



A rapid and sensitive LC–MS/MS assay for the determination of propafenone and its active metabolite 5–hydroxy propafenone in human plasma using hybrid SPE precipitation technology

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ABSTRACT

The quality of sample extraction had a significant impact on mass spectrometry results. The presence of phospholipids in the sample extracts resulted in poor quantitation and also it decreases the method robustness. Here we adopted a novel sample preparation Hybrid SPE phospholipid technology to extract plasma samples for improved phospholipid removal. This new method allowed simultaneous quantification of propafenone and 5-OH propafenone at lower levels 0.5 and 0.25 ng/mL respectively. The phospholipid free filtrate obtained through Hybrid SPE-Phospholipid cartridge was chromatographed onto Gemini C18 column (75 x 4.6 mm, 3.0 μ m). An isocratic mobile phase of a mixture of 10mM ammonium formate (pH 3.0 adjusted with formic acid) and methanol (20:80%V/V) at a flow rate of 0.5 mL/min was used. Precursor ion and product ion transition for analytes and IS were monitored on a TSQ Vantage triple quadrupole mass spectrometer, operated in the positive ionization mode. Method was validated over a concentration range of 0.50-500.00 ng/mL for propafenone, 0.25-250.00 ng/mL for 5-OH propafenone. The intra- and inter-day precision over the concentration range for propafenone and 5-OH propafenone were lower than 6.1 and 14.2% (coefficient of variation, %CV), and accuracy was between 99.5–108.7 and 94.6–108.3%, respectively. By using this new Hybrid SPE-Phospholipid technology the risk of phospholipid accumulation on column was knocked out completely and resulted in good peak shape with excellent column performance.

Keywords: Propafenone, 5-OH propafenone, LC-MS/MS, Hybrid SPE phospholipid technology

INTRODUCTION

Atrial fibrillation is the most common, clinically significant arrhythmia [1, 2]. Propafenone is a chiral antiarrhythmic drug used clinically as a racemate mixture of (S) and (R) propafenone. Although both enantiomers are equally potent in their activity as sodium channel blockers the (S)-enantiomer exhibits β -blocking activity approximately 100 times higher [3]. Propafenone undergoes extensive first pass metabolism by cytochrome P450 2D6 into two active metabolites 5-hydroxypropafenone and N-despropylpropafenone. Both the metabolites have antiarrhythmic activity comparable to that of propafenone with a negligible β -adrenergic activity [4].

Several chromatographic methods including HPLC-UV [5-9], gas chromatography-mass spectrometry (GC-MS) [10] have been reported for the determination of propafenone and 5-OH propafenone in biological matrices. Most of the reported HPLC-UV methods have a very long run time of more than 25 min and have used a high volume of plasma (1 mL) with tedious liquid-liquid extraction steps. The HPLC method developed by Minoo afshar *et al* [11] has used a simple protein precipitation method with a minimal plasma volume of 250 μ l but the run time was 25 min.

Among the published LC-MS/MS methods [12-14] the method developed by Liping Pan *et al* [15] is the only method which has short run time of 6 min and with a limit of quantification of 1.5 ng/mL for propafenone from sustained release formulations. This method was developed in beagle dog plasma and didn't focus on separation and quantitation of its active metabolite 5-OH propafenone.

To the best of our knowledge this is the first LC-MS/MS method developed for the simultaneous quantification of propafenone and its active metabolite 5-OH propafenone in human plasma in a short runtime of 4 min and with limit of quantification of 0.5 and 0.25 ng/mL respectively. Hybrid-SPE-Precipitation technology was used as sample preparation technique to achieve a sensitivity which is 10 folds higher compared to recently published method.

MATERIALS AND METHODS

Chemicals and materials

The reference sample of propafenone hydrochloride (>98.0%) and 5-hydroxy propafenone hydrochloride (99.75%) were purchased from Clear synth labs (Mumbai, India) and Propafenone d5 hydrochloride (98.22%) from Vivan Lifesciences Ltd, India and their chemical structures were shown in Fig 1. Hybrid-SPE phospholipid cartridges were purchased from Sigma (Sigmaaldrich, UK). Water used for the LC-MS/MS analysis was collected from Milli Q water purification system procured from Millipore (Bangalore, India). HPLC grade acetonitrile and methanol were purchased from J.T Baker (Phillipsburg, USA). Analytical grade formic acid and ammonium formate were purchased from Merck, (Mumbai, India). The control human plasma sample was procured from Deccan's Pathological Labs, (Hyderabad, India).

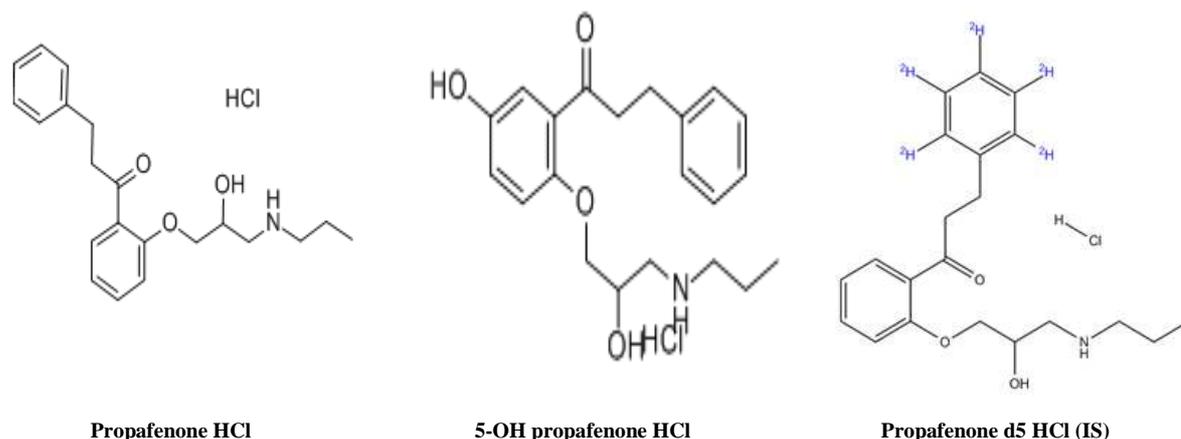


Fig.1 Chemical structures of propafenone, 5-OH propafenone and internal standard

Chromatographic conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a binary LC-20AD prominence pump and an auto sampler (SIL-HTc) and a solvent degasser (DGU-20A3) were used for the study. The separation of analytes was performed on Gemini C18 column (75 x 4.6 mm, 3.0 μ m) (Phenomenex, Cheshire, UK). Aliquots of the processed samples (20 μ L) were injected onto the column, which was maintained at 35°C. An isocratic mobile phase of a mixture of 10mM ammonium formate (pH 3.0 adjusted with formic acid) and methanol (20:80% V/V) was delivered at a flow rate of 0.5 mL/min into the mass spectrometer.

Mass spectrometer conditions

A TSQ Vantage triple stage quadrupole mass spectrometer (Thermo Scientific, USA) with a heated electro spray ionization (HESI-II) probe operated in positive ion mode at a spray voltage of 4.0 kv, capillary temperature of 270 °C and vaporizer temperature of 350 °C. Sheath and auxiliary gas pressures were set at 50 and 10 units respectively. Compound optimisation was done manually using Thermo TSQ Tune Master 2.1.0.1028 (Thermo Scientific, USA) by infusion into the mass spectrometer and the scan parameters were shown in Table 1. Selective Reaction Monitoring (SRM) mode was used for data acquisition. Peak integration and calibration were carried out using LC Quan 2.5.2 software.

Table 1: Mass Spectrometry Parameters

Analyte	Parent ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Collision Energy (V)	Tube lens
Propafenone	342.2	116.1	27	105
5-OH propafenone	358.3	116.2	31	95
Propafenone-d5	347.1	121.1	28	105

Preparation of stock, working solutions, calibration and quality control samples

Primary stock solutions (1 mg/mL) of propafenone, 5-hydroxy propafenone, and internal standard were prepared in methanol separately and these stocks were stored at 2–8 °C. Two sets of working solutions containing a mixture of propafenone and 5-OH propafenone were prepared in methanol and water (50:50, v/v; diluent) separately for the preparation of calibration standards and quality control samples.

Calibrations standards (CC) containing a mixture of two analytes were prepared by 2% addition of working solution in human blank plasma (20 µL of working solution was added to 1 mL human plasma) giving final concentrations of 0.500, 1.270, 3.750, 12.500, 24.990, 49.980, 99.960, 249.900, 425.000, 500.000 ng/mL for propafenone and 0.250, 0.640, 1.870, 6.250, 12.500, 24.990, 49.980, 124.950, 212.950, 250.000 ng/mL for 5-hydroxy propafenone. The CC samples were analyzed along with the quality control (QC) samples for each batch of plasma samples. The QC samples were prepared at five different concentration levels of 0.520 (lower limit of quantification, LLOQ), 1.350 (low quality control, LQC), 26.800 (middle quality control, MQC–1), 125.000 (MQC–2) and 375.000 (high quality control, HQC) ng/mL for propafenone and 0.260 (LLOQ), 0.700 (LQC), 12.500 (MQC–1), 65.800 (MQC–2) and 187.500 (HQC) ng/mL for 5-hydroxy propafenone. All the prepared plasma samples were stored at -40 ± 10 °C. A separate working solution of internal standard (100 ng/mL) was also prepared in the diluent.

HybridSPE-Phospholipid Technology (Sample preparation)

HybridSPE-Phospholipid cartridges were used for effective removal of phospholipids and for accurate and reproducible LC-MS/MS Analysis. Protein precipitation was performed offline by adding 100 µL of spiked plasma followed by 300 µL of 1% formic acid in acetonitrile containing internal standard (100 ng/mL) into 1.5 mL eppendorf tube. Samples were thoroughly mixed up by vortexing for 30 sec. 400 µL of the above mixture was transferred into a Hybrid SPE cartridge. Samples were passed through the Hybrid SPE cartridge by applying a positive vacuum. The filtrate was analyzed directly using the analytical conditions.

Method validation

The validation of the above method was carried out as per US FDA guidelines [16]. The parameters included selectivity, specificity, sensitivity, matrix effect, linearity, precision and accuracy, recovery, dilution integrity, and stability.

RESULTS AND DISCUSSION**Method development**

The goal of this work is to develop a simple, rapid and a sensitive LC-MS/MS method for the simultaneous determination of propafenone and 5-hydroxy propafenone suitable for pharmacokinetic and bioequivalence studies. Hence, during method development different options were evaluated to optimize detection and chromatography parameters. Tuning was done in positive and negative ionization modes using ESI source. The signal intensities obtained in the positive ion mode was much higher for the analytes than the negative mode. Data in the SRM mode were considered, which showed better selectivity.

Once the mass spectrometer conditions were set, chromatographic conditions such as mobile phase, flow rate, column type and injection volume were monitored to obtain the good resolution from the endogenous components which in turn affect sensitivity and reproducibility of the method. The mobile phase composition was optimized with acetonitrile and methanol by varying its proportion with volatile buffers like ammonium acetate, ammonium formate as well as acid additives like acetic acid and formic acid in varying strength. Symmetric peak shape, better separation and best sensitivity were achieved with 10mM Ammonium formate (pH 3.0 adjusted with formic acid): Methanol (20:80% V/V) as the mobile phase. Among the various chromatographic columns tested for their suitability Gemini C₁₈, 75 × 4.6 mm, 3.0µm column gave good peak shape and response even at lowest concentration level for both the analytes. The retention time of propafenone, 5-hydroxy propafenone, and internal standard (2.18, 1.71, and 2.17 min, respectively) were short enough allowing a small run time of 4.0 min with a mobile phase flow rate of 0.5 mL/min. Stable labeled internal standard is suggested for bioanalytical assays to increase assay precision and limit variable recovery between analyte and the IS. Propafenone-d5 was used as internal standard in this method.

When used a standard protein precipitation method with acetonitrile or methanol with and without acid resulted in increased back pressure of the column after few injections. Steps were taken to back flush the column before start of each run. This didn't improve the performance of column and this explains the elution of phospholipids intermittently from the column affecting the reproducibility and peak shape. Liquid Liquid extraction with different extraction solvents yielded poor recovery for 5-OH propafenone. Before moving onto Solid phase extraction for better cleaner extracts a new technology for removal of phospholipids namely HybridSPE-Phospholipid which combines the simplicity of protein precipitation with the selectivity of solid phase extraction (SPE) for the targeted removal of phospholipids in biological plasma was evaluated. The mean recovery was more than 80% for both propafenone and 5-OH propafenone which was more than the LLE and S/N ratio was 10 times higher compared to standard protein precipitation, suggesting that the risk of phospholipid accumulation was knocked out completely and resulted in good recovery with excellent column performance using Hybrid SPE-Phospholipid technology.

Method Validation

Selectivity

The selectivity of the method was examined by analyzing extracted blank human plasma samples from six different sources. As shown in Fig.2 and 3 for individual analytes, no significant interference in the processed blank plasma samples were observed at the retention times of the analytes and internal standard.

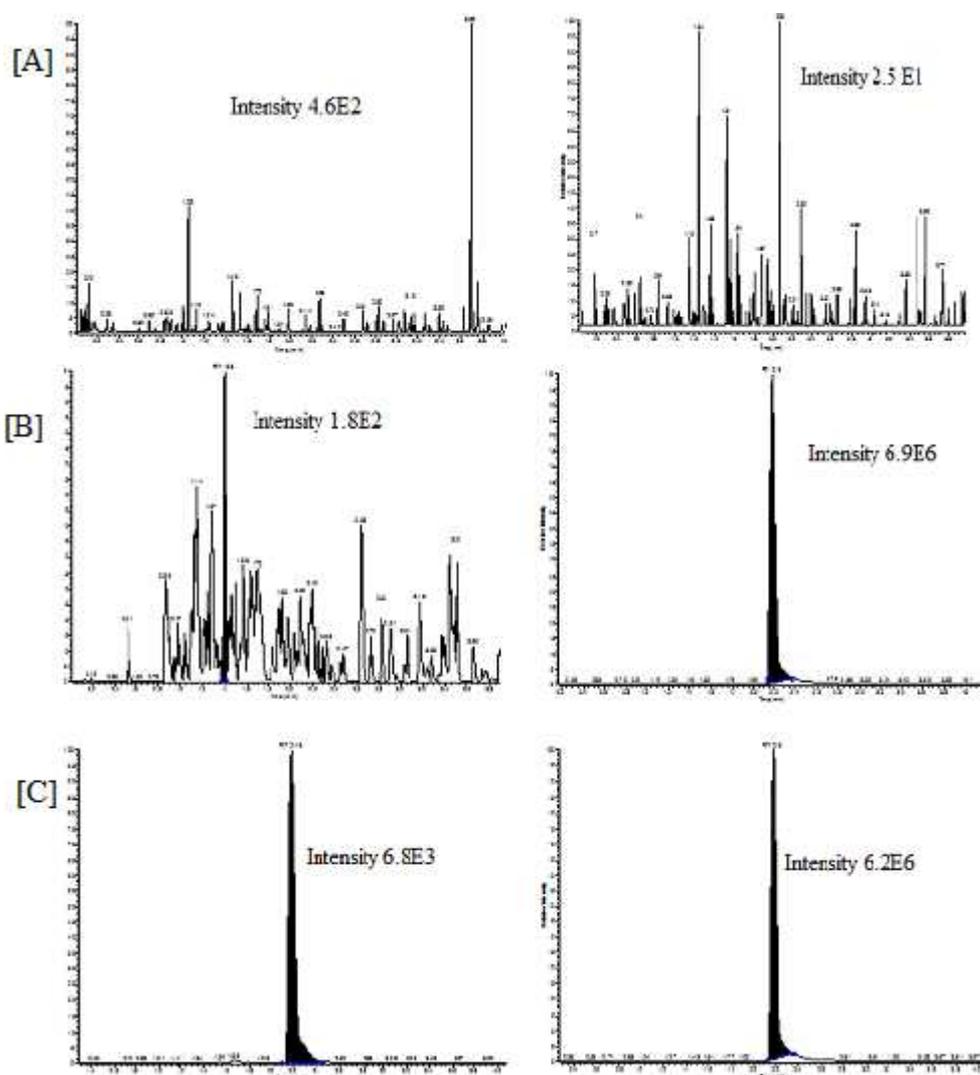


Fig.2. Typical chromatogram of propafenone (left panel) and IS (right panel) in human blank plasma [A], plasma spiked with internal standard [B] and lower limit of quantification sample along with IS [C]

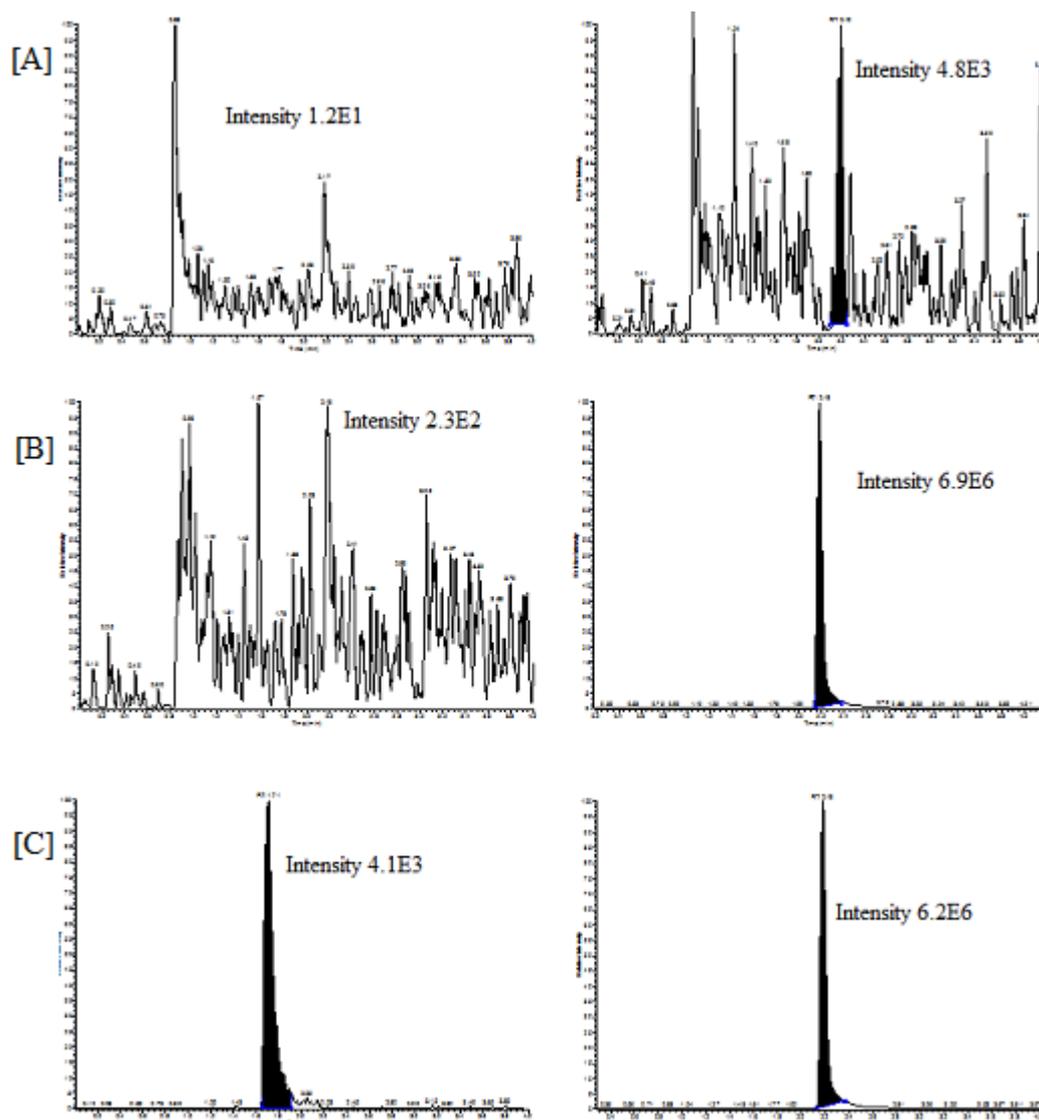


Fig.3. Typical chromatogram of 5-OHpropafenone (left panel) and IS (right panel) in human blank plasma [A], plasma spiked with internal standard [B] and lower limit of quantification sample along with IS [C]

Sensitivity

The reliable lowest limit of quantification (LLOQ) for the propafenone and 5-hydroxy propafenone was set at the concentration of 0.5 ng/mL and 0.25 ng/mL, respectively. At this concentration, the precision and accuracy results were found to be 5.38% and 106.2% and 8.17% and 99.8% for propafenone and 5-hydroxy propafenone. The signal-to-noise ratio (S/N) was measured at LLOQ concentration and found to be ≥ 5 for both the analytes.

Matrix effect

Matrix effect assessment was done with the aim to check the effect of different lots of plasma on the back calculated value of QC's nominal concentration. The precision and accuracy for propafenone at LQC concentration were found to be 5.28% and 107.2%, and at HQC level they were 4.34% and 108.2%, respectively. Similarly, the precision and accuracy for 5-hydroxy propafenone at LQC concentration were found to be 8.07% and 108.3%, and at HQC level they were 4.92% and 97.9%, respectively. These results indicate that no significant matrix effect was observed in all the six batches of human plasma for the analytes at low and high quality control concentrations.

Linearity, Precision and Accuracy

Ten-point calibration curve was found to be linear over the concentration range of 0.50–500 ng/mL for propafenone 0.25–250 ng/mL for 5-hydroxy propafenone. After comparing the two weighting models ($1/x$ and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration–detector response relationship for both the analytes in human plasma. The mean correlation

coefficient of the weighted calibration curves generated during the validation was ≥ 0.99 for both the analytes.

The results for intra-day and inter-day precision and accuracy in plasma quality control samples are summarized in Table 2. The intra-day and inter-day precision values were within 15% of the relative standard deviation (RSD) at low, middle 1, middle 2 and high quality control level, whereas LLOQ QCs level was within 20%. The intra-day and inter-day accuracy deviation values were within $100 \pm 15\%$ of the actual values at low, middle 1, middle 2 and high quality control level, whereas LLOQ QCs level was within $100 \pm 20\%$. The results revealed good precision and accuracy.

Table 2: Intra day and inter day precision and accuracy of the method

Analyte	Conc. (ng/mL)	Intra-day Precision and Accuracy (n=12; 6 from each batch)			Inter-day Precision and Accuracy (n=30; 6 from each batch)		
		Conc. Found (Mean \pm SD; ng/mL)	Precision (%CV)	Accuracy (%)	Conc. Found (Mean \pm SD; ng/mL)	Precision (%CV)	Accuracy (%)
Propafenone	0.520	0.5159 \pm 0.02339	4.53	99.2	0.5174 \pm 0.03168	6.12	99.5
	1.350	1.3936 \pm 0.08118	5.83	103.2	1.3938 \pm 0.07837	5.62	103.2
	26.800	27.4575 \pm 1.67744	6.11	102.4	27.3270 \pm 1.52385	5.52	101.9
	125.000	135.9283 \pm 4.12760	3.04	108.7	134.2490 \pm 5.15488	3.84	107.4
	375.000	389.6733 \pm 18.87515	4.84	103.9	390.5323 \pm 18.3624	4.70	104.1
5-OH Propafenone	0.260	0.2530 \pm 0.03061	12.10	97.3	0.2580 \pm 0.01691	6.55	99.2
	0.700	0.7482 \pm 0.10682	14.28	106.8	0.7284 \pm 0.05307	7.29	104.0
	12.500	13.0683 \pm 1.04510	8.00	104.5	13.4524 \pm 0.94297	7.01	107.6
	65.800	71.2833 \pm 3.77403	5.29	108.3	69.6413 \pm 3.48566	5.01	105.8
	187.500	184.5042 \pm 19.05371	10.33	98.4	177.4487 \pm 9.30913	5.25	94.64

Recovery and dilution integrity

The recoveries of analytes and the internal standard were good and reproducible. The mean recovery (%) was 85.05, 80.22 and 87.58% for propafenone, 5-OH propafenone and internal standard with CV (%) less than 5%.

Table 3: Stability data for propafenone and 5-OHpropafenone

Analyte	Stability test	QC (spiked conc. (ng/mL))	Mean \pm SD (ng/mL)	Precision (%CV)	Accuracy/Stability (%)
Propafenone	Bench top ^a	1.350	1.3983 \pm 0.05670	4.05	103.5
		375.000	403.0500 \pm 33.04217	8.20	107.4
	Freeze Thaw ^b	1.350	1.3248 \pm 0.11838	8.94	98.1
		375.000	405.2500 \pm 22.76015	5.62	108.0
	Wet Extract ^c	1.350	1.2962 \pm 0.05388	4.16	96.0
		375.000	384.8370 \pm 45.44420	11.81	102.6
	Auto Sampler ^d	1.350	1.2587 \pm 0.08864	7.04	93.2
		375.000	359.4095 \pm 36.28007	10.09	95.8
	Re injection ^e	1.350	1.4033 \pm 0.01366	0.97	101.3
		375.000	403.8767 \pm 25.26407	6.26	95.4
	Long term ^f	1.350	1.3367 \pm 0.14685	10.99	99.0
		375.000	388.3633 \pm 47.80075	12.31	103.5
5-OH Propafenone	Bench top ^a	0.700	0.6880 \pm 0.05217	7.58	98.29
		187.500	178.6317 \pm 10.32239	5.78	95.27
	Freeze Thaw ^b	0.700	0.7302 \pm 0.03173	4.35	104.31
		187.500	177.9167 \pm 14.17185	7.97	94.89
	Wet Extract ^c	0.700	0.6840 \pm 0.08473	12.39	97.71
		187.500	177.8133 \pm 16.22439	9.12	94.83
	Auto Sampler ^d	0.700	0.7100 \pm 0.04599	6.48	101.43
		187.500	206.3133 \pm 14.05315	6.81	110.03
	Re injection ^e	0.700	0.7065 \pm 0.02561	3.62	103.9
		187.500	187.1900 \pm 7.89803	4.22	101.6
	Long term ^f	0.700	0.6682 \pm 0.04208	6.30	95.4
		187.500	197.8887 \pm 21.84971	11.40	105.5

^a after 7 h at room temperature; ^b after 3 Freeze thaw cycles; ^c after 50 h at 2–8°C; ^d after 72 h at 2–8°C; ^e after 52 h at 2–8°C; ^f at –40°C for 45 days

The upper concentration limits can be extended to 875.0 ng/mL for propafenone and 437.5 ng/mL for 5-hydroxy propafenone and further diluted by 1/2 and 1/4 dilutions with screened human blank plasma. The precision and accuracy for propafenone at 1/2 dilution were found to be 4.55% and 92.6%, and at 1/4 dilution they were 6.35% and 90.6%, respectively. Similarly, the precision and accuracy for 5-hydroxy propafenone at 1/2 dilution were found to be 3.98% and 106.7%, and at 1/4 dilution they were 1.67% and 111.5%, respectively.

Stability studies

Various stability experiments carried out namely bench top stability (7 h), autosampler stability (72 h), repeated freeze-thaw cycles (3 cycles), reinjection stability (52 h), wet extract stability (50 h at 2–8 °C) and long-term stability at –40 °C for 45 days. The mean % nominal values of the analytes were found to be within $\pm 15\%$ of the predicted concentrations for the analytes at their LQC and HQC levels (Table 3). Thus, the results were found to be within the acceptable limits during the entire validation.

CONCLUSION

Sample preparation plays an important role to address problems encountered during method development for quantification of small molecules from biological samples by LC-MS/MS. Matrix effect due to endogenous phospholipids results in poor sensitivity and specificity. Here we adopted a new sample preparation technique called Hybrid SPE phospholipid for cleaner extracts. The proposed method has several advantages compared to standard protein precipitation and LLE and also this is a simple single step method and can be used as alternative to Solid phase extraction for sample clean up to eliminate matrix related effects on LC-MS/MS with improved S/N ratio.

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