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ABSTRACT

In the work presented, an high-performance liquid chromatography method was developed and validated for the estimation of seratrodast (SER) in spiked human plasma, using flurbiprofen as an internal standard (IS). The drug and IS were extracted from pooled human plasma sample using 5 mL of tert-butyl methyl ether with 200 µL of 1% v/v formic acid. Both SER and IS were well separated and resolved from each other and plasma interferents using 20 mM phosphate buffer (pH 3.0) with acetonitrile in the proportion of 30: 70% v/v, in an isocratic mode at 1 mL/min on a C18 column and detection was carried out at 267 nm. FLB was retained at 4.656 ± 0.15 min and SER at 7.482 ± 0.18 min, respectively. A linear response was observed over 0.5–11 µg/mL using $1/x^2$ weighted linear regression. Further, the method was validated with respect to selectivity, accuracy, precision, recovery, stability, and carry over as per US-FDA guidance for bioanalytical method validation.

Keywords: Human plasma, High-performance liquid chromatography, Liquid-liquid extraction, method Validation, Seratrodast

INTRODUCTION

Chemically, seratrodast (SER) is 7-phenyl-7-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl) heptanoic acid [Figure 1].[1] It is a potent stereospecific thromboxane A2 and prostaglandin-endoperoxide receptor antagonist. It is used as an antiasthmatic and antithrombotic.[2]

Several analytical methods for estimation of SER in bulk and pharmaceutical dosage forms have been reported in the literature, these include high-performance liquid chromatography (HPLC) methods,[3,4] HPTLC method.[5] Besides these methods, there are several HPLC methods reported for the estimation of SER along with its related substances[6,7] and isolation and structure elucidation of major photodegradation products of SER.[8]

With respect to the reported work, there have been previous attempts to estimate the SER and its metabolites in serum, urine[9] and in plasma[10, 11] by HPLC. Although, these methods claimed to be sensitive to the wide calibration range, however, lack attention on the heteroscedasticity of the calibration data. Most bioanalytical assays usually have to cover a broad concentration range and the variance is more likely to increase at higher concentration ranges. This heteroscedasticity of data reduces the accuracy of the method even with an acceptable regression coefficient.[12] To minimize this, several authors[13-15] suggested the use of weighted regression models with different weighting factors which not only provides better curve fitting with random scatter in residual plots but also, minimizes the variance in slope and intercept for the calibration curve (CC) obtained over serval days with increased accuracy over the selected range.

In the context of this, in the present paper, an attempt was made to develop and validate a simple, economical, accurate, precise, and specific HPLC method for the estimation of SER in spiked human plasma. SER and IS were extracted using tert-butyl methyl ether (TBME) with 1% v/v formic acid. The CC range was selected as per the US-FDA guidance for Bioanalytical Method Validation, May 2018.[16] Weighted regression models in the CC experiment were evaluated using different weighting factors to minimize the heteroscedasticity

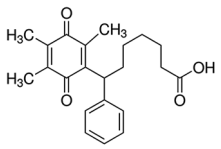

Figure 1: Chemical structure of seratrodast

in the calibration data. Further, the developed method was validated with respect to specificity, accuracy, precision, recovery, and stability of the SER in different stability cycles.

MATERIALS AND METHODS

Reagents and Chemicals

SER was generously supplied as a gift sample by Precise Chemi Pharma Pvt. Ltd., Nashik, India, certified to contain 99.97% *w/w* on a dried basis. Blank human plasma samples from six different sources were procured from Dr. Vasantrao Pawar Medical College, Hospital and Research Center, Nashik, India. The pooled plasma sample was prepared by thoroughly mixing the obtained samples of plasma. Acetonitrile and methanol used in the analysis were of HPLC grade. All the other chemicals used which include TBME and potassium dihydrogen phosphate were of analytical grade. All the above chemicals were purchased from Merck Life Sciences Pvt. Ltd., Mumbai, India. Freshly prepared double distilled water was used in the analysis, which was obtained using the All Glass Double Distillation Assembly, purchased from Borosil India Pvt. Ltd., Mumbai, India. The obtained double distilled water was further filtered through Dura Pore 0.45 μ × 47 mm membrane filter papers, purchased from Millipore India Pvt. Ltd., Bengaluru, India.

Instrumentation and Chromatographic Conditions

The analysis was carried out using a JASCO HPLC System having dual PU-2080 *plus* pumps, Ultraviolet (UV)-2075 multichannel UV detector equipped with Rheodyne (7725*i*) injection system and a 100 μL sample loop. The processing of the obtained analytical data was done using Borwin Chromatography Software (version 1.50).

All separations were carried out using 20 mM phosphate buffer (pH 3.0): acetonitrile (30:70% *v/v*) in an isocratic mode at a flow rate of 1 mL/min. Detection was carried out at 267 nm.

Liquid-Liquid Extraction (LLE)

An aliquot of 1.0 mL of pooled human plasma was taken in a 20 mL stoppered glass tube. To this tube 100 μL of 100 μg/mL SER solution and 100 μL of 100 μg/mL FLB (IS) (certified to contain 99.63%, *w/w*) solutions were added. The entire mixture was vortex mixed for 3 min. To it, 200 μL of 1% formic acid was added and the entire content was vortex mixed for 3 min. At the end, 5 mL of extracting solvent was added to the above mixture and again vortex mixed for 5 min. The tubes were then centrifuged at 3000 rpm for 10 min at 4 ° C in a cooling centrifuge to effect phase separation. The organic layer was then transferred to an Eppendorf tube and the solvent was evaporated under the stream of nitrogen. The residue was further reconstituted with 500 μL of the mobile phase. An aliquot of 100 μL of this solution was then injected into the HPLC system.

Preparation of Standard Stock Solution, CC Standards and Quality Control (QC) Samples

A stock solution of SER and IS (1000 μg/mL) was prepared in methanol. The prepared stock solution of SER was diluted further with methanol to get 10 different working standard solutions of 5, 15, 30, 40, 50, 60, 70, 85, 100, and 110 μg/mL, respectively, and IS was diluted to get 50 μg/mL. To prepare CC standards, 100 μL of above prepared working standard solutions of SER and IS were taken in separate 20 mL glass stoppered tubes containing 1.0 mL of pooled blank plasma samples to get CC standards of 0.5, 1.5, 3, 4, 5, 6, 7, 8.5, 10 and 11 μg/mL, respectively. The obtained CC standards were further processed as per the procedure described in the LLE section and were finally injected into the HPLC system under optimized chromatographic conditions in six replicates. The chromatograms of all the CC standards were processed to obtain peak areas of SER and IS. QC samples were prepared and processed similarly as per the procedures mentioned above. QC samples consist of Low-**QC** (LQC: 1.5 μg/mL), Middle **QC** (MQC: 3 μg/mL) and High- **QC** (HQC: 8.5 μg/mL).

Selection of Regression Model

All chromatograms of CC standards were processed and the peak area ratios of SER to IS were determined and plotted against the respective concentration of SER. Regression models with weighting factors of 1, 1/x, $1/x^2$ and $1/\sqrt{x}$ were evaluated.

Each calibration model was evaluated for the % Relative Error (RE), which was calculated as

$$\%RE = \frac{(Nominal\,Concenration - Interpolated\,Concentration)}{(Nominal\,Concentration)} \times 100$$

The regression model with minimum % RE and uniform scatter over residual plot was selected and further used in analyses.

Validation Study

The developed bioanalytical method was validated as per the US-FDA guidelines for Bioanalytical Method Validation May 2018 with respect to selectivity, accuracy, precision, recovery, stability, and carry over.

Selectivity was evaluated at the lower limit of quantitation (LLOQ) i.e. 0.5 μg/mL. The drug peak area for LLOQ was noted. Similarly, blank plasma samples from six different sources were analyzed and the detector responses of the blank were noted at the retention time of the drug. Accuracy and precision were evaluated by analyzing five batches over five successive days. Each batch consisted of, one blank, all CC standards and five replicates of LQC, MQC, and HQC samples. The accuracy and precision of the

method were evaluated with respect to the % RE and % RSD, respectively. The recovery of the method was estimated by comparing the peak areas of processed QC samples with standard dilutions in five replicates. The stability studies conducted were stability at room temperature, stability at -20° C, bench-top stability, freeze-thaw stability, and long-term stability. The % nominal and % RSD were calculated for each type of stability. In the carry over study, a series of samples were injected into the HPLC system in the order of blank solution, an unextracted upper limit of quantitation (ULOQ), blank solution, extracted blank matrix, extracted ULOQ, and extracted blank matrix.

RESULTS AND DISCUSSION

Different blends of mobile phases were tried to get adequate retention of SER and IS with an acceptable resolution of SER from plasma interferents and from IS. Finally, mobile phase with 20 mM phosphate buffer: acetonitrile (30: 70% v/v) on a C18 column (250 × 4.6 mm, 5 m gave satisfactory retention at 4.656 ± 0.15 min for IS and 7.482 ± 0.18 min for SER, respectively, with acceptable system suitability parameters. The flow rate was kept constant at 1 mL/min and all eluents were detected at 267 nm.

LLE experiments were optimized using different organic solvents with the aim to obtain a clean extract and maximum recovery of SER and IS. From Table 1, it can be observed that good recovery was obtained for SER and IS using 5 mL of TBME with the addition of 200 µL of 1% v/v formic acid compared to other solvents. The chromatograms of extracted blank plasma and SER with IS are depicted in Figure 2a and b, respectively.

The obtained CC standard data [Table 2] when subjected to linear regression, a CC equation of $y = 0.00027x + 0.04452$ with $r^2 = 0.9971$ was obtained. However, when the calibration data were subjected to the test of homoscedasticity [Table 3], the $F_{exp} = 41.92$ was found considerably greater than $F_{critical}$, reveals the heteroscedasticity. Furthermore, when the obtained calibration data were subjected to regression analyses with different weights ($1/x$, $1/x^2$, and $1/\sqrt{x}$), it was observed that the regression model with $1/x^2$ weight was obtained with less % RE.

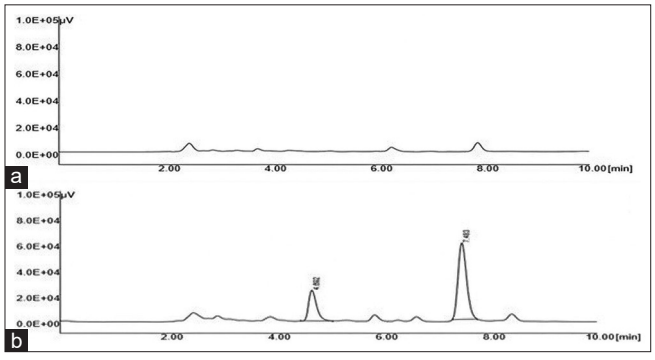

Figure 2: Representative chromatogram of (a) blank plasma (b) seratrodast and internal standard extracted with tert-butyl methyl ether with 1% *v/v* formic acid

The selectivity of the developed method was proved at the LLOQ concentration of 0.5 µg/mL. The chromatogram of blank plasma did not show any significant peak at the retention time of SER and IS. The responses of the blank plasma samples from six different sources were <20% of the LLOQ response [Table 4].

Table 1: Recovery of SER and IS with various organic solvents in LLE

Organic solvent	% Recovery of SER	% Recovery of IS
Ethyl acetate	35.64	29.56
Dichloromethane	24.87	44.75
Chloroform	30.67	31.25
n-hexane	12.54	27.32
Diethyl ether	34.84	35.26
ter-butyl methyl ether (TBME)	49.63	37.52
ter-butyl methyl ether with 1% *v/v* formic acid	54.22	45.04

Table 2: Calibration curve data for SER

Concentration (µg/mL)	Area Ratio (mean±SD) (*n*=6)	%RSD
0.5	0.21±0.01	4.25
1.5	0.48±0.01	7.16
3.0	0.95±0.03	2.02
4.0	1.09±0.03	6.7
5.0	1.43±0.06	4.2
6.0	1.69±0.02	1.72
7.0	1.97±0.03	1.62
8.5	2.43±0.04	6.79
10.0	2.92±0.02	0.62
11.0	3.10±0.05	2.95

Table 3: Regression parameters for unweighted and weighted regression with different weighting factors

Sr. No.	Weighting factor	a	b	% RE	*F (exp)*
1	1	0.04452	0.00027	116.48	41.92
2	1/x	0.06813	0.00028	−6.37×10−13	0.0866
3	1/x2	0.07388	0.00028	−8.57×10−13	0.0002
4	1/√x	0.0595	0.00030	37.33	1.90

* a: Intercept and b: Slope of calibration curve

Table 4: Blank Responses and peak areas SER at LLOQ

Sr. No.	Blank Response (µV.s)	Peak area of SER at LLOQ (µV.s)	% Peak area in Blank
1	1258	78552	1.60
2	1259	76589	1.64
3	1412	75849	1.86
4	1155	78975	1.46

Table 5: Accuracy and precision studies of SER

QC Level	Conc. added (μg/mL)	Inter day (*n*=5) mean conc. found (μg/ml)	%RE	%RSD	Intraday (*n*=5) mean conc. found (μg/ml)	% RE	% RSD
LLOQ	0.5	0.47	−1.89	1.92	0.53	−1.23	1.15
LQC	1.5	1.48	−1.07	1.67	1.45	−2.05	1.18
MQC	3.0	4.14	−3.14	3.19	4.01	0.22	2.80
HQC	8.5	8.53	0.19	1.56	8.57	−2.34	2.31

Table 6: Stability of SER

Stability at Room Temperature	LQC		HQC	
	% Nominal	% RSD	% Nominal	% RSD
2 h	98.81	1.91	101.23	2.33
4 h	98.46	1.17	100.47	1.49
6 h	98.73	1.51	101.62	1.60
Stability at −20°C				
10 Days	99.70	1.01	100.51	1.50
20 Days	99.10	1.12	101.06	1.27
30 Days	99.72	1.04	101.73	1.71
Freeze-thaw stability				
FT1	99.01	1.16	100.51	2.77
FT2	99.34	1.15	101.79	1.91
FT3	98.83	0.74	101.12	1.53
Bench Top Stability	99.76	0.98	101.12	1.07
Long-term stability	98.81	1.48	100.09	1.30

Table 7: Results of Carry-over test for SER

Sr. No.	Samples	Area	
		SER	FLB (IS)
1	Blank DI	0	0
2	AQ-ULOQ	1656942.30	598453.71
3	BLANK DI	0	0
4	AQ-ULOQ	1594422.62	601556.36
5	Blank DI	0	0
6	Blank plasma	0	0
7	Extracted ULOQ	829486.51	267854.24
8	Blank plasma	0	0
9	Extracted ULOQ	827945.20	264846.63
10	Blank plasma	0	0

The developed method was found to be accurate and precise as the % RE obtained was between ±15%and the % RSD was <15%. The results of the accuracy and precision study are shown in Table 5.

From the results of stability studies depicted in Table 6, it can be seen that the % nominal values were between 85 and 115% and the % RSD values were <15% of all the stability samples. Thus, it can be concluded that the drug remained stable after the stability cycles.

When a carry-over study was conducted as per the mentioned sequence for SER, no residue of the previous samples was observed in the subsequent samples. Thus, it can be concluded that no carry-over effect was seen in the developed method [Table 7].

CONCLUSIONS

With the present work, a simple, economical, accurate, precise, and specific HPLC method was developed and validated for the estimation of SER in spiked human plasma. Although the recovery was up to 54.22% and 45.04% for SER and IS, respectively, the clean extract was obtained with good and reproducible recovery when SER was extracted with 5 mL of TBME with 1% *v/v* formic acid. Successful separation of SER from IS was achieved in reversed-phase mode with no interference from the plasma components proved the selectivity of the method. In the previously reported method,[9] the total run time was about 50 min, which was cost and time-intensive. With the present method, the total run time was <10 min. Hence, the developed method was found economic. The observed heteroscedasticity in the linearity experiments was minimized using a weighted linear regression model with a weighting factor of $1/x^2$. The acceptable values of accuracy, precision, stability, and carry-over proved that the method can be used for the estimation of SER in human plasma.

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