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DESIGN OF EXPERIMENT IN THE BIO-ANALYTICAL DETERMINATION OF QUETIAPINE FUMARATE IN HUMAN PLASMA BY A RP-HPLC METHOD

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Keywords:

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ABSTRACT: A new, simple, sensitive, accurate and precise RP-HPLC method was developed for the estimation of quetiapine fumarate in human plasma. Full factorial design was used for the optimization of an extraction method. The main effect of volume of deproteinating agent, speed of centrifugation, time of centrifugation and temperature of centrifugation was found to be significant at P<0.0001 on all the responses. After deproteinization, the drug was analyzed on a C18 (150 × 4.6mm, 5 µm) column using UV detector. The mobile phase consisting acetonitrile and phosphate buffer (pH 3) in the ratio of 50:50 (v/v) at a flow rate of 1.0 ml/min. The standard calibration curve was constructed in the concentration range of 5 µg/ml to 30 µg/ml and linearity was found to be 0.999. Irbesartan was used as the internal standard. The retention time of quetiapine fumarate and the internal standard was found to be 5.42 and 2.89 min, respectively. No interference peak was perceived. The high performance liquid chromatography method was successfully demonstrated as rapid and sensitive method which can be used as an alternative for the analysis of quetiapine fumarate in plasma samples.

INTRODUCTION: Quetiapine fumarate (QTF) is an atypical antipsychotic agent indicated for the treatment of schizophrenia and for the treatment of acute manic episodes associated with bipolar disorder. It is a selective monoaminergic antagonist [1]. However, this effect is mediated through antagonism of dopamine type 2 (D_2) and serotonin type 2 ($5HT_2$) receptors. QTF is a dibenzothiazepine derivative and is chemically 2, (2-[2-(4-Dibenzo [*b,f*] [1,4]thiazepin-11-yl-1-piperazinyl) ethoxy] ethanol) fumarate.

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QTF belongs to the same family as clozapine and olanzapine, which are classified as a typical anti-psychotic and do not cause major extrapyramidal side effects. The generic name is Seroquel. It is white or almost white powder, moderately soluble in water and soluble in methanol and 0.1 N HCl. It is available in tablets form in dosage level of 25 mg, 50 mg, 100 mg, 200 mg, 300 mg and 400 mg. Maximum daily dosages is 800 mg in adults. This drug is rapidly absorbed after oral administration with peak plasma concentration attained within 1.50 hrs. Bioavailability of tablet formulation is 100% relative to an oral solution, which may be marginally affected by food. Plasma protein binding of QTF is 83 %. The drug is extensively metabolites, principally through CYP3A4. The drug is having half-life period of approximately 6 hours.

Quetiapine fumarate is not official in any pharmacopoeia [2]. Literature survey reveals that LC-MS/MS [3], LC-Electrospray-Tandem Mass Spectroscopy [4], HPLC [5] methods have been reported for determination of QTF in human plasma. However, these existing methods involved multiple steps of extraction and expensive. Hence, there is a need to develop and validate a simpler, easier, faster yet economical new bio-analytical method for the extraction and estimation of quetiapine in plasma samples using HPLC with a UV detector. It is also necessary that the extraction process be simpler as rapid as possible. Although, the extraction process has been well established, it is affected by the choice of de-proteinizing agent used, volume of the extracting solvent, simplicity of the extraction and the protein separating process [3-4].

Furthermore, in the estimation process, short analysis time, sensitivity, robustness, precision, accuracy, sharpness of the peak and other economic aspects should be considered. These factors can be achieved with appropriate extraction conditions, appropriate buffer selection, pH and UV detector wavelength and flow rate, stationary and mobile phase. A slightly volatile buffer with a suitable pH should be used with respect to the pKa value of drug for appropriate elution, while composition of the mobile phase and flow rate should be adjusted to reduce the retention time [6].

The aim of the present study was to develop and validate a sensitive, simple, easy, fast, reproducible, precise and economical bio-analytical HPLC method for the estimation of QTF in human plasma. To achieve the aim of the present study, design of experiment (DOE) approach has been embraced for the statistical optimization of de-proteinizing agent, volume of de-proteinizing agent, centrifugation speed and time as a part of the extraction process. Further, the extracted drug samples were analysed with the newly developed and validated HPLC bio-analytical method.

Chemicals and Reagents: Active pharmaceutical ingredient (API) working standards of Quetiapine fumarate, was obtained as gift sample from Lupin Limited, Pune, India. HPLC grade acetonitrile, methanol and orthophosphoric acid were obtained from Merck, Mumbai, India Limited. HPLC grade water was obtained from Molychem, Thane, India. The blank human plasma was obtained from Blood bank (healthy human volunteer).

Instrumentation: The HPLC system (Cyberlab LC 100) consisting of binary gradient pump, micro-sorb-MV 100-5 C- 18 column (250 × 4.6mm, 5 µm), UV detector was employed for analysis. Chromatographic data was acquired using WS-100 Workstation software. Microsorb MV 100-5 C-18 column (150 mm×4.6 mm, 5µm) was used as a stationary phase. A Millipore glass filter (Millipore filter cellulose nitrate gridded with 0.22 µ size and 47 mm diameter) assembly attached with vacuum pump was used to filter mobile phase. Samples were sonicated for 20 min using ultrasonic cleaner-Equitron-Medica Instrument Mfg. Co., Mumbai, India) to remove dissolved gases from the mobile phase. A Genie-2 Spinix model vortex mixer, a cooling centrifuge (Remi, Germany) was used for extraction of the drug from human plasma. Eqiuptronics pH meter Instrument using a glass electrode was used to adjust the pH of buffer.

Chromatographic Conditions: The isocratic mobile phase consisting of a mixture of phosphate buffer (pH 3.0 adjusted with ortho-phosphoric acid) and acetonitrile in the ratio of 50:50 (v/v) was used on microsorb MV 100-5 C-18 column (250 mm × 4.6 mm, 5 µm) as a stationary phase. The flow rate of the mobile phase was 1.0 ml/min. Detector signal was monitored at a wavelength of 292 nm using UV detector while keeping the 10 min run time for chromatographic analysis. Prior to the injection of the drug solution, the column was equilibrated for at least 30 min. The column temperature was kept ambient and injection volume was 20 µl.

Preparation of Stock Solutions, Calibration Standards and Quality Control Sample: Quetiapine fumarate standard stock solution was prepared by transferring 10 mg of QTF working standard into a 100 ml volumetric flask, approximately 25 ml of diluent was added and sonicated for 20 min. The volume was made up to 100 ml with diluent. This solution was filtered through a 0.45 µm pore size Nylon 66 membrane filter. The subsequent dilutions were prepared by diluting stock solution with the mobile phase. Irbesartan was used as an internal standard (IS) in the study.

Accurately weighed amount of irbesartan (10 mg) was taken in a 100-ml glass volumetric flask and the volume was made up to the mark with mobile phase to produce 100 µg/ml concentrations. Further, appropriate dilution was made to produce a working concentration of irbesartan. A 10 µl aliquot of each spiking solution was added to 490 µl of plasma to provide calibration concentrations of 5, 10, 15, 20, 25, 30 µg/ml. Similarly, quality control samples of 15, 20 and 25 µg/ml were prepared. QC and sample concentrations were calculated from the calibration curve using a linear regression analysis.

Technique for Extraction: A solvent deproteinization technique was used for extraction of quetiapine from human plasma. A 490 µl volume of plasma was transferred to a 2 ml micro centrifuge tubes, and then 10 µl of QTF working solution and 10µl of IRB was spiked. After vortexing for 30 s, then the pre-optimized deproteinizing agent was added. The sample-containing micro centrifuge tubes were centrifuged at pre-optimized speed for an optimized time and temperature. Then the supernatant liquid was transferred to prelabled HPLC vial for analysis.

Optimization of Extraction Process: The volume of deproteinizing agent, speed of centrifugation, time of centrifugation and temperature of centrifugation may play an important role in the extraction of drug. Hence, in this research work, these four factors were optimized using DOE (Design Expert® v.10.0.6.0 software). In the present study, ACN was used as deproteinizing agents. The deproteinizing agent was used at two different levels, such as 500 µl and 1000 µl. Two different centrifugation speeds such as 10000 and 15000 rpm were used for the separation of supernatant. Similarly, centrifugation time and temperature were also studied at two different levels, such as 10 and 15 min. whereas 5 and 10 ºC respectively.

Optimization of independent variables with their interactions became easier with the applications of design of experiments [7 - 9]. With the help of design of experiment, it is possible to study the effect of abundant variables at a time. This research work involves the use of central composite design for optimization of independent variables. In this study, four independent variables with two levels were used, which suggested total 16 runs to execute the experiment. The volume of de-proteinizing agent (A), speed of centrifugation (B), time of centrifugation (C) and temperature of centrifugation (D) were used as independent variables. These independent variables were projected at two different levels, such as minimum (−1) and maximum (+1). Total 30 experimental runs were executed and the obtained response like % extraction of Drug from plasma (RS1), the number of theoretical plates (USP) (RS2) and drug tailing factor (RS3) was fed into the software to study the interaction between the independent variables and for optimization.

Factors and levels used in central composite design for extraction of quetiapine from human plasma are shown in **Table 1** and the composition of experimental runs are shown in **Table 2**. The significance of independent variables was estimated using analysis of the variance (ANOVA) and the Quadratic Equation, $RS = \beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_4D+\beta_{12}AB+\beta_{13}AC+\beta_{14}AD+\beta_{23}BC+\beta_{24}BD+\beta_{34}C\ D+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{44}D^2$, where, RS is the response, β represents as regression coefficients and A, B, C and D are, volume of deproteinizing agent, speed of centrifugation, time of centrifugation and temperature of centrifugation respectively.

Validation of HPLC method: Optimized and developed method was validated as per the USFDA guidelines. The method was validated with the following steps such as selectivity, linearity, accuracy, precision, recovery and stability [10].

Selectivity: Selectivity is related to the absence of interference on the retention time of drug peak by the proteins and/or other impurities. The rat blank plasma samples and plasma with drugs (for LLQC, LQC, MQC and HQC samples) were extracted with the same above given procedure for drug extraction and were analysed by HPLC with six replicate injections of each.

Linearity: Calibration plot was constructed with blank samples (plasma without drug) and eight different concentrations from 5 µg/ml - 30.0 µg/ml. Each concentration was repeated in triplicates. Drug concentration was plotted against drug/IS peak area ratio to find the linear regression and correlation coefficient.

Accuracy: Accuracy was determined at three different quality control concentrations such as LQC, MQC and HQC. Percent accuracy was calculated using nominal concentrations and average back calculated concentrations using the formula given in Eqn. Average back calculated concentration was determined, from six replicate injections of each quality control concentration. Percent accuracy = BC/NC × 100, where, BC represents the average back calculated concentration and NC represents the nominal concentration of drug.

Precision: Study was conducted as intra-day precision and inter-day precision. In this study three quality control levels, such as LQC, MQC and HQC were considered. Intra- day precision of the developed method was performed for these three quality controls by means of six replicates of each two times in a day (*i.e.*, morning and evening). Similarly, inter-day precision study was performed for these three quality controls by means of six replicates of each, two times on two different days (*i.e.*, 1 and 2 day). The drug/IS peak area ratio was measured and percent relative standard deviation (% RSD) was calculated.

Recovery: Absolute recovery of quietiapine from spiked human plasma samples (extracted samples) was compared with analytical standards of the same concentration (un-extracted samples). In the present study, the mean percent recovery was calculated at three different levels of concentration, such as LQC, MQC and HQC. For each concentration level, the extracted and un- extracted samples were analysed in triplicate and the mean was calculated. The value of recovery towards 100 % indicates that the selected solvent has very high extraction efficiency and it also indicates that the method is robust. The formula used to calculate the percent recovery is given in Equation Percent recovery = AE/AU×100, where, AE is the peak area of extracted samples and AU is the peak area of un-extracted samples.

Limit of Detection and Limit 0f Quantitation: Limit of detection and limit of quantitation for quetiapine was determined at a signal-to-noise ratio of 3:3 and 10:1, respectively, by using serial dilutions of known concentration.

Stability Studies: Stability of quietiapine in human plasma was evaluated using LQC and HQC samples under different stress conditions. The percent drug remaining in the plasma was calculated using the formula given in Eqn. Percent drug remaining = final concentration/initial concentration ×100.

Bench Top Stability Study: Bench top stability study of valsartan in human plasma was performed by keeping three replicates of each low and high plasma quality control concentrations for 24 h at room temperature. After 24 h, the samples were processed and the drug was extracted. The extracted samples were analysed by HPLC system and the concentration was calculated.

Freeze Thaw Stability Study: Freeze thaw stability of valsartan in rabbit plasma was assessed by analysing three replicates of each low and high quality control concentrations after three freeze and thaw cycles. For each cycle, the samples were kept at -70 °C for 12 h for frozen and thaw at room temperature for 2 to 3 h. After third cycle, the samples were extracted and were analysed using HPLC. The concentration was calculated using linear equation obtained from calibration plot.

Long-Term Stability Study: This is the study which gives the information about the one-month stability of drug in plasma at -70 °C. Long- term stability of valsartan was determined after storage of three replicates of each low and high quality control concentrations at -70 °C for 30 d. After 30 d, the samples were processed for extraction of drugs and were analysed using HPLC. Then the final concentration of drug in samples was calculated.

TABLE 1: CENTRAL COMPOSITE DESIGN FOR EXTRACTION OF QUIETIAPINE FROM HUMAN PLASMA

Independent factors	Levels	
	-1	+1
Volume of deproteinating agent (µl)	500	1000
Speed of centrifugation (rpm)	10000	15000
Time of centrifugation (min)	10	15
Temperature of centrifugation (ºC)	5	10

TABLE 2: EXPERIMENTAL RUNS OBTAINED FROM CENTRAL COMPOSITE DESIGN

Run	Independent Variables			
	Volume of deproteinating agent (µl)	Speed of centrifugation (rpm)	Time of centrifugation (min)	Temperature of centrifugation (ºC)
1	500	10000	10	5
2	1000	10000	10	5
3	500	15000	10	5
4	1000	15000	10	5
5	500	10000	15	5
6	1000	10000	15	5
7	500	15000	15	5
8	1000	15000	15	5
9	500	10000	10	10
10	1000	10000	10	10
11	500	15000	10	10
12	1000	15000	10	10
13	500	10000	15	10
14	1000	10000	15	10
15	500	15000	15	10
16	1000	15000	15	10
17	250	12500	12.5	7.5
18	1250	12500	12.5	7.5
19	750	7500	12.5	7.5
20	750	17500	12.5	7.5
21	750	12500	7.5	7.5
22	750	12500	17.5	7.5
23	750	12500	12.5	2.5
24	750	12500	12.5	12.5
25	750	12500	12.5	7.5
26	750	12500	12.5	7.5
27	750	12500	12.5	7.5
28	750	12500	12.5	7.5
29	750	12500	12.5	7.5
30	750	12500	12.5	7.5

RESULT AND DISCUSSION:

Optimisation of Extraction of Drug from Plasma: The extraction process was optimized using the Design Expert® v.10.0.6.0 (Stat-Ease, Inc., Mumbai) by employing the central composite design. The main and complexed effects of independent factors on responses were analysed. The effect of independent variables on responses was assessed with the help of quadratic equation and perturbation plots constructed with the software as shown in **Fig. 1**.

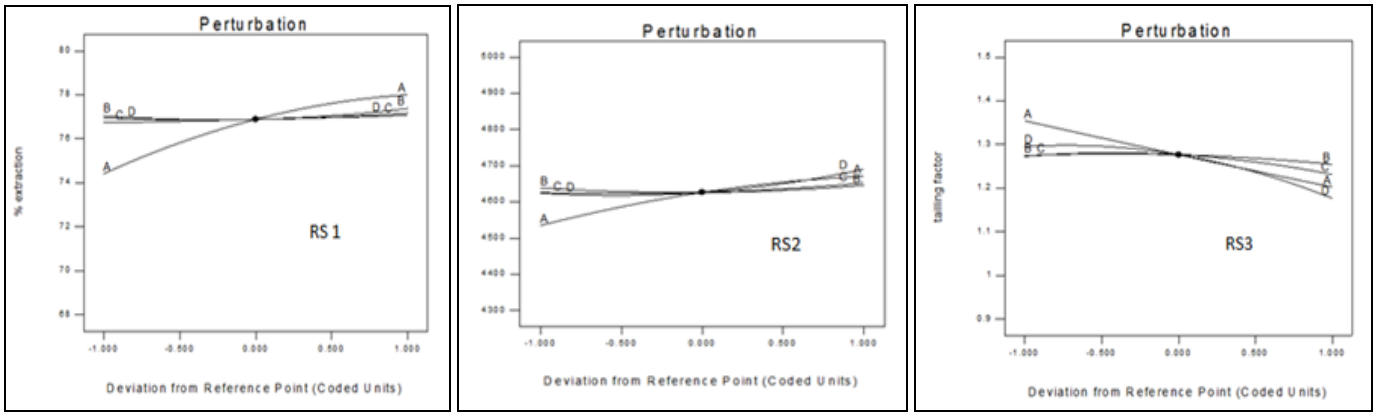

FIG 1: EFFECT OF FACTORS, VOLUME OF DEPROTEINISING AGENT (A), SPEED OFCENTRIFUGATION (B), TIME OF CENTRIFUGATION (C) AND TEMPERATURE OF CENTRIFUGATION (D) ON % DRUG EXTRACTION (RS1), NUMBER OF THEORETICAL PLATES (RS2) AND TAILING FACTOR (RS3)

Effect of independent factors on % extraction of Drug from plasma (RS1) shows that the volume of deproteinizing agent (A) showed a significant effect indicating that volume of deproteinizing agent increases the % drug extraction. Similarly, the centrifugation speed (B) and Centrifugation Temperature (D) slightly decreases the % extraction of drug. However centrifugation time (C) had no effect on extraction of the drug from the plasma. The perturbation plot of number of theoretical plates (RS2) indicates that there is no effect of speed of centrifugation (B) and time of centrifugation (C) on the number of theoretical plates whereas the volume of deprotenizing agent (A) increases the number of theoretical plates and temperature of centrifugation (D) slightly increases the number of theoretical plates. The volume of deproteinizing agent (A), time of centrifugation (C) and temperature of centrifugation (D) decreases the tailing factor (RS3), however the speed of centrifugation has no effect on the tailing factor.

Responses obtained, such as a % extraction of Drug (RS1), the number of theoretical plates (RS2) and drug tailing factor (RS3) were entered in the Design Expert software and were analyzed using ANOVA were A, B, C and D are volume of deproteinizing agent, speed of centrifugation , time of centrifugation and temperature of centrifugation respectively. The equations for the response factors are given below:

% drug Extraction (RS1) = +76.89 +1.79 * A +0.16 * B +0.17 * C +0.11 * D +0.30 * AB -0.57 * AC -0.28 * AD -0.13 * BC -0.30 * BD -0.066 * CD -0.68 * A2+0.33 * B2 +9.479E-003 * C2 +0.17 * D2

Number of theoretical Plates (RS2) = +4626.50 +67.83 * A +3.50 * B +13.25 * C +32.92 * D -28.62 * AB +16.00 * AC +31.00 * AD +2.38 * BC -36.87 * BD +24.50 * CD -23.48 * A2+16.15 * B2 +12.65 * C2 +31.15 * D2

Tailing Factor (RS3) = +1.28 -0.076 * A -0.010 * B -0.021 * C -0.059 * D +0.029 * AB+2.500E-003 * AC+8.750E-003 * AD+0.040 * BC-1.250E-003 * BD-0.012 * CD+1.875E-003 * A2 -0.012 * B2-0.024 * C2-0.041 * D2

Validation of optimized factors, responses obtained from optimized independent factors such as volume of deproteinating agent, speed of centrifugation, time of centrifugation and temperature of centrifugation were validated by comparing the observed responses and predicted responses. The difference between the predicted responses and observed responses was found to be within ± 2.32 % as shown in the **Table 3**. The percent residual value is calculated using the formula: percent residual = (predicted responses-observed responses/ predicted responses)×100, desirability value was found to be 0.885. The desirability is an indicative for the assessment of suitability of method and the value was found towards 1.0, which indicates that the process of extraction is strong.

Validation of Bioanalytical Method: The method was validated as per the USFDA guidelines. The validated method was simple, economical and rapid due to its short run time of quitiepine with 5.42 min and that of internal standard irbesartan was 2.89 min. Specificity studies confirmed that the spiked blank plasma sample showed no peak on or near the retention time of drug and internal standard. The chromatographs of drug and internal standard are shown in **Fig. 2**.

Correlation coefficient value obtained from linearity indicated a strong correlation of 0.9962. The linear regression obtained from a calibration plot was found as y=0.8254x+0.0812. Where, y is representative of drug/IS peak area ratio and x is representative of drug concentration. Accuracy results suggested that the method was accurate with % RSD from 2.65 % to 4.78 %. Percent accuracy was also found within the range such as from 96.08 to 100.14 % as shown in **Table 4**. Precision results specified that this method has been precise %RSD from 2.46 % to 3.5 %. Mean recovery of valsartan from spiked human plasma (extracted) samples was compared with un-extracted samples of same concentration and found to be within 77.53 to 79.31% with percent RSD values between 0.47-0.69%. Recovery results are reported in **Table 5**.

TABLE 3: VALIDATION OF OPTIMIZED INDEPENDENT VARIABLES

Response	Predicted results	Observed result	Residual values
% extraction of Drug from plasma	77.84	79.65	-2.32
Number of theoretical plates	4908	`4856	1.05
Tailing factor	0.987	0.977	1.01

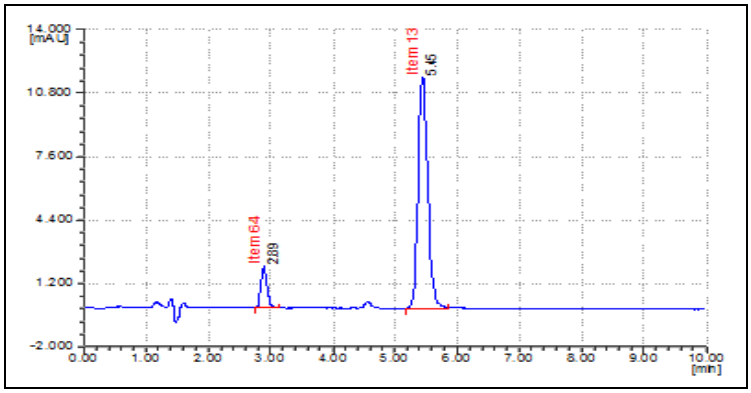

FIG. 2: CHROMATOGRAM OF QUITIEPINE AND IRBESARTAN (IS)

TABLE 4: PRECISION AND ACCURACY RESULTS OF PROPOSED METHOD (n=6)

Quality control level	Nominal concentration (µg/ml)	Precision (% RSD)		Accuracy	
		Intra-day	Inter-day	% Accuracy	% RSD
LQC	5	3.5	3.02	96.08	4.78
MQC	10	3.33	2.46	99.04	2.65
HQC	25	2.92	3.22	100.14	3.46

TABLE 5: RECOVERY FROM HUMAN PLASMA (n=3)

Quality control level	Nominal concentration (µg/ml)	Percent mean recovery (%)		% RSD
		Mean	SD	
LQC	5	77.53	0.53	0.69
MQC	10	79.31	0.51	0.64
HQC	25	78.85	0.37	0.47

TABLE 6: RESULTS OF STABILITY STUDIES

Quality control level	Percent Drug (%)		
	Bench Top Stability	Freeze Thaw Stability	Long Term Stability
LQC	98.8	96.6	97.8
HQC	99.0	97.2	98.2

Limits of detection and limit of quantitation were found to be 5.2 and 16 mg/ml, respectively. Stability studies result investigated at two levels such as LQC and HQC is reported in the **Table 6**. The stability studies revealed that the quitiepine was stable.

CONCLUSION: A HPLC method was developed and optimized successfully by implementing Design expert version 10.0.6.0 software. High significant effect of independent variables has been noticed in the form of perturbation plots. This study concludes that the DoE is a highly efficient tool for the optimization of independent variables for a bio-analytical method development. The method has been validated as per USFDA guidelines and the results obtained indicated that the present method was a novel, simple, accurate, precise, economical and robust.

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CONFLICTS OF INTEREST: Nil

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