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ANALYSIS OF HUMAN PLASMA FOR GROWTH IN PHARMACEUTICAL MEDICINES

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ABSTRACT

Bioanalytical chemistry is the willpower of medicines substances in biological fluids like plasma, serum in terms of qualitative and measurable analysis. It plays important role in evaluation of pharmacokinetic parameters which required for bioavailability and bioequivalence studies. The validation of developed method required to assure common level of quality therefore use of validated methods increased in current practice. Analytical methods plays important role in research and development of new product. Full method validation will be start after promising results of method development because the method developer doesn't have idea about the actual method conditions during method validation. Method development mainly involves ordinal. The important stages in method development are preparation of sample, separation of prepared sample by using chromatography, detection of separated sample by using suitable detection method. To start method development of newer analyt extensive literature survey required. After literature survey the primary. Literature survey information will be used to select instrument that is comfortable for analysis. This includes analytical column, high performance liquid select suitable internal standard as per analyte parameters economical as industrial prospective. Two factors mainly consider for willpower of quality are recovery and standardization of method. Recovery of analyte into. Comparison between matrix content and pure solvent is nothing but relative and suitability of chromatographic methods.

DEVELOPMENT AND OPTIMIZATION OF LC-MS/MS METHOD

1 Selection of internal standard

Various medications like pantoprazole, quitapine, lansoprazole were attempted as inner standard, however lansoprazole was chosen as IS a result of its basic, physicochemical and pharmacological closeness to febuxostat.

2 Development of chromatographic conditions

Methanol and formic corrosive focus were utilized for readiness of versatile stage in the

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wake of taking different preliminaries like methanol: 0.01% formic corrosive (90:10% v/v), methanol: 0.1% formic corrosive (90:10% v/v). Formic corrosive focus was enhanced to 0.1% subsequent to utilizing different fixations. The proportion of the 0.1% formic corrosive was expanded to take into consideration better pinnacle shape and goals in plasma. Best outcomes were acquired by utilizing the proportion; methanol: 0.1% formic corrosive (80:20 % v/v). Hypurity C18 (100 x 4.6 mm), 5 µ section was chosen for decrease of run time. Low pace of stream was chosen to 1.0 ml/min to expand the effectiveness of section and to decrease the use of portable stage.

3. Optimization of extraction technique

- Liquid-Liquid extraction (LLE)

The LLE strategy was finished by utilizing different natural solvents like tertiary butyl methyl ether (TBME), diethyl ether, n-hexane, dichloromethane, ethyl acetic acid derivation and the different mixes of above solvents in different proportions had been

endeavored. In any case, exceptionally less recuperation got for analyte and IS. So LLE was precluded.

- Solid phase extraction

Desert spring HLB 1cc cartridges were utilized. Molding of cartridges was finished utilizing 1.0 ml of methanol and 2.0 ml of milli q water. For washing, water and methanol in water (5, 10, 15 and 20%) in fluctuating extents were endeavored. The eluent was then vanished under nitrogen weight for drying. After complete drying it reconstituted with versatile stage. Recuperation gotten by this extraction strategy was discovered great yet technique was discovered very tedious and exorbitant.

- Protein precipitation method

Different protein hastening specialists like acetonitrile, methanol and 5% perchloric corrosive were endeavored. Be that as it may, great outcomes were acquired with methanol as a protein encouraging operator. No obstructions were seen from the natural grid at maintenance time of the analyte and inside standard. Great recuperation was additionally gotten for the analyte and IS. Henceforth, the protein precipitation technique was chosen for the present investigation.

4. Final chromatographic conditions

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- Chromatographic Mode : Reversed Phase
- Type of Method : Isocratic
- Column : Hypurity C_{18} (100x4.6mm), 5 µ.
- Injection Volume : 2 µl
- Flow Rate : 0.45 ml/min
- Column Oven Temperature : 40° C
- Run Time : 3.3 minutes
- Mobile Phase : Methanol: 0.1% formic acid (80:20)
- Diluent : Methanol: mill q water (50: 50)
- Retention Time : 2.3 min. for febuxostat (Analyte)

1.2 min. for lansoprazole (IS)

- Extraction Technique : Protein Precipitation

5. LC-MS/MS-detector condition

- Spraying probe position : X Axis - 5, Y Axis - 5
- Mass Tuning Parameters : As mentioned in Table 3.4

Table 1: Mass tuning parameters

Parameters	Febuxostat	Lansoprazole
Q1	317.1	370.1
Q3	261.0	251.9
CUR	20	20
CAD	6	6
GS1	45	45
GS2	55	55
IS	5500	5500
TEM	350	350
EP	10	10
DP	70	45
CE	24	11
CXP	7	12

Preparation of reagents and solutions

1. Preparation of 0.1% formic acid solution

100 µL of formic acid was transferred into 100 ml of milli q water to made 0.1% formic acid solution.

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2. Preparation of mobile phase

800 ml of methanol (HPLC grade) and 200 ml of 0.1% formic acid were transferred into 1000 ml volumetric flask and sonicated to mix well.

3. Diluent

250 ml of methanol and 250 ml of milli q water were transferred into a 500 ml of volumetric flask and sonicated to mix well.

4. Rinsing solution

250 ml of methanol and 250 ml of milli q water were prepared in into a 500 ml of volumetric flask and sonicated to mix well.

5. Preparation of febuxostat stock solution (1.0 mg/ ml)

25 mg of febuxostat was transferred into 25 ml of volumetric flask and made volume with methanol.

6. Preparation of lansoprazole stock solution (0.5 mg/ml)

5 mg of lansoprazole was transferred into 10 ml of volumetric flask and made volume with methanol.

7. Preparation of samples for calibration curve

The working solutions of febuxostat were prepared using diluent (Table 3.5). Similarly LQC (lower quality control), MQC (middle quality control), HQC (higher quality control), LLOQ (lower limit of quantification) samples were prepared as per Table 3.6.

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Table 2: Preparation of samples for calibration curve

Sr. No.	Concentration Before Dilution (ng/ml)	Stock Solution ID	Stock Volume (ml)	Diluent Volume (ml)	Final Concentration (ng/ml)	Solution ID
1	986361.600	Main stock	5.10	4.90	50304.4164	WS-08
2	503044.416	WS-08	8.00	2.00	402435.33	WS-07
3	402435.533	WS-07	8.20	1.80	329997.137	WS-06
4	329997.137	WS-06	7.00	3.00	230997.996	WS-05
5	230997.996	WS-05	4.00	6.00	92399.198	WS-04
6	92399.198	WS-04	3.00	7.00	27719.759	WS-03
7	27719.759	WS-03	1.80	8.20	4989.557	WS-02
8	4989.557	WS-02	5.00	5.00	2494.779	WS-01

Table 3.6: Preparation of QC samples

Sr. No.	Concentration Before Dilution (ng/ml)	Stock Solution ID	Stock Volume (ml)	Diluent Volume (ml)	Final Concentration (ng/ml)	Solution ID
1	986361.600	Main Stock	4.00	6.00	394544.640	WS-HQC
2	394544.640	WS-HQC	6.20	3.80	244617.677	WS-MQC
3	244617.677	WS-MQC	1.52	8.48	37181.887	WS-INT QC
4	37181.887	WS-INTQC	2.00	8.00	7436.377	WS-LQC
5	7436.377	WS-LQC	3.40	6.60	2528.368	WS-LLOQ

8. Preparation of spiked calibration curve standards:

Required quantities of tests of centralization of febuxostat going from 49.896 to 10060.888 ng/ml were set up by make up the volume with medication free plasma and named them as STD-1 to STD-8 depicted according to Table 3.7.

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Table 3: Preparation of spiked calibration curve standards

Sr. No	Stock Solution	Stock Concentration (ng/ml)	Stock Volume (ml)	Volume of Plasma (ml)	Final Volume (ml)	Febuxostat Final Concentration (ng/ml)	Stock ID
1	Ws-08	503044.416	0.2	9.8	10	10060.888	STD 8
2	Ws-07	402435.533	0.2	9.8	10	8048.711	STD 7
3	Ws-06	329997.137	0.2	9.8	10	6599.943	STD 6
4	Ws-05	230997.996	0.2	9.8	10	4619.960	STD 5
5	Ws-04	92399.198	0.2	9.8	10	1847.984	STD 4
6	Ws-03	27719.759	0.2	9.8	10	554.395	STD 3
7	Ws-02	4989.557	0.2	9.8	10	99.791	STD 2
8	Ws-01	2494.779	0.2	9.8	10	49.896	STD 1
9	WS-HQC	394544.640	0.2	9.8	10	7890.893	HQC
10	WS-MQC	244617.677	0.2	9.8	10	4892.354	MQC
11	WS-LQC	7436.377	0.2	9.8	10	148.728	LQC
12	WS-LLOQ	2528.368	0.2	9.8	10	50.567	LOQQC

SAMPLE PREPARATION

Precisely pipetted 50 µl of test into pre marked vials and 1.0 ml of IS was included and blended for 2 min (For clear example 1.0 ml of methanol arrangement was included rather than Internal Standard arrangement). Methanol in lansoprazole arrangement was utilized for protein precipitation. Centrifuged the examples at 4600 RPM at less than 5°c for 15 min. at that point supernant layer was moved into pre named HPLC vial. At that point 2.0 µl was infused into HPLC framework utilizing auto sampler.

VALIDATION STUDIES

1 Bioanalytical method validation

The following parameters were considered for the bioanalytical method validation for the febuxostat quantification:

(i) System suitability

System suitability was performed independently before beginning of each new cluster. Six infusions of fluid equal MQC arrangement blended with the ISTD working arrangement were performed. The mean, S.D, and % CV for the pinnacle zone proportion and for the maintenance time of analyte and ISTD were determined.

Acceptance criteria

- % CV for peak area ratio should be ≤ 5.0
- % CV for retention time for both the analyte and ISTD should be ≤ 5.0%.

(ii) Selectivity and specificity

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Selectivity is capacity of created technique to deliver a reaction for focused particle which recognizing from obstructions like endogenous and exogenous sources. Endogenous sources are analyte metabolite, corruption items, synthetic concoctions happening in organic liquids, co-directed medications and exogenous sources grimy lab products, contaminations in reagents, chemicals10 . This was deciding by checking six unique heaps of supported plasma for any obstruction at the maintenance time of the medication and inner standard by utilizing the chose technique conditions. The clear plasma was handled and keep running for 45 min to watch any meddling pinnacles.

Acceptance criteria

- Any meddling pinnacle whenever found ought not be available inside ±10% of the maintenance times of medication and ISTD.

- If impedance saw at the maintenance times of medication and ISTD, it ought to be ≤ 20% and ≤ 5% of reaction of the mean separated LLOQ and mean removed ISTD individually at the focus to be utilized in study.

- at least 75% plasma parcels utilized for the explicitness should meet the above criteria.

(iii) Sensitivity

Sensitivity is the particular reaction which relies upon the fixations to be estimated in organic network for the particular atom. It very well may be communicated as slant of direct of the straight relapse alignment bend and it gauged at the time as the linearity test 5. This was controlled by contrasting removed clear plasma and separated LLOQ tests (5 recreates) with ISTD utilizing the proposed bioanalytical technique conditions.

HYPOTHESIS

H1: Bio analytical methods for pharmaceutical industry by using novel techniques for quantification of medicines molecules such as liquid chromatography– tandem mass spectrometry.

H2: Analyze the developed bio analytical method should be fully validated for quantifying medicines molecules fast, selective and reproducible. Validation experiments included study of matrix effect, anticoagulant effect from different biological matrixes lots, precision and accuracy, selectivity/specificity, sensitivity metabolite interferences and various stability tests such as freeze thaw stability, stock solution stability, and bench top stability.

H3: The Febuxostat is categorised as a xanthine oxidase inhibitor. On comprehensive literature review of febuxostat, it was observed that there was not a single analytical or bioanalytical method reported for estimation of febuxostat. No stability indicating assay method is published to reveal specificity and degradation study.

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H4: The aim of present work was to develop simple isocratic bio analytical method to estimate Clebopride from human plasma with due consideration of accuracy, sensitivity, rapidity, economy, selectivity, stability according to regulatory guidelines.

H5: The Darifenacin is antimuscarinicsmedicines. On complete literature review of darifinacin, it was observed that there was few bioanalytical method reported for determination of darifenacin from human, which was found time consuming and costly.

RESULTSANDDISCUSSION

FEBUXOSTAT

(i) System suitability

To confirm that the investigative framework would work appropriately and would giveexact and exact outcomes, the framework appropriateness parameters were performedbefore each approval parameter and results were found inside acknowledgment criteria.The outcomes acquired were in acknowledgment criteria as the % CV for pinnacleproportionswasbetween2.4% to4.8% and%CV for the maintenance time wasbetween 0.1 to 0.2 % and 0.0 to 0.1 % for the analyte and IS individually. After effectsoframework reasonableness areportrayedin Table 4.1 to 4.3.

Table 4:System suitability-IforLC-MS/MSmethod

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Sr. No.	Drug		IS		
	Area	RT	Area	RT	Area Ratio
System suitability-I	1145819	2.24	575685	1.426	1.990
	1155793	2.25	582706	1.428	1.983
	1115376	2.25	592069	1.427	1.884
	1145233	2.24	590759	1.427	1.939
	1168880	2.24	588144	1.427	1.987
	1166584	2.24	579848	1.427	2.012
Mean	1149614	2.243	584869	1.427	1.966
S.D.	19509.87	0.00	6503.73	0.00	0.05
% CV	1.7	0.1	1.1	0.0	2.4

Table 5:System suitability-IIforLC-MS/MSmethod

Sr. No.	Drug		IS		
	Area	RT	Area	RT	Area Ratio
System suitability-II	1156389	2.346	707729	1.441	1.634
	1137633	2.341	709390	1.442	1.604
	1137008	2.339	686405	1.442	1.656
	1144493	2.333	663056	1.441	1.726
	1190927	2.334	693954	1.442	1.716
	1146680	2.339	673109	1.440	1.704
Mean	1152188	2.339	688941	1.441	1.673
S.D.	20249.40	0.00	18565.55	0.00	0.05
% CV	1.8	0.2	2.7	0.1	3.0

Table 6:System suitability-IIIforLC-MS/MSmethod

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Sr. No.	Drug		IS		
	Area	RT	Area	RT	Area Ratio
System suitability-III	795695	2.175	1143132	1.408	0.696
	812509	2.174	1110451	1.408	0.732
	807601	2.175	1116894	1.407	0.723
	809393	2.176	1086036	1.408	0.745
	807213	2.176	1060398	1.405	0.732
	876138	2.176	1061059	1.406	0.723
Mean	818092	2.175	1096328	1.407	0.728
S.D.	29004.06	0.00	33026.74	0.00	0.04
% CV	3.5	0.0	3.0	0.1	4.8

(i) Selectivity

Screening and selectivity was finished utilizing six bunches of plasma and the outcomewas found inside as far as possible. The acquired pinnacles were great shape. Theselectivity of the strategy is displayed in the Table 4.4. The chromatogram of the clearplasma is as appeared in Figure 4.1. The outcomes got were inside acknowledgmentcriteria of ≤20% impedance at maintenance time of medication and≤ 5% atthemaintenancetime of IS.

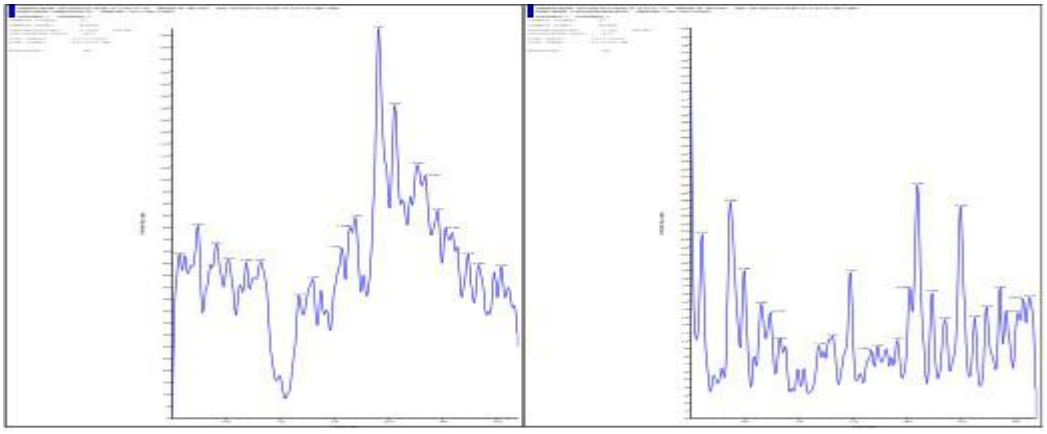

Figure 1:Chromatogram of blank plasma

CONCLUSION

The coefficient of the analyte and the internal standard are very similar is the main assumption consider for selection of internal standard. Now a day's structural isotope analogue of targeted analyt used as internal standard. The structural and isotopic analogue

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of analyteis added to the biological sample prior to sample pretreatment to calculate the area ratio between analyte and internal standard is back calculated against standard curve which determine the concentration of medicines. The selection of suitable instrument and there make is an important issue during method development. The difference betweendifferent makes of instrument is not surprising and should be considering is nothing but lowest concentration of an analyte in a sample that can operation condition of the method. These parameters associated with signal to noise ratio. The signal is measured in blank plasma and measured frombase line to peak of apex and divided by peak to peak. The calibration curve is determined between lowest concentration of analytic to the highest required concentration as per. The minimum six concentrations are required to define relationship between analytic response and concentration and it should be reproducible at all stages of method. The more concentration may be required in case of nonlinear relationship. The most appropriate weighing factor is 1/x, 1/x2 commonly observed. Among the seven none zero standard satleast should be pass the criteria

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