Development of Highly-sensitive method and its validation for the determination of Formoterol in human Plasma by UPLC-MS/MS

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Abstract: A Bio-analytical method was developed and validated to determine Formoterol in plasma in the range from 0.4 to 150 pg/mL by UPLC-MS/MS due to the lack of efficient methods to determine very low levels of Formoterol in plasma. Plasma was diluted by methanol and mixed with the internal standard (Formoterol D6). Formoterol and internal standard were extracted using a Zorbax sb-Aq (100 × 4.6 mM, 3.5 µ). After evaporation of the elution liquid the residue was re-dissolved and analyzed by HPLC–MS/MS with electrospray ionization (ESI) in positive mode. A gradient between 10 mM ammonium formate and acetonitrile were used. Extraction Procedure developed is the combination of LLE+PPT. The inter-batch precision of the calibration standards ranged from 0.6 % to 10.7%. The inter-batch accuracy of the calibration standards ranged from 95.3% to 111.1. This method has been used widely for quantifying Formoterol after inhalation of 24 µg of the drug.

Key Words: Formoterol, LC-MS/MS, PPT, LLE, PPT, ESI, Accuracy, Precision, Recovery, Stability

Introduction [1-6]:

Formoterol is used in management of asthma and/or in COPD. Inhaled formoterol is a long-acting β-2-agonist which works similar to other β-2-agonist which cause bronchodilatation through relaxation of the smooth muscle in the airway in order to treat aggravation of asthma. Formoterol is practically insoluble in water, slightly soluble in methanol, ethanol, soluble in acetone (0.1% solution in water has a pH 5.5- 6.5).

Formoterol stimulates beta2-adrenergic receptors which activates adenyl cyclase, the enzyme that catalyzes the conversion of adenosine triphosphate (ATP) to cyclic-3',5'-adenosine monophosphate (cyclic AMP). Increased cyclic AMP levels cause relaxation of bronchial smooth muscle and inhibits the release of pro-inflammatory mast-cell mediators such as histamine and leukotrienes.

Liquid chromatography–mass spectrometry (LC-MS) is the technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS).Official analytical method for formoterol is available in IP-2016, BP-2013.

Up to now, only a few published methods exist regarding the determination of Formoterol in human serum or plasma: a HPLC method with an electrochemical detector with a LLOQ of in ng/mL. Our results show that Formoterol can be quantitated with a LLOQ of 0.4 pg/mL in human plasma which is 10 times more sensitive than presented in the literature.

Products and reagents:

Formoterol was provided as (E)-but-2-enedioic acid;N-[2-hydroxy-5-[(1S)-1-hydroxy-2 [[(2S)-1-(4-methoxyphenyl)propan-2yl]amino]ethyl]phenyl]formamide;hydrate.FormoterolD6 (deuterium labels on the methoxyphenyl group) used as internal standard. Methanol: 0.1% formic acid in 5mM ammonium formate solution (40:60) used as mobile phase. Purified water (ASTM-I grade) was produced in-house. Other reagents used were Ammonium Formate (AR/Emparta, Merck life science), Formic Acid (Emparta, Merck life science), Di-chloro Methane (HPLC, Merck life science), Methanol (HPLC/LC-MS, Merck life science), and Acetonitrile (HPLC/LC-MS, Bio solve).

Preparation of solution:

Formoterol working/reference standard equivalent to about 1 mg of formoterol D6 was weighed and transferred it into a 10 mL volumetric flask, dissolved and made up to the volume with methanol to produce the stock solution between 9000000 to 11000000 pg/mL intermediate ISTD dilution of formoterol D6.

Diluted the stock solution with methanol to acquire about 10000 pg/mL intermediate ISTD dilution of formoterol D6 and stored the stock solution and intermediate ISTD dilution in a freezer at -22±5ºC.

Apparatus:

The UPLC-MS/MS system was a Shimadzu 8060 equipped with degasser (DGU-20A) pump (LC-30AD) and autosampler (SIL-30AC). The column employed was a Zorbax sb-Aq (100 × 4.6 mM, 3.5 µ). Vials were cooled at 06 ◦C and the column was maintained at 30 ◦C. The mobile phase consisted of (A) Methanol and (B) 0.1% formic acid in 5mM ammonium formate solution. Gradient elution at a flow rate of 0.400 mL/min was performed. Total analysis time per sample was 9 min. The LC effluent was pumped to a Quantum Vantage mass spectrometer (Thermo) equipped with an ESI source, operated in the positive ionization mode. The capillary temperature was 450 ºC. Nitrogen was used as sheath gas and auxiliary gas. The sheath gas flow rate was set to 50 units. Auxiliary gas was set to 20. The mass spectrometer was operated in Multiple reaction monitoring (MRM) mode and transitions are presented in Table 1.

Table 1. Mass and Tuning Parameter

Compound Name	Ionization Type	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	m/z	Dwell (msec)
Formoterol	ESI	Positive	345.100	149.100	345.05>149.20	250
Formoterol D6 (ISTD)			351.100	152.100	351.05>152.30	150

State file parameter ID	State file parameters for Formoterol	State file parameters for Formoterol D6
CID GAS (kPa)	270	270
Interface voltage (kV)	3.00	3.00
Q1 Pre-Bias (V)	-14.0	-10.0
Collision Energy (CE)	-20.0	-20.0
Q3 Pre-Bias (V)	-27.0	-11.0
Nebulizer gas Flow (L/Min)	3.00	3.00
Heating Gas flow (L/Min)	18.00	18.00
Interface Temperature (ºC)	350	350
DL Temperature (ºC)	200	200
Heat Block Temperature (ºC)	450	450
Drying Gas Flow (L/min)	5	5

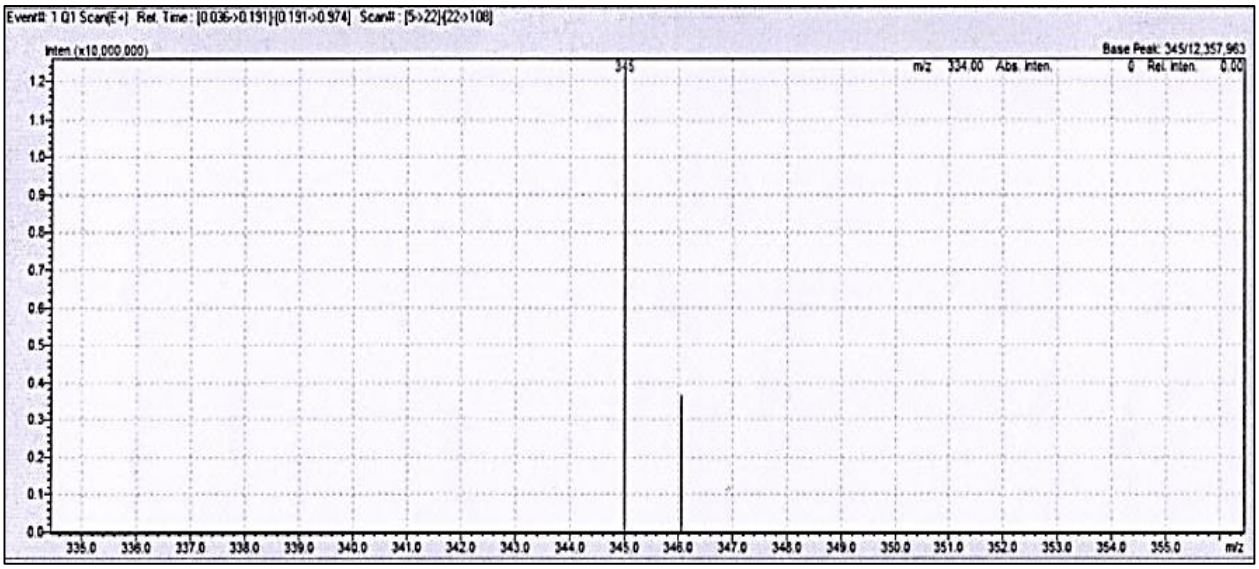

Figure 1. Formoterol Parent Ion Q1 MS Q1

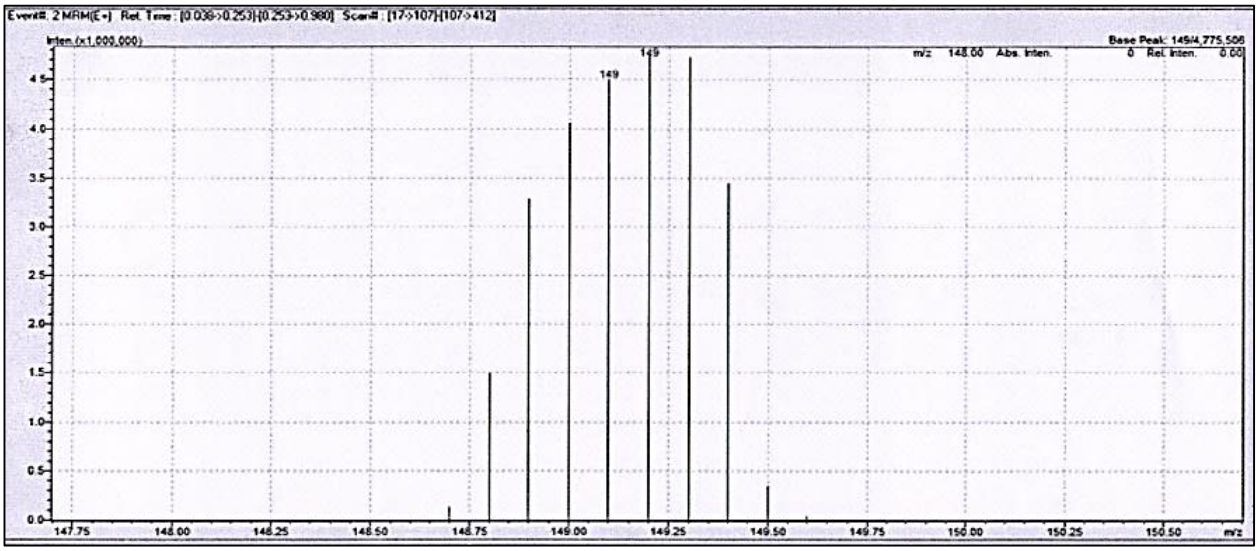

Figure 2. Formoterol Product Ion Q3 MS Q3

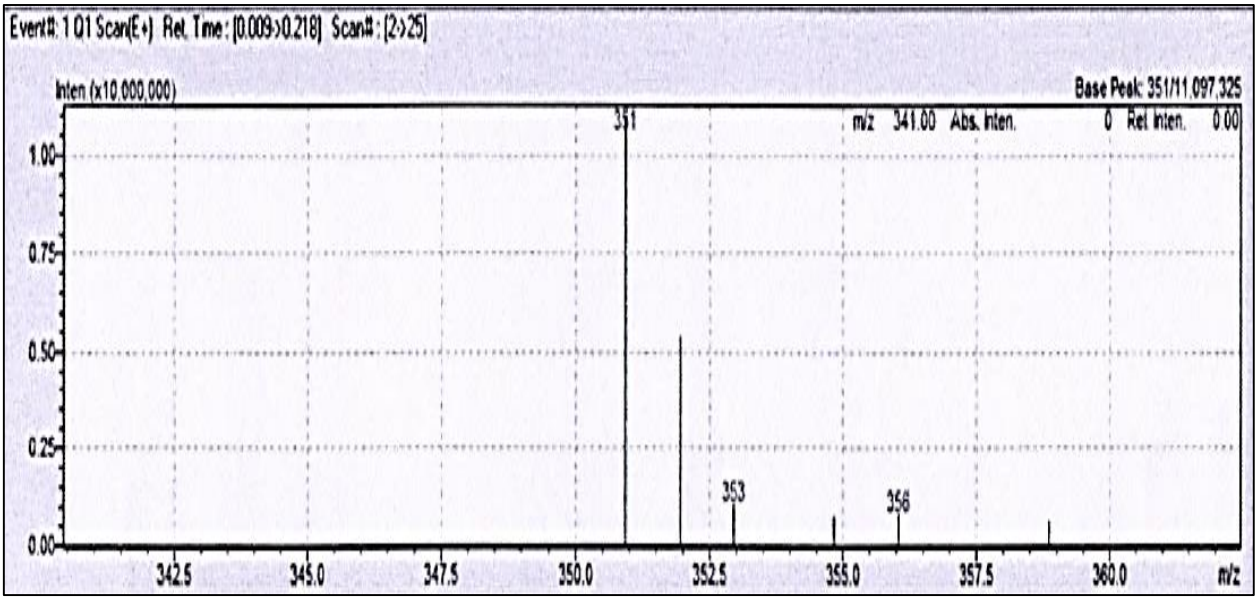

Figure 3. Formoterol D6 Parent Ion Q1 MS Q1

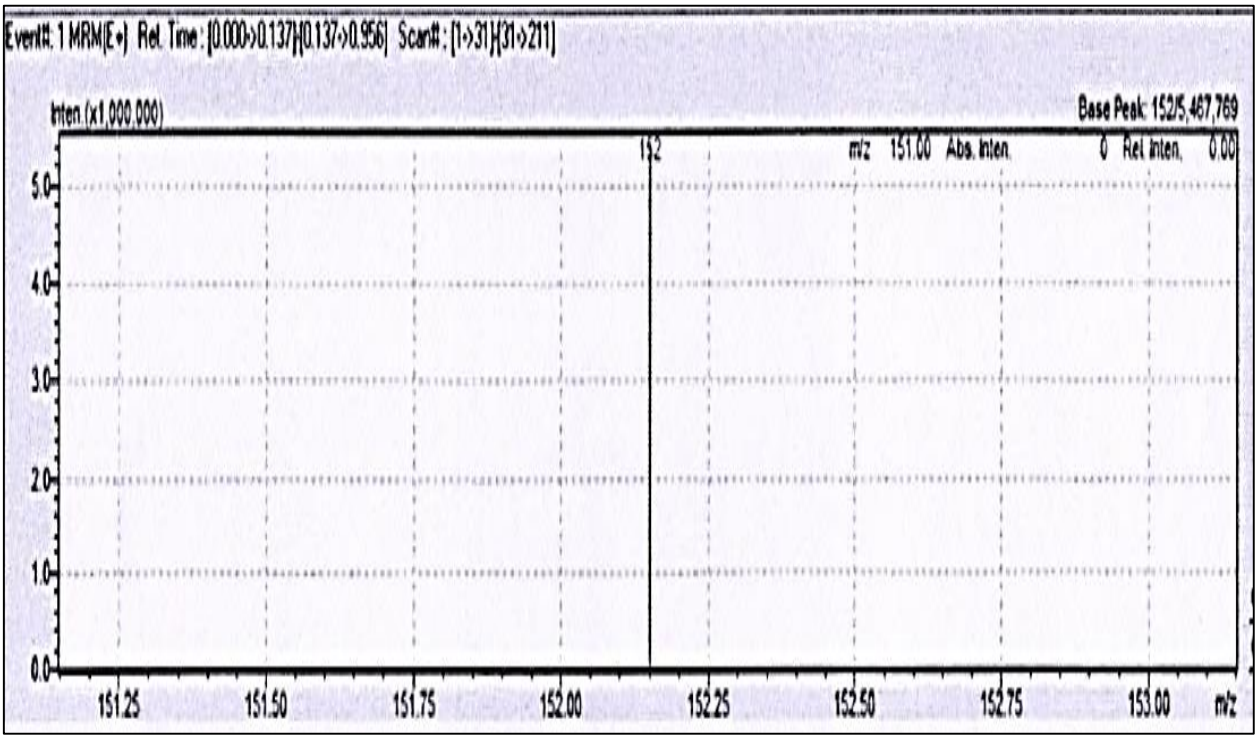

Figure 4. Formoterol D6 Product Ion Q3 MS Q3

Method Development:

METHOD SUMMARY	
Extraction Procedure	PPT+SPE
Sample Preparation	
Blank Matrix	0.500 µL blank plasma
Zero Standard	0.500 µL blank plasma + 0.100 µL of ISTD
Calibration Standards & Quality Control Samples	0.500 µL plasma containing known concentration of analyte + 100 µL of ISTD
Mixing	Vortex for 3-5 Minute
Addition	750 µl Ammonium Formate
Mixing	Vortex for 10 minutes
Centrifugation	Centrifuge the samples at 4345±150 rcf for 5 minutes at ≤10 ℃
Conditioning	1 mL of water on SPE manifold on gravity
Sample loading	Transfer 0.950 mL of supernatant
Evaporation	30±2 ºC temperature under nitrogen gas stream (Approx. drying time 10-35 minutes)
Reconstitution	200 µL of 100 mM ammonium formate buffer and vortex it

Table 2. Method Summary

Method Validation:

Compound and source parameter were optimized during method development to get maximum and stable response below parameter has been finalized.

A. LINEARITY

STD was prepared by spiking appropriate volume of methanol. To mimic the study sample condition and to avoid any dilution factor during the study sample analysis additional 10% of buffered plasma was added after making up final volume (i.e. Additional 10 mL of buffered plasma was added into 100mL of spiked plasma).

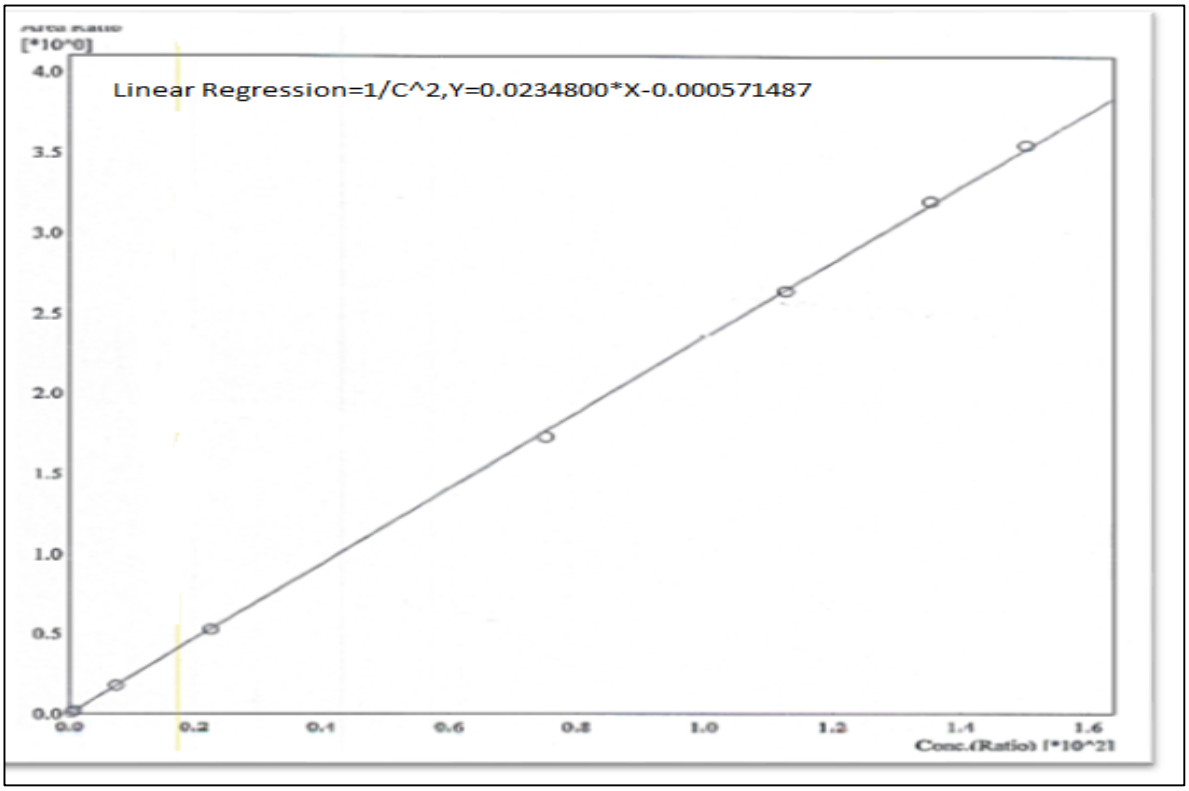

Figure 5. Calibration Curve of Formoterol

B. SELECTIVITY

Selectivity was performed for Formoterol and Formoterol D6 by using ten different sources of blank plasma and among them six sources of normal biological matrix, two Haemolyesd and two Lipemic. All the different lots of blank plasma was extracted using above mentioned extraction procedure along with one calibration curve set and injected as per above mentioned LC-MS condition.

The response of the interfering peak at the retention of Formoterol and Formoterol D6 in blank sample was compared with response of STD1(Lowest calibration standard).

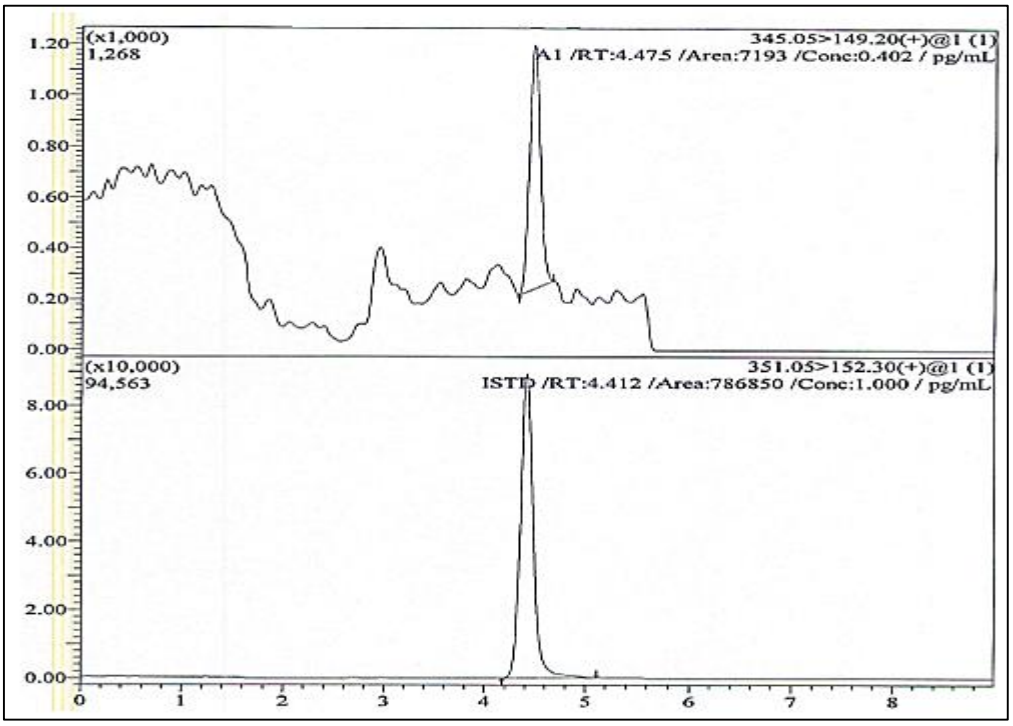

Figure 6. Representative Chromatogram of LLOQ

Sr. No	Sample ID	Area	RT	ISTD Area	ISTD RT	Area Ratio	S/N
01	STD1	7193	4.475	786850	4.412	0.009	34.9
02	BLK1	-	-	-	-	-	-
03	BLK2	-	-	-	-	-	-
04	BLK3	-	-	-	-	-	-
05	BLK4	-	-	-	-	-	-
06	BLK5	-	-	-	-	-	-
07	BLK6	-	-	-	-	-	-
08	HEMO1	-	-	-	-	-	-
09	HEMO2	-	-	-	-	-	-
10	LYP1	-	-	-	-	-	-
11	LYP2	-	-	-	-	-	-

Table 3. Selectivity Evaluation for Formoterol and ISTD

C. ACCURACY & PRECISION

The sample of each P & A batch were analysed using the following batch organization pattern:

- Standard Blank
- Standard Zero
- Calibration Standards
- Quality control sample in following sequence:

  LOQ QC, LQC, LMQC, MQC, DQC, HQC, Blank QC

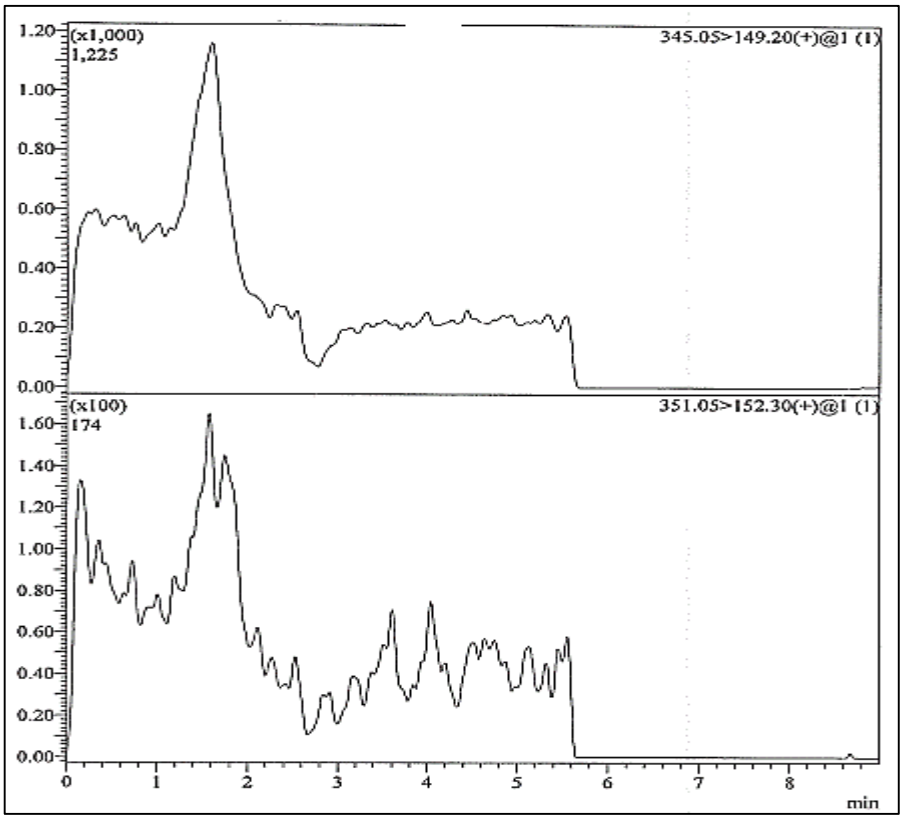

Figure 7. Representative Chromatogram of Blank Plasma

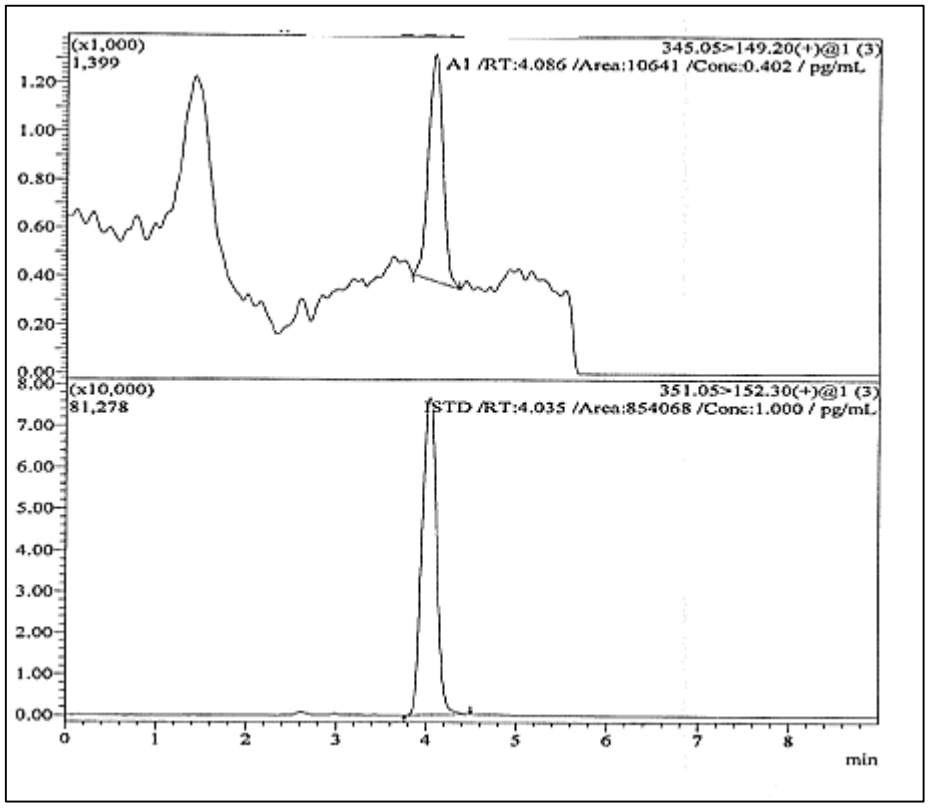

Figure 8. Representative Chromatogram of LQC

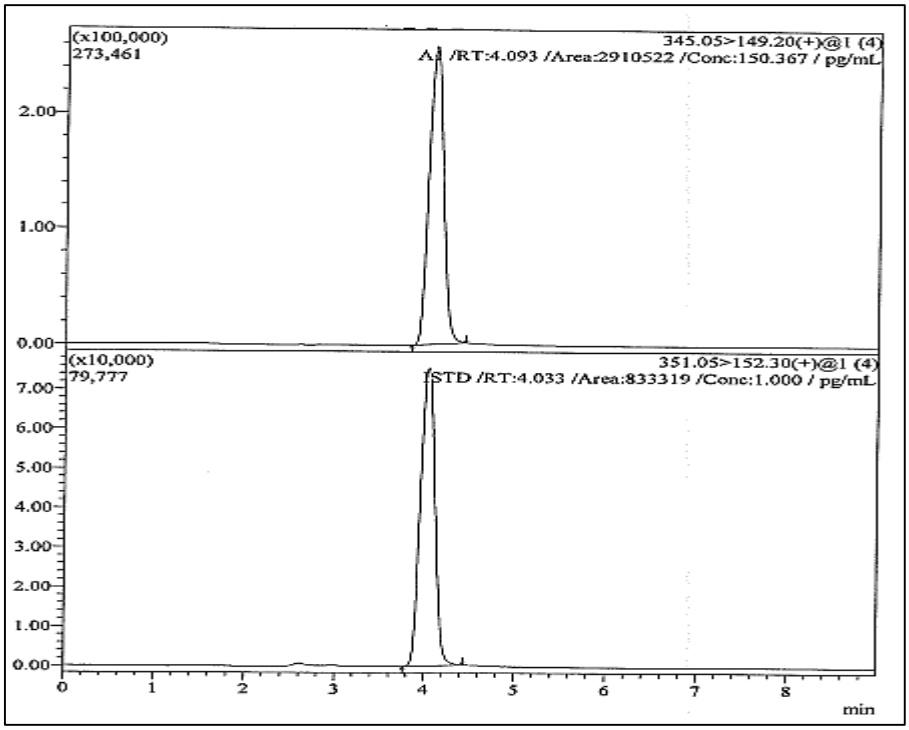

Figure 9. Representative Chromatogram of HQC

Batch	Parameter	DIL QC (1/5)	HQC	MQC	LMQC	LQC	LOQQC
PA-I	**Precision (%CV)**	1.0	1.4	0.9	2.6	3.1	10.7
PA-I	**Accuracy (%)**	104.7	106.4	97.7	103.3	99.3	103.3
PA-II	**Precision (%CV)**	0.6	0.7	0.7	1.3	3.5	6.9
PA-II	**Accuracy (%)**	101.0	103.6	95.3	100.9	99.1	105.0
PA-III	**Precision (%CV)**	2.2	2.3	1.2	1.3	6.9	10.2
PA-III	**Accuracy (%)**	110.1	111.1	100.5	105.9	100.8	109.5

Table 4. Result Precision and Accuracy of formoterol

Conclusion:

Quantitation of samples deriving from pharmacokinetic studies after inhalative administration of 24 µg Formoterol required a more sensitive method (below 1 pg/mL serum) in order to calculate reliable pharmacokinetic characteristics. Our results show that Formoterol can be quantitated with a LLOQ of 0.40 pg/mL in plasma which is 10 times more sensitive than presented in the literature published. This has been achieved by the combination of a very efficient and selective sample clean-up method (PPT+SPE)with an ultra-sensitive LC-MS/MS instrument.

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