Chapter 9

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Survey of literature revealed that tetrasubstituted thiophene compounds had proved to be very effective anti inflammatory agents. From the reported newly synthesized anti-inflammatory agents few showed anti-inflammatory activity at a very low dose as compared to both standard drugs mefenamic acid and ibuprofen. FT1 is the best candidate among whole series which showed maximum anti-inflammatory activity at all the three graded doses employed, 71% at 10 mg/kg, 72% at 20 mg/kg and 76% at 40 mg/kg. For FT2 the % protection was found to be 50% at 10 mg/kg, 63% at 20 mg/kg, and 70% at 40 mg/kg, while ibuprofen showed 33% at 20 mg/kg and mefenamic acid showed 39% at 100 mg/kg which is less as compared to FT1 and FT2. From these compounds two agents FT1 and FT2 were selected for the purpose of method development and pharmacokinetic study. These agents were synthesized by reported procedures (Scheme I) and were characterized by IR and NMR. Spectral interpretation gives confirmation of structure of synthesized material.

For both the drugs spectrophotometric methods and chromatographic methods were developed. Spectrophotometric method involves simple UV spectroscopic method and derivative spectroscopic method while chromatographic method involves HPLC method for estimation of both the drugs in chemical sample as well as estimation of drugs in plasma. All the assay methods were validated according to ICH guidelines and bioanalytical method by USFDA guidelines.

UV spectroscopic methods both simple and derivative, for FT1, obeys linearity within the concentration range of 2-10μg/ml as the correlation coefficient was found to be 0.9996, 0.9999 and 0.9993 at 228nm, 378nm and 360.6/398.6nm respectively which is very near to 1. From the results for intraday precision, it was found that the %RSD values were found to be ≤1.235% at all the wavelengths. For all the three wavelengths, %RSD values for Intermediate Precision were found to be ≤1.855% for different days and ≤1.704% for different analyst. It was found that the percentage recovery values were between 98.20%
to 101.90% for simple as well as derivative spectroscopy methods which indicates that both the proposed methods are accurate.

For FT2, it was found that drug displays different ranges for simple UV method i.e. concentration range is 4-20μg/ml at 229nm, 6-18 μg/ml at 373nm. For derivative method range is 4-20μg/ml at 360/396nm. Correlation coefficients were found to be 0.9998, 0.9999 and 0.9980 at 229nm, 373nm and 360/396nm respectively which indicates that methods are linear. From the results for repeatability (precision), it was found that the %RSD values were found to be ≤1.901% at all the wavelengths. For all the three wavelengths, %RSD values for Intermediate Precision were found to be ≤1.878% for different days and ≤1.664% for different analyst. It was found that the percentage recovery values were between 98.43% to 101.42% for simple as well as derivative spectroscopy methods which indicates that both the proposed methods are accurate.

For both the drugs FT1 and FT2, HPLC method was developed which is reverse phase HPLC method. Method utilised C18 column with packing 5μ. Wavelength selected for estimation was 230nm and Flow rate was 1mL/min. Acetonitrile:Water (90:10) was selected as mobile phase. For both the drugs FT1 and FT2, system suitability test was performed and the parameters like %RSD of RT(≤0.7227) and %RSD of tailing factor(≤1.2783) was found to be less than 2. For FT1, RP-HPLC method is linear within the range 0.5-20 µg/ml with the correlation coefficient of 0.9999 which is very near to 1. FT2 shows linearity within the range of 1-30 µg/ml with the correlation coefficient of 0.9999. Regression equation was found to be y = 108.8x + 13.99 for FT1 and y=93.07x+25.87 for FT2. From the results of intraday precision, it was found that the %RSD for intraday precision was less than 0.315 for FT1 and 0.209 for FT2. For FT1 interday precision was found to be ≤ 0.315 and for FT2 it was found to be ≤1.449. Robustness of HPLC method for both the drugs was determined by implementing small but deliberate changes in flow rate and wavelength. The results of %RSD for change in flow rate was found to be ≤ 0.534 for FT1 and ≤ 0.196 for FT2. For change in wavelength, %RSD was ≤ 0.811 and ≤ 0.115 for FT1 and FT2 respectively. Mean %accuracy values were found in to be 100.47% for FT1 and 100.29% for FT2. LOD was 0.02183μg/ mL for FT1 and 0.0105μg/ mL for FT2. Limit of Quantification was 0.00721μg /mL for FT1 and 0.0318μg /mL for FT2. The proposed method is simple,
sensitive and reliable. Method is linear, precise and accurate. Hence, this method can be used for the routine determination of the agents.

Bioanalytical method developed was just the modification of the HPLC method developed for assay of drug in chemical sample. All the chromatographic parameters are same as that of HPLC assay method but in bioanalytical method Pantoprazole is used as internal standard. System suitability was checked by observing theoretical plates, resolution, asymmetry and %RSD of RT of drug as well as internal standard. All the system suitability parameters are within the acceptance limit. Bioanalytical method is selective as plasma peak does not interfere with the peak of internal standard and drug. Matrix effect is also checked for six different lots of plasma and %CV of concentration of LLOQ was found to be within the limit for both the drugs. Methods are sensitive as LLOQ are 25ng/ml for FT1 and 35ng/ml for FT2. For FT1, method is linear within the range 25 to 5000 ng/ml with \( r^2 \) - 0.9980 and regression equation \( y = 0.00009x + 0.001 \). For FT2, method is linear within the range 35 to 5000ng/ml with \( r^2 \) - 0.9994 and regression equation \( y = 0.00009x + 0.003 \).

Bioanalytical method for both the drugs is precise as %RSD for within batch and between batch precision was found to be \( \leq 10.78\% \) which is under the acceptance criteria (less than 15% and Less than 25% at LLOQ level). %Accuracy was found to be 86.37% to 94.90% and 81.48% for LLOQ. For FT2 %Accuracy was found to be 92.06% to 107.41% (including LLOQ). which is under the limit (\( \pm 15\% \)). %Recovery is also within the range for both the methods. Stock solution stability and stability of drug in plasma were performed for both the methods. Short term stock solution stability for drug and ISTD was performed for both the drugs and they were found to be stable for 12hrs. Long term stock solution stability for drug and ISTD was determined after the storage for 1 month and both the drugs were found to be stable for 1 month. Short Term-Bench Top (BT) stability was performed for 8 hrs at ambient temperature and both the drugs were found to be stable. Freeze Thaw stability (FT) was also performed for both the drugs and it was found that both the drugs withstands the freeze- thaw cycle. Both the drugs were found to be stable at 8°C under long term stability study. Method was proved to be rugged with change in external variables like analysts, change in equipments and change in columns for both the drugs.
The bioanalytical method described above was applied to quantify the concentration of FT1 and FT2 in plasma. Pharmacokinetic study was conducted on six wistar albino rats. The protocol was approved by Institutional Ethical Committee. Pharmacokinetic study was performed by taking blood samples at 0.0, 0.5, 1, 2, 3, 4, 5, 6, 9 and 12 h time points. Separated plasma then utilized to quantify the drugs by developed bioanalytical method. Pharmacokinetic parameters were calculated. $T_{\text{max}}$ were found to be 1h for both FT1 and FT2. $C_{\text{max}}$ were found to be 157 ng/ml and 147 ng/ml for FT1 and FT2 respectively. For FT1, $AUC_{(0\rightarrow12)}$ was found to be 546.25 ng h/ml and For FT1, it was found to be 537.375 ng h/ml. $AUC_{(0\rightarrow\infty)}$ were 608.233 ng h/ml and 687.736 ng h/ml for FT1 and FT2 respectively. $T_{1/2}(h)$ was 1.3859h and 2.3687h for FT1 and FT2 respectively.