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Application News

Liquid Chromatograph Mass Spectrometer LCMS-8060NX

Highly Sensitive and Selective Method for Estimation of Formoterol at Sub-pg/mL in Human Plasma Using Shimadzu LCMS-8060NX

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User Benefits

- Simple, selective and highly sensitive method with lower limit of quantification of 0.2 pg/mL
- Linear dynamic range suitable for pharmacokinetic studies, spanning from 0.2 pg/mL to 100 pg/mL
- Single-step sample extraction method that increases sample productivity

1. Introduction

Formoterol is an inhaled long-acting beta2-adrenergic receptor agonist used as a bronchodilator in the management of asthma and COPD (refer Fig.1 for structure of formoterol). It acts on bronchial smooth muscle to dilate and relax airways and is administered as a racemic mixture of its active (R;R)- and inactive (S;S)-enantiomers. A major clinical advantage of formoterol over other inhaled beta-agonists is its rapid onset of action (2-3 minutes), which is at least as fast as salbutamol, combined with a long duration of action (12 hours) . Following single/multiple dose administration of formoterol, the drug exhibits very low bioavailability and requires a highly sensitive and selective quantification of formoterol from plasma. In this work, a highly sensitive, and selective method was developed for the accurate quantification of formoterol from plasma.

We therefore developed a new LCMS method to solve problems like low bioavailability and low extraction recovery. The developed method has a high level of accuracy and can detect very low concentration of formoterol in human plasma. We focused on making the extraction process easier, improving the chromatography, and increasing sensitivity. This helped us to develop the most sensitive bioanalytical LCMS method for formoterol in human plasma.

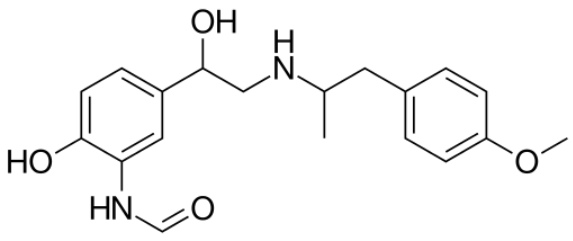


Fig. 1 Structure of Formoterol [1)]

2. Salient Features

- Quantitative method for estimation of Formoterol in human plasma was developed and partially validated as per US major Guidelines (results are presented in Table 1)
- Highly sensitive method to detect and quantify the analyte of interest at very low concentrations.
- Rapid turnaround time for sample processing and data generation.
- Exhibits a linear relationship between the analyte concentration and the analytical response.
- Straightforward sample preparation with minimal steps and handling.
- Partially validated method for quick implementation of assay

Table 1 Method Validation Summary

Calibration curve range		0.20 pg/mL to 100 pg/mL
Intraday precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)	113.60
	Precision (% RSD)	4.8
Intraday precision and accuracy (For LQC, MQC and HQC)	Accuracy (% Nominal)	95.89 to 108.82
	Precision (% RSD)	2.10 to 4.70
Global precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)	105.43
	Precision (% RSD)	11.55
Global precision and accuracy (For LQC, MQC and HQC)	Accuracy (% Nominal)	98.82 to 101.43
	Precision (% RSD)	5.99 to 9.82
Global % recovery	Recovery (%)	62.09
	Precision (% RSD)	5.33
Matrix effect	Mean Matrix Factor	1.01

Note: LLOQ QC- Lower Limit of Quantification Quality Control, LQC- Lower Quality Control, MQC- Middle Quality Control and HQC- Higher Quality Control

3. Experimental

3.1. Sample preparation and analytical conditions

- Two hundred microliters of extraction buffer was added to plasma samples and vortexed to mix for 30 seconds.
- After vortexing the samples were processed by using solid phase extraction technique. The sample extraction protocol is mentioned below:

Extraction protocol

- Conditioning and equilibration- 1mL methanol followed by 1 mL water
- Sample loading
- Wash 1 - 1.000 mL 0.05 % ammonia in water
- Wash 2 - 1.000 mL milli Q water
- Dry the cartridges for few seconds.
- Elution - 0.200 mL of 10% acetonitrile in water
- Transfer the solution into the prelabelled HPLC vials for analysis.

3.2. Instrument parameters on LCMS-8060NX

Refer to Table 2 for analytical conditions and instrument parameters. Refer to Table 3 for MRM transition.

Table 2 Analytical conditions and instrument parameters

Parameter	**HPLC**
Column	Shim-pack Velox™ C18 100 × 2.1 mm, 2.7 µm (P/N: 227-32009-03)
Mobile Phase	Gradient – Acetonitrile (B): 0.1% formic acid in 5 mM Ammonium Acetate (A)
Flow Rate	0.3 mL/min
Oven Temp	50 ˚C
Injection	20 µL
Parameter	**MS**
Interface	ESI
Interface voltage and temp	1 kV and 300 ˚C
MS Mode	MRM, Positive
Heat Block Temp	100 ˚C
DL Temp	200 ˚C
CID Gas	270 kPa
Nebulizing Gas	3 L/min
Drying Gas	10 L/min
Heating Gas	10 L/min

Table 3 MRM transition and parameters of Formoterol on LCMS

Compound	**MRM (*m/z*)**	**CE (V)**
Formoterol	345.35-149.10	-15.0

4. Result and Discussion

4.1. Method Development

Mass Spectrometry Optimization Conditions:
The analysis was conducted in positive electrospray ionization (ESI) mode to achieve efficient ionization of formoterol. Various mobile phase compositions, including different concentrations of formic acid and ammonium formate, were evaluated to optimize formoterol ionization. Source parameters, such as temperature, capillary voltage, and desolvation gas flow, were optimized to maximize signal intensity and reproducibility. The protonated molecular ion [M+H]+ at m/z 345.35 was selected as the precursor for formoterol, as it provided the highest response. The most abundant and specific product ion was identified at m/z 149.1 for quantification purpose, and for formoterol D6 the MRM was set at m/z 351 > 152. The collision energy was optimized to obtain the highest signal for the selected product ions, ensuring robust and reliable quantification.

Chromatographic Conditions:
Reversed-phase high-performance liquid chromatography (RP-HPLC) was selected as the chromatographic technique, employing a Shimadzu Shim-pack Velox C18, 2.1 x 100 mm, 2.7 µm particle size for excellent peak shape and resolution. The mobile phase consisted of 0.1% formic acid in 5 mM ammonium acetate (solvent A) and 0.1% formic acid in acetonitrile (solvent B), with a gradient elution program starting at 10% B and increasing to 90% B over 5 minutes, followed by a 2-minute hold. The flow rate was set at 0.3 mL/min, and the column temperature was maintained at 50°C to improve peak symmetry and reproducibility. The injection volume was optimized at 20 µL.

Sample preparation:
The samples were prepared using a slightly changed version of an already published method. Because we used plasma as a matrix and formoterol binds strongly to plasma proteins, we had to break this binding before extraction to get the best results. Usually, pure methanol is used for this, but because the sample sticks to the sorbent through hydrophobic interaction, using too much methanol can cause problems. So, we treated the plasma samples with 20% methanol to make the proteins settle. After spinning the samples in a centrifuge, we put the clear liquid onto a cartridge for cleaning. We found that up to 50% methanol could be used on the cartridge without losing the compounds. So, we used 50% methanol to wash away unwanted substances. Finally, we used pure acetonitrile to get the compounds of interest. This method allowed us to recover over 60% of the compounds and removed most of the unwanted substances.

Fig. 2 Nexera™ X2 with LCMS-8060NX system

4.2. Method Validation Summary

The bioanalytical method demonstrated acceptable selectivity, with no interfering peaks observed at the retention time of the analyte or the internal standard. The method was linear over the concentration range of 0.2 to 100 pg/mL, with a correlation coefficient (r2) of 0.9967. The accuracy and precision of the method were within the acceptance criteria, with intra-day accuracy ranging from 95.89% to 108.82% and intra-day precision (% CV) less than 5.0%. The global accuracy and precision were also within the acceptable limits, with values ranging from 98.82% to 101.43% and % CV less than 10.0%, respectively. The recovery of the analyte from the matrix was consistent, with an average recovery of 62.09%. The matrix effect assessment showed that the matrix did not significantly influence the analyte response, with a matrix factor of 1.04 ± 0.08. Overall, the partially validated bioanalytical method was demonstrated to be selective, linear, accurate, precise, and is suitable for the quantification of the analyte in the matrix samples

Table 4 Selectivity

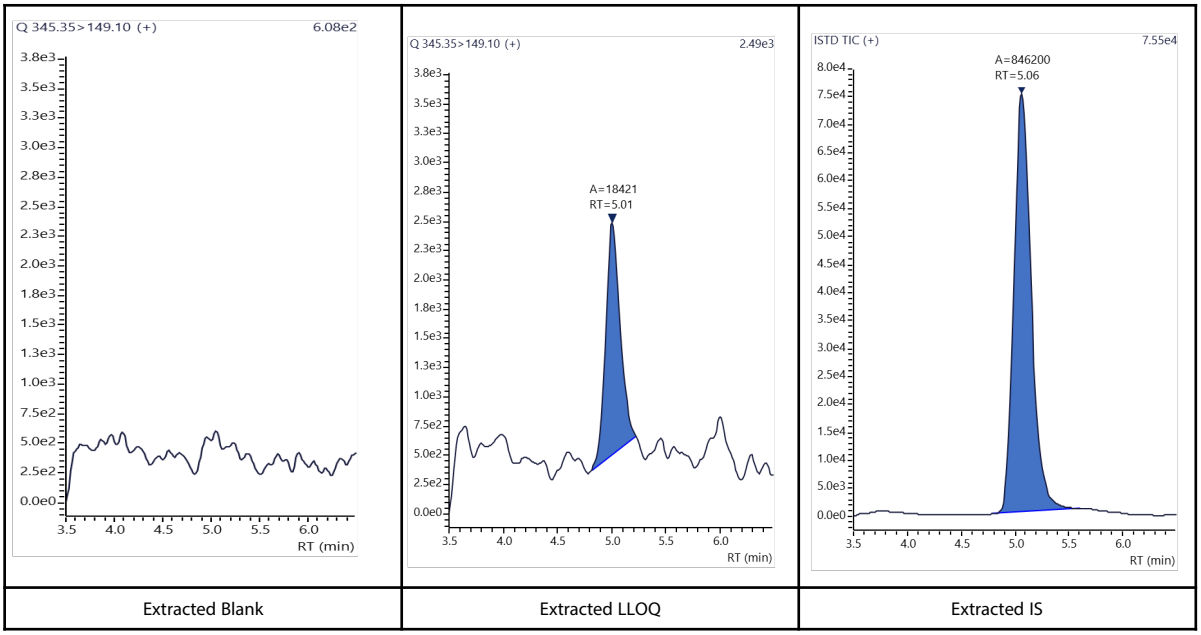
Plasma lot no.	Formoterol		
	Blank Plasma	LLOQ area	% Interference
V1102	0	19,359	0.00
V8245	0	17,936	0.00
V6132	0	17,594	0.00
V11886	0	18,480	0.00
V11782	0	23,070	0.00
V11911	0	19,115	0.00

Table 5 Intra-day precision and accuracy

Intra-day (n=6)			
Nominal Conc (pg/mL)	Observed Conc (pg/mL)	Accuracy (%)	Precision (% RSD)
LLOQ QC (0.20 pg/mL)	0.23	113.60	4.80
LQC (1.57 pg/mL)	1.71	108.82	4.70
MQC (10.00 pg/mL)	9.59	95.89	4.20
HQC (50.00 pg/mL)	49.95	99.90	2.10

Table 6 Global precision and accuracy

Inter-day (n=18)			
Nominal Conc (pg/mL)	Observed Conc (pg/mL)	Accuracy (%)	Precision (% RSD)
LLOQ QC (0.20 pg/mL)	0.21	105.43	11.55
LQC (1.57 pg/mL)	1.55	98.82	9.82
MQC (10.00 pg/mL)	10.14	101.43	9.05
HQC (50.00 pg/mL)	50.62	101.24	5.99



Extracted Blank | Extracted LLOQ | Extracted IS

Fig. 3 Chromatograms of Formoterol (Extracted Blank, extracted LLOQ and extracted IS)

Table 7 Recovery

Sr.No.	Ext- Sample	PE-Sample	Ext- Sample	PE-Sample	Ext- Sample	PE-Sample
	LQC		MQC		HQC	
1	41,604	64,591	3,43,507	5,37,660	18,07,964	28,65,374
2	46,653	72,793	3,21,080	5,44,004	16,34,704	28,97,701
3	43,708	73,741	3,43,610	5,29,191	16,58,957	27,33,750
4	40,121	68,189	3,32,688	5,06,571	15,86,493	28,23,271
5	48,724	67,748	3,36,506	5,23,299	15,64,788	27,71,630
6	45,982	64,346	3,18,991	5,28,119	16,74,796	29,03,347
AVERAGE	44,465	68,568	3,32,730	5,28,141	16,54,617	28,32,512
STD DEV	3,251.35	3,976.64	10,707.57	12,900.07	86,079.02	69,127.51
% RSD	7.31	5.80	3.22	2.44	5.20	2.44
% Recovery	64.85		63.00		58.42	
Note: Read Ext-Sample as extracted sample and PE-Sample as post extracted sample						

Table 8 Global Recovery

QC level	Recovery
LQC (n=6)	64.85
MQC (n=6)	63.00
HQC (n=6)	58.42
Mean	**62.09**
SD	**3.31**
% RSD	**5.33**

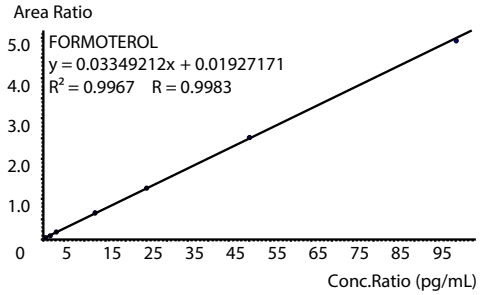


Fig. 4 Calibration curve of formoterol

Table 9 Matrix effect

Formoterol	Aqueous sample	Post extracted sample	Matrix factor	Formoterol-D6	Aqueous sample	Post extracted sample	Matrix factor	IS Normalized Matrix Effect
LQC	59,541	67,353	0.88	LQC	11,63,053	13,67,542	0.85	1.04
	52,732	73,516	0.72		11,51,676	15,03,566	0.77	0.94
	53,781	72,457	0.74		11,34,897	14,93,306	0.76	0.98
	55,868	74,502	0.75		11,24,465	15,10,743	0.74	1.01
	58,883	73,438	0.80		11,23,858	15,03,789	0.75	1.07
	60,559	68,817	0.88		11,20,498	14,70,161	0.76	1.15
Mean								**1.03**
SD								**0.08**
% RSD								**7.45**

Formoterol	Aqueous sample	Post extracted sample	Matrix factor	Formoterol-D6	Aqueous sample	Post extracted sample	Matrix factor	IS Normalized Matrix Effect
HQC	30,43,248	28,33,021	1.07	HQC	12,26,890	11,81,358	1.04	1.03
	30,52,981	29,09,235	1.05		12,33,019	12,14,213	1.02	1.03
	29,95,738	28,31,373	1.06		12,43,741	11,76,539	1.06	1.00
	30,00,218	28,51,836	1.05		12,48,614	11,61,478	1.08	0.98
	30,30,254	28,46,925	1.06		12,31,934	11,38,770	1.08	0.98
	26,20,942	30,16,549	0.87		9,95,497	10,55,653	0.94	0.92
Mean								**0.99**
SD								**0.04**
% RSD								**4.23**

5. Conclusion

In this study, a sensitive, rapid, and less plasma volume LC-MS method was developed and validated for the quantification of the formoterol in human plasma. The method utilized a simple sample preparation and provided a short chromatographic runtime, enabling efficient high-throughput analysis. The assay demonstrated excellent analytical performance characteristics, including good linearity (0.2 pg/ml to 100 pg/ml, precision, accuracy, and selectivity. The method's low LLOQ of 0.2 pg/ml and minimal matrix effects make it a valuable tool for supporting PK studies.

6. References

1) https://www.chemspider.com/Chemical-Structure.2340731.html (Accessed Feb 13,2024)

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