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Development and validation of an enantioselective liquid-chromatography/tandem mass spectrometry method for the separation and quantification of eslicarbazepine acetate, eslicarbazepine, R-licarbazepine and oxcarbazepine in human plasma

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ABSTRACT

The purpose of this study was develop and validate a sensitive and specific enantioselective liquid–chromatography/tandem mass spectrometry (LC–MS/MS) method, for the simultaneous quantification of eslicarbazepine acetate (ESL), eslicarbazepine (S-Lic), oxcarbazepine (OXC) and R-licarbazepine (R-Lic) in human plasma. Analytes were extracted from human plasma using solid phase extraction and the chromatographic separation was achieved using a mobile phase of 80% n-hexane and 20% ethanol/isopropyl alcohol (66.7/33.3, v/v). A Daicel CHIRALCEL® OD-H column (5 μm, 50 mm × 4.6 mm) was used with a flow rate of 0.8 mL/min, and a run time of 8 min. ESL, S-Lic, R-Lic, OXC and the internal standard, 10,11-dihydrocarbamazepine, were quantified by positive ion electrospray ionization mass spectrometry. The method was fully validated, demonstrating acceptable accuracy, precision, linearity, and specificity in accordance with FDA regulations for the validation of bioanalytical methods. Linearity was proven over the range of 50.0–1000.0 ng/mL for ESL and OXC and over the range of 50.0–25,000.0 ng/mL for S-Lic and R-Lic. The intra- and inter-day coefficient of variation in plasma was less than 9.7% for ESL, 6.0% for OXC, 7.7% for S-Lic and less than 12.6% for R-Lic. The accuracy was between 98.7% and 107.2% for all the compounds quantified. The lower limit of quantification (LLOQ) was 50.0 ng/mL for ESL, S-Lic, OXC and R-Lic in human plasma. The short-term stability in plasma, freeze–thaw stability in plasma, frozen long-term stability in plasma, autosampler stability and stock solution stability all met acceptance criteria. The human plasma samples, collected from 8 volunteers, showed that this method can be used for therapeutic monitoring of ESL and its metabolites in humans treated with ESL.

1. Introduction

Eslicarbazepine acetate (ESL) [(S)-(−)-10-acetoxy-10,11, dihydro-5H-dibnz[b,f]azepine-5-carboxamide] is a novel voltage-gated sodium channel blocker that has documented efficacy as an adjunct therapy in patients with inadequately controlled partial-onset seizures [1–3]. ESL, carbamazepine (CBZ) and oxcarbazepine (OXC) have a similar dibenzazepine nucleus bearing the 5-carboxamide substituent, but with structural differences at the 10,11-position. This molecular variation results in differences in metabolism, preventing the formation of toxic epoxide metabolites such as carbamazepine-10,11 epoxide. In humans, ESL is rapidly and nearly completely reduced by esterases in the intestine and liver to eslicarbazepine (S-Lic). This is the active form and accounts for approximately 92% of the total ESL excreted in urine, whether in its unchanged form (66%) or conjugated with glucuronic acid (33%). In plasma following an oral dosing of ESL, only approximately 2% of S-Lic undergoes glucuronidation. R-licarbazepine (R-Lic) and OXC are also minor metabolites in human plasma [4].

In recent years several methods to measure ESL and its metabolites in human plasma have been reported [5–8]; however, the published methods were not sufficiently sensitive for quantification of OXC and R-Lic, with a lower limit of quantification ranging

Abbreviations: ESL, eslicarbazepine acetate; S-Lic, eslicarbazepine; R-Lic, R-licarbazepine; OXC, oxcarbazepine; CNS, central nervous system; AED, antiepileptic drugs; SPE, solid phase extraction; DMSO, dimethylsulfoxide; LC–MS/MS, high performance liquid chromatography–mass spectrometry; ESI, electrospray ionization; LLOQ, lower limit of quantification; FDA, Food and Drug Administration; MeCN, acetonitrile; MeOH, methanol; IS, internal standard; HPLC, high performance liquid chromatography; UV, ultraviolet; QC, quality control; LQC, low quality control; MQC, medium quality control; HQC, high quality control; DiQC, dilution quality control; CV, coefficient of variation.

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Table 1
ESL and OXC linearity, accuracy and precision of calibration standards (3 analytical runs).

	Calibration samples (ng/mL)									
	50.0	100.0	200.0	300.0	400.0	500.0	600.0	700.0	850.0	1000.0
ESL										
Mean (ng/mL)	50.6	100.7	197.1	318.9	373.0	496.3	609.2	671.9	826.9	1046.2
S.D. (ng/mL)	1.9	0.9	3.9	6.2	7.3	30.5	30.2	–	43.8	17.2
CV%	3.7	0.9	2.0	2.0	2.0	6.1	5.0	–	5.3	1.6
Bias%	101.2	100.7	98.5	106.3	93.2	99.3	101.5	96.0	97.3	104.6
OXC										
Mean (ng/mL)	49.1	102.3	200.7	318.9	376.3	488.3	619.6	636.9	856.0	1031.0
S.D. (ng/mL)	2.7	3.3	0.8	11.6	17.2	23.6	32.8	–	47.3	18.7
CV%	5.4	3.3	0.4	3.6	4.6	4.8	5.3	–	5.5	1.8
Bias%	98.3	102.3	100.3	106.3	94.1	97.7	103.3	91.0	100.7	103.1

from 0.1 to 0.4 μg/mL, employing ultraviolet (UV) detection with a run time of approximately 25–30 min [7,9]. Existing HPLC with UV detector methods hardly reach the limit of quantification required for reliable measurements of minor metabolites at the levels found in circulation. Moreover, the low specificity/selectivity of UV detection involved a long analysis time which is not compatible with the necessary higher speed analysis for clinical samples. The LC–MS/MS technique herein described, on the other hand, has an exceptional sensitivity and specificity compared to UV detection and reduced the analysis time. The mass spectrometry detector can be programmed to select and fragment certain ions and, as long as there are no interferences or ion suppression, the LC separation can be rapid and it is now common to have analysis times of 1 min or less by MS/MS detection.

In order to overcome the limitations of UV analysis, we established a novel approach for the detection of ESL and its metabolites in human plasma. Compounds were analyzed using a positive electrospray tandem mass spectrometry method following sample clean-up by solid phase extraction. This new sensitive and selective method for determination of ESL and its metabolites provides higher sensitivity and shorter analysis time than the previously reported methods. The method was validated according to "Guidance for Industry-Bioanalytical Method Validation" recommended by the Food and Drug Administration (FDA) of the United States and according to recommendations from Validation Groups Workshops [10,11].

2. Materials and methods

2.1. Chemicals and reagents

ESL, S-Lic and R-Lic were synthesized at BIAL Laboratory of Chemistry Research (S. Mamede Coronado, Portugal), with purity > 99.5%. Oxcarbazepine was synthesized and provided by Farchemia (Italy). 10,11-Dihydrocarbamazepine was purchased from Sigma–Aldrich (St. Louis, MO). HPLC grade acetonitrile (MeCN), HPLC grade methanol (MeOH), n-hexane, ethanol and isopropyl alcohol were purchased from Merck KGaA (Darmstadt, Germany). Ammonium acetate was purchased from Sigma–Aldrich (St. Louis, MO) and milliQ water (HPLC grade, >15MΩ, Millipore) was produced in house. Heparinised human plasma was obtained from Biological Specialty Corporation, 2165 N. Line Street, Colmar, PA 18915-9703, USA.

2.2. Calibration standard and quality control sample preparation

Standard stock solutions of ESL and OXC were prepared individually at 500.0 μg/mL in MeCN in polypropylene tubes. Combined standard spiking solutions of ESL and OXC at 250.0 and 50.0 μg/mL in MeCN were prepared in polypropylene tubes by diluting the standard stock solutions. Standard stock solutions of S-Lic and R-Lic were prepared individually at 3.2 mg/mL in MeCN in polypropylene tubes. Combined standard spiking solutions at 1600.0, 1000.0 and 50.0 μg/mL in MeCN were prepared in polypropylene tubes by diluting from the standard stock solutions. The stock solutions and the spiking solutions were stored at a temperature of 4 °C nominal. A standard stock solution of 10,11-dihydrocarbamazepine (IS) was prepared at 1.0 mg/mL in MeCN in a volumetric flask. Internal standard working solution at 500.0 ng/mL was prepared in a polypropylene tube by diluting 10,11-dihydrocarbamazepine standard stock solution in H_2O with 3% MeCN (v/v). These solutions were stored at a temperature of 4 °C nominal. Calibration standards at 50.0, 100.0, 200.0, 300.0, 400.0, 500.0, 600.0, 700.0, 850.0 and 1000.0 ng/mL for ESL and OXC and at 50.0, 100.0, 500.0, 1000.0, 3750.0, 6250.0, 10,000.0, 17,500.0, 21,248.0 and 25,000.0 ng/mL for S-Lic and R-Lic were prepared by adding known amounts of the appropriate combined spiking solutions to blank human plasma. Quality control (QC) samples at low 150.0 ng/mL (LQC), mid 450.0 ng/mL (MQC), and high 800.0 ng/mL (HQC), for ESL and OXC and at 150.0 ng/mL (LQC), 4500.0 ng/mL (MQC), and 20,000.0 ng/mL

Table 2
Eslicarbazepine and R-licarbazepine linearity, accuracy and precision of calibration standards (3 analytical runs).

	Calibration samples (ng/mL)									
	50.0	100.0	500.0	1000.0	3750.0	6250.0	10000.0	17504.0	21248.0	25000.0
Eslicarbazepine										
Mean (ng/mL)	50.0	100.0	483.1	1097.9	3596.2	6240.1	10610.4	17069.2	20582.3	24164.2
S.D. (ng/mL)	0.7	2.7	13.9	19.6	244.2	295.7	481.5	–	1210.7	429.3
CV%	1.3	2.7	2.9	1.8	6.8	4.7	4.5	–	5.9	1.8
Bias%	99.9	100.0	96.6	109.8	95.9	99.8	106.1	97.5	96.9	96.7
R-licarbazepine										
Mean (ng/mL)	50.0	99.3	491.2	1122.7	3692.2	6159.0	10568.0	16873.9	20114.8	23828.6
S.D. (ng/mL)	1.5	5.1	15.2	22.3	183.7	277.7	345.1	–	1546.1	760.1
CV%	2.9	5.1	3.1	2.0	5.0	4.5	3.3	–	7.7	3.2
Bias%	100.0	99.3	98.2	112.3	98.5	98.5	105.7	96.4	94.7	95.3

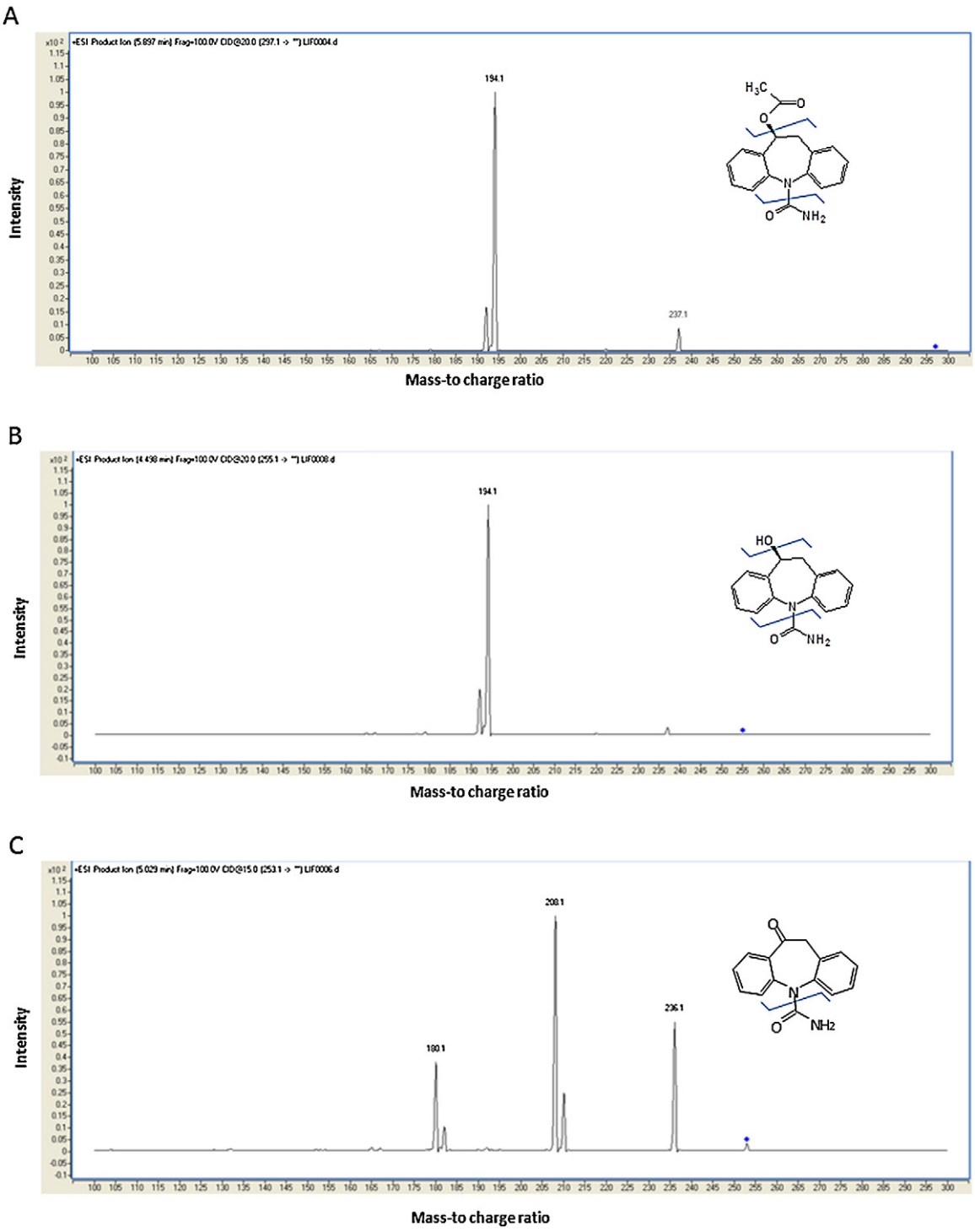

Fig. 1. Product ion mass spectrum of (A) $[ESL+H]^+$, (B) $[S\text{-}Lic+H]^+$ and $[R\text{-}Lic+H]^+$, and (C) $[OXC+H]^+$ in positive ion ESI mode.

(HQC) for S-Lic and R-Lic were prepared independently in the same matrix (blank human plasma).

2.3. Sample preparation procedure

Aliquots of human plasma (100.0 μL) were added to 500 μL of internal standard working solution (500.0 ng/mL 10,11-dihydrocarbamazepine), except the blank sample to which was added 500 μL of water 3% of MeCN (v/v) solution. Samples placed into (16 mm × 125 mm) glass culture tubes were vortex-mixed and loaded (500 μL) into Varian Bond-Elut C_{18} cartridges (100 mg/1 mL), which were previously conditioned with 1 mL of MeOH, 1 mL of MeCN and 1 mL of water 3% of MeCN (v/v) solution. After sample elution, the loaded cartridges were centrifuged at 500 rpm for 2 min at 22 °C nominal, acceleration low, deceleration high. Cartridges were rinsed with 500 μL of water 3% MeCN (v/v) solution and centrifuged at 1500 rpm for 3 min at 22 °C nominal, acceleration low, deceleration high. Following transfer into clean glass culture tubes (13 mm × 100 mm), 250 μL of MeCN was added to the cartridges and centrifuged at 500 rpm for 1 min at

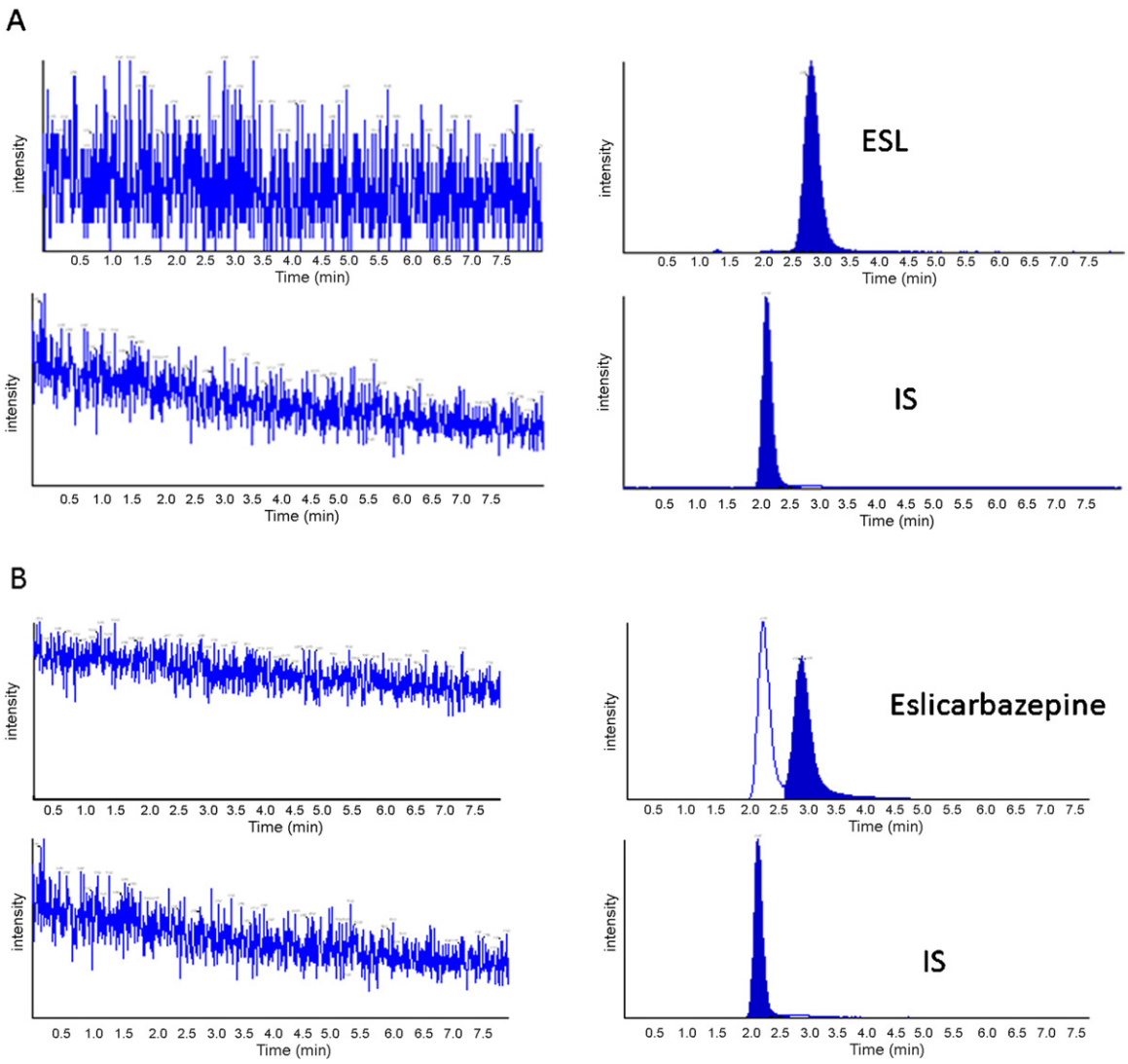

Fig. 2. Chromatograms of extracted human plasma resulting from SRM detection showing extracted blank plasma and (A) ESL with internal standard, (B) S-Lic with internal standard, (C) R-Lic with internal standard and (D) OXC with internal standard. Analytes were separated using a mobile phase of 80% of n-hexane and 20% of ethanol/isopropyl alcohol (66.7/33.3%, v/v). A Daicel CHIRALCEL® OD-H (5 μm, 50 mm × 4.6 mm) was used with a flow rate of 0.8 mL/min, and a run time of 8 min. (A) ESL has the retention time of 3.019 min; (B) S-Lic has the retention time of 3.019 min; (C) R-Lic has the retention time of 2.374 min; (D) OXC the retention time of 4.097 min and the internal standard the retention time of 2.298 min.

22 °C nominal, acceleration low, deceleration high. A second cartridge wash was performed with MeCN followed by centrifugation at 3000 rpm for 2 min at 22 °C nominal, acceleration low, deceleration high. Cartridges were then discarded and the eluted sample evaporated in a TurboVap with the temperature control set at 40 °C for approximately 10 min. Residue was reconstitute with 1 mL of n-hexane:isopropyl alcohol (90/10, v/v) solution, vortex mixed and transferred to polypropylene vial to be injected.

2.4. Liquid chromatography and mass spectrometry

The analysis was performed using an LC–MS/MS system consisting of a 1100 HPLC (Hewlett Packard) and an API 3000 MS/MS detector (PE Biosystems). A Chiralcel® OD-H column (50 mm × 4.6 mm, 5 μm, Daicel) was employed. HPLC mobile phase A was n-hexane, mobile phase B was ethanol:isopropyl alcohol (66.7/33.3%, v/v) with a isocratic mixture of 80% mobile phase A: 20% mobile phase B and the HPLC flow rate was 0.8 mL/min. A sample volume of 20 μL was injected. The autosampler cooler was maintained at 4 °C and the column temperature was kept at 30 °C for a run time of 8 min. Electrospray ionisation in positive mode was used for the mass spectrometer methods. The multiple reaction monitoring were m/z 297.3 → 194.4, collision energy of 31 eV for ESL; m/z 255.2 → 194.4, collision energy of 31 eV for S-Lic and R-Lic; m/z 253.2 → 208.3, collision energy of 31 eV for OXC; m/z 239.4 → 194.4, collision energy of 31 eV for 10,11-dihydrocarbamazepine. The retention times for each compound were: 3.02 min for ESL; 3.02 min for S-Lic; 2.37 min for R-Lic; 4.10 min for OXC; 2.30 min for 10,11 dihydrocarbamazepine.

2.5. Validation procedure

Validation of the assay was conducted using calibration standards and QC samples prepared by fortifying human plasma with the analytes. The linearity, accuracy and precision of the assay were assessed in three separate analytical batch runs.

The linearity of the analytical run was assessed using calibration standards at 10 different concentrations within the range of 50.0–1000.0 ng/mL for ESL and OXC and 50.0–25,000.0 ng/mL for R-Lic and S-Lic. Ten point calibration curves were prepared in human plasma for each analyte at concentrations of 50.0, 100.0,

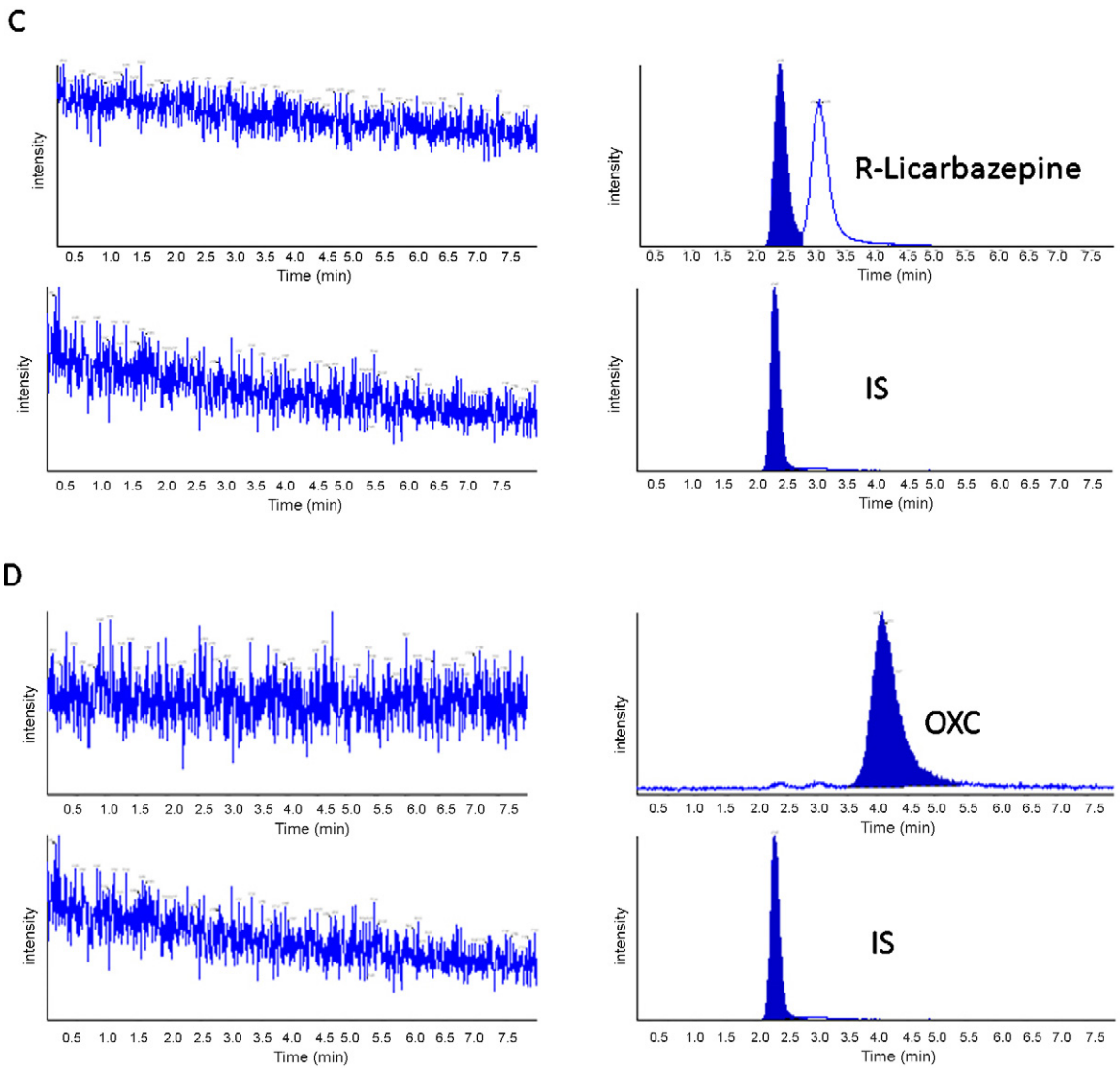

Fig. 2. (*Continued*).

200.0, 300.0, 400.0, 500.0, 600.0, 700.0, 850.0 and 1000.0 ng/mL for ESL and OXC and 50.0, 100.0, 500.0, 1000.0, 3750.0, 6250.0, 10,000.0, 17,504.0, 21,248.0 and 25,000.0 ng/mL for R-Lic and S-Lic. The standard curve from each of 3 batches was regressed separately and the values for QCs were calculated by the internal standard method using peak area ratios. The data were subject to a $1/x$ weighted linear regression for ESL and OXC and $1/x^2$ weighted linear regression for R-Lic and S-Lic. To determine intra-day ($n = 6$) and inter-day ($n = 6$ over a period of 3 separate days) precision and accuracy, analytes were spiked into human plasma at low, mid, and high quality control concentrations. To evaluate the dilution integrity was prepared a QC pool at the concentration twice the upper limit of quantification (DiQC) and diluted 5-fold with blank human plasma before extraction to be quantified over the analytical range validated. Each batch included a standard curve and six replicates of the lowest concentration of the calibration curve (LLOQ) and of each level of low, mid and high QC. For ESL and OXC concentrations of 50.0, 150.0, 450.0, 800.0 and 2000.0 ng/mL (to evaluate dilution integrity) were prepared. For S-Lic and R-Lic the concentrations prepared were 50.0, 150.0, 4500.0, 20,000.0 and 40,000.0 ng/mL (to evaluate the dilution integrity). Precision was calculated from the coefficient of variation (%CV) of replicates and accuracy was calculated by comparison of the measured levels of spiked analyte with expected concentrations (%Bias). A %CV and %Bias of 20% was considered acceptable for accuracy and precision at the LLOQ and for the other concentrations 15% was considered acceptable. Specificity of the assay was confirmed by analyzing 6 different lots of blank human plasma (obtained from different individuals) for chromatographic interferences. Any chromatographic peak at the retention time of the analyte in its SRM channel with an area greater than 20% (5% for internal standard) of the LLOQ sample was considered significant interference. The matrix effect was assessed by employing 3 independent lots of plasma for the preparation of low and the high QC samples. The matrix effect was acceptable if both precision and accuracy criteria were met.

The recovery of ESL, S-Lic, R-Lic, OXC and internal standard was evaluated by comparing the peak response of 6 replicates of extracted QC samples versus the peak response of samples prepared post plasma extraction (samples represent 100% recovery). ESL, S-Lic, R-Lic and OXC were prepared at low, medium and high concentrations and internal standard was prepared at the concentration used in assay. Recovery of the analyte does not need be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible.

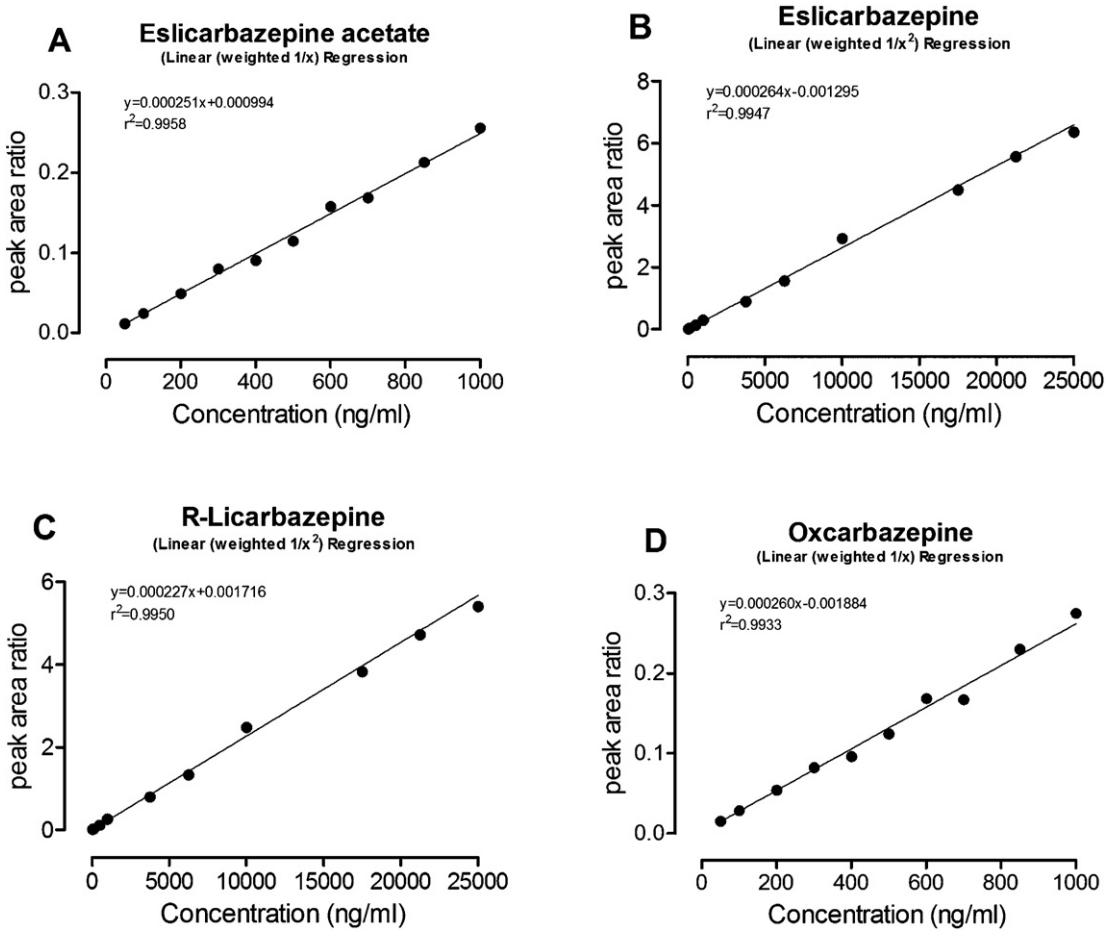

Fig. 3. Representative calibration curves for (A) eslicarbazepine acetate, (B) eslicarbazepine, (C) R-Licarbazepine and (D) oxcarbazepine in human plasma.

2.6. Stability evaluation procedure

Stock solution stability of ESL and metabolites in MeCN was evaluated at the temperatures of 22 °C and −20 °C by comparing 6 replicates of a stability reference solution prepared from a stock solution maintained at tested temperatures (22 °C or −20 °C), versus 6 replicates of a comparison reference solution freshly prepared. The ESL and OXC stock solution stability was assessed at concentrations of 500.0 μg/mL and 50.0 μg/mL, S-Lic and R-Lic stock solution stability was assessed at concentrations of 3.2 mg/mL and 50.0 μg/mL. For internal standard, the stock solution stability was measured at concentrations of 1.0 mg/mL and 500.0 ng/mL. Stability was evaluated over 17 h and 16 days at 22 °C and −20 °C, respectively.

Short-term temperature stability in human plasma samples was assessed by preparing 6 replicates at LQC and HQC levels and kept at room temperature (22 °C) before extraction. Mean concentrations were calculated and compared against nominal concentrations and against 6 replicates at LQC and HQC levels freshly prepared. Freeze–thaw stability was evaluated at LQC and HQC levels (6 replicates each) by freezing samples completely at −20 °C then allowing them to thaw completely at room temperature for three cycles. Samples were extracted with freshly prepared standard curves and QC samples. Mean concentrations of the tested samples were calculated and compared against nominal concentrations and against freshly prepared QC samples. Processed sample/autosampler stability was evaluated by re-injecting extracted LQC and HQC samples (in 6 replicates each) that had been kept in the autosampler for approximately 48 h. Mean concentrations of the re-injected QCs were calculated using a freshly prepared standard curve and QC samples and were compared against nominal concentrations and against freshly prepared QC samples.

Long-term storage stability of each analyte in human plasma at −20 °C was evaluated up to 31 months. The LQC and HQC were extracted in six replicates with a freshly prepared standard curve and QC samples. Mean determined concentrations for the QCs were compared against nominal concentrations and against freshly prepared QCs.

2.7. Sample analysis

Blood samples for the assay of plasma ESL and its metabolites (S-Lic, R-Lic, OXC) were taken from 8 human healthy subjects receiving 600 mg of ESL enrolled in a clinical trial. Samples were taken at the following times: pre-dose, and 1/2, 1, 1 1/2, 2, 3, 4, 6, 8, 12, and 24 h post-dose. Blood samples of 7 mL were taken into lithium heparin tubes and centrifuged at approximately 1500 × *g* for 10 min at 4 °C. The resulting plasma was stored at −20 °C until required for analysis. Samples were analyzed using the method described above.

3. Results and discussion

3.1. Validation results

The product ion fragment and the corresponding structure for each compound analyzed is illustrated in Fig. 1 and an example

Table 3
Mean ($n = 6$) calculated concentrations (ng/mL) of analytes in QC samples in three analytical runs.

	Mean (ng/mL)	S.D. (ng/mL)	CV%	Bias%
ESL				
LLOQ	49.3	4.8	9.7	98.7
LQC	149.8	5.3	3.6	99.8
MQC	469.9	12.0	2.5	104.4
HQC	822.6	22.3	2.7	102.8
DiQC (diluted 5×)	2079.0[a]	22.8	5.5	104.0
Eslicarbazepine				
LLOQ	52.8	4.1	7.7	105.5
LQC	148.0	6.9	4.7	98.7
MQC	4822.8	161.0	3.3	107.2
HQC	20102.0	606.6	3.0	100.5
DiQC (diluted 5×)	53253.0[a]	561.3	5.3	106.5
R-licarbazepine				
LLOQ	51.5	6.2	12.1	102.9
LQC	151.2	11.9	7.9	100.8
MQC	4799.5	128.4	2.7	106.7
HQC	19736.1	836.4	4.2	98.7
DiQC (diluted 5×)	54050.5[a]	660.8	6.1	108.1
OXC				
LLOQ	52.1	3.0	5.7	104.3
LQC	154.5	7.8	5.0	103.0
MQC	461.6	17.6	3.8	102.6
HQC	825.7	32.9	4.0	103.2
DiQC (diluted 5×)	2015.4[a]	24.3	5.9	102.6

[a] Mean × dilution factor of 5.

Table 4
Short-termplasma stability at 22 °C.

	Short-term stability			
	Mean	%CV	%Bias	%Desviation fresh prep. solution
ESL—17.1 h				
LOQ	128.0	4.9	85.3	−9.5
HQC	751.5	5.0	93.9	−8.4
Eslicarbazepine—21.6 h				
LQC	167.0	9.4	111.4	14.6
HQC	20681.1	3.5	103.4	2.0
R-licarbazepine—21.6 h				
LQC	146.9	7.0	97.9	−2.7
HQC	20353.7	2.6	101.8	1.0
OXC—21.6 h				
LQC	131.1	11.6	87.4	−12.7
HQC	706.6	4.1	88.3	−13.0

of chromatograms with the extracted blank and spiked samples is illustrated in Fig. 2.

The linearity of the calibration curves was determined over the specified range shown in Table 1 for ESL and OXC and Table 2 for S-Lic and R-Lic. For each compound, the calibration curve showed a coefficient of determination (R^2) greater than 0.992. The %CV of the standards was ≤7.7% and the %Bias was within ±12.3% for all four compounds at all concentrations. Representative calibration curves for each compound analyzed are presented in Fig. 3.

Table 5
Freeze–thaw plasma stability.

	3-Freez–thaw			
	Mean	%CV	%Bias	%Deviation fresh prep. solution
ESL				
LOQ	142.7	2.9	95.1	−7.4
HQC	798.8	3.1	99.9	−1.9
Eslicarbazepine				
LQC	147.1	3.4	98.0	−0.3
HQC	18742.2	2.3	93.7	−4.7
R-licarbazepine				
LQC	146.0	7.7	97.3	−3.7
HQC	18115.5	2.4	90.6	−4.1
OXC				
LQC	140.3	1.5	93.5	−6.7
HQC	782.9	4.5	97.9	−5.5

Inter-assay accuracy and precision were assessed over three separate days by preparing a daily standard calibration curve along with six replicates of LLOQ, LQC, MQC, HQC and DiQC samples. Analysis of the accuracy and precision is presented in Table 3. The inter-assay %CV was ≤12.1% and the %Bias was within ±7.2% for all the analytes. For the DiQC samples the measured concentrations were multiplied by a factor of 5 to get the original plasma concentrations as shown in Table 3.

The intra-day precision and accuracy in human plasma were determined within a single batch with a %CV ≤ 12.6% and the %Bias was within ±11.5% for all the analytes and all concentrations.

No significant interference was observed at the retention time and mass transition of ESL, S-Lic, R-Lic, OXC or internal standard in any of 6 different batches of human plasma tested.

The three different lots spiked with LQC and HQC show acceptable accuracy and precision which indicate that different matrix samples had no effect on the quantification of the four compounds. The mean relative recoveries ranged from 73.0 to 75.0% for ESL, 75.2 to 80.8% for S-Lic, 72.2 to 82.3% for R-Lic, 69.2 to 72.5% for OXC and were 82.0% for internal standard.

3.2. Sample stability

Stock solution stability of ESL, S-Lic and R-Lic in MeCN were demonstrated at the temperature of 22 °C for at least

Table 6
Long-term plasma stability.

	13 months				18 months				31 months			
	Mean	%CV	%Bias	%Desv[a]	Mean	%CV	%Bias	%Desv[a]	Mean	%CV	%Bias	%Desv[a]
ESL												
LOQ	130.62	3.4	93.3	−4.2	134.36	3.5	96.0	−8.7	131.4	5.2	93.7	−5.7
HQC	754.40	1.7	94.3	−3.8	807.88	5.8	101.0	−2.1	720.65	5.1	90.1	−10.3
Eslicarbazepine												
LQC	141.02	5.0	100.7	1.4	154.11	3.1	110.1	5.6	145.44	7.6	103.9	14.9
HQC	7394.93	4.3	92.4	−2.0	8314.02	6.2	104.0	4.3	8287.57	4.9	103.6	2.7
R-licarbazepine												
LQC	138.99	4.4	99.3	−1.7	159.12	1.8	113.7	2.4	136.32	8.9	97.4	2.6
HQC	7556.38	3.5	94.5	−2.0	8402.82	3.8	105.0	3.8	8128.98	3.7	101.6	0.9
OXC												
LQC	74.61	12.3	53.3	−42.4	79.87	3.0	57.1	−51.7	57.47	7.1	41.0	−56.6
HQC	350.30	4.1	43.8	−56.0	314.98	6.3	39.4	−68.4	192.37	7.5	24.0	−75.4

[a] Deviation from the freshly prepared solution.

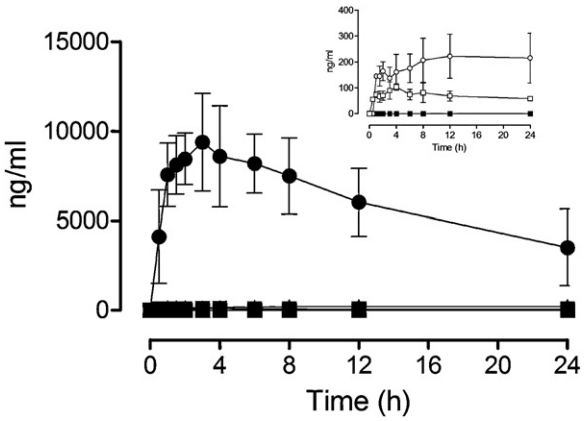

Fig. 4. Mean (±SD) plasma concentration profiles of eslicarbazepine acetate (■), eslicarbazepine (●), R-licarbazepine (○) and oxcarbazepine (□) in healthy male subjects ($n = 8$) after a single oral dose of eslicarbazepine acetate (600 mg). The inset shows plasma levels eslicarbazepine acetate (■), R-licarbazepine (○) and oxcarbazepine (□). The limit of quantification for eslicarbazepine acetate, eslicarbazepine, R-licarbazepine and oxcarbazepine was 50 ng/mL.

17 h. OXC and the internal standard were considered stable in MeCN stored at the temperature of 22 °C for 12 h. In addition, ESL and OXC stock solutions can be stored in MeCN for 7 days at −20 °C, R-Lic and S-Lic for 15 days and the internal standard for 16 days at −20 °C, with acceptable stability.

Overall, ESL, S-Lic, R-Lic, and OXC demonstrated excellent stability in human plasma under the tested conditions, including short-term plasma stability at room temperature (Table 4) for at least 21.6 h (except for ESL which was 17.1 h), freeze–thaw stability (Table 5) for 3 cycles and the processed sample stability at 4 °C for 48 h. The %Bias from nominal concentration was within ±14.7% for all compounds, at all concentrations, under all tested conditions. ESL, S-Lic and R-Lic were considered stable in human plasma stored at −20 °C for 31 months; however, OXC was only considered stable for 8 days with a %Bias from nominal concentration of ±9.3% and a deviation from the freshly prepared samples of −12.4% (Table 6).

3.3. Sample analysis

The analysis of ESL, S-Lic, R-Lic and OXC levels in human plasma samples collected from 8 healthy volunteers show that S-Lic was the major metabolite, OXC and R-Lic were minor metabolites and ESL was below the limit of quantification (Fig. 4). The results indicate that method validation over the analytical range of 50–1000 ng/mL for OXC and ESL and 50–25,000 ng/mL for S-Lic and R-Lic is appropriated to quantify the levels of these compounds present in circulation following an oral administration of 600 mg. In addition, the levels of ESL were always below the limit of quantification, which is compatible with its hydrolysis shortly after absorption.

4. Conclusion

Recently, some studies have shown that S-Lic, R-Lic presented a enantioselectivity in dog and mouse biodisposition [12,13] which encouraged the need for therapeutic drug monitoring of these two enantiomers in patients, using a robust and fast methodology. In this study, a sensitive and selective method for the quantification of ESL, S-Lic, R-Lic and OXC in human plasma based on LC–MS/MS technology was established. The assay demonstrated excellent accuracy, precision, linearity and specificity for the intended purpose of ESL, S-Lic, R-Lic and OXC quantification in clinical trial samples. The stability of analytes was thoroughly investigated in the current study and it was found that in plasma ESL, S-Lic, R-Lic and OXC demonstrated good short-term stability, freeze–thaw stability and long-term stability of 31 months, with the exception of OXC that can only be maintained for 8 days at −20 °C. The limited short stability of OXC does not affect the quantification of the other compounds.

Comparing the method validated herein with recently publications on ESL and metabolites quantification, the use of MS/MS detection provides several advantages over the UV detection: it provides faster sample analysis, higher selectivity and sensitivity allowing improved detection of compounds. Using tandem mass spectrometry, it was possible to unambiguously identify each analyte avoiding interference of contaminates at the same retention time. Importantly, the method described herein is tremendously time-saving since one run last only 8 min compared to 25–30 min using existing methods [7] making it optimal for analysis of clinical samples. In addition, this analytical methodology allows preparation and analysis of larger numbers of samples in a relatively short period of time, which is extremely important considering short-term OXC stability at −20 °C.

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