# DEVELOPMENT OF HIGHLY-SENSITIVE METHOD AND ITS VALIDATION FOR THE DETERMINATION OF FLUTICASONE IN HUMAN PLASMA BY UPLC-MS/MS

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**ABSTRACT:**

A highly sensitive and selective liquid chromatography Electron spray ionization tandem mass spectrometry assay was developed and validated for determination of Fluticasone Propionate (FP) in Human plasma. The drugs were isolated from human plasma using solid-phase extraction method. An improved method with a lower limit of quantitation (LLOQ) of at least 1 pg/ml (pg $ml^{-1}$) was needed to measure the low levels of FP present in human plasma following inhalation administration of doses in the range 50–250 mg twice daily. Fluticasone propionate was extracted from Human plasma using Oasis MAX cartridges. The separation was achieved by Reprosil Gold 100 C18 x BD, 2 µm, 100 x 2 mm, Methanlo: 1mM Ammonium Triflouro Acetate buffer (90: 10 v/v) as mobile phase, at a flow rate of 0.2 ml/minute. Retention time of Fluticasone propionate was found to 8.183 min. The method used an isotopically labelled internal standard ($^{13}C_3$ -FP) as an internal standard. The method was shown to be specific, sensitive and reliable in the analysis of clinical samples. The inter and intra batch precision (% CV) of the quality controls samples were less than 15%. The method was linear in the concentration of 1.009-200.45 pg/mL for Fluticasone with a correlation coefficient of 0.999.

**KEY WORDS:** Fluticasone, LC-MS/MS, Chromatography, Validation

## INTRODUCTION [1-6]:

Fluticasone propionate (FP) is a Tri-fluorinated glucocorticoid based on the androstane nucleus. Fluticasone propionate is an established corticosteroid administered intranasally for the treatment of rhinitis or by oral inhalation for the management of asthma.

The inhaled form is used in the long-term management of asthma. The nasal spray is used for allergic rhinitis and nasal polyps. The topical (for the skin) form used to treat the inflammation and itching caused by a number of skin conditions such as allergic reactions, eczema, and psoriasis.

On March 18, 1996, Central Drug Standard Control Organization approved Fluticasone Propionate Cream (0.05%), Fluticasone Propionate Ointment (0.005%), and Fluticasone Propionate Nasal Spray (0.05%).

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 Fluticasone is available in IP-2014, BP-2013.

Up to now, only a few published methods exist regarding the determination of Fluticasone in human serum or plasma: a HPLC method with an electrochemical detector with a LLOQ of in ng/mL. Our results show that Fluticasone can be quantitated with a LLOQ of 1 pg/mL in human plasma which more sensitive than presented in the literature.

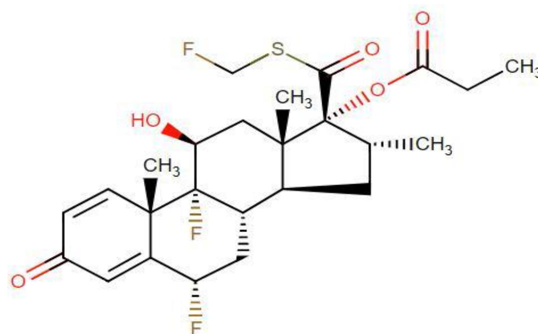


**Figure 1.0 the structure of Fluticasone**

## MATERIALS AND METHOD

- **Sample Collection and Preparation**:

The biological fluid that include the analyte are usually blood, plasma, urine, serum. Blood is usually collected from human subjects by vein puncture with a hypodermic syringe up to 5 to 7 ml (depending on the assay sensitivity and the total number of samples taken for a study being performed). The venous blood is withdrawn into tubes with an anticoagulant, e.g. Sodium heparin. Plasma is obtained by centrifugation at 3000 - 4000 rpm for a specific period of time (~15 min.). About 30 % to 50 % of the volume is collected.
Sample preparation has a key role in bio-analysis to get clean extract with high extraction efficiency. Additionally, choose of detector depends on the analyte concentration range.
The purpose of sample preparation is to clean up the sample before analysis and to concentrate the sample. A goal with the sample preparation is also to exchange the analyte from the biological matrix into a solvent suitable for injection into the chromatographic system.

- **Solid phase extraction (SPE):**

Solid phase extraction is a sample preparation method having high efficiency along with other major advantages like it's economic, easy to operate and high-reproducibility.

SPE involves bounding of analyte with a solid support where the interface is washed off with elution of analyte

Solid phase extraction method comprises of four steps i.e.

**a) Conditioning**: In conditioning step, column acts as a wetting agent on the packing material and forms a bonded complex with the functional groups of the sorbent. A Wetting agent can be activated by an organic solvent e.g. Water or aqueous buffer for proper adsorption.

**b) Sample Loading:** Once the pH is adjusted, the sample is loaded on the column through gravity feed, pumping or by vacuum.

**c) Washing:** Here, the analytes are retained and interferences from the matrix are removed.

**d) Elution:** This step involves interaction between loosely bonded complex and analyte using proper solvent removing very little interferences.

**PRODUCTS AND REAGENTS:**

Fluticasone was provided as (1R,2S,8S,10S,11S,13R,14R,15S,17S)-1,8-difluoro-14-{[(fluoromethyl)sulfanyl]carbonyl}-17-hydroxy-2,13,15-trimethyl-5-o xotetracyclo [8.7.0.$0^{2,7}$.$0^{11,15}$] heptadeca-3,6-dien-14-yl propanoate. FluticasoneD5 (deuterium labels on the methoxyphenyl group) used as internal standard. Methanol: 1mM Ammonium Triflouro Acetate buffer (90:10) 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:**

Weigh Fluticasone Propionate to about 1 mg of Fluticasone Propionate and transfer into a 10 ml volumetric flask and dissolve and make up the volume with methanol to produce the stock solution between 9000000 to 1100000 pg/ml of Fluticasone Propionate.

Dilute the above stock solution with Methanol to produce the stock dilution about 1000000 pg/ml of Fluticasone propionate. Dilute the above stock dilution 01 with Methanol to produce the stock dilution 02 about 10000 pg/ml of Fluticasone Propionate.

**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 Reprosil Gold 100 C18 x BD, 2 µm, 100 x 2 mm.

Vials were cooled at 6 °C and the column was maintained at 50 °C. The mobile phase consisted of (A) Methanol and (B) 1mM Ammonium Triflouro Acetate buffer solution. Gradient elution at a flow rate of 0.200 mL/min was performed. Total analysis time per sample was 8 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 400 ◦C. 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.

**Tuning of Fluticasone Propionate & Fluticasone Propionate D5 in MS tuning solution (100 ng/ml)**

**Table 1.0 MS Condition**

| Compound Name | Ionization Type | Polarity | Q1 Mass (amu) | Q3 Mass (amu) | m/z | Dwell (msec) |
|---|---|---|---|---|---|---|
| **Fluticasone** | ESI | Positive | 501.00 | 293.20 | 501.00>293.20 | 400 |
| **Fluticasone D6 (ISTD)** | | | 506.10 | 293.20 | 506.10>293.20 | 250 |

**Table 2.0 Mass and Tuning Parameters**

| State File Parameter | State File Parameter FP | State File Parameter FP D5 |
|---|---|---|
| CID Gas (kPa) | 250 | 250 |
| Interface Voltage | 3.00 | 3.00 |
| Q1 Pre Bais (V) | -20.00 | -20.0 |
| Collision Energy (CE) | -17.0 | -17.0 |
| Q3 Pre Bias (V) | -21.0 | -21.0 |
| Nebulizer gas flow (L/Min.) | 3 | 3 |
| Heating gas flow (L/Min.) | 10.00 | 10.00 |
| Interface Temperature (°C) | 250 | 250 |
| DL Temperature | 200 | 200 |
| Heat block Temperature | 400 | 400 |
| Drying Gas flow | 10 | 10 |

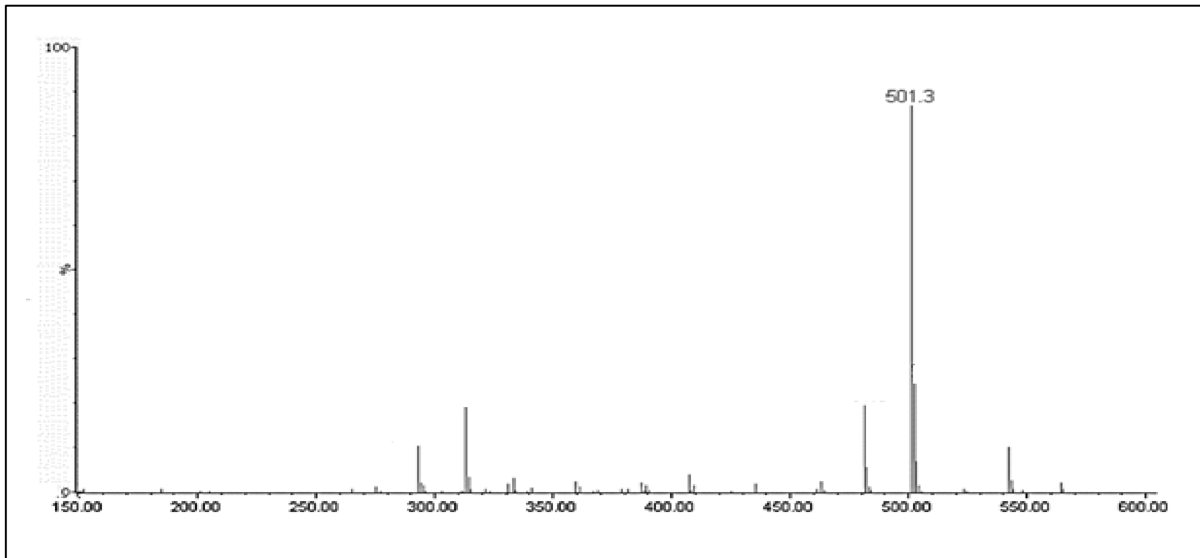


**Figure 1. Fluticasone Parent Ion Q1 MS Q1**

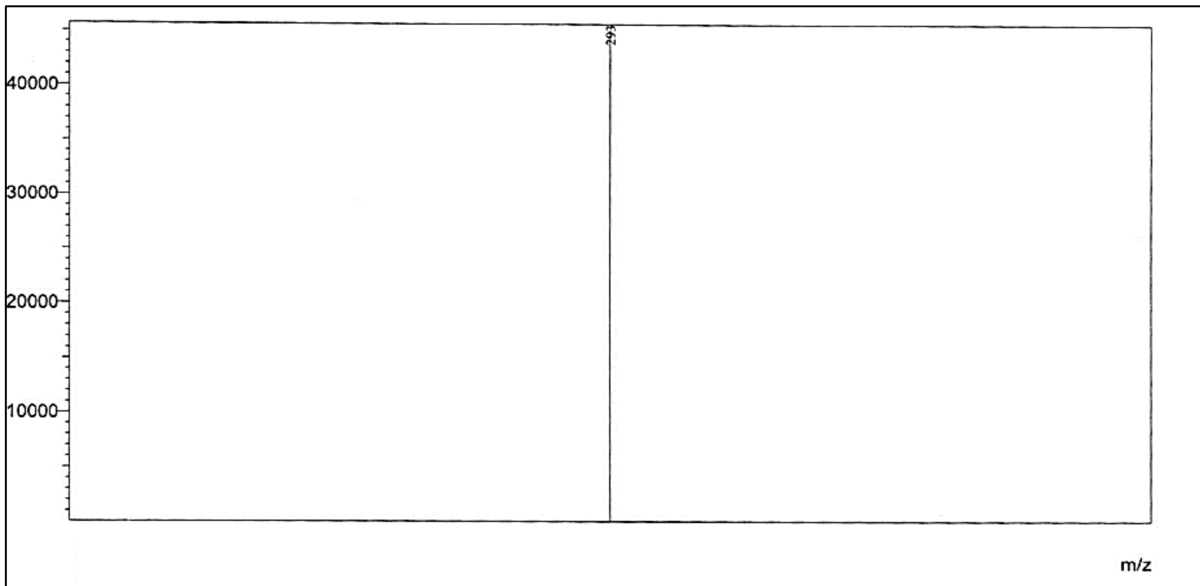


**Figure 2. Fluticasone Product Ion Q3 MS Q3**

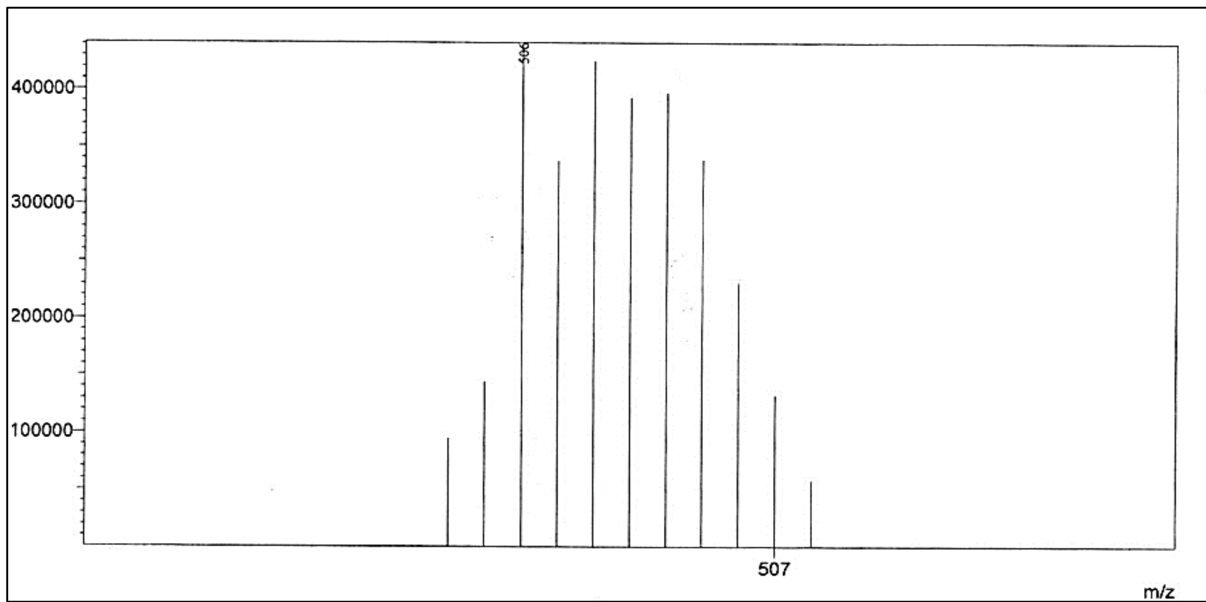


**Figure 3. Fluticasone D5 Parent Ion Q1 MS Q1**

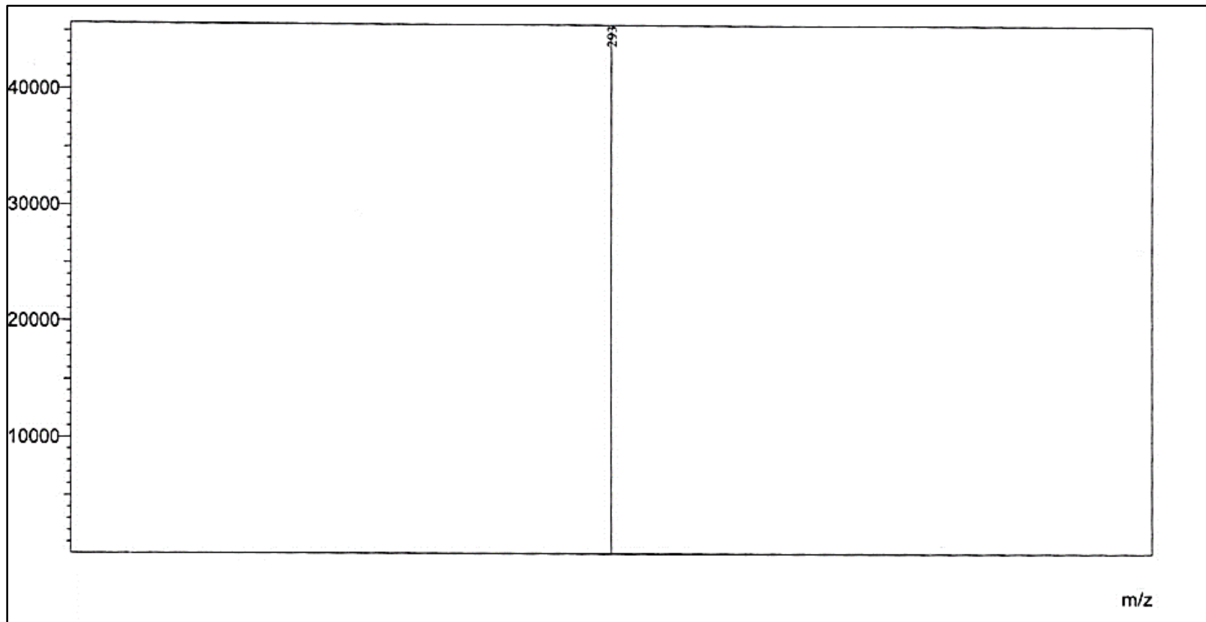


**Figure 4. Fluticasone D5 Product Ion Q3 MS Q3**

**Method Development:**

**Table 3.0 Method Summary**

| METHOD SUMMARY | |
|---|---|
| Extraction Procedure | SPE |
| **Sample Preparation** | |
| Blank Matrix | 0.500 µL blank plasma |
| Zero Standard | 0.500 µL blank plasma + 50 µL of ISTD |
| Calibration Standards & Quality Control Samples | 050 µl of ISTD Dilution except STD BL and BLK QC samples, add 50 µl of water to STD BL and BLK QC samples |
| Addition | 0.05% Ammonia solution |
| Centrifuge | 3345 ± 150 RPM for 5 minute |
| Conditioning | 1mL of methanol followed by 1 mL of water on SPE manifold on gravity |
| Sample loading | Transfer 0.950 mL of supernatant |
| Washing | Wash the cartridges with 400 ml of water and 400 ml of 40:60 (v/v) (laboratory frame). The transitions m/z5501 to 313 aqueous methanol and dry the cartridges under full pressure. Wash the cartridges 1 mL of 20% Acetonitrile solution and dry the cartridges under full pressure for 5 minutes. |
| Elution | 1 ml MeOH twice. |

**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).

Condition for Spiking in Plasma: Ice cold water bath (Below 4 °C).

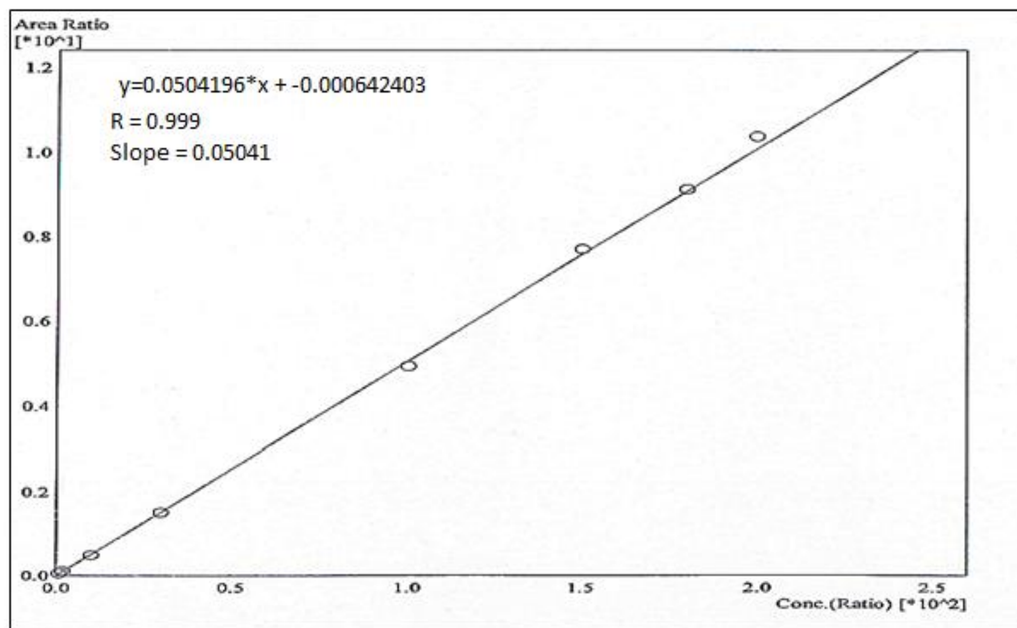


**Figure 5. Calibration Curve of Fluticasone**

## B. Selectivity

Selectivity was performed for Fluticasone and Fluticasone D5 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 Fluticasone and Fluticasone D6 in blank sample was compared with response of STD1 (Lowest calibration standard).

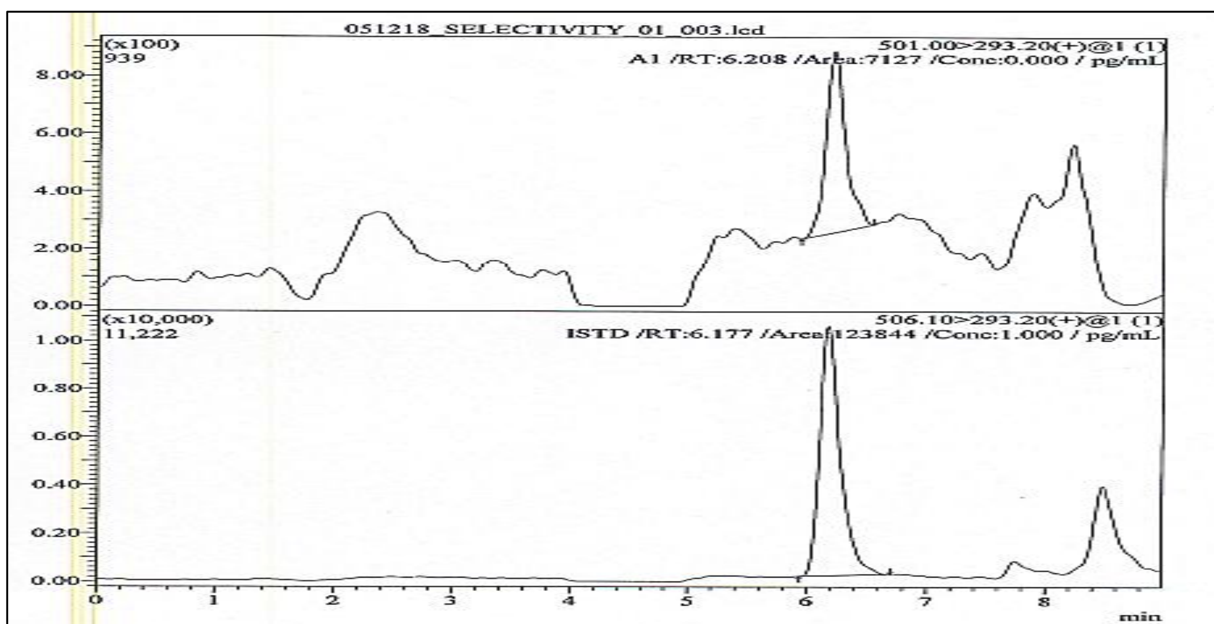


**Figure 6. Representative Chromatogram of LLOQ**

**Table 4. Selectivity Evaluation for Fluticasone and ISTD**

| Sr. No | Sample ID | Area | RT | ISTD Area | ISTD RT | Accu (%) | S/N |
|---|---|---|---|---|---|---|---|
| 01 | STD1 | 7127 | 6.208 | 123844 | 6.177 | 0.009 | 212.5 |
| 02 | BLK1 | - | - | - | - | - | - |
| 03 | BLK2 | - | - | - | - | - | - |
| 04 | BLK3 | - | - | - | - | - | - |
| 05 | BLK4 | - | - | - | - | - | - |
| 06 | BLK5 | - | - | - | - | - | - |
| 07 | BLK6 | - | - | - | - | - | - |
| 08 | HEMO1 | - | - | - | - | - | - |
| 09 | HEMO2 | - | - | - | - | - | - |
| 10 | LYP1 | - | - | - | - | - | - |
| 11 | LYP2 | - | - | 413 | 6.036 | - | - |

### 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

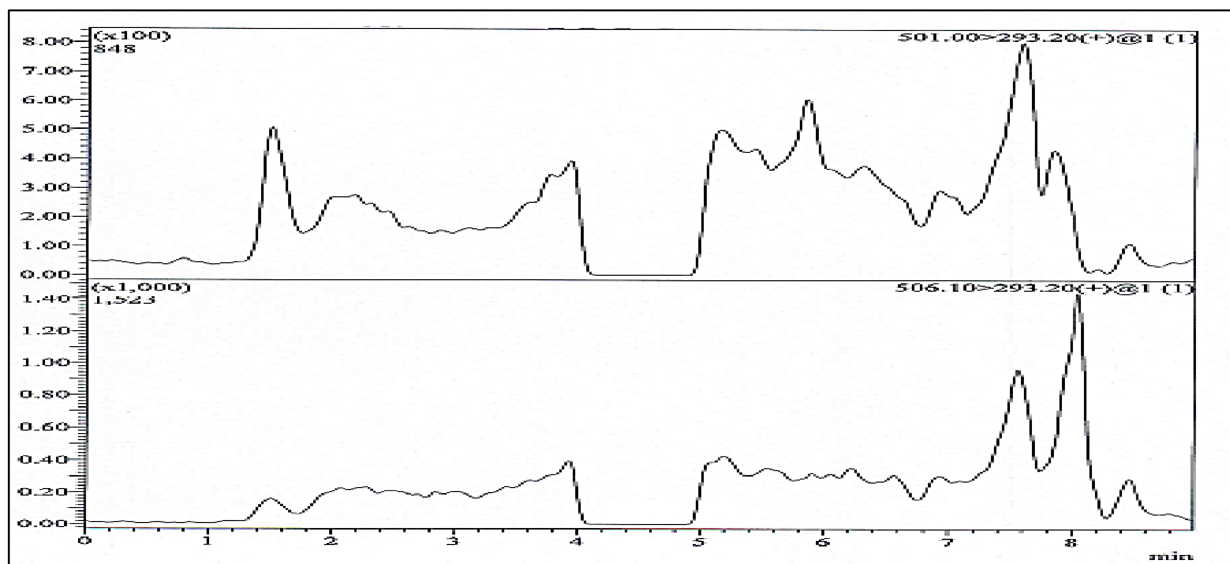


**Figure 7. Representative Chromatogram of Blank Plasma**

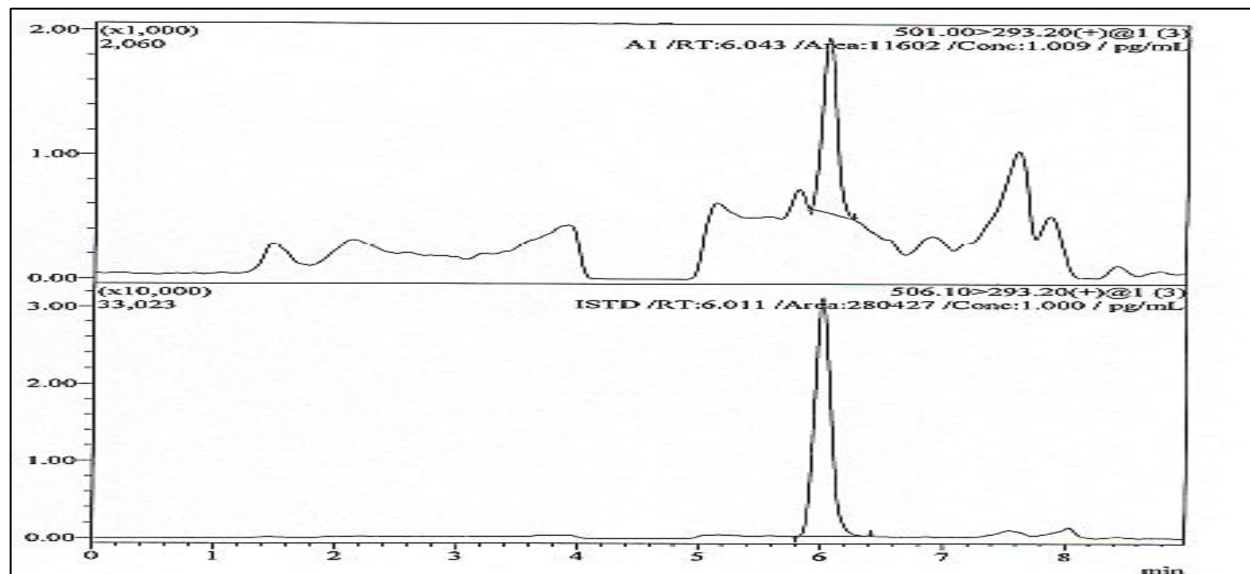


**Figure 8. Representative Chromatogram of LQC**

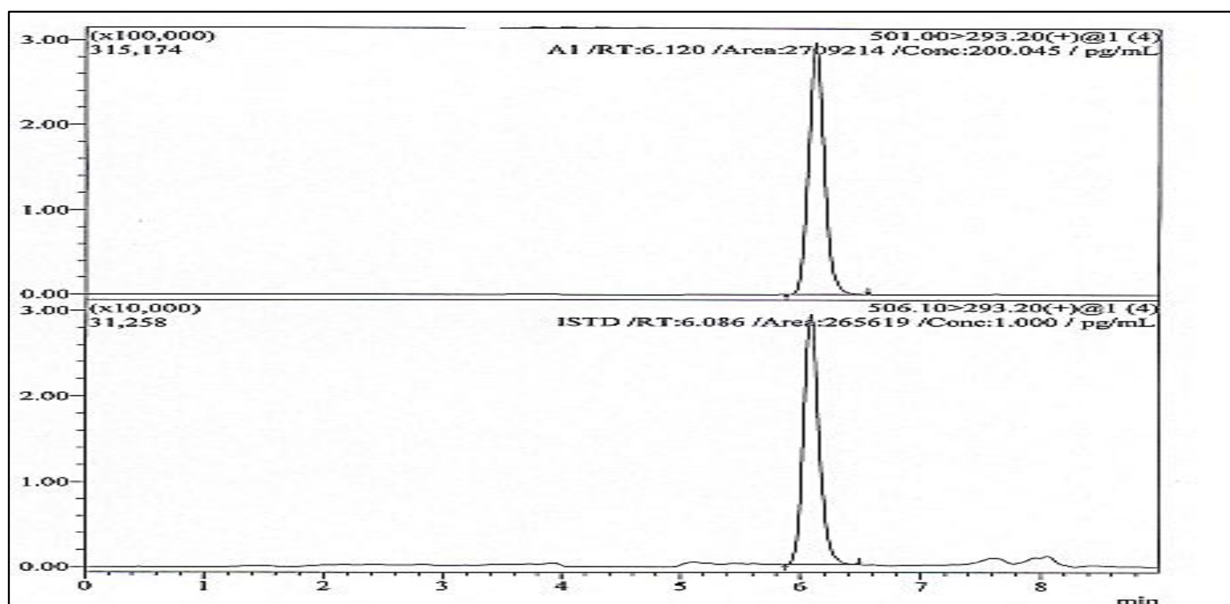


**Figure 9. Representative Chromatogram of HQC**

**Table 5. Result Precision and Accuracy of Fluticasone**

| Batch | Parameter | DIL QC (1/5) | HQC | MQC | LMQC | LQC | LOQQC |
|---|---|---|---|---|---|---|---|
| **PA-I** | **Precision (%CV)** | 4.2 | 3.2 | 0.7 | 2.4 | 3.1 | 6.6 |
| **PA-I** | **Accuracy** | 102.6 | 99.0 | 105.6 | 108.6 | 98.8 | 114.7 |
| **PA-II** | **Precision (%CV)** | 8.8 | 0.5 | 0.7 | 0.9 | 1.3 | 4.3 |
| **PA-II** | **Accuracy** | 104.0 | 100.8 | 109.0 | 104.1 | 97.6 | 106.6 |
| **PA-III** | **Precision (%CV)** | 2.7 | 1.4 | 0.7 | 1.6 | 2.2 | 3.2 |
| **PA-III** | **Accuracy** | 102.5 | 101.7 | 107.9 | 104.3 | 99.7 | 109.7 |

**Conclusion:**

A sensitive, robust and high throughput LC–MS/MS method has been developed for the glucocorticoid FP in human plasma. The LC–MS/MS method offers increased specificity and sensitivity over the previous method, with an LLOQ of 1.008 pg/ml from only 0.5 ml of plasma which is more sensitive than presented in the literature published. This approach to automated SPE has proved to be a very successful replacement to manual extraction offering reduced sample preparation times. This has been achieved by the combination of a very efficient and selective sample clean-up method (SPE) with an ultra-sensitive MS/MS instrument.

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