Jack Zheng[1]
Michael W. Jann[2]
Yuen Yi Hon[3]
Shahab A. Shamsi[1]

[1]Department of Chemistry, Center of Biotechnology and Drug Design, Georgia State University, Atlanta, GA, USA
[2]Department of Clinical and Administrative Science, Mercer University, Southern School of Pharmacy, Atlanta, GA, USA
[3]Clinical Pharmacokinetics, Research Laboratory, Clinical Center Pharmacy Department, National Institutes of Health, Bethesda, MA, USA

Development of capillary zone electrophoresis-electrospray ionization-mass spectrometry for the determination of lamotrigine in human plasma

A method of coupling capillary zone electrophoresis (CZE) with electrospray ionization-mass spectrometry (ESI-MS) detection has been developed for monitoring an antiepileptic drug, lamotrigine (LTG) in human plasma. The CZE-MS was developed in three stages: (i) CZE separation and ESI-MS detection of LTG and tyramine (TRM, internal standard) were simultaneously optimized by studying the influence of CZE background electrolyte (BGE) pH, BGE ionic strength, and nebulizer pressure of the MS sprayer; (ii) sheath liquid parameters, such as pH, ionic strength, organic modifier content, and flow rate of the sheath liquid, were systematically varied under optimum CZE-MS conditions developed in the first stage; (iii) MS sprayer chamber parameters (drying gas temperature and drying gas flow rate) were varied for the best MS detection of LTG. The developed assay was finally applied for the determination of LTG in plasma samples. The linear range of LTG in plasma sample assay was between 0.1–5.0 μg/mL with a limit of detection as low as 0.05 μg/mL and run time less than 6 min. Finally, the concentration-time profile of LTG in human plasma sample was found to correlate well when CZE-ESI-MS was compared to a more established method of high-performance liquid chromatography with ultraviolet detection.

Keywords: Capillary zone electrophoresis / Electrospray ionization-mass spectrometry / Lamotrigine / Therapeutic drug monitoring DOI 10.1002/elps.200305823

1 Introduction

Lamotrigine (LTG, [3,5-diamino-6(2,3-dichlorophenyl)-1,2,4-triazine]) is one of the recently developed antiepileptic drugs [1]. This drug has a therapeutic range (steady-state through plasma LTG concentration) of 1–4 mg/L LTG and a mean elimination half-life of 25 h [2, 3]. However, due to common interaction with the other drugs, the mean elimination half-life of LTG could vary from 15 h to 59 h [4]. In order to maintain the therapeutic plasma concentrations and to avoid toxicity of LTG, therapeutic drug monitoring has been studied for LTG-treated patients [5]. Therefore, it is important to develop a reliable analytical method for LTG assay.

Several immunological methods, including radioimmunoassay [6] and immunofluorimetic assay [7], have been reported for the determination of LTG in human plasma. However, because lack of reliable commercialized procedure, these methods are not commonly used. A number of high-performance liquid chromatography (HPLC) [5, 8–15] and capillary zone electrophoresis (CZE) [16, 17] methods, both with ultraviolet (UV) detection have been reported for measurement of LTG in human plasma. Although HPLC-UV method is more popular than CZE-UV method for LTG assay, the drawbacks, such as poor efficiency, large-volume sample consumption, and tedious sample preparation of the former method, make it problematic. Based on electrophoretic separation mechanism, with migration time as the basic for identification, the CZE-UV method was developed by Shihabi and Oles [16] and recently validated by Theurillat *et al.* [17]. The CZE-UV method appears to be rugged and robust [16, 17]. However, it shows lack of sensitivity and specificity due to the on-column UV detection, along with the interference from the matrix which hinder the application of CZE-UV method [16, 17]. In addition, for the analysis of plasma samples, reproducible migration times are somewhat problematic due to irreversible adsorption on the capillary surface. Thus, there is a growing interest for developing a replacement method with high sensitivity, high selectivity, high specificity, and high throughput for LTG assay. The CZE coupled with electrospray mass spectrometric detection (ESI-MS) seems to have a potential to serve as an alternative method applied for LTG assay. This is because CZE-ESI-MS combines high sen-

Correspondence: Professor Shahab A. Shamsi, Department of Chemistry, Center of Biotechnology and Drug Design, Georgia State University, Atlanta, GA 30303, USA
E-mail: chesas@panther.gsu.edu
Fax: +404-651-2751

Abbreviations: LTG, lamotrigine; **TRM**, tyramine

sitivity, high specificity, and high selectivity provided by ESI-MS with high efficiency, high resolution, and high throughput of CZE. Thus, CZE-ESI-MS could be proved as one of promising technique in drug and pharmaceutical analysis [18–27]. In this study, the feasibility of CZE-ESI-MS method was investigated for the LTG assay. To our knowledge, this is the first report in which CZE-ESI-MS was developed and applied for LTG assay.

2 Materials and methods

2.1 Standards and chemicals

LTG was a kind gift of GlaxoSmithKline (Philadelphia, PA, USA). Ammonium acetate (as 7.5 M NH_4OAc solution), thiourea (used as electroosmotic flow (EOF) marker) and tyramine hydrochloride (TRM, as internal standard) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) and methanol (MeOH), both HPLC-grade, were purchased from Burdick & Jackson (Muskegon, MI, USA). Ammonium hydroxide ($NH_3 \cdot H_2O$), acetic acid (HOAc), and hydrochloric acid (HCl) were supplied by Fisher Scientific (Springfield, NJ, USA). Water used in all of the experiments was triply deionizied and obtained from Barnstead Nanopure II water system (Barnstead International, Dubuque, IA, USA).

2.2 Preparation of buffer and sample solution

All aqueous buffers were obtained by first adjusting the pH of NH_4OAc buffer to the desired value with HOAc (for pH $<$ 6.8) or $NH_3 \cdot H_2O$ (for pH $>$ 6.8). The pH of the aqueous buffer was checked and adjusted with an Orion 420A pH meter (Beverly, MA, USA). The sheath liquids were prepared by mixing aqueous NH_4OAc buffer (adjusted to the desired pH value prior to mixing) with an appropriate volume ratio of MeOH. The final CZE buffer or sheath liquid was degassed for 30 min and filtered with 0.45 μm PTFE membrane before use. The stock solutions of LTG, TRM, and thiourea were prepared at 1.0 mg/mL by dissolving an appropriate amount of each in 50% v/v MeOH and diluted to desired concentration before use.

2.3 Plasma sample preparation

The sample preparation procedure for plasma was similar to the procedure reported by Shihabi and Oles [16]. The plasma samples were collected over a period of 96 h from a subject after oral administration of 50 mg LTG [15]. Both blank human plasma samples and the subject plasma samples were obtained from Mercer University Southern School of Pharmacy (Atlanta, GA, USA) and stored under −78°C until analysis. In order to set up the calibration curve, each 100.0 μL aliquot of plasma was spiked with the desired volume of LTG solution at levels of 0.1, 0.25, 0.5, 1.0, 2.0, 3.0, and 5.0 μg/mL. To each of the 100 μL aliquot of plasma (spiked blank plasma or plasma from the subject) contained in a 1.5 mL microcentrifuge tube, 3 μL of the internal standard solution (containing 10 μg/mL TRM), and 200 μL of ACN were added. The tube was vortexed for 1 min and then centrifuged at 10k rpm for 10 min. After centrifugation, the supernatant was transferred into a Spin-x microcentrifuge filter cartridge (0.22 μm Nylon filter; Corning, NY, USA) followed by centrifugation of the cartridge at same rpm for another 3 min. The filtrate was combined with 5 μL HOAc and an aliquot of each mixture was used for CZE-MS analysis.

2.4 Instrumentation

The CZE-UV-MS experiments were carried out with an Agilent Capillary Electrophoresis system (including an autosampler and a diode-array detector) interfaced to a quadrupole mass spectrometer, Agilent 1100 series MSD, a G1603A CE-MS adapter kit, and a G1607 CE-ESI-MS sprayer kit, all from Agilent Technologies (Palo Alto, CA, USA). A 120 cm long fused-silica capillary (OD 363 μm, ID 75 μm; Polymicro Technologies, Phoenix, AZ, USA) was used for CZE-UV-MS separation. The UV detection window was fabricated by burning 1–2 mm segment of polyimide coating of the capillary at 30 cm from the inlet side. For CZE coupled with ESI-MS detection only, shorter capillaries (60–65 cm long, OD 363 μm, ID 75 μm) were used since the diode-array detector was bypassed. The capillary outlets for both CZE-UV-MS and CZE-MS have 1–2 mm segment coating removed for exposure to the atmospheric pressure ionization source of the electrospray. An Agilent 1100 series HPLC pump equipped with 1:100 splitter was used to deliver the sheath liquid. The Agilent ChemStation and CE-MS add-on software were used for instrument control and data analysis.

2.5 CZE-MS conditions

After installation of CE column on the cartridge and mounting in the nebulizer, the column was flushed for 40 min with ~ 2 M $NH_3 \cdot H_2O$ followed by flushing for 40 min with CE run buffer. Injection was made by applying 0.435 psi (30 mbar) pressure for 2 s. The separation voltage was set at 20 kV, employing a voltage ramp of 3 kV/s. Between each run, the capillary was flushed with

run buffer for 3 min. Unless otherwise stated, the following ESI-MS conditions were used to obtain optimum simultaneous separation of LTG and TRM in CZE. Sheath liquid, MeOH-H_2O (90:10 v/v) containing 50 mM NH_4OAc, pH 6.8; sheath liquid flow rate, 7.5 μL/min; capillary voltage, 4000 V; fragmentor voltage, 70 V; drying gas flow rate, 5 L/min; drying gas temperature, 150°C; nebulizer pressure, 4 psi. The MS detection was performed in the selective ion monitoring (SIM) mode. Since both LTG and TRM exist as cations in acidic solutions, positive $[M+H]^+$ ions were monitored at 256.0 and 138.0 *m/z*, respectively. The EOF velocity was determined by injecting thiourea sample solution under similar conditions except positive SIM mode was used at 77.0 *m/z.*

2.6 Direct infusion method

The electrospray ionization (ESI) mass spectrum of LTG was obtained by the direct infusion method, which involves continuously flushing 1 mg/mL LTG through a 60 cm long, 75 μm ID open tubular capillary at 0.725 psi (50 mbar) to the ESI interface. Sheath liquid and MS spray chamber parameters were same as described in Section 2.5. Data were collected in positive scan mode over the range of 50–500 *m/z.*

2.7 Calculations

The resolution (R_s) and selectivity (α) between LTG and TRM as well as the separation efficiency (*N*) of analytes were calculated with Agilent Chemstation software (V9.0) as reported in [28]. All electropherograms shown were smoothed with a factor of 0.1 min. The noise level was determined using peak-to-peak noise method for a selected time range between 1 min to 2 min with the Chemstation software. The signal-to-noise ratio (S/N) was obtained by the ratio of LTG peak height over the noise level. The LTG calibration curve was obtained by plotting the peak area ratio of LTG to the internal standard (TRM) *versus* LTG concentration. To assess linearity, the line of best fit was determined by least squares regression.

3 Results and discussion

For LTG assay, TRM was chosen as the internal standard according to the study of Shihabi and Oles [16] who used a background electrolyte (BGE) containing 130 mM sodium acetate-acetic acid at pH 4.8. However, due to the nonvolatility of the sodium acetate, such a BGE is not suitable for the ESI-MS detection. Thus, a BGE containing volatile NH_4OAc-HOAc is used for the CZE-MS study.

3.1 Comparison of CZE-UV detection with CZE-ESI-MS detection

Initially, a 120 cm long CE capillary was employed for simultaneous UV and ESI-MS detection of LTG and internal standard, TRM as well as to compare the limit of detection (LOD) of these two techniques (Fig. 1). The CZE-UV at 214 nm showed a shorter analysis time of ~ 4 min due to shorter effective separation capillary

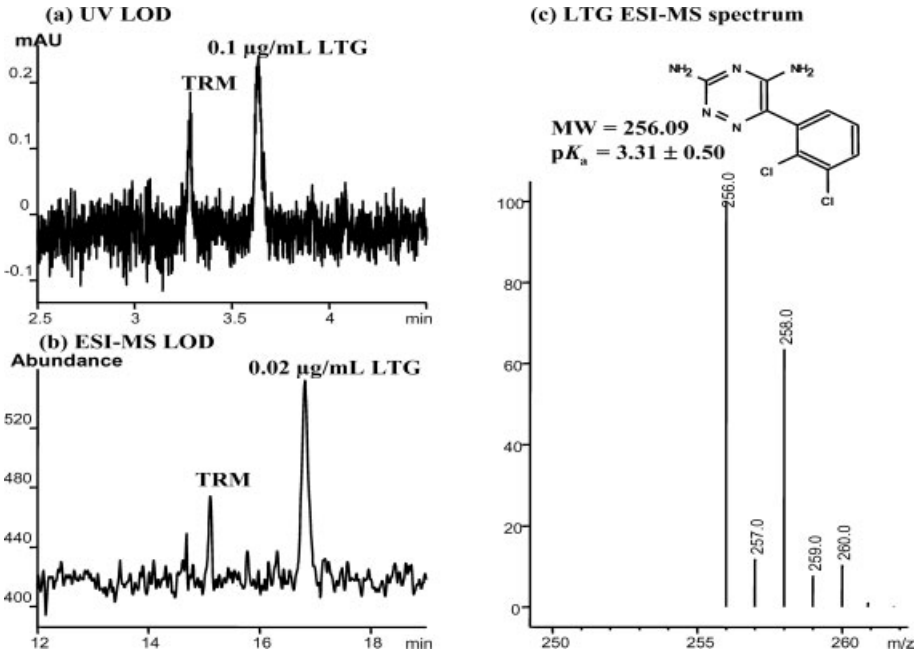

Figure 1. Electropherograms of CE-UV-MS showing the simultaneous separation at the LOD of LTG and TRM at (a) UV LOD (0.1 μg/mL LTG) and (b) ESI-MS LOD (0.02 μg/mL LTG). Conditions: 120 cm long, 75 μm ID open tubular capillary (UV window located 30 cm from the inlet); run buffer, 20 mM NH_4OAc, pH 3.0. For other conditions, see Section 2. (c) ESI-MS spectrum of LTG obtained by direct infusion method.

length (~ 30 cm from the injection end) as compared to the ESI-MS detection (~ 120 cm from the injection end). However, it is important to note that ESI-MS detection showed *ca.* 5 times better sensitivity (~ 0.02 μg/mL, Fig. 1b) as compared to UV detection (~ 0.1 μg/mL, Fig. 1a). In addition, CZE-UV detection suffers due to the matrix interferences. For example, the presence of endogenous compounds in blank plasma or patient samples as well as the appearance of the solvent peaks was a problem with UV detection (data not shown). As discussed in Section 3.5, matrix interference from plasma samples also influences the ionization in ESI-MS, but such interference has much less effects on CZE coupling with ESI-MS since selected ion monitoring mode (SIM, positive ion, *m/z* 256.0, Fig. 1c) used in ESI-MS provides a highly specific and selective detection. Therefore, UV detection was not employed in the following experiments and only ESI-MS detection was carried for the CZE separation. For CZE-MS experiments, the capillary was cut to reduce the length to ~ 60 cm. Consequently, as shown in the following sections, faster separation (with analysis time equivalent to CZE-UV) was achieved with a high sensitivity for ESI-MS.

3.2 Optimization of CZE-ESI-MS separation

To optimize a CZE-MS method, pH and concentration of the volatile BGE as well as nebulizer gas pressure are essential parameters that influence both the CZE separation as well as the MS detection sensitivity. Therefore these parameters are optimized first, followed by optimizing the sheath liquid parameters and the spray chamber parameters (drying gas temperature and drying gas flow rate).

3.2.1 Effect of BGE pH

Because LTG and TRM are weak bases, a series of BGEs containing 20 mM NH_4OAc-HOAc were studied over the range of pH between 4.0 and 6.8. As demonstrated in Figs. 2a–e, TRM has a shorter migration time than LTG

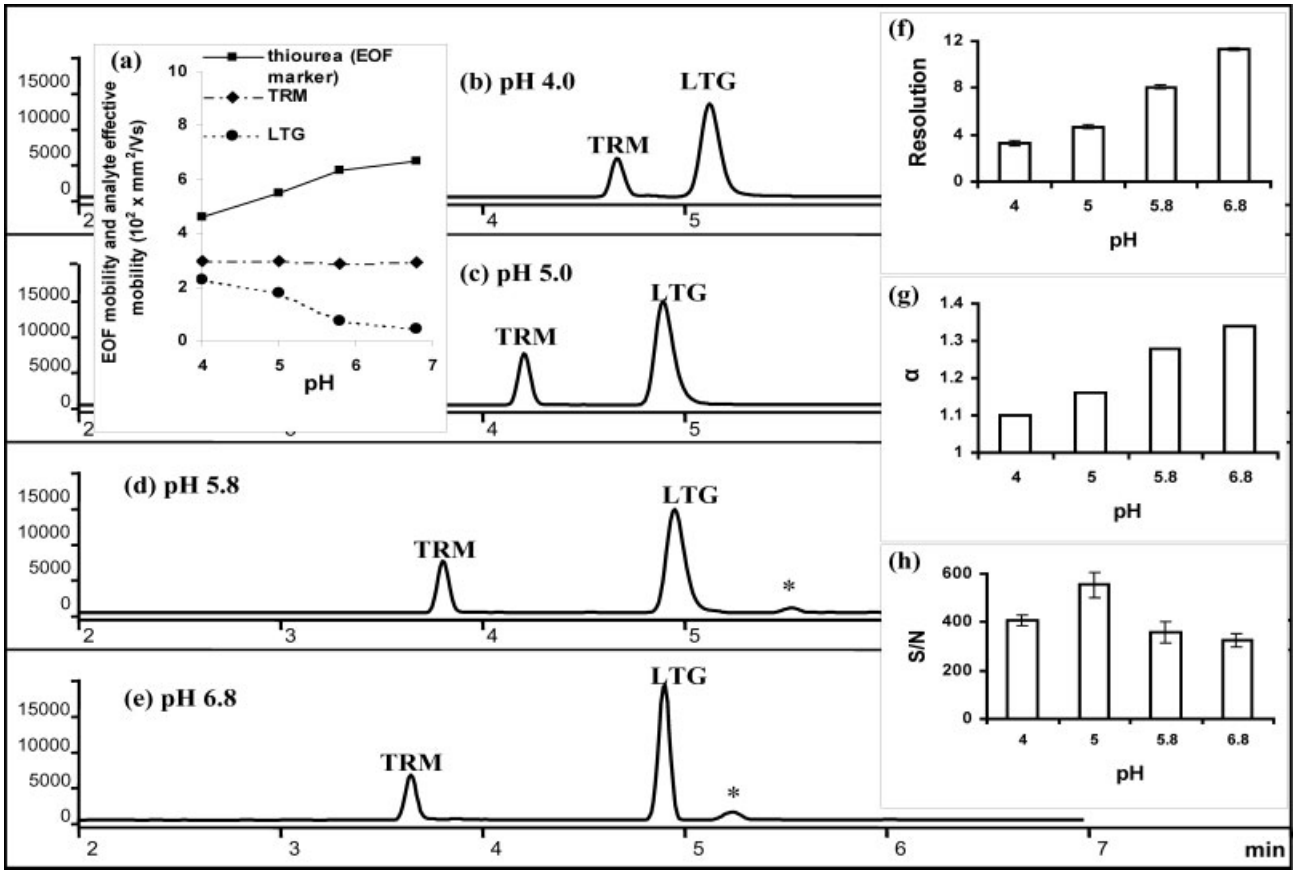

Figure 2. (a) Effects of buffer pH for EOF velocity and effective mobility of TRM and LTG. Electropherograms (b)–(e), bar plots (f)–(h) effects of buffer pH for separation of TRM and LTG. The bar plots (f)–(h) represent (f) resolution, (g) selectivity, and (h) S/N. Conditions: 65 cm long, 75 μm ID open tubular capillary; run buffer, 20 mM NH_4OAc at pH (b) 4.0, (c) 5.0, (d) 6.0, and (e) 6.8. For other CZE-ESI-MS conditions see Section 2.

since its basicity (pK_a = 10.67) is higher than LTG (pK_a = 3.31). Consequently, as the BGE pH increased from 4.0 to 6.8, the effective mobility of these two analytes showed different trends (Fig. 2a). The migration time of TRM showed a significant decrease while the migration time of LTG was more or less the same (Figs. 2b–e). This observation is associated with different electrophoretic mobility (which is dependent on effective positive charge) of these two analytes as well as electroosmotic flow (EOF) at different pH. Thus, TRM, which is predominantly cationic within the investigated pH range, showed increase migration time at lower pH mainly due to lower EOF, even though its effective electrophoretic mobility does not change significantly over the studied pH range (Fig. 2a). In contrast, LTG, which acquires more positive charge when decreasing the BGE pH from 6.8 to 4.0, its effective electrophoretic mobility towards the anodic end (MS detector end) is enhanced. However, at the same time, the decrease of BGE pH also increases the BGE ionic strength (due to the addition of HOAc), which in turn decreases EOF. Therefore, there is no net change in the migration time of LTG due to a balance between electrophoretic mobility of LTG and EOF. As a result of increasing selectivity between these two analytes (Fig. 2g), the resolution (R_s) between these two analytes increases from 3.3 to 11.3 as the BGE pH was increased from 4.0 to 6.8 (Fig. 2f). The BGE pH also influences the detection sensitivity. As shown in Fig. 2h, a BGE at pH 5.0 provides the highest S/N due to lower noise level at this pH. Thus, the BGE containing 20 mM NH_4OAc-HOAc at pH 5.0 was selected for the further study since it provided a reasonable compromise between resolution and detection sensitivity.

3.2.2 Effect of BGE ionic strength

The ionic strength effects were investigated with a series of BGEs containing different concentrations (5.0, 10.0, 20.0, and 30.0 mM) of NH_4OAc-HOAc at pH 5.0. As demonstrated in the electropherograms (Fig. 3a–d), when the BGE concentration was increased from 5 mM

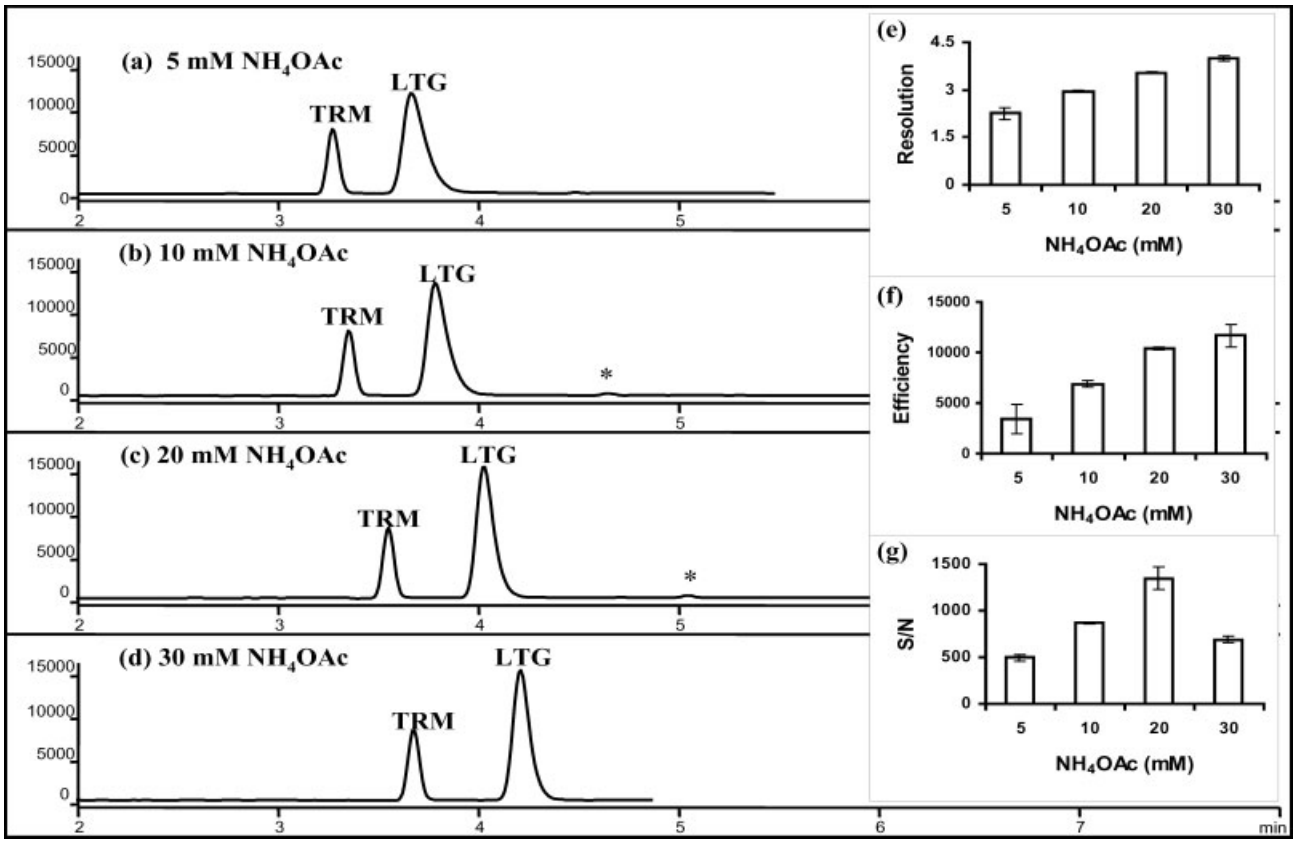

Figure 3. Electropherograms (a)–(d), bar plots (e)–(g) effects of buffer concentration (ionic strength) for the separation of TRM and LTG. The bar plots (e)–(g) represent (e) resolution, (f) efficiency, and (g) S/N. Conditions: 60 cm long, 75 μm ID open tubular capillary; run buffer, (a) 5 mM, (b) 10 mM, (c) 20 mM, and (d) 30 mM NH_4OAc, pH 5.0. For other CZE-ESI-MS conditions, see Section 2.

to 30 mM, the migration time of both analytes decreased, which could be associated with decrease of EOF. Since the double layer is composed of compact and diffuse layer, an increase of ionic strength increases the thickness of compact layer with a concurrent decrease of the diffuse layer thickness. Because the zeta potential is predominantly controlled by the diffuse layer thickness, a decrease in EOF is observed at higher BGE concentration. In addition, several observations in the bar plots (Fig. 3e–g) are noteworthy. As the concentration of NH_4OAc/HOAc increased, the R_s between LTG and TRM increased mainly due to the concomitant increase in efficiency (*N*). For the same reason, the S/N at 20 mM is higher than the other two lower concentrations. However, it should be noted that at the highest concentration, *i.e.*, 30 mM NH_4OAc, the current is close to 50 μA (the upper limit of the CE-MS instrument used in this study). Most probably the Joule heating effect contributes to a much higher noise level at the highest concentration of BGE. Therefore, a 20 mM NH_4OAc-HOAc at pH 5.0 was selected for further optimization.

3.2.3 Effect of nebulizer gas pressure

The nebulizer gas pressure is also a key parameter needed to consider for optimizing the CZE-ESI-MS separation. The nebulizer gas pressure was varied from 2 psi to 10 psi while the other parameters (including BGE pH and concentration) were kept constant. As expected, the migration time of analytes decreased while increasing the nebulizer gas pressure (Figs. 4a–e) because the nebulizer gas pressure has the potential to generate a suction force at the capillary outlet [29]. As a result, a laminar flow is formed inside the capillary, thus the separation efficiency drops and so does the R_s between two analytes

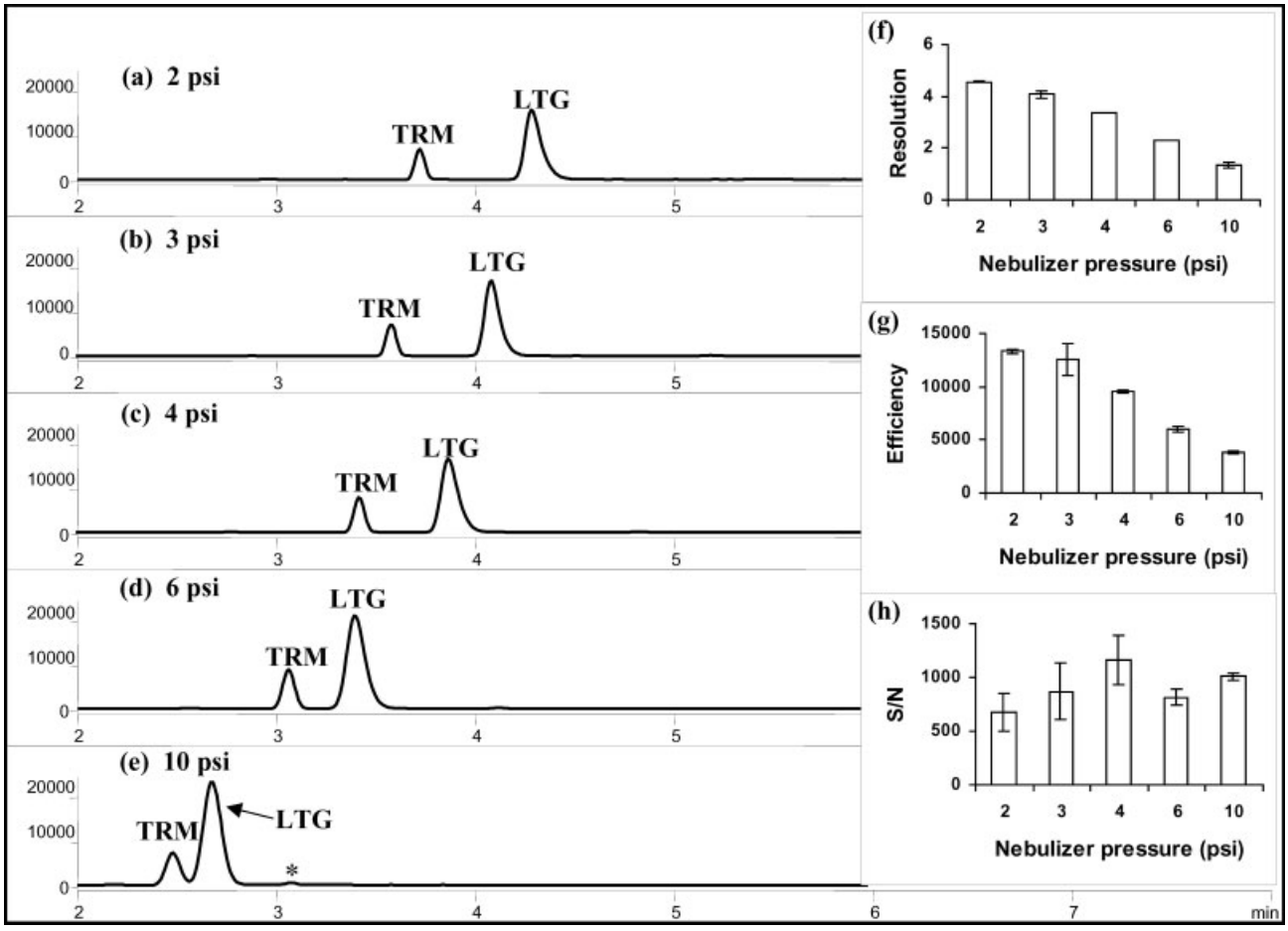

Figure 4. Electropherograms (a)–(e), bar plots (f)–(h) effects of nebulizer gas pressure for the separation of TRM and LTG. The bar plots (f)–(h) reprsent (f) resolution, (g) efficiency, and (h) S/N. Conditions: 60 cm long, 75 μm ID open tubular capillary; run buffer, 20 mM NH_4OAc, pH 5.0; nebulizer gas pressure at (a) 2 psi, (b) 3 psi, (c) 4 psi, (d) 6 psi, and (e) 10 psi. For other CZE-ESI-MS conditions see Section 2.

(Figs. 4f, g). It was also observed that the nebulizer gas pressure not only affects the CZE separation, but also the detection sensitivity. As the nebulizer gas pressure increased from 4 psi to 10 psi, the abundance of LTG improved by about 20%, but the S/N ratio at 10 psi was 20% lower than at 4 psi due to higher noise level (Fig. 4h). This indicates that high nebulizer gas pressure could influence the stability of the electrospray [25]. As a compromise for analysis time, resolution and S/N, the nebulizer gas pressure was set to a moderate value of 4 psi.

3.3 Optimization of the sheath liquid parameters

In a normally designed CE-ESI-MS system, the flow rate through the CE column (10–100 nL/min) is too low for supporting a stable electrospray (typically a few μL/min flow rate required for the electrosprayer used in this study). Hence, the sheath liquid is introduced at post-column as the make-up liquid to stabilize the electrospray. In addition, similar to CEC-ESI-MS [30], the sheath liquid for CZE-ESI-MS not only serves to establish an electrical connection between the outlet end of the CZE column and electrosprayer, but also serves as a terminal pH and electrolytic reservoir. Therefore, it is important to optimize the sheath liquid parameters for achieving high ESI-MS sensitivity. In this section, the sheath liquid ionic strength was studied first, followed by organic modifier composition, pH and flow rate of the sheath liquid. At the first stage, the impact of these parameters on the separation was investigated by varying these parameters but kept the other separation parameters constant. As expected, none of sheath liquid parameters have significant effects on the separations (data not shown). Their effects on the detection sensitivity are discussed as follows.

3.3.1 Effect of the sheath liquid ionic strength

The effect of sheath liquid ionic strength was investigated by increasing the sheath liquid NH_4OAc concentration from 5 mM to 50 mM but keeping other conditions constant. As shown in Fig. 5a, the lowest NH_4OAc concentration (*i.e.*, 5 mM) in the sheath liquid gives the highest abundance probably due to higher ion transmission efficiency [31]. Although the magnitude of error bars suggests that the S/N value is not significantly different at different concentration of NH_4OAc in sheath liquid (Fig. 5b), the 50 mM NH_4OAc buffer was chosen as the optimum since higher NH_4OAc concentration stabilizes the electrospray which leads to a relatively lower noise level [31].

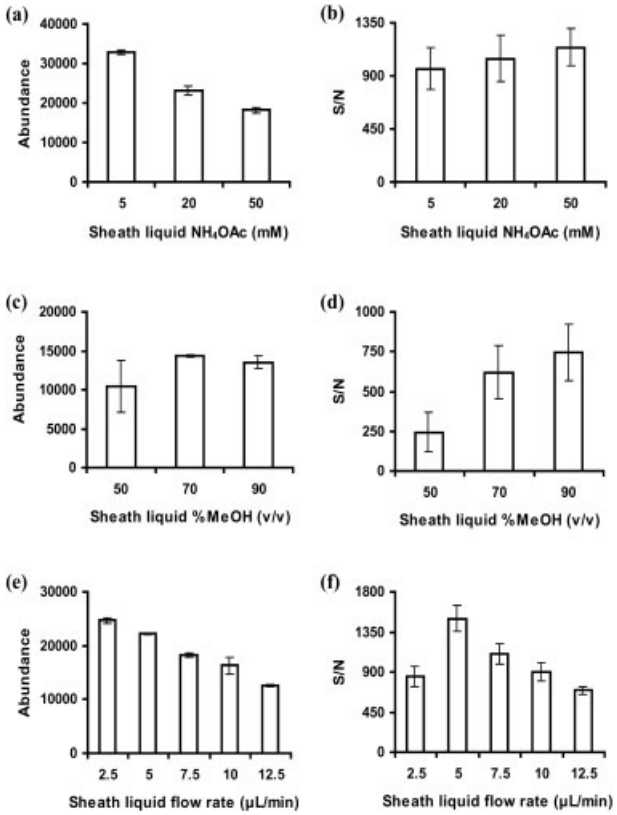

Figure 5. Effects of sheath liquid (a), (b) NH_4OAc concentration, (c), (d) MeOH content, and (e), (f) sheath liquid flow rate upon abundance and S/N for LTG. The error bar in each plot represents one standard deviation (SD) of three measurements. Conditions: nebulizer gas pressure, 4 psi; sheath liquid in (a), (b) containing various concentration of NH_4OAc in 90% v/v MeOH; flow rate, 7.5 μL/min. Sheath liquid in (c), (d): 50 mM NH_4OAc in various % v/v MeOH; flow rate, 7.5 μL/min. Sheath liquid in (e), (f): 50 mM NH_4OAc in 90% v/v MeOH; flow rate, 2.5–10.0 μL/min. Other conditions as in Fig. 4 or described in Section 2.

3.3.2 Effect of the sheath liquid pH

The effect of sheath liquid pH was studied by gradually increasing the sheath liquid pH from 4.0 to 8.5. However, no significant effects were found by varying the sheath liquid pH in this range (data not shown). This is despite of the fact that in the aqueous solution, the pK_a value of LTG is around 3.3. One possible interpretation of this result supports the hypothesis that the solution-phase basicity (*e.g.*, in aqueous solution) is not related to the gas-phase proton affinities (*e.g.*, in the electrospray ionization source) [31]. A sheath liquid pH 6.8 was chosen because no acid or base is required to adjust the pH value of the NH_4OAc (pH 6.8 being the natural pH of NH_4OAc).

3.3.3 Effect of the sheath liquid organic modifier composition

The effect of sheath liquid organic modifier composition was studied by varying the volume fraction of MeOH from 50–90% v/v. Again, a number of trends are noteworthy. First, the use of 50% v/v MeOH in the sheath liquid provides ~ 40% less response as compared to 70% or 90% v/v MeOH. This could be explained by the fact that the solution containing a large content of MeOH decreases the droplet size which in turn results in higher efficiency for desolvation [31]. Unlike the abundance, the S/N keeps increasing as the MeOH composition increases from 50% v/v to 90% v/v (Fig. 5d). The S/N increase could be due to low liquid surface tension that stabilizes the electrospray at such high content of MeOH, thereby causes a decrease in the noise level. Therefore, a sheath liquid containing 90% v/v MeOH was chosen for the further study.

3.3.4 Effect of the sheath liquid flow rate

The sheath liquid flow rate was varied from 2.5 μL/min to 12.5 μL/min. It was observed that the LTG response was decreased while the flow rate increased due to the dilution effect (Fig. 5e). In contrast to the abundance, a maximum S/N was found at 5 μL/min (Fig. 5f). At the lower end of the sheath liquid flow rate (*i.e.*, 2.5 μL/min) high noise was observed since this low flow rate is unable to support a stable electrospray, although this flow rate provided the highest abundance. As a result, 5 μL/min was selected as the optimum sheath liquid flow rate.

3.4 Optimization of the MS spray chamber parameters

Because the effect of nebulizer gas pressure has been investigated in Section 3.2.3, we mainly focused on the drying gas flow rate and drying gas temperature. As expected, these two parameters were found to have no significant impact on the CZE separation. On the other hand, their influences on the MS detection sensitivity were noticed.

3.4.1 Effect of drying gas flow rate

The effect of drying gas flow rate was studied by varying the flow rate from 2.5 to 10 L/min under optimum CZE separation and sheath liquid conditions. The abundance of LTG was more or less the same, which indicated that the LTG signal intensity could be independent of the drying gas flow rate. Unlike the abundance, S/N of LTG showed the highest response at 5 L/min due to low noise level at this flow rate. Consistent with the results obtained by Huikko *et al.* [32], our data suggest that drying gas flow rate could influence the stability of electrospray, which in term affects the noise level. Thus, a drying gas flow rate at 5 L/min was chosen for the further study.

3.4.2 Effect of drying gas temperature

The effect of drying gas temperature was studied by varying the temperature from 150 to 350°C but keeping other conditions constant. As shown in Fig. 6c, the LTG abundance showed only a slightly increase upon increasing the drying gas temperature from 150 to 350°C. This is due to higher efficiency for desolvation at higher drying gas temperature. The S/N of LTG was slightly higher at 200 and 250°C compared to lower (150°C) or higher (300 and 350°C) drying gas temperature (Fig. 6d). This is because higher noise was experienced at lower or higher drying gas temperature. It should be noted that drying gas temperature at 200°C was chosen as the optimum since the magnitude of the error bar was significantly less at 200°C than 250°C (Fig. 6d).

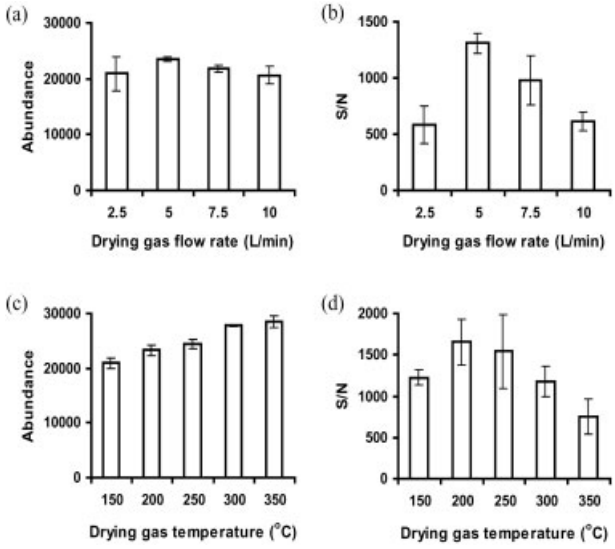

Figure 6. Effects of (a), (b) drying gas flow rate and (c), (d) drying gas temperature upon abundance and S/N of LTG. The error bar in each plot represents one standard deviation (SD) of three measurements. Conditions: nebulizer gas pressure, 4 psi; sheath liquid, 50 mM NH_4OAc in 90% v/v MeOH; flow rate, 5.0 μL/min. (a), (b) Drying gas flow rate, 2.5–10.0 L/min; constant drying gas temperature, 150°C. (c), (d) Drying gas temperature, 150°C–350°C; constant drying gas flow rate, 5.0 L/min. Other conditions as in Fig. 4 or described in Section 2.

3.5 LTG assay for human plasma samples

Human plasma is a complex matrix containing various amounts of protein and salt. Therefore, it is necessary to employ a deproteinization process before injecting the plasma samples into the capillary for CZE-ESI-MS analysis. We chose a protein precipitation process by mixing plasma sample with ACN [16] followed by an ultrafiltration step (for removal of large molecular weight protein and sub-μm particles). Furthermore, to obtain good peak shapes, a small amount of HOAc (5 μL) was added in the deproteinized sample before injection.

In order to set up the calibration curve, blank plasma was spiked with desired volume of LTG solution at levels of 0.1, 0.25, 0.5, 1.0, 2.0, 3.0, and 5.0 μg/mL, and TRM solution at 3 μg/mL. After aforementioned sample preparation and the CZE-ESI-MS analysis, the peak area ratios of LTG to TRM were plotted *versus* the LTG concentration. The calibration curve (Fig. 7a inset) shows good linearity within the studied concentration range (R^2 = 0.9996). The LOD of LTG in plasma sample was found to be as low as

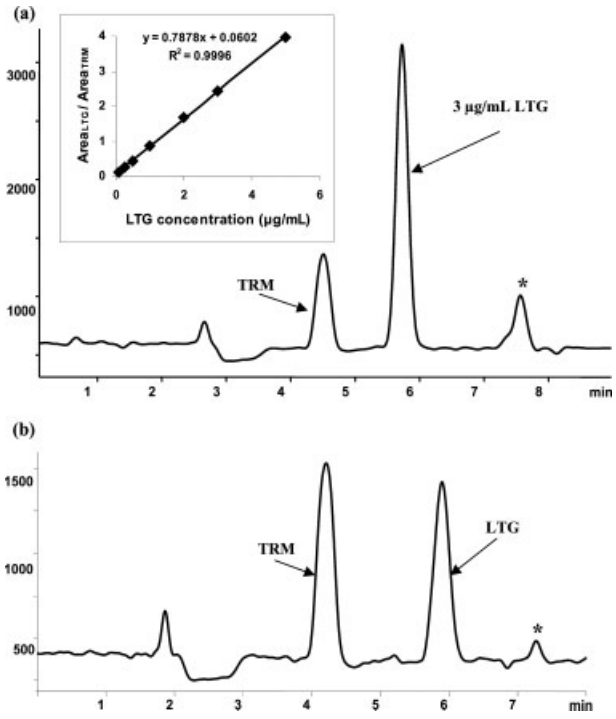

Figure 7. (a) Blank human plasma spiked with 3 μg/mL LTG and TRM. The inset shows the calibration curve of LTG using TRM as the internal standard. (b) Subject plasma sample (collected at 3 h after oral administration of 50 mg LTG). Conditions: 63 cm long, 75 μm ID open tubular capillary; run buffer, 20 mM NH_4OAc, pH 5.0, 30 kV; sheath liquid, 50 mM NH_4OAc in 90% v/v MeOH; flow rate, 5.0 μL/min; nebulizer gas pressure, 4 psi; drying gas flow rate, 5.0 L/min; drying gas temperature, 200°C. Other conditions are described in Section 2.

0.05 μg/mL (data not shown) which was slightly poorer than LOD observed in pure standard solutions (Fig. 1b). It should be noted that the electropherograms of plasma sample spiked with LTG and TRM (Fig. 7a) show slightly longer migration times of LTG and TRM compared to the electropherograms obtained using pure standards of the same two analytes (Figs. 2–4). This could be attributed to the unavoidable modification of the CZE capillary wall when analyzing the plasma samples. In addition, an extra peak marked with the asterisk (*) in Fig. 7a was observed after LTG peak. This peak was eluted after both cationic TRM and LTG, and it was observed in both pure standard (peak marked with the asterisk, see Figs. 2–4) and blank plasma as well as the subject plasma sample (Fig. 7b). Because the appearance of this peak is even independent to the *m/z* setting for SIM (data not shown), we hypothesize that this peak is presumably associated to result from solvent (*e.g.*, ACN) displacement. Hence, further study is required to evaluate the potential of using this peak as the EOF marker in CZE-MS.

The 14 human plasma samples were collected from a subject over a period of 96 h after oral administration of 50 mg LTG. By applying the developed CZE-ESI-MS method, these samples were analyzed. As demonstrated in Fig. 7b, the electropherogram of subject plasma showed high specificity for LTG and TRM (the peak marked with an asterisk was also present). The concentrations of LTG determined by using both CZE-ESI-MS and HPLC-UV method are compared in Fig. 8b. Despite the fact that 24-h and 96-h samples for the two methods are different, it is clear that the two methods showed a reasonable correlation of the concentration profile of LTG. The differences in LTG concentration obtained by the two methods suggest a detailed validation process of the newly developed CZE-ESI-MS is warranted.

Although these two methods have similar detection limits (Jann *et al.*, submitted), in terms of sample throughout, the CZE-ESI-MS method demonstrates significant advantages over the HPLC-UV method. For example, 15 min of sample preparation and *ca.* 6 min of separation/sample were needed in CZE-ESI-MS and due to processing the samples in batches (24 samples/batch), the average time required for each sample preparation can be reduced to less than 1 min/sample. In contrast, 40 min of sample preparation and 15 min of separation/sample make the HPLC-UV method very time-consuming. The longer sample preparation time in HPLC-UV is mainly due to tedious liquid-liquid extraction followed by additional steps that involve evaporation to dryness and reconstitution of the dry residue in a suitable solvent. Thus, overall faster sample throughput with better separation efficiency and higher specificity were achieved with CZE-ESI-MS.

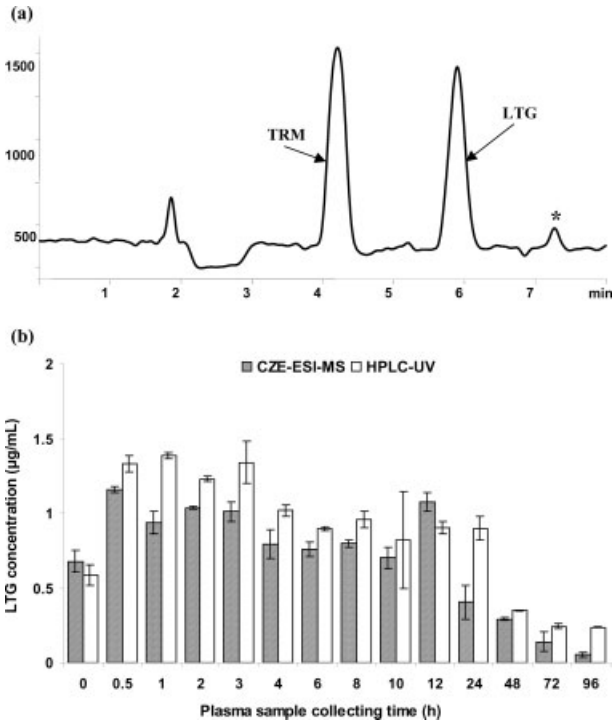

Figure 8. Variation of LTG concentration as a function of time in a series of plasma samples (collected from the subject over a period of 96 h after oral administration of 50 mg LTG) using CZE-ESI-MS and HPLC-UV methods [15]. The CZE-ESI-MS conditions are as in Fig. 7.

4 Concluding remarks

In this study, CZE separation coupled with ESI-MS detection was developed and applied for LTG assay. Several conclusions are noteworthy. (i) The BGE pH and concentration of NH_4OAc as well as nebulizer gas pressure were found to be the key parameters that influence both the CZE separation and the MS detection sensitivity. The BGE pH influenced migration time of LTG along with the resolution between LTG and internal standard, TRM by affecting the electrophoretic mobility and EOF. The NH_4OAc concentration in the BGE affected the resolution and migration time through affecting the EOF velocity and separation efficiency. The nebulizer gas pressure influenced the resolution and migration time as well as the separation efficiency by generating a suction force at the outlet of the CE capillary. In addition, it was revealed that nebulizer gas pressure influences the stability of the electrospray, in turns of the detection sensitivity. (ii) The sheath liquid parameters (ionic strength, MeOH content, and flow rate) had no impact on the separation, but mainly influence the detection sensitivity for LTG. However, the sheath liquid pH shows no impact on the detection sensitivity for LTG despite of the fact that in the aqueous solution, the pK_a value of LTG is $\sim$ 3.3. (iii) As observed for the sheath liquid parameters, the electrospray chamber parameters (drying gas flow and drying gas temperature) mainly influence the detection sensitivity for LTG. The drying gas flow rate influences the stability of electrospray, which in term affects the noise level; the drying gas temperature affects the abundance of LTG by influencing the desolvation efficiency.

Under the optimum conditions (*ca.* 60 cm long, 75 μm ID capillary; BGE containing 20 mM NH_4OAc-HOAc, pH 5.0; injection, 0.435 psi (30 mbar) for 2 s; 90% v/v CH_3OH sheath liquid containing 50 mM NH_4OAc (pH 6.8), flow rate 5 μL/min; drying gas flow rate, 5 L/min; drying gas temperature, 200°C; nebulizer pressure, 4 psi; capillary voltage, 4000 V; fragmentor voltage, 70 V; SIM, positive ion mode at 256.0 and 138.0 *m/z*), the CZE-ESI-MS method was applied to assay blank plasma samples spiked with LTG. Good linearity of calibration curve and limit of detection (0.05 μg/mL LTG) were achieved. Finally, the developed method was applied to analysis of the plasma samples collected from an LTG-treated subject. The LTG concentrations measured by the CZE-ESI-MS method showed reasonable correlation with the results determined by the HPLC-UV method [15]. In addition, the CZE-ESI-MS demonstrated higher chromatographic efficiency, higher specificity, and fast throughout over the HPLC-UV method. The extensive validation of this CZE-ESI-MS method is currently under investigation in our laboratory.

Financial support for this project was provided by the National Institutes of Health (Grant No. GM 62314-02). The authors would like to thank GlaxoSmithKline (Philadelphia, PA, USA) for donating the LTG standard.

Received October 16, 2003

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