CHAPTER 5

Summary and Conclusions
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In this thesis, the development and validation of liquid chromatography-tandem mass spectrometric (LC-MS/MS) methods for the quantitative bioanalysis of selected drugs (anti-depressants, anti-hypertensives, and anti retroviral drugs) are described. The monitoring of these drugs in biological fluids (human plasma) is important during both pre-clinical and clinical development and often in routine clinical use. Traditionally, liquid chromatography (LC), in combination with ultraviolet (UV), fluorescence, or electrochemical detection is employed for this purpose. The successful hyphenation of liquid chromatography (LC) and mass spectrometry (MS), however, has dramatically changed this. Mass spectrometric detection provides better sensitivity and selectivity than UV detection and, in addition, is applicable to a significantly larger group of compounds than fluorescence or electrochemical detection.

LC-MS/MS has now become the method of first choice for the quantitative bioanalysis of many pharmaceutical drugs (anti-depressants, anti-hypertensives, and anti retroviral agents) as is demonstrated the large number of articles that have appeared on the subject so far.

In chapter 1 an overview of depression, hypertension and HIV/AIDS epidemic treatment and care through types of antidepressant drugs, beta blocking agents and anti retroviral drugs through validated analytical methods, and the instrumentation has been described. In almost every case, MS-MS detection provides better sensitivity than other detection techniques. Furthermore, due to the selectivity of MS-MS detection less chromatographic separation is required and thus shorter run times can be achieved. As a result high-throughput strategies are explored by an increasing number of researchers.

In chapter 2, methods for the determination of anti-depressant agents are described. Venlafaxine and its major metabolite O-desmethylvenlafaxine were shows anti-depressant activity. A sensitive method was developed and validated for the quantitative analysis of venlafaxine and O-desmethylvenlafaxine in human
plasma using LC-MS/MS as described in chapter 2. Liquid chromatography performed with a mobile phase containing HPLC grade methanol and 5 mM ammonium acetate was used for the separation of analytes, resulting in no significant matrix effects were observed. Addition of acetonitrile cannot reduce matrix effects, and peak shape was distorted by the addition of formic acid. However, ammonium acetate reduced matrix effects without decreasing response and was included in the final mobile phase. Employing mobile phase containing ammonium acetate provided an excellent response for venlafaxine and O-desethylvenlafaxine in the positive ion mode.

Plasma samples were prepared for LC-MS/MS by liquid-liquid extraction (LLE) using methyl tertiary butyl ether (MTBE) on C18 cartridges. Fluoxetine was used as internal standard. The lower limit of quantification (LLOQ) using a 500 μL sample volume has 4 ng/mL, and the linear dynamic range extended to 400 ng/mL for venlafaxine, and 5 ng/mL and the linear dynamic range extended to 500 ng/mL for O-desethylvenlafaxine.

The validated method was applied to the determination of VEN and ODV in plasma samples from an open label balanced randomized two-treatment two-sequence two-period two-way crossover single dose comparative bioavailability study under fasting condition. Plasma samples were periodically collected up to 72 hrs after oral single dose administration of 75 mg tablet to 32 healthy male volunteers in each phase. The 90% confidence intervals of Cmax, AUC0−t and AUC0−∞ are within the 80-125% acceptance range.

Internal standards are very important in quantitative MS assays, since MS is not quantitative by nature. The greater advantage of this method is the shortening of run time to 3.0 minutes and also simple sample processing technique (Liquid-liquid extraction with MTBE). The method provided excellent specificity and linearity with a limit of quantification of 4 ng/ml for venlafaxine, and 5 ng/ml of ODV. Thus, the method well suits for the rapid and rugged analysis of bioavailability/bioequivalence (BA/BE) study sample.

In chapter 3, a novel analytical method for the simultaneous determination of bisoprolol, and hydrochlorothiazide (anti-hypertensive drugs) in human plasma was
developed and validated successfully. Determinations were made on a triple-quadrupole mass spectrometer (API 4000 LC-MS/MS) equipped with electrospray ionization (ESI) turbo ion source used in the positive/negative ion switching scan mode and operated with the Analyst™ Version 1.4.1 software. Tramadol and zidovudine (see Figure. 1(C) and 1(D)) were chosen as the internal standards, because similar retention to the bisoprolol and hydrochlorothiazide. Different percentages of acetonitrile were tested, such as 90, 80, 70 and 60%, for the influence on the retention time and sensitivity of both analytes. The sensitivity of bisoprolol was promoted with increasing amounts of acetonitrile up to 90% and that of hydrochlorothiazide was not affected significantly. The addition of ammonium acetate achieved more symmetrical peak shape.

The different concentrations of ammonium acetate buffer solution at levels of 10, 20 and 30 mM were tested in the mobile phase to get better chromatographic peak shapes of analytes. The test results showed that the concentration of 10 mM ammonium acetate buffer was satisfying enough to get better chromatographic peak shapes of bisoprolol, hydrochlorothiazide and internal standards and the symmetric chromatography peaks were obtained under this buffer condition. The aqueous solution containing 15% ammonium acetate and 85% acetonitrile was thus selected for the simultaneous elution, and the determination of the analytes. Since the two compounds did not interfere at each others mass trace, this simultaneous elution was not an impeding factor. The isocratic elution was performed to ensure that the analytes were eluted with proper retention and sharp peak shape. Under the present chromatographic conditions, the run time of each sample was 4 minutes only. Even though the internal standards having different chemical structures, the extraction efficiency using liquid-liquid extraction technique with MTBE proved similar and consistent for both the analytes and internal standards. Highest sensitivity was observed with ESI instead of APCI (atmospheric pressure chemical ionization) for both analytes for mass detection. The mass spectrometric conditions (ESI scan mode, ion source voltage, and collision energy) were optimized to obtain maximum sensitivity. Bisoprolol is a weak basic and medium-polarity compound; the ESI in positive ion mode was applied for the LC-MS/MS determination of bisoprolol. The LC-ESI-MS/MS was performed in the multiple reactions monitoring (MRM) mode.
Hydrochlorothiazide has respective polar groups; the presence of amido-group makes it facile to produce good mass spectrometric responses in negative ionization mode.

The results revealed that bisoprolol and tramadol (IS) were more sensitive in positive mode, while hydrochlorothiazide and zidovudine (IS) had higher response in negative mode. The calibration curves of each drug showed a good linearity in a range of concentration between 1.023 - 511.350 ng/mL for bisoprolol and 1.019 - 509.335 ng/mL for hydrochlorothiazide. The recovery comparison samples (non-extracted) of bisoprolol and hydrochlorothiazide were compared with extracted samples of LQC, MQC, and HQC. The recovery comparison samples of internal standards (tramadol and zidovudine) were compared with the response of internal standard in MQC level. The mean overall recoveries of bisoprolol and hydrochlorothiazide were 69.485% with the precision of 4.98% and 69.705% with the precision of 2.33% respectively.

The results revealed that bisoprolol and hydrochlorothiazide were stable in plasma for at least 6 hrs at room temperature, and 3 days at -20°C temperature. It was confirmed that repeated freeze and thawing (three cycles) of plasma samples spiked with bisoprolol and hydrochlorothiazide at LQC and HQC level did not affect the stability of bisoprolol and hydrochlorothiazide. The obtained results indicate that no significant degradation of bisoprolol and hydrochlorothiazide were observed under the tested conditions.

The developed and validated analytical method was successfully applied to compare the single oral dose bioavailability of test product, Bisoprolol 10 mg and hydrochlorothiazide 6.25 mg combination tablet under fasting condition. The 90% confidence intervals of C\text{max}, AUC\text{0→∞} and AUC\text{0→∞} are with in the 80-125% acceptance range. Thorough validation following FDA guideline indicated that the developed method had good sensitivity, consistency, specificity and excellent efficiency with a total run time of 4 min per sample and a limit of quantification of 1.023 ng/mL for bisoprolol and 1.019 ng/mL for hydrochlorothiazide. Thus, the method well suits for the rapid and rugged analysis of comparative bioavailability study sample.
In chapter 4, a simple and rapid analytical method for the determination of protease inhibitors lopinavir and ritonavir in human plasma was described. The development of a simultaneous method for lopinavir and ritonavir, an isocratic elution method was developed to attain good peak shape and higher sensitivity for the analytes. It was found that an isocratic elution with a mobile phase system consisting of 5 mM ammonium acetate buffer and acetonitrile (20:80 v/v) could accomplish this function and was finally assumed as the mobile phase for this method. A flow-rate of 0.8 mL/min produced sharp peak shapes and a short run time of 2.0 min.

Fluoxetine was a suitable internal standard for this method. Before choosing fluoxetine as an internal standard, several compounds were investigated to find an appropriate internal standard, but their CC's and QC's were not within acceptance criteria. The protein precipitation technique provides simplicity and is relatively cheap to perform and acetonitrile gave tolerable recovery of the analytes studied.

Quantification of drugs in biological matrices by LC-MS/MS is becoming more common due to the improved sensitivity and selectivity of this technique. [M + H]⁺ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The lowest limit of reliable quantification for lopinavir and ritonavir was set at the concentration of the LLOQ, 49.04 ng/mL and 5.024 ng/mL, respectively. The precision and accuracy results obtained for this method were found within the acceptance criteria. The correlation coefficients ($r^2$) of the calibration curves were greater than 0.995 in the concentration range of 50.67 ng/mL to 1000.82 ng/mL for lopinavir and greater than 0.996 in the concentration range of 5 ng/mL to 1000.693 ng/mL for ritonavir as determined by least-squares analysis. The standard curves for each drug were linear in the calibration range.

The stability in human plasma was determined during four freeze-thaw cycles of lopinavir and ritonavir. The stability data of the analytes in plasma over four freeze–thaw cycles indicate that the analytes are stable in human plasma for four freeze–thaw cycles, when stored at below –50°C and thawed to room temperature. The validated method was successfully applied to a single-dose bioequivalence study of lopinavir/ritonavir tablets 200/50mg under fasting condition. The
90% confidence intervals of $C_{\text{max}}$, $\text{AUC}_{0-\infty}$ and $\text{AUC}_{0-t}$ are with in the 80-125% acceptance range.

This method provided an excellent specificity and linearity with a limit of quantification of 49.04 ng/mL for lopinavir, and 5.024 ng/mL for ritonavir. The better advantages of this method; the shortening of run time to 2 minutes for each sample, made it possible to analyze more than 400 human plasma samples per day, and simple, rapid extraction procedure (Protein precipitation technique) requiring only 100 µL of plasma. By using protein precipitation technique, the absolute results not affected by matrix effects, the lower LOQ also not compromised. The High extraction efficiency and low limit of quantification make this a suitable method for use in clinical trials and for therapeutic drug monitoring of protease inhibitors.

From the analytical methods presented in chapter 2 to 4, it can be concluded that LC-MS/MS is very suitable for sensitive and selective quantitation of selected pharmaceutical drugs in biological samples. Furthermore, liquid chromatography with an appropriate mobile phase in combination with mass spectral detection in both positive and negative modes, appeared very useful for the analysis of selected drugs (venlafaxine, O-desmethylvenlafaxine, bisoprolol, hydrochlorothiazide and protease inhibitors lopinavir and ritonavir) described in this thesis. This system may be applicable to other basic drugs.

In this thesis the development and validation of LC-MS/MS methods for selected pharmaceutical drugs are described. These assays were developed in order to support clinical pharmacological studies. LC-MS/MS appeared to be very suitable for this purpose. Hopefully, this thesis has made a relevant contribution to the depression, hypertension and HIV/AIDS pharmacotherapy.
From:-
The Drugs Controller General (India),
Directorate General of Health Services

FDA Shavan, Kotla Road,
New Delhi-110002.
Dated 12 JUN 2009.

To,
M/s Wellquest Clinical Research,
(A Division of Nicholas Piramal India Ltd.)
4th floor, Mirra Kamsetty Mall,
Ramanthapur, R.R. Dist, Hyderabad-500013.

Sub: Approval of Bioavailability/Bioequivalence Study—regarding.

Sir,

Please refer to your letter no. Nil dated 2nd January 2009 and 10th July 2009 on the above subject.

This Directorate will continue to accept the bioavailability/bio-equlivalence protocols and the report of the studies with the new drugs from your laboratory situated at M/s Wellquest Clinical Research, (A Division of Nicholas Piramal India Ltd.) 4th floor, Mirra Kamsetty Mall, Ramanthapur, R.R. Dist, Hyderabad-500013, subject to the condition that specific protocol for conducting BE/BA studies be got approved by Institutional Ethics Committee and then from this Directorate on case to case basis.

Yours faithfully,

(A.B. RAMTEKE)
Joint Drugs Controller (India)
For Drugs Controller General (I)

Copy to:-
The Dy. Drugs Controller (I),
CDSCO (South Zone),
2nd Floor, Shastril Shavan Annex,
26, Haddows Road, Chennai-6