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DEVELOPMENT AND VALIDATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRIC METHOD FOR ESTIMATION OF DEXMETHYLPHENIDATE FROM HUMAN PLASMA USING LIQUID-LIQUID EXTRACTION

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ABSTRACT

A novel, simple, specific, sensitive and reproducible liquid chromatography mass Spectrometric (LC/MS-MS) assay method has been developed and validated for estimation of Dexmethylphenidate in human plasma biological matrix. The LC/MS-MS method includes use of D Threo-Methylphenidate-D10 as an internal standard (IS). Samples were extracted using liquid-liquid extraction methodology. The detection was performed using API 3200 (AB Sciex) and PC based data system with Analyst 1.4.2 software. Chromatographic separation was achieved on ZORBAX× SB -C8, 100×4.6mm, 3.5µm column using isocratic mobile phase composition with (5 mM Ammonium acetate Solution with 0.1 % Formic Acid: Acetonitrile: 7:3 V/V) at a flow rate of 0.8 mL/min at 40ºC column oven temperature, without splitter over a total run time of 3 min. Method validation was performed as per USFDA guidelines and the results met the pre-defined acceptance criteria. The calibration curve was linear over a concentration range of 0.205 to 80.195 ng/mL for Dexmethylphenidate (using D Threo-Methylphenidate-D10 as internal standard) with correlation value $r^2 \geq 0.995$. Inter-batch quality control samples accuracy for Dexmethylphenidate ranged from 98.47 to 103.94% with inter-batch precision values of 0.98% to 4.78% during the course of validation, demonstrating acceptable assay accuracy and precision. Further experiments like; Matrix Effect, Bench-Top, Freeze-Thaw, Dry extract, Wet extract, Effect of Haemolysis,

anticoagulant, Lipemic plasma, etc were conducted to assess the suitability of proposed method for intended usage. Method was found to be suitable for quantitation of Dexmethylphenidate clinical study samples.

KEYWORDS: Dexmethylphenidate; LCMS-MS; Human plasma, Method validation.

INTRODUCTION

Dexmethylphenidate (also named as d-threo-methylphenidate) is Central nervous system (CNS) stimulant of piperidine and phenethylamine class of drugs. These class of drugs are used in treatment of special disease class known as attention deficit hyperactivity disorder (ADHD) and narcolepsy. The drug also has been recommended for the off-label treatment of other disorders such as lethargy. Being a Central nervous system (CNS) stimulant drug abuse has also increased and has lead to its testing in forensic context. The MPH molecule contains a piperidine ring, methylester and benzene group (Figure 1). According to IUPAC nomenclature drug is named as 'Methyl 2-phenyl-2-(2-piperidyl) acetate'. Methylphenidate contains two stereogenic centers, resulting in a total of four possible stereoisomers: dextro(d-) and levo (l-) enantiomers of erythro- and threo-methylphenidate. Most recent pharmaceutical formulations of the drug contain the two threo enantiomers as a racemic mixture, because only the threo enantiomers result in the desired pharmacological effect, whereas the erythro enantiomers exert only hypertensive and toxic effects. Newer studies have indicated that of the two threo enantiomers, only the d-form is responsible for the desired therapeutic effects. Dexmethylphenidate shows considerable first-pass metabolism consisting by hydrolysis of the ester group, resulting in the de-esterified, pharmacologically inactive metabolite Ritalinic acid. The hydrolysis is catalyzed by carboxylesterase 1 (CES1), which in humans is primarily expressed in the liver and adipose tissue. In addition to in-vivo metabolism, there seems to be an enzyme-mediated hydrolysis in human plasma ex-vivo also. The hydrolysis in human plasma is characterized by the opposite enantioselectivity compared to CES1-mediated hydrolysis in the liver because d-methylphenidate is preferred over l- methylphenidate.

Few research articles are available on quantization of non-stereo selective quantization approach for methylphenidate. On contrary, a recent publication by Thomsen and coworkers demonstrates stereo-selective quantitation of methylphenidate and ritalinic acid in whole blood. Approach involves solid phase extraction of protein precipitated blood samples, followed by chromatography on chiral-AGP column coupled with tandem triple quad mass spectrometer. Total run time was nearly 10 minutes.

Literature survey shows that there is no LC-MS/MS method available for quantitative analysis of Dexmethylphenidate in biological samples (human plasma) using liquid-liquid extraction strategy. Hence in present research, we demonstrate an analytical strategy for quantitation of Dexmethylphenidate in biological samples. Evaluation of experiments related to stability and the assessment of drug at different concentration levels were also performed. Further the method was validated as per regulatory guidelines recommendations and employed for clinical studies.

MATERIALS AND METHOD

Chemicals and reagents

Dexmethylphenidate was procured from Clear Synth Labs, Mumbai (India) and D Threo-Methylphenidate-D10 (IS) was procured from Toronto Research center, Canada. LCMS grade acetonitrile and methanol were obtained from JT Baker. Formic Acid - AR Grade and Tert-butyl methyl ether was procured from Merck India. All other chemicals/reagents were of analytical grade and used without further purification.

HPLC-MS operating conditions

Mass spectrometric detection was carried out on an API 3200 triple quadrupole instrument equipped with a heated nebulizer (ESI) source operated in the positive ion mode. Dexmethylphenidate was selectively isolated from 200µL human plasma aliquot by liquid - liquid extraction using Tert- Butyl Methyl Ether as Extraction solvent. Estimation was done by mass spectrometric method and chromatographed using a ZORBAX× SB-C8, 100×4.6mm, 3.5µm column. An isocratic mobile phase was used consisting of 5 mM Ammonium acetate Solution with 0.1 % Formic Acid**:** Acetonitrile:: 7:3 V/V ratio of two solutions. The flow rate was 0.8 ml/min, without splitter under ambient temperature. The auto sampler temperature was maintained at 10°C ± 4°C and the injection volume was 10 µL. The run time was 3 min. During the optimization of the mass spectrometric parameters, strong and stable signals of analytes and internal standards were monitored and selected for quantitation. Ion transitions m/z 234.3/84.1 and 244.3/93.2 were selected for the MRM of Dexmethylphenidate and D Threo-Methylphenidate-D10 (IS) respectively. The source/gas parameters were optimized as follows: curtain gas: 30, collision Activated Dissociation (CAD): 7, ion source gas-1: 55, ion source gas-2: 45, ion spray voltage: 5500 V and temperature: 500°C.

Sample preparation

To 200µL of sample aliquot, 50µL of internal standard working solution (D Threo-Methylphenidate-D10) was added and vortexed to ensure complete mixing. Further 2.5 mL of extraction solvent was added. Samples were vortexed for 10 minutes at 2500 rpm (revolutions per minute) to ensure complete extraction of analytes in solvent layer. 2.0 mL extraction solvent layer was transferred in another tube and evaporated under nitrogen flow (water bath at 40ºC). Dried residue was reconstituted in 250 µL of mobile phase. Samples were analysed as per chromatographic conditions described in above section.

Note: To avoid hydrolysis of analytes in plasma, a stabilizing agent was added at the sample collection time itself.

Validation procedures

A full validation according to the FDA guidelines (US DHHS, FDA, CDER, 2001) was performed for the assay of Dexmethylphenidate in human plasma.

RESULTS AND DISCUSSION

Specificity and selectivity

Plasma selectivity was evaluated by analyzing six normal, one Haemolysed and one Lipemic K2EDTA human plasma lots obtained from independent sources. No interferences were observed at the retention times of analyte and IS (D Threo-Methylphenidate-D10) in all the six lots evaluated and % CV of selectivity was 8.26%, demonstrating acceptance criteria were met.

Matrix Effect

The potential for co-extracted matrix components to influence the detector response of analyte and IS was evaluated in six normal, one Haemolysed and one Lipemic K2EDTA human plasma lots obtained from independent sources. Analyte and IS (D_Threo-Methylphenidate-D10) detector responses in matrix samples spiked post-extraction were compared with detector responses observed in unextracted standards prepared at the same concentrations.

For matrix effect experiments performed at low QC concentration level

Matrix factors ranged from 1.03 to 1.14 (% CV 3.64%) for Dexmethylphenidate and from 1.02 to 1.09 (% CV 1.87%) for IS (D_Threo-Methylphenidate-D10). Matrix effects were 110.27% for Dexmethylphenidate and 107.26% for IS (D_Threo-Methylphenidate-D10),

Matrix factor of IS-normalized at low QC level ranged from 0.99 to 1.05 (% CV 1.94%) for Dexmethylphenidate.

For matrix effect experiments performed at high QC concentration level

Matrix factors ranged from 1.00 to 1.05 (% CV 1.96%) for Dexmethylphenidate and from 1.00 to 1.05 (% CV 1.96%) for IS D Threo-Methylphenidate-D10. Matrix effects were 102.27% for Dexmethylphenidate and 102.27% for IS D Threo-Methylphenidate-D10, Matrix factor of IS-normalized at ULOQ level ranged from 0.99 to 1.01 (% CV 1.00%) for Dexmethylphenidate.

Matrix Factor and Matrix Effect were within acceptance criteria indicating absence of significant matrix effect.

Carryover

Carryover is the appearance of an analyte and internal standard signal in blank sample peaks after the analysis of samples with a high analyte concentration.

No carryover was observed for Dexmethylphenidate of the extracted analyte at LLOQ sample response, and IS (D Threo-Methylphenidate-D10). Both values were within acceptance limits.

Linearity

Linearity was established by preparing an eight-point standard calibration curve in K2EDTA human plasma covering the concentration range 0.205 to 80.195 ng/mL using (D Threo-Methylphenidate-D10) as internal standard. Calibration standards were prepared and six batches of precision and accuracy were analyzed. Calibration curves were calculated by least-squares linear regression analysis of the response ratios (analyte/IS) in calibration standards with 1/x2 weighting. Back-calculated concentrations of analyte in calibration standards were determined using the best-fit regression curve calculated for each batch.

Inter-batch calibration standard accuracy for Dexmethylphenidate ranged from 97.56% to 101.92% with inter-batch precision values of 0.73% to 2.91% during the course of validation, demonstrating acceptable assay linearity. Correlation coefficient (r) was consistently greater than 0.995.

Weighting Scheme

Three calibration curves were analyzed by least-squares linear regression analysis with weighting factors of l/x and $1/x^2$. The relative errors of the back-calculated Dexmethylphenidate calibration standards for the three curves were tabulated, and the sum of the absolute values of the relative errors was calculated for each weighting factor. The weighting factor providing the smallest sum value was selected for use for this method.

Linear regression with 1 /x2 weighting was selected as the weighting scheme.

Sensitivity

The Sensitivity was assessed by considering the LLOQ concentration from standard curve. LLOQ Standard concentration was spiked in 6 replicates as QCs and these samples are analyzed along with calibration curve standard of precision and accuracy batch. The mean Dexmethylphenidate accuracy and %CV were 111.22% and 9.65%.

Precision and Accuracy

Intra-batch, Inter-batch and intra-day precision and accuracy values were determined for six PA batches by analyzing six replicates of quality control at lower limit of quantification (QCLLQ), low (QCL), middle (QCM) and high (QCH) Quality Control samples in each batch. One batch of precision and accuracy were performed for inclusion of QCM1.

Intra-batch precision (% CV) for Dexmethylphenidate in QCLLQ samples ranged from 2.16% to 9.00% across six precision and accuracy batches. Intra-batch precision (% CV) for Dexmethylphenidate in QCL, QCM and QCH samples ranged from 0.42% to 5.01%. Intra-batch precision (% CV) for Dexmethylphenidate samples was 0.98% in QCM1 samples.

Intra-batch accuracy (% nominal) for Dexmethylphenidate in QCLLQ samples ranged from 93.40% to 112.26% across six precision and accuracy batches. Intra-batch accuracy (% nominal) for Dexmethylphenidate in QCL, QCM and QCH samples ranged from 94.75% to 107.05%. Intra-batch accuracy (% nominal) for Dexmethylphenidate samples was 98.47% in QCM1 samples.

Inter-batch precision (% CV) for Dexmethylphenidate was 8.84% in QCLLQ samples and ranged from 1.88% to 4.78% in QCL, QCM, and QCH samples. Intra-batch precision (% CV) for Dexmethylphenidate was 0.98% in QCM1 samples.

Inter-batch accuracy (% nominal) for Dexmethylphenidate was 101.42% in QCLLQ samples and ranged from 100.70% to 103.94% in QCL, QCM and QCH samples. Intra-batch accuracy (% nominal) for Dexmethylphenidate was 98.47% in QCM1 samples.

Intra-day precision (% CV) for Dexmethylphenidate in QCLLQ samples ranged from 4.20% to 9.00% across six precision and accuracy batches. Intra-day precision (% CV) for Dexmethylphenidate in QCL, QCM and QCH samples ranged from 0.97% to 5.01%. Intra-batch precision (% CV) for Dexmethylphenidate samples was 0.98% in QCM1 samples.

Intra-day accuracy (% nominal) for Dexmethylphenidate in QCLLQ samples ranged 93.87% to 112.26% across six precision and accuracy batches. Intra-day accuracy (% nominal) for Dexmethylphenidate in QCL, QCM and QCH samples ranged from 94.75% to 106.10%. Intra-batch accuracy (% nominal) for Dexmethylphenidate samples was 98.47% in QCM1 samples.

Intra-batch, inter-batch and intra-day precision and accuracy values were within established acceptance limits.

Recovery

Recovery of Dexmethylphenidate from K2EDTA human plasma was determined by comparing peak areas of extracted QCL, QCM and QCH samples with peak areas determined from freshly prepared unextracted (aqueous) samples prepared at similar concentrations in mobile phase.

Recovery of IS (D Threo-Methylphenidate-D10) from K2EDTA human plasma was determined by comparing the peak areas of blank matrix samples spiked with IS (extracted samples) using this method with peak areas determined from freshly prepared IS (unextracted samples) in mobile phase at the concentration level required for the method.

Mean overall %recovery were 69.25% and overall %CV 5.05% for Dexmethylphenidate and %recovery was 72.55 and %CV was 4.71% for IS D_Threo-Methylphenidate-D10.

Reinjection Reproducibility

Reinjection reproducibility of Dexmethylphenidate in K2EDTA human plasma samples was evaluated by reinjecting the calibration curve standards and QCL and QCH samples from an accepted precision and accuracy batch.

Mean calculated Dexmethylphenidate concentrations in reinjected samples were 103.39%, 106.88% and 101.40% (% Nominal) and 2.13%, 1.01% and 1.02% (%CV) at QCL, QCM and QCH concentrations, respectively.

Dilution Integrity

Dilution integrity of Dexmethylphenidate in K2EDTA human plasma samples was evaluated using samples prepared at approximately 1.6 times the concentration of the highest calibration concentration level.

Mean calculated Dexmethylphenidate concentrations at two time and six time dilution levels were 98.95% and 107.65% (% nominal) and 1.10% and 0.96% (% CV) of the expected concentration, demonstrating acceptable sample dilution integrity.

Bench-Top Stability

Bench-top stability of Dexmethylphenidate in K2EDTA human plasma was evaluated at QCL and QCH concentrations. Six replicates of stability samples were stored unprocessed on the bench-top at room temperature for 5 hours 05 minutes. Samples were processed and analyzed against freshly spiked calibration curve standards and compared with bulk spiked QCL and QCH samples (comparison samples).

Mean calculated Dexmethylphenidate concentrations in stability samples were 108.81% and 101.09% (% nominal) and 8.26% and 0.90% (%CV) at QCL and QCH concentrations, respectively. In addition, Dexmethylphenidate concentrations in stability samples were 101.90% and 99.81% relative to bulk spiked samples at QCL and QCH concentrations. Dexmethylphenidate samples were stable on the bench-top for 5 hours 05 minutes at room temperature.

Freeze-Thaw Stability

Six replicates of Dexmethyl Phenidate samples at QCL and QCH concentrations in K2EDTA human plasma were analyzed after four freeze-thaw cycles (-70±10ºC at room temperature). Samples were analyzed against freshly spiked calibration curve standards and compared with bulk spiked QCL and QCH samples (comparison samples).

Mean calculated Dexmethylphenidate concentrations in stability samples were 106.95% and 101.55% (% nominal) and 1.11% and 0.67% (%CV) at QCL and QCH concentrations, respectively. In addition, Dexmethylphenidate concentrations in stability samples were

100.16% and 100.27% relative to bulk spiked samples at QCL and QCH concentrations, respectively. Dexmethylphenidate was stable in K2EDTA human plasma for four freeze-thaw cycles.

Dry Extract Stability

Six replicates of Dexmethylphenidate QCL and QCH samples were processed and stored as dry extract in the refrigerator between (2-8°C) for 53 hours 01 minutes. After reconstitution, samples were analyzed against freshly spiked calibration curve standards and compared with bulk spiked QCL and QCH samples (comparison samples).

Mean calculated Dexmethylphenidate concentrations in stability samples were 107.63% and 101.57% (% nominal) and 3.15% and 0.48% (%CV) at QCL and QCH concentrations. In addition, Dexmethyl phenidate concentrations in stability samples were 100.79% and 100.29% relative to bulk spiked samples at QCL and QCH concentrations, respectively.

Wet Extract Stability (in refrigerator)

Six replicates of Dexmethylphenidate QCL and QCH samples were processed and stored after reconstitution in the refrigerator between (2-8ºC) as wet extract in autosampler vials for 53 hours in refrigerator. These samples were analyzed against freshly spiked calibration curve standards and compared with bulk spiked QCL and QCH samples (comparison samples).

Mean calculated Dexmethylphenidate concentrations in stability samples were 107.97% and 101.16% (% nominal) and 2.67% and 0.31% (%CV) at QCL and QCH concentrations, respectively. In addition, Dexmethylphenidate concentrations in stability samples were 101.11% and 99.88% relative to bulk spiked samples at QCL and QCH concentrations, respectively. Dexmethylphenidate samples were stable as wet extracts when stored for 53 hours in refrigerator between (2-8°C).

Wet Extract Stability (at room temperature)

Six replicates of Dexmethylphenidate QCL and QCH samples were processed and stored after reconstitution at room temperature as wet extract in autosampler vials for 5 hours 56 minutes. These samples were analyzed against freshly spiked calibration curve standards and compared with bulk spiked QCL and QCH samples (comparison samples).

Mean calculated Dexmethylphenidate concentrations in stability samples were 107.12% and 101.93% (% nominal) and 3.01% and 0.47% (%CV) at QCL and QCH concentrations, respectively. In addition, Dexmethylphenidate concentrations in stability samples were 100.32% and 100.64% relative to bulk spiked samples at QCL and QCH concentrations, respectively. Dexmethylphenidate samples were stable as wet extracts when stored for 5 hours 56 minutes at room temperature.

In-Injector Stability

Six replicates of Dexmethylphenidate QCL and QCH samples were processed, reconstituted and stored in the autosampler (10°C) for 84 hours 26 minutes. Samples were analyzed against freshly spiked calibration curve standards and compared with bulk spiked QCL and QCH samples (comparison samples). QCL, QCM and QCH samples of PA01 stored at 10°C in the autosampler for analyzing In-Injector stability.

In-injector stability of D Threo-Methylphenidate-D10 (IS) was determined by storing reconstituted QCM samples in the autosampler (10°C) for 84 hours 26 minutes. Peak area ratios (IS/analyte) in stored samples were compared with peak area ratios of bulk spiked QCM samples.

Mean calculated Dexmethylphenidate concentrations in stability samples were 105.76% and 101.83% (%nominal) and 1.92% and 0.66% (%CV) at QCL and QCH concentrations, respectively. In addition, Dexmethylphenidate concentrations in stability samples were 99.05% and 100.54% relative to bulk spiked samples at QCL and QCH concentrations, respectively.

Mean internal standard (D Threo-Methylphenidate-D10) concentration in QCM samples were 100.18% relative to bulk spiked samples and the %CV was 0.80%.

Dexmethylphenidate and D Threo-Methylphenidate-D10 (IS) were stable in mobile phase stored at 10°C in the autosampler for 84 hours 26 minutes.

Effect of Haemolysis

Six replicates of Dexmethylphenidate at QCL and QCH samples were spiked in haemolysed blood and processed. After reconstitution, samples were analyzed and compared these QC samples against calibration curve prepared using non haemolysed plasma.

Mean calculated Dexmethylphenidate concentrations in stability samples were 92.05% and 100.78% (% nominal) and 2.76% and 1.81% (%CV) at QCL and QCH concentrations, respectively.

Effect of Sodium Heparin

Six replicates of Dexmethylphenidate QCL and QCH samples were spiked in sodium heparin plasma and processed. After reconstitution, samples were analyzed and compared these QC samples against any calibration curve containing K2EDTA.

Mean calculated Dexmethylphenidate concentrations in stability samples were 86.63% and 94.79% (% nominal) and 4.30% and 1.43% (%CV) at QCL and QCH concentrations, respectively.

Effect of Lipemic Plasma

Six replicates of Dexmethylphenidate QCL and QCH samples were spiked in lipemic plasma and processed. After reconstitution, samples were analyzed and compared these QC samples against calibration curve prepared using non lipemic plasma.

Mean calculated Dexmethylphenidate concentrations in stability samples were 89.68% and 94.86% (% nominal) and 4.72% and 0.74% (%CV) at QCL and QCH concentrations.

Whole Blood Stability

Six replicates of Dexmethylphenidate QCL and QCH samples were spiked in whole blood and were stored at room temperature for 4 hours and processed. After reconstitution, samples were analyzed against freshly spiked calibration curve standards and compared with freshly spiked QCL and QCH samples in whole blood.

Mean calculated Dexmethylphenidate concentrations in stability samples were 89.34% and 95.20% (% nominal) and 2.08% and 1.00% (%CV) at QCL and QCH concentrations, respectively. In addition, Dexmethylphenidate concentrations in stability samples were 92.63% and 93.22% relative to freshly samples at QCL and QCH concentrations. Dexmethylphenidate samples were stable as whole blood when stored for 4 hours at room temperature.

Table 1: Summary of the Validation Parameters of Dexmethylphenidate in human plasma

Experimental Parameters	Results
Analyte	Dexmethylphenidate
Biological Matrix	Human Plasma
Specificity and Selectivity	
% CV	8.26%
Matrix Effect Test at QCL Level	
(i) Matrix factor :	
% CV	3.64% (Analyte) 1.87% (IS)
(ii) % Matrix Effect :	110.27% (Analyte) 107.26% (IS)
(iii) IS Normalised Matrix Factor	
% CV	1.94%
Matrix Effect Test at ULOQ Level	
(i) Matrix factor :	
% CV	1.96% (Analyte) 1.96% (IS)
(ii) % Matrix Effect :	102.27% (Analyte) 102.27%% (IS)
(iii) IS Normalised Matrix Factor	
% CV	1.00%
Carry Over Test	
Dexmethylphenidate	0.00%
D_Threo-Methylphenidate-D10	0.00%
Analytical range	0.205 ng/ml to 80.195 ng/ml
Sensitivity :	
Precision	9.65%
Accuracy	111.22%
Precision	
Intra batch - (QCLLQ)	2.16% to 9.00%
Intra batch - (QCL, QCM, QCH)	0.42% to 5.01%
Intra batch - (QCM1)	0.98%
Inter batch - (QCLLQ)	8.84%
Inter batch - (QCL, QCM, QCH)	1.88% to 4.78%
Inter batch - (QCM1)	0.98%
Intra Day - (QCLLQ)	4.20% to 9.00%
Intra Day - (QCL, QCM, QCH)	0.97% to 5.01%
Intra Day - (QCM1)	0.98%
Continued	

Experimental Parameters	Results
Accuracy	
Intra batch - (QCLLQ)	93.40% to 112.26%
Intra batch - (QCL, QCM, QCH)	94.75% to 107.05%
Intra batch - (QCM1)	98.47%
Inter batch - (QCLLQ)	101.42%
Inter batch - (QCL, QCM, QCH)	100.70% to 103.94%
Inter batch - (QCM1)	98.47%

Intra Day - (QCLLQ)	93.87% to 112.26%
Intra Day - (QCL, QCM, QCH)	94.75% to 106.10%
Intra Day - (QCM1)	98.47%
Recovery	
Dexmethylphenidate	
OVERALL % CV	5.05%
OVERALL % RECOVERY	69.25%
D_Threo-Methylphenidate-D10	
% CV	4.71%
% RECOVERY	72.55%
Reinjection Reproducibility	
% CV (Reinjected Samples)	1.01% to 2.13%
Against calibration curve standards	101.40% to 106.88%
Dilution Integrity :	
2 fold: Precision	1.10%
Accuracy	98.95%
6 fold: Precision	0.96%
Accuracy	107.65%
Continued	

Experimental Parameters	**Results**
Bench Top Stability (5hr 05min) at room temperature	
%CV (Stability Samples)	0.90- 8.26%
Against freshly prepared calibration curve standards	101.09- 108.81%
Against comparison samples (bulk spiked) quality control samples	99.81- 101.90%
Freeze Thaw Stability (FT4 : at -70°C±10°C) at room temperature	
%CV (Stability Samples)	0.67- 1.11%
Against freshly prepared calibration curve standards	101.55-106.95%
Against comparison samples (bulk spiked) quality control samples	100.16-100.27%
Processed Sample Stability (Dry Extract : 53 hr 01 min) in refrigerator 2-8ºC	
%CV (Stability Samples)	0.48-3.15%
Against freshly prepared calibration curve standards	101.57-107.63%
Against comparison samples (bulk spiked) quality control samples	100.29- 100.79%
Processed Sample Stability (Wet Extract : 53 hr00 min) in refrigerator 2-8ºC	
%CV (Stability Samples)	0.31- 2.67%
Against freshly prepared calibration curve standards	101.16- 107.97%
Against comparison samples (bulk spiked) quality control samples	99.88- 101.11%
Processed Sample Stability (Wet Extract : 05 hr 56min) at room temperature	
%CV (Stability Samples)	0.47%-3.01%
Against freshly prepared calibration curve standards	101.93- 107.12%

Against comparison samples (bulk spiked) quality control samples	100.32- 100.64%
In-Injector Stability (10°C for84 hr 26 min) - Dexmethylphenidate	
%CV (Stability Samples)	0.66- 1.92%
Against freshly prepared calibration curve standards	101.83- 105.76%
Against comparison samples (bulk spiked) quality control samples	99.05- 100.54%
In-Injector Stability (10°C for 84 hr 26 min) - (D_Threo-Methylphenidate-D10)	
%CV (Stability Samples)	0.80- 1.04%
Against comparison samples (bulk spiked) quality control samples	100.18%
Effect of Haemolysis :	
%CV (Stability Samples)	1.81- 2.76%
Against freshly prepared calibration curve standards	92.05- 100.78%
Continued	

Experimental Parameters	**Results**
Effect of Sodium Heparin :	
%CV (Stability Samples)	1.43% to 4.30%
Against freshly prepared calibration curve standards	86.63% to 94.79%
Effect of Lipemic Plasma :	
%CV (Stability Samples)	0.74% to 4.72%
Against freshly prepared calibration curve standards	89.68% to 94.86%
Whole Blood Stability : (4 hrs)	
%CV (Stability Samples)	1.00% to 2.08%
Against freshly prepared calibration curve standards	89.34% to 95.20%
Against freshly spiked quality control samples	92.63% to 93.22%

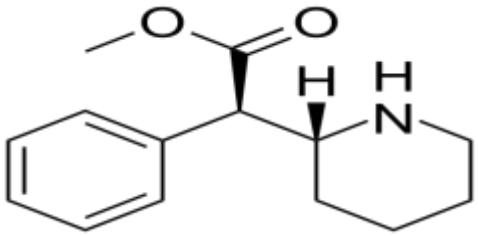

Figure: 1. Structural representation of Dexmethylphenidate

Molecular structure of Dexmethylphenidate

IUPAC Nomenclature: Methyl 2-phenyl-2-(2-piperidyl) acetate

Empirical formula: $C_{14}H_{19}NO_2$

Molecular weight: 233.31 g/mol

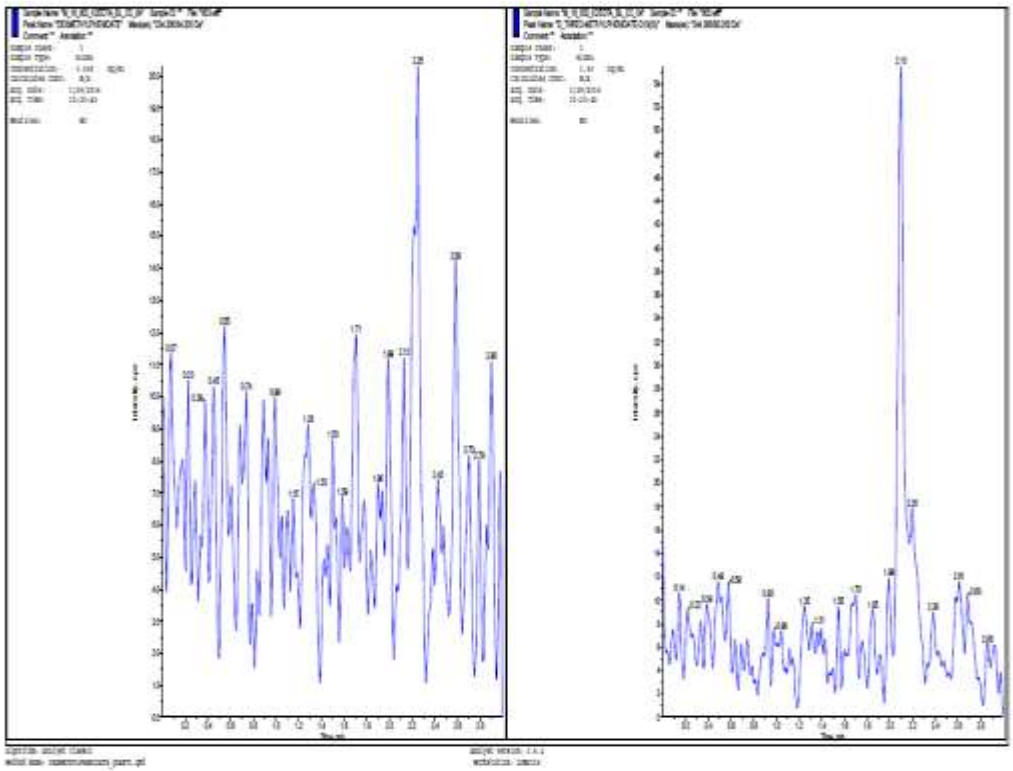

Figure 2: Typical MRM chromatograms of Dexmethylphenidate (left panel) and IS (right panel) for blank human plasma sample

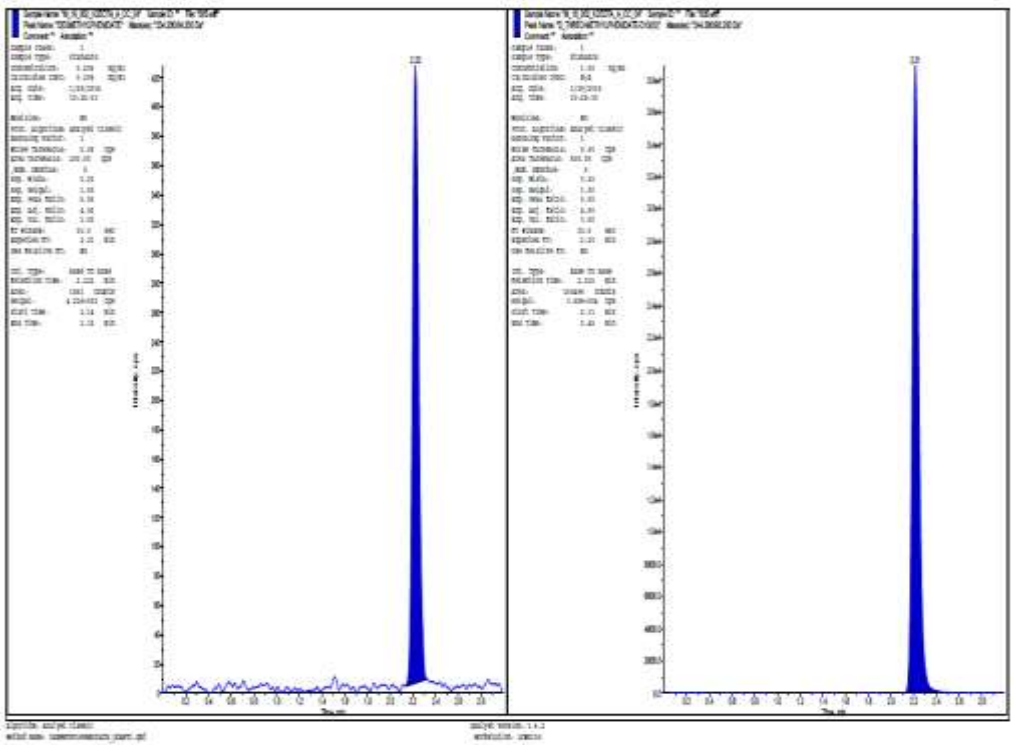

Figure 3: Typical MRM chromatograms of Dexmethylphenidate (left panel) and IS (right panel) for Dexmethylphenidate at LLOQ concentration level and Internal standard.

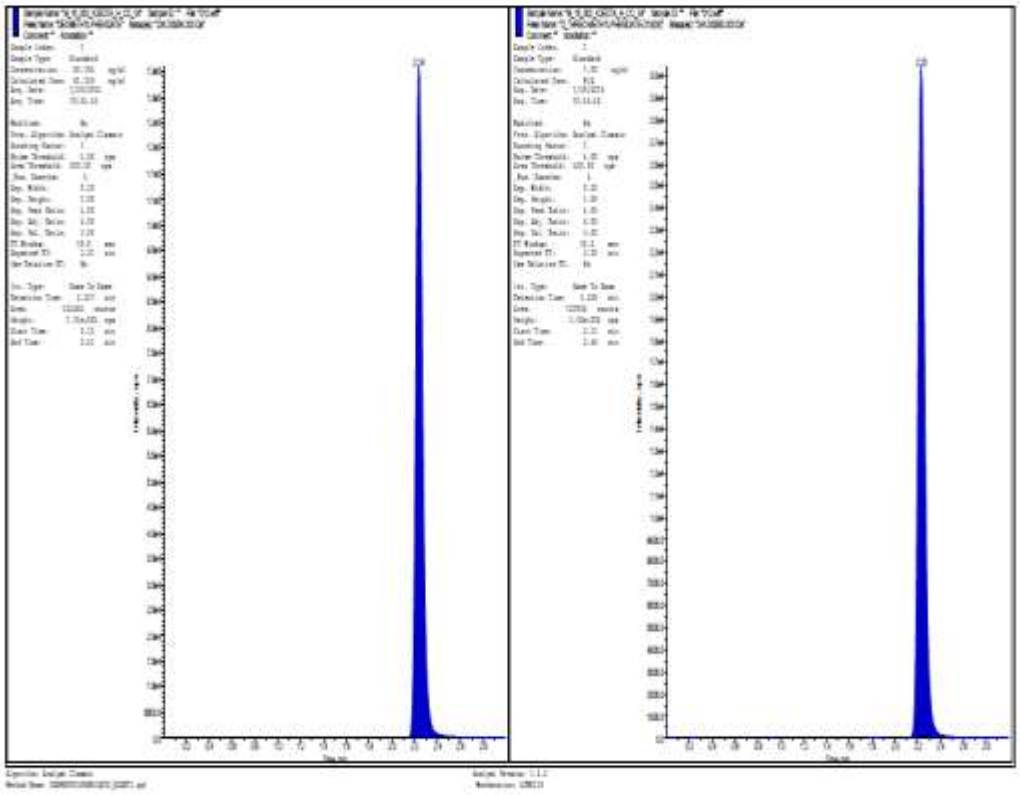

Figure 4: Typical MRM chromatograms of Dexmethylphenidate (left panel) and IS (right panel) for Dexmethylphenidate at ULOQ concentration level and Internal standard

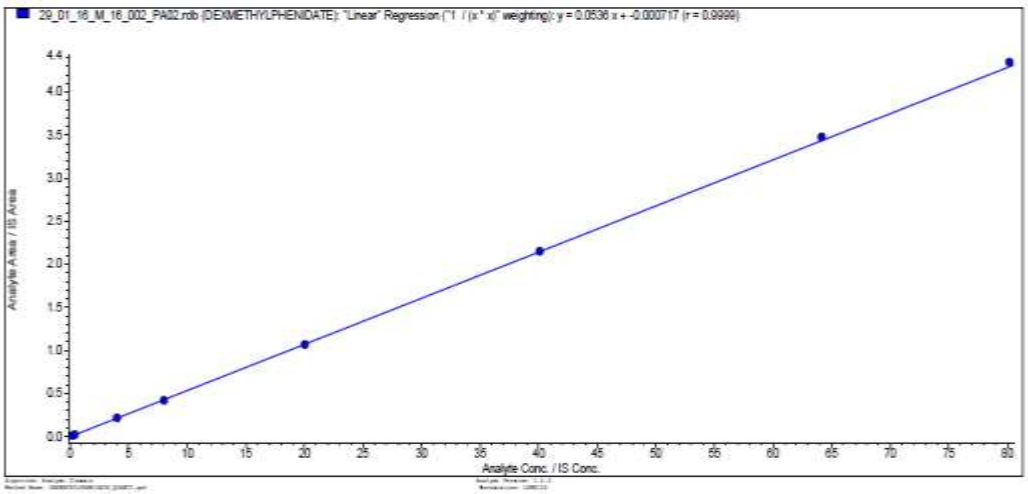

Figure: 5. Representative Calibration Curve for Dexmethylphenidate in human plasma.

CONCLUSION

A sensitive and selective LC-MS/MS method to quantitate Dexmethylphenidate in human plasma over the concentration range 0.205 to 80.195 ng/mL was successfully validated. This

method is suitable for study sample analysis to support bioequivalence / bioavailability and /or pharmacokinetic studies involving dosage of Dexmethylphenidate formulations.

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REFERENCES

1. D.J. Schlenger *et.al.* Misuse of methamphetamine and prescription stimulants among youths and young adults in the community. Drug and Alcohol Dependence, 2007; 89: 195–205.
2. D.R. Doerge *et.al.* Analysis of methylphenidate and its metabolite ritalinic acid in monkey plasma by liquid chromatography/electrospray ionization mass spectrometry. Rapid Communications in Mass Spectrometry, 2000; 14: 619–623.
3. D. Tuerck *et.al.* Dose-proportional pharmacokinetics of d-threo-methylphenidate after a repeated-action release dosage form. Journal of Clinical Pharmacology, 2007; 47: 64–69.
4. D. Andersen *et.al.* Validation of a fully automated robotic setup for preparation of whole blood samples for LC-MS toxicology analysis. Journal of Analytical Toxicology, 2012; 36: 280–287.
5. E. Marchei *et.al.* Development and validation of a liquid chromatography-mass spectrometry assay for hair analysis of methylphenidate. Forensic Science International, 2008; 176: 42–46.
6. E. Marchei *et.al.* Liquid chromatography-electrospray ionization mass spectrometry determination of methylphenidate and ritalinic acid in conventional and non-conventional biological matrices. Journal of Pharmaceutical and Biomedical Analysis, 2009; 49: 434–439.
7. F.T. Peters *et.al.* Validation of new methods. Forensic Science International, 2007; 165: 216–224.
8. H.J. Zhu *et.al.* Enantiospecific determination of DL-methylphenidate and DL-ethylphenidate in plasma by liquid chromatography-tandem mass spectrometry: Application to human ethanol interactions. Journal of Chromatography B, 2011; 879: 783–788.
9. H.J. Zhu *et.al.* Two CES1 gene mutations lead to dysfunctional carboxylesterase 1 activity in man: Clinical significance and molecular basis. The American Journal of Human Genetics, 2008; 82: 1241–1248.

10. J.S. Markowitz *et.al.* Ethylphenidate formation in human subjects after the administration of a single dose of methylphenidate and ethanol. Pharmacology, 2000; 28: 620–624.
11. J.S. Markowitz *et.al.* Advances in the pharmacotherapy of attention-deficit-hyperactivity disorder: focus on methylphenidate formulations. Pharmacotherapy, 2003; 23: 1281–1299.
12. J. Eichhorst *et.al.* Urinary screening for methylphenidate (Ritalin) abuse: A comparison of liquid chromatography-tandem mass spectrometry, gas chromatography-mass spectrometry and immunoassay methods. Clinical Biochemistry, 2004; 37: 175–183.
13. K.S. Patrick *et.al.* Influence of ethanol and gender on methylphenidate pharmacokinetics and pharmacodynamics. Clinical Pharmacology and Therapeutics, 2007; 81: 346–353.
14. L. Ramos *et.al.* Liquid-liquid extraction using 96-well plate format in conjunction with liquid chromatography/ tandem mass spectrometry for quantitative determination of methylphenidate (ritalin) in human plasma. Rapid Communications in Mass Spectrometry, 2000; 14: 740–745.
15. L. Ramos *et.al.* Liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry enantiomeric separation of dl-threomethylphenidate (Ritalin) using a macrocyclic antibiotic as the chiral selector. Rapid Communications in Mass Spectrometry, 1999; 13: 2054–2062.
16. L. Szporny and P. Gorog Investigations into the correlations between monoamine oxidase inhibition and other effects due to methylphenydate and its stereoisomers. Biochemical Pharmacology, 1961; 8: 263–268.
17. M.C. Ritz *et.al.* Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science, 1987; 237: 1219–1223.
18. M. Koehm *et.al.* Influence of ethanol on the pharmacokinetics of methylphenidate's metabolites ritalinic acid and ethylphenidate. Arzneimittel-Forschung, 2010; l-60: 238.
19. M.H. Teicher *et.al.* Methylphenidate blood levels and therapeutic response in children with attention-deficit hyperactivity disorder: I. Effects of different dosing regimens. Journal of Child and Adolescent Psychopharmacology, 2006; 16: 431.
20. M. Josefsson and I. Rydberg; Determination of methylphenidate and ritalinic acid in blood, plasma and oral fluid from adolescents and adults using protein precipitation and liquid chromatography tandem mass spectrometry–A method applied on clinical and forensic investigations. Journal of Pharmaceutical and Biomedical Analysis, 2011; 55: 1050–1059.

21. N. Barbarin *et.al.* High-throughput selected reaction monitoring liquid chromatography mass spectrometry determination of methylphenidate and its major, 2003. metabolite, ritalinic acid, in rat plasma employing monolithic columns. Journal of Chromatography B, 783: 73–83.
22. N.R. Srinivas *et.al.* Enantioselective pharmacokinetics of dl-threo-methylphenidate in humans. Pharmaceutical Research, 1993; 10: 14–21.
23. N.R. Srinivas *et.al.* In vitro hydrolysis of RR,SS-threo-methylphenidate by blood esterases—Differential and enantioselective interspecies variability. Chirality, 1991; 3: 99–103.
24. N.R. Srinivas *et.al.* Stereoselective disposition of methylphenidate in children with attention-deficit disorder. Journal of Pharmacology and Experimental Therapeutics, 1987; 241: 300–306.
25. N.B. Modi *et.al.* Dose-proportional and stereospecific pharmacokinetics of methylphenidate delivered using an osmotic, controlled-release oral delivery system. Journal of Clinical Pharmacology, 2000; 40: 1141.
26. O.A. Ismaiel *et.al.* Investigation of endogenous blood plasma phospholipids, cholesterol and glycerides that contribute to matrix effects in bioanalysis by liquid chromatography/mass spectrometry. Journal of Chromatography B, 2010; 878: 3303–3316.
27. R. Bakhtiar *et.al.* Toxicokinetic assessment of methylphenidate (ritalin) in a 13-week oral toxicity study in dogs. Biomedical Chromatography, 2004; 18: 45–50.
28. R. Bakhtiar and F.L.S. Tse, Toxicokinetic assessment of methylphenidate (ritalin) enantiomers in pregnant rats and rabbits. Biomedical Chromatography, 2004; 18: 275–281.
29. R. Thomsen *et.al.* Enantioselective determination of methylphenidate and ritalinic acid in whole blood from forensic cases using automated solid-phase extraction and liquid chromatography–tandem mass spectrometry. Journal of Analytical Toxicology, 2012; l-00 1-9.
30. Ramirez Fernandez *et.al.* Liquid chromatography-tandem mass spectrometry method for the simultaneous analysis of multiple hallucinogens, chlorpheniramine, ketamine, ritalinic acid, and metabolites, in urine. Journal of Analytical Toxicology, 2007; 31: 497–504.
31. S.K. Teo *et.al.* A single-dose, two-way crossover, bioequivalence study of dexmethylphenidate HCl with and without food in healthy subjects. Journal of Clinical Pharmacology, 2004; 44173–178.

32. S. Patrick *et.al.* Pharmacology of the enantiomers of threo-methyphenidate. Pharmacology, 1987; 241: 158.
33. T. Shinozuka *et.al.* Solid-phase extraction and analysis of 20 antidepressant drugs in human plasma by LC/ MS with SSI method. Forensic Science International, 2006; 162: 108–112.
34. T. Aoyama *et.al.* Nonlinear kinetics of threo-methylphenidate enantiomers in a patient with narcolepsy and in healthy volunteers. European Journal of Clinical Pharmacology, 1993; 44: 79–84.
35. Y.S. Ding *et.al.* Chiral drugs: Comparison of the pharmacokinetics of $^{[11C]}$ d-threo and l-threo-methylphenidate in the human and baboon brain. Psychopharmacology, 1997; 131: 71–78.
36. Y.S. Ding *et.al.* Brain kinetics of methylphenidate (ritalin) enantiomers after oral administration. Synapse, 2004; 53: 168–175.