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A Rugged Bioanalytical Method Development and Validation of Disopyramide and N-Despropyl disopyramide in Human serum using Gas Chromatography with Tandem Mass Spectrometry

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ABSTRACT

A simple, sensitive and rugged quantitative method for the determination of Disopyramide (DIS), and N-Despropyl disopyramide (N-DIS), in human serum using a gas chromatography-tandem mass spectrometric (GC-MS/MS) method has been developed and validated. Disopyramide-d^5 (DIS d^5) and N-Despropyl disopyramide-d^5 (N-DIS d^5) were used as internal standards. Analytes and the internal standards were extracted from human serum by liquid-liquid extraction technique using dichloromethane and methyl tertiary-butyl ether as extraction solvents. The reconstituted samples were chromatographed on an Ultra 1, 12m X 0.2mm, 0.33µM film thickness by using helium as the carrier gas. The method was validated over the concentration range of 100 to 6000 ng/mL for DIS and 25 to 1500 ng/mL for N-DIS. Waters Quattro Micro mass spectrometer was operated under the multiple reaction-monitoring mode (MRM) for quantification of ion transitions at m/z 212>195, 217>200, 280>194 and 285>201 for DIS, DIS d^5, N-DIS, N-DIS d^5 respectively. The results of the intra and inter batch precision and accuracy studies were well within the acceptable limits. The method has been proved to be simple, sensitive, fast, reliable, rugged and reproducible. A run time of 16.0 min for each sample made it possible to analyze more than 36 serum samples per day. The validated method can be applied for the estimation of the drug in real time serum samples for pharmacokinetic studies.

Keywords: Disopyramide (DIS), N-Despropyl disopyramide (N-DIS), Disopyramide-d^5 (DIS d^5), N-Despropyl disopyramide-d^5 (N-DIS d^5), Validation, Human serum.

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INTRODUCTION

Disopyramide [4-diisopropylamino-2-(2-pyridyl) butyramide] is a widely used anti-arrhythmic drug, having pharmacological effects on the heart that are quantitavely similar to those of quinidine and procainamide. Disopyramide phosphate is available for oral administration in extended-release capsules equivalent to 150 mg of Disopyramide present as the phosphate. The base content of the phosphate salt is 77.66%. Disopyramide phosphate extended-release capsules, USP are designed to afford a gradual and consistent release of Disopyramide. Thus, for maintenance therapy, Disopyramide phosphate extended-release capsules, USP provide the benefit of less frequent dosing (every 12 hours) as compared with the every-6-hour dosage schedule of immediate-release capsules.

Disopyramide determination in serum by GC with N_2 selective detector, HPLC and GC-MS has been published by some workers [1-11]. Although GC with N_2 selective detector is good for total Disopyramide determination, the procedure could not determine desalkayl metabolite due to interference. To overcome this problem a different detector has to be used for the determination of total Disopyramide and its metabolite. In this paper we report the application of gas chromatography-tandem mass spectrometry (GC-MS/MS) to the determination of total Disopyramide (DIS) and N-Despropyl disopyramide (N-DIS) in human serum. Our results show that GC-MS/MS is a powerful tool to study the *in vitro* metabolism of drugs, allowing the identification of parent and metabolite in presence of other drugs interferences at very low level (in ng/mL range). The structural formula of Disopyramide (DIS), N-Despropyl disopyramide (N-DIS) was given in Fig .1 (a,b).

Fig1a: Chemical structures of Disopyramide

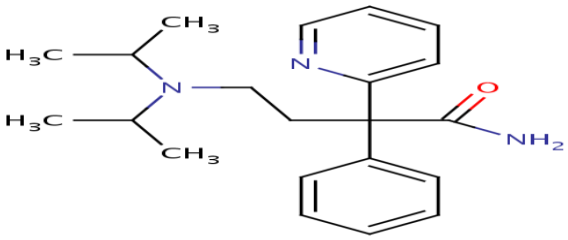
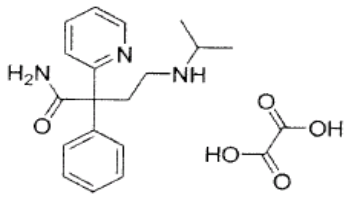


Fig1b: Chemical structures of N-Despropyl Disopyramide



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MATERIALS AND METHODS

Serum Samples

Serum was collected from commercially procured human whole blood. Serum blanks from six different donors were chromatographically screened for interfering substances prior to use.

Chemicals and Reagents

Disopyramide (DIS) reference standard (99.0%) was procured from Sigma Aldrich, Germany, N-Despropyl disopyramide (N-DIS) (99.5) oxalic acid salt, internal standards Disopyramide- d^5 (DIS d^5) and N-Despropyl disopyramide-d^5 oxalic acid salt (N-DIS d^5) reference standards were procured from Syncom, Germany. Methanol, dichloromethane, methyl tertiary-butyl ether (MTBE), water (all HPLC grade) and ethyl acetate (GR grade) were procured from Merck, Germany. Sodium hydroxide (GR grade) from Qualigens was used.

Instrumentation and Chromatographic Conditions

A GC system (Agilent 6890N, USA) equipped with MS/MS (Waters Quattro Micro Mass Spectrometer) consisting of an Ultra 1, 12m X 0.2mm, 0.33µM film thickness column was used for the validation. Aliquots of the processed samples (1.0 µL) were injected onto the column with helium as the carrier gas. Quantitation was achieved with MRM (MS/MS data acquisition mode) in positive ion mode for both the analytes and their respective internal standards. The tuning parameters were summarized in Table 1. Detection of the ions were carried out in the MRM, by monitoring the transition pairs of m/z 212.00/195.00, 217.00/200.00, 280/194 and 285/201 for DIS, for DIS d^5, N-DIS and N-DIS d^5 respectively. The analysis data obtained were processed by Masslynx – Quanlynx software v 4.1.

Standard Solutions

Primary stock solutions of DIS and N-DIS for preparation of standard and quality control (QC) samples were prepared from separate weighings. The primary stock solutions of 1000.900 µg/mL and 1012.538 µg/mL of DIS and N-DIS analyte was prepared in methanol and stored at 2-8 ºC. From the stock solution, appropriate dilutions were made using a 50:50 v/v mixture of methanol and water as a diluent to produce working standard solutions of 4003.600, 10009.000, 24021.600, 40036.000, 80072.000, 120180.000, 200180.000 and 240216.000 ng/mL of DIS and 1012.539, 2430.093, 6075.232, 10125.387, 20250.774, 30736.161, 50626.934 and 60752.231 ng/mL of N-DIS. These solutions were used to prepare the relevant calibration curve (CC) standards. Another set of working solutions of DIS were prepared in the diluent (from primary stock) at concentrations of 4051.620, 12004.800, 37014.800, 112044.800 and 216086.400 ng/mL for DIS and 1013.074, 3039.222, 9117.665, 28366.068 and 54705.988 for N-DIS, to be used as quality control (QC) samples. The primary stock solutions of DIS d^5 (1284.972

ug/mL) and N-DIS d^5 (9641.854 µg/mL) were prepared in methanol. A working concentration of the internal standard (51.940 µg/mL of DIS d^5 and 19.284 µg/mL N-DIS d^5) solution was prepared in the diluent. These working solutions were stored at 2-8 ºC for 15 days.

The calibration curve and quality control samples were prepared by spiking 25 µL of the working solution into 975 µL of control serum. Calibration standards for DIS and N-DIS were made at concentrations of 100.090, 250.225, 600.540, 1000.900, 2001.800, 3002.700, 5004.500, 6005.400 ng/mL and 25.313, 60.752, 151.881, 253.135, 506.269, 759.404, 1265.673, 1518.808 ng/mL respectively. Quality control samples for DIS and N-DIS were prepared at concentrations of 5402.160, 1367.650 (higher quality control, HQC), 2801.120, 709.152 (middle quality control, MQC1), 925.370, 227.942 (geometric mean quality control GMQC), 300.120, 75.981 (lower quality control, LQC) and 101.291, 25.327 (lower limit quality control, LLOQ QC) ng/mL respectively.

Table 1: Tuning parameters

Electron Impact Ionization (+ve)	Settings	Analyser Parameter	Settings	GC Agilent 6890 Parameter	Settings
Ion source	Electron Impact Ionization (+ve)	Mass	Waters Quattro Micro Mass Spectrometer	Column	Ultra 1, 12m X 0.2mm, 0.33µM film thickness
Electron Energy (eV)	70	Data	Centroid	Column oven temp.	100°C (hold 1.00min) to 280°C @20°C/min (hold 6.00min)
Emission (µA)	200	Mode	MRM	Injector	7683 Series Agilent technologies
Extraction Lens (V)	4	DIS	212>195 CE 5ev	Injector temp	275°C
Focus Lens 1 (V)	35	N-DIS	280>194 CE 10ev	Carrier gas	Helium
Focus Lens2 (V)	60	d^5-DIS (ISTD for DIS)	217>200 CE 5ev	Flow rate	1.0 mL/min.
Source Temperature	200°C	d^5-N-DIS (ISTD for N-DIS)	285>201 CE 10ev	Volume of injection	1.0 µL
GC Interface	250°C	Inter Scan Delay	0.07 secs	Total run time	16.00 minutes
		Solvent Delay	4 min	Retention time	
				DIS	8.86 min (± 0.5 min)
				N-DIS	7.34 min (± 0.5 min)
				d^5-DIS (ISTD for DIS)	8.84 min (± 0.5 min)
				d^5- N-DIS (ISTD for N-DIS)	7.36 min (± 0.5 min)

Calibration curve

A series of human serum based calibration curve standards (100, 250, 600, 1000, 2000, 3000, 5000 & 6000 ng/mL for DIS and 25,60,150,250,500,750,1250, & 1500 ng/mL for N-DIS) and quality control sample (300, 925, 2800, & 5400 ng/mL for DIS and 75, 225, 700 & 1350 ng/mL for N-DIS) were prepared. All calibration curve standard and quality control solutions were stored at -80 °C in freezer. A working internal standard solution mixture of DIS-d^5 and N-DIS-$d^5$was prepared in methanol

Sample processing

A 1000-µL volume of the serum sample was transferred to a 5-mL ria vial, and 50.0 µL of internal standard mixture solution (51.940 µg/mL + 19.284 µg/mL) was spiked, vortexed for 30 sec. Added 30 µL of extraction buffer (4M sodium hydroxide solution), vortexed for another 30 sec, and add extraction solvent (80:20 DCM: MTBE) 2.5 mL using dispensette organic (Finnpipette). The sample was shaken for 15 min using a multiplus vortexer and centrifuged all the ria vials at 4000 rpm, at 20 ?C for 10 mins, using a Haeraus centrifuge. The organic layer (2.0 mL) was transferred to a prelabelled ria vial and evaporated at 45ºC under a stream of nitrogen (Turbo Vap LV, Zymark (Hopkinton, MA, USA). Added 0.100 mL of reconstitution solution to all the Ria vials and vortexed for about 2 min. Transfered the reconstituted solution into pre-labelled auto sampler vials and injected 1.0 µL onto GC-MS/MS.

Method validation

Method validation of DIS and N-DIS was carried out as per the US FDA guidelines (FDA, 2001). The method was validated for selectivity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity, ruggedness, effect of potentially interfering drugs on the method and stability. Selectivity of the method was assessed by analyzing six blank human serum samples. The responses of the interfering substances or background noises at the retention times of the DIS and N-DIS analytes and their internal standards are acceptable if they are less than 20% of the response of the lowest standard curve point, and less than 5% of the response of the internal standard respectively.

The sensitivity of the method was evaluated by analyzing 6 LLOQ samples. At least 67% (4 out of 6) of LLOQ samples should be within 80-120%. Matrix effect was investigated to ensure that precision, selectivity, and sensitivity are not compromised by the matrix. Matrix effect was checked with six different lots of serum. Three replicate samples of quality control (low and high) were prepared from different lots of serum. The QCs should be within acceptance limit 85.00 - 115.00 % (36 QC samples in total).

Linearity was tested for DIS and N-DIS in the concentration range of 100.090-6005.400 ng/mL and 25.313- 1518.808 ng/mL. Linearity was determined by using a $1/x^2$ weighed least square regression analysis of standard plots associated with eight-point standard curve. To

confirm blank interference in each of the standard curves, blank serum samples were also analyzed. The acceptance limit of accuracy for each of the back-calculated concentrations is ±15%, except for LLOQ where it is ±±20%. For a calibration run to be accepted at least 75% of the standards, including the LLOQ and ULOQ are required to meet the acceptance criterion otherwise, the calibration curve is rejected [12]. The samples were run from low to high concentration.

Intra-assay precision and accuracy were determined by analyzing six replicates at five different QC levels on two different days. Inter-assay precision and accuracy were determined by analyzing six replicates at five different QC levels. The acceptance criteria included accuracy within ±15% deviation (SD) from the nominal values, except LLOQ QC, where it should be ±20% and a precision of ±15% relative standard deviation (RSD), except for LLOQ QC, where it should be ±20%[12].

Recovery of the analyte was determined by comparing the peak areas of the analyte in spiked serum samples (six each of low, medium, and high QCs) with the those of the analyte in samples prepared by spiking the extracted drug-free serum samples with the same amounts of the analyte at the step immediately prior to chromatography. Similarly, recovery of the internal standard was determined by comparing the mean peak areas of the extracted QC samples (n=6) with those of the internal standard prepared by spiking the extracted drug-free serum samples with the same amounts of internal standards at the step immediately prior to chromatography.

The dilution integrity exercise was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations above the ULOQ during real time analysis. Dilution integrity experiment was carried out at 1.5 times the ULOQ concentration for the analyte. Six replicates each of dilution factor (DF) 5 concentrations were prepared and their concentrations were calculated by applying the DF 5.

Ruggedness of the method was evaluated by using different lot of the same column manufacturer and a different analyst. The precision and accuracy for the quality control samples at HQC, GMQC, MQC, LQC and LLOQ QC concentration levels were found to be within the acceptance limit.

Stability tests were conducted to evaluate the analyte stability in stock solutions and in serum samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2-8 °C) was performed by comparing the area response of the analyte (stability samples) with the response of the sample prepared from fresh stock solution. Bench top stability [6.0 hrs], processed samples stability [autosampler stability for 16 hrs, wet extract stability at room temperature 8 hrs, dry extracted stability (23 ± 2^{0}C) 08 hrs and reinjection reproducibility (70 hrs, 15 mins), Freeze and thaw stability (three cycles)] were performed at low and high QC levels using six replicates at each level and stability of analyte in serum has been proven at room temperature (12 hrs) and refrigerator temperature (20 hrs). Long term stability (12 days) was performed at low and high QC levels using six replicates at each level.

Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15% SD) and precision (?15% RSD).

RESULTS AND DISCUSSION

Method Development

Mass spectrometer parameters were tuned in positive ionization mode using electron impact ionization for the analytes and the internal standards. For the data acquisition MRM mode was used to get better selectivity.

Separation has been achieved by a various combination of GC parameters with MS detector in positive ionization mode. Helium was used as the carrier gas and an Ultra 1 column (12m X 0.2mm, 0.33µM film thickness) gave a good peak shape for both analyte and internal standard and at LLOQ level signal to noise ratio was found to be good. The retention time of DIS, N-DIS, DIS d^5 and N-DIS d^5 were 7.57, 9.11, 7.56 and 9.09 min. respectively.

Liquid-liquid extraction (LLE) technique was employed for the extraction of drug and internal standard. LLE is helpful in producing a spectroscopically clean sample when compared to protein precipitation and avoiding the introduction of serum components and non-volatile materials onto the GC and MS system.

An internal standard must mimic the analyte during extraction as well as during the ionization. For GC-MS/MS analysis, use of stable isotope-labeled molecule as internal standard proved to be helpful when there is a significant matrix effect. The use of DIS d^5 and N-DIS d^5 isotopically labeled internal standard is to compensate sample-to-sample variability and recovery.

Selectivity and Chromatography

The specificity of the GC-MS/MS method was established by screening the standard blanks of different lots from commercially available human serum. Ten different lots of serum were screened for the specificity experiment. All the investigated human serum lots were found to be free of interferences at the retention time of drug and the internal standard. Area of the peak at the retention time of drug in standard blank samples was ≤ 20.00% of the area of the analyte in the extracted LLOQ sample; area of the peak at the retention time of internal standard in standard blank samples was ≤ 5.00 % of the area of the internal standard in the extracted LLOQ sample. Positive product ion mass spectra of DIS, N-DIS and DIS d^5, N-DIS d^5 are given in Fig. 2,3 (a,b). MRM chromatograms of DIS and N-DIS at serum blank, LLOQ, LQC, GMQC, MQC and HQC are given in Figures 4 – 9 respectively.

Sensitivity

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The sensitivity of the method was evaluated by analyzing 6 LLOQ samples. The LLOQ was 100.291 ng/mL for DIS and 25.327 ng/mL for N-DIS. The precision and accuracy for DIS and N-DIS at LLOQ level were found to be 101.23 %, 99.66% and 4.56%, 4.05% respectively.

Fig 2a: Product ion mass spectra of $[M+H]^+$ of Disopyramide

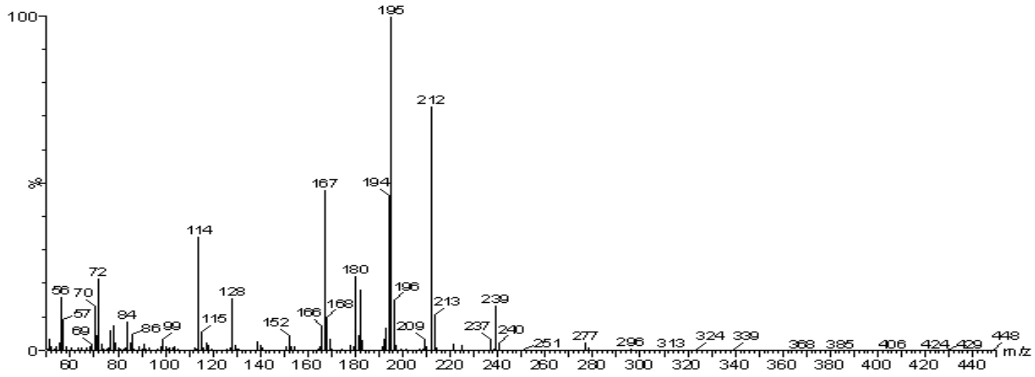

Fig 2b: Product ion mass spectra of $[M+H]^+$ of N-despropyldisopyramide

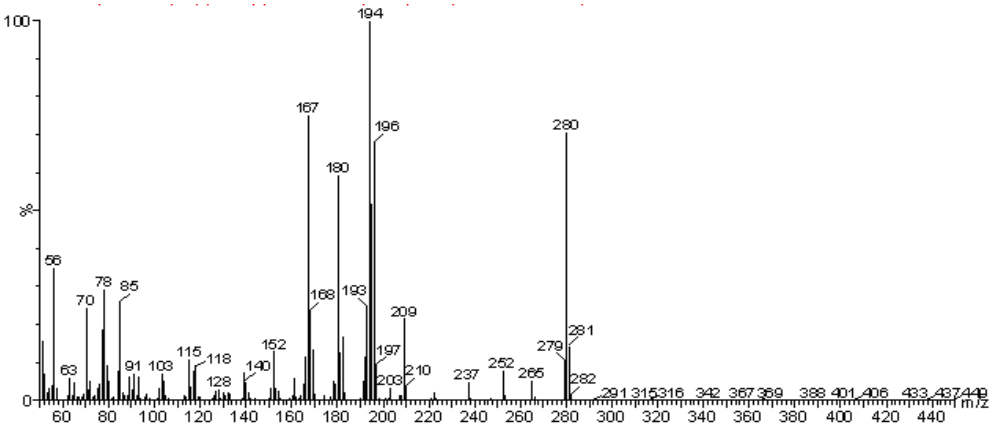

Fig 3a: Product ion mass spectra of $[M+H]^+$ of Disopyramide-d^5

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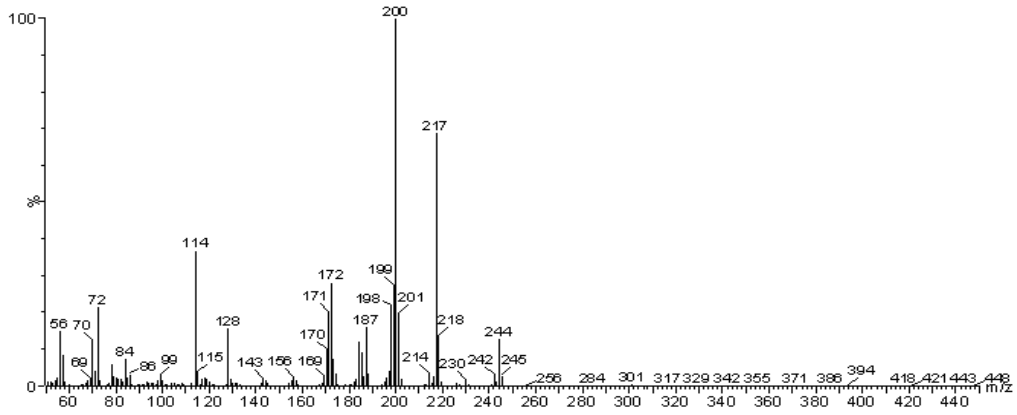

Fig 3b: Product ion mass spectra of $[M+H]^+$ of N-despropyldisopyramide -d^5

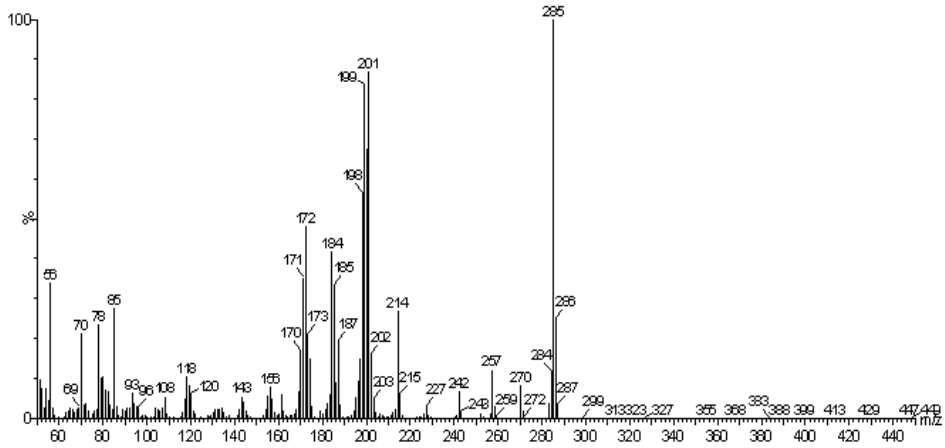

Fig.4: Typical MRM chromatograms of Disopyramide (left panel) and N-**Despropyl disopyramide (right panel) Serum Blank.**

Standard Zero sample (Serum Blank) Disopyramide, N-Despropyl disopyramide (Upper panel) and IS (Lower panel)

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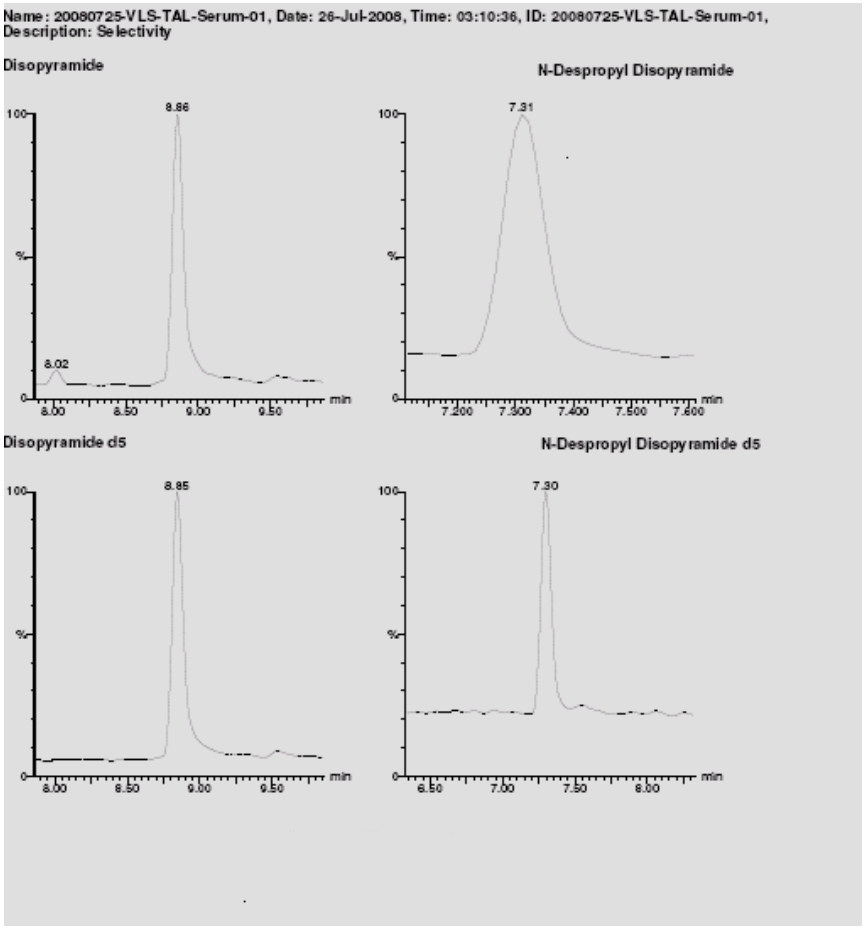

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Fig-5. Typical MRM chromatograms of Disopyramide, N-Despropyl disopyramide (Upper panel) and IS (Lower panel) at Lower Limit of Quantitation

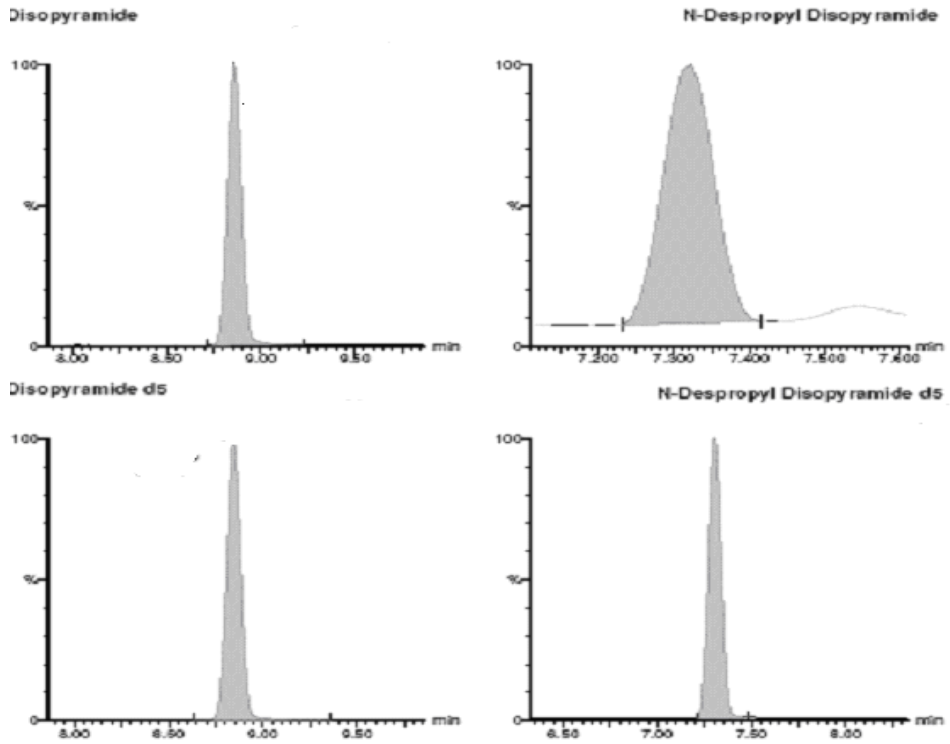

Fig6: Typical MRM chromatograms of Disopyramide, N-Despropyl disopyramide (Upper panel) and IS (Lower panel) at LQC

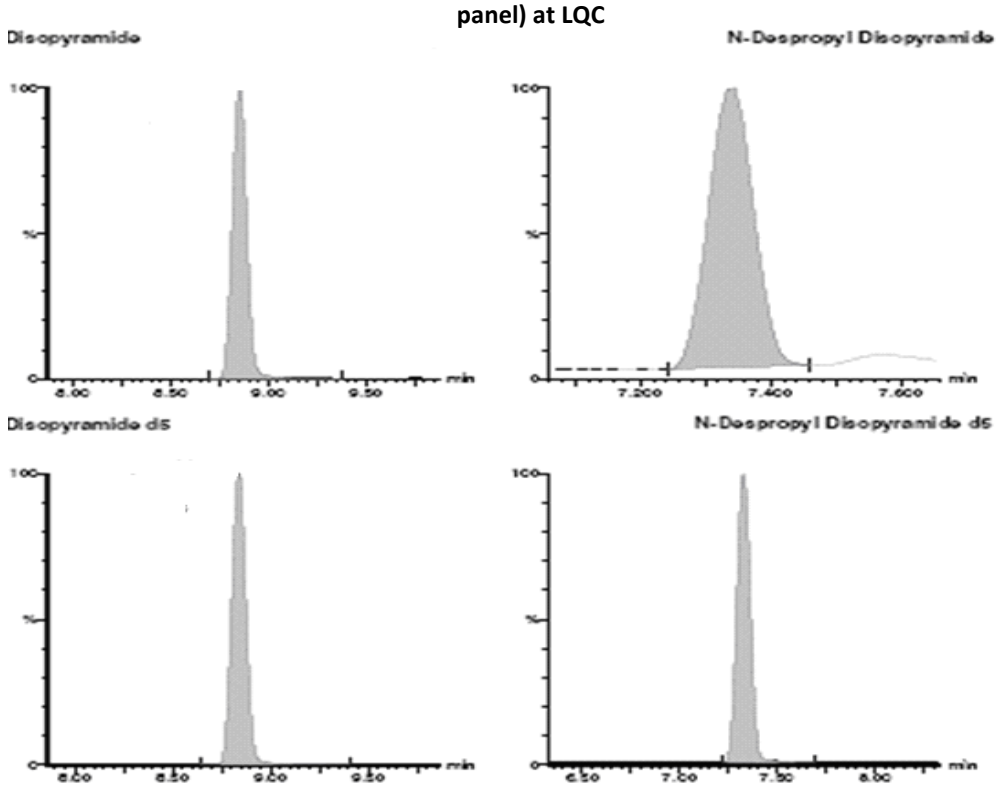

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Fig 7. Typical MRM chromatograms of Disopyramide, N-Despropyl disopyramide (Upper panel) and IS (Lower panel) at GMQC

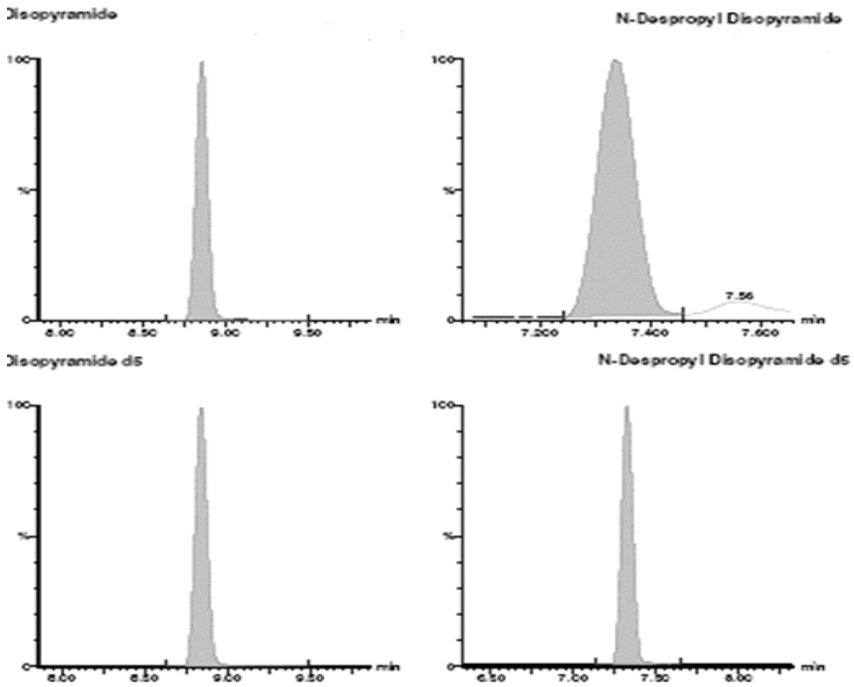

Fig 8. Typical MRM chromatograms of Disopyramide, N-Despropyl disopyramide (Upper panel) and IS (Lower panel) at MQC

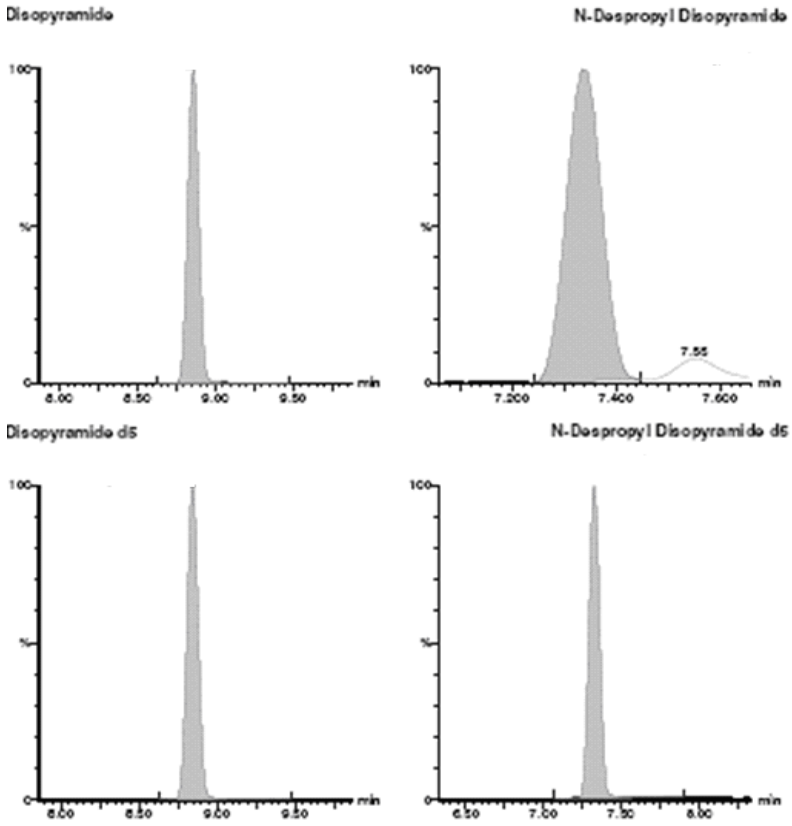

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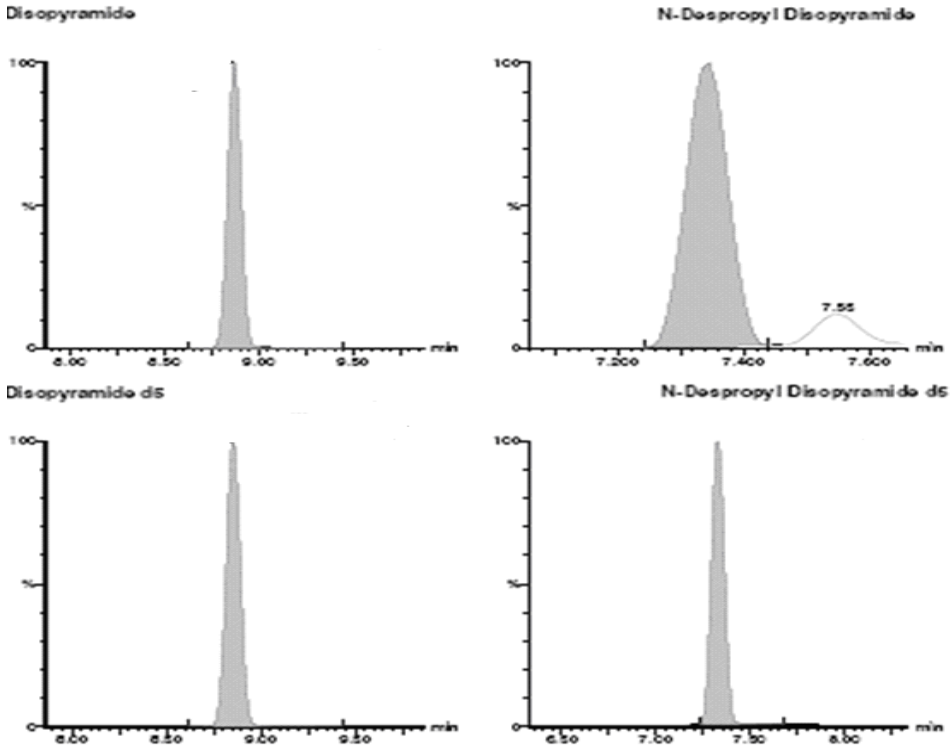
Fig 9. Typical MRM chromatograms of Disopyramide, N-Despropyl disopyramide (Upper panel) and IS (Lower panel) at HQC



Matrix Effect

No significant matrix effect was observed in all the six batches of human serum for the analyte at low and high QC concentrations. The precision and accuracy for DIS and N-DIS at low QC concentration was found to be 3.13%, 8.69% and at high QC level is 8.47%, 2.10% respectively.

Linearity

The linearity was determined by using a $1/x^2$(DIS) and 1/x(N-DIS) weighted least square regression analysis of standard plots associated with a eight-point standard curve. All the three calibration curves analyzed during the course of validation were found to be linear over the concentration ranging from 100.090 – 6005.400 ng/mL for DIS and 25.313 to 1518.808ng/mL for N-DIS. The correlation coefficient (r) was observed to be ≥ 0.99 and ≥ 0.99 The overall % mean accuracy for the CC standards was found to be between 92.58 – 106.43 % and 92.66 – 107.24 % the precision is 0.51 - 4.02 % and 1.00 – 5.43% respectively.

Precision and Accuracy

As shown in Table 2(a), 2(b), 3(a), 3(b), 4(a), 4(b), 5(a) and 5(b) the precision and accuracy of the method was evaluated by the % CV and % accuracy respectively, at different

concentration levels corresponding to LQC, GMQC, MQC and HQC during the course of validation. The precision and accuracy of analyte in the intra-batch and inter-batch runs were within ± 15%.

Table 2a: Intra-batch precision and accuracy for Disopyramide

Spiked QC Concentration (ng/mL)	Concentration found (mean; ng/mL)	Precision (%)	Accuracy
300.120	287.949	2.99	95.94
925.370	932.563	1.61	100.78
2801.120	2758.986	1.64	98.50
5402.160	5087.492	3.41	94.18

Table 2b: Intra-batch precision and accuracy for N-Despropyl Disopyramide

Spiked QC Concentration (ng/mL)	Concentration found (mean; ng/mL)	Precision (%)	Accuracy
75.981	79.206	4.08	104.24
227.942	210.818	1.71	92.49
709.152	706.519	2.24	99.63
1367.650	1368.496	1.68	100.06

Table 3a: Intra-batch precision and accuracy for Disopyramide

Spiked QC Concentration (ng/mL)	Concentration found (mean; ng/mL)	Precision (%)	Accuracy
300.120	294.154	4.27	98.01
925.370	936.780	2.78	101.23
2801.120	2864.973	2.34	102.28
5402.160	5391.119	4.88	99.80

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Table 3b: Intra-batch precision and accuracy for N-Despropyl Disopyramide

Spiked QC Concentration (ng/mL)	Concentration found (mean; ng/mL)	Precision (%)	Accuracy
75.981	77.511	3.79	102.01
227.942	204.606	2.13	89.76
709.152	701.119	3.48	98.87
1367.650	1336.908	1.93	97.75

Table 4a: Intra-batch precision and accuracy for Disopyramide

Spiked QC Concentration (ng/mL)	Concentration found (mean; ng/mL)	Precision (%)	Accuracy
300.120	290.368	1.76	96.75
925.370	896.517	1.44	96.88
2801.120	2659.958	2.42	94.96
5402.160	4855.773	1.42	89.89

Table 4b: Intra-batch precision and accuracy for N-Despropyl Disopyramide

Spiked QC Concentration (ng/mL)	Concentration found (mean; ng/mL)	Precision (%)	Accuracy
75.981	79.405	2.77	104.51
227.942	212.343	2.47	93.16
709.152	718.537	3.02	101.32
1367.650	1418.355	2.80	103.71

Table 5a: Inter-batch precision and accuracy for Disopyramide

Spiked QC Concentration (ng/mL)	Concentration found (mean; ng/mL)	Precision (%)	Accuracy
300.120	290.824	3.13	96.90
925.370	921.953	2.79	99.63
2801.120	2761.306	3.72	98.58
5402.160	5111.461	5.58	94.62

Table 5b: Inter-batch precision and accuracy for N-Despropyl Disopyramide

Spiked QC Concentration (ng/mL)	Concentration found (mean; ng/mL)	Precision (%)	Accuracy
75.981	78.707	3.55	103.59
227.942	209.256	2.59	91.80
709.152	708.725	2.97	99.94
1367.650	1374.586	3.26	100.51

Extraction Efficiency

Six replicates at low, medium and high quality control concentration for DIS and N-DIS was prepared for recovery determination. The recovery comparison samples of analytes were compared against the response of analyte in the mid QC level. The mean recovery of DIS and N-DIS at different levels of HQC, MQC, and LQC were found to be 97.20% and 92.69%. The mean recovery of internal standards was found to be 93.30% and 76.19% respectively.

Dilution Integrity

The upper limit of quantitation can be extended to 9008.100 ng/mL for DIS, 2278.212 ng/mL for N-DIS. The dilution integrity of the method was evaluated for DF 5 (1777.680 for DIS and 455.642 for N-DIS) with screened human blank serum. The precision for DF 5 was found to be 3.84, 3.94 and the % mean accuracy for DF 5 was found to be 92.37 % and 91.76% which are within acceptance limit of 85.00 - 115.00 %. The results are summarized in the Table 6.

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Table 6: Dilution Integrity

Analyte	Dilution Factor	DIQC (spiked concentration (ng/mL)	Concentration found (mean; ng/mL)	Mean Accuracy (%)	Precision (%)
DIS	**5**	1777.680	1924.547	92.37	3.84
NDIS	**5**	455.642	418.102	91.76	3.94

Ruggedness

Ruggedness was performed by using a different lot of the same column manufacturer and different analyst. The precision and % mean accuracy for the quality control samples at HQC, GMQC, MQC and LQC concentration levels were found to be within the acceptance limit of 15.00 %. For all the samples of LLOQ QC was found to be within the acceptance limit of ≤ 20.00 %. The results are summarized in the Table 7 (a,b) and Table 8 (a,b).

Table 7a: Ruggedness Precision and Accuracy for Disopyramide Different column

QC (spiked concentration (ng/mL)	Concentration found (mean; ng/mL)	Mean Accuracy (%)	Precision (%)
300.120	294.154	4.27	98.01
925.370	936.780	2.78	101.23
2801.120	2864.973	2.34	102.28
5402.160	5391.119	4.88	99.80

Table7b: Ruggedness Precision and Accuracy for Disopyramide Different analyst

QC (spiked concentration (ng/mL)	Concentration found (mean; ng/mL)	Mean Accuracy (%)	Precision (%)
300.120	290.368	1.76	96.75
925.370	896.517	1.44	96.88
2801.120	2659.958	2.42	94.96
5402.160	4855.773	1.42	89.89

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Table 8a: Ruggedness Precision and Accuracy for N-Despropyl Disopyramide Different column

QC (spiked concentration (ng/mL)	Concentration found (mean; ng/mL)	Mean Accuracy (%)	Precision (%)
75.981	77.511	3.79	102.01
227.942	204.606	2.13	89.76
709.152	701.119	3.48	98.87
1367.650	1336.908	1.93	97.75

Table 8b: Ruggedness Precision and Accuracy for N-Despropyl Disopyramide Different analyst

QC (spiked concentration (ng/mL)	Concentration found (mean; ng/mL)	Mean Accuracy (%)	Precision (%)
75.981	79.405	2.77	104.51
227.942	212.343	2.47	93.16
709.152	718.537	3.02	101.32
1367.650	1418.355	2.80	103.71

Stability

In the different stability experiments carried out viz. bench top stability (06 hrs), autosampler stability (16 hrs), repeated freeze-thaw cycles (3 cycles), reinjection reproducibility (70 hrs), 15 mins), and wet extract stability at room temperature (8 hrs) dry extract stability (08 hrs -23±2^{0}C), the mean % nominal values of the analyte were found to be within ?15% of the predicted concentrations for the analyte at their low and high QC levels. Thus the results were found to be within the acceptable limits during the entire validation. Long term stability at –70?C for 12 days, the mean % nominal values of the analyte was found to be within ?15% of the predicted concentrations for the analyte at their low and high QC levels. The results are summarized in Table 9 (a,b).

The sample preparation technique, described offers a rapid means of processing samples for the assay of both total Disopyramide (DIS), and N-Despropyl disopyramide (N-DIS), thus making the assay simple, rapid and rugged. The use of GC-MS/MS technology enables the performance of highly accurate analysis, thus making it sensitive, specific and selective in the range validated. This GC-MS/MS method developed and validated, is not only suitable for pharmacokinetic studies for humans, but also plays an extremely important role in therapeutic drug monitoring in the development and clinical application to treat cardiac rhythm disturbances.

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Table 9a: Stability Samples Results for Disopyramide

Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Accuracy/ Stability (%)	Precision (%)
Autosampler [a]	**300.120**	278.948	92.95	1.73
	5402.160	5158.340	95.49	6.62
Wet extract (RT)[b]	**300.120**	302.839	100.91	10.15
	5402.160	5013.200	92.80	2.21
Bench top[c]	**300.120**	281.698	93.86	8.17
	5402.160	4964.229	91.89	3.14
FT[d]	**300.120**	274.838	91.58	8.77
	5402.160	5178.822	95.87	5.49
Dry extract[e]	**300.120**	283.587	94.49	9.16
	5402.160	4994.507	92.45	1.66
Long-term[f]	**300.120**	289.416	96.43	1.13
	5402.160	4782.152	88.52	2.01

[a] 16 hrs, in auto sampler at 23±2°C; [b] after 21 hrs at room temperature; [c] 10 hrs on bench; [d] after three freeze and thaw cycles; [e] processed sample stability 21 hrs at 23±2^{0}C; [f] 12 days stable in Serum

Table 9b: Stability Samples Results for N-Despropyl Disopyramide

Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Accuracy/ Stability (%)	Precision (%)
Autosampler [a]	**75.981**	67.865	89.32	2.55
	1367.650	1370.788	100.23	7.92
Wet extract (RT)[b]	**75.981**	77.378	101.84	5.36
	1367.650	1353.892	98.99	2.54
Bench top[c]	**75.981**	77.058	101.42	7.93
	1367.650	1258.503	92.02	2.53
FT[d]	**75.981**	74.077	97.49	10.18
	1367.650	1289.126	94.26	3.75
Dry extract[e]	**75.981**	78.688	103.56	11.61
	1367.650	1332.200	97.41	6.28
Long-term[f]	**75.981**	71.007	93.45	2.55
	1367.650	1348.108	98.57	1.41

[a] 16 hrs, in auto sampler at 23±2°C; [b] after 21 hrs at room temperature; [c] 10 hrs on bench; [d] after three freeze and thaw cycles; [e] processed sample stability 21 hrs at 23±2^{0}C; [f] 12 days stable in Serum

CONCLUSION

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