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DEVELOPMENT AND VALIDATION OF CHROMATOGRAPHIC TECHNIQUE FOR ANALYSIS OF PROPAFENONE

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Abstract

Objective: A simple, sensitive and rapid performance liquid chromatography/positive ion electrospray tandem mass spectrometry method was to be developed and validated for quantification of propafenone (PPF) and its major metabolite 5-hydroxy propafenone (5-OHP) in human plasma.

Methods: L Analytes were separated on an Thermo Betabasic C8, 100 mm X 4.6 mm, 5µ, column maintained at temperature of 40°C. An isocratic flow-rate of 1.0 mL/minute with 1:1 splitted post column with mobile phase Methanol and MilliQ water (80:20 v/v) with 0.1% formic acid of total volume was used for chromatographic separation.. The multiple reaction monitoring transitions were set at 342.2 > 116.2 (m/z), 358.2 > 116.2 (m/z), and 349.2 > 123.2 (m/z) Propafenone, 5-Hydroxypropafenone and Propafenone D7 respectively.

Results: The analytical method described above is valid for the analysis of Propafenone , and 5-Hydroxypropafenone in human plasma over a range of 0.499 ng/mL to 1502.841 ng/mL and 0.496 ng/mL to 504.079 ng/mL, respectively, in human plasma.

Conclusion: The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetics, bioavailability and bioequivalence studies.

Keywords: Stability, 5-hydroxy propafenone,Validation, LC-MS

INTRODUCTION

Propafenone hydrochloride (PPF) is an antiarrhythmic drug with a phenyl propanolamine nucleus that is common to various beta blocking agents. PPF is known to be effective in the treatment of supraventricular and ventricular arrhythmias [1]. It is known to act by blocking the fast inward sodium impulse in the cardiac muscle and all other excitable tissues. PPF also possesses some beta blocking action as well as a weak calcium channel blocking effect [2]. PPF is primarily metabolized by hydroxylation of the ring to form 5-hydroxy propafenone [3] (5-OHP). The excretion of PPF is mainly in the form of glucuronide and sulfate conjugates of PPF, 5-OHP and NDP [4]. While the primary metabolite 5-OHP exerts pharmacological activity equivalent to the parent drug [5].

Several methods have previously been reported for the determination of PPF and its metabolites in human plasma using liquid-liquid extraction and solid phase extraction techniques [6-9]. Most of these methods either have very long run times or tedious extraction processes involved for analysis of the sample mixture. A few methods including the one developed by Lipig Pan *et al.* [10] and Sheshagiri Rao *et al.* [11] are known to have smaller run times of 6 and 4 min respectively. This paper describes a very simple liquid chromatography-mass spectroscopy (LC-MS) method for estimation of PPF, 5-OHP and NDP in human plasma using liquid-liquid

extraction technique and a very short acquisition time using amlodipine besylate as the internal standard (IS).

EXPERIMENTAL PROCEDURES

Instrumentation

Shimadzu HPLC equipped with dual pump auto sampler and Column Oven, Mass spectrometer AB SCIEX API 5500 LC-MS/MS and data acquisition system Software Analyst were used for the quantitative determination of Propafenone and 5- Hydroxypropafenone in human plasma.

Reagents / Materials

The reagents / materials used during analysis include Acetonitrile (HPLC Grade), Methanol (HPLC Grade), Formic acid (AR Grade), Milli-Q Water / HPLC Grade water

.Analytical standards of Propafenone Hydrochloride, 5-Hydroxypropafenone Hydrochloride, and Propafenone D7 HCl with >98% purity were used.

Stock Solutions

Propafenone Stock Solution

Approximately 2 mg of Propafenone Hydrochloride was weighed and transferred to 2 mL volumetric flask to make approximately 1.0 mg/mL stock solution with HPLC grade methanol. This stock solution was transferred to a reagent bottle, labeled and stored at 2°C to 8°C. Further dilutions of Propafenone were prepared in dilution solution for spiking in plasma. This solution was used within 7 days from the date of preparation.

5-Hydroxypropafenone Stock Solution

Approximately 2 mg of 5-Hydroxypropafenone Hydrochloride was weighed and transferred to 2 mL volumetric flask to make approximately 1.0 mg/mL stock solution with HPLC grade methanol. This stock solution was transferred to a reagent bottle, labeled and stored at 2°C to 8°C. Further dilutions of 5-Hydroxypropafenone were prepared in dilution solution for spiking in plasma. This solution was used within 7 days from the date of preparation.

Propafenone D7 Stock Solution

Approximately 2 mg of Propafenone D7 Hydrochloride was weighed and transferred to 2 mL volumetric flask to make approximately 1.0 mg/mL stock solution with HPLC grade methanol. This stock solution was transferred to a reagent bottle, labeled and stored at 2°C to 8°C. This solution was used within 9 days from the date of preparation.

Further stock dilutions of Propafenone D7 were prepared in dilution solution to get desired concentration to be used as internal standard dilution. All other dilutions (e.g. aqueous mixture, recovery dilutions etc.) of Propafenone and 5-Hydroxypropafenone were prepared using mobile phase.

Biological Matrix

Human plasma batches for Propafenone and 5-Hydroxypropafenone were commercially procured from blood bank. These plasma batches containing K_2EDTA as anticoagulant were chromatographically screened and those found free of any significant interference were pooled and used to prepare CC standards and QC samples.

Buffers and Solutions

Rinsing Solution

A mixture of acetonitrile and Milli-Q water was prepared in the volume ratio of 70:30 as rinsing solution. It was sonicated in ultrasonicator for approximately 5 to 10 minutes. The solution was labeled and used within 4 days from the date of preparation.

Mobile phase

A mixture of methanol and milli-Q-water solution was prepared in the volume ratio of 80:20, in which 0.1% of formic acid of total volume was added. It was then sonicated in ultrasonicator for 5 to 10 minutes and labelled appropriately. The solution was used within 4 days from the date of preparation.

Dilution solution

A mixture of Methanol and milli-Q water was prepared in the volume ratio of 50:50. It was sonicated in ultrasonicator for approximately 5 to 10 minutes. The solution was labeled and used within 4 days from the date of preparation.

Ammonia solution

2 mL of ammonia (25%V/V) was transferred into 100 mL volumetric flask and diluted with milli-Q-water up to the mark. It was then sonicated in ultrasonicator for 5 to 10 minutes. The solution was labelled appropriately. It was used within 4 days from the date of preparation.

Calibration Curve (CC) Standards and Quality Control (QC) Samples Calibration curve standards of Propafenone and 5-Hydroxypropafenone, concentrations ranging from 0.499 ng/mL to 1502.841 ng/mL and 0.496 ng/mL to 504.079 ng/mL, were prepared respectively. The QC samples for Propafenone at concentrations of 0.499 ng/ mL (LLOQ QC), 1.348 ng/mL

(LQC), 245.08 ng/mL (M1QC), 612.697 ng/mL (MQC) and 1141.398 ng/mL (HQC) were prepared. The QC samples for 5- Hydroxypropafenone at concentrations of 0.496 ng/mL (LLOQ QC), 1.478 ng/mL (LQC), 83.900 ng/mL (M1QC), 214.030 ng/mL (MQC) and 433.210 ng/mL (HQC) were prepared. These samples were stored in deep freezer below –50°C until use.

Chromatographic Conditions

Analytes were separated on an Thermo Betabasic C8, 100 mm X 4.6 mm, 5µ, column maintained at temperature of 40°C. An isocratic flow-rate of 1.0 mL/minute with 1:1 splitted post column with mobile phase Methanol and MilliQ water (80:20 v/v) with 0.1% formic acid of total volume was used for chromatographic separation. Following 5µL injection analytes were separated. The overall run time was around 3.5 min. A mixture of acetonitrile and Milli-Q water in the volume ratio of 70:30 was used as needle wash solution.

Mass Spectrometry

Mass spectrometric detection was performed on an Applied Biosystems MDS Sciex (Concord, Ontario, Canada) API 5500 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface. Electro spray ionization was performed in the positive ion mode. The tandem mass spectrometer was operated at unit resolution in the selected reaction monitoring mode (SRM), The multiple reaction monitoring transitions were set at 342.2 > 116.2 (m/z), 358.2 > 116.2 (m/z), and 349.2 > 123.2 (m/z) Propafenone, 5-Hydroxypropafenone and Propafenone D7 respectively (Figure 3.1,3.2a&b).

The mass spectrometric conditions were optimized for Propafenone,5- Hydroxypropafenone and Propafenone D7 by continuous infusion of the standard solution at the rate of 10 µL min^{-1} using a Harvard infusion pump. The ion source temperature was maintained at 500°C. The ion spray voltage was set at 5,000 V. The curtain gas (CUR) was set at 40 and the collision gas (CAD) at 7. The optimal collision energy (CE) was 21 V. The following most favorable parameters of ion path were used for analysis, declustering potential (DP) at 50 V and entrance potential (EP) at 10V. The quantification was performed via peak area ratio. Data acquisition and processing were accomplished using the Applied Biosystems Analyst software. Calibration curves were generated using peak area ratios of the components to internal standards versus the known concentrations with a linear regression equation of $1/concentration^2$.

Chromatographic Conditions summary:

Column	: Betabasic C8, 100 mm X 4.6 mm, 5µ,
Mobile Phase	: Methanol: MilliQ water (80:20 v/v) with 0.1% formic acid
Rinsing solution	: Acetonitrile: Milli-Q water (70:30 v/v)
Flow Rate	: 1.0 mL/minute with the splitter (1:1 v/v)
Column Oven Temperature	: 40°C ± 1°C
Injection Volume	: 5 µL

Mass Spectrometric Parameters summary

Mass spectrometer	: LC-MS/MS (API 5500) Ion
source	: Turbo V Source
Polarity	: Positive
Mass Transitions (Parent / Fragment) m/z	
Propafenone	: 342.2 / 116.2
5-Hydroxypropafenone	: 358.2 / 116.2
Propafenone D7	: 349.2 / 123.2

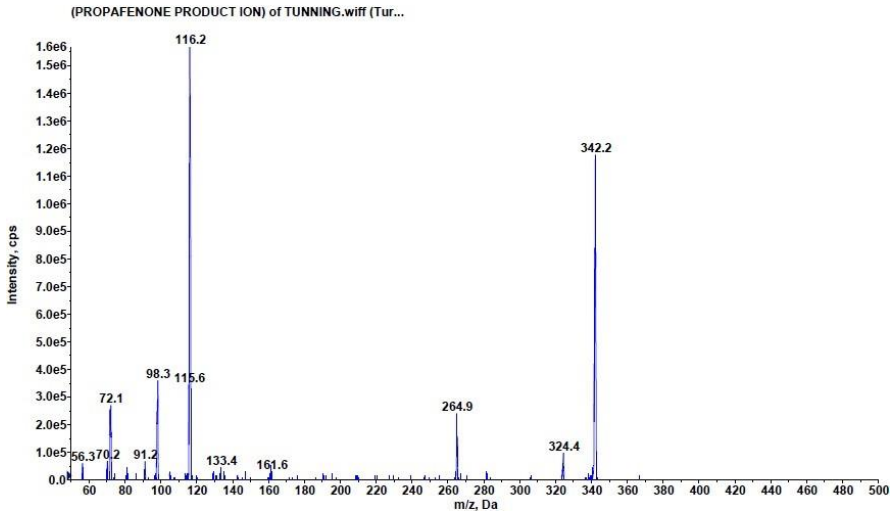

Figure 3.1 Product ion spectra of Propafenone

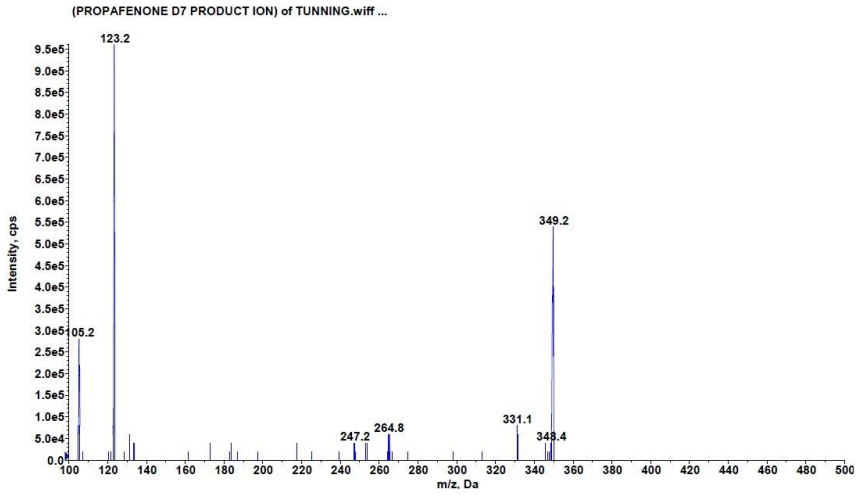

Figure 3.2a Product ion spectra of Propafenone D7

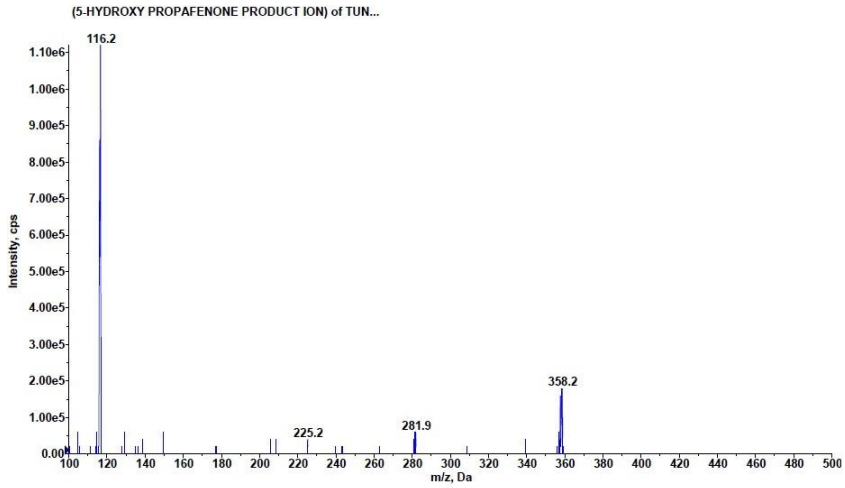

Figure 3.2b Product ion spectra of 5-Hydroxypropafenone

Sample extraction procedure

The previously spiked plasma samples were retrieved from the deep-freezer and thawed in a water bath at room temperature. The thawed samples were vortexed to ensure complete mixing of the contents. A 50 μL of internal standard dilution (approximately 500 ng/mL of Propafenone D7) was taken in prelabeled polypropylene tubes except in blank samples wherein 50 μL of dilution solution was added. A 250 μL of the sample was added to it and tubes were vortexed followed by addition of 100 μL of ammonia solution and vortexed

again. 2 ml of tertiary butyl methyl ether was added into the above samples and vortexed for approximately 1 minute. It was centrifuged for 2 minutes at 4090 rcf, 10°C. 1 mL supernatant was taken out into prelabeled glass tubes. The supernatant was dried at 50°C under the stream of nitrogen and reconstituted with 500 µL of mobile phase. It was transferred into auto sampler vials and analyzed using LC-MS/MS system.

Note: Propafenone and 5-Hydroxypropafenone are light sensitive hence all sample processing related activities were carried out under monochromatic light conditions.

METHOD VALIDATION RESULTS

Selectivity

Representative chromatogram of extracted blank plasma sample is given in Figure 3.3 a&b for Propafenone and Figure 3.3 c&d for hydroxypropafenone, any significant interference from endogenous components was not observed at retention time of analyte and IS in all the human plasma batches screened.

Sensitivity

Propafenone (Table 3a)

The lowest limit of quantification (LLOQ) was set at the concentration of 0.499 ng/mL. The precision and accuracy at LLOQ were found to be 8.91 % and 101.2 % respectively (Figure 3.4 a&b).

5-Hydroxypropafenone (Table 3b**)**

The lowest limit of quantification (LLOQ) was set at the concentration of 0.490 ng/mL. The precision and accuracy at LLOQ were found to be 5.06 % and 98.79 % respectively (Figure 3.4 c&d).

Matrix Effect

Propafenone (Table 1a)

It ensures that method reproducibility is not affected in different matrix lots due to ion suppression or ion enhancement. This unintended peak area response alteration appears due to co-eluting matrix component at the retention time and MRM of the analyte(s) and internal standard is interferes with analyte peaks.

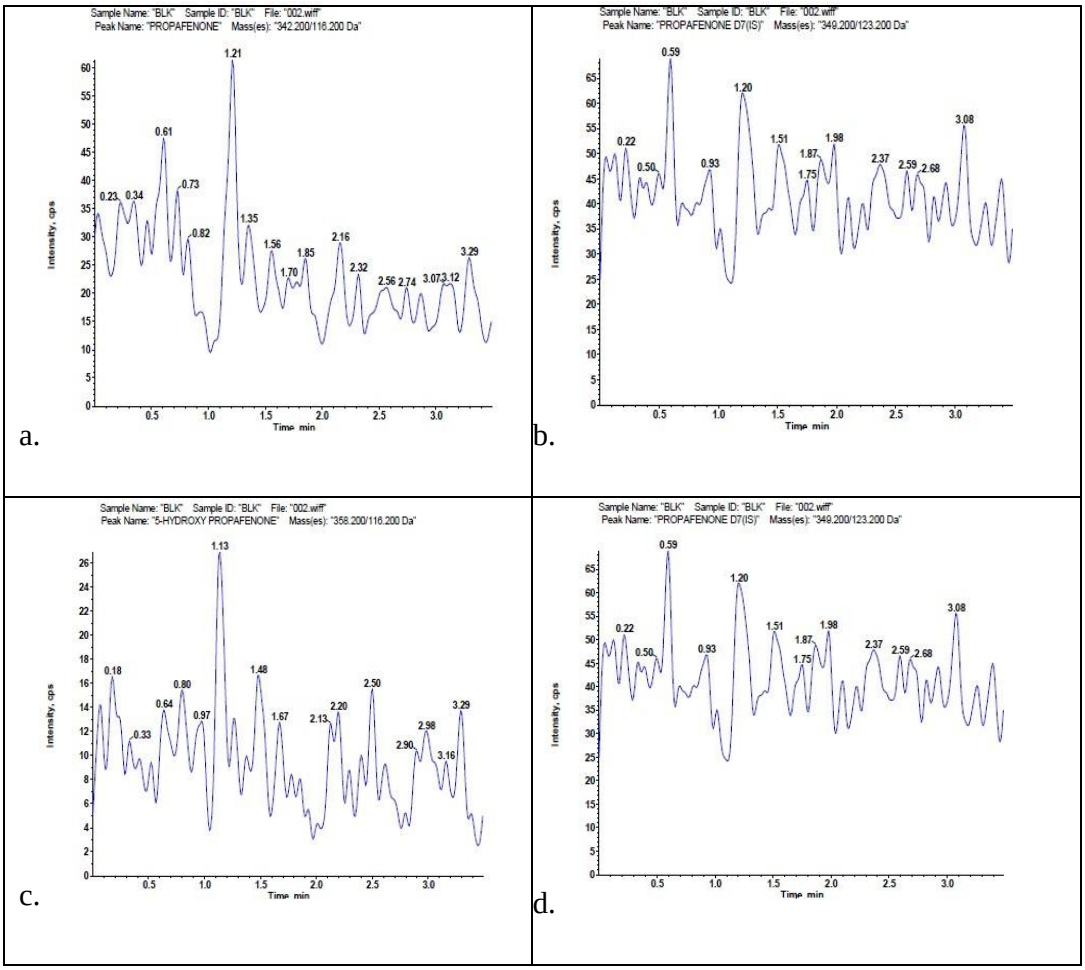

Figure 3.3 a. Blank Chromatogram of Propafenone b. Blank Chromatogram of Propafenone D7 c. Blank Chromatogram of 5-Hydroxypropafenone d. Blank Chromatogram of Propafenone D7

In this experiment 8 different plasma lots were processed and prepared equivalent to LQC and HQC for Propafenone concentration for extracted blank samples. Unextracted samples equivalent to LQC and HQC Propafenone concentration were also prepared and injected in 6 replicates. The mean precision of QC samples ranged from 2.21 % to 5.0 %. The % CV of mean peak area response ratio of post spiked sample was found to be < 15%.

5-Hydroxypropafenone **(Table 1 b)**

It ensures that method reproducibility is not affected in different matrix lots due to ion suppression or ion enhancement. This unintended peak area response alteration appears due to co-eluting matrix component at the retention time and MRM of the analyte(s) and internal standard is interferes with analyte peaks.

In this experiment 8 different plasma lots were processed and prepared equivalent to LQC and HQC for 5-Hydroxypropafenone concentration for extracted blank samples. Unextracted samples equivalent to LQC and HQC 5-Hydroxypropafenone concentration were also prepared and injected in 6 replicates. The mean precision of QC samples ranged from 3.37 % to 5.5 %. The % CV of mean peak area response ratio of post spiked sample was found to be < 15%.

Table 1 a. Matrix effect data of Propafenone

Sr. No.	Matrix ID	LQC			HQC		
		Area Ratio of Extracted Samples	Area Ratio of Unextracted Samples	Matrix Factor	Area Ratio of Extracted Samples	Area Ratio of Unextracted Samples	Matrix Factor
1	Blank-1	0.011	0.010	1.16	7.768	7.430	1.06
2	Blank-2	0.010	0.010	1.05	7.895	7.491	1.08
3	Blank-3	0.009	0.009	0.95	7.553	7.178	1.03
4	Blank-4	0.010	0.009	1.05	7.480	7.201	1.02
5	Blank-5	0.010	0.009	1.05	7.361	7.463	1.01
6	Blank-6	0.010	0.010	1.05	7.466	7.089	1.02
7	Blank Haemolysed	0.010	-	1.05	7.430	-	1.02
8	Blank Lipemic	0.010	-	1.05	7.491	-	1.02
Mean			0.0095	1.501		7.3087	1.033
SD (±)		-		0.0525	-		0.0228
C.V. (%)				5.0			2.21

Table 1 b. Matrix Effect data of 5-Hydroxypropafenone

Sr. No	Matrix ID	LQC			HQC		
		Area Ratio of Extracted Samples	Area Ratio of Unextracted Samples	Matrix Factor	Area Ratio of Extracted Samples	Area Ratio of Unextracted Samples	Matrix Factor
1	Blank-1	0.006	0.006	1.00	2.081	1.995	1.06
2	Blank-2	0.006	0.006	1.00	1.923	1.952	0.98
3	Blank-3	0.006	0.006	1.00	2.119	2.033	1.07
4	Blank-4	0.006	0.006	1.00	1.997	1.924	1.01
5	Blank-5	0.006	0.006	1.00	1.923	1.999	0.98
6	Blank-6	0.006	0.006	1.00	1.997	1.926	1.01
7	Blank Haemolysed	0.007	-	1.17	2.089	-	1.06
8	Blank Lipemic	0.006	-	1.00	2.078	-	1.05
Mean			0.0060	1.02		1.9715	1.03
SD (±)		-		0.0562	-		0.0346
C.V. (%)				5.5			3.37

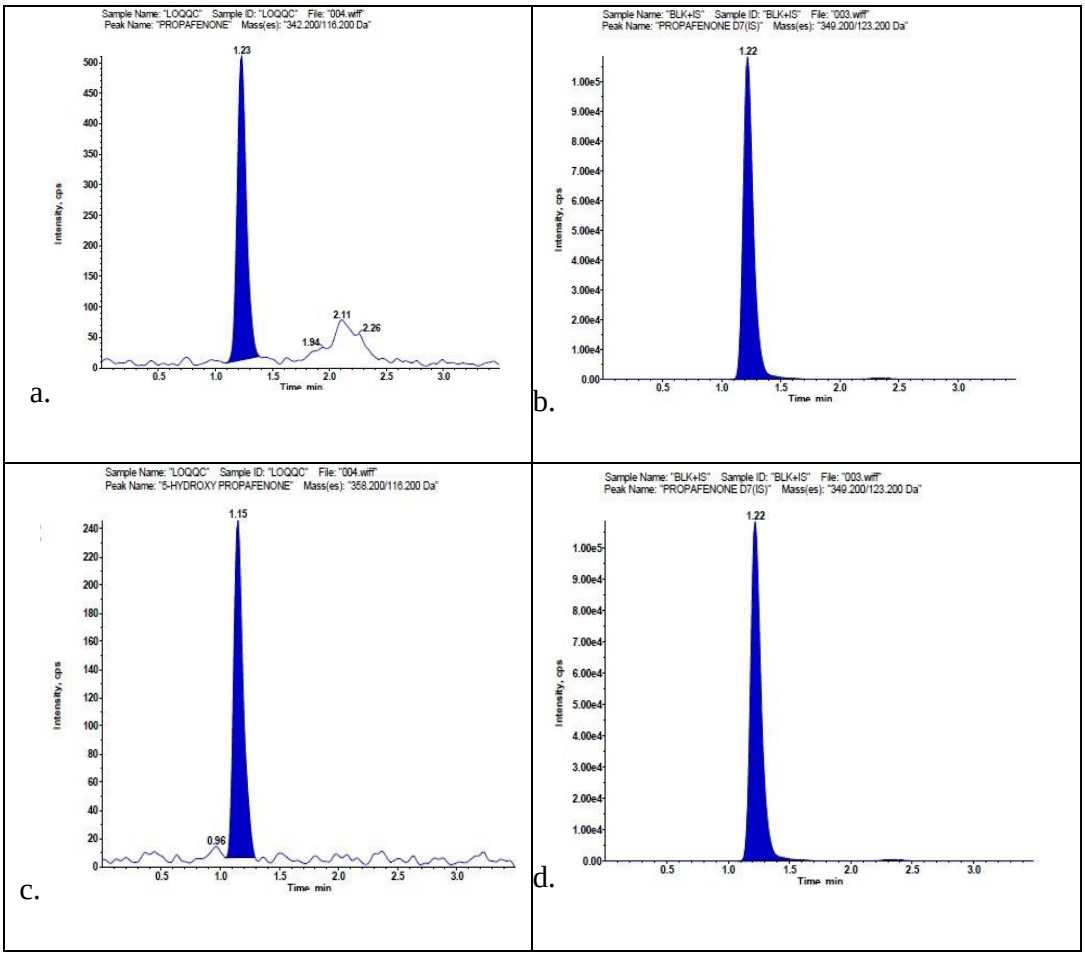

Figure 3.4 a. LLOQ Propafenone b. Propafenone D7(IS) c. LLOQ 5-Hydroxypropafenone d. Propafenone D7(IS)

Linearity

Propafenone (Table 2a)

A regression equation with a weighting factor of 1/ (concentration^2) was applied to produce the best fit for the concentration-detector response relationship for Propafenone in human plasma. The representative calibration curve for regression analysis is illustrated in Figure 3.5a Coefficient of determination (r^2) was greater than 0.997 in the concentration range from 0.499 ng/mL to 1502.841 ng/mL.

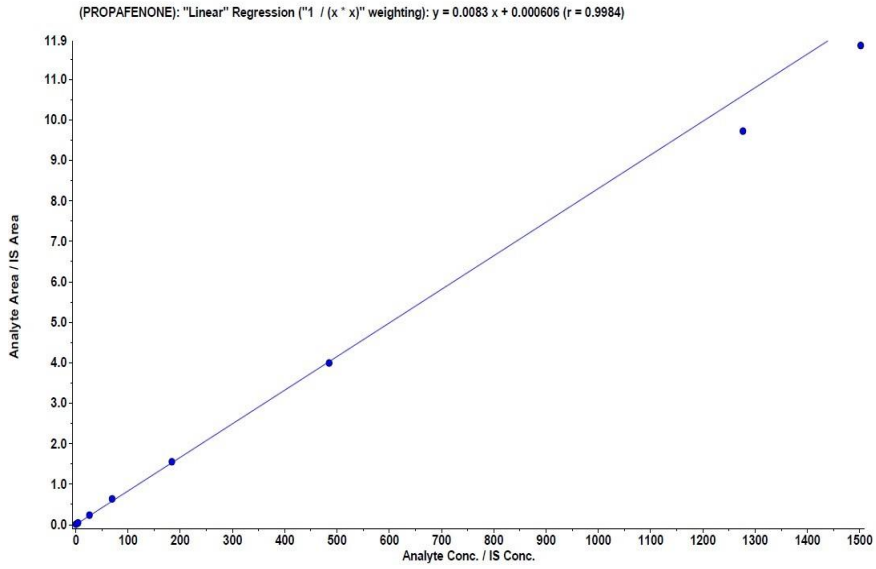

Figure 5 a. Propafenone linearity

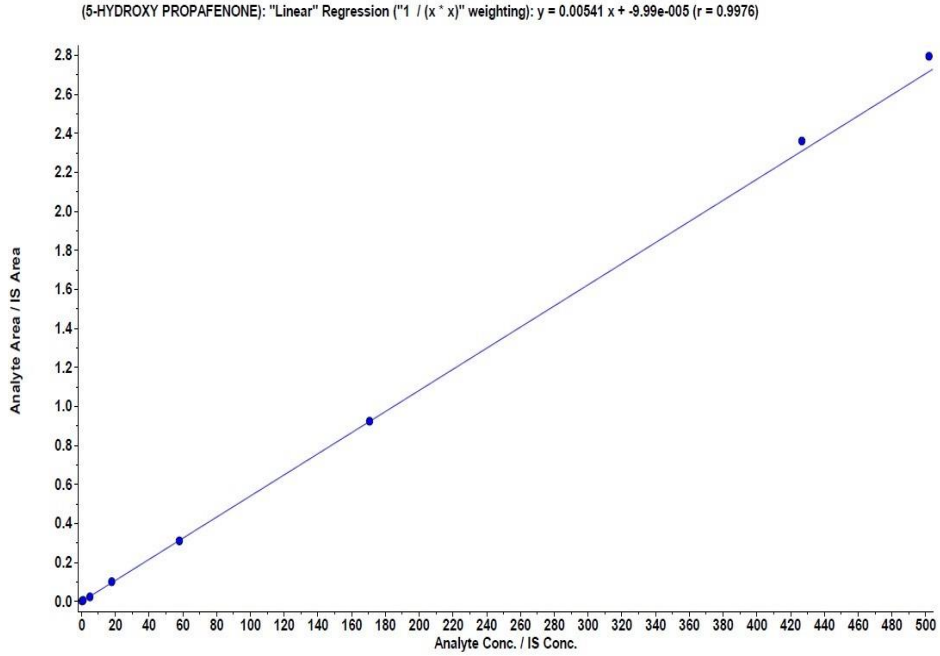

Figure 5 b. 5-Hydroxypropafenone linearity

Table 2 a. Concentration-Response Linearity data of Propafenone

	Nominal Concentration (ng/mL)								Slope	Intercept	r^2
	STD A	STD B	STD C	STD D	STD E	STD F	STD G	STD H			
	0.499	1.996	7.984	31.935	127.741	510.966	1277.415	1502.841			
PA-01	0.501	1.920	8.620	31.900	134.000	525.000	1230.000	1370.000	0.00830	0.000606	0.998
PA-02	0.500	1.970	8.200	34.010	132.110	505.340	1249.290	1378.440	0.00121	0.000874	0.997
PA-03	0.511	1.880	6.750	32.800	153.000	545.000	1140.000	1510.000	0.00109	0.000427	0.998
Mean	0.504	1.923	7.857	32.903	139.703	525.113	1206.430	1419.480			
SD (±)	0.0061	0.0451	0.9811	1.0588	11.5540	19.8302	58.3330	78.5061			
CV (%)	1.21	2.34	12.49	3.22	8.27	3.78	4.84	5.53	-		
% Nominal	101.00	96.36	98.41	103.03	109.36	102.77	94.44	94.45			
N	3	3	3	3	3	3	3	3			

5-Hydroxypropafenone (Table 2b)

A regression equation with a weighting factor of 1/ (concentration^2) was judged to produce the best fit for the concentration-detector response relationship for 5-Hydroxypropafenone in human plasma. The representative calibration curve for regression analysis is illustrated in Figure 3.5b. Coefficient of determination (r^2) was greater than 0.996 in the concentration range from 0.496 ng/mL to 504.079 ng/mL.

Table 2 b. Concentration-Response Linearity data of 5-Hydroxypropafenone

	Nominal Concentration (ng/mL)								Slope	Intercept	r^2
	STD A	STD B	STD C	STD D	STD E	STD F	STD G	STD H			
	0.496	1.837	7.348	20.995	59.985	171.387	428.468	504.079			
PA-01	0.501	1.800	7.030	19.800	61.900	178.000	438.000	513.000	0.00541	0.0000999	0.999
PA-02	0.500	1.820	7.000	20.580	61.190	168.420	453.650	509.070	0.00101	0.0000874	0.997
PA-03	0.504	1.810	5.710	21.700	66.000	175.000	410.000	555.000	0.00103	0.000107	0.996
Mean	0.502	1.810	6.580	20.693	63.030	173.807	433.883	525.690			
SD (±)	0.0021	0.0100	0.7536	0.9551	2.5965	4.9002	22.1143	25.4592			
CV (%)	0.41	0.55	11.45	4.62	4.12	2.82	5.10	4.84	-		
% Nominal	101.15	98.53	89.55	98.56	105.08	101.41	101.26	104.29			
N	3	3	3	3	3	3	3	3			

Precision and Accuracy

The precision of the assay was measured by the percent coefficient of variation over the concentration range of LLOQ QC, LQC, M1QC, MQC and HQC samples respectively,

during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the LLOQ QC, LQC, M1QC, MQC and HQC samples to their respective nominal values, expressed in percentage (Figure 3.6 a for Propafenone and Figure 3.6 b. for 5-Hydroxypropafenone).

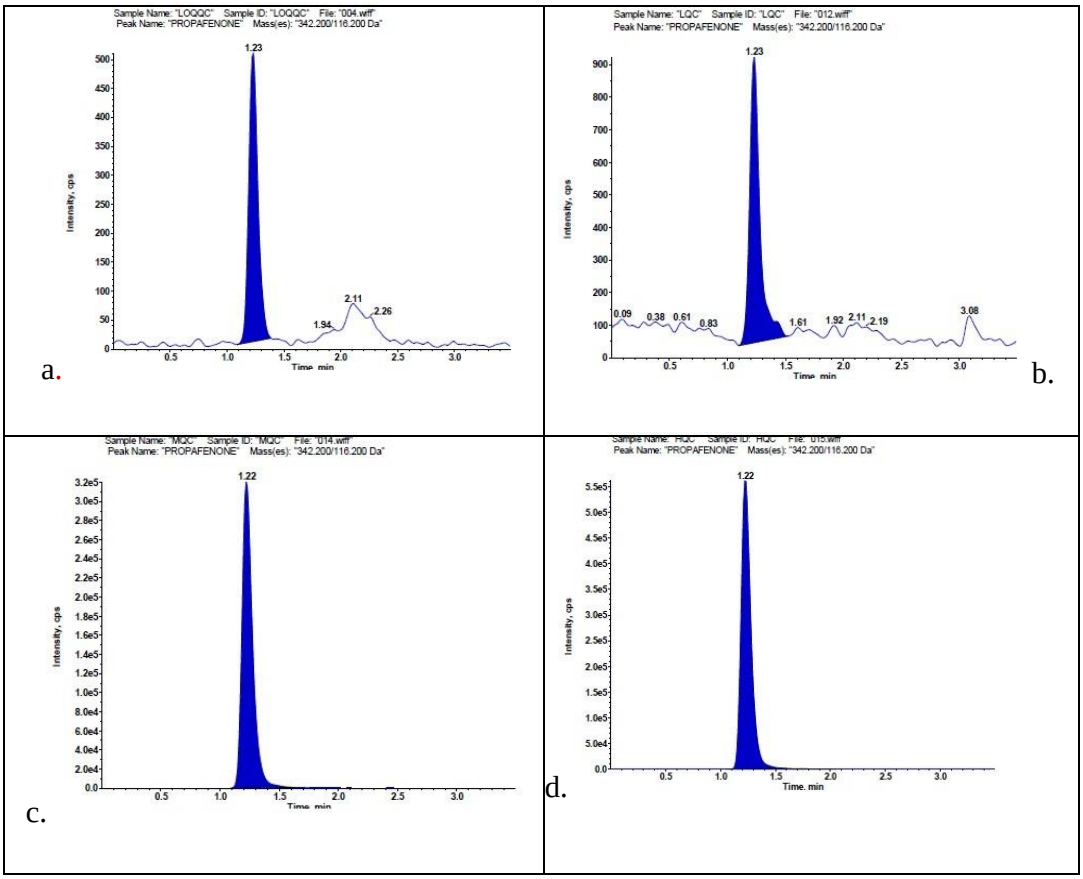

Figure 3.6 a. Propafenone chromatograms a. LLOQ b. LQC c. MQC d. HQC

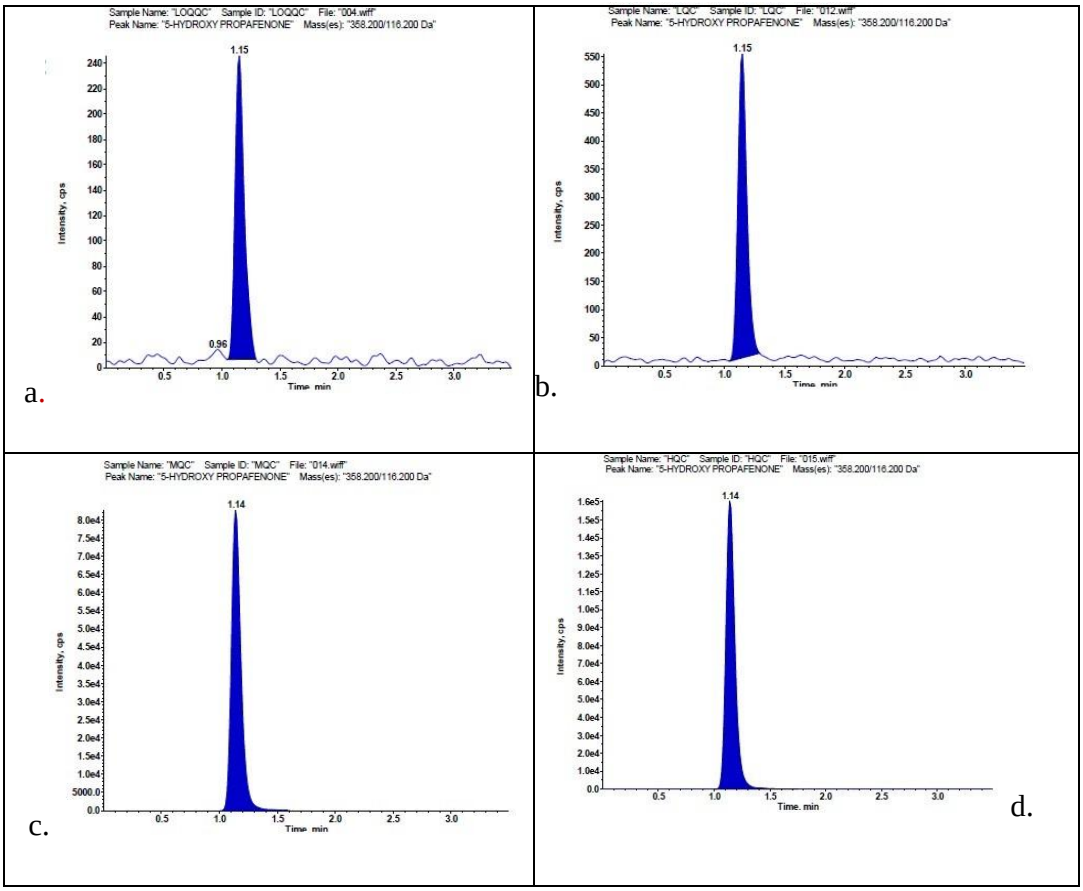

Figure 3.6 b. 5-Hydroxypropafenone chromatograms a. LLOQ b. LQC c. MQC d. HQC

Within-batch precision and accuracy

Propafenone (Table 3a)

Within-batch precision ranged from 0.76 % to 8.91 % and the within batch accuracy ranged from 93.0 % to 106.45 %. % CV & % Nominal were within the acceptance criteria; hence no outlier observed.

Table 3 a. Within Batch Precision and Accuracy data of Propafenone

QC	Nominal Concentration (ng/mL)				
	LLOQ QC	LQC	M1QC	MQC	HQC
	0.499	1.348	245.08	612.697	1141.398
001	0.565	1.460	230.560	565.480	1110.980
002	0.482	1.270	231.010	589.290	1090.830
003	0.460	1.210	233.380	577.900	1079.770
004	0.463	1.240	224.540	565.870	1086.100
005	0.552	1.230	231.760	580.520	1091.230
006	0.508	1.320	238.950	564.330	1095.730
Mean	0.505	1.288	231.700	573.898	1092.440
SD (±)	0.04502	0.09239	4.65682	10.23366	10.57673
CV (%)	8.91	7.17	2.01	1.78	0.97
% Nominal	101.2	95.57	94.54	93.67	95.71
N	6	6	6	6	6
007	0.512	1.470	232.070	570.770	1091.220
008	0.497	1.460	228.290	581.280	1082.300
009	0.462	1.490	230.880	569.280	1078.770
010	0.565	1.460	228.660	549.670	1088.320
011	0.501	1.410	228.080	585.620	1088.470
012	0.503	1.320	231.380	563.650	1063.640
Mean	0.507	1.435	229.893	570.045	1082.120
SD (±)	0.03336	0.06221	1.74937	12.85265	10.14236
CV (%)	6.58	4.34	0.76	2.25	0.94
% Nominal	101.54	106.45	93.8	93.04	94.81
N	6	6	6	6	6
013	0.485	1.320	224.410	556.720	1091.510
014	0.519	1.360	229.260	573.300	1057.670
015	0.473	1.469	226.760	566.980	1094.180
016	0.469	1.387	229.200	568.050	1071.180
017	0.509	1.240	229.040	574.420	1069.030
018	0.510	1.390	228.900	589.000	1128.800
Mean	0.494	1.361	227.928	571.412	1085.395
SD (±)	0.02123	0.07682	1.9654	10.66537	25.44452
CV (%)	4.3	5.64	0.86	1.87	2.34
% Nominal	99.04	100.96	93.0	93.26	95.09
N	6	6	6	6	6

5-Hydroxypropafenone (Table 3b)

Within-batch precision ranged from 1.41 % to 9.13 % and the within batch accuracy ranged from 88.52 % to 103.06 %. % CV & % Nominal were within the acceptance criteria; hence no outlier observed.

Table 3b. Within Batch Precision and Accuracy data of 5-Hydroxypropafenone

QC	Nominal Concentration (ng/mL)				
	LLOQ QC	LQC	M1QC	MQC	HQC
	0.496	1.478	83.900	214.030	433.210
001	0.470	1.350	85.030	213.250	452.290
002	0.530	1.360	82.210	218.890	422.420
003	0.493	1.400	85.480	204.260	422.810
004	0.460	1.340	82.500	215.180	432.370
005	0.502	1.410	83.720	219.840	427.870
006	0.485	1.430	87.640	214.760	440.710
Mean	0.490	1.382	84.430	214.363	433.078
SD (±)	0.02481	0.03656	2.04568	5.56104	11.61032
CV (%)	5.06	2.65	2.42	2.59	2.68
% Nominal	98.79	93.48	100.63	100.16	99.97
N	6	6	6	6	6
007	0.580	1.410	82.140	208.860	441.850
008	0.460	1.430	85.910	217.950	436.630
009	0.502	1.420	84.980	203.380	436.670
010	0.485	1.490	83.210	220.930	436.770
011	0.473	1.460	86.510	224.440	447.370
012	0.462	1.410	89.240	220.710	451.010
Mean	0.494	1.437	85.332	216.045	441.717
SD (±)	0.04508	0.03204	2.5223	8.14564	6.23148
CV (%)	9.13	2.23	2.96	3.77	1.41
% Nominal	99.54	97.21	101.71	100.94	101.96
N	6	6	6	6	6
013	0.582	1.330	82.810	219.050	427.870
014	0.472	1.220	85.100	224.030	436.670
015	0.514	1.330	87.720	205.740	436.770
016	0.497	1.340	81.030	221.430	447.370
017	0.474	1.220	85.460	229.070	430.940
018	0.510	1.410	88.660	224.130	448.620
Mean	0.508	1.308	85.130	220.575	438.040
SD (±)	0.04022	0.07468	2.87997	7.99842	8.44173
CV (%)	7.91	5.71	3.38	3.63	1.93
% Nominal	102.46	88.52	101.47	103.06	101.11
N	6	6	6	6	6

Intra-day precision and accuracy

Propafenone (Table 4a)

Intra-day precision ranged from 1.04 % to 7.88 % and the intra-day accuracy ranged from 93.35 % to 101.36 %. % CV & % Nominal were within the acceptance criteria; hence no outlier observed.

Table 4 a. Intra-day Precision and Accuracy data of Propafenone

QC	Nominal Concentration (ng/mL)				
	LLOQ QC	LQC	M1QC	MQC	HQC
	0.499	1.348	612.697	245.08	1141.398
1	0.565	1.460	565.480	230.560	1110.980
2	0.482	1.270	589.290	231.010	1090.830
3	0.460	1.210	577.900	233.380	1079.770
4	0.463	1.240	565.870	224.540	1086.100
5	0.552	1.230	580.520	231.760	1091.230
6	0.508	1.320	564.330	238.950	1095.730
7	0.512	1.470	570.770	232.070	1091.220
8	0.497	1.460	581.280	228.290	1082.300
9	0.462	1.490	569.280	230.880	1078.770
10	0.565	1.460	549.670	228.660	1088.320
11	0.501	1.410	585.620	228.080	1088.470
12	0.503	1.320	563.650	231.380	1063.640
Mean	0.5058	1.3617	571.9717	230.7967	1087.2800
SD (±)	0.03779	0.10727	11.25787	3.48403	11.25402
CV (%)	7.47	7.88	1.97	1.51	1.04
% Nominal	101.36	101.02	93.35	94.17	95.26
N	12	12	12	12	12

5-Hydroxypropafenone (Table 4b)

Intra-day precision ranged from 2.28 % to 7.07 % and the intra-day accuracy ranged from 95.35 % to 101.17 %. % CV & % Nominal were within the acceptance criteria; hence no outlier observed.

Table 4 b. Intra-day Precision and Accuracy data of 5-Hydroxypropafenone

QC	Nominal Concentration (ng/mL)				
	LLOQQC	LQC	M1QC	MQC	HQC
	0.496	1.478	83.900	214.030	433.210
1	0.470	1.350	85.030	213.250	452.290
2	0.530	1.360	82.210	218.890	422.420
3	0.493	1.400	85.480	204.260	422.810
4	0.460	1.340	82.500	215.180	432.370
5	0.502	1.410	83.720	219.840	427.870
6	0.485	1.430	87.640	214.760	440.710
7	0.580	1.410	82.140	208.860	441.850
8	0.460	1.430	85.910	217.950	436.630
9	0.502	1.420	84.980	203.380	436.670
10	0.485	1.490	83.210	220.930	436.770
11	0.473	1.460	86.510	224.440	447.370
12	0.462	1.410	89.240	220.710	451.010
Mean	0.4918	1.4092	84.8808	215.2042	437.3975
SD (±)	0.03475	0.04358	2.23958	6.70730	9.96365
CV (%)	7.07	3.09	2.64	3.12	2.28
% Nominal	99.15	95.35	101.17	100.55	100.97
N	12	12	12	12	12

Between batch / Inter-day precision and accuracy

Propafenone (Table 5a)

Between batch / Inter day precision ranged from 1.44 % to 7.71 % and the between batch / Inter day accuracy ranged from 93.32 % to 101.71 %. % CV & % Nominal were within the acceptance criteria, hence no outlier observed.

Table 5 a. Between Batch / Inter-day Precision and Accuracy data of Propafenone

QC	Nominal Concentration (ng/mL)				
	LLOQ QC	LQC	M1QC	MQC	HQC
	0.499	1.348	245.08	612.697	1141.398
001	0.565	1.460	230.560	565.480	1110.980
002	0.482	1.270	231.010	589.290	1090.830
003	0.460	1.210	233.380	577.900	1079.770
004	0.463	1.240	224.540	565.870	1086.100
005	0.552	1.230	231.760	580.520	1091.230
006	0.508	1.320	238.950	564.330	1095.730
007	0.512	1.470	232.070	570.770	1091.220
008	0.497	1.460	228.290	581.280	1082.300
009	0.462	1.490	230.880	569.280	1078.770
010	0.565	1.460	228.660	549.670	1088.320
011	0.501	1.410	228.080	585.620	1088.470
012	0.503	1.320	231.380	563.650	1063.640
013	0.485	1.320	224.410	556.720	1091.510
014	0.519	1.360	229.260	573.300	1057.670
015	0.473	1.530	226.760	566.980	1094.180
016	0.469	1.500	229.200	568.050	1071.180
017	0.509	1.240	229.040	574.420	1069.030
018	0.510	1.390	228.900	589.000	1128.800
Mean	0.502	1.371	229.841	571.785	1086.652
SD (±)	0.0330	0.1057	3.3055	10.7489	16.5290
CV (%)	6.57	7.71	1.44	1.88	1.52
% Nominal	100.58	101.71	93.78	93.32	95.20
N	18	18	18	18	18

5-Hydroxypropafenone (Table 5b)

Between batch / Inter day precision ranged from 2.11 % to 7.31 % and the between batch / Inter day accuracy ranged from 93.07 % to 101.39 %. % CV & % Nominal were within the acceptance criteria; hence no outlier observed.

Table 5 b. Between Batch / Inter-day Precision and Accuracy data of

5-Hydroxypropafenone

QC	Nominal Concentration (ng/mL)				
	LLOQQC	LQC	M1QC	MQC	HQC
	0.496	1.478	83.900	214.030	433.210
001	0.470	1.350	85.030	213.250	452.290
002	0.530	1.360	82.210	218.890	422.420
003	0.493	1.400	85.480	204.260	422.810
004	0.460	1.340	82.500	215.180	432.370
005	0.502	1.410	83.720	219.840	427.870
006	0.485	1.430	87.640	214.760	440.710
007	0.580	1.410	82.140	208.860	441.850
008	0.460	1.430	85.910	217.950	436.630
009	0.502	1.420	84.980	203.380	436.670
010	0.485	1.490	83.210	220.930	436.770
011	0.473	1.460	86.510	224.440	447.370
012	0.462	1.410	89.240	220.710	451.010
013	0.582	1.330	82.810	219.050	427.870
014	0.472	1.220	85.100	224.030	436.670
015	0.514	1.330	87.720	205.740	436.770
016	0.497	1.340	81.030	221.430	447.370
017	0.474	1.220	85.460	229.070	430.940
018	0.510	1.410	88.660	224.130	448.620
Mean	0.497	1.376	84.964	216.994	437.612
SD (±)	0.0363	0.0725	2.3874	7.3968	9.2354
CV (%)	7.31	5.27	2.81	3.41	2.11
% Nominal	100.26	93.07	101.27	101.39	101.02
N	18	18	18	18	18

Recovery

Propafenone (Table 6a)

The peak areas of extracted LQC, MQC and HQC samples of precision and accuracy batch were compared against the peak areas of respective Unextracted QC samples. The overall mean recovery of Propafenone was found to be 81.47 % with precision of 5.05 %.

Table 6 a. Recovery data of Propafenone from Human Plasma

	Propafenone Response					
	LQC		MQC		HQC	
	Extracted	UnExtracted	Extracted	UnExtracted	Extracted	UnExtracted
001	2596	3246	835379	1101487	1563193	1960276
002	3026	3309	807898	1058214	1479423	1835998
003	2550	3887	883920	954223	1470657	1816968
004	2658	3724	881927	951561	1532753	1694307
005	2856	3145	808562	974769	1464633	1685341
006	2283	3503	788053	941473	1430579	1650675
Mean	2661.5	3469	834289.8	996954.5	1490206.3	1773927.5
SD (±)	257.17	290.24	40570.76	66538.28	48672.67	118160.2
CV (%)	9.66	8.37	4.86	6.67	3.27	6.66
N	6	6	6	6	6	6
% Recovery	76.72		83.68		84.01	

Overall Propafenone Recovery: N=3, Mean 81.47%, SD(±) 4.1169, CV 5.05(%) 5-Hydroxypropafenone (Table 6b)

The peak areas of extracted LQC, MQC and HQC samples of precision and accuracy batch were compared against the peak areas of respective Unextracted QC samples. The overall mean recovery of 5-Hydroxypropafenone was found to be 91.41 % with precision of 5.74 %.

Table 6 b. Recovery data of 5-Hydroxypropafenone from Human Plasma

	5-Hydroxypropafenone Response					
	LQC		MQC		HQC	
	Extracted	UnExtracted	Extracted	UnExtracted	Extracted	UnExtracted
001	2319	2747	369704	382654	652480	683348
002	2118	2705	367974	373494	652728	685386
003	2413	2779	336677	361666	643382	694611
004	2381	2827	355787	385862	652565	697597
005	2201	2561	359194	390784	651615	681964
006	2535	2741	371689	384926	655518	712601
Mean	2327.8	2726.7	360170.8	379897.7	651381.3	692584.5
SD (±)	150.46	90.93	13088.71	10586.48	4137.09	11660.35
CV (%)	6.46	3.33	3.63	2.79	0.64	1.68
N	6	6	6	6	6	6
% Recovery	85.37		94.81		94.05	

Overall 5-Hydroxypropafenone Recovery: N=3, Mean 91.41%, SD(±) 5.2446, CV5.74 (%)

Propafenone D7 (Table 6c)

The peak areas of extracted MQC samples of precision and accuracy batch were compared against the peak areas of respective Unextracted QC samples. The recovery of Propafenone D7 was found to be 87.5 % with precision ranged from 2.51 % to 5.17 %.

Propafenone (Table 7a)

Dilution integrity samples were prepared by spiking about 1.6 times the highest standard concentration of Propafenone (2460.24 ng/mL). Six sets of dilution integrity samples were processed by diluting them twice and another six sets were processed by diluting them four times. These QC samples were analysed along with CC standards and were calculated using appropriate dilution factor. The precision and accuracy for a dilution factor of 2 were found to be 3.05 % and 93.32 % respectively. Similarly, the precision and accuracy for a dilution factor of 4 were found to be 3.8 % and 91.32 % respectively.

Table 6 c. Recovery data of Propafenone D7 (IS) from Human Plasma

	Propafenone D7 Response	
	MQC	
	Extracted	UnExtracted
001	327369	377441
002	336498	373710
003	331251	343045
004	316388	400759
005	334844	385327
006	319409	366414
Mean	327626.5	374449.3
SD (±)	8219.86	19343.31
CV (%)	2.51	5.17
N	6	6
% Recovery	87.5	

Dilution Integrity

Table 7 a. Dilution Integrity data (Two and Four times dilution) of Propafenone

2x DI QC	Concentration (ng/mL)	4x DI QC	Concentration (ng/mL)
	2460.24		2460.24
2 DIQC 01	2199.24	4 DIQC 01	2203.63
2 DIQC 02	2345.12	4 DIQC 02	2241.95
2 DIQC 03	2274.23	4 DIQC 03	2302.36
2 DIQC 04	2369.21	4 DIQC 04	2200.78
2 DIQC 05	2235.21	4 DIQC 05	2145.85
2 DIQC 06	2352.36	4 DIQC 06	2385.44
Mean	2295.895	Mean	2246.6683
SD (±)	69.97245	SD (±)	85.44445
CV (%)	3.05	CV (%)	3.8
% Nominal	93.32	% Nominal	91.32
N	6	N	6

5-Hydroxypropafenone (Table 7b)

Dilution integrity samples were prepared by spiking about 1.6 times the highest standard concentration of 5-Hydroxypropafenone (860.324 ng/mL). Six sets of dilution integrity samples were processed by diluting them twice and another six sets were processed by diluting them four times. The precision and accuracy for a dilution factor of 2 were found to be 3.06 % and 94.59 % respectively. Similarly, the precision and accuracy for a dilution factor of 4 were found to be 3.57 % and 95.27 % respectively.

Table 7 b.Dilution Integrity data (Two and Four times dilution) of 5-Hydroxypropafenone

2x DI QC	Concentration (ng/mL)	4x DI QC	Concentration (ng/mL)
	860.321		860.324
2 DIQC 01	823.456	4 DIQC 01	790.425
2 DIQC 02	800.204	4 DIQC 02	826.234
2 DIQC 03	820.396	4 DIQC 03	812.754
2 DIQC 04	798.201	4 DIQC 04	825.416
2 DIQC 05	785.302	4 DIQC 05	792.456
2 DIQC 06	855.209	4 DIQC 06	870.246
Mean	813.7947	Mean	819.5885
SD (±)	24.86671	SD (±)	29.24961
CV (%)	3.06	CV (%)	3.57
% Nominal	94.59	% Nominal	95.27
N	6	N	6

Stability Studies

Freeze-Thaw stability

Propafenone (Table 8)

The stability of Propafenone in human plasma was determined during 3 freeze-thaw cycles. Six sets of QC (LQC & HQC) samples were analyzed after FT-3 cycle. Six sets of freshly spiked QC (LQC & HQC) samples were prepared on the day of experiment and injected along with the stability QC samples and quantified against the freshly spiked CC standards. The precision ranged from 0.58 % to 3.33 % and accuracy ranged from 95.32 % to 99.14 %. The freshly spiked CC standards and QC samples were found within acceptance criteria.

5-Hydroxypropafenone (Table 8)

The stability of 5-Hydroxypropafenone in human plasma was determined during 3 freeze- thaw cycles. Six sets of QC (LQC & HQC) samples were analyzed after FT-3 cycle. Six sets of freshly spiked QC (LQC & HQC) samples were prepared on the day of experiment and injected along with the stability QC samples and quantified against the freshly spiked

CC standards. The precision ranged from 1.31 % to 5.11 % and accuracy ranged from 88.5 % to 98.99 %. The freshly spiked CC standards and QC samples were found within acceptance criteria.

Table 8 Freeze-Thaw Stability (FT-3 Cycle) data of Propafenone and 5-Hydroxypropafenone

QC	Propafenone Concentration (ng/mL)		5-Hydroxypropafenone Concentration (ng/mL)	
	LQC	HQC	LQC	HQC
	0.499	1141.398	1.478	433.210
001	0.502	1091.230	1.331	422.420
002	0.508	1095.730	1.229	422.817
003	0.498	1091.220	1.333	432.373
004	0.497	1082.300	1.340	427.876
005	0.462	1078.770	1.224	436.679
006	0.501	1088.320	1.391	430.943
Mean	0.495	1087.928	1.308	428.851
SD (±)	0.01646	6.29776	0.06688	5.59957
CV (%)	3.33	0.58	5.11	1.31
% Nominal	99.14	95.32	88.5	98.99
N	6	6	6	6

Auto sampler stability for 48 hrs

Propafenone (Table.9)

In assessing the auto sampler stability, six sets of QC (LQC & HQC) samples from PA Batch: 01 exercise) samples were processed and placed in the auto sampler. They were injected after a period of 48 hrs. Six sets of freshly spiked QC (LQC and HQC) samples were prepared on the day of experiment and injected along with the stability QC samples and quantified against the freshly spiked CC standards.The results demonstrate that the processed samples were stable up to 48 hrs. The precision ranged from 2.92 % to 4.59 % and accuracy ranged from 94.15 % to 98.46 %. The freshly spiked CC standards and QC samples were found within acceptance criteria.

5-Hydroxypropafenone (Table 9)

In assessing the auto sampler stability, six sets of QC (LQC & HQC) samples from PA Batch: 01 exercise) samples were processed and placed in the auto sampler. They were injected after a period of 48 hrs. Six sets of freshly spiked QC (LQC and HQC) samples were prepared on the day of experiment and injected along with the stability QC samples and quantified against the freshly spiked CC standards. The results demonstrate that the processed samples were stable up to 48 hrs. The precision ranged from 1.60 % to 2.97 % and accuracy ranged from 96.5 % to 97.69 %. The freshly spiked CC standards and QC samples were found within acceptance criteria.

Table 9 Auto Sampler Stability of Propafenone and 5-Hydroxypropafenone for 48 hrs

QC	Propafenone Conc. (ng/mL)		5-Hydroxypropafenone Conc. (ng/mL)	
	LQC	HQC	LQC	HQC
	0.499	1141.398	1.478	433.210
001	0.473	1058.680	1.442	425.686
002	0.518	1095.190	1.481	427.907
003	0.487	1072.190	1.451	424.805
004	0.459	1070.040	1.410	430.927
005	0.509	1029.810	1.417	414.922
006	0.502	1121.800	1.357	414.844
Mean	0.491	1074.618	1.426	423.182
SD (±)	0.02253	31.426	0.04243	6.767
CV (%)	4.59	2.92	2.97	1.60
% Nominal	98.46	94.15	96.5	97.69
N	6	6	6	6

Short Term Room Temperature Stability for 8 hrs

Propafenone (Table 10)

Short-term room temperature stability, using six sets of QC (LQC & HQC) samples was determined at room temperature. Six sets of freshly spiked QC (LQC & HQC) samples were prepared on the day of experiment and injected along with the stability QC samples and quantified against the freshly spiked CC standards. Propafenone was found to be stable up to 8 hrs. The precision ranged from 2.01 % to 4.34 % and the accuracy ranged from 95.27

% to 98.74 %. The freshly spiked CC standards and QC samples were found within acceptance criteria.

5-Hydroxypropafenone (Table 10)

Short-term room temperature stability, using six sets of QC (LQC & HQC) samples was determined at room temperature. Six sets of freshly spiked QC (LQC & HQC) samples were prepared on the day of experiment and injected along with the stability QC samples and quantified against the freshly spiked CC standards. 5-Hydroxypropafenone was found to be stable up to 7 hrs. The precision ranged from 0.96 % to 4.44 % and the accuracy ranged from 83.38 % to 97.93 %. The freshly spiked CC standards and QC samples were found within acceptance criteria

Table 10 Short term Room Temperature Stability of Propafenone and 5-Hydroxypropafenone for 7 hrs

QC	Propafenone Conc. (ng/mL)		5-Hydroxypropafenone Conc. (ng/mL)	
	LQC	HQC	LQC	HQC
	0.499	1141.398	1.478	433.210
001	0.462	1078.770	1.296	426.311
002	0.505	1088.320	1.226	421.457
003	0.501	1088.470	1.204	420.035
004	0.469	1071.180	1.283	425.878
005	0.509	1069.030	1.239	421.102
006	0.510	1128.800	1.146	430.631
Mean	0.493	1087.428	1.232	424.236
SD (±)	0.0214	21.86764	0.0547	4.07654
CV (%)	4.34	2.01	4.44	0.96
% Nominal	98.74	95.27	83.38	97.93
N	6	6	6	6

Long Term Stability data (below −50°C) for 92 days

Propafenone (Table 11)

The stability of Propafenone, for plasma samples stored below −50°C, was generated for 92 days by quantifying six sets of QC (LQC & HQC) samples against freshly spiked CC

standard. Six sets of freshly spiked QC (LQC & HQC) samples were prepared on the day of experiment and injected along with the stability QC samples and quantified against the freshly spiked CC standards. The precision of the calculated concentrations of QC samples ranged from 0.75 % to 8.50 % and accuracy ranged from 94.89 % to 96.59 %. The freshly spiked CC standards and QC samples were found within acceptance criteria.

5-Hydroxypropafenone (Table 11)

The stability of Hydroxybupropion, for plasma samples stored below −50°C, was generated for 92 days by quantifying six sets of QC (LQC & HQC) samples against freshly spiked CC standard. Six sets of freshly spiked QC (LQC & HQC) samples were prepared on the day of experiment and injected along with the stability QC samples and quantified against the freshly spiked CC standards. The precision of the calculated concentrations of QC samples ranged from 1.54 % to 2.6 % and accuracy ranged from 95.2 % to 98.89 %. The freshly spiked CC standards and QC samples were found within acceptance criteria.

Table 11 Long-Term Stability data of Propafenone and 5-Hydroxypropafenone at below -50°C for 92 days

	Propafenone Conc. (ng/mL)		5-Hydroxypropafenone Conc. (ng/mL)	
	LQC	HQC	LQC	HQC
	0.499	1141.398	1.478	433.210
001	0.460	1079.770	1.391	427.870
002	0.463	1086.100	1.402	440.710
003	0.462	1078.770	1.441	420.192
004	0.565	1088.320	1.384	411.091
005	0.473	1094.180	1.399	433.904
006	0.469	1071.180	1.425	436.770
Mean	0.482	1083.053	1.407	428.423
SD (±)	0.04095	8.13543	0.0217	11.12778
CV (%)	8.50	0.75	1.54	2.6
% Nominal	96.59	94.89	95.2	98.89
N	6	6	6	6

DATA PROCESSING

The chromatograms were acquired using the computer based Analyst Software. The slopes, intercepts and goodness of fit were determined by linear regression analyses using the ratios of analyte / IS peak areas of the calibration curve standards. A weighting factor of $1/x^2$ ($1/concentration^2$) was used in the calculation of the linear regression line and the concentrations of QC samples were calculated by computer based Analyst software.

y = mx + b; Where, y = Peak area ratio of Propafenone to Propafenone D7, 5- Hydroxypropafenone to Propafenone D7, m = slope of the calibration curve, x = concentration of Propafenone, and 5-Hydroxypropafenone in ng/mL, b = y-axis intercept of the calibration curve.

CONCLUSION

The analytical method described above is valid for the analysis of Propafenone , and 5-Hydroxypropafenone in human plasma over a range of 0.499 ng/mL to 1502.841 ng/mL and 0.496 ng/mL to 504.079 ng/mL, respectively, using specified Bioanalytical conditions with the High Performance Liquid Chromatography Mass Spectrometric method.

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