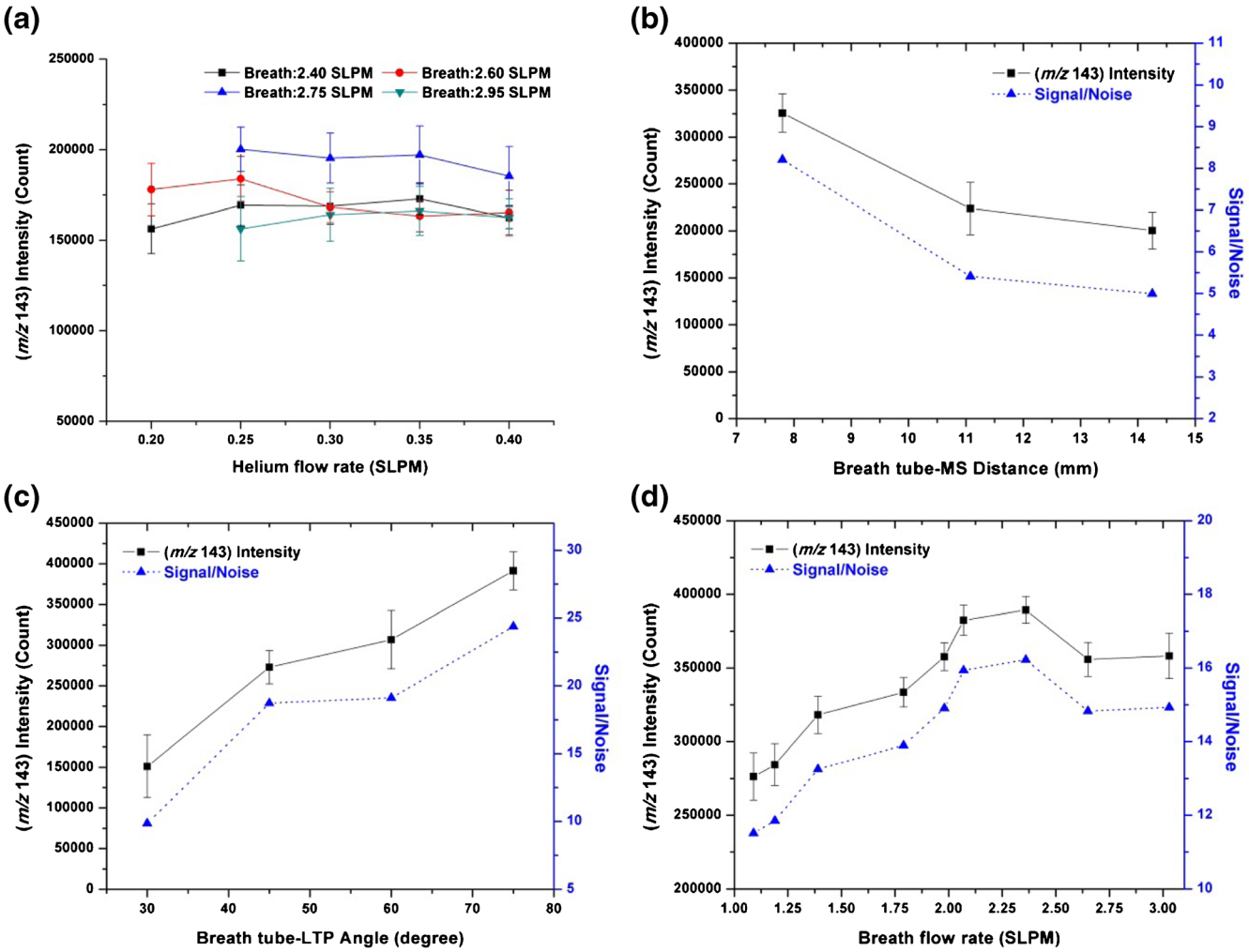

Figure 4. Signal intensity and signal/noise as a function of: (**a**) helium flow rate under different breath flow rates, (**b**) breath tube-MS distance, (**c**) breath tube-LTP angle, and (**d**) breath flow rate

rate was 2.75 SLPM or 2.95 SLPM, there was no signal. This was most probably due to the turbulence disturbing the plasma. The result (see Figure 4a) demonstrated that the signal intensity was not significantly affected by the helium flow rate in a large range at different breath flow rates. The trend could be due to the amount of reagent ions not being a limiting factor within these operating conditions. Another possibility is that a potential increase in reagent ions at higher helium flow rates is offset by the lower residence time in the reaction volume. In summary, helium flow rate can be chosen in the range between 0.2 and 0.4 SLPM.

Geometry of Breath Tube and Breath Flow Rate

The effects of the distance between the breath tube and MS inlet (7.81–14.25 mm, Figure 4b) and the angle between LTP probe and breath tube (30°–75°, the angle range is geometrically constrained) on the signal of interest were explored within the accessible range limits. Other conditions were: applied voltage, 3.0 kV; frequency, 26 kHz; inter-electrode distance, 4 mm; helium flow rate, 0.4 SLPM; breath flow rate, 2.75 SLPM; angle between breath tube and LTP source, 35°. The breath tube was moved back and forth towards the MS-inlet and the value at 7.81 mm gave the highest response (Figure 4b). With these optimal distances (LTP probe-MS inlet: 0.63 mm, breath tube-MS inlet: 7.81 mm), the signal intensity was highest when the angle between LTP probe and breath tube was 75° (Figure 4c). At smaller angles, part of breath stream is blocked by the LTP glass tube so that less analyte reaches the ionization volume, which results in a lower signal. It has also been found that at 75° between LTP and breath tube, the breath tubing can be moved closer to the MS inlet but limited to 5 mm geometrically, and at 5 mm gave a higher signal compared with 7.81 mm (data is not shown). With all the optimal conditions (here, helium flow rate was chosen as 0.4 SLPM), the breath flow rate was further evaluated. Figure 4d clearly demonstrates that the breath flow rate at 2.0 and 2.36 SLPM gave higher signal intensity compared with other breath flow rates. It was noticed that the trend in Figure 4d is different from the result of Figure 4a, in which breath flow rate at 2.75 SLPM shows higher signal intensity; this change is due to different geometry of the breath tube. In summary, the optimal geometry for LTP

source was as follows: the distance between LTP probe and MS inlet was 0.63 mm; the angle between LTP probe and breath tube was 75°; the distance between breath tube and MS inlet was 5 mm. The best breath flow rate range can be chosen between 2.0 and 2.36 SLPM.

Identification of γ-Valprolactone by MS/MS

MS/MS is usually very useful for exclusion of false positive signals, especially when analyzing with complex matrices. MS^2 spectrum acquired for the molecular ion *m/z* 143 ($[M + H]^+$) via collision induced dissociation (CID) (Figure 5a) shows major fragments at *m/z* 125 and 97. The fragmentation pattern is consistent with previous literature on protonated γ-valprolactone ions (*m/z* 143) using extractive electrospray ionization [13]. The parent ions of *m/z* 143 could lose H_2O to yield the fragment of *m/z* 125, which loses CO to generate the fragment of *m/z* 97. The MS/MS confirms the identity of signal at *m/z* 143 corresponds to γ-valprolactone. The normalized collision energy was also optimized at 20, where it gives the highest intensity of the most abundant fragment ion (*m/z* 97) (Figure 5b). No fragmentation is observed when the collision energy is at 5 units. As the energy value increases, the signal

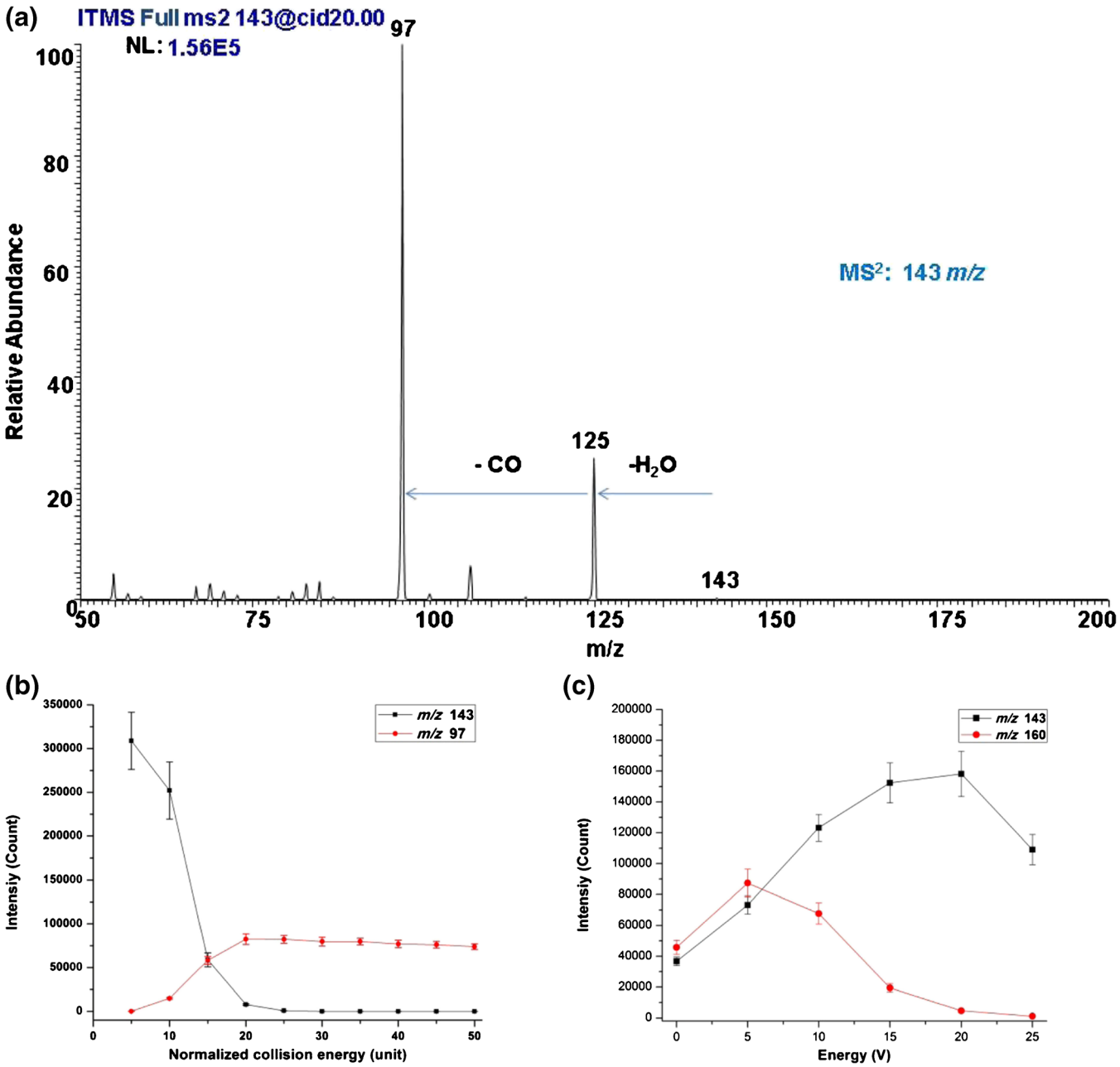

Figure 5. LTP MS/MS for: (**a**) CID spectra of protonated γ-valprolactone in exhaled breath (MS/MS: *m/z* 143 to *m/z* 97 and *m/z* 125), (**b**) intensity of *m/z* 143 and *m/z* 97 as a function of the instrumental CID energy, (**c**) in-source fragmentation characteristics of adduct ions (*m/z* 160). Plotted ions are *m/z* 160 (red line) and *m/z* 143 (black line)

intensity of *m/z* 143 decreases, whereas the intensity of *m/z* 97 is increased until a maximum is reached at 20 collision energy units. Higher collision energy leads to similar intensities at *m/z* 97. In addition, MS/MS has another advantage that it will result in significant noise decrease at the *m/z* of interest. Consequently, it is advantageous to use the characteristic fragment at *m/z* 97 for the following quantitative analysis.

Quantitative Analysis of γ-Valprolactone in Exhaled Breath

In-Source Fragmentation It has already been shown that there are two major peaks at *m/z* 143 (protonated γ-valprolactone molecular ions) and *m/z* 160 (ammonium adduct ions) in the original breath spectra. In-source fragmentation was implemented to fragment ammonium adduct ions (*m/z* 160) into protonated γ-valprolactone ions (*m/z* 143) before getting to the ion trap. The main resulting advantages are obtaining higher signal intensity at *m/z* 143 and also overcoming the signal variability due to person-to-person breath ammonia concentration, which can range from 248 to 2935 ppb [24]. In-source fragmentation occurs in the intermediate pressure part of the mass spectrometer, between the atmospheric pressure source and the high vacuum of the mass analyzer. The ions are accelerated by applying specified voltage, which prompts collisions with the surrounding neutrals to generate fragmentations [25, 26]. The signal intensity of *m/z* 160 and *m/z* 143 were studied at increasing instrument settings of fragmentation energies (0, 5, 10, 15, 20, 25 V). As shown in Figure 5c, when the fragmentation energy is increased from 0 to 5 V, the intensity of these two ions goes up due to improved ion transmission efficiency. No fragmentation is observable when the energy is at 5 V. As the value of the fragment energy is increased, the signal intensity of *m/z* 160 decreases, whereas, the intensity of *m/z* 143 is increased. This is because more and more ions at *m/z* 160 are fragmented to *m/z* 143. The fragment ion intensity (*m/z* 143) reaches the maximum when the fragment energy is 20 V. Higher voltages lead to lower intensities at *m/z* 143; this is due to fragmentation, which was evident by the increasing signal intensity of fragment ion *m/z* 97 (data not shown). Thus, the optimal in-source fragmentation voltage to maximize the signal at *m/z* 143 is 20 V, which was chosen for the following quantitative analysis.

Standard Addition Method The standard addition method was applied to identify and overcome possible matrix interferences by spiking exhaled breath from the individual (after 14–16 h ingestion of 2 g VPA) with 5 μL head-space gas sample from the prepared standard sample vials (with final γ-valprolactone concentration in spiked breath of 1.4, 2.8, and 5.6 ng/L), and 5 μL gas-phase DMSO as blanks, respectively. After mixing for 5 s, the gas mixture was transported by the patient exhaled breath to the LTP source. Here, the optimal parameters were applied for this measurement: applied voltage, 3.0 kV; frequency, 26 kHz; helium flow rate, 0.20 SLPM; breath flow rate, 2.0 SLPM; the LTP source was positioned coaxial with MS capillary; distance between the mixing chamber exit tube and MS inlet was 5 mm; angle between the mixing chamber exit tube and LTP was 75°; distance between LTP and MS was 0.63 mm. The in-source fragmentation was implemented with a fragment energy set to 20 V to fragment *m/z* 160 to *m/z* 143. In addition, the CID was implemented with a normalized collision energy set to 20 units to fragment ions at *m/z* 143 and obtain the highest signal intensity at *m/z* 97.

A satisfactory relative standard deviation (RSD) of 8% was obtained for γ-valprolactone in breath with 10 measurements within 5 min. A decrease in signal was observed when the sample breath was spiked with pure gas-phase DMSO; this would indicate that the presence of DMSO is causing ionization suppression. After addition of 5 μL gas-phase DMSO, the DMSO was diluted continuously by sample breath. In this dilution process, the DMSO concentration in the mixing flask was decayed exponentially and can be calculated based on the ideal exponential dilution equation [27]. The suppression effect was evaluated by taking into account that the analyte concentration in exhaled breath is constant during the DMSO dilution and by plotting the signal (*m/z* 97) suppression percentage against DMSO concentration. The equation was $y=0.3407x - 0.0559$ ($R^2=0.99967$), which shows a linear relationship between the suppression percentage and DMSO concentration. The signal (*m/z* 97) of each standard sample can be compensated based on the suppression percentage at different DMSO concentration in each calibration gas. The quantification can be obtained by plotting the compensated signal against the spiking standard concentration of γ-valprolactone. It was found that the calibration equation was $y = 7353.9x + 35512$ with a good linearity coefficient $R^2=0.9973$ (shown in Figure 6a). The linear regression curve can be used to extrapolate the concentration of γ-valprolactone in exhaled breath, which is estimated to be 4.83 (±0.32) ng/L. This is the first time the concentration of γ-valprolactone is quantified in exhaled breath. If we assume this concentration value of γ-valprolactone in exhaled breath represents the average concentration in a single day, it is possible to approximate the total daily exhalation to be 54 μg day^{-1}. This is a very rough estimation because the relative amount of γ-valprolactone in exhaled breath has been shown to vary throughout the day [13]. Nevertheless, it is instructive to get an idea of the scale of excretion of γ-valprolactone through breath.

Calibration Curve Here a similar protocol to the standard addition method was followed except five γ-valprolactone gas standards (with the final concentration in the gas mixture of 0.7, 1.4, 2.8, 5.6, and 22.5 ng/L) injected into the mixing chamber, which was full of exhaled breath from the healthy volunteer, as well as transported by the breath from healthy volunteer to the LTP source. Blanks (injection with 5 μL gas-phase DMSO) and calibration gas sample were run consecutively. The calibration curve was obtained based on the analysis of these five standard samples. The compensated blank

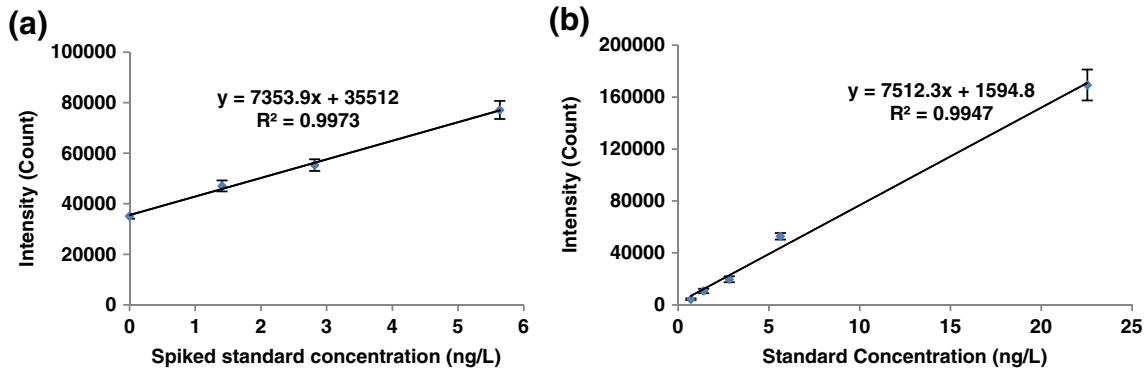

Figure 6. Quantification curves: (**a**) Standard addition curve for the fragment ion intensity (*m/z* 97) as a function of added standard gas concentration to patient exhaled breath. (**b**) Calibration curve for the fragment ion intensity (*m/z* 97) as a function of gas standard concentration mixed with healthy volunteer exhaled breath

subtracted signals (*m/z* 97) were quantified and plotted against the concentration of the standard gas sample to generate the calibration curve of γ-valprolactone. According to Figure 6b, it was found that the calibration equation was y=7512.3x + 1594.8. The linearity was satisfactory with coefficient of determination R^2=0.9947. A linear range was obtained from 0.7 to 22.5 ng/L. Accordingly, the limit of detection (LOD) of γ-valprolactone in exhaled breath was determined to be 0.18 ng/L, and the limit of quantitation (LOQ) was 0.59 ng/L. The therapeutic range reported for total VPA in human plasma is 50–100 μg/mL, with the corresponding mean free concentration ranges between 1.2 and 6.1 μg/mL [28]. In the exhaled breath pharmacokinetic studies by EESI [13], the results showed linear relationship between of the signal of *m/z* 143 and the free VPA fraction in blood. The authors also observed that the signal intensity at *m/z* 143 in exhaled breath can vary within a day for a person maintaining the free VPA fraction blood concentration within the therapeutic range (the peak intensity being up to three times higher than the minimum intensity). This indicates that the LOD and LOQ of γ-valprolactone in exhaled breath by LTP-MS are well suited for monitoring VPA.

Based on the calibration curve, the content of γ-valprolactone in the exhaled gas of the volunteer under VPA treatment (after 14 h ingestion of VPA) was determined to be equivalent to 4.46 (±0.84) ng/L. Compared with the result based on the standard addition method, the discrepancy was as low as 7.7%. Thus, the calibration curve protocol is validated. Furthermore, it is also shown that it is possible to study the LTP-MS figures of merit for analysis of exhaled breath only by using exhaled breath from healthy individuals mixed with the standard gaseous analytes of interest, without having access to patients. This is important because access to patients can be limiting.

Conclusions

In this study, a novel platform based on LTP mass spectrometry was constructed for real-time quantitative analysis of valproic acid in exhaled breath, using a homemade LTP source coupled to a commercially available linear ion trap mass spectrometer. The effect of working conditions and geometry of the LTP source on the ion signal of interest was systematically characterized by using the exhaled breath from individuals with VPA intake. The optimized conditions were: applied voltage, 3.0 kV; frequency, 26 kHz; helium flow rate, 0.20–0.40 SLPM; breath flow rate range between 2.0 and 2.36 SLPM; the LTP source was positioned coaxial with MS capillary; inter-electrode distance, 4 mm; distance between breath tubing and MS inlet was 5 mm, angle between breath tubing and LTP was 75°, distance between LTP and MS was 0.63 mm. Quantitative analysis of γ-valprolactone in exhaled breath was achieved for the first time. Under the optimal conditions, the limit of detection was found to be 0.18 ng/L. Agreement between quantitation with a calibration curve and a standard addition curve show the possibility of determining the figures of merit for breath analytes without having access to patient breath. LTP-MS has proven to be a fast, noninvasive, and quantitative technique for exhaled breath analysis. Current and future work is aimed at extending the capabilities to monitor other exhaled metabolites. This technology has great potential for clinical diagnostics and drug monitoring.

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