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A Comprehensive Review on analytical methods used for analysis of Steroid genesis inhibitors

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ABSTRACT: Liquid chromatography (LC) is the most widely used analytical separation method. The development and validation of analytical methods is important for drug discovery, development and production research. More than 85% of drugs are analyzed using high performance liquid chromatography (HPLC), one of the most popular analytical techniques. The design should be as simple as possible and allow the use of advanced technology. Reducing the amount of cortisol produced by the adrenal cortex is an important part of many medications used to treat Cushing's syndrome, such as steroidogenic enzyme inhibitors. Ketoconazole, metryapone, mifepristone, mitotane, etomidate, and osilodrostat are steroidogenic enzyme inhibitors. UV, HPLC, LC-MS/MS, etc. many articles have been published on methods of producing these chemicals using a variety of analytical methods, including the main purpose of this review is to review the methods used to evaluate drugs belonging to the family of steroidogenic enzyme inhibitors. It will also widen the gap between current research and the urgent need to develop new technologies for certain drugs with little available technology.

KEYWORDS: Liquid Chromatography, Analytical Methods, HPLC, Steroidogenesis Inhibitors

I. INTRODUCTION

Liquid chromatography (LC) is the most widely used separation method. The method is widely used for business and various research important products, and its precision, willingness to adapt to various orders, ease of automation, suitability for the separation of non-volatile products, and suitability for automation are the reasons for this position [1]. Chemical separation is done by chromatographic methods and to a lesser extent by electrophoretic methods. Separation during chromatography is based on the variation of the distribution between the two separate phases (stationary and mobile). Separation of analytes based on changes in measured mobility in liquid media is the basis of electrophoretic techniques. The main purpose of chromatographic separation is to separate substances (for example) in the sample. The distribution of the solvent between the two separate phases (mobile and stationary) is the main part of the process used for each individual chromatographic column. Each time, the mobile time moves the sample mixture from the end of the sample injection to the other end of the line [2].

Method Development

The analytical development, production and discovery of pharmaceuticals depends on the development and validation of analytical methods [3]. Before creating a method, it is necessary to choose its requirements and decide which tools to use and why [4]. An appropriate analysis strategy must be chosen to manage the editing sequence. Analytical strategies should be developed using the methods and validation procedures outlined in ICH Guidelines Q2(R1)before being implemented in a GMP and GLP environment [5].

Important requirements for the development of analytical methods for new drugs

- If the drug or combination of drugs is not listed in a any pharmacopoeias
- If there is a patent issue that prevents the use of appropriate analytical methods
- Analytical methods for drugs in design will not be available due to the influence of formulation excipients.

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- Analytical techniques may not be available to measure drug concentrations in biological fluids or to mix drugs with other drugs [3].

HPLC Method Development

One of the most widely used methods for is High Performance Liquid Chromatography (HPLC). More than 85% of drugs are analyzed by HPLC. HPLC is a separation module consisting of a stationary phase and a mobile phase of different polarity, both of which are usually equipped with a high pressure pump. Stationary and mobile phases work together for separation. Appropriate selection of stationary and mobile phases should create the desired separation. The pH of the cell, various parameters, column temperature, sample diluent, detection wavelength and many other factors are important in the design process. To achieve the desired results, the number of experiments required to establish a good method should be kept to a minimum. Finally, it should be possible to create a process as simple as possible while allowing the use of technological tools such as computer modeling [6].

When developing a High Performance Liquid Chromatography (HPLC) system, the following steps are typically performed:

Step 1: Choosing the HPLC procedure and starting system
Step 2: Choosing the initial circumstances
Step 3: Method Development
Step 4: Method Validation

Steps for HPLC method development

Step 1: Choosing the HPLC procedure and starting system

In HPLC method development, the design was not full housing when you are not encountering. Avoiding useless experiments saves time. When choosing an HPLC system, it should be able to identify samples with high probability. For example, if a sample contains polar analytes, reverse phase HPLC provides adequate retention and resolution, while conventional HPLC is less efficient [7].

Sample collection and processing

Preferably, the sample should be dissolved in the first mobile phase. If stability or solubility problems prevent this, the solubility of the sample can be increased by adding formic acid, acetic acid or salt. Additives generally do not affect separation as long as the volume of the injected sample is equal to the volume. After sample injection, more than one or two peaks can be separated in a blank volume using only large samples. Sample preparation is an important step in HPLC analysis as it creates a homogeneous solution suitable for injection into the chromatography column. The purpose of sample preparation is to create aliquots of samples with the following properties: is compatible with the specified HPLC method with little interference, does not damage the column, and dissolves heavy sample in the mobile phase without affecting sample retention or solubility [4].

Column Selection:

Column is an essential part of the HPLC system. High quality silica and bonding will be repeatable and consistent with guaranteed accuracy. Each of the RP C18 (USP L1), C8 (USPL8), Phenyl (USP L11) and Cyno (USP L18) columns are widely used. There is no good or bad columns. The two different phases of bone showed a large variation in selectivity when using the same mobile phase. Different manufacturers have different pore volumes, pore sizes, surface areas, particle sizes, carbon charges and whether they are capped or not. In addition, line length has an important effect on solving the separation problem [6].

Step 2: Choosing the initial circumstances

In particular, it ensures that there is no less than zero screening test. 5 (weak fuse causes conflict) or more than 10-15 (over fuse causes long search time, high altitude and is not recommended). This step determines the best conditions for adequate storage of each parameter. [8]

Step 3: Method Development

Finding the "weaknesses" of the method will help you develop using test models. Know how the process accommodates variables, metrics, and model types [9]. In the optimization of the development of high-performance liquid chromatography technology, the development of high-performance liquid chromatography conditions has been most popular. It is necessary to take into account the composition of the stationary and mobile phases. Optimization of the mobile phase is not always given priority as it is more efficient and easier than the development of the stationary phase [10]. Some of the different tests to check are: HPLC parameters such as % organic

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content, pH, flow rate, temperature and age. Sample preparation considerations include sample size, aging time, organic content, pH, sonication and agitation. Calculation and normalization methods include wavelength integration, response modulation, and quantitative methods [11].

Step 4: Method Validation

The concept of validation was introduced in the United States in 1978. Over time, the word "validation" has evolved to encompass many activities, such as computer systems used in clinical research, control procedures or labels, and analytical procedures used to determine the efficacy of medical components and medical devices. The best way to think about usability is the importance and importance of cGMP. The word "verification" describes the process of determining whether something is true or valid. Members of various factory departments work together in the witnessing process. The verification method is a "evidence building method" that provides a high degree of assurance that a product (device) will meet the requirements of the proposed analytical application [12].

The validation parameters are as follows:

1. Accuracy
2. Precision
3. Linearity
4. Limit of detection
5. Limit of quantitation
6. Specificity
7. Range
8. Robustness
9. Ruggedness

Accuracy: The accuracy of the analytical method is defined as the degree of agreement between the observed value and the value considered to be the actual or reference value. The accuracy of the method is defined as the fit of the amplified method (also known as spiking) and the difference between the measured values of the reinforced and non-reinforced samples.

Precision: The precision of the analytical method is defined as the consistency of the method measuring several samples of the same sample under different conditions. Reproducibility, intermediate precision, and reproducibility are the three types of precision.

1. Repeatability: Repeatability is a measure of the accuracy of measurements made over a short period of time during the same process.
2. Intermediate precision: This level of precision can be used for example different days, analysts, products, etc. represents change in the laboratory due to many factors.
3. Reproducibility: Reproducibility refers to agreement between laboratories.

Linearity: Linearity is the ability of an analytical method to produce test results that are directly proportional to the concentration (amount) of the analyte in the sample.

Limit of Detection: The limit of detection for a given analytical method is the lowest amount of analyte that can be detected in a sample but does not need to be measured accurately.

Limit of Quantitation: The limit of quantitation of analytical methods refers to the minimum number of analytes that can be counted with accuracy and precision in the sample.

Specificity: The ability to clearly determine whether a test has the required properties is now called specificity.

Range: The range of the analytical method is the difference between the top and bottom (range) of the analyte in the sample containing the concentrations the sample has shown to have the required sensitivity, accuracy, and linearity.

Robustness: The term "robustness" refers to the ability of a method to remain unaffected by small but deliberate changes in the process. This tool can be used as usual to measure the reliability of the method.

Ruggedness: The United States Pharmacopoeia (USP)10 (USP) defines ruggedness as the degree of consistency of test findings generated by the analysis of the same samples under a variety of common test circumstances, including as separate labs, different analysts, and different batches of reagents. The ability of test results to be duplicated throughout laboratories and analysts in normal, foreseeable operational circumstances is measured by ruggedness [2].

Importance of validation

1. Guaranteed high standard.
2. Limitation of time.enhancing the process.

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3. Minimum batch product failure,
4. Improved productivity and manufacturing.
5. Costs for quality fell.
6. Rejection fell off.
7. The yield rises.
8. Less complaints regarding problems with the process.
9. Quick and realistic equipment startup.
10. Increased process awareness among the workforce [13].

Guidelines for validation

1. Definitions and terminology from the ICH Q2A book on the validation of analytical processes (March 1995).
2. Methodology for ICH Q2B validation of analytical techniques (June 1997).
3. FDA (Draft) Industry Guidance: Analytical Procedures and Method Validation
4. United States Pharmacopoeia (USP) and European Pharmacopoeia (EP) pharmacopoeias [5].

Steroidogenic Inhibitors

Although the various steroidogenic enzyme inhibitors used in CS have different modes of action, they all reduce cortisol produced by the adrenal cortex. With many years of clinical experience, steroidogenic enzyme inhibitors are still the drug of choice for the treatment of hypercortisolemia in CS. The use of steroidogenic enzyme inhibitors has been recently reconsidered. Although its efficacy and adverse effects are greater than expected and less than expected, it is still an important agent in the treatment of hypercortisolemia in various CS, as it has not undergone rigorous clinical trials. They are used to block other methods or dose titration. In dose titration, the drug is given in an initial dose and then the dose is increased according to the biochemical response. To avoid adrenal insufficiency, block and change the target to avoid cortisol completely. Generally, higher doses are initiated, immediately maximized, and then replaced with glucocorticoids [14].

Some journals show the development of research for many drugs belonging to the class of steroidogenesis inhibitors. The following are drugs that fall in class above,

i. Ketoconazole
ii. Metryapone
iii. Mitotane
iv. Etomidate
v. Osilodrostat

Ketoconazole

Chemically, Ketoconazole is Cis-1-acetyl-4-[4-[2-(2,4-dichlorophenyl)-2H-imidazolyl] methyl]-1,3-dioxolan-4-yl] methoxy] phenyl]-piperazine. It is an imidazole derivative, originally used as an antibiotic. It works by inhibiting various enzymes (cholesterol side chain cleavage complex, 17,20-lyase, 11-hydroxylase and 17-hydroxylase) involved in adrenal steroidogenesis, thereby reducing cortisol synthesis. The drug is approved to treat CS patients 12 years of age and older. In addition to the adrenal blocking effect, ketoconazole can directly affect corticotropic tumor cells in CD patients. Ketoconazole, an imidazole derivative that has been shown to be effective in the treatment of fungal infections, is given orally at a dose of 200-1200 mg two or three times a day[15]. Ketoconazole is a poor drug that is insoluble at higher pH. Ketoconazole has a Biopharmaceutical Classification System (BCS) Class II designation with poor solubility and high permeability [16].

Viviane Annisa et al. Estimation of Ketoconazole in Phosphate Buffered Salt Water at pH 6.8 by modified isocratic separation method using RP-HPLC, UV technique and reverse phase columns in the year 2022. The method should be validated against ICH requirements for resolution studies that require linear, accurate, precise, specific and analytical measurements. HPLC was performed using a Phenomex Luna (250 4.6 mm, 5 µm) column with a mobile phase containing acetonitrile:water (50:50) and TEA 0.15% pH 3.3 with UV detection at 232 nm. The difference coefficient is set to 0.5 and 12 g/mL at r2 = 0.9997. The return rate varies between 100% and 101%. The %RSD of watches is less than 1.74%. LOD and LOQ are 0.15 and 0.45 g/mL, respectively [17].

Saurabh Shrivastava et al. Determination of ketoconazole content in pharmaceutical preparations in 2020 using a new UV spectrophotometric method. Phosphate Buffer 6. It is the solvent used to create the 208 nm in the wavelength range of 200 to 400 nm. Beer's law is followed in the concentration range of 10 to 60 g/mL. The aim is to develop and implement methods based on ICH requirements, including robustness, linearity, accuracy and detection and

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quantitation limits. The average correlation coefficient was found to be 0.999. It has been found to be accurate between 98.99% and 99.32%. Ketoconazole was shown to have a 0.10 to 0.56 intraday RSD and a 0.13 to 0.55 interday RSD. The method is considered correct because the RSD percentage is less than 0.2%. The results showed that the LOD and LOQ were 1.37 and 4.07 g/mL, respectively [18].

Metyrapone

A pyridine derivative called Metyrapone reduces cortisol synthesis by inhibiting several enzymes involved in adrenal steroidogenesis (cholesterol side chain cleavage, 17-hydroxylase, 18-hydroxylase, and especially the 11-hydroxylase complex). The drug is approved for the treatment of patients with CS. Metyrapone is an oral medication that should be taken in doses of 500 to 6000 mg four to six times a day[15]. About 50-60% of patients with Cushing's syndrome receive short-term metyropone before surgery or while awaiting adrenalectomy or radiation therapy (if pituitary surgery fails) [19].

Brett C. McWhinney et al. developed a high-performance liquid chromatography-tandem mass spectrometry (UHPLC MS/MS) method with ultraviolet detection for the simultaneous measurement of cortisol, cortisone, 11-deoxycortisol, prednisolones, and thyroxine. The aim is to detect a variety of endogenous and exogenous glucocorticoids in a variety of biological matrices using quick and easy sample preparation. The chromatography process takes 3 minutes. The limit for cortisol and cortisone in plasma is 3.75 nmol/L and goes up to 2000 nmol/L. For cortisol 0.6 nmol/L is the limit for the amount of saliva and plasma ultrafiltrates. The measurement limits for prednisolone, 11-deoxycortisol, and dexamethasone are all 5 nmol/L. For all analyzes in all matrices, the CV test is 5% and the CV test is 10%. The correlation coefficient for comparison with immunoassay (IA) plasma cortisol is UHPLC = 0.79 IA + 31.12, R2 = 0.960 (p = 0.0001). The regression equation for comparison with high performance liquid chromatography (HPLC) cortisol is UHPLC = 1.06 HPLC + 9.82, R2 = 0.992 (p 0.0001) [20].

Neila M. Cassiano et al. have designed and validated the direct injection high pressure liquid chromatography (HPLC) method for measurement of metyrapole enantiomers and metyrapone in human blood by colon transfection. The technique used consists of an amylose tris (3,5-dimethoxyphenylcarbamate) chiral column connected to a bovine serum albumin (BSA) octyl column with restricted access medium (RAM). The eluent used for the conversion and analysis of metyrapole enantiomers and metyropone was acetonitrile-water (30:70 v/v) with UV detection at 260 nm. Plasma proteins are first eluted with water at a flow rate of 1 milliliter per minute for the first five minutes. The entire screening took about 32 minutes. Calibration curves for each enantiomer and metyrapone are linear over concentration 0.075-0.75g/ml and 0.150-1.50g/ml, respectively. Three positive controls, one for low plasma concentrations (0.18 g/ml), one for medium concentration (0.75 g/ml) and one for high concentration (1.35 g/ml), were used to measure mean day-to-day precision and accuracy. The amount limit of quantifiation for metyrapone and both enantiomers is 0.045 g/ml [21].

Mitotane

Mitotane is a medicine made from amphenone B used to treat advanced or terminal adrenocortical cancer. It is mainly cytotoxic to the adrenal glands, but also blocks the enzyme that breaks down the side chains of triglycerides. Many side effects of mitotane include neurotoxicity, nausea, and vomiting. It is used only in some special cases of CD after careful consideration [22]. An off-label treatment for CS is called mitotane. It reduces cortisol synthesis by blocking several enzymes involved in adrenal steroidogenesis (cholesterol side chain cleavage, 11-hydroxylase, 18-hydroxylase, and 3-hydroxysteroid dehydrogenase). Oral doses of mitotane range from 1 to 12 grams per day [15].

Giacomo Luci et al. In 2021, he achieved a simple and effective HPLC-UV method using human blood samples to identify mitotane (DDD) and its major metabolites. Dichlorodiphenylethene (DDE) and dichlorodiphenylacetic acid ester (DDA) are its two main metabolites. Fixed level C18 column (250 mm x 4.6 mm x 5 µm) for chromatographic separation at 350 Â°C. The mobile phase is water with a volume ratio of 10:90 and acetonitrile, a flow rate of 1.0 mL/min, and absorbance measured at 226 nm. DDD (r2 = 0.9988, range 1-50 mg/L) and DDE (r2 = 0.9964, range 1-40 mg/L), with an average recovery of 95% for all chemicals analyzed. The lower and upper limits of calculation for DDD and DDE are

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0.102 and 0.310 mg/L and 0.036 and 0.108 mg/L respectively. The retention times of DDD, DDE, and IS were 7.06, 9.42, and 12.60 minutes, respectively [23].

Motozumi Ando et al. 2019 adopts GC-EI-MS (Gas Chromatography-Electron Ionization-Mass Spectrometer) technique. The aim is to develop a method to measure mitotane in plasma using gas chromatography, electron ionization and mass spectrometry. Total run time was 12 minutes with mitotane and IS retention time of 8.2 and 8.7 minutes, respectively. The standard deviation is 0.25 g/mL. The calibration curve is linear from 0.25 to 40 g/mL with a coefficient of determination of 0.992. The % recovery of this method was found to be true [24].

Etomidate

Etomidate as a chemotherapy drug is an imidazole. Another potent steroid hormone production inhibitor is etomidate. Inhibits cortisol synthesis by 11-hydroxylase. Parenteral administration started in a short time[22]. Etomidate potently inhibits 11b-hydroxylase and to a lesser extent 20,22-desmolase. It is given by injection into a vein and works very quickly. An anesthetic dose of 0.04 mg/kg over 30 minutes reverses 11-hydroxylase inhibition, blunts the cortisol response to ACTH, and increases the cortisol precursor (11-deoxycortisol) [25].

K. Bhavyas et al. have Developed and validated the simple UV spectrophotometric and stress test for the determination of etomidate in bulk and commercial formulations. When using 75:25 distilled water and methanol as the diluent, etomidate exhibits maximum absorption at a wavelength of 243 nm. The drug is linear at concentrations between 0.1 and 34 g/ml. The correlation coefficient for this method is 0.9994. The percentage of etomidate recovered using this formula decreased from 50%, 100%, and 150% to between 98% and 100.89%. The % RSD precision value was found to be less than 2%. After examining the acid hydrolysis, alkaline hydrolysis, oxidation, photolysis and forced degradation of thermal processes, a decrease in absorbance was observed [26].

Yu-Kyung Jung et al. used liquid chromatography-tandem mass spectrometry (LC-MS/MS). Simultaneous determination of etomidate (ET) and etomidate acid (ETA) in urine in 2019 by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Samples were separated at 6.5 minutes using reverse phase chromatography with gradient elution for all analytes. ETA was isolated and fixed using graphite carbon fibers held at a temperature of 300 Â°C. The coefficient of determination (r2) is greater than 0.9958. Calibration curve is linear over the concentration range of 0.4-120.0 ng/mL (ET) and 1.0-300.0 ng/mL (ETA). The lower limit of product was found to be 0.4 ng/mL (ET) and 1.0 ng/mL (ETA); Actual values for all drugs for intra-day (n=6) and inter-day (n=24) measurements were below 10.2% and 8.4%, respectively [27].

Kalpana Gopagani et al. developed and validated a liquid chromatography (HPLC) method for the determination of etomidate (ETO) injection. The method was developed using a Waters HPLC system on a Develosil-ods-UG column with dimensions of 300 x 3.9 mm and a particle size of 5 µm. The mobile phase consists of acetonitrile and phosphate buffer at a volume flow rate of 1.5 ml and a volume of 1.5 µL in a ratio of 40:60, v/v. The shelf life of the test is 12.061 minutes, measurement wavelength is 254 nm. The difference between etomidate is 40-240 g/mL. Studies of etomidate recovery have shown from 98% to 102% [28].

Osilodrostat

Osilodrostat is a small dose oral 11-hydroxylase inhibitor developed by Novartis for the treatment of Cushing's disease. The enzymes 11-hydroxylase [cytochrome P450 (CYP11B1)] and aldosterone synthase (CYP11B2, steroid 18-hydroxylase) that catalyze the last step of cortisol synthesis are strongly inhibited by osilodrostat[29]. Osilodrostat is a new drug that acts on the adrenal glands. Because it reduces cortisol production by inhibiting 11-hydroxylase, it has a new and possible treatment for CD and theoretically all types of CS. The most common side effects of this drug are fatigue, gastrointestinal problems, adrenal insufficiency, headache, and hyperandrogenism [15].

Janki Patel et al. 2023 with a stable reverse-phase high-performance liquid chromatography (RP-HPLC) method. The aim is to develop and validate an RP-HPLC method for Osilodrostat estimation in API form. Chromatographic separation was performed on a stationary phase C18 column (250 mm x 4.6 mm x 5 µm) and the mobile phase was made of

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phosphate buffer pH 3.2 and formaldehyde at a ratio of 40:60 with a flow rate of 1.0 mL/min. The absorbance was measured at a wavelength of 240 nm. Osilodrostat has been shown to have a retention time of 5.160 minutes. A range of 10 to 30 g/ml was found for o Osilodrostat. LOD and LOQ were found to be 0.0015 g/ml and 0, respectively.048 g/ml [30].

David Balakirouchenane et al. He developed the LC-MS/MS method. He developed and implemented a liquid chromatography tandem mass spectrometry (LC-MS/MS) method in 2023 for the simultaneous measurement of Osilodrostat and metyrapone in human blood. On KinetexÂ® HILIC (4.6, 50 mm; 2.6 µm) analytical column, chromatographic separation by isocratic elution at 2.0 min run time. The process is linear from 0.5 to 250 ng/mL for ODT and 2.5 to 1250 ng/mL for MTP. Precisions within and between tests were 7.2%, with a 95.9%–114.9% accuracy range. While the IS-normalized matrix effect ranged from 106.0% to 123.0% (ODT) and from 107.0% to 123.0% (MTP), respectively, the IS-normalized extraction recovery ranged from 84.0 to 101.0% for ODT and 87.0 to 101.0% for MTP. The LC-MS/MS method was successfully applied to patient plasma samples (n = 36), and the trough concentrations of ODT and MTP varied from 2.7 ng/mL to 8.2 ng/mL and from 10.8 ng/mL to 27.8 ng/mL, respectively [31].

Wenkui Li et al. developed and used a new liquid chromatography system with tandem mass spectrometry discovery(LC- MS/ MS) for the determination of LCI699, a steroid 11- hydroxylase asset with dynamic ranges of0.0500-50.0 ng/ mL and1.00- 1000 ng/ mL using0.0500 mL and0.100 mL, independently, of mortal tube. Both the standard(M 6) LCI699 and LCI699 deduced from mortal blood were used. Chromatographic separation was performed on an ACE C18(50mm x 4.6mm, 3µm) column with a 30 methanol waterless result containing 0.5 acetic acid and 0.05 TFA as the mobile phase at a flow rate of 1.0 mL/ min. The entire check cycle takes about 3.5 twinkles each time. For the low wind range(0.0500-50.0 ng/ mL), the LOQs' delicacy and perfection were 13.0 to2.0 bias and 3.4-19.2 CV, independently. For several QC samples(0.100,6.00,20.0, and40.0 ng/ mL), the perfection ranged from 1.2 to 9.0 and from 3.8 to 8.8 CV, independently. The LOQs(1.00 ng/ mL) for the high wind range(1.00- 1000 ng/ mL) had delicacy and perfection of 1.0-15.0 bias and 7.4-9.2 CV, independently. In the intra-day and inter-day evaluations for the fresh QC samples(3.00,20.0, 200, and 750 ng/ mL), the perfection ranged from0.8 to7.0 and from1.9 to5.2 CV, independently [32].

II. CONCLUSION

Steroidogenesis inhibitors include Metryapone, Mifepristone, Mitotane, Etomidate, and Osilodrostat. Reducing the amount of cortisol produced by the adrenal cortex is an important part of many medications used to treat Cushing's syndrome, such as steroidogenic enzyme inhibitors. There have been a number of studies published on the method development for these medications utilizing different analytical techniques as UV, HPLC, LC-MS/MS, etc. Therefore, we decided that there weren't many ways to focus on these types of drugs. Currently, there is only one HPLC and few other analytical methods for osilodrostat. There is still a lot of room for research in the future on steroidogenesis enzyme inhibitors, especially osilodrostat, and much room for development using a variety of tests.

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