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Research Article

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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ABSTRACT

Objective: Objective of this work is to evaluate the levels in plasma of pyridostigmine impurity B (metabolite of pyridostigmine) in humans after administration of pyridostigmine formulations.

Methods: Plasma concentrations of pyridostigmine impurity B were estimated using by liquid chromatography coupled with electron spray ionization triple quad mass spectrometer technique, lamivudine is used as an internal standard. Multiple reaction at 109.9/95.2 (pyridostigmine impurity B) and 230.2/112.2 (lamivudine) were monitored. Chromatography was optimized using acetonitrile: Buffer (10 millimolar ammonium acetate) (85:15) on an Inertsil C18, 150 mm×4.6 mm, 5 µ analytical column. Linear Calibration curve set between 1.434 ng/ml and 39.637 ng/ml. Sample extraction was conducted using solid phase extraction method using mixed mode cation exchange cartridges. The method was developed on API 4000.

Results: Method was developed, tested for system suitability, carryover, selectivity, matrix effect, intra-inter precision and accuracy, recovery, linearity, and various stabilities in aqueous as well as plasma as matrix. The method was validated for all the above validation parameters mentioned as per European medical agency guideline on method validation.

Conclusions: The method is successfully applied to analyze 1890 subject samples after administration of pyridostigmine 180 mg as per Independent Ethics Committee approved protocol. Incurred sample reanalysis was revealed a great reproducibility of method. Statistical analysis was also conducted to compare test formulation with innovator formulation. Test formulation concentrations of pyridostigmine impurity B are similar to those obtained from innovator formulation.

Keywords: Pyridostigmine impurity B, Pyridostigmine impurity B in plasma, Pyridostigmine impurity B by liquid chromatography coupled with tandem mass spectrometer, Pyridostigmine, Safety of pyridostigmine metabolite, Pyridostigmine metabolite.

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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 are 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 during bioequivalence studies. High 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].

Pyridostigmine impurity B molecular formula is C_6H_8NO with molecular weight is 190.04 g/mol.

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 other methods [3-11]. No method is published on public domain 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_2EDTA 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, IS 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 2 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*x). 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_2EDTA 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, acetonitrile 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 $1/_{x}^{2}$ found to be simple, so that was selected.

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

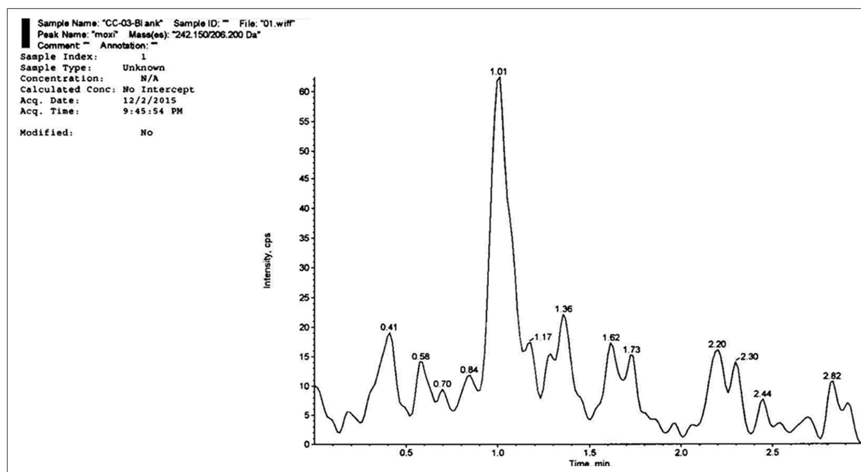

Fig. 1: Pyridostigmine impurity B blank chromatogram

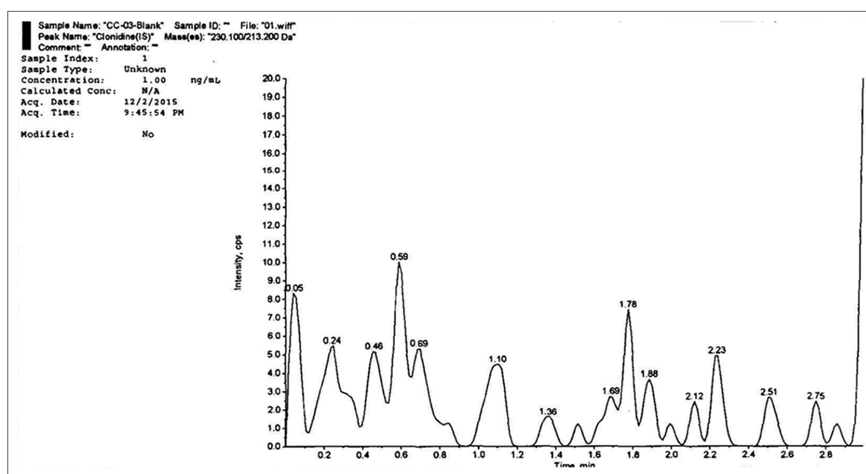

Fig. 2: ISTD blank chromatogram

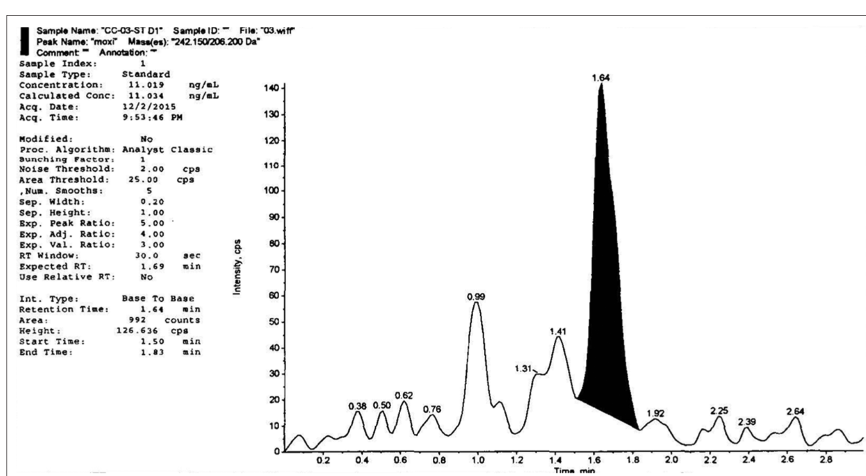

Fig. 3: Pyridostigmine impurity B lower limit of quantification chromatogram

Table 1: LC‑MS/MS conditions

Name	Q1 mass (amu)	Q3 mass (amu)	Dwell (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
Pyridostigmine Impurity B	109.9	95.1	200	70	8	35	5
Lamivudine	230.2	112.2	200	36	10	20	5
CUR (psi)	**CAD (psi)**	**Ion spray voltage (KV)**	**TEM (°C)**	**GAS 1 (psi)**	**GAS 2 (psi)**	**Scan type**	**Polarity**
15	High	5500	500	30	50	MRM	Positive
Column	**Mobile phase**			**Flow rate**		**Injection volume**	
Inertsil C18, 100 mm×4.6 mm, 5 µ	Acetonitrile: 10 mm ammonium acetate) (85:15)			1.00 ml/min		10 µl	

LC‑MS/MS: Liquid chromatography coupled with tandem mass spectrometer

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:

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

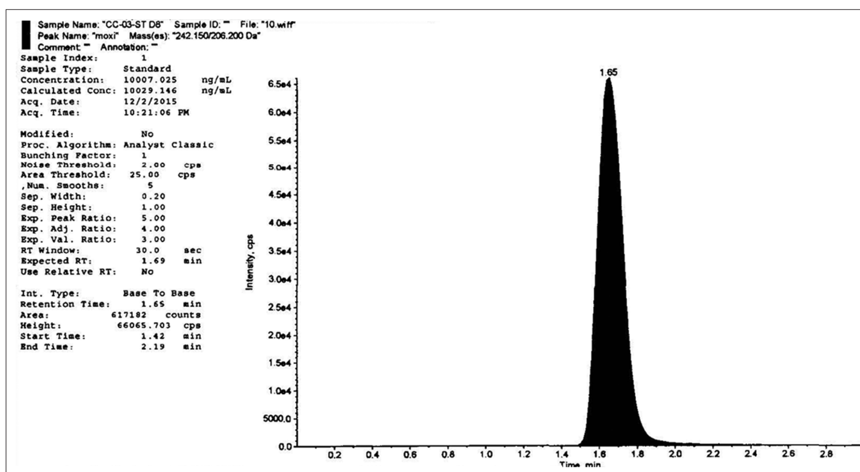

Fig. 4: Pyridostigmine impurity B upper limit of quantification chromatogram

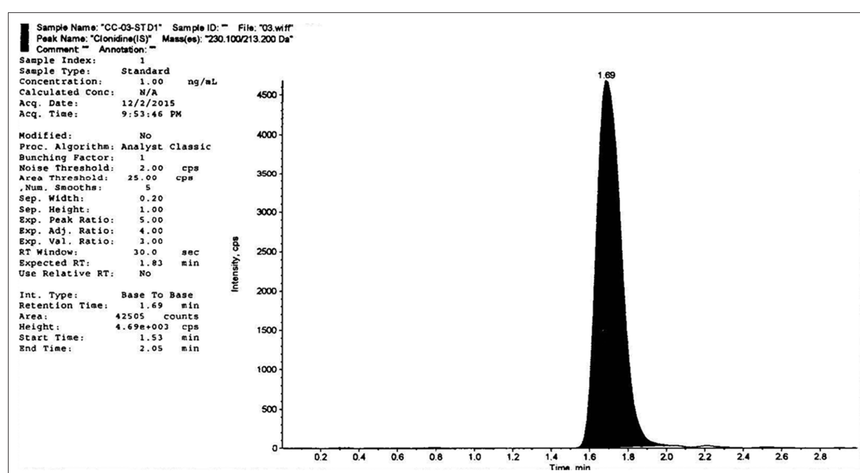

Fig. 5: ISTD chromatogram

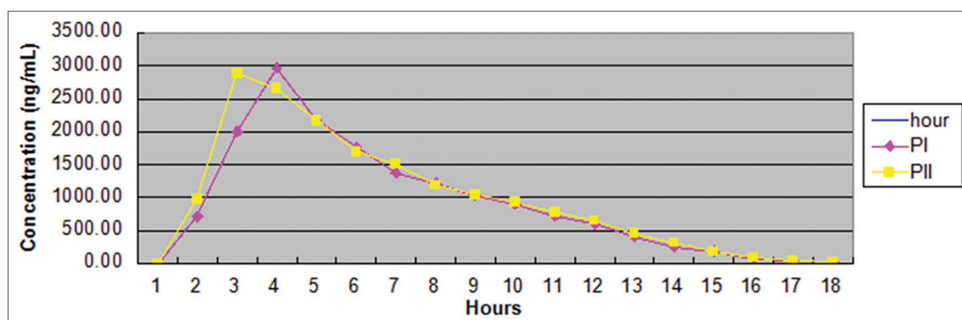

Fig. 6: Pharmacokinetic mean graph for pyridostigmine impurity B tablets 0.2 mg test (PI) vs. reference formulation (PII)

Table 3: Stability durations

Stability condition	Duration
Bench top stability at ambient temperature	6 h
Freeze-Thaw stability	4 cycles
Evaporation stability	1 h
Wet extract stability	25 h
Long-term stability in plasma	24 days
Long-term stock solution stability	7 days
Autosampler stability	28 h

is conducted under GLP environment. Mean graph is presented under Fig. 6.

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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