HIGHLY SENSITIVE AND RAPID EVALUATION OF PYRIDOSTIGMINE IMPURITY B IN HUMAN PLASMA BY LIQUID CHROMATOGRAPHY COUPLED WITH TANDEM MASS SPECTROMETER AFTER ADMINISTRATION OF PYRIDOSTIGMINE TO HEALTHY VOLUNTEERS IN A PHARMACOKINETIC STUDY

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INTRODUCTION

The key concern by any regulator on pharmaceutical formulations is whether the formulation is safe to administer? Especially are the endogenous impurities safe? As per United States product monograph, accepted limit of endogenous pyridostigmine impurity B as impurity in the pyridostigmine formulation is 0.2%, more than 0.2% impurity may raise safety concerns. Regulatory interested to know the level of pyridostigmine impurity B in humans (exogenous+endogenous) when administered innovator formulation as well as test formulation as impurity in the pyridostigmine formulation is 0.2%, more than 0.2% impurity may raise safety concerns. Regulatory interested to know the level of pyridostigmine impurity B in plasma may cause potential health hazard.

Pyridostigmine impurity B is a metabolite of pyridostigmine. Pyridostigmine is an orally active cholinesterase inhibitor. Pyridostigmine is useful in the treatment of myasthenia gravis. Pyridostigmine inhibits the destruction of acetylcholine by cholinesterase and thereby permits freer transmission of nerve impulses across the neuromuscular junction [1].

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No methods are published in the public domain for the determination of pyridostigmine impurity in plasma by liquid chromatography coupled with tandem mass spectrometer (LC-MS/MS), one method was published using high-performance liquid chromatography [2]. There were multiple methods published for the quantitation of pyridostigmine impurity B in plasma but none of them is sensitive and selective, rapid. These methods are not sensitive (10 pg/ml limit of quantitation) and selective, rapid (3 min), multiple reaction monitoring method has been developed and validated for the estimation of pyridostigmine impurity B in plasma by LC-MS/MS with 10 pg/ml and 3 min run time.

Our work is first ever for the determination of pyridostigmine impurity B in plasma by LC-MS/MS. Highly sensitive (10 pg/ml limit of quantitation) and selective, rapid (3 min), multiple reaction monitoring method has been developed and validated for the estimation of pyridostigmine impurity B in human plasma (K_{EDTA} as an anticoagulant) by simple isocratic liquid chromatography with 10 µl injection on ESI electron spray ionization tandem mass spectrometry system, method involves sample processing on solid phase extraction with very low matrix effects and highly selective and rapid, lamivudine used as internal standard.

Pyridostigmine impurity B is a low molecular weight basic polar drug. Basic functional groups are active for extraction and ionization. Due to the drug polar in nature, we got matrix effect problems but lamivudine (internal standard) found compensating matrix effects, 15% normalized matrix factor is within limits. Recovery found ~40% due to matrix effects, but recovery is consistent and reproducible. Selectivity, precision, and accuracy found acceptable.
MATERIALS AND METHODS

Materials
Pyridostigmine impurity B and lamivudine (as internal standard) working reference standards are purchased from our vendor Vivan Life sciences, India, Hypurit mixed-mode cation exchange 1cc/30 mg cartridges were purchased from the National Chromatography Inc., USA. Positive pressure solid phase extraction manifolds of Orochem (USA) were utilized for processing. In-house collected blank plasmas are used for the preparation of calibration curve and quality control samples. Bioequivalence study was conducted based an Independent Ethics Committee approved protocol.

Methods
1 mg/ml stock solutions of pyridostigmine impurity B and lamivudine were prepared in methanol as solvent, further working dilutions and spiking solutions are prepared in 50% methanol-water solution as diluent. Stock and working dilutions are stored in refrigerator maintained at 2–8°C for storage. Spiking solutions were bulk spiked in screened blank plasma for calibration curve standards and quality control samples. Eight point calibration curve for the range 1.4–39.6 ng/ml is prepared, and four levels of quality control are also prepared within calibration curve range. Samples are stored in a deep freezer at −70°C until completion of analysis.

Plasma extraction procedure
Add 50 µl of lamivudine working dilution to all samples except a blank sample where add 50 µl of diluent into pre-labeled vials.

Aliquot 0.200 ml of sample and then vortex. Add 0.200 ml of 5% hydrochloric acid in water solution to all samples and vortex. Centrifuge the samples for 5 min at 10±2°C for 4000 rpm. Condition the cartridges with 1 ml methanol followed by equilibrate the cartridges with 1 ml of 5% hydrochloric acid in water. Load the prepared sample in the cartridges and pass with low pressure. Wash cartridges two times with 1 ml of 5% hydrochloric acid in water solution. Wash cartridges two times with 1 ml of methanol and dry the cartridges for 2 min at maximum pressure. Elute the samples with 1 ml of the 5% ammonia in methanol in pre-labeled set of vials. Dry the samples using nitrogen evaporator at 50±2°C for 20 min. Reconstitute the samples with 0.300 ml of mobile phase and vortex. Transfer the samples into pre-labeled autosampler vials and load for analysis. (Table 1).

Data processing
Acquire the data and calculate the concentration of samples using linear regression analysis with weighing factor (1/x^2). Sample concentrations are back-calculated using the formula y=mx+c, where y is the area ratio, m is the slope of curve, c is intercept of curve, and x is the unknown sample concentration.

RESULTS AND DISCUSSION

Method development
Method development was initiated, aqueous, and extracted standards (K$_{2}$EDTA an anticoagulant) (10 pg/ml to 10 ng/ml) using liquid-liquid extraction technique (ethyl acetate with basification) and found standards acceptable with a limit of quantification (LOQ) area of ~2000 on API 4000 Q trap system, acetoniitrite and 10 mm ammonium acetate was used for mobile phase in the ratio of 80:20 with 30 µl injection on Inertsil ODS 150 mm column, 242.05/206.1 ions were monitored using turbo ion source. Procedure tested and found acceptable over a small duration, but method performance is found lower over the long period (more than 50 injections). So decided, liquid-liquid extraction was replaced with solid phase extraction and found suitable with no drop in the response, but lower limit of quantification (LLOQ) response is between 1000 and 2000; hence, we thought to introduce another daughter ion to use sum of multiple ions to get area for Std 1 minimum 2000.

System suitability was conducted every day using pure aqueous upper LOQ samples, % CV found within acceptance limit 5% for area, area ratio, and retention times.

Autosampler carryover test is conducted on the first day using pure as well as a extracted sample and no found carry over from autosampler.

Selectivity of the method is tested by using 6 plasma lots processed as blank and LLOQ and compared the blank response with that of LLOQ, no endogenous or exogenous interferences are found at the retention times. Sample chromatograms are presented in Figs. 1-5.

Matrix factor for method has been established using LQC and HQC level samples prepared in post-extracted blanks and compared with that of un-extracted pure samples, matrix factor is found between 0.85 and 1.15 with maximum internal standard normalized matrix factor % CV of 11.5.

Intra- and inter-day precision and accuracy along with ruggedness has been tested by analyzing three precision and accuracy batches (each batch containing 6 each of 4 levels of quality controls) on different days by different analysts on different columns. Intra- and inter-precision and accuracy found acceptable.

Recovery of method found reproducible and consistent over the entire calibration curve range. (Table 2).

Calculations are done for simple weighing factor and regression selection using three calibration curves data and linear regression with 1/y found to be simple, so that was selected.

Stability of the method is further tested at different stability conditions. (Table 3).

Fig. 1: Pyridostigmine impurity B blank chromatogram

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This method has many advantages than the previously published methods, this method is highly sensitive (10 pg/ml Limit of Quantitation) and selective, rapid (3 min), multiple reaction monitoring.

**Application to bioequivalence study sample analysis**

The method is applied to analyze bioequivalence study consist of 52 volunteers dosed with 180 mg of pyridostigmine as per approved protocol. A total of 1890 samples were collected before and after administration of pyridostigmine, plasma separated and stored at −70°C deep freezer. These samples analyzed for pyridostigmine impurity B concentrations using calibration curve and interspersed quality control samples. Standards and quality control samples during the study analysis showed accuracy and precision within the acceptance limits stated in guideline on bio-analytical method validation. Study analysis

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**Table 1: LC-MS/MS conditions**

| Name            | Q1 mass (amu) | Q3 mass (amu) | Dwell (ms) | DP (V) | EP (V) | CE (V) | CXP (V) |
|-----------------|---------------|---------------|------------|--------|--------|--------|--------|
| Pyridostigmine  | 109.9         | 95.1          | 200        | 70     | 8      | 35     | 5      |
| Impurity B      | 230.2         | 112.2         | 200        | 36     | 20     | 5      |
| Lamivudine      | 230.2         | 112.2         | 200        | 36     | 20     | 5      |
| CUR (psi)       | 15            | High          | 5500       | 500    | 30     | 50     | MRM    |
| CAD (psi)       | 15            | High          | 5500       | 500    | 30     | 50     | MRM    |
| Ion spray voltage (KV) | 15   | 15           | 5500       | 500    | 30     | 50     | MRM    |
| TEM (°C)        | 30            | High          | 5500       | 500    | 30     | 50     | MRM    |
| GAS 1 (psi)     | 50            | High          | 5500       | 500    | 30     | 50     | MRM    |
| GAS 2 (psi)     | 50            | High          | 5500       | 500    | 30     | 50     | MRM    |

**Table 2: Precision and accuracy results**

| Description | LLOQQC | LQC | MQC | HQC |
|-------------|--------|-----|-----|-----|
| Nominal     | 11.019 | 29.668 | 4262.66 | 7750.291 |
| Observed    | 11.179 | 28.603 | 4370.51 | 7948.344 |
| % Accuracy  | 101.5  | 96.4 | 102.5 | 102.6 |
| % Precision | 11     | 5    | 8     | 2    |

LQC: Low-quality control, MQC: Medium-quality control, HQC: High-quality control, LLOQQC:
CONCLUSION

Pyridostigmine impurity B is a low molecular weight basic polar drug. Basic functional groups are active for extraction and ionization. Due to the drug polar in nature, we got matrix effect problems but lamivudine (internal standard) is compensating those, internal standard normalized matrix factor is within limits. Recovery found 40% because of matrix effects but recovery is consistent and reproducible. Selectivity, precision, and accuracy found acceptable. Method can be used for intended purpose.

Successfully developed and validated a simple, rapid bio-analytical method for the estimation of pyridostigmine impurity B in plasma by LC-MS/MS. Sample extraction using solid phase extraction while separation was achieved on a Hypurity C18 column, acquired in multiple
reaction monitoring mode and quantified using analyst 1.6.2 software. Method found selective, no endogenous or exogenous interference found either at analyte or internal standard retention times. Matrix factor ratio found around 1. Signal to noise ratio found always more than 5. Precision and accuracy, ruggedness and all stability experiments found acceptable. The method has successfully validated and applied to analyze bioequivalence study samples of 1890.

CONFLICT OF INTEREST
Nil.

AUTHORS’ CONTRIBUTION
Our research concept was thought of all authors, first two authors were conducted the research whereas the third author has guided. The first author has drafted the manuscript whereas the other authors have reviewed. Hence, all authors are equally contributed for this research.

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