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Development and Validation of a Sensitive LC-MS/MS Method for Simultaneous Quantitation of Fluticasone and Salmeterol in Human Plasma

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ABSTARCT

A simple, robust bioanalytical method (BAM) was developed and validated for simultaneous quantification of fluticasone (FCS) and salmeterol (SMR) in biological matrix (BM). The solid phase extraction (SPE) procedure was use to extract FCS and SMR from BM. The developed method was validated as per regulatory guidelines with calibration range of FCS and SMR with were 0.500-150.200pg/mL and 1.00-250.218pg/mL. The mass transition ions for FCS and SMR were 501.300→293.000, 415.900→232.200 with column Discovery C18, gradient mobile phase 0.1mM ammonium trifluoro acetate, acetonitrile with injection volume 15µL. The linear regression $1/X^2$ was confirmed with three precision and accuracy (P&A) batches and the best r^2 value among three P&A was 0.9994 and 0.9996. The developed BM was showed no carry over, free of inter conversion between FCS & SMR and no matrix effect. The extraction procedure showed more than 80% recovery from spiked samples. The stabilities of FCS and SMR in aqueous and matrix were met the acceptance criteria as per guidelines. The stability in aqueous samples was 36 hrs at 2-8℃ and stability in matrix was up to 38 days at -70℃ and -20℃. In conclusion, developed BM will useful in pharmacokinetic studies of FCS and SMR either individual and combined pharmaceutical products.

KEY WORDS: *emerging diseases, analytical methods, quantification, LC-MS/MS, regulatory guidelines.*

1. INTRODUCTION

The developments in science and technology have been adding various biopharmaceutical products in medicine to treat different ailments over last few decades [1]. As the medicines are developing, it is vital to know about their bioavailability, pharmacokinetic and pharmacodynamic activities in the human body [2,3]. This making the essential to develop efficient analytical methods to know their purity, potency, quantification, separation and stability in their development studies with complies to regulatory guidelines [4-5]. The developed new analytical methods are crucial to know the bioavailability of new drug molecules and bioequivalence studies of generic drugs to establish their quantity in systematic circulation of the body [6]. The emerging and blowout of new diseases and spreading of current diseases around the world, insisting the researchers to develop new drugs and generic drugs to meet the needs to treat different diseases. The developed new drugs (innovator medicines) are high costs compared to the generic drugs as the innovator companies are spends more money and time on research and development [7-9]. As above said, new analytical methods are playing vital role in development of drugs. In this point of view, the current study was carried out to establish a new liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) method for simultaneous quantification of salmeterol and fluticasone in human plasma.

Asthma is one of the major chronic lung diseases in the all-ages people. There were many causes for asthma in people starting from birth to environmental conditions such as low birthweight, prematurity, allergic to pollution, dust and overweight [10-11]. The symptoms of asthma are varying from reason to reason and treatments are available either with modern medicines and natural medicines. One of the main drugs have been using in treatment for asthma are corticosteroids and beta2-adrenergic agonists [12-15]. Fluticasone (FCS) and Salmeterol (SMR) are two drugs belongs to these categories, the combination of both drugs have been using to treat asthma [16-18]. FCS is a corticosteroid, which reduces the inflammation in lungs and SMR will help in relaxation of smooth muscles in lungs and controls the hypersensitivity against allergenic agents. The urbanization and multiple life style factors enhanced the rate of asthma patients around the world and depends on it need recently US food and drug administration given approval for its generic medicines and it is 56^{th} most commonly prescribing medicine in United States and there were very few bioanalytical methods to quantify FCS and SMR simultaneously [19]. So, there is a need to develop new methods to quantify FCS and SMR in human matrices in development of generic medicines as there were less methods available on their quantification in different matrices using LC-MS/MS.

2. MATERIALS AND METHODS

2.1 Chemicals, Reagents and Instrumentation

The chemicals and reagents used in current research were analytical grade. The drugs fluticasone (FCS), salmeterol (SMR), fluticasone-D5 (FCSD5), Salmeterol-D3 (SMRD3)) were procured from Vivan Lifesciences Pvt. Ltd, Mumbai. The human plasma with anticoagulant dipotassium ethylene diamine tetra acetic acid (K_2EDTA) was from M/S Laxmi Sai Chemical lab, Hyderabad, India. The LC-MS/MS equipped with LC from Shimadzu (Japan) and Triple Quad Sciex 6500^+ (Sciex, Canada) was used. The analytical column Discovery C18 (150X4.6mm, 5µ) from Merck (USA) and the cartridges HLB Cartridges from Waters (USA) were used.

2.2 Preparation of standard and Internal standard stock solutions

The drugs FCS and FCSD5 were prepared as 0.200 mg/mL in dimethyl sulfoxide (DMSO): methanol (50:50v/v) mixture and SMR and SMRD3 were prepared as 0.200mg/mL in methanol. These stock solutions were used for further dilutions and are prepared using methanol: water (50:50v/v) mixture as diluent to spike the plasma as 2% spiking. The eight non-zero calibration curve (CC) points were used for FCS and SMR with the range 0.500-150.200 pg/mL and 1.00-250.218 pg/mL respectively. The quality control (QC) samples for FCS and SMR were prepared as lower limit of quantitation QC (LLOQ QC) equal or greater than lower point of CC, lower QC (LQC) as equal or less than three times of lower point of CC, middle QC (MQC) as 30-50% of ULOQ, high QC (HQC) as 75-80% of ULOQ and dilution integrity QC (DQC) as two times of ULOQ. The QC samples' concentrations for FCS are 0.501, 1.496, 47.100, 114.888 and 300.400 pg/mL. The QC samples' concentrations for SMR are 1.010, 2.986, 78.456, 191.480 and 500.200 pg/mL. The DIQC samples were processed using dilution factor 4. The IS dilutions of FCSD5, SMRD3 were used at 100ng/mL concentration. The spiked samples in plasma were stored at -70°C and -20°C for validation of developed LC-MS/MS methods as per U.S. Food and Drug Administration (USFDA) bioanalytical method validation guidelines [20].

2.3 LC-MS/MS parameters optimization

The quantification of any drugs using LC-MS/MS, first the ions have to be identified for the targeted compounds with source and compound parameters. The ions used and identified for FCS, SMR and their ISs (FCSD5 and SMRD3) are in positive mode as Table 1 and figures 1 and 2. The source and compound parameters were optimized and used as showed in Table 2.

Once the mass parameters were optimized, it is essential to optimize LC conditions for chromatographic separation, good peak shape and response of the targeted compounds. Diverse columns and mobile phases were tested to get best chromatographic conditions and finalized parameters were showed in Table 3.

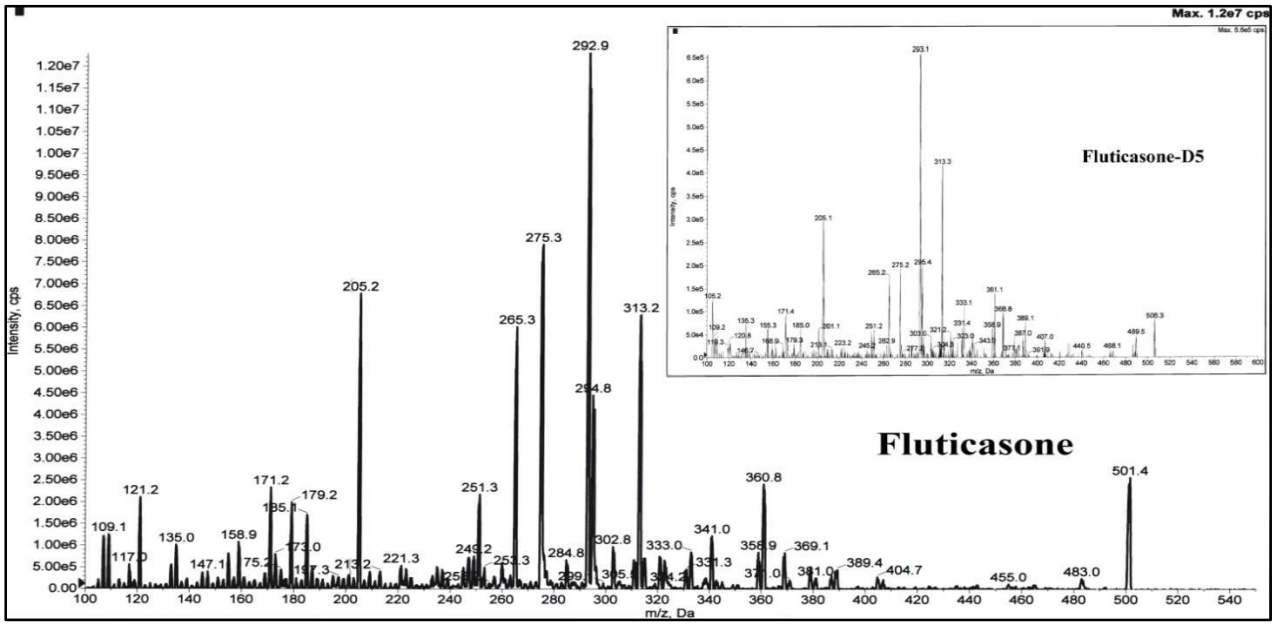

FIGURE 1: Mass spectra of Fluticasone and Fluticasone-D5

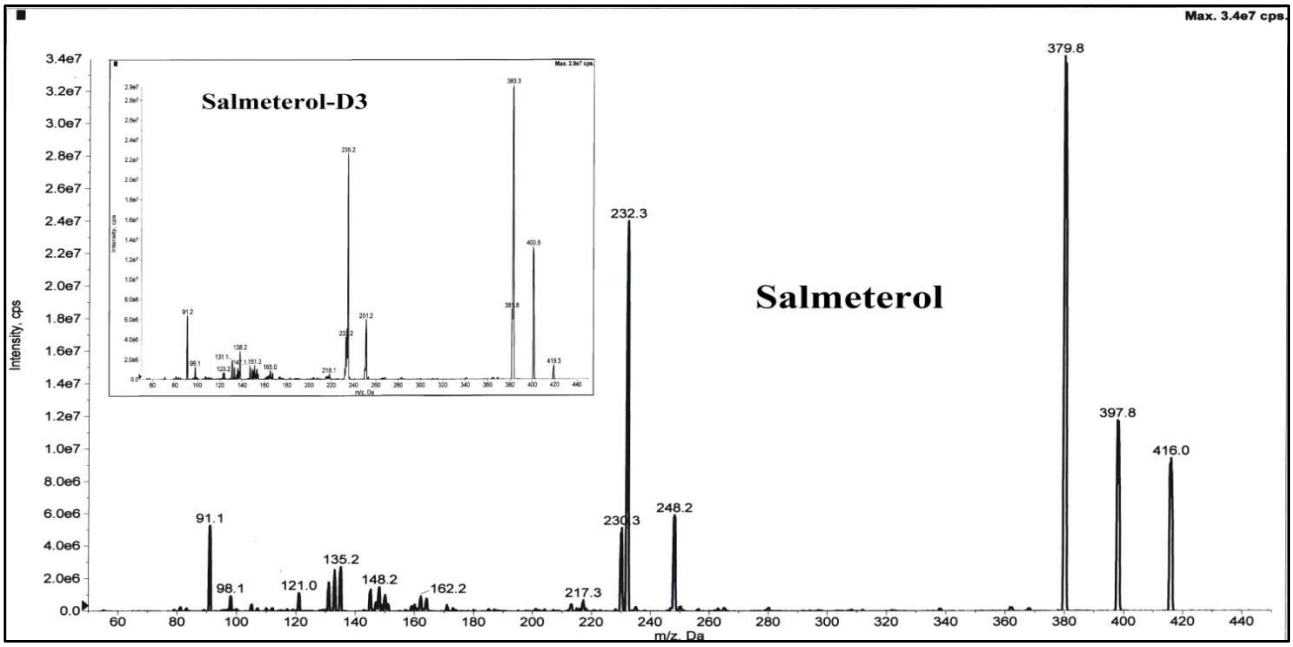

FIGURE 2: Mass spectra of Salmeterol and Salmeterol-D3

TABLE 1: The multiple reaction monitoring (MRM) ions (Q1/Q3 m/z)

Compound name	Q1 Mass	Q3 Mass
Fluticasone	501.300	293.000
Fluticasone D5	506.200	293.000
Salmeterol	415.900	232.200
Salmeterol D3	419.200	235.200

TABLE 2: The optimized mass spectrometry parameters

Name of the optimized parameter	Name of the compound			
	Fluticasone	Fluticasone D5	Salmeterol	Salmeterol D3
	Parameters value			
Ion spray voltage (IS)	5500.00	5500.00	5500.00	5500.00
Temperature (°C)	550.00	550.00	550.00	550.00
Curtain gas (CUR)	35.00	35.00	35.00	35.00
Collision gas (CAD)	6.00	6.00	6.00	6.00
Gas 1	50.00	50.00	50.00	50.00
Gas 2	55.00	55.00	55.00	55.00
Declustering potential (DP)	60.00	60.00	85.00	85.00
Collision Energy (CE)	30.00	30.00	25.00	35.00
Collision cell exit potential (CXP)	12.00	12.00	10.00	10.00
Entrance potential (EP)	10.00	10.00	10.00	10.00
Dwell time (in msec)	200	200	200	200

TABLE 3: The optimized chromatographic conditions

Column	150×4.6 mm, C18, 5µ Discovery Supelco
Mobile phase (Gradient program)	**Pump A:** 0.1mM Ammonium trifluoroacetate 40 parts and Acetonitrile 60 parts **Pump B:** 0.1mM Ammonium trifluoroacetate 60 parts and Acetonitrile 40 parts. 0.01-4min Pump B 0%; 4.01-10.00min Pump B 100%; 10.01-12.00 Pump B 0%.
Run Time	12 minutes
Injection volume	15µL
Sample cooler Temperature	5±2°C
Column oven Temperature	40±2°C
Retention Time (RT)	FCS and FCSD5 : 8.90 and 8.80±1.0min SMR and SMRD3: 3.20 and 3.10±1.0min
Regression and Weighing factor	Linear regression and $1/X^2$

The LC-MS/MS parameters were optimized with the aqueous samples. The development method was targeted to quantify FCS and SMR from biological matrices. So, the aqueous samples (50 times dilutions) were spiked in human K2 EDTA plasma as 2% spiking, and then tried different extraction procedures like solid phase extraction (SPE), liquid-liquid extraction (LLE) and precipitation technique (PPT) to extract targeted drugs from spiked plasma. The SPE method was finalized for extraction from the extraction trails as the best peak shape, less matrix effect (ME), no inter conversion between analytes, ISs and best recovery was obtained. The extraction procedure was as follows, added 50.0µL of IS dilution (ISD) to samples except blank (to blank added 50.0µL of diluent), then added 0.300mL of plasma samples and vortex. After the vortex added 0.300mL pretreatment solution (2% orthophosphoric acid) and vortex to mix the samples. Then, on SPE setup used waters HLB cartridges and conditioned with 1mL methanol (MeOH) and 1mL HPLC grade water (H-GW). After conditioning the cartridges loaded the samples, followed by washed the cartridges with 1mL of H-GW two times and followed by 1mL of washing solution (10% Methanol in H-GW). Then dried the cartridges for few minutes and then eluted the samples from cartridges with 1mL MeOH. The eluted samples were dried under nitrogen evaporator at 50°C with 15psi pressure until dryness. The dried samples were re constituted with 0.3mL reconstitution solution Mobile Phase-A.

2.4 Method validation

The developed LC-MS/MS method for FCS and SMR was validated as per USFDA guidelines [20]. As part of method validation (MV) different parameters were validated such as autosampler carryover (ASCO), Aqueous linearity (AQL), Selectivity (SEL), Specificity (Spec), Precision and accuracy (P&A), Matrix effect (ME), Recovery (REC), Concomitant Medication (CM), Batch size determination (BSD) and stabilities. The stabilities were including stock solution and dilution, extraction stabilities Freeze thaw (FT), Bench top (BT), Autosampler (AS), Dry extract (DE), Wet extract (WE), Short-term (ST), Long-term (LT) and Whole blood (WB).

2.5 Data processing

The data generated on LC-MS/MS was using Analyst software 1.7.3. as peak area ratio. The $1/X^2$ regression was selected from the precision and accuracy batches and the same was used for all the data.

3. RESULTS AND DISCUSSION

The analytical methods have been playing vital role in modern drug developments in their qualitative and quantitative analysis. The BAMs are mainly using in quantitative analysis i.e. BA and BE studies of different pharmaceutical products in various biological matrices. The BAMs are helpful to know the pharmacokinetic and pharmacodynamic characteristics of drugs in the body. So, the developed BAMs to identify pharmaceutical drugs should be specific, precise, sensitive, accurate and reproducible. As discussed earlier on need of current research, developed a simple, sensitive, rugged bioanalytical method for the simultaneous estimation of FCS and SMR in human plasma using LC-MS/MS.

The developed BAM was for estimation of FCS and SMR have the high sensitivity with LLOQ 0.500 pg/mL and 1.000 pg/mL. The method has no carryover from preceding injection to frontward injection as the LLOQ was very low and the experiment was done with extracted samples at higher level to blank injections and response was compared with the LLOQ level. The results were within acceptance criteria as per regulatory guidelines i.e. less than 20% of analyte area at analyte RT and less than 5% area to IS area at IS RT in blank sample after injection of the ULOQ sample. The specificity is very important in LC-MS/MS methods as the using analyte and IS are free from interference from each other and in case multiple analytes they should be interference free from one analyte to another analyte. In the current study, the specificity was done with selectivity experiment using extracted samples as injecting ULOQ sample without IS, LLOQ samples with IS and compared the results with respect to analyte response in LLOQ samples and IS response in LLOQ with IS sample. The results showed there was no interference between analyte to IS and vice versa.

The selectivity experiment was performed as per the regulatory guideline using minimum 6 blank plasma lots and each 2 hemolytic and lipemic lots. Prepared each one blank and LLOQ samples from individual lots and were quantified under freshly prepared CC. The selected all lots met acceptance criteria and all are interference free.

The selected lots were used to perform three precision and accuracy batches on intra and inter days by using freshly spiked 8 non-zero standards and each 6 replicates of HQC, MQC, LQC LLOQ QC and DQC with dilution factor 4. There was no interference was observed in blank and zero samples in all P&A batches. The chromatograms were provided in Figures 3 and 4. The global accuracies of FCS at LLOQQC 100.6%, LQC: 103.5 %, MQC: 107.6 %, HQC: 106.5% and DQC: 107.8% and global precision (%CV) at LLOQQC: 9.6%, LQC: 5.1 %, MQC: 1.5 %, HQC: 1.4%, DQC: 1.1% respectively. The global accuracies of SMR at LLOQQC: 98.7 %, LQC: 98.5 %, MQC: 102.8 %, HQC: 102.0% and DQC 109.1% and global precision (%CV) LLOQQC: 4.0%, LQC: 2.8 %, MQC: 2.7%, HQC: 2.4% and DQC 3.6%. The correlation Coefficient was varying from three batches of FCS was 0.9986 to 0.9994 (Figure 5), for SMR was varies to 0.9992 to 0.9996 (Figure 6). The global CC results showed in Table 4. The intraday and interday P&A results of QC samples were showed Table 5 and 6. The reinjection reproducibility (RIRP) was performed at LQC and HQC levels form the accepted under prepared CC samples. The RIRP was met acceptance criteria until 25 hours.

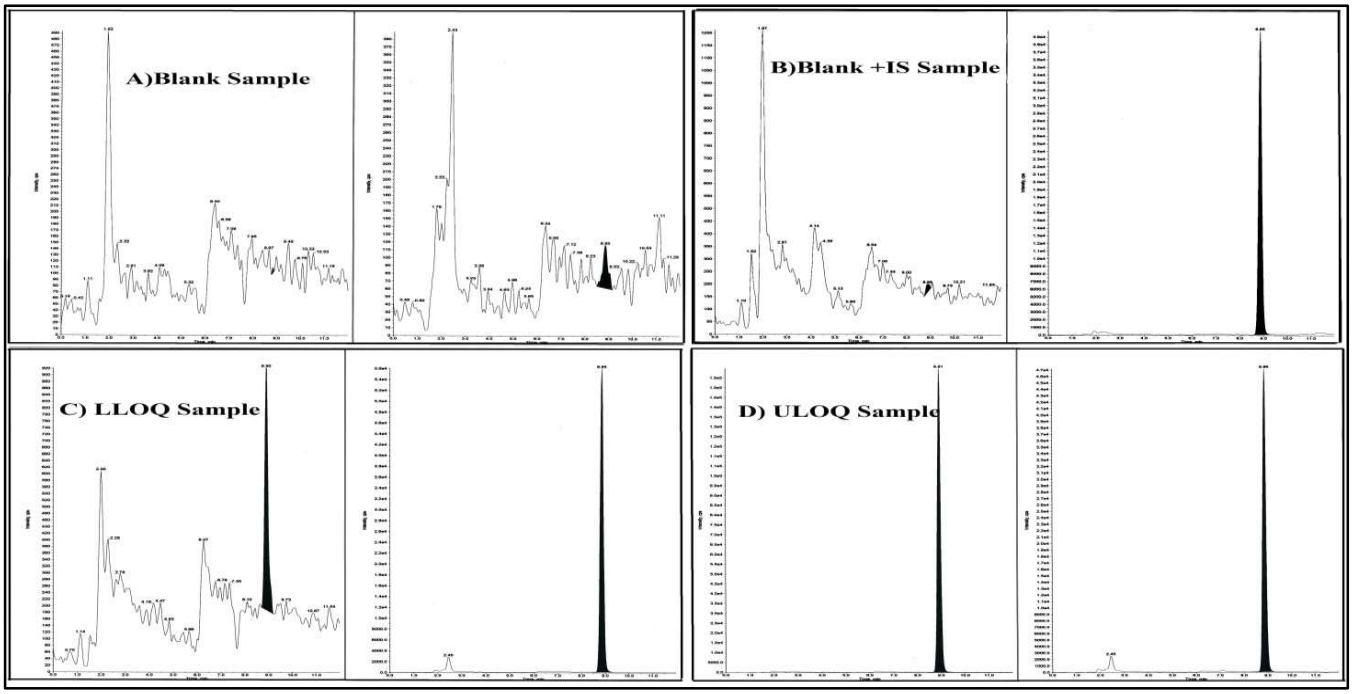

FIGURE 3: The chromatograms of Fluticasone A) Blank sample; B) Blank +IS sample; C) LLOQ sample; D) ULOQ sample

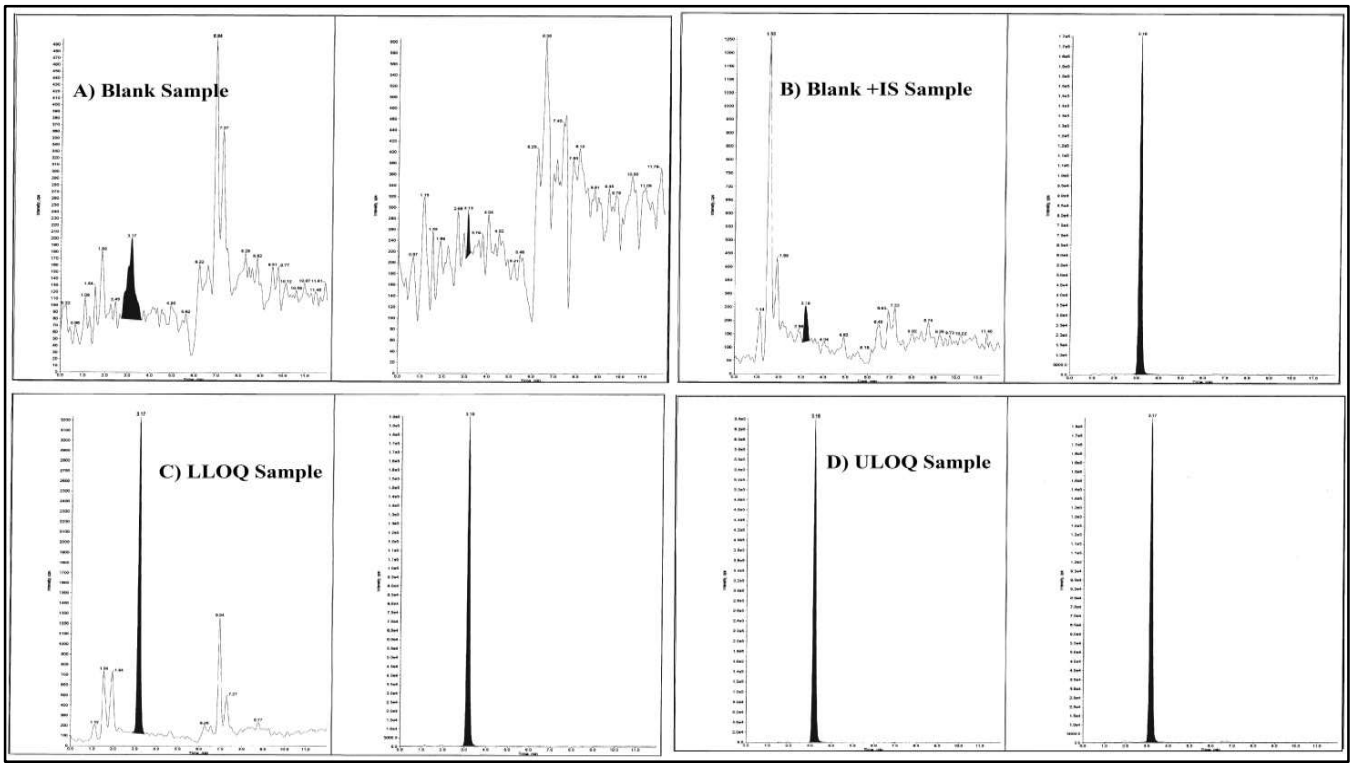

FIGURE 4: The chromatograms of Salmeterol A) Blank sample; B) Blank +IS sample; C) LLOQ sample D) ULOQ sample

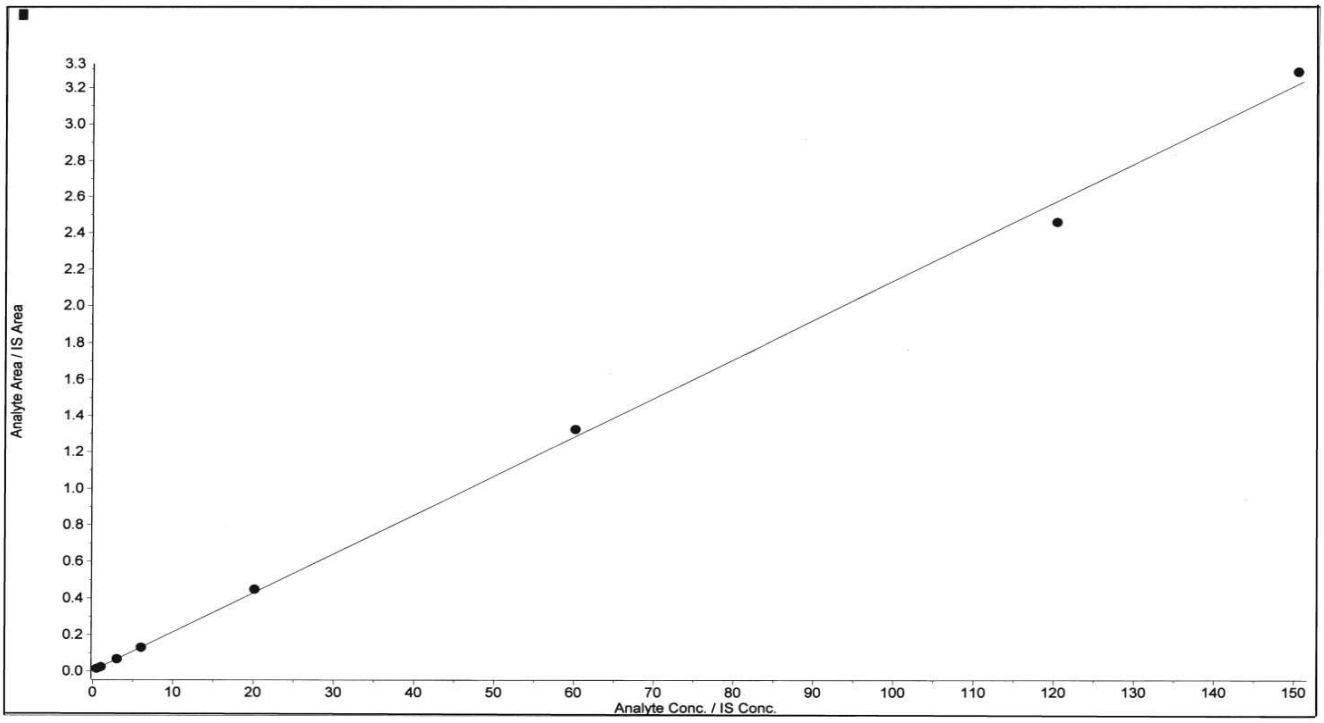

FIGURE 5: Fluticasone linearity curve

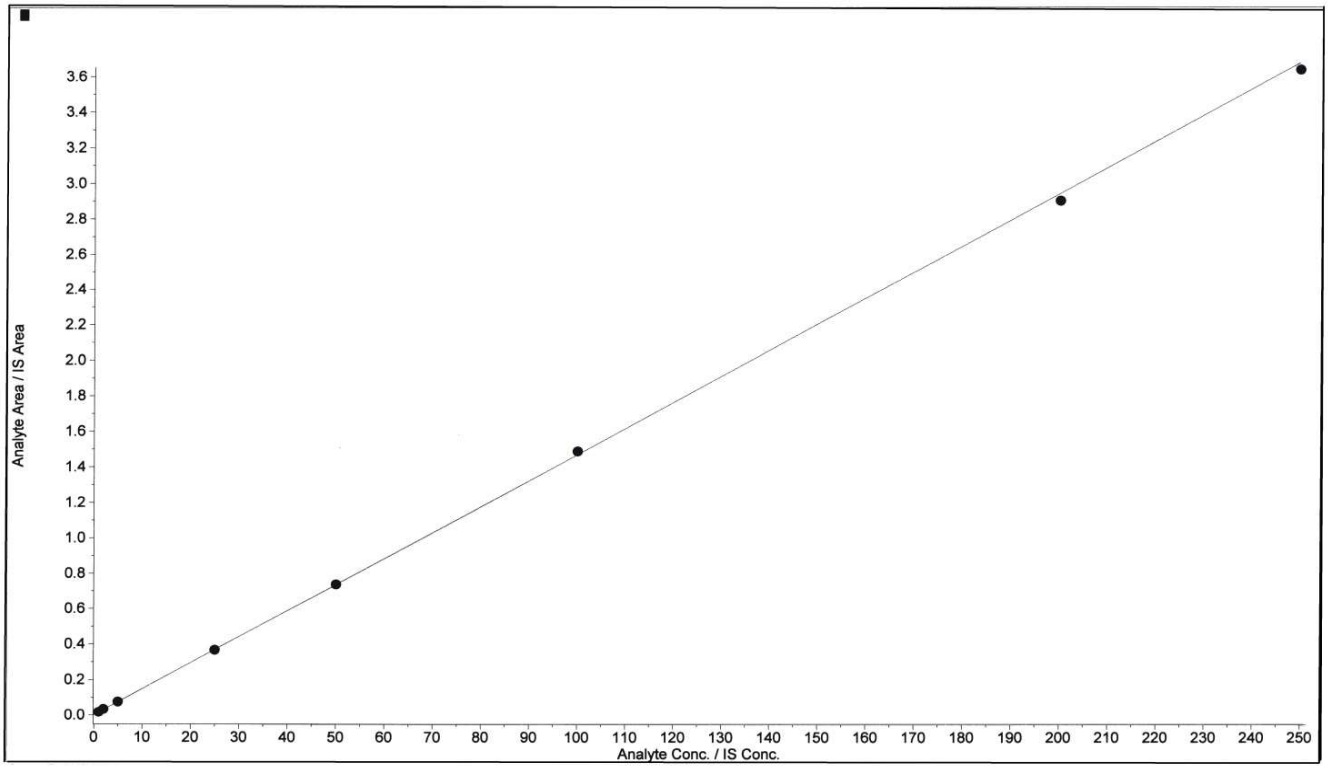

FIGURE 6: Salmeterol linearity curve

TABLE 4: Global calibration curve results of precision and accuracy batches

	STD ID.	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
FCS	Nominal Conc. (pg/mL)	0.500	1.010	3.021	6.042	20.140	60.108	120.206	150.200
	Mean	0.5037	0.9960	3.0000	6.0090	20.6780	61.6333	112.7830	154.5790
	±SD	0.01644	0.06879	0.01400	0.10513	0.28015	0.73982	1.87927	1.40697
	%CV	3.3	6.9	0.5	1.7	1.4	1.2	1.7	0.9
	%Nominal	100.7	98.6	99.3	99.5	102.7	102.5	93.8	102.9
SMR	Nominal Conc. (pg/mL)	1.000	2.000	5.000	25.000	50.000	100.000	200.000	250.218
	Mean	0.9967	2.0100	4.9233	25.2100	51.1367	100.4867	197.2860	247.8200
	±SD	0.02517	0.09000	0.11719	0.30643	0.89891	0.80008	1.30901	2.50621
	%CV	2.5	4.5	2.4	1.2	1.8	0.8	0.7	1.0
	%Nominal	99.7	100.5	98.5	100.8	102.3	100.5	98.6	99.0

TABLE 5: Intraday results of precision & accuracy batches

QC ID			LLOQQC	LQC	MQC	HQC	DIQC
	Nominal Conc. (pg/mL)		**0.501**	**1.496**	**47.100**	**114.888**	**300.400**
Intraday P&A (n=12; 6 from each batch)	FCS	Mean(pg/mL)	0.4978	1.5329	50.4485	121.9440	323.0834
		±SD	0.05055	0.08152	0.83242	1.86971	4.02333
		%CV	10.2	5.3	1.7	1.5	1.2
		% Nominal	99.4	102.5	107.1	106.1	107.6
	Nominal Conc. (pg/mL)		**1.010**	**2.986**	**78.456**	**191.480**	**500.200**
	SMR	Mean(pg/mL)	0.9958	2.9433	80.6850	196.5975	544.3925
		±SD	0.04814	0.08532	2.65069	3.94836	10.06630
		%CV	4.8	2.9	3.3	2.0	1.8
		% Nominal	98.6	98.6	102.8	102.7	108.8

TABLE 6: Interday results of precision & accuracy batches and extraction efficiency

QC ID			LLOQQC	LQC	MQC	HQC	DIQC
	Nominal Conc. (pg/mL)		**0.501**	**1.496**	**47.100**	**114.888**	**300.400**
Interday P&A (n=18; 6 from each batch)	FCS	Mean(pg/mL)	0.5039	1.5485	50.6867	122.3196	323.7248
		±SD	0.04846	0.07873	0.78248	1.69839	3.68830
		%CV	9.6	5.1	1.5	1.4	1.1
		% Nominal	100.6	103.5	107.6	106.5	107.8
	Nominal Conc. (pg/mL)		**1.010**	**2.986**	**78.456**	**191.480**	**500.200**
	SMR	Mean(pg/mL)	0.9967	2.9418	80.6478	195.3839	545.5278
		±SD	0.04015	0.08092	2.20700	4.72482	19.84535
		%CV	4.0	2.8	2.7	2.4	3.6
		% Nominal	98.7	98.5	102.8	102.0	109.1
% of Recovery		FCS	-	85.8	84.1	83.1	-
		SMR	-	85.8	85.1	84.7	-

Ruggedness was performed with the different solvents, different column of same make and different analyst on same model LC-MS/MS as precision and accuracy batch (spiked CC and 6 replicates of QCs). The regression linearity was checked with the none, 1/X and $1/X^2$ from three P&A batches. The 1/X2 linear regression was found low mean difference value to nominal concentrations. The best r^2 value of FCS was 0.9994 and r value of SMR was 0.9996 among three P&A batches.

The Matrix Effect (ME) was performed with selected lots during selectivity including lipemic and hemolyzed matrices at HQC and LQC levels by comparing the post spiked samples to aqueous samples. The ME met the acceptance criteria and found there was no ions suppression and enhancement on processed samples using the developed extraction process compared to the response of analytes and ISs in aqueous samples. The mean IS normalized matrix factor (ISMF) for FCS at LQC and HQC levels was 0.923 and 1.039 with %CV 5.1% and 5.2%, for SMR was 1.019 and 0.991with %CV 4.7% and 2.4%, respectively.

The recovery of FCS and SMR was compared with post spiked samples to spikes extracted samples processed using the developed extraction procedure at HQC, MQC, LQC levels for analytes and ISs recovery was evaluated at MQC level. The extraction procedure showed consistent recovery at lower (LQC) to higher (HQC). The mean recovery percentage of FCS was 84.36±1.22 and SMR was 85.18±0.46, SMRD3 was 87.30% and FCSD5 was 86.90%. The results showed in Table 6.

The concomitant medication effect on selected in biological matrices was evaluated with the commonly used medications such as Paracetamol, Ibuprofen, Aceclofenac, Ranitidine, Ondansetron, Caffeine and Nicotine were spiked in blank plasma and their interference was compared with spiked CM analytes along with FCS,

SMR spiked LQC level. There was no impact in LQC samples and no interference was observed in blank samples. The all LQC samples were met the accuracy under freshly processed CCs. The mean accuracies of FCS and SMR were 104.4 and 97.6 with %CV 5.2 and 2.8.

The stabilities were performed of FCS and SMR were performed in both aqueous and biological matrix (plasma). The all stabilities were met acceptance criteria as per regulatory guidelines. The stock dilution stabilities were performed at higher and lower levels. The % stability at lower level for FCS and SMR was 102.8%, 97.6% up to 36.40hrs. The % stability at higher level for FCS and SMR was 103.4% and 100.6% up to 36.20hrs. The ISTD stock dilution stability was performed at higher levels of related stocks. The % stability of FCS D5 and SMR D3 was 99.5% and 101.2% up to 36.20hrs. The short-term stock solution stability of FCS, SMR and FCS D5, SMR D3 were performed at higher levels (ULOQ) up to 16.22hrs. The %stability of FCS, SMR were 98.5%, 99.3% and FCS D5, SMR D3 were 99.6%, 100.1%.

The stability of FCS and SMR were measured in whole blood (WBS) at room temperature (RT) and below 10°C (<10°C) with LQC and HQC level concentrations. The stability was measured time gap between freshly spiked and spiked samples kept at both conditions. The FCS and SMR were found stable up to 2hrs. The %stability at LQC, HQC were 102.8, 100.3 at RT and 96.2, 99.1 at <10°C for FCS. The % stability of SMR were 100.2, 100.7 at RT, 100.9, 100.1 at <10°C.

The stabilities of FCS and SMR in matrix were performed for FT, BT, LTM at -70°C & -20°C, ASS(In-injector) at below 10°C, WE at 2-8°C, DE at 2-8°C at HQC and LQC levels. The stability samples were processed along with freshly spiked CC curve and QC samples (n=6) of stability samples and batch acceptance fresh QC samples. The QC samples of different stabilities were met the accuracy i.e. 85-115%. The mean calculated concentrations, %CV of stabilities were within the acceptance criteria i.e. less than 15%. The % stabilities of FCS and SMR were 101.5%, 103.1 and 102.6%, 101.3% at LQC and HQC levels with 6 FT cycles. The BT stability of FCS and SMR were met the acceptance up to 14.50hrs with % stability 104.9, 104.8 to FCS, 105.2, 101.9 to SMR at LQC and HQC. The autosampler stability (<10°C) was up to 36.38hrs with % stability 104.8, 103.9 for FCS and 106.1, 107.7 for SMR. The processed sample stability (DE stability) at 2-8°C was up to 28.82hrs with % stability 99.4, 102.9 for FCS and 109.1, 103.8 for SMR. The LTM % stability of FCS at -70°C was 102.6, 103.8, at -20°C was 100.9, 99.7 at LQC and HQC level. The LTM % stability of at SMR -70°C was 105.4, 105.0, at -20°C was 98.3, 106.0 at LQC and HQC level up to 38.83 days.

The developed and validated method for the simultaneous quantification of FCS and SMR was very robust, simple with best recovery from biological matrix. The method was validated as per the world most accepted USFDA regulatory guidelines. So, the developed method is very useful to carry out different bioequivalence/bioavailability studies on FCS &SMR either individually or mixed combinations of different pharmaceutical products.

Conclusion

A new simple and very sensitive method was developed and validated for FCS & SMR. The developed methods had no matrix effect, carry over and cross conversion and very less aliquot volume has been used for the extraction procedure. The validated parameters as per regulatory guidelines were met the acceptance criteria and mainly the extraction procedure was simple and is reproduceable. In conclusion, the developed method will helpful in different pharmacokinetic studies of FCS, SMR either in combination or individually.

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Conflict of interest

There was no conflict of interest.

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