Development and Validation of a Liquid Chromatography Coupled with Atmospheric-Pressure Chemical Ionization Orbitrap Mass Spectrometric Method for Simultaneous Determination of Hydrocortisone, Cortisone, Corticosterone

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Abstract - A sensitive method has been developed and validated for the simultaneous determination of Hydrocortisone, Cortisone and Corticosterone in human serum using Triethyl Phosphate as an internal standard (IS). After Hydrocortisone, Cortisone and Corticosteronein human serum were extracted with ethyl acetate, the extracts were separated on an XDB C18 column (30 mm × 2.1-mm i.d., 3 µm) using a mobile phase of acetic acid/ammonium acetate (5 mmol/L, pH 4.5)/acetonitrile/methanol in gradient elution. Detection was performed by high-performance liquid chromatography coupled with atmospheric-pressure chemical ionization ion trap mass spectrometry in negative multiple reaction monitoring mode. The transition ions m/z 362 → 363, m/z 360 → 361, m/z 346 → 347 were selected for the quantification of Hydrocortisone, Cortisone, and Corticosterone respectively. The linear range was 1.0–1000.0 ng/mL, the absolute recoveries were between 98.3, 99.1 and 100.2%, the relative recoveries were between 98.1,101.02 and 94.6%, and the limits of quantification in human serum were 0.5–1.0 ng/mL for the three target compounds. The intra and interday relative standard deviations were less than 11.9% and 13.0%, respectively. This method was found to determine trace amount of Hydrocortisone, Cortisone, and Corticosterone in human blood serum effectively and simultaneously and can be suitable for clinical and toxicological studies.

Keywords: Corticosteroids, LC, APCI, Orbitrap-MS and Blood Serum.

I. INTRODUCTION

Corticosteroids are produced by the cortex of the adrenal glands.[1] There are two main forms-glucocorticoids and mineralocorticoids. The actions of glucocorticoids include gluconeogenesis, fat deposition, sodium retention, decreased protein synthesis and decreased immune response. Examples of glucocorticoids include Cortisol (Hydrocortisone), Prednisolone and Dexamethasone. Mineralocorticoids, such as Fludrocortisone, mainly act on the extracellular balance of sodium and potassium in the distal tubule of the kidney.

Glucocorticoids are commonly used within palliative care in a variety of doses to tackle both specific and non-specific symptoms of advanced cancer. [2] They are commonly referred to as steroids although as explained above they are one form of several corticosteroids. The corticosteroid used most commonly in palliative care is Dexamethasone.[3] The use of corticosteroids within the general medical population is extremely closely monitored and there have been some concerns within the literature that this is not appropriately translated into palliative care patients. [4,5]

Corticosteroids (often referred to simply as steroids but are not to be confused with body-building "steroids") were first introduced as therapy for inflammatory bowel disease (IBD) in the 1950s. Since that time, these powerful and fast-acting anti-inflammatory drugs have been frequently used in the treatment of acute flare-ups of disease. Corticosteroids reduce inflammation in both the intestine and throughout the body, including the joints, skin, and eyes. While 20-30% of patients with acute symptoms of IBD will not respond to corticosteroids, most patients notice an improvement in symptoms within days of starting these medications. [6]

Steroids are best suited for short-term control of IBD symptoms and disease activity and should not be used as primary therapy for long periods of time. Despite many patients experiencing improvement in symptoms with steroid use, corticosteroids are not effective in preventing complications or progression of disease. Other reasons for discouraging long-term steroid use are the side effects and risks, which increase with repeated or long-term use. The need for repeated courses of steroids often indicates that a patient's primary IBD medication regimen is insufficient and that a change is likely needed. While some unique situations may require long-term steroid use, generally, corticosteroids should be used sparingly at the lowest effective dose.

Side effects of Corticosteroids are exerting their anti-inflammatory effect by suppressing immune system activity. As a result, steroids can leave individuals more susceptible to some infections, especially yeast infections

of the mouth (thrush), female reproductive organs, and occasionally urinary tract infections.

In addition, steroids also impact several other systems and exert additional side effects. The risk and severity of these side effects are related to increasing dose and duration of steroid use. These side effects often resolve when the steroid is stopped, or the dose is reduced. Exceptions are osteoporosis (weakening of the bones) and cataracts, which require additional treatment to correct should they occur.

Side effects include:

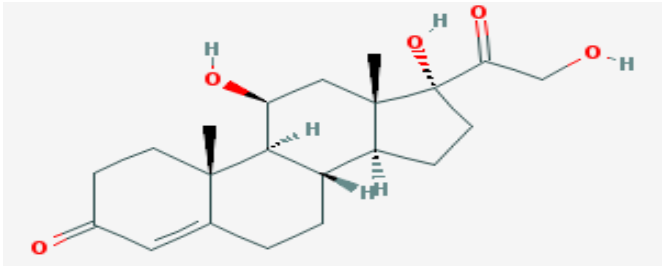
- high blood pressure (hypertension)
- high blood sugar levels
- cataracts
- weight gain
- stretch marks
- acne
- growth disturbance in children
- rounding of the face (“moon face”)
- increased facial hair
- insomnia (difficulty sleeping)
- mood swings
- psychosis and other psychiatric symptoms
- weakened bones (osteoporosis)

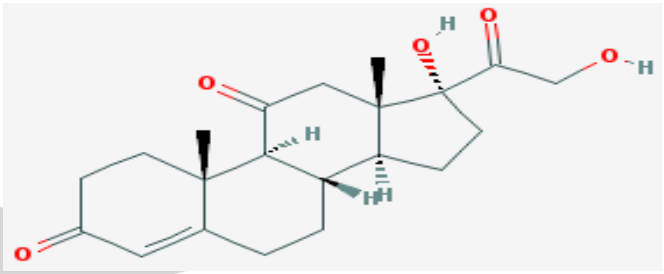
Corticosteroidscan beanalyzed by using liquid chromatography (LC) coupled to a mass spectrometer (MS) as a detector.LC offers good separation efficiency and a choice of MS detectors, including single or triple quadrupoles. Quadrupole mass analyzers are selective, sensitive, and cost-effective instruments that operate at nominal mass resolution. When using quadrupole MS, the selectivity required to separate target drugs from chemical background is achieved using either selected ion monitoring (SIM) or selected reaction monitoring (SRM). Both SIM and SRM are used in targeted experiments in which the mass spectrometer is pre-programmed using a list of preselected drugs. How-ever, targeting specific compounds during acquisition limits the scope of analysis and can result in false negative results (non-detection) for both unknown and untargeted compounds, which may be of concern with respect to food safety[7-9].

Thislimitation has led to increased interest in developing methods using MS analyzers that can operate in full scan with a higher mass resolving power than triple quadrupoles but provide similar levels of selectivity and quantitative performance. Until now, high-resolution, accurate-mass GC-MS instruments have not gained wide acceptance due to their limited ability to provide full scan selectivity and quantitative performance comparable to triple quadrupole instruments operated in SRM. [10-13]

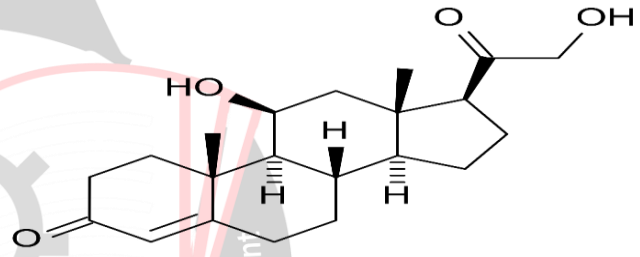
In this work, we demonstrate the use of LC coupled to Orbitrap™ MS technology for fast, high throughput corticosteroid residues analysis in blood serum samples, with an almost unlimited scope in the analysis through full scan acquisition.

Hydrocortisone **Cortisone**





Corticosterone



II. MATERIALS AND METHODS

Chemicals:

Hydrocortisone, Cortisone, and Corticosteroneanalytical standards (purity >99%), DSC C18, Triethyl Phosphate, Milli-Q water and Methanolwere purchased from Sigma Aldrich.

EXPERIMENTAL PROCEDURE

Apparatus

Thermo Scientific-Exactive LC- Orbitrap,Centrifuge Model: RC -8C,Micro Spatula, 15ml Centrifuge Tubes, Vortex, Sonicator, Rotary Evaporatorwas used for analysis.

Mobile phase preparation

Mobile Phase A:

0.1% Formic Acid in water is Prepared. The mixture is filtered, Sonicated, and degassed for 15mins.

Mobile Phase B:

0.1% Formic Acid in methanol is Prepared. It is filtered, Sonicated, and degassed for 15mins.

Standard preparation:

Accurately weight and transfer each about 1mg of Hydrocortisone, Cortisone, and Corticosterone into 2mL vial, add 1mL of mobile phase and sonicate to dissolve.

Preparation of stock solutions and working standards:

Individual standard stock solutions were prepared at 1 mg/mL by dissolving 10 mg of each pesticide in 10 mL of acetonitrile and were stored at -20^0C until use. Working standard solutions at different concentration levels were prepared in ethyl acetate

Chromatographic conditions

Column C-18 (33 mmx4.6 mm I.D; particle size 3µm) was used for analysis at 300°c column temperature. The mobile phase was pumped through the column at a flow rate of 800 µL/min. The sample injection volume was 1µL. Mass Spectrometer was used as detector and Chromatographic runtime was 10 minutes.

Data Processing

Data was acquired and processed using Thermo Scientific™ TraceFinder™ software. TraceFinder software allows the analyst to build acquisition and processing methods for high throughput screening and quantitative analysis and incorporates library searching capabilities as well as easy data reviewing and data reporting. Results and Discussion The objective of this study was to evaluate the utility of Orbitrap-based GC-MS technology for fast pesticides screening and quantification to increase sample throughput and laboratory productivity.

III. RESULTS AND DISCUSSION

Method development

To develop a suitable and robust LC-MS method for the determination of target Analytes, different mobile phases were employed to achieve the best separation and resolution. Finally, the method development was achieved with Symmetry C-18 (33 mmx4.6 mm I.D; particle size 3µm) with the 0.1% of formic acid in water as mobile phases A and 0.1% of formic acid in methanol as mobile phase B. Gradient mode was initially maintained as 20-95% mobile phase B for 0-5mins, 95% for 5-7mins and 20% mobile phase B for 7.1-10mins. The flow rate was maintained 800µL/min. For APCI, Sheath gasflow rate is 60µL/min, Auxillary gas flow rate is 30µL/min, Capillary temperature is 350^0 C, Heater temperature is 300^0 C.

The targeted analyte mixture standards were injected and achieved good separation by the method parameters. The retention time for Hydrocortisone is 4.25mins, Cortisone is 4.05mins and Corticosteroneis 4.60mins were achieved. Finally, the method was good enough to separate targeted analytes by using proposed method. Chromatograms are represented in Figure 1-3.

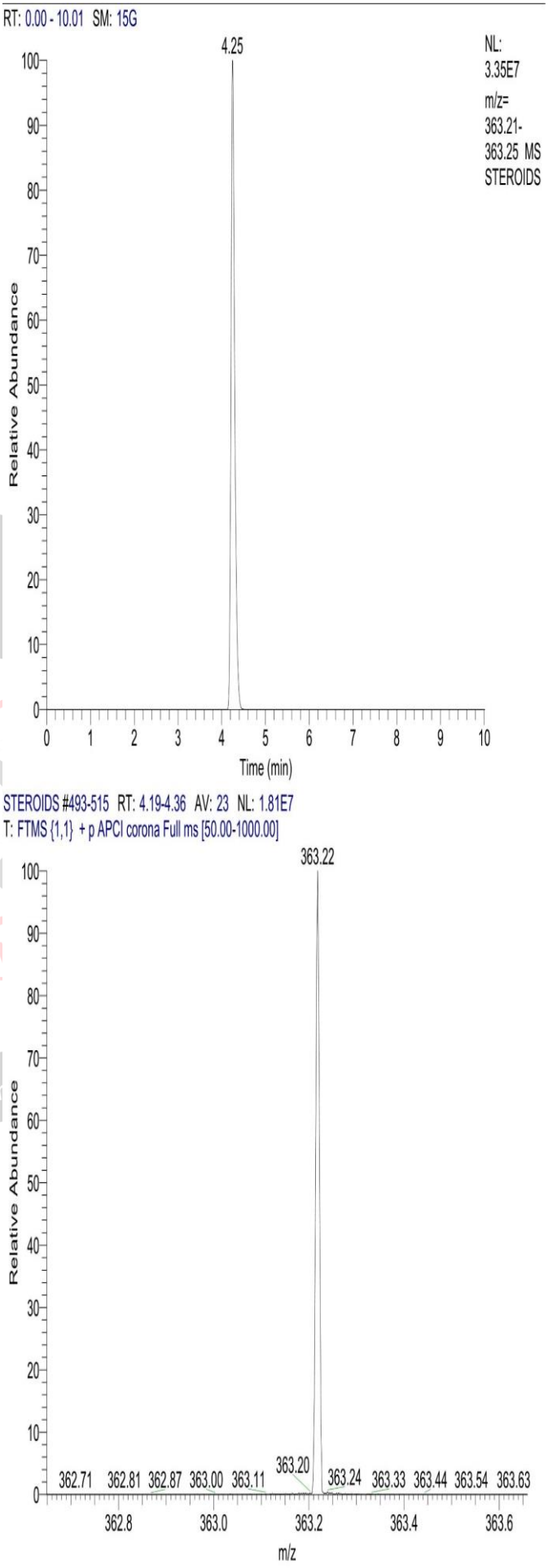


Figure 1: Chromatogram for Hydrocortisone

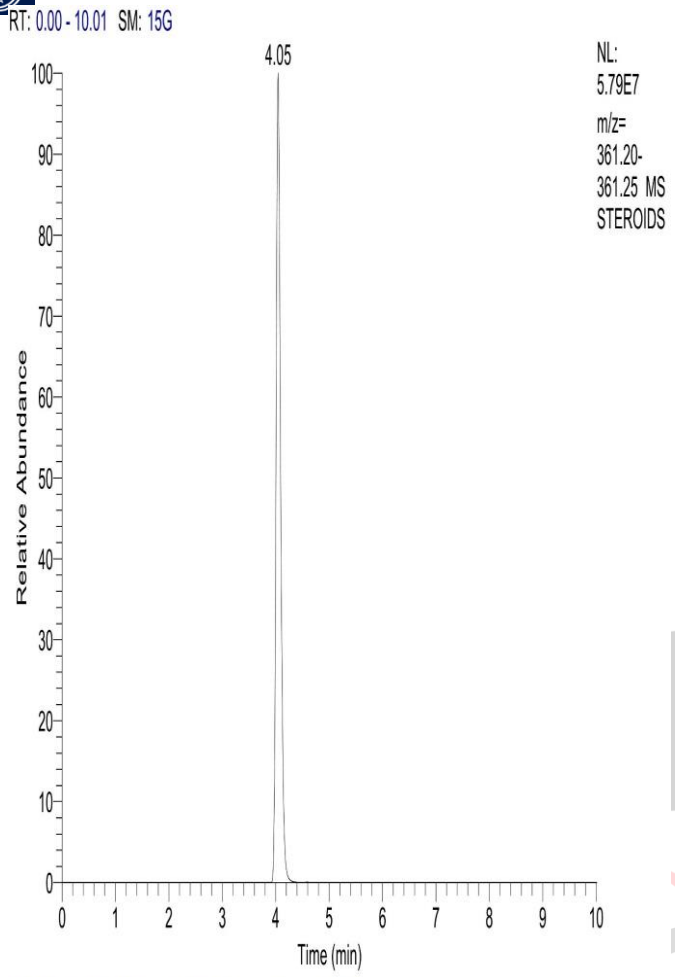


Figure 2: Chromatogram for Cortisone

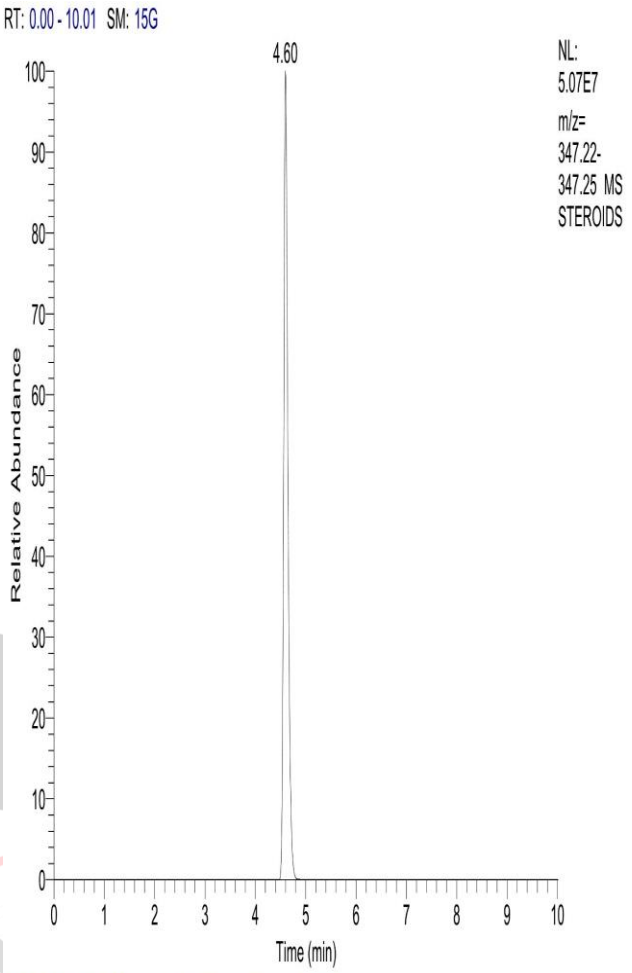


Figure 3: Chromatogram for Corticosterone

METHOD VALIDATION

For method validation, from the stock solutions serial dilutions were done ranging from 100ppm-0.01pm. Triethyl Phosphate is used as an Internal Standard (IS) to normalize the signal. IS were taken about 1ppm Concentration.

SPECIFICITY

Blank Interference

1µL of blank sample wasinjected into the LC-MS system as per the proposed test method. Evaluated the interference of blank at the retention time of targeted analyte peaks and foundno peaks at the retention time of targeted peaks. The results are Figure-4.

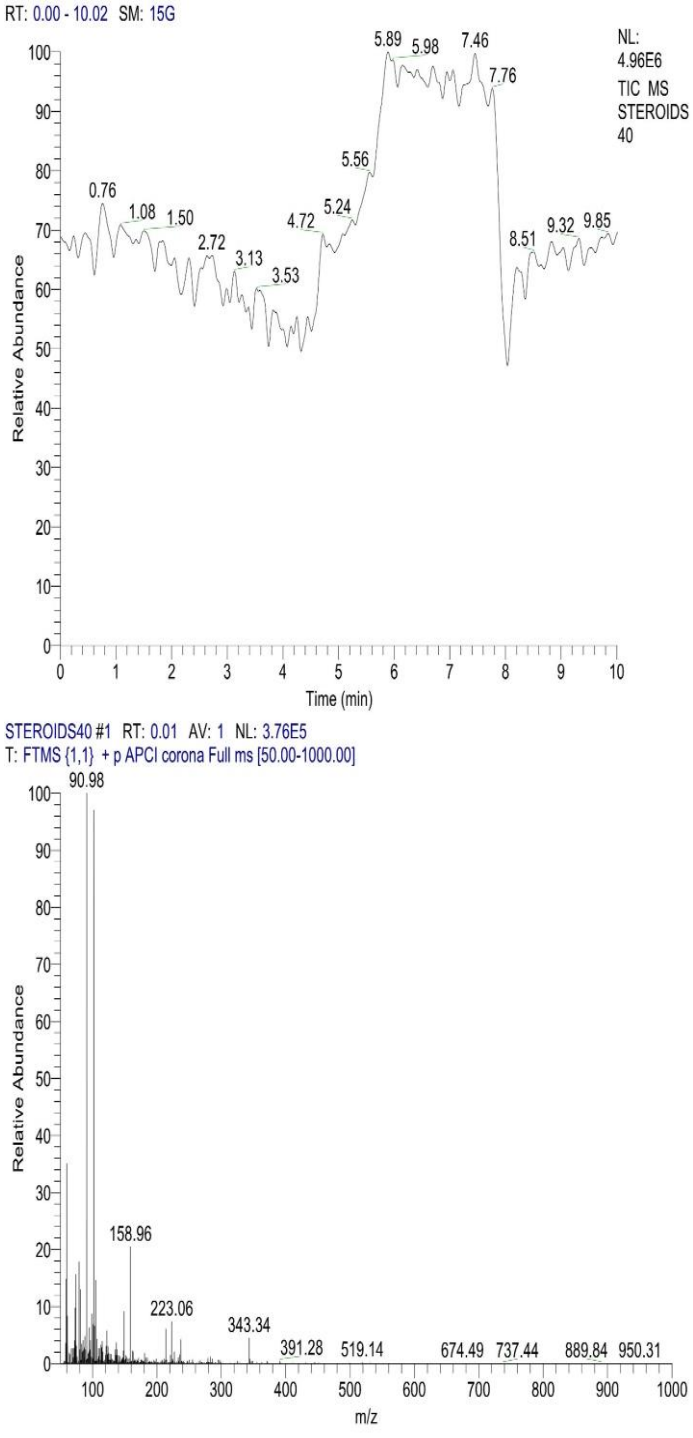


Figure 4: Chromatogram for Blank showing no interferences

ESTABLISHMENT OF LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

A study was conducted to establish the limit of detection (LOD) and limit of quantification (LOQ) of Hydrocortisone, Cortisone, and Corticosteronebased on slope method. Prepared a series of solutions from 10ppm to 0.01ppm of standard concentration of Hydrocortisone, Cortisone, and Corticosterone. These solutions were injected into the LC-MS system as per methodology.

Plotted a graph by taking concentration on X-axis and area on Y-axis, calculated the standard error and slope of the calibration curve. The predicted LOQ concentration and LOD concentration are calculated by using formula given below.

$$LOQ = \frac{10 \times \sigma}{S} \qquad LOD = \frac{3.3 \times \sigma}{S}$$

σ = Standard Error of the calibration curve
S = Slope of the calibration curve

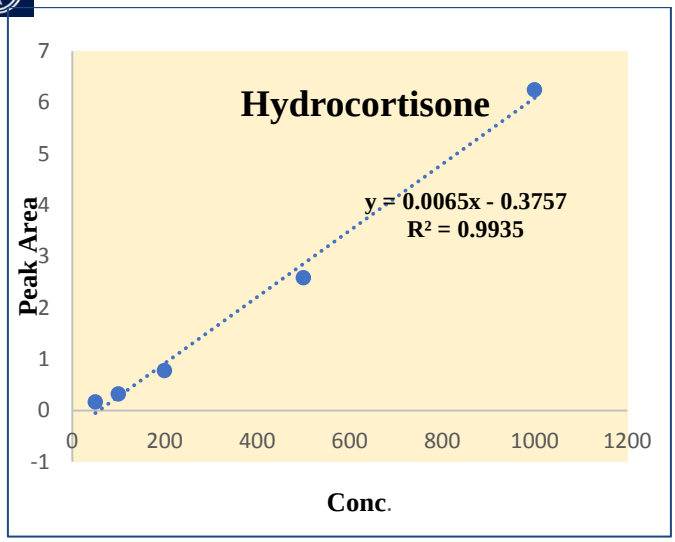
LINEARITY

Linearity is carried out under LOD-LOQ establishment experiment, the same linearity establishment data can be used to deduce the linearity from LOQ level to 150% (0.01ppm) specification level. A graph was plotted to concentration in ppm on X-axis versus response on Y-axis. Calculated % y-intercept and correlation coefficient which were shown in Graph 1-3.

LINEARITY GRAPHS ARE REPRESENTED AS FOLLOWS.

S.No.	Conc. in ppm	Area Response
1.	50	0.166116
2.	100	0.32175
3.	200	0.772807
4.	500	2.585433
5.	1000	6.243323

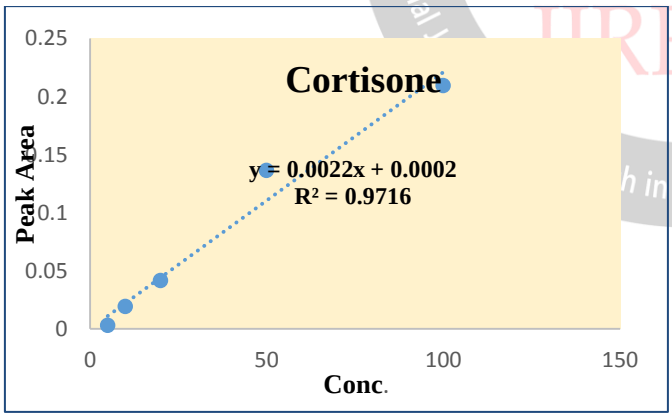
Table 1: Conc. in ppm and Peak Area response showing for Hydrocortisone



Graph 1: Linearity for Hydrocortisone

CORTISONE		
S.No.	**Conc. in ppm**	**Area Response**
1.	5	0.003033
2.	10	0.019308
3.	20	0.04147
4.	50	0.136336
5.	100	0.209421

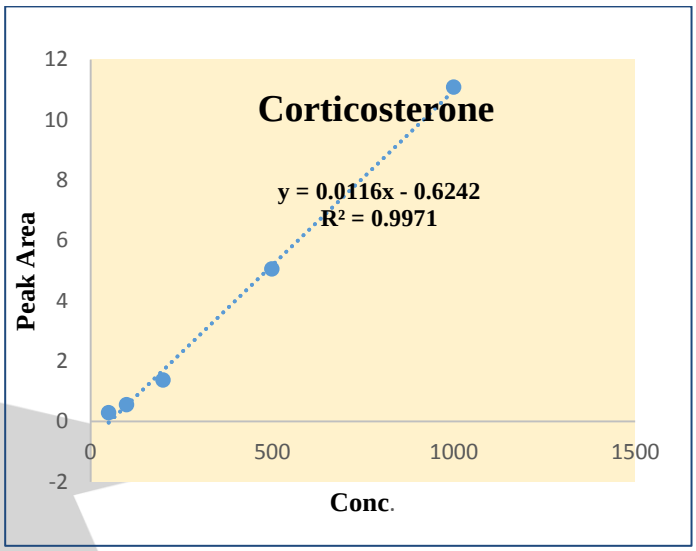
Table 2:Conc. in ppm and Peak Area response showing for Cortisone



Graph 2: Linearity forCortisone

CORTICOSTERONE		
S.No.	**Conc. in ppm**	**Area Response**
1.	50	0.286297
2.	100	0.547835
3.	200	1.365449
4.	500	5.05465
5.	1000	11.08481

Table 3: Conc. in ppm and Peak Area response showing for Corticosterone



Graph 3: Linearity for Corticosterone

PRECISION

Intra-day precision, or within-day reproducibility, is expressed as the average of the relative standard deviation (RSD%) of the areas obtained for each analyte after the replicate (n=6) analysis. The results were shown that within the acceptable limits as per ICH guidelines.

ACCURACY

Accuracy is carried out by taking Concentration ranges as 5ppm, 50ppm and 500ppm. Results shown that average recoveries not less than 80% and the %RSD are within the limited ranges which are shown in Table 4-6.

HYDROCORTISONE			
S.No.	**5ppm**	**50ppm**	**500ppm**
1.	0	103.95	88.94
2.	69.09	110.53	105.19
3.	101.68	97.56	103.19
4.	124.04	101.90	95.96
5.	111.33	113.68	99.15
Average (NLT 80%)	101.54	105.53	98.49
SD	23.49	6.53	6.42
%RSD	23.13	6.19	6.52

Table 4: Accuracy for Hydrocortisone

CORTISONE			
S.No.	**5ppm**	**50ppm**	**500ppm**
1.	0	105.26	93.17
2.	75.19	104.13	103.00
3.	97.72	90.60	108.44
4.	86.47	99.42	107.35
5.	100.26	118.34	93.94
Average (NLT 80%)	89.91	103.55	101.18
SD	11.50	10.08	7.26
%RSD	12.79	9.74	7.17

Table 5: Accuracy for Cortisone

CORTICOSTERONE			
S.No.	**5ppm**	**50ppm**	**500ppm**
1.	0	105.26	93.17
2.	106.22	104.13	103.00
3.	130.70	90.60	108.44
4.	125.36	99.42	107.35
5.	116.22	118.34	93.94
Average (NLT 80%)	119.63	103.55	101.18
SD	10.75	10.08	7.26
%RSD	8.99	9.74	7.17

Table 6: Accuracy for Corticosterone

ROBUSTNESS

Similarly, Robustness also evaluated and found that the method is robust enough for various robustness parameters such as flow variation, column temperature variation, mobile phase composition variation.

All the system suitability criteria are meeting in all the robust parameters, this indicates that the proposed analytical method is robust enough for the estimation of Hydrocortisone, Cortisone, and Corticosterone by using the analytical method.

APPLICATION TO REAL-SAMPLE ANALYSIS

SAMPLE PREPARATION

The developed method was applied successfully to the Blood Serumsamples collected for monitoring multiresidue analysis. 200µL of Blood Serumis taken in a vial and store it at -20^0 C temperature.

EXTRACTION PROCEDURE OPTIMIZATION

200µL of Blood Serum is taken in a vial, added 400µL ACN and 20µL of Triethyl Phosphate as an Internal Standard of 1ppm concentration to it and sonicated it for 10mins, shake it by using horizontal shaker at the speed of 250ppm for 15mins and centrifuged it for 15mins. Remove the supernatant, add 10ml of Milli Q water and centrifuged it and add 10ml of methanol and again centrifuge for 15mins and collect the supernatant. Evaporate the solvent by using rotary evaporator.1µL is injected in the LC-MS and run the whole method as discussed above. The results obtained is that of targeted compounds is well detected in the Blood Serum residue by using developed method.

IV. RESULTS AND DISCUSSION

A simple, economic, accurate and precise LC-MS method was successfully developed. In this method, it was carried out by using Column C18, (33× 4.6mm) with 3µm particle size. Injection volume of 1µl is injected and eluted with the mobile phase A and B over gradient program, which is pumped at a flow rate of 0.8 ml/min. Detection, was carried out by mass spectrometry. All the compounds are well resolved from blank peak and there is no interference from blank. The results obtained were accurate and reproducible. The method developed was statistically validated in terms of Selectivity, accuracy, linearity, precision, robustness.

For Selectivity, the chromatograms were recorded for standard and sample solutions of Hydrocortisone, Cortisone, and Corticosterone. Selectivity studies reveal that the peak is well separated from each other. Therefore, the method is selective for the determination of Hydrocortisone, Cortisone, and Corticosterone.

The limit of detection (LOD) and limit of quantitation (LOQ) was found to be for 0.001µg/ml, 0.003µg/ml, 0.006µg/ml, 0.001µg/ml, 0.002µg/ml, 0.001µg/ml and 0.004 µg/ml respectively for Hydrocortisone, Cortisone, and Corticosterone. The linearity results for Hydrocortisone, Cortisone, and Corticosteronein the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.95. Calibration curve was plotted and correlation co-efficient for Hydrocortisone is 0.9935,Cortisone is 0.9716, and Corticosterone is 0.9971.

The accuracy studies were shown as % recovery for Hydrocortisone, Cortisone, and Corticosteroneat 50%, 100% and 150%. The limit of % recovered shown is not less than 80% and the results obtained were found to be within the limits. Hence the method was found to be accurate. The accuracy studies showed % recovery of the Hydrocortisone, Cortisone, and Corticosterone in the range 98-100% respectively.

For Precision studies six (6) replicate injections were performed. %RSD was determined from the peak areas of Hydrocortisone, Cortisone, and Corticosterone The acceptance limit should be not more than 13 %RSD, and the results were found to be within the acceptance limits. For intermediate precision, the bias is not more than ± 1.0.

In the real sample, the targeted is detected in Blood Serum about 0.04ppm trace levels. Hence, the chromatographic method developed for Hydrocortisone, Cortisone, and Corticosterone are rapid, simple, sensitive, precise, and accurate. Therefore, the proposed method can be successfully applied for the routine analysis of the steroid residue analysis for assurance of its presence in matrices.

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