EXECUTIVE SUMMARY OF THE PROJECT

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Title of the Research Project **"Development and Validation of A Precise single HPLC Method For Determination of APIs in pharmaceutical formulation"**

A simple, selective, linear, precise, and accurate RP-HPLC method was developed and validated for the simultaneous estimation of Clopidogrel Bi Sulphate and Aspirin from bulk drug. Chromatographic separation was achieved isocratically on a Shimadzu Phenomenex Luna, C18 column (250×4.6 mm, 5 µ particle size) using a mobile phase, (0.3% ortho phosphoric acid (v/v)-acetonitrile (40:60 v/v). The flow rate was 1 ml/min and effluent was detected at 226 nm and 20ìl of sample was injected. The retention time of Clopidogrelbisulphate and Aspirin were 6.6 and 8.4 min respectively. Linearity was observed in the concentration range of 0.030-0.120 mg/ml for aspirin and 0.015-0.060 mg/ml for clopidogrel. Percent recoveries obtained for for aspirin was 99.12-99.83% and 98.20-100.35 % for clopidogrel. The percentage RSD for precision and accuracy of the method was found to be less than 2%. The method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision, LOD and LOQ. The method developed was successfully applied for the analysis of simultaneous estimation of Clopidogrel Bi Sulphate and Aspirin bulk drug. A simple, selective, linear, precise, and accurate RP-HPLC method was developed and validated for the estimation of Aceclofenac from bulk drug. Chromatographic separation was achieved isocratically on a Phenomenex, C8 column (250×4.6 mm, 3 µ particle size) using a mobile phase, (0.01M ammonium acetate buffer with 2 ml triethylamine, (v/v)-acetonitrile (68:32 v/v) pH was adjusted to 6.5 with glacial Acetic acid. The flow rate was 1.2 ml/min and effluent was detected at 270 nm and 20ìl of sample was injected. The retention time of Aceclofenac was 6.4 min. Linearity was observed in the concentration range of 8-16 µg/ml. Percent recoveries obtained for Aceclofenac was 99.65-99.93%. The percentage RSD for precision and accuracy of the method was found to be less than 1%. The method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision, LOD and LOQ. The method developed was successfully applied for the analysis & estimation of Aceclofenac in bulk drug.

A rapid, stability-indicating reversed phase ultra-performance liquid chromatographic (RP-UPLC) method was developed for the determination of paliperidonepalmitate (PP), in depot injectable dosage form. The chromatographic separation was achieved on an Acquity BEH C18 (50 mm × 2.1 mm, 1.7 µm) column, with a mobile phase consisting of ammonium acetate buffer, and acetonitrile at a ratio of 10:90 (v/v) and a flow rate of 0.6 mL/min. The eluted compound was monitored at a wavelength of 238 nm using a UV detector. The method described herein separated paliperidonepalmitate from all other formulation components and

two major known degradation products (N-Oxide and paliperidone) within a run time of 2.5 min. The method also generated linear results over a PP concentration range of 156 to 468 µg/mL. The stability indicating capability of the method was established by performing forced degradation experiments. The RP-UPLC method that was developed was validated according to the International Conference on Harmonization (ICH) guidelines. This method was successfully applied in the quantitative determination of PP in a stability study of paliperidonepalmitate depot injection. The procedure described herein is simple, selective, and reliable for routine quality control analysis as well as stability testing.

(A)Clopidogrelbisulphate.:

Clopidogrel is an inhibitor of platelet aggregation. A variety of drugs that inhibit platelet function have been shown to decrease morbid events in people with established cardiovascular atherosclerotic disease as evidenced by stroke or transientischemic attacks, myocardial infarction, unstable angina or the need for vascular by-pass or angioplasty. This indicates that platelets participate in the initiation and/or evolution of these events and that inhibiting them can reduce the event rate.

(1)Intial HPLC Condition:

Mobile phase: Buffer-ACN (60: 40, v/v) Buffer: 0.3% orthophosphoric acid

Column: Phenomenex, C8 (250 mm x 4.6 mm i.d., 5µ particle size)

Flow rate: 1 ml/min

Wavelength: 226 nm

Injection volume: 20 µl

Diluent: Mobile phase

(2) Sample Preparation:

(I) Standard Preparation

Standard solution containing aspirin (0.075 mg/ml) and clopidogrel (0.0375 mg/ml) were prepared by dissolving 37.5 mg aspirin and 24.46 mg clopidogrelbisulphate (equivalent to 18.5 mg clopidogrel) in 100 ml volumetric flask by mobile phase (stock standard solution). Pipette out 10 ml stock solution into 50 ml volumetric flask and dilute up to mark with mobile phase (standard solution).

(II) Test Preparation

Twenty tablets were weighed and the average tablet weight was determined. Tablets were crushed by mortar and pastel. Tablet powder was weighed equivalent to five times of average weight and transfer in to 200 ml volumetric flask. About 170 ml mobile phase was added and

sonicated for of 30 min time interval with intermittent shaking. Content was brought back to room temperature and dilute to volume with mobile phase (stock solution). The stock solution was filtered through 0.45 µm nylon syringe filter. Pipette out 2 ml filtered stock solution in to 100 ml volumetric flask and diluted with mobile phase (test solution). The concentration obtain was 0.075 mg/ml of aspirin and 0.0375 mg/ml of clopidogrel.

(B)Aceclofenac:

The mode of action of aceclofenac is largely based on theinhibition of prostaglandin synthesis. Aceclofenac is potent inhibiter of the enzymecyclo-oxygenase, which is involved in the production of prostaglandins. Aceclofenac hasbeen shown to exert effects on a variety of mediators of inflammation.

(1)Initial HPLC Condition:

Mobile phase: Buffer-ACN (68: 32, v/v)

Buffer: 0.01M ammonium acetate buffer with 2 ml triethylamine,pH 6.5 with glacial Acetic acid.

Column: Phenomenex, C18 (250 mm x 4.6 mm i.d., 5µ particle size)

Flow rate: 1 ml/min

Wavelength: 270 nm

Injection volume: 20 µl

Diluent: Water: ACN (50: 50, v/v)

(2)Sample Preparation

(I) Standard Preparation

Standard solution containing tramadol hydrochloride (0.0375 mg/ml) and aceclofenac (0.100 mg/ml) were prepared by dissolving 18.75 mg tramadol hydrochloride and 50 mg aceclofenac in 50 ml volumetric flask by diluent (stock standard solution). Pipette out 5 ml stock solution into 50 ml volumetric flask and dilute up to mark with diluent (standard solution).

(II) Test Preparation

Twenty tablets were weighed and the average tablet weight was determined. Tablets were crushed by mortar and pastel. Tablet powder was weighed equivalent to five times of average weight and transfer in to 500 ml volumetric flask. About 50 ml methanol and 300 ml mobile phase was added and sonicated for of 20 min. time interval with intermittent shaking. Content was brought back to room temperature and dilute to volume with diluent (stock test solution). The stock solution was filtered

through 0.45 µm nylon syringe filter. Pipette out 5ml filtered stock solution in to 50 ml volumetric flask and dilute with diluent (test solution). The concentration obtain was 0.0375 mg/ml of tramadol hydrochloride and 0.100 mg/ml of aceclofenac.

Development and Optimization of the HPLC Method

In the presence work, an analytical method based on LC using UV detection was developed and validated for assay determination of aspirin and clopidogrel in tablet formulation. The analytical conditions were selected, keeping in mind the different chemical nature of aspirin and clopidogrel. The development trials were taken by using the degraded sample of each component was done, by keeping them in various extreme conditions.

The column selection has been done on the basis of backpressure, resolution, peak shape, theoretical plates and day-to-day reproducibility of the retention time and resolution between aspirin and clopidogrel peak. After evaluating all these factors, C8 (2) (250 mm 4.6 mm i.d., 5 µm particle size) column was found to be giving satisfactory results. The selection of buffer based on chemical structure of both the drugs. The acidic pH range was found suitable for solubility, resolution, stability, theoretical plates and peak shape of both components. Best results were obtained with 0.3% orthophosphoric acid solution improved the peak shape of aspirin and clopidogrel. Finally, by fixing 0.3% orthophosphoric acid (v/v) and mobile phase composition consisting of a mixture of 0.3% orthophosphoric acid (v/v)-acetonitrile (65:35, v/v). Optimized mobile phase proportion was provide good resolution between aspirin and clopidogrel and also for degradation product which is generated during force degradation study. For the selection of organic constituent of mobile phase, acetonitrile was chosen to reduce the longer retention time and to attain good peak shape. Figure 3 and Figure 4 represent the chromatograms of standard and test preparation respectively.

Method Validation

Specificity: The specificity of the method was determined by checking the interference of placebo with analyte and the proposed method were eluted by checking the peak purity of aspirin and clopidogrel during the force degradation study. The peak purity of the aspirin and clopidogrel were found satisfactory under different stress condition. There was no interference of any peak of degradation product with drug peak.

Linearity: For linearity seven points calibration curve were obtained in a concentration range from 0.030-0.120 mg/ml for aspirin and 0.015-0.060 mg/ml for clopidogrel. The response of the drug was found to be linear in the investigation concentration range and the linear regression equation for aspirin was y= 60026378.57x + 51410.11 with correlation coefficient 0.9999(Figure 10) and for clopidogrel was y = 44544414.03x -1890.29 with correlation coefficient 0.9999 (Figure 11). Where x is the concentration in mg/ml and y is the peak area in absorbance unit.

LOD and LOQ: The limit of detection and limit of quantification were evaluated by serial dilutions of aspirin and clopidogrel stock solution in order to obtain signal to

noise ratio of 3:1 for LOD and 10:1 fro LOQ. The LOD value for aspirin and clopidogrel were found to be 0.05 ppm and 0.15 ppm, respectively and the LOQ value 0.2 ppm and 0.3 ppm, respectively.

Result of precision study:

Set	Aspirin (%Assay)		Clopidogrel (%Assay)	
	Intraday (n = 6)	Interday (n = 6)	Intraday (n = 6)	Intraday (n = 6)
1	99.1	100.2	99.3	99.6
2	100.0	99.9	98.7	99.6
3	99.6	100.5	98.6	100.1
4	99.5	100.3	99.0	100.1
5	100.3	101.0	100.0	100.6
6	99.1	100.8	99.5	100.7
Mean	99.6	100.5	99.2	100.1
Standard deviation	0.48	0.40	0.53	0.47
% RSD	0.48	0.40	0.53	0.47

Accuracy: Recovery of aspirin and clopidogrel were determined at three different concentration levels. The mean recovery for aspirin was 99.12-99.83 % and 98.20-100.35 % for clopidogrel (Table 2). The result indicating that the method was accurate. Chromatogram obtain during accuracy study were shown in following figures.

Result of accuracy study:

	Level (%)	Amount Added Concentration[a] (mg/ml)	Amount Found Concentration[a] (mg/ml)	% Recovery[a]	% RSD[a]
Aspirin	50	0.03751	0.03721	99.22	0.07
	100	0.07497	0.07432	98.12	0.23
	150	0.11250	0.11232	99.83	0.05
Clopidogrel	50	0.01874	0.01840	98.20	0.19
	100	0.03748	0.03695	98.59	0.14
	150	0.05627	0.05647	100.35	0.24

Solution stability study: Table 3 shows the results obtain in the solution stability study at different time intervals for test preparation. It was concluded that the test

preparation solution was found stable up to 48 h at 2-5 ^{0}C and 36 h at ambient temperature with the consideration of < 2.0% in %assay value difference of inteval value against initial value.

Evaluation data of solution stability study:

Intervals	% Assay for Test Solution Stored at 2 –5 ^{0}C		% Assay for Test Solution Stored at Ambient Temperature	
	Aspirin	Clopidogrel	Aspirin	Clopidogrel
Initial	101.3	100.0	101.3	100.0
12 h	100.9	99.3	100.1	99.5
24 h	100.3	99.6	99.8	99.0
36 h	100.1	99.0	99.8	98.6
48 h	99.9	98.7	98.1	98.7

Robustness: The result of robustness study of the developed assay method was established in Table 4 and Table 5. The result shown that during all variance conditions, assay value of the test preparation solution was not affected and it was in accordance with that of actual. System suitability parameters were also found satisfactory; hence the analytical method would be concluded as robust. Chromatogram obtain during robustness study were shown in following figures.

Evaluation data of robustness study of aspirin

Robust Conditions	% Assay	System Suitability Parameters		
		Theoretical Plates	Asymmetry	% RSD
Flow 0.9 ml/min	100.5	6460	1.05	0.22
Flow 1.1 ml/min	100.3	5661	1.05	0.09
0.28 % H_3PO_4-ACN (65:35, v/v)	100.0	6117	1.00	0.40
0.32 % H_3PO_4-ACN (65:35, v/v)	99.7	5588	1.02	0.30
0.3% H_3PO_4-ACN (63:37,v/v)	100.2	5475	1.12	0.19
0.3% H_3PO_4-ACN (67:33,v/v)	100.1	5838	1.04	0.20
Column change	100.4	5425	1.05	0.34

Evaluation data of robustness study of clopidogrel

Robust Conditions	% Assay	System Suitability Parameters			
		Theoretical Plates	Asymmetry	% RSD	Resolution
Flow 0.9 ml/min	98.5	6975	1.07	0.67	5.75
Flow 1.1 ml/min	100.0	5992	1.06	0.39	5.14
0.28 % H_3PO_4-ACN(65:35,v/v)	98.8	6899	1.03	1.03	6.35
0.32 % H_3PO_4-ACN(65:35,v/v)	99.2	6113	1.03	0.69	5.62
0.3% H_3PO_4-ACN(63:37,v/v)	99.5	5850	1.11	0.70	4.31
0.3% H_3PO_4-ACN (67:33,v/v)	99.0	6185	1.04	0.30	5.71
Column change	100.0	5996	1.04	0.25	4.97