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Fast chiral chromatographic method development and validation for the quantitation of eszopiclone in human plasma using LC/MS/MS

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ABSTRACT

Traditional chiral chromatographic separation method development is time consuming even for an experienced chromatographer. This paper describes the application of computer software ACD Lab® to facilitate the development of chiral separation for the quantitation of eszopiclone using LC–MS/MS technology. Assisted by ACD/Chrom Manager and LC Simulator software, the optimal chiral chromatographic development was completed within hours. The baseline chiral separation was achieved with a total cycle time of 3 min. For sample extraction method development, a Waters Oasis® Sorbent Selection Plate containing four different sorbents was utilized. Optimal conditions were determined using a single plate under various load, wash and elution conditions. This was followed by a GLP validation which demonstrated excellent intra- and inter-day accuracy and precision for the quantitation of eszopiclone in human plasma at 1.00–100 ng/mL range using LC/MS/MS technology. This method was utilized to support multiple clinic bioequivalence studies.

1. Introduction

Since its approval for the treatment of insomnia in 1988, zopiclone has been sold in 85 countries. Zopiclone has two enantiomeric isomers, which are metabolized at different rates [1]. Eszopiclone (S-zopiclone, (+)-(5S)-6-(chloropyridin-2-yl)-7-oxo-6,7-dihydro-5H-pyrrolo [3,4-b] pyrazin-5yl 4-methylpiperazine-1-caroxylate) is much more pharmacologically active than the R-zopiclone isomer (Fig. 1). In 2005, eszopiclone was approved by the FDA for the treatment of both transient and chronic insomnia [2–3].

During the last two decades, several papers have been published for the quantitation of racemic zopiclone in biological matrices using HPLC–UV [4–6], GC [7], GC–MS [8], and LC/MS/MS techniques [9–12]. However, only four papers were published for the separation or quantitation of the enantiomers of zopiclone in biological matrices. In 1994, Foster et al. reported a normal phase chiral HPLC methodology to quantify enantiomers of zopiclone using chlordiazepoxide as internal standard [13]. Because the chemical structure of chlordiazepoxide is considerably different from that of zopiclone, this resulted in much earlier elution time than zopiclone. In 1995, Piperaki et al. published a research work to elucidate solvent selectivity in chiral chromatography using eight generic drugs and zopiclone is one of model compounds [14]. A complete baseline separation between R- and S-zopiclone was achieved at retention time of 27.0 and 29.0 min using methanol as strong solvent and a cyclobond I, 250 mm × 4.6 mm column. In 2002, Gebauer et al. reported a modification of the Foster method using a much more closely related structural analogue as the internal standard [15]. The total HPLC cycle time of this method is 35 min with 4-mL aliquot of the plasma sample in order to achieve a 1.00 ng/mL lower level of quantitation (LLOQ). In 2004, Blaschke et al. reported a preparative and analytical separation of zopiclone enantiomers [16]. It also required large sample volumes and long HPLC cycle times.

There were various sample extraction methods utilized in the early publications for the purification of zopiclone or eszopiclone from biological samples such as blood, serum, plasma or urine. Liquid–liquid extraction using various organic solvents under basic condition was reported in most publications [4,5,9,11–13,15]. Solid-phase extraction was utilized in two publications [7,10]. All of them were tedious manual extraction and required large sample volume (up to 4 mL).

Although eszopiclone is an enantiomer and directly administered to patient, in order to monitor potential *in vivo* racemization, a chiral LC–MS/MS assay was requested per FDA. The goal of the present experiments was to develop a selective and specific chiral LC/MS–MS assay to quantify eszopiclone in order to support GLP clinical studies. Several important issues were identified prior to the initiation of the actual laboratory work. To meet the study time-

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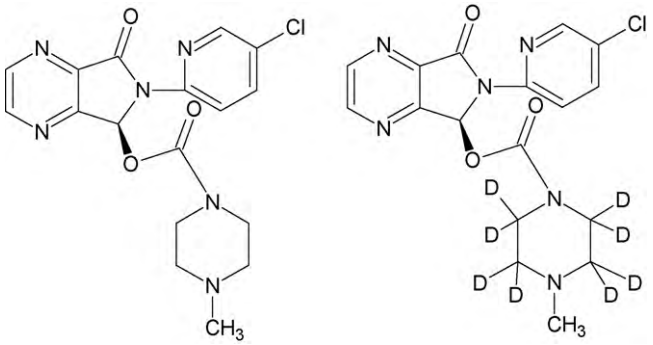

Fig. 1. Chemical structure of eszopiclone and eszopiclone-D8, I.S.

lines, the method development had to be conducted efficiently. To maintain high throughput, the HPLC cycle time had to be shorter than 5 min. Because this method was to be utilized to support large GLP sample studies, it had to be selective, specific and reproducible.

To meet these criteria, we decided to use computer software to accelerate chiral HPLC development. Computer software assisted HPLC development has been reported since the late 1970s. Currently, a number of computer software programs are commercially available and each year new papers using these are published [17–20]. Among them, the most popular software programs are DryLab (LC Resources, USA) and ChromSword (Merck KGaA, Germany). ACD Lab software (Advanced Chemistry Development, Canada) was introduced to the industry during last decade. In comparison, DryLab software assisted separations are optimized by simulating new runs based on two or more experimental runs. ChromSword and ACD Lab software can predict retention data based on one initial experimental chromatographic condition and molecular structural data [21]. For this study, ACD Lab software was utilized to facilitate the chiral HPLC method development.

Historically, the method development for biological sample clean-up prior to bioanalysis is time consuming and requires significant experience. The typical approach is a linear trial and error process starting from simple extractions such as protein precipitation, liquid–liquid extraction and move to more complicated procedures such as solid-phase extraction. Even after solid-phase extraction is selected, several days may be required to explore different retention mechanisms, i.e., reverse-phase mode, ion exchange mode, or reverse-ion exchange mix mode. This process began to change when manufacturers started to introduce SPE method development plates containing multiple bed masses in one single 96-well plate. In this study, we chose the Waters Oasis Sorbent Selection Plate which comprises of MAX, MCX, WAX and WCX providing four different retention mechanisms for plasma sample clean-up method development. In our experience, the generic wash and elution conditions provided by the manufacturer are practical and work well for most applications. It ensures the highest recovery using the weakest wash solvent and the strongest elution solvent. However, it cannot remove all endogenous materials adequately, in particular, phospholipids. In 2003, Bennett et al. first reported the identification of the phospholipids as the major contributor to ion suppression when using electrospray ionization in positive ion mode [22]. Although these matrix effects can be avoided by manipulating chromatography, the preferable approach is to remove them prior to injection and produce the cleanest possible extracts. Particularly for SPE extraction, the cleanest extract can be obtained using the strongest wash solvent and weakest elution solvent. This approach was successfully demonstrated in our previous publications and presentations and utilized in this study as well [23–24].

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (MeCN), methanol (MeOH), isopropyl alcohol (IPA) and N,N-dimethylformamide (DMF) were HPLC grade along with formic acid, 98%, ACS grade and purchased from EMD Chemicals (Gibbstown, NJ, USA). Ammonium acetate, HPLC reagent grade, ≥98% was purchased from Sigma–Aldrich (St. Louis, MO, USA). Eszopiclone [(S)-(+)-zopiclone] (>99.9% chemical purity) was purchased from SynFine Inc. (Richmond Hill, ON, Canada). Deuterium-labeled eszopiclone used as an internal standard, eszopiclone-D8 (Fig. 1) >98% chemical purity, >99% isotopic purity) was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Deionized water used for reagent preparation was Type I, typically 18.2 MΩ cm, generated in house by a Milli-Q water system from Millipore (Billerica, MA, USA). Human plasma (K_3EDTA anticoagulant) was purchased from BioReclamation (Hicksville, NY, USA).

2.2. Standard and quality control (QC) solution preparation

Two stock solutions of eszopiclone were prepared by dissolving the reference material in DMF to yield approximate concentration at 0.500 mg/mL and stored at 4 °C. After precision check, one solution, designated as standard stock, was used for standard calibrator preparation. Another solution, designed as quality control (QC) solution, was used for QC preparation.

Standard working spiking solution was subsequently diluted in DMF from standard stock solution to obtain eight spiking solution at 20.0, 40.0, 100, 200, 500, 1000, 1700 and 2000 ng/mL. These solutions were used for daily preparation of standard calibrators in human plasma at concentration of 1.00, 2.00, 5.00, 10.0, 25.0, 50.0, 85.0 and 100 ng/mL. The final percentage of plasma in standards is 95%.

QC working spiking solution was subsequently diluted in DMF from QC stock solution to obtain five spiking solution at 20.0, 60.0, 800, 1600, and 8000 ng/mL. Dilutions were performed to prepare LLOQ, low, medium, high and dilutional QC in human plasma at concentration of 1.00, 3.00, 40.0, 80.0 and 400 ng/mL. The final percentage of plasma in QC is 95%. The QC pools were aliquoted immediately and stored at −20 °C along with study samples.

2.3. Sample preparation

An aliquot of 50.0 μL of the plasma sample (standard, quality control sample, or subject sample) was spiked with 250 μL of working Internal Standard (I.S.) eszopiclone-D8 [10.0 ng/mL in 100 mM ammonium acetate in water (pH unadjusted)]. Oasis® MAX mixed-mode polymeric anion-exchange sorbent 96-well plate, 1-mL/10 mg format from Waters (Milford, MA, USA) was used for SPE sample extraction. The automated SPE was performed on a Tomtec Quadra 96® (Hamden, CT, USA) and a CEREX® Multi-Channel SPE System 96™ positive pressure SPE manifold from SPEware Corp. (San Pedro, CA, USA). After conditioning and equilibrating the SPE plate with 0.5 mL of acetonitrile and 0.5 mL of 100 mM ammonium acetate in water (pH unadjusted), the samples were loaded onto SPE plate. The SPE plate was then washed with 0.5 mL of 100 mM ammonium acetate in water (pH unadjusted) and dried at maximum pressure (~25 psi) for approximately 5 min. After drying, samples were eluted slowly with 0.4 mL of 5% formic acid in 30/70 acetonitrile/water (v/v) and evaporated in a Zymark Turbovap® 96 (Hopkinton, MA, USA) at 50 °C until completely dry. The sample was then reconstituted in 300 μL of 85/15 (10 mM ammonium acetate in water (pH unadjusted)/MeOH)

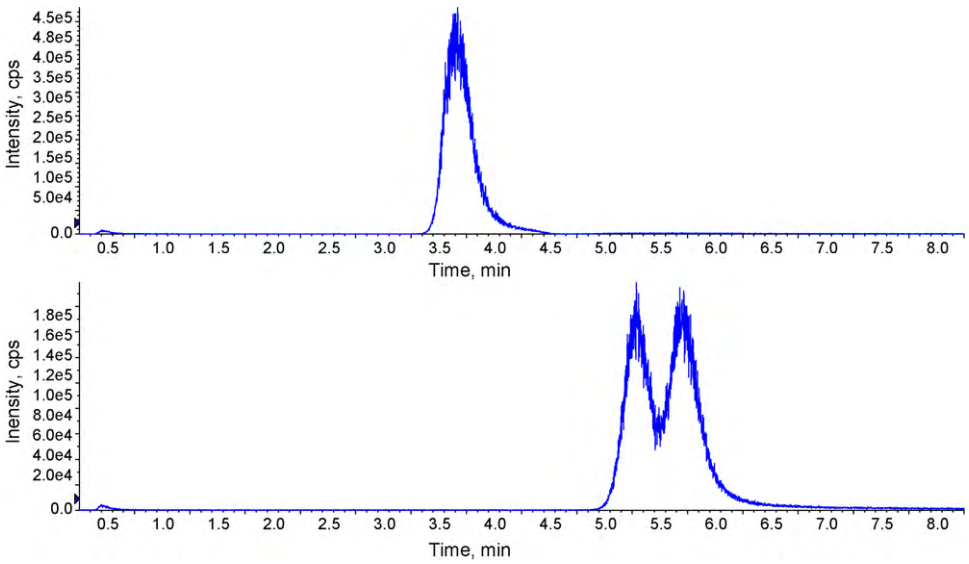

Fig. 2. Experimental method development chromatograms (top panel: 20/80 IPA/10 mM ammonium acetate in water; bottom panel: 10/90 IPA/10 mM ammonium acetate in water). *Note*: R-zopiclone and S-zopiclone were mixed at 1:1 ratio.

(v/v). After centrifugation, the extracts were analyzed directly by LC/MS/MS.

2.4. LC/MS/MS analysis

2.4.1. Software assisted chiral chromatography

ACD/Structure Designer, ChromManager and LC Simulator software (v. 10.0) were purchased from Advanced Chemistry Development, Inc. (Toronto, Canada) and were utilized for chiral chromatography development. Structure Designer software was utilized for the calculation of physical chemical properties, i.e., structure, pK_a value and solubility information. ChromManager software was utilized for data editing and processing. LC Simulator software was utilized for LC chromatography simulation and predictions. Using the ACD Lab software approach, two initial LC/MS/MS chromatographic conditions were acquired in the laboratory using recemic mixture of R-zopiclone and S-zopiclone at 1:1 ratio. The conditions were randomly selected and the enantiomeric peaks either completely coeluted or were partially resolved. The raw data from the two injections were then uploaded into the ACD/ChromManager software. After peak editing, i.e., peak picking and labeling, structure and LC condition assignment, these two chromatograms were imported into the ACD/LC Simulator software. The predicted separation conditions were obtained instantly by manipulating the column dimension, flow rate, mobile phase composition within the software. The computational results were then utilized as the guidance for further experimental work in the laboratory. This procedure was repeated until satisfactory chromatography was achieved.

2.4.2. LC separation conditions

After extraction, the resulting extracts are injected onto an HPLC system consisting of one Shimadzu LC-10AD high pressure pump, a SCL-10A system controller (Shimadzu, Columbia, MD, USA), a CTC PAL autosampler (Leap Technologies, Chapel Hill, NC, USA) equipped with a 50-μL sample loop and a 50-μL syringe, a Cera LC Column Heater 150 (Baldwin Park, CA, USA). LC separation was performed with an AGP chiral analytical column (50 mm × 2.0 mm, 5 μm particle size, ChromTech Ltd, Cheshire, UK). The analytical column was temperature controlled at 30 °C. Five to ten microliters of the extracted sample was injected into the HPLC system. The mobile phase was pre-mixed at a ratio of 85/15 10 mM ammonium acetate in water (pH unadjusted)/MeOH (v/v). The LC flow rate was 0.5 mL/min and the LC pump was operated under isocratic condition. The total cycle time was 3.0 min. To minimize carryover, the autosampler was equipped with two wash solvents. The first wash solvent was 0.1% formic acid in 90/10 acetonitrile/water (v/v) and the second wash solvent was 85/15 10 mM ammonium acetate in water (pH unadjusted)/MeOH (v/v) which matched the composition of the mobile phase. A series of injection syringe washes and injector valve washes were performed after each injection (first wash solvent followed by the second wash solvent).

2.4.3. Mass spectrometric condition

Eszopiclone and eszopiclone-D8 were detected by tandem mass spectrometric detection with a TurboIonSpray™ interference. The Sciex API4000 triple-quadrupole mass spectrometer (MDS Sceix, Toronto, Canada) was operated in positive ion mode with selected reaction monitoring (SRM) mode. Electrospray ionization conditions were optimized by infusion of eszopiclone and eszopiclone-D8 (500 ng/mL, 5 μL/min) into a stream of mobile phase at the chromatographic flow rate and composition. Both analyte and internal standard produce the same predominate product ion at m/z 245. The following MRM transitions were selected for further experiment: 389.2 → 245.0 for eszopiclone and 397.2 → 245.0 for eszopiclone-D8 using 200 ms dwell times. The MS conditions were as follows: curtain gas: 25, desolvation gas 1 and 2: 70 and 70, ion spray potential: 4000 eV, desolvation temperature: 500 °C, nitrogen collision gas: 12 a.u. (arbitrary units), declustering potential: 100 eV, entrance potential: 10 eV, collision cell exit potential: 22 eV, collision energy: 28 eV. The data was collected and processed through Analyst 1.4.2 software (MDS Sceix, Toronto, Canada) and Bioanalytical LIMS Watson 7.2.

2.5. Method validation

Acceptance criteria for all validation tests were based on current U.S. FDA Guidelines to Industry for Bioanalytical Method Validations [25].

The method was evaluated in terms of inter- and intra-assay precision and accuracy using Laboratory Information Management System Watson® LIMS (version 7.2.0.03). The precision was obtained by one-way analysis of variance (ANOVA) testing and was expressed as percent coefficient of variation (%CV). The acceptance

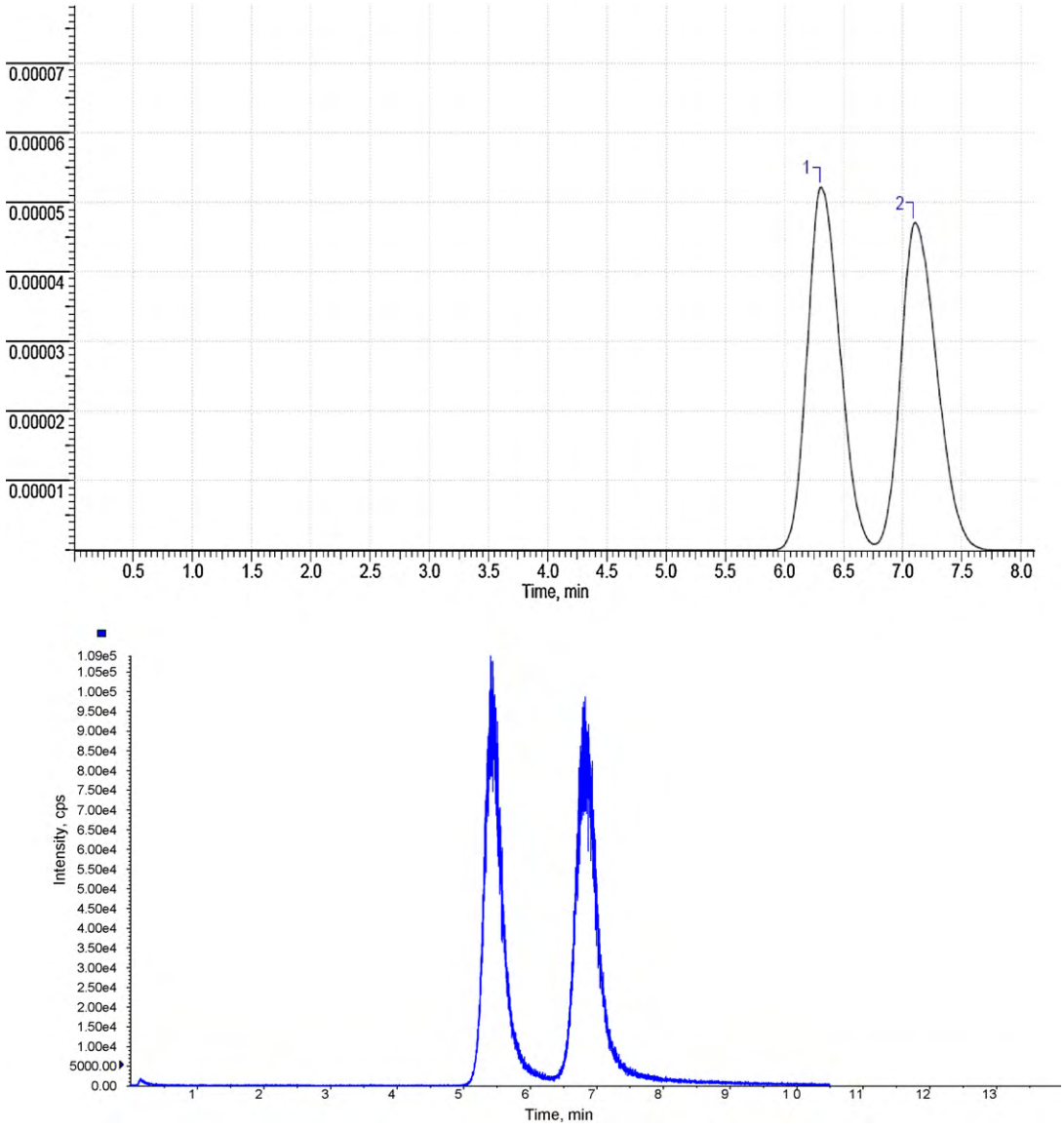

Fig. 3. Computational and experimental method development chromatograms based on ACD Lab predication (top panel: ACD Lab predication; bottom panel: 5/95 IPA/10 mM ammonium acetate in water). *Note*: R-zopiclone and S-zopiclone were mixed at 1:1 ratio.

for the accuracy has to be within ±15% of the nomination value (20% for standard one and LLOQ QC samples).

The method was also evaluated for selectivity and specificity (matrix effects) in six individual donors of human plasma at LLOQ and control double blank. To conduct this experiment, six selectivity LLOQ samples were prepared by spiking 50 μL of the standard spiking solution (20.0 ng/mL) into 950 μL six individual donor blank plasma matrix. 50 μL aliquot of the same six blank plasma lots were transferred out and extracted as selectivity blank. For six selectivity LLOQ samples, five out of six samples have to be within ±20% of the nomination value. For six selectivity blank samples, all six samples have to show either no peak at the retention time of the analyte of the interest or a minor peak less than 20% of the lowest acceptable standard LLOQ peak.

Extraction recovery was evaluated at three concentration levels (3.00, 40.0, and 80.0 ng/mL) by comparing the instrument response (analyte/I.S. area ratio) of plasma QC samples spiked with analyte prior to extraction to those spiked to extracted plasma blank samples after the extraction. The pre-extraction samples were prepared by the procedure described in Section 2.3. The post-extraction samples were control blanks and then spiked with neat solution after elution and prior to evaporation.

The method was also evaluated for various stabilities. Short-term and long-term stability in plasma was demonstrated at room temperature, 4 °C and −20 °C. Similarly, the short-term and long-term stability in DMF solvent was demonstrated at room temperature and 4 °C. Plasma stability against four freeze/thaw cycles at room temperature was evaluated. Additionally, whole blood stability during sample collection at room temperature up to 2 h was also demonstrated. To conduct whole blood stability experiment, eszopiclone was spiked into fresh whole blood at medium QC level. The treated whole blood samples were then placed at room temperature and wet ice bath up to 2 h. The plasma sample from the whole blood samples were harvested, extracted and analyzed following the procedure described in Section 2.3.

Autosampler reinjection reproducibility was evaluated by re-injecting a previously extracted validation batch containing

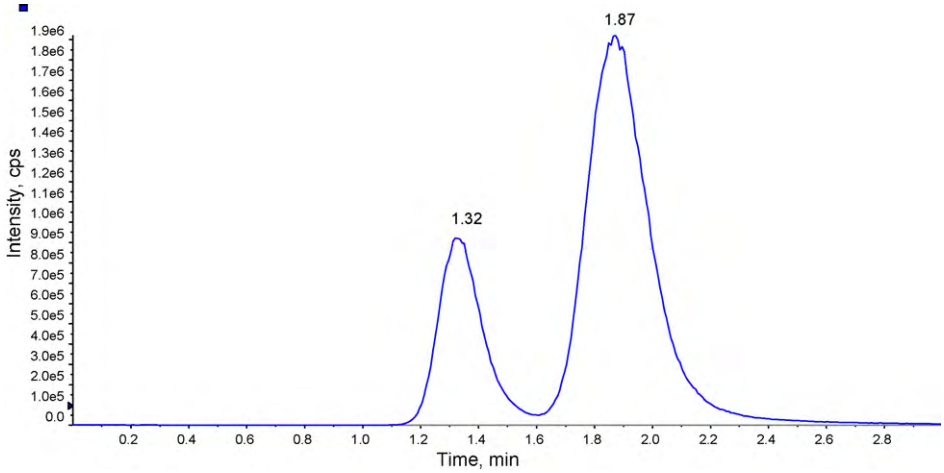

Fig. 4. Experimental method development chromatograms (15/85 MeOH/10 mM ammonium acetate in water). *Note*: R-zopiclone and S-zopiclone were mixed at 1:1 ratio.

standard calibration curve ($n = 2$) and low, medium and high QC ($n = 6$) after a period of storage at room temperature.

2.6. Relative bioavailability study of eszopiclone 3 mg Tablet Versus Lunesta® 3 mg Tablet

A randomized, single dose, two-way, open-label crossover study under fed conditions was conducted with twenty-Four (24) healthy adult (male and female) subjects.

Eszopiclone 3 mg Tablet given as a single dose with 8 fl. oz. of room temperature water 30 min after initiation of a standardized, high-fat and high-calorie breakfast preceded by an overnight fast of at least 10 h. Lunesta® 3 mg Tablet given as a single dose with 8 fl. oz. of room temperature water 30 min after initiation of a standardized, high-fat and high-calorie breakfast preceded by an overnight fast of at least 10 h. 17 blood samples per subject each period were collected for bioanalysis.

Pharmacokinetic sampling occur within 90 min prior to administration of study product to the first study participant (Hour 0 only) and at post-dose Hours 0.5, 1, 1.5, 1.75, 2, 2.25, 2.5, 3, 4, 6, 8, 10, 12, 14, 18, and 24 h. The eszopiclone plasma concentrations were measured using the validated method according to FDA Guidelines. The plasma concentration was calculated using Laboratory Information Management System Watson® LIMS (version 7.2.0.03) and the Plasma concentration profile was in processed in Microsoft Office Excel 2003.

3. Results and discussion

3.1. Chiral LC/MS/MS method development

Because it is less prone to ionization effects, atmospheric pressure chemical ionization (APCI) was evaluated first but abandoned due to inadequate sensitivity. Electrospray ionization (ESI) using a TurboIonSpray™ source was used in MS detection of eszopiclone and eszopiclone-D8.

Chiral chromatography development is time consuming and challenging even for a very skillful and experienced chromatographer. For this study, we chose ACD/LC Simulator software approach to assist chiral chromatographic development. The initial conditions were selected using a Chiral AGP 2 mm × 100 mm column and 10 mM ammonium acetate in water and IPA as mobile phases at flow rate of 0.4 mL/min. A test sample containing mixture of 1:1 ratio of R- and S-zopiclone at concentration of 100 ng/mL was injected. As shown in Fig. 2, the first two injections performed had either no separation using 20/80 IPA/10 mM ammonium acetate in water (pH unadjusted) (v/v) (Fig. 2, top panel) or partial separation at 10/90 IPA/10 mM ammonium acetate in water (pH unadjusted) (v/v) (Fig. 2, bottom panel). These two chromatograms were then imported into the ACD/LC Simulator software. The software simulated the conditions and predicted the best separation at 5/95 IPA/10 mM ammonium acetate in water (pH unadjusted) (v/v) (Fig. 3, top panel). Based on the computational parameters, a third injection was performed in the laboratory and the experimental results matched the simulated conditions (Fig. 3, bottom panel). Although the chiral separation is excellent, the long cycle time was not desirable because our goal was to achieve total cycle time less than 5 min. In the past, without the computer simulation, scientist would continue working in the laboratory in hope to adjust the existing condition slightly to obtain ultimate separation. Using ACD/LC Simulator software, we can easily conclude that it is impossible to achieve baseline separation less than 5 min using IPA as strong solvent. Immediately, a decision was made to replace IPA with MeOH. The optimal conditions were quickly obtained using an AGP column 50 mm × 2 mm and 15/85 MeOH/10 mM ammonium acetate in water (pH unadjusted) (v/v) in the laboratory without assistance of ACD computer software (Fig. 4). After the optimal results were produced in the laboratory, we verified it using the ACD Lab software. A test sample containing mixture of 1:1 ratio of R- and S-zopiclone at concentration of 100 ng/mL were prepared. Two random conditions, i.e., 30/70 and 25/75 MeOH/10 mM ammonium acetate in water (pH unadjusted) (v/v), were selected in which the R- and S-zopiclone enantiomers were either no or partial separation. These two chromatograms were then imported into the ACD/LC Simulator software. The computational results predicted similar chromatograms to those obtained in the laboratory.

Using MeOH as strong mobile phase, the baseline resolution was achieved with total cycle time less than 3 min. It was speculated that due to more acidic nature of active hydrogen in MeOH, relative to IPA, it is increased H-bonding capabilities (compared to IPA) allow for more interaction with the stationary phase (competition with the analyte) and the analyte, which will reduce it is retention while preserving the interaction mechanism on the stationary phase, thus allowing for chiral separation.

In this study, we validated the accurate predication of the ACD software via forward validation "using IPA as solvent" and backward validation "using MeOH as solvent". The efficiency in the method development provided by the software is clear. As shown in Fig. 2, it is not mandate to achieve partial separation for initial condition in order to use LAC Lab software. However, like any other computer software, it cannot be relied upon as a sole MD strategy.

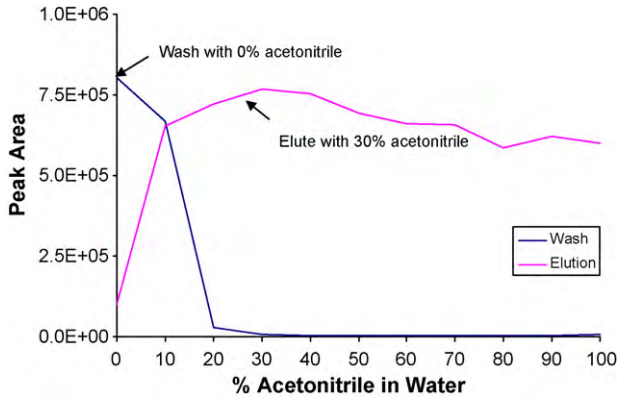

Fig. 5. Optimization of MAX mixed-mode anion-exchange conditions for the SPE of eszopiclone from human plasma.

The software only works with the imported chromatograms and mobile phase provided by the user. In this case, it failed to consider the separation mechanism and suggest more suitable solvent such as MeOH for better separation. As a result, due to these limitations it can only serve as a method development aid. Sound scientific judgment and laboratory experiment is still required to select the appropriate starting condition.

3.2. Sample extraction method development

SPE was chosen as the method for sample preparation because of its greater selectivity, the wide selection of separation mechanisms, greater potential for minimizing matrix effects, and lower solvent consumption. Eszopiclone is a neutral drug with overall pK_a of ~6.7 calculated by ACD/Structure Designer software. Based on the structure and pK_a value, we expected a weak cation-exchange or reversed phase extraction mechanism as the most appropriate for the drug extraction from a biological matrix. We performed a screening test using a Waters Oasis Sorbent Selection Plate 96-well plate under multiple loading, elution and pH conditions. Similar high recovery (>80%) was obtained using WCX column under acidic wash and MAX column under neutral wash condition. For both bed mass, the pH of the elution condition appears less critical, varying from 90 to 100% recovery from neutral to basic to acidic condition. The likely reason for this is that under these conditions, the eszopiclone molecule is neutral. Although the molecule is retained on MAX and WCX column, the main mechanism of the retention is reverse-phase interactions with the packing material. For this study, MAX column was selected for further experiment.

Optimization of the wash and elution steps in the SPE procedure was performed in a similar fashion as reported by Čápka et al. [23]. Briefly, 0.1 mL of eszopiclone fortified human plasma samples were extracted by SPE as outlined in Section 2. For the wash optimization experiment, the samples were washed with 1 mL of solvent with progressively increasing wash strength (0% acetonitrile to 100% acetonitrile in water in 10% increments). The samples were eluted under the same condition using 5% formic acid in 90/10 acetonitrile and water (v/v). In the elution optimization experiment, the samples were washed under the same condition using 100 mM ammonium acetate in water (pH unadjusted) and followed by elution with 1 mL of solvent with progressively increasing elution strength (0% acetonitrile to 100% acetonitrile in 5% FA in water in 10% increments). The extract was then evaporated and reconstituted in 0.5 mL of mobile phase and analyzed by LC/MS/MS. Fig. 5 shows the results of this optimization. The optimized SPE wash solvent composition was 100% 100 mM ammonium acetate in water (pH unadjusted), whereas the maximum signal was achieved using 5% formic acid in 30/70 acetonitrile/water (v/v) as an elution solvent. Analyte signal decreased slightly at compositions of greater than 30% acetonitrile presumably due to an increase in co-extraction of endogenous matrix components, e.g., phospholipids, consequently causing matrix effects and ion suppression in the MS source [22–24].

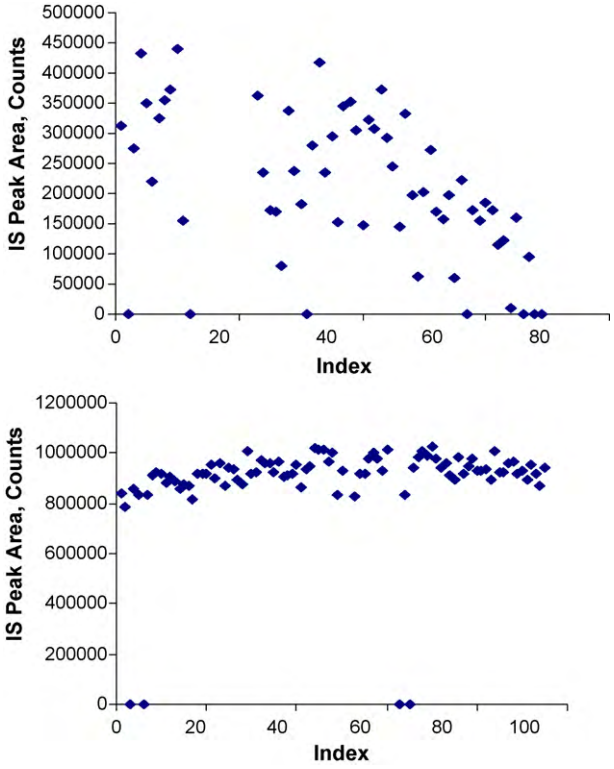

Fig. 6. Internal standard area plot (top panel: using less than optimal SPE conditions initial method development condition; bottom panel: using final and optimized SPE condition).

In addition to focusing on elution and washing solvent, various experiments was conducted to evaluate the pH and the ionic strength of the buffer, the reconstitute solvent, the volume of aliquot size, the evaporation temperate and time, etc. Throughout method development period, the internal standard area plot of the test batch was monitored closely. It provides useful information for extraction recovery and instrument consistence. Fig. 6 showed the comparison of the eszopiclone-D8 area response from a test batch using initial SPE extraction vs. a validation batch using optimized SPE extraction.

3.3. Method validation

Validation for determination of eszopiclone in human plasma (K_3EDTA) by LC/MS/MS in the range of 1.00–100 ng/mL was performed according to current US FDA guidelines [25]. Representative chromatograms, i.e., LLOQ, Control blank, Mix QC from the validation are shown in Fig. 7. Because the standard, QC and future study samples were only prepared from eszopiclone, in order to monitor the adequate separation between the isomers, a single test sample (Mix QC) containing recemic mixture of zopiclone was freshly spiked into plasma at final concentration of 500 ng/mL and processed along with other standard and QC samples. The Mix QC was included in every method validation and sample analysis run. There is no quantitative acceptance criterion and it serves only for chromatography monitoring purpose. Fig. 8 is a representative chromatogram of the Mix QC.

While re-evaluating this method now, it appears to us that the better alternative approach is to utilize recemic zopiclone-D8

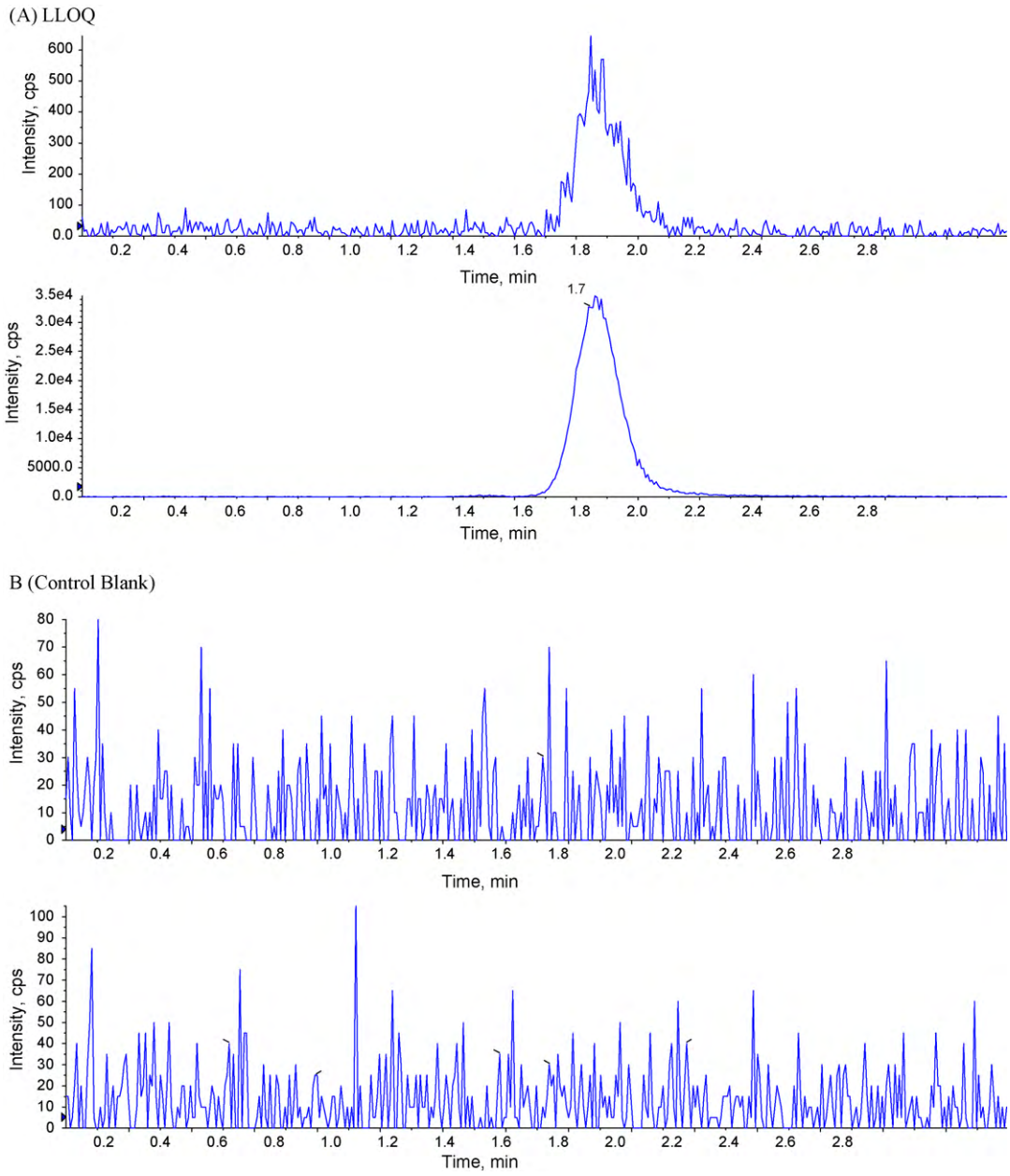

Fig. 7. Chromatogram of control Blank (top panel: eszopiclone SRM channel; bottom panel: eszopiclone-D8 SRM channel) and chromatogram of LLOQ at 10.0 ng/mL (top panel: eszopiclone SRM channel; bottom panel: eszopiclone-D8 SRM channel).

as internal standard for following two reasons: (1) the recemic zopiclone-D8 is less expensive than eszopiclone-D8; (2) using eszopiclone-D8 as internal standard and analyzing Mix QC in the beginning of the batch, it only guarantee adequate chiral separation prior to initialing batch. It is unknown if the adequate separation has maintained throughout entire batch. In contrast, using recemic zopiclone-D8 as internal standard, the chiral separation in each sample throughout entire batch can be monitored instantly. Because of the apparent advantage, it is currently under consideration to replace eszopiclone-D8 with recemic zopiclone-D8, although it requires a full GLP revalidation.

Table 1
Back-calculated concentration of calibration standards for eszopiclone. All concentrations are expressed as ng/mL.

Run date	Run number							
	1.00	2.00	5.00	10.0	25.0	50.0	85.0	100
Mean	1.01	1.99	4.80	10.1	25.0	50.3	85.6	102
S.D.	0.0455	0.0787	0.188	0.130	0.472	1.34	1.86	1.40
%CV	4.5	4.0	3.9	1.3	1.9	2.7	2.2	1.4
%Bias	1.0	−0.5	−4.0	1.0	0.0	0.6	0.7	2.0
n[a]	6	6	6	6	6	6	6	6

[a] *n* value over three validation runs.

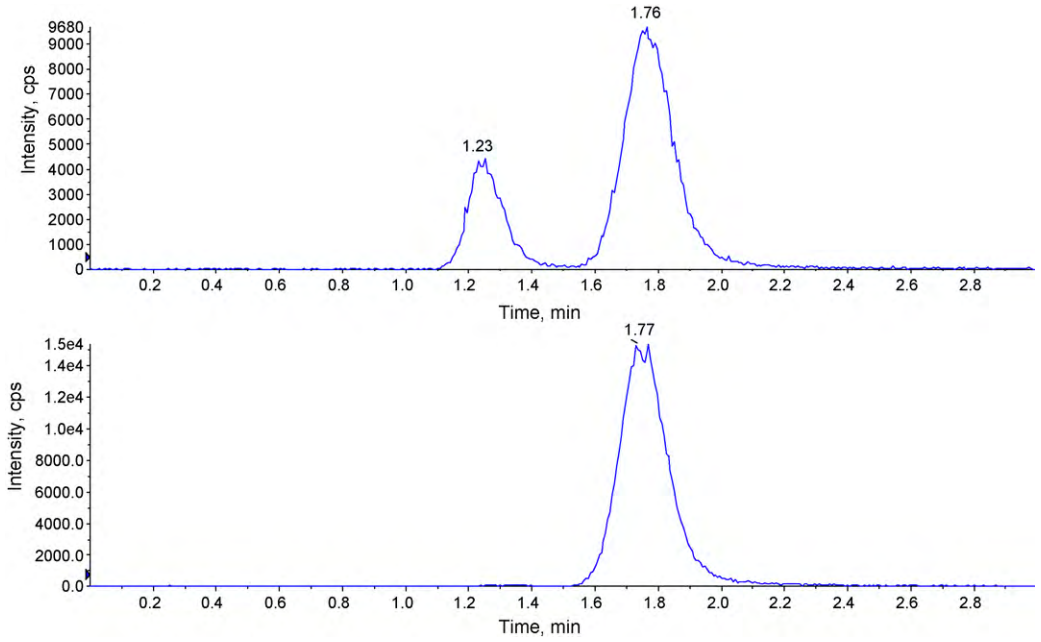

Fig. 8. Chromatogram of SST-Mix zopiclone at 100 ng/mL (top panel: eszopiclone SRM channel; bottom panel: eszopiclone-D8 SRM channel). *Note*: R-zopiclone and S-zopiclone were mixed at 1:1 ratio.

Table 2
Accuracy and precision quality control samples from ANOVA for eszopiclone.

Nominal conc.	LLOQ QC 1.00 ng/mL	Low QC 3.00 ng/mL	Medium QC 40.0 ng/mL	High QC 80.0 ng/mL
Mean observed conc.	0.965	2.96	36.8	76.9
%Bias	−3.5	−1.3	−8.0	−3.9
Between run precision (%CV)	8.5	1.0	3.7	4.0
Within run precision (%CV)	5.9	3.1	2.6	2.1
Total variation (%CV)	10.4	3.2	4.5	4.6
n[a]	18	18	18	18
Number of runs	3	3	3	3

[a] *n* value over three validation runs.

Intra- and inter-day accuracy and precision were evaluated in three separate validation runs on three different days at four concentration levels (including LLOQ, low, medium and high levels) at $n = 6$. The back-calculated calibration data over three accuracy/precision runs are shown in Table 1. Intra- and Inter-day accuracy and precision of quality control data are shown in Table 2.

Method selectivity was evaluated by analysis of control blank extracts of the human plasma from six individual donors. In addition, QC samples at the LLOQ of the assay (1.00 ng/mL) using the six different lots were tested. All six selectivity LLOQ met the acceptance criteria with the average %theoretical of 109.0% and mean %CV of 5.8%. No peaks were detected at the retention time of eszopiclone and the I.S. in any of the tested plasma lots. It was also noticed that there is no peak at the retention time of R-zopiclone while only eszopiclone was spiked indicating that there is no conversion from eszopiclone to R-zopiclone. Because the standard, QC and future study samples were only prepared from eszopiclone, the possibility of the conversion from R-zopiclone to eszopiclone was not evaluated.

Short-term stability in plasma was evaluated and demonstrated acceptable results against four freeze/thaw cycles, 3 and 6 h bench top stability in matrix (ambient room temperature and 4 °C). The comparison of the long-term stability in plasma stored at −20 °C and −70 °C was also evaluated and demonstrated acceptable results for 107 days (Table 3).

The following experiments were evaluated and demonstrated acceptable results: (1) ability to dilute the samples by diluting plasma samples at 400 ng/mL by a dilution factor of 10 (%theoretical of 86.5% and mean %CV of 2.4%). (2) Extraction recovery was evaluated at three concentration levels (3.00, 40.0, and 80.0 ng/mL, $n = 6$ at each level) as described in Section 2. The mean extraction recovery from all levels was 90% with mean %CV of 5.8%. No concentration dependency of the recovery was observed. (3) Processed extract stability at room temperature where a previously extracted validation experiment batch was re-injected after 167 h of storage at ambient temperature. (4) Stability in whole blood during sample collection at room temperature up to 2 h. (5) Solution stability for short-term room temperature and long-term reduced temperature (4 °C) condition for up to 6 h at room temperature and 105 days at 4 °C condition.

Table 3
Various matrix stability at the low and high QC level for eszopiclone.

Experiment	Low QC (3.00 ng/mL)		High QC (800 ng/mL)	
	%Theoretical	%CV	%Theoretical	%CV
Freeze–thaw $n = 4$ cycles	96.7	−3.3	99.4	−0.6
Bench-top 3 h, RT	92.0	−8.0	86.8	−13.3
Bench-top 6 h, 5 °C	97.0	−3.0	99.3	−0.8
Long-term 107 days, −70 °C	104.7	2.5	102.8	0.5
Long-term 107 days, −20 °C	104.0	2.7	100.3	2.1

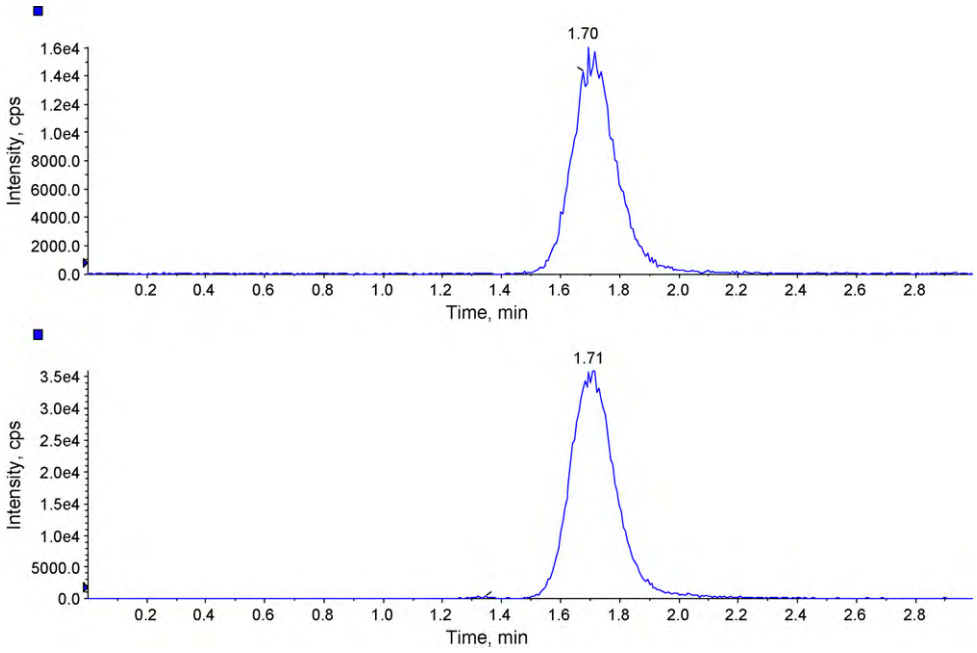

Fig. 9. Chromatogram of a human subject sample after inhaled administration of a therapeutic dose of eszopiclone (top panel: eszopiclone SRM channel; bottom panel: eszopiclone-D8 SRM channel).

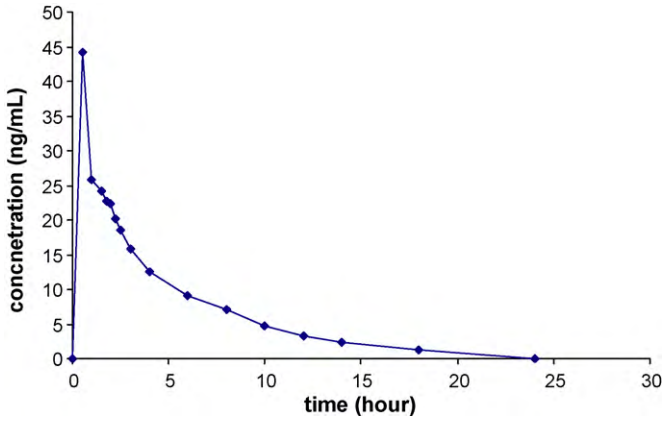

Fig. 10. Plasma concentration profile of eszopiclone after oral administration of a therapeutic dose (3 mg Tablet) of eszopiclone to a human subject.

3.4. Relative bioavailability study of eszopiclone 3 mg Tablet Versus Lunesta® 3 mg Tablet

After this method was fully validated, several clinic bioavailability studies were conducted to compare eszopiclone 3 mg Tablet Versus Lunesta®. Figs. 9–10 shows a representative chromatogram and the plasma concentration profile of eszopiclone after oral administration of a therapeutic tablet (3 mg) to a human subject. In all clinic studies, there was no *in vivo* racemization observed in patient samples.

4. Conclusion

Computer software has been utilized to facilitate the chromatography development for decades. It was especially useful for spectrometric method in which the baseline separation of complicated ingredients is essential. While LC–MS/MS technique has gain more and more popularity, the requirement for baseline separation became less important because of the mass filtration function of tandem mass spectrometer. Using LC–MS/MS technique, the analytes (i.e., pharmaceutical drug) and its related degrading product (i.e., metabolites) can be quantified while coeluting or partially coeluting as long as their SRM transitions are unique. The exception is chiral compounds, for enantiomers possess same molecular weight. Therefore, the chromatographic separation of the enantiomers is still essential for chiral LC–MS/MS assay. In this study, we presented an example that computer software can accelerate the method development of chiral LC/MS/MS assay for the determination of eszopiclone in human plasma.

It is well recognized that the separation of enantiomers is expensive, challenging and time consuming. Without computer software assistance, often the primary goal is to achieve resolution by trial and error and it is commonly acceptable to have long cycle time for a chiral LC assay as long as the resolution is achieved. Because of the high cost of chiral LC column, chiral chromatography method development was hindered by the availability of the various chiral LC columns in the laboratory. Chiral LC column manufacturers recognize the limitation and start to offer charged chiral column screening service. In our laboratory, we had pursued this approach but the outcome was always less satisfactory. The chiral column screen experiment by the manufacturer is often conducted using LC–UV technique and big chiral LC column. Either the cycle time is too long (>10 min) or MS non-compatible buffer (i.e., sodium or potassium phosphate buffer) was utilized. In the past, we always have to revamp the screening condition completely and redo method development using the screening condition as starting reference.

Although the cycle times of all previously published chiral HPLC assays for zopiclone are more than 20 min, we aggressively explored the possibility of shortening the cycle time using ACD Lab software as a tool. The starting experiment condition using IPA and AGP chiral LC column was a random choice. Although we obtained a promising partially resolution using IPA as strong solvent, the decision of change of organic solvent from IPA and MeOH was very prompt, for ACD LC simulator showed that none of the change, i.e., composition, column dimension and flowrate, would improve the cycle time. Using the same strategy, three GLP chiral LC–MS/MS assays for the quantitation of armodafinil, ramelteon and dexlansoprazole were successfully developed shortly [26]. The time for

all three chiral chromatographic developments was very minimal (less than 1 day) and all cycle times for all three chiral assays were less than 5 min. At this point, we only explored simulation functions such as composition and column dimension change under isocratic conditions. More simulation functions including the effect of pH, ionic strength, and gradients were available but not tested in this study. In short, the ACD/LC Simulator can be successfully used to assist chiral chromatographic method development.

For sample extraction method development, we focused not only on the reduction or elimination of matrix effect components, but we also focused on the recovery and reproducibility of the analyte and internal standard. Often, when a stable isotope labeled compound is used as the internal standard, large variability of the internal standard response is common, yet widely accepted by the industry because the quantitative results still pass acceptance criteria. However, we believe that consistent internal standard response is essential to demonstrate and confirm assay robustness and ruggedness regardless of whether a stable isotope labeled internal standard or structural analogue internal standard is used. Although a stable isotope labeled eszopiclone-D8 was used as an internal standard, inconsistent internal standard area response was obtained initially. Normally, extremely widely variable internal standard response is an indicator of low recovery or adsorption or matrix effects. In our case, the source was inconsistent recovery. Following the above philosophy, we identified and resolved several other issues such as divergent curves and high back pressure.

The diligent, systematic and persistent method development and troubleshooting strategy was demonstrated during the GLP validation. Over the entire validation, except one dilution QC, the remaining 287 standard calibrators and quality control samples passed with excellent precision and accuracy. This method was utilized to support multiple clinic bioavailability studies successfully.

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