APPLICATION OF SENSITIVE, RAPID AND VALIDATED LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY METHOD FOR SIMULTANEOUS DETERMINATION OF LETROZOLE AND METFORMIN IN HUMAN PLASMA.

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ABSTRACT
A rapid and sensitive liquid chromatography tandem mass spectrometry method has been developed and validated for the simultaneous determination of metformin and letrozole in human plasma. The solid-phase extraction technique was used for the extraction of metformin and letrozole from human plasma. Anastrozole was used as the internal standard (IS). Chromatography was performed on a Hypurity C18, 5 μm, 50 mm × 4.6 mm column, with the mobile phase consisting of 5mM ammonium formate and acetonitrile (in a 20:80 ratio), followed by detection using mass spectrometry. The method involves a simple reversed isocratic chromatography condition and mass spectrometry detection, which enables detection at sub-nanogram levels. The method was validated and the lower limit of quantification for metformin and letrozole was found to be 5.0 ng mL⁻¹ and 0.5 ng mL respectively. The mean recovery for letrozole and metformin ranged from 90.1 to 95.6%. This method increased the sensitivity and selectivity; resulting in high-throughput analysis of letrozole and metformin using single IS in a single experiment for bioequivalence studies, with a chromatographic run time of 2.5 min only.

KEY WORDS: letrozole, metformin, UFLC-MS/MS, human plasma

INTRODUCTION
Metformin hydrochloride (C₄H₁₁N₅·HCl) [N,N-di-methylimidodicarbonimidicdiamide hydrochloride] is an antidiabetic agent (Kar and Chaudhury, 2009). Metformin is an insulin-sensitizing agent with potent antihyperglycemic properties (Dalia, 2012). Its efficacy in reducing hyperglycemia in type 2 diabetes mellitus is similar to that of sulfonylureas, thiazolidinediones, and insulin (Mistria et al., 2007). Metformin used for the treatment of polycystic ovary syndrome (PCOS) in reproductive medicine and also reduce inflammation and have an effect on steroidogenesis inovarian granulosa cells and thecal cells (Batukan and Baysal, 2001). Metformin in PCOS patients by improving pregnancy rate and the metabolic situation and by decreasing the complications of pregnancy
such as gestational diabetes (Vandermolen et al., 2001). Letrozole\(\text{C}_{17}\text{H}_{11}\text{N}_5\)\(4,4'-(1\text{H}-1,2,4\text{-triazol}-1\text{-yl})\text{methylene})\text{dibenzonitrile}\) is a potent and selective non-steroidal aromatase inhibitor (Acharjya et al., 2010). It is approved for use in post-menopausal women who have breast cancer that has progressed after antiestrogen therapy (Jana et al., 2012).

Polycystic ovary disease (PCOD) is the major cause of an ovulation, the incidence of which has been reported to reach 6% in infertile females (Fleming et al., 2002). Letrozole is a newly designed selective aromatase inhibitor, which can be used to induce ovulation in infertile women with polycystic ovary syndrome (PCOS) (Sohrabvand et al., 2006).

A variety of methods have been employed for the detection of the individual component or combination in plasma, including HPLC, HPLC-UV, LC–MS or LC–MS/MS. (Sekar et al., 2009; Laha et al., 2008; Arayne et al., 2010; Rao et al., 2011; Chenga et al., 2001; Koseki et al., 2005; Yuen et al., 1998). However, these reported methods required more time, laborious extraction procedures, relatively large sample volume, long chromatographic analysis time and also showed low sensitivity which were not adequate for pharmacokinetic and bioequivalence studies. Recently, a LC–MS/MS method was reported for the determination of letrozole in human plasma by protein precipitation extraction with a lower limit of quantitation (LLOQ) of 0.40 and 50.0 ng/mL. The method achieved sufficient sensitivity in long run time, which is not useful for high throughput analysis. No previous methods with a simultaneous determination of the letrozole and metformin had been described. Therefore, a highly sensitive and simple LC–MS/MS method was developed and validated for the simultaneous determination of metformin and letrozole in human plasma. This developed method offered higher sensitivity, more simple procedure, smaller sample volume and shorter run time; resulting in high-throughput analysis of letrozole and metformin using single IS in a single experiment for bioequivalence studies, with a chromatographic run time of 2.5 min only.

EXPERIMENTAL

2.1 Materials and chemicals

A Hypurity C18, (50 x 4.6mm), 5µ column obtained from Thermo Scientific, Mumbai, India was used for the compound retention. The reference standards of metformin (98.04%), letrozole (98.00%) and anastrozole (99.24%) were obtained from Glenmark Pharmaceuticals Nashik, India. High purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore, Bangalore, India. HPLC grade methanol and acetonitrile were purchased from J.T.Baker., Mumbai, India. Extra pure ammonium acetate was purchased from Fluka, Germany. Drug free (blank) human plasma containing K3-EDTA as the anticoagulant was obtained from Yash Laboratory, Thane, India. The plasma thus obtained was stored at –20°C prior to use.

2.2 Preparation of standards and quality control samples

Two separate stock solutions of metformin and letrozole were prepared for bulk spiking of calibration curve and quality control samples for the method validation exercise. metformin, letrozole and anastrozole (IS) stock solutions were prepared by dissolving them in methanol to obtain a desired concentration. These stock solutions of metformin and letrozole thus prepared were used to spike a pool of blank human plasma which was serially diluted with blank human plasma eight times to obtain calibration standards spanning a range between 5 to 2000 ng/mL for metformin and 0.5 to 200
ng/mL for letrozole. Similarly, quality control standards (QC’s) were prepared (using the same methodology) at five different concentrations namely, 5 (LLOQ), 30 (LQC), 300 (LMQC), 950 (MQC) and 1500 (HQC) ng/mL for metformin and 0.5 (LLOQ), 3 (LQC), 30 (LMQC), 100 (MQC) and 150 (HQC) ng/mL for letrozole. Sufficient calibration standards and quality control standards were prepared to validate the method, only four levels of controls were prepared as in LQC (lower quality control), LMQC (lower middle quality control), MQC (middle quality control) and HQC (higher quality control).

2.3 Extraction Procedure
The plasma samples (100 µl) were transferred to 2.0-mL Eppendorf® microfuge tubes (Eppendorf, Hamburg, Germany) and 25µl of the diluted working solution of internal standard (50 ng/mL of anastrozole). The samples were then vortexed for two minutes and centrifuged at 15000 RPM (Rotations per Minute) for 5 minutes. The entire supernatant sample after centrifugation was passed through Phenomenex Strata-X 30mg/1cc extraction cartridge that was pre-conditioned with 1mL methanol followed by 5mM ammonium formate buffer. The extraction cartridge was then washed 1 mL of 10% v/v methanol in deionised water, followed by 1ml of deionised water. Both the analytes and the internal standard were eluted with 1.5 mL of the mobile phase. 1.0 µL of the eluant was injected into the LC-MS/MS system through the autosampler.

2.4 LC–MS/MS conditions
All experiments were performed using a triplequadrapole system (Applied Biosystems, Ontario, Canada) mass spectrometer fitted with a Turbolon Spray source. The front end included a Shimadzu autosampler and a Shimadzu Prominence series (Kyoto, Japan) binary pump. Data acquisition and quantitation was performed using Analyst® 1.5 software.

The chromatographic separation was achieved on a Hypurity C18, (50 x 4.6mm), 5µ column obtained from Thermo Scientific Mumbai, India. The mobile phase consisted of acetonitrile-5mM ammonium formate buffer (80:20, v/v), delivered at 0.5 mL min⁻¹ at ambient temperature. The total run time was 2.5 min and the injection volume was 1.0 µl. Moreover, the column and autosampler temperature were maintained at 30°C and 5°C, respectively.

Electrospray ionization (ESI) was performed in the positive ion mode with nitrogen as the collision gas. The spray voltage and source temperature were kept at 5500 kV and 500°C respectively. Nitrogen was used as the collision gas. The Declustering Potential (DP), Collision Energy (CE), Entrance potential (EP), Cell Exit Potential (CXP) were optimized during tuning as 48, 30.5, 10, 12.5; 41, 17, 10, 12; 98, 31, 10, 14 eV for metformin, letrozole and anastrozole respectively. The collision activated dissociation (CAD) gas was set at 6 psi, while the curtain gas was set at 45 psi.

The mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion m/z 129.90→70.90 for metformin, m/z 286.20→217.00 for letrozole and m/z 294.10→224.90 for the internal standard, anastrozole. The instrument response was optimized for metformin and letrozole by infusing a constant flow of a solution of the drug dissolved in mobile phase via a T-piece into the stream of mobile phase eluting from the column. The same methodology was used to optimize the response of the instrument for the internal standard.

Figs.1, 2 and 3 show the product ion mass spectra obtained from collision-induced dissociation of the protonated molecular ions of metformin, letrozole and anastrozole respect-
tively. The instrument was interfaced with a computer running Applied Biosystems Analyst version 1.5 software.

2.5 Assay validation
The method was validated in terms of selectivity, accuracy, precision, recovery, calibration curve, matrix effect and reproducibility according to the FDA guidelines for validation of bioanalytical method.

The method was validated by analyzing plasma quality control samples six times at five different metformin and letrozole concentrations, i.e. 5, 30, 300, 930 and 1550 ng/mL for metformin and 0.5, 3, 30, 95 and 150 ng/mL for letrozole to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve containing eight different concentrations spanning the concentration range of 5 to 2000 ng/mL and 0.5 to 200 ng/mL for metformin and letrozole respectively.

Calibration graphs were constructed using a linear regression (weighted with 1/concentration)$^2$ of the drug peak-area ratios of the product ions of the analyte to the internal standard versus the nominal drug concentrations. Several regression types were tested and the linear regression (weighted with 1/concentration)$^2$ was found to be the simplest regression, giving the best results ($r^2 \geq 0.9982$, for inter-batch validation).

The intra-batch and inter-batch accuracy were determined by replicate analysis of the four quality control levels along with the LLOQ (Lower Limit of Quantitation) level that were extracted from the sample batch. In each of the precision and accuracy batches, six replicates at each quality control level inclusive of the LLOQ level were analysed. Accuracy is defined in terms of % recovery and was calculated using the formula, % recovery = mean found concentration/nominal concentration] x 100. Assay precision was calculated by using the formula \(\% \text{ RSD} = \frac{(SD/M)}{M} \times 100\) where $M$ is the mean of the experimentally determined concentrations and $SD$ is the standard deviation of $M$.

The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) was performed by processing six lots of different normal controlled plasma samples in quadruplet ($n=4$). LQC and HQC working solutions were spiked post extraction in duplicate for each lot. The results found were well within the acceptable limit set i.e. the RSD of area ratio to be within ±15% at each level tested. Also, the minor suppression of analyte signal due to endogenous matrix interferences does not affect the quantification of analyte and IS peak which was confirmed by post-column infusion.

Absolute recoveries of the analyte were determined at the four different quality control levels viz. LQC, LMQC, MQC and HQC, by comparing the peak areas of the extracted plasma samples with those of the unextracted standard mixtures (prepared in the injection vehicle at the same concentrations as the extracted samples) representing 100% recovery.

RESULTS AND DISCUSSION
3.1 Selectivity
The selectivity of the method was investigated by analyzing human plasma of six different sources, two each of lipemic and heamolysed plasma. Any interference obtained was compared versus six replicates of extracted samples at the LLOQ level prepared in one of the plasma lots with the least interference at the retention time of metformin, letrozole and anastrozole. No endogenous interference peaks were found at the retention times of all analytes and IS. Representative chromatogram obtained from blank plasma and plasma spiked with LLOQ(CS01) standard for
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3.2 Linearity and sensitivity
The assay was linear over the concentration range of 5–2000 ng/mL for metformin and 0.5–200 ng/mL for letrozole. The correlation coefficients for the calibration regression curve were 0.998 or greater. The calibration standards were back-calculated from the responses. The deviations from the nominal concentrations and coefficient of variation (CV) were less than 15% for all concentrations.

The current assay had an LLOQ of 5 ng/mL for metformin and 0.5 ng/mL for letrozole. These limits are sufficient for clinical pharmacokinetic studies following oral administration of therapeutic dose.

3.3 Accuracy and precision
A summary of inter- and intra-day precisions and accuracies at QC concentrations are shown in Table 1. The intra- and inter day precisions were in between 1.82% and 6.51% for metformin and 3.17% and 7.49% for letrozole. The accuracies were in range of 97.44% to 101.62% for metformin and 92.50% to 104.35% for Letrozole. The results indicated that the assay had remarkable reproducibility with acceptable accuracy and precision.

3.4 Recovery
To calculate the absolute recovery, six replicates of spiked plasma QC samples of low, lower medium, medium and high concentrations were extracted under the conditions noted above. The integrated peak area response of each analyte was compared with those obtained from the standard solutions of equivalent concentration subjected to the same extraction. The mean absolute recoveries of metformin determined at 5, 30, 90 and 1550 ng/mL were 92.55, 90.09, 95.62 and 94.65 %, respectively. The mean absolute recovery of letrozole determined at 0.5, 3, 30, 95 and 150 ng/mL were 91.59, 93.54, 92.87 and 92.23%, respectively. The mean absolute recovery of anastrozole was 88.05 %.

3.5 Matrix effect
No matrix effect for metformin and letrozole were observed for the six different plasma lots tested. The RSD of the area ratios of post spiked recovery samples at LQC and HQC levels were less than 2.71% for metformin and less than 3.03% for letrozole. For the internal standard the RSD of the area ratios over both LQC and HQC levels was less than 5.39%. This indicated that the extracts were “clean” with no co-eluting compounds influencing the ionization of the analytes and the internal standard.

3.6 Stability
The metformin and letrozole were found to be stable under following conditions: in plasma at room temperature for at least 24 h, in the auto sampler at 5 °C for 48 h and three freeze–thaw cycles. The long term stability results also indicated that metformin and letrozole were stable in matrix for up to 30 days at a storage temperature of -50°C. This period of long term stability was sufficient enough to cover the entire period right from first day of storage of the plasma samples to the last day of analysis.

3.7 LC–MS/MS method optimization
During method development quantification was performed using multiple reactions monitoring (MRM) in the positive ion mode and different options were evaluated to optimize sample like extraction, detection parameters and chromatography. Electro spray ionization (ESI) was evaluated to get better response of analytes as compared to APCI (Atmospheric Pressure Chemical Ionization) mode. It was found that the best signal was achieved with the ESI positive ion mode. Fig. 1, 2 and 3 displays the product ion spectra of [M+H] + ions of metformin, letrozole
and anastrozole (IS). For metformin, the product mass spectrum was recorded from the precursor ion m/z 129.90 [M+H] + and the most abundant fragment was monitored at m/z 70.90. For letrozole, the product mass spectrum was recorded from the precursor ion m/z 286.20 [M+H] + and the most abundant fragment was monitored at m/z 217.00 and Internal standard anastrozole the product mass spectrum was recorded from the precursor ion m/z 294.10 [M+H] + and the most abundant fragment was monitored at m/z 224.90. A mobile phase with different buffer solution and acetonitrile in varying combinations was tried during the initial development stages. But the best signal for metformin and letrozole was achieved using a mobile phase containing 5mM ammonium formate buffer in combination with acetonitrile (20:80 v/v). Use of a Hypurity C18, (50mm x 4.6mm), 5µ column resulted in better separation and reduced run time. The retention times for metformin, letrozole and anastrozole were ~ 1.09 minutes, ~ 1.36 minutes and ~ 1.37 minutes, respectively. LC–MS/MS chromatograms showed that the analytes and IS were separated with no interference from each other.

CONCLUSIONS
To our knowledge, this is the first fully validated LC–MS/MS method for the simultaneous quantification of metformin and letrozole in human plasma. The method was proved to be sensitive, accurate, precise and reproducible. Sample preparation showed high recovery for the quantitative determination of these two analytes in human plasma. The method allows for a much higher sample throughput due to the short chromatography time (2.5 minutes) and simple sample preparation. Robust LC–MS/MS performance was observed, with acceptable variation in instrument response within batches. This method is an excellent analytical option for rapid quantification of metformin and letrozole in human plasma.

REFERENCES


Figure 1. Representative spectra of product ion of Metformin

Figure 2. Representative spectra of product ion of Letrozole
Figure 3. Representative spectra of product ion of Anastrozole

Figure 4. Representative chromatogram of blank plasma of Metformin, Letrozole and its Internal Standard Anastrozole.
Figure 5. Representative chromatogram of blank plasma spiked with Metformin (5.0 ng ml\(^{-1}\)), Letrozole (0.5 ng ml\(^{-1}\)) and its Internal Standard Anastrozole at the lower limit of quantification.
Table 1: Precision, accuracy and extraction recovery for metformin and letrozole in plasma QC samples.

<table>
<thead>
<tr>
<th>Components</th>
<th>Nominal concentration (ng/mL)</th>
<th>Intra-day (n = 6)</th>
<th>Inter-day (n = 18)</th>
<th>Extract recovery (%)</th>
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<tr>
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<td>Nominal concentration (ng/mL)</td>
<td>Measured conc. (ng/mL)</td>
<td>RSD (%)</td>
<td>Bias (%)</td>
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<tr>
<td>metformin</td>
<td></td>
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<td></td>
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<td></td>
<td>0.52</td>
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<td>3.67</td>
<td>-0.98</td>
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Table 2: Stability of metformin and letrozole in human plasma under different storage conditions (n = 6).

<table>
<thead>
<tr>
<th>Components</th>
<th>Nominal concentration(ng/mL)</th>
<th>Room temperature for 24 h</th>
<th>Autosampler at 4°C for 48 h</th>
<th>Freeze-thaw Stability third cycle</th>
<th>Storage at -50 °C</th>
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<tbody>
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<td>metformin</td>
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<td>156.8</td>
<td></td>
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All values are represented as the percent of mean deviation from nominal concentration, bias% = (measured concentration−nominal concentration)/nominal concentration×100%