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IJPBS |Volume 3| Issue 2 |APR-JUN |2013|247-257

Research Article

Pharmaceutical Sciences

RAPID AND SENSITIVE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRIC METHOD FOR THE SIMULTANEOUS ESTIMATION OF THE ANTIASTHEMATIC DRUG SALBUTAMOL SULPHATE AND BECLOMETHASONE DIPROPIONATE, IN HUMAN PLASMA

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ABSTRACT

A highly sensitive, specific and rapid LC–ESI-MS/MS method has been developed and validated for simultaneous quantification of Salbutamol and Beclomethasone in human plasma (200 µl) using Anastrozole respectively, as an internal standard (IS) as per the regulatory guidelines. ***Results:*** *The LLE method was used to extract the analytes and IS from human plasma. The chromatographic resolution of Salbutamol, Beclomethasone and Anastrozole IS was achieved using an isocratic flow on a RP8 column. The total chromatographic run time was 3.5 min. A linear response function was established for the range of concentrations 23 to 4000 ng/mL and 8 to 1500 ng/mL for Salbutamol and Beclomethasone, respectively, in human plasma.* ***Conclusion:*** *The intra- and inter-day accuracy and precision values for Salbutamol and Beclomethasone met the acceptance as per regulatory guidelines.*

KEY WORDS

Salbutamol, Beclomethasone, Anstrozole, LC MS/MS, Human Plasma

1. INTRODUCTION

Salbutamol is chemically 2-(hydroxymethyl)-4-[1-hydroxy- 2-(tert-butylamino) ethyl] phenol. It is a short-acting β2-adrenergic receptor agonist used for the relief of bronchospasm in conditions such as asthma and COPD [1-3]. It is usually given by the inhaled route for direct effect on bronchial smooth muscle. This is usually achieved through a metered dose inhaler, Nebulizer or other proprietary delivery devices. In these forms of delivery, the effect of Salbutamol can take place within 5 to 20 minutes of dosing. It can also be given orally or intravenously. Despite the fact that SS is well absorbed, its systemic bioavailability is only 50% due to extensive presystemic metabolism in the gastrointestinal tract and liver [4].

Beclomethasone dipropionate (BDP) is atopically active corticosteroid used in the treatment of asthma and rhinitis. Beclomethasone dipropionate (BDP) is a potent pro-drug to beclomethasone (BOH) and is used in the treatment of chronic and acute respiratory disorders in the horse. The therapeutic dose of BDP (325 µg per horse) by inhalation results in very low plasma and urinary concentrations of BDP and its metabolites that pose a challenge to detection and confirmation by equine forensic laboratories.

Early inflammatory lesions and bronchial hyper responsiveness are characteristics of the respiratory distress in premature neonates and are susceptible to aggravation by assisted ventilation. We hypothesized that treatment with inhaled salbutamol and beclomethasone might be of clinical value in the prevention of bronchopulmonary dysplasia (BPD) in ventilator-dependent premature neonates. Many patients with chronic asthma require long-term treatment with both an inhaled bronchodilator and an inhaled corticosteroid. A combination inhaler which contains both types of drugs would

be convenient for the patient, and by reducing the number of different therapies might improve compliance while also reducing the number of inhalations required for each dose.

Worldwide, Salbutamol and Beclomethasone dipropionate (BDP) are frequently prescribed together. Recently, a combination inhaler containing these two drugs has been developed, so that with each actuation the same amount of each drug is delivered as from one actuation of each of the two separate inhalers.

To solve this problem, a method involving the use of a liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) was developed for the detection, confirmation and quantification of the analytes in equine samples.

The literature reports many analytical methods for the quantitative determination of Salbutamol Sulphate and Beclomethasone Dipropionate alone or in combination with other drugs including spectroscopic (13) and chromatographic methods (2-5, 8, 10-12, 14)

There are no reported articles on quantification of these drugs in combination from human plasma using any modern analytical technique. Hence at our laboratory we have developed a method for quantifying in human plasma using liquid chromatography tandem mass spectrometry. The developed method was fully validated and to indicate its suitability.

2. EXPERIMENTAL

2.1 Materials and chemicals

Symmetry Sheild RP8, (3.9 x 150mm), 5µ column obtained from Waters, Mumbai, India was used for the compound retention. The reference standards of Salbutamol Sulphate (99.00%), Beclomethasone Dipropionate (98.50%) and Anastrozole (99.24%) were obtained from Glenmark pharmaceuticals Nashik, India. HPLC grade water was used by a Milli-Q water purification system obtained from Millipore, Bangalore, India. HPLC grade methanol and acetonitrile were purchased from J.T.Baker., Mumbai, India. Ammonium acetate was obtained from Fluka, Germany. Human blank plasma containing K3-EDTA as the anticoagulant was obtained from Yash Laboratory, Thane, India. The plasma thus obtained was stored at –20° C prior to use.

2.2 Preparation of standards and quality control samples

Two separate stock solutions of Salbutamol and Beclomethazone were prepared for bulk spiking of calibration curve and quality control samples for the method validation exercise. Salbutamol, Beclomethazone and Anastrozole (IS) stock solutions were prepared by dissolving them in methanol to obtain a desired concentration. These stock solutions of Salbutamol and Beclomethazone thus prepared were used to spike a pool of blank human plasma which was serially diluted with blank human plasma eight times to obtain calibration standards spanning a range between 23 to 4000 ng/mL for Salbutamol and 8 to 1500 ng/mL for Beclomethazone. Similarly, quality control standards (QC's) were prepared (using the same methodology) at four different concentrations namely, 23(LLOQ), 65 (LQC), 1300 (MQC) and 3500 (HQC) ng/mL for Salbutamol and 8(LLOQ), 25 (LQC), 500 (MQC) and 1300 (HQC) ng/mL for Beclomethazone. Sufficient calibration standards and quality control standards were prepared to validate the method and to serve as standards and controls during the study, only three levels of controls were prepared as in LQC (lower quality control), MQC (middle quality control) and HQC (higher quality control). The spiked plasma samples (standard and QC samples) were pretreated and detected in each analytical batch along with the unknown samples. Aliquots of the standards and quality controls were stored together with study

samples at −50 °C until used for sample processing.

2.3 Extraction Procedure

An aliquot of 200 µL of the human plasma sample was mixed with 25 µL of the internal standard working solution (1000 ng/mL of Anastrozole). To this, 50 µL of the potassium dihydrogen phosphate buffer (1M) was added. After vortexing for 20 s, a 4 mL aliquot of the MTBE was added using eppedorf 5ml pipette. The sample was shaken for 10 min using a reciprocating shaker (reciprocating motion, Digita) and then centrifuged for 4 min at 4000 rpm using a Hettich Zentrifugen Rotanta 460 R centrifuge. The organic layer (3.0 mL) was transferred into a 10 mL glass test tube and evaporated at 40ºC under a stream of nitrogen. The dried extract was reconstituted in 250 µl of the mobile phase and transferred into autosampler vials. From these, a 20 µl aliquot was injected into the chromatographic system.

The assay method was developed and validated and applied successfully to the analysis of Salbutamol and Beclomethasone.

2.4 LC–MS/MS conditions

All experiments were performed using a triplequadrapole system (Applied Biosystems, Ontario, Canada) mass spectrometer fitted with a TurboIonSpray source. The front end included a Shimadzu autosampler and a Shimadzu Prominence series (Kyoto, Japan) binary pump. Data acquisition and quantitation was performed using Analyst® 1.5 software.

The chromatographic separation was achieved on a Symmetry sheild RP8, (3.9 x 150mm), 5µ column obtained from Waters Mumbai, India. The mobile phase consisted of acetonitrile-2mM ammonium acetate buffer (80:20, *v/v*), delivered at 0.5 mL min^{-1} at ambient temperature. The total run time was 3.5 min and the injection volume was 10.0 µl. Moreover, the column and autosampler temperature were maintained at 30°C and 5°C, respectively.

Electrospray ionization (ESI) was performed in the positive ion mode with nitrogen as the collision gas. The spray voltage and source temperature were kept at 5500 kV and 500°C respectively. Nitrogen was used as the collision gas. The Declustering Potential (DP), Collision Energy (CE), Entrance potential (EP), Cell Exit Potential (CXP) were optimized during tuning as 64, 19, 10, 11; 64, 25, 10, 11; 110, 50, 10, 11 eV for Salbutamol, Beclomethazone and Anastrozole respectively. The collision activated dissociation (CAD) gas was set at 6 psi, while the curtain gas was set at 45 psi.

The mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion m/z 240.1→148.1 for Salbutamol, m/z 521.2→337.4 for Beclomethazone and m/z 294.10→224.90 for the internal standard, anastrozole. The instrument response was optimized for Salbutamol and Beclomethazone by infusing a constant flow of a solution of the drug dissolved in mobile phase via a T-piece into the stream of mobile phase eluting from the column. The same methodology was used to optimize the response of the instrument for the internal standard.

Figs.1, 2 and 3 show the product ion mass spectra obtained from collision-induced dissociation of the protonated molecular ions of Salbutamol, Beclomethazone and anastrozole respectively. The instrument was interfaced with a computer running Applied Biosystems Analyst version 1.5 software.

2.5 Assay validation

The method was validated in terms of selectivity, accuracy, precision, recovery, calibration curve, matrix effect and reproducibility according to the FDA guidelines for validation of bioanalytical method.

The method was validated by analyzing plasma quality control samples five times at four different Salbutamol and Beclomethazone concentrations, i.e. 23, 65, 1300 and 3500 ng/mL for Salbutamol and 8, 25, 500 and 1300 ng/mL for Beclomethazone to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve containing eight different concentrations spanning the concentration range of 23 to 4000 ng/mL and 8 to 1500ng/mL for Salbutamol and Beclomethazone respectively.

Calibration graphs were constructed using a linear regression (weighted with $1/concentration)^2$ of the drug peak-area ratios of the product ions of the analyte to the internal standard versus the nominal drug concentrations. Several regression types were tested and the linear regression (weighted with $1/concentration)^2$ was found to be the simplest regression.

The intra-batch and inter-batch accuracy were determined by replicate analysis of the four quality control levels along with the LLOQ (Lower Limit of Quantitation) level that were extracted from the sample batch. In each of the precision and accuracy batches, six replicates at each quality control level inclusive of the LLOQ level were analysed. Accuracy is defined in terms of % recovery and was calculated using the formula, % recovery = mean found concentration/nominal concentration] x 100. Assay precision was calculated by using the formula % RSD = (SD/M) (100) where M is the mean of the experimentally determined concentrations and SD is the standard deviation of M.

The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) was performed by processing six lots of different normal controlled plasma samples in quadruplet (*n*=4). LQC and HQC working solutions were spiked post extraction in duplicate for each lot. The results found were well within the acceptable limit set i.e. the RSD of area ratio to be within ± 15% at each level tested. Also, the minor suppression of analyte signal due to endogenous matrix interferences does not affect the quantification of analyte and IS peak which was confirmed by post-column infusion.

Absolute recoveries of the analyte were determined at the four different quality control levels viz. LQC, MQC and HQC, by comparing the peak areas of the extracted plasma samples with those of the unextracted standard mixtures (prepared in the injection vehicle at the same concentrations as the extracted samples) representing 100% recovery.

3. RESULTS AND DISCUSSION

3.1 Selectivity

The selectivity of the method was investigated by analyzing human plasma of six different sources, two each of lipemic and heamolysed plasma. Any interference obtained was compared versus six replicates of extracted samples at the LLOQ level prepared in one of the plasma lots with the least interference at the retention time of Salbutamol, Beclomethazone and Anastrozole. No endogenous interference peaks were found at the retention times of all analytes and IS. Representative chromatogram obtained from blank plasma and plasma spiked with LLOQ standard for Salbutamol and Beclomethazone is presented in Figs. 4 and 5, respectively.

3.2 Linearity and sensitivity

The assay was linear over the concentration range of 23–4000 ng/mL for Salbutamol and 8–1500 ng/mL for Beclomethazone. The correlation coefficients for the calibration regression curve were 0.997 or greater. The calibration standards were back-calculated from the responses. The

deviations from the nominal concentrations and coefficient of variation (CV) were less than 15% for all concentrations.

The current assay had an LLOQ of 23 ng/mL for Salbutamol and 8 ng/mL for Beclomethazone. These limits are sufficient for clinical pharmacokinetic studies following oral administration of therapeutic dose.

3.3 Accuracy and precision

A summary of intra and inter-day precisions and accuracies at QC concentrations are shown in **Table 1**. The intra- and inter day precisions were ≤4.02% and ≤6.22% for Salbutamol and ≤2.60% and ≤7.78% for Beclomethazone.The accuracies were in range of 90.66% to 106.10% for Salbutamol and 96.54% to 105.35% for Beclomethazone . The results indicated that the assay had remarkable reproducibility with acceptable accuracy and precision.

Table 1: Precision, accuracy and extraction recovery for Salbutamol and Beclomethasone in plasma QC samples

Components	Nominal concentration (ng/mL)	Intra-day (n = 6)			Inter-day (n = 18)			Extract recovery (%)
		Measured concentration (ng/mL)	RSD (%)	Bias (%)	Measured concentration (ng/mL)	RSD (%)	Bias (%)	
Salbutamol	23.651	24.779	3.31	4.77	24.372	4.048	3.05	-
	67.573	61.263	4.13	-9.34	62.320	4.623	-7.77	98.13
	1299.480	1339.857	6.65	3.11	1355.922	6.217	4.34	95.82
	3332.000	3535.171	2.44	6.10	3458.652	4.454	3.80	96.15
Beclomethaso	9.126	9.168	2.60	0.46	9.235	3.798	1.20	-
	26.074	25.171	4.04	-3.46	25.137	3.957	-3.59	95.72
	501.423	528.261	2.69	5.35	530.958	3.398	5.89	98.88
	1285.7	1304.769	7.77	1.48	1326.019	7.357	3.14	98.65

3.4 Recovery

To calculate the absolute recovery, six replicates of spiked plasma QC samples of low, lower medium, medium and high concentrations were extracted under the conditions noted above. The integrated peak area response of each analyte was compared with those obtained from the standard solutions of equivalent concentration subjected to the same extraction. The mean absolute recoveries of Salbutamol determined at 67, 1300 and 3300 ng/mL were 98.13, 95.82 and 96.15 %, respectively. The mean absolute recoveries of Beclomethazone determined at 25, 500 and 1300 ng/mL were 95.72, 98.88, and 98.65 %, respectively. The mean absolute recovery of anastrozole was 97.13 %.

3.5 Matrix effect

No matrix effect for Salbutamol and Beclomethasone were observed for the six different plasma lots tested. The RSD of the area of post spiked recovery samples at LQC and HQC levels were less than 1.00% for Salbutamol and less than 3.37% for Beclomethasone. For the internal standard the RSD of the area over both LQC and HQC levels was less than 6.60%.This indicated that the extracts were “clean” with no co-eluting compounds influencing the ionization of the analytes and the internal standard.

3.6 Stability

The Salbutamol and Beclomethazone were found to be stable under following conditions: in plasma at room temperature for at least 24 h, in

the autosampler at 5 °C for 48 h and three freeze–thaw cycles. The long term stability results also indicated that Salbutamol and Beclomethazone were stable in matrix for up to 30 days at a storage temperature of -50°C. This period of long term stability was sufficient enough to cover the entire period right from first day of storage of the plasma samples to the last day of analysis.

3.7 Bench Top Stability:

The stability of Salbutamol and Beclomethazone in plasma at room temperature was examined by storing six sets of LQC and HQC level at room temperature for 24 Hrs. After 24 Hrs the stability samples were analysed and compared with freshly prepared samples and back calculated against freshly prepared calibration curve standards.

The **Table No. 2** shows the statistical data of the back-calculated values of fresh and stability samples for Salbutamol and Beclomethazone respectively. There is no significant difference in concentration after 24 Hrs, thus it can be concluded that Salbutamol and Beclomethazone plasma samples are stable at room temperature for at least 24 Hrs.

Table 2:Stability of Salbutamol and Beclomethasone in human plasma under different storage conditions (n = 6).

Components	Nominal concentration(ng/mL)	Room temperature for 24 h	Autosampler at 4◦C for 48 h	Freeze-thaw Stability third cycle	Storage at –50 ◦C
Salbutamol	67.573	-4.82	-4.20	-7.11	-7.34
	3332.000	0.43	0.81	-3.09	-5.17
Beclomethasone	26.074	-6.86	-5.90	-9.64	-9.76
	1285.7	-2.03	-1.45	-8.32	3.95

All values are represented as the percent of mean deviation from nominal concentration, bias% = (measured concentration−nominal concentration)/nominal concentration×100%

3.8 Stability after Repeated Freezing / Thawing Cycles

Freeze thaw stability in plasma was assayed using freshly prepared LQC and HQC samples against six replicates of LQC and HQC samples previously frozen and thawed over three and five cycles. The freeze and thaw stability was evaluated at the end of third and five cycles. The back calculated concentrations were obtained was freshly spiked calibration curve standards.

There is no significant difference in concentration of freshly prepared and stability samples after three cycles.

Refer to **Table No.2** for Freeze thaw stability results after three cycles for Salbutamol and Beclomethazone, respectively.

3.9 Process stability (Autosampler Stability)

The stability of Salbutamol and Beclomethazone in processed samples at autosampler conditions was examined by extracting six sets of LQC and HQC samples and storage of these samples for 48 Hrs in autosampler at 4^0C. These samples were compared with freshly prepared samples.

The following **Tables No. 2** show the statistical data of the results of autosampler stability for of Salbutamol and Beclomethazone, respectively. There is no significant change in concentration, so it can be concluded that of Salbutamol and

Beclomethazone is stable in processed samples for at least 48 Hrs at autosampler temperature

3.10 Long Term Stability in Matrix (at –70°C ± 5°C)

This exercise was performed at two concentration levels of Salbutamol and Beclomethazone i.e. LQC and HQC. Stability sample containing LQC and HQC concentrations in plasma were prepared and stored at –70°C ± 5°C .Six replicates of LQC and HQC samples was processed and analyzed on LC-MS/MS against a calibration curve. (Refer table no.2 for of Salbutamol and Beclomethazone respectively, PA batch-I analysed on next day of bulk spiking) This was analysis of initial long-term stability. Remaining sets of spiked samples were stored at –70°C ± 5°C.

After 30 days, six replicates of stored samples was processed and analyzed against fresh calibration standards.

Back calculated concentrations were obtained from calibration curve. Average of back calculated concentration obtained for samples of stability exercise were compared to the average of back-calculated concentration for samples of initial day to calculate % difference. This period of long term stability in matrix was sufficient to prove stability over the entire period of date of first sample collection in the clinics to the last day of analysis.

3.11 LC–MS/MS method optimization

During method development quantification was performed using multiple reactions monitoring (MRM) in the positive ion mode and different options were evaluated to optimize sample like extraction, detection parameters and chromatography. Electro spray ionization (ESI) was evaluated to get better response of analytes as compared to APCI (Atmospheric Pressure Chemical Ionization) mode. It was found that the best signal was achieved with the ESI positive ion mode. **Fig. 1, 2 and 3** displays the product ion spectra of [M+H] + ions of Salbutamol, Beclomethazone and Anastrozole(IS). For Salbutamol, the product mass spectrum was recorded from the precursor ion m/z 240.1 [M+H] + and the most abundant fragment was monitored at m/z 148.1 For Beclomethazone, the product mass spectrum was recorded from the precursor ion m/z 521.2 [M+H] + and the most abundant fragment was monitored at m/z 337.4 and Internal standard anastrozole the product mass spectrum was recorded from the precursor ion m/z 294.10 [M+H] + and the most abundant fragment was monitored at m/z 224.90. A mobile phase with different buffer solution and methanol in varying combinations was tried during the initial development stages. But the best signal for Salbutamol and Beclomethazone was achieved using a mobile phase containing 2mM ammonium Aacetate buffer in combination with Acetonitrile (20:80 *v/v*). Use of a Symmetry sheild RP8, (3.9 x 150mm), 5µ column resulted in better seperation and reduced run time. The retention times for Salbutamol, Beclomethazone and Anastrozole were ~ 2.05 minutes, ~ 2.91 minutes and ~ 1.92 minutes, respectively. LC–MS/MS chromatograms showed that the analytes and IS were separated with no interference from each other.

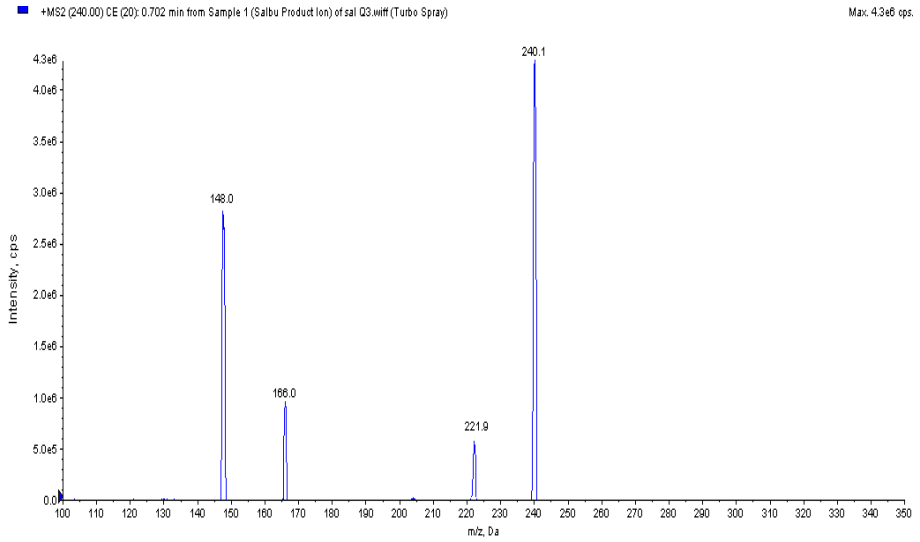

Figure.1. Representative spectra of product ion of Salbutamol

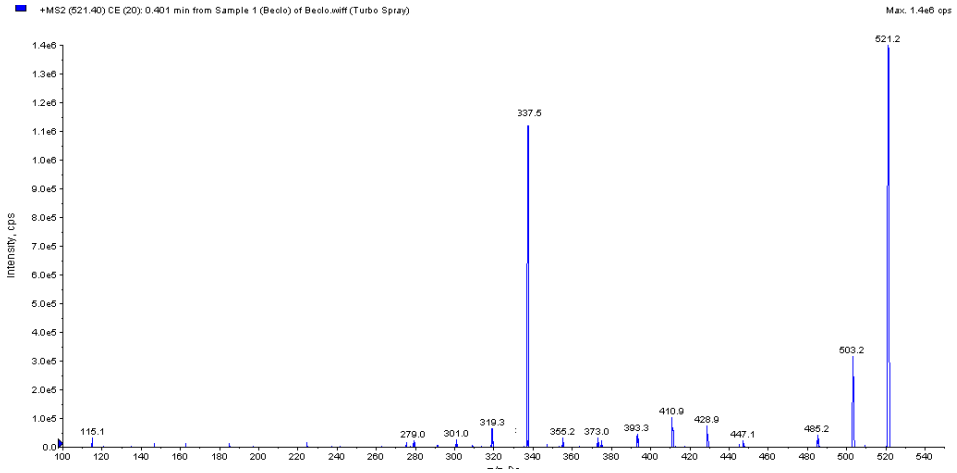

Figure.2. Representative spectra of product ion of Beclomethasone

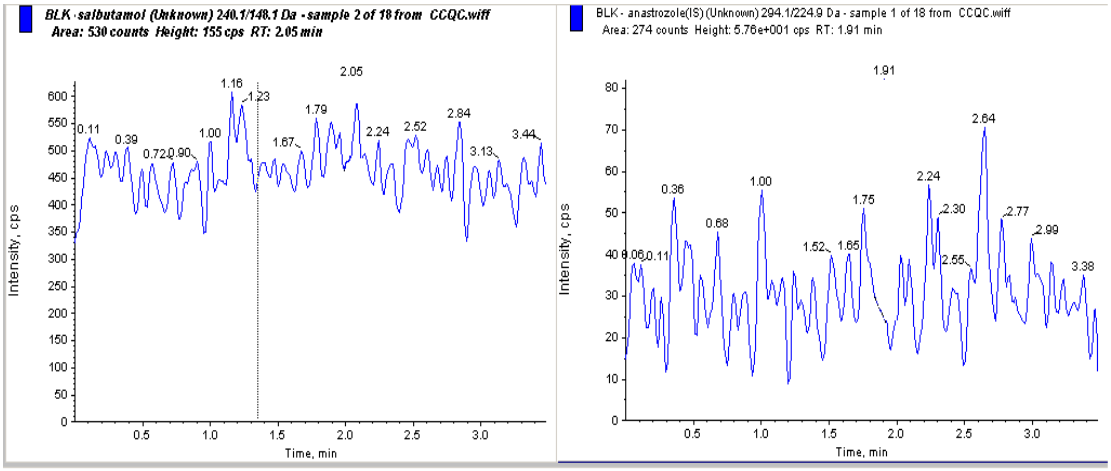

Figure.3. Chromatogram of blank plasma of Salbutamol and its Internal Standard Anastrozole

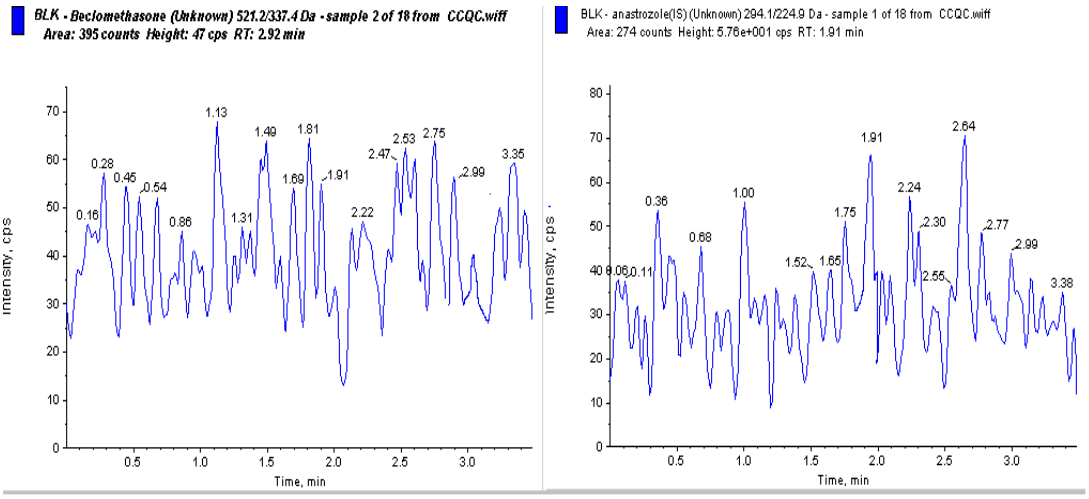

Figure.4. Chromatogram of blank plasma of Beclomethasone and its Internal Standard Anastrozole

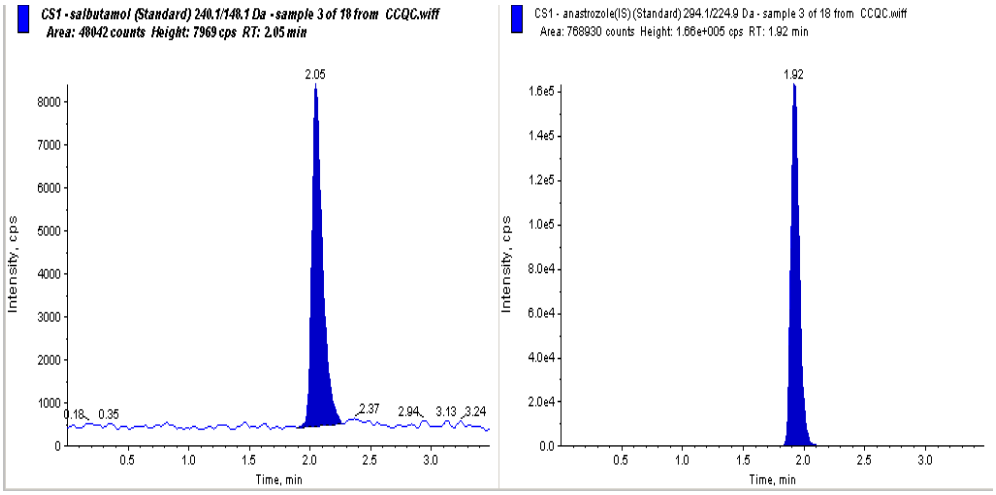

Figure 5. Chromatogram of Salbutamol and its Internal Standard Anastrozole at the lower limit of quantification

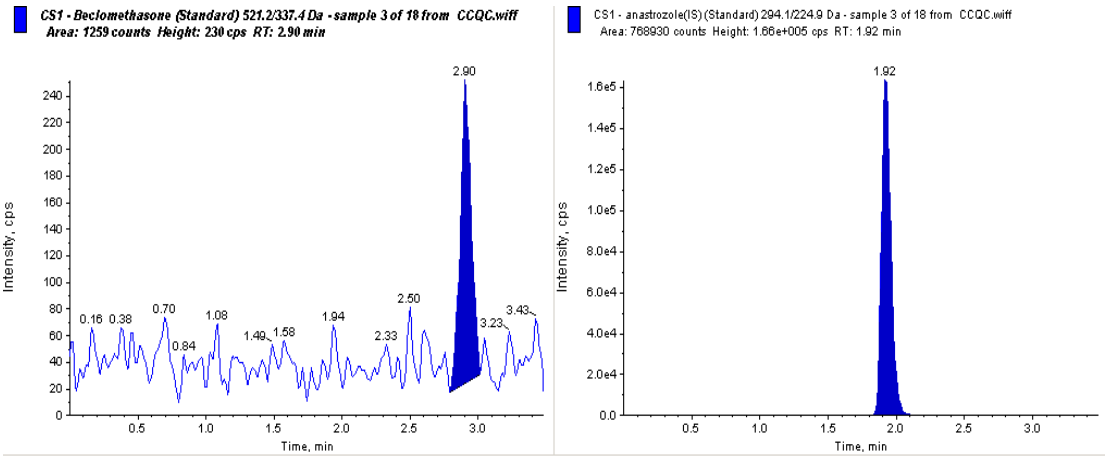

Figure 6. Chromatogram of Beclomethasone and its Internal Standard Anastrozole at the lower limit of quantification

4. CONCLUSIONS

To our knowledge, this is the first fully validated LC–MS/MS method for the simultaneous quantification of Salbutamol and Beclomethazone in human plasma. The method was proved to be sensitive, accurate, precise and reproducible. Sample preparation showed high recovery for the quantitative determination of these two analytes in human plasma. The method allows for a much higher sample throughput due to the short chromatography time (3.5 minutes) and simple sample preparation. Robust LC-MS/MS performance was observed, with acceptable variation in instrument response within batches. This method is an excellent analytical option for rapid quantification of Salbutamol and Beclomethazone in human plasma.

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