Review Article

Improved Method for Differentiation of Synthetic and Natural Endogenous Anabolic Steroids using Gas Chromatography Isotope Ratio Mass Spectrometry (GC/C/IRMS) followed by Two-Fold High Performance Liquid Chromatography (HPLC) Cleanup Method: A Perspective

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ABSTRACT

Background: Androgenic Anabolic Steroids (AAS) are also synthetic derivatives of testosterone, modified to improve its anabolic actions. The misuse of AAS is of particular concern in sports and society. Gas chromatography-mass spectrometry had some limitations and allows identification and characterization of steroids and their metabolites in the urine but may not be able to distinguish between pharmaceutical (Exogenous) and endogenous origin. Thus, it is of great importance to discriminate endogenous steroids such as testosterone or testosterone prohormones from their chemically identical synthetic copies. The abuse of Androgenic Anabolic Steroids (AAS) by sports person is banned by World Anti-doping Agency (WADA) as per the WADA Prohibited list 2019. **Methodology:** The gas chromatography-combustion/isotope ratio mass spectrometry (GC/C/IRMS) technique differentiates between natural and synthetic endogenous steroids by comparing compounds specific $^{13}C/^{12}C$ ratio. However, the analytes have to be efficiently isolated and purified prior to GC/C/IRMS analysis. **Results and Discussion:** HPLC Cleanup method prior to analysis by GC–C/IRMS needs to be developed and validated for discriminating the origin of anabolic androgenic steroids. These methods involves the solid-phase extraction, enzymatic hydrolysis with β-glucuronidase, HPLC-fractionation for the cleanup and analysis by GC–C/IRMS. The difference ($\Delta^{13}C$) of urinary $\delta^{13}C$ values between synthetic analogues and Endogenous Reference Compounds (ERC) by GC-C/IRMS would be used to elucidate the origin of steroids. The present perspective gives an overview of the use of anabolic-androgenic steroids in sport and methods used in anti-doping laboratories for their detection in urine, with special emphasis on GC–C/IRMS technique after two-fold HPLC cleanup.

Key words: Endogenous Steroids, Exogenous Steroids, HPLC Cleanup, Doping, GC/C/IRMS.

Submission Date: 03-05-2019;
Revision Date: 04-09-2019;
Accepted Date: 30-09-2019

DOI: 10.5530/ijper.53.4s.139

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INTRODUCTION

The misuse of synthetic endogenous steroid copies is one of the most important issues in sports. Athletes may abuse steroids or manipulate metabolic pathways in an attempt to increase concentrations of biologically active steroids with the intent of enhancing athletic performance through increased muscle mass and more rapid recovery from injury or intense training.[1] Therefore, the administration of steroids has been strictly prohibited by World Anti-Doping Agency (WADA) and it is necessary to discriminate endogenous steroids from their chemically identical synthetic copies

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for elucidating the origin of steroids.[2] Traditionally, the GC-MS method has been used for the detection of steroids.[3] However, this method has limitation on distinguishing endogenous steroids such as testosterone or testosterone prohormones from the chemically identical endogenous synthetic copies. In steroids, differences in ^{13}C content arise because synthetic steroids are derived from plant sterols such as stigmasterol and sitosterol obtained from C-3 plants that constitute about 90% of all plants.[4] They usually exhibit depleted $^{13}C/^{12}C$ ratio ($\delta^{13}C$) values in range −25.9‰ to −35.6‰ in comparison with the values for endogenous steroids.[5] Based on 13C isotopic differences, the measurement of δ13C of steroids is highly regarded to elucidate the origin of steroids and the use of gas chromatography combustion isotope ratio mass spectrometry (GC-C/IRMS) has proven to be the unique analytical technique of choice in this field.[6-10] Thus, the analysis and difference ($\Delta^{13}C$) of urinary $\delta^{13}C$ values between synthetic analogues and Endogenous Reference Compounds (ERC) such as 11-keto-etiocholanolone, 11β-OH-androsterone and pregnanediol allows endogenous steroids to be distinguished from their synthetic analogues in the urine and provides significant information that they have not administrated synthetic analogues of endogenous steroids.[11-13] The detection of endogenous steroids (found naturally in the body) is a challenge faced by Doping Control Laboratories across the world. In the present study, we validated a comprehensive GC-C/IRMS method combined with HPLC cleanup for the discrimination of endogenous steroids and successfully applied to the urine samples of endogenous synthetic steroids abusers especially in the cases when confirmation for the low concentration metabolites was to be made. Analytes have to be efficiently isolated and purified before GC/C/IRMS analytes in order to avoid any co-elution of compounds. Therefore, extensive sample preparation followed by HPLC cleanup was employed. Moreover, it was reviewed in literature that HPLC Cleanup was accurate and reproducible enough to be successfully applied to the test of urine sample from suspected anabolic steroid abusers.

Methods for Urinary Steroid Analysis

Reagents and Chemicals

Reference standards of endogenous steroids and deuterated internal standards were procured from Sigma-Aldrich, USA, or National Measurement Institute, Australia. 11-keto-etiocholanolone (11-keto) was obtained from German laboratory. C_{18} sample preparation cartridges were procured from RFCL Ltd and 3 M Empore, Varion. β-glucuronidase enzyme from *E. coli* was from Roche Diagnostics, USA. Methanol (MeOH) (HPLC-grade) and acetonitrile (ACN) (HPLC-grade) was purchased from Merck (USA). HPLC grade water was prepared using a Milli-Q purification system (Millipore, Bedford, MA).

Urine steroid profile determination

Routine screening procedure consisting of solid phase clean up, enzymatic hydrolysis, solvent extraction and derivatization, followed GC-MSD analysis was implemented to determine the concentration of Androgenic Anabolic Steroids (AAS), as described previously.[14-15]

Sufficient clean up of all analytes was achieved by a two fold HPLC fractionation comprising first of underivatized and then acetylated steroids. The clean up was performed on Waters Alliance 2695 separation module with automated fraction collector WFC-3, equipped with Merck analytical column (LiChroCART 250 x 4 mm i.d., 5 µm particle size) and guard column (LiChroCART 25 X 4 mm i.d., 5 µm particle size). For the first run, a linear gradient increasing from acetonitrile: water (30:70) to 100% acetonitrile in 25 min was used. After 5 min at 100 % acetonitrile, the column was re-equilibrated for 5 min; 50 µL injection volume and flow rate of 1 ml/min. Before each batch of samples, a standard comprising of 100 µg/ml of 11-keto-etiocholanone (11-keto), Testosterone (T), Epi-testosterone (EpiT), Androsterone (A), Etiocholanone (E), Pregnanediol (PD) and β-Estradiol-3, 17 diacetate (EST) were injected twice to determine the retention times for fraction collection. HPLC chromatograms of the first clean up along with fraction collection window are shown in Figure 1. Fraction I, IV and V were acetylated and injected onto GC-C-IRMS. Fraction II and III from the first run was acetylated and purified again to achieve sufficient clean up.

For second HPLC clean-up, a different gradient was used. From 70/30 acetonitrile/water, a linear increase to 100% acetonitrile was accomplished in 33 min and

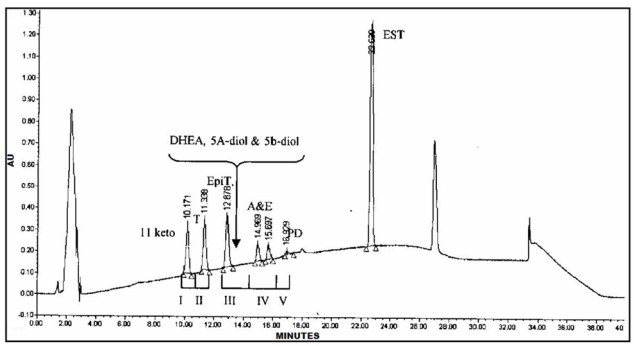

Figure 1: HPLC Chromatogram of standard injection for 1st Fraction Collection at wavelength of 192 nm with fraction collection.

maintained for 5 min. subsequently the column was re-equilibrated for 5 min. A standard mixture containing TAc, RSTDAc, EST, 5a (5α-androstane-3α,17β-diol) Ac, 5b (5β-androstane-3α,17β-diol) Ac, DHEA (dehydroepiandrosterone) Ac, EpiT Ac (100 ug/ml) in 70:30 acetonitrile: water in HPLC to know the RTs for fraction collection. 17β-estradiol-diacetate (RSTD) was used as reference standard for HPLC to control the acetylation step. Figure 2 shows the HPLC chromatogram of second clean up along with fraction collection windows.

Determination of Carbon Isotope Ratio (CIR)

Analytes have to be efficiently isolated and purified before GC/C/IRMS analytes in order to avoid any co-elution of compounds. Therefore, extensive sample preparation followed by HPLC clean up was employed. The samples were tested on GC/C/IRMS and $^{13}C/^{12}C$ ratios of endogenous steroids (Androsterone, Etiocholanolone, 5a and 5b-androstan-3a,17b diols, Testosterone DHEA and Epitestosterone and ISTD-RSTD) along with ERCs (11 Keto-etiocholanolone, Pregnanediol and ISTD-RSTD) were measured. The QC Negative and QC Positive used were Drug Free Urine from a healthy male volunteer and pooled excretion study samples (1-61 hr) from healthy male volunteer by administration of Oral Testosterone undecanoate (40 mg).

GC/MS Identification

In order to detect and identify co-elutions and to ensure the absence of any disturbing matrix components in all fractions it was necessary to scan all samples on a GC-MS system using chromatographic conditions equivalent to the IRMS set-up during method development. For this purpose, GC Agilent 6890 coupled to a mass selective detector was used. The MSD data was acquired in scan mode from m/z 40 to 400 and mass spectral data was compared to standards.

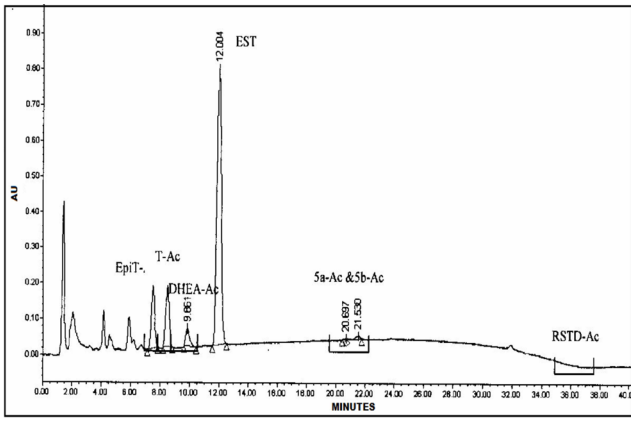

Figure 2: HPLC Chromatogram of standard injection for second clean up at 192nm with Fraction collection.

RESULTS AND DISCUSSION

The endogenous steroids which are excreted in low concentration show improved separation by HPLC Cleanup procedure as it removes all the interfering co-eluting compounds and thus proves to be more useful for the confirmation of suspicious samples in routine testing. The different purified fractions along with their R_T is shown in Table 1.

The aim of this study to develop and validate the GC/C/ IRMS method for the discrimination of endogenous steroids and demonstrate that the present method is useful for the identification of the anabolic androgenic steroids abusers was achieved. An initial effort for optimizing the sample preparation was made to achieve more complete enzymatic hydrolysis to convert glucuronide conjugated metabolites of the target endogenous steroids into their free form and clean isolation of the hydrolyzed target analytes to minimize the endogenous interferences. For these purposes, the solid-phase extraction was employed prior to the enzymatic hydrolysis and HPLC-cleanup of the hydrolyzed sample. Seven fractions were collected for the isolation of 10 endogenous steroids using the HPLC-cleanup condition described in Table 2. After dryness and reconstitution of fractions, the resulting samples were analyzed by GC−C/ IRMS with derivatization step. Figure 3-11 illustrates representative chromatograms of each fraction obtained from

Table 1: Fraction collection program for pure form of urinary steroids purified by HPLC 1st Cleanup.

HPLC fraction No.	Compound Names	Retention times (min.)
F-1	11 Keto Etiocholanolone	9.4 – 10.4
F-2	Testosterone	11 - 12
F-3	Epitestosterone, 5a-ADiol, 5b-ADiol, DHEA	12.2 – 14.2
F-4	Androsterone and Etiocholanonone	14.4 – 16.1
F-5	Pregnanediol	16.3 – 17.4

Table 2: Fraction collection program for acetate urinary steroids purified by HPLC 2nd Cleanup.

HPLC fraction No.	Compound Names	Retention times (min.)
F-2	Testoaterone -Acetate	8.3 – 9.3
F-3-1	Epitestosterone-Acetate	7.2 – 8.3
F-3-2	Dehydroepiandrosterone-Acetate	9.1 – 10.1
F-3-3	5a-ADiol-Diacetate, 5b-ADiol-Diacetate	20 – 22

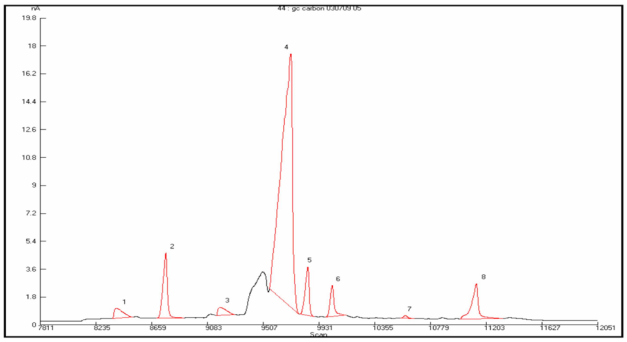

Figure 3: IRMS chromatogram of a sample injected previously without HPLC cleanup showing problem of co elution and absence of various compounds/peaks of interest.

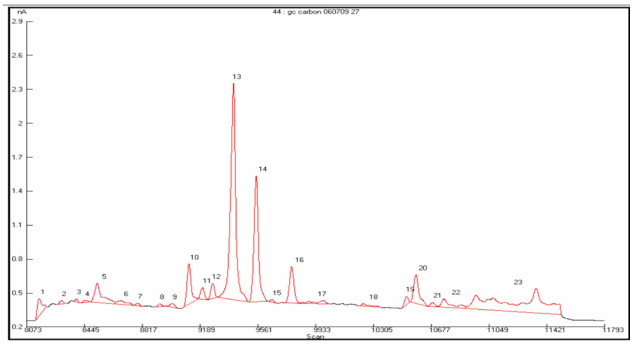

Figure 4: IRMS chromatogram of a sample injected previously without HPLC cleanup showing problem of uneven baseline and various interfering peaks.

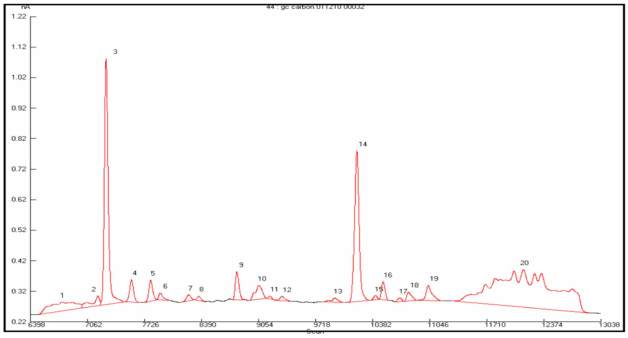

Figure 5: Fraction 1 from first fractionation showing concentrated and separate peaks of RSTD (3) and 11-Keto-etio (14).

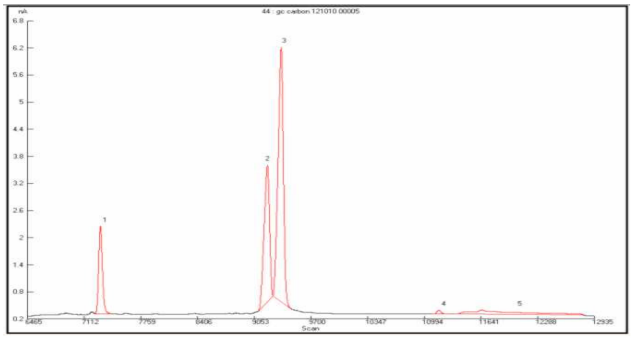

Figure 6: Fraction 4 from First Fractionation showing concentrated separate peaks of Androsterone (3) and Etiocholanolone (2) along with RSTD (1).

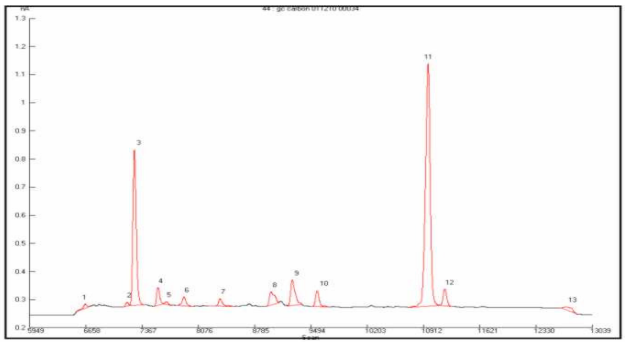

Figure 7: Fraction 5 from First Fractionation showing concentrated separate peaks of Pregnandiol (11) along with RSTD (3).

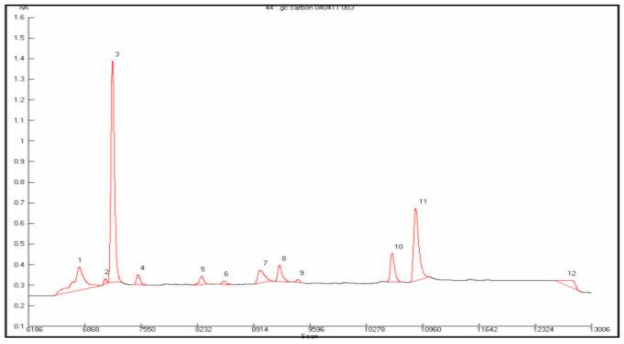

Figure 8: Pooled fraction 2 after second fractionation showing concentrated separate peaks of Testosterone (11) along with RSTD (3).

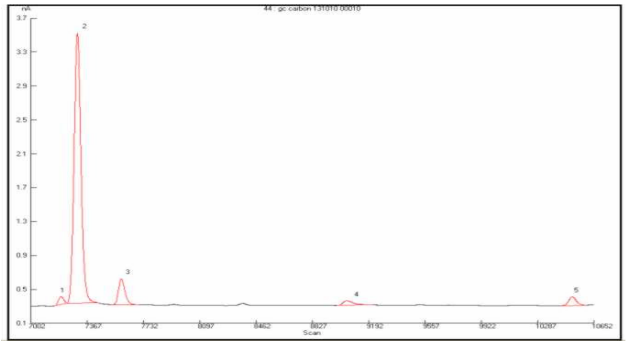

Figure 9: 1st fraction of Fraction 3 after second fractionation showing concentrated separate peaks of Epitestosterone (5) along with RSTD (2).

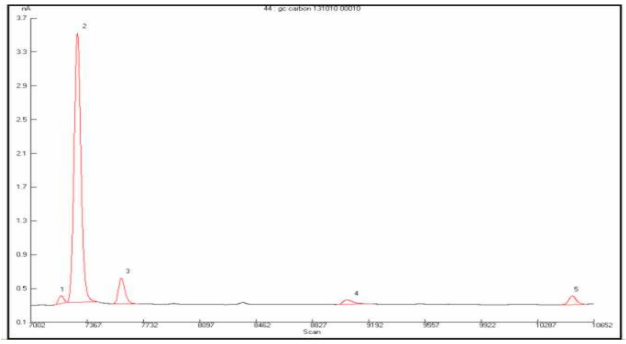

Figure 10: 2nd fraction of Fraction 3 after second fractionation showing concentrated separate peaks of DHEA (5) along with RSTD (2).

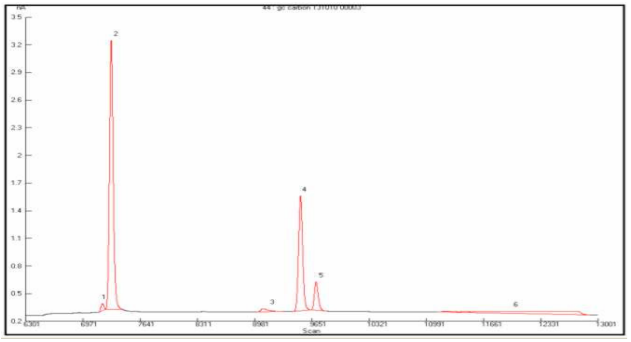

Figure 11: 3rd fraction of Fraction 3 after second fractionation showing concentrated separate peaks of 5b-ADiol-acetate (4), 5a-ADiol-acetate (5) along with RSTD (2).

the urine samples. No significant interfering peak of urine matrix was observed at the retention time of all steroids and the retention times of target analytes were consistent with those of standards. Identification of target urinary steroids was confirmed by comparison with mass spectra of standards obtained from GC-MS analysis.[16,17] The present method for the discrimination of endogenous steroids was validated. The urinary $\delta^{13}C$ values for 10 endogenous steroids were obtained and intra- and inter-day precision and isotopic fractionation were evaluated to be reproducible in the measurement of urinary $\delta^{13}C$ values. Sample preparation steps such as the SPE and HPLC-cleanup may seriously give rise to the isotopic fractionation phenomenon affecting the accuracy of IRMS analyses. To determine potential ^{13}C isotopic fractionation of the steroids during the sample preparation, the isotopic fractionation test was conducted. When comparing with $\delta^{13}C$ values of standards, $\delta^{13}C$ values of the analytes from urine samples were consistent with those of standards (RSTD) suggesting that no significant isotope fractionation effect was observed.

CONCLUSION

In continuation of earlier reviews, the present study summarizes the various approaches undertaken in expanding knowledge and improving method for GC-C-IRMS in with regard to human doping controls. GC-C/IRMS method combined with HPLC-cleanup has been developed and validated for the discrimination of endogenous steroids in human urine. The method is much better than the previous method using Solid Phase Extraction (SPE) only. This method was successfully applied to analysis of the urine samples from the suspected synthetic testosterone abusers in CWG 2010 and SYOG 2010 in presence of experts from national and international laboratories. As results, the present method is useful to elucidate the origin of the endogenous steroids even with metabolites which are very low in concentration.

ACKNOWLEDGEMENT

The author wishes to acknowledge the Ministry of Youth Affairs and Sports, Govt. of India for their continuous support.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

AAS: Androgenic Anabolic Steroids; **GC/C/IRMS:** Gas chromatography-combustion/isotope ratio mass spectrometry; **WADA:** World Anti-Doping Agency; **ERC:** Endogenous Reference Compounds; **HPLC:** High Performance Liquid Chromatography; **GC-MSD:** Gas Chromatography-Mass Selective Detector; **SPE:** Solid Phase Extraction; **CIR:** Carbon Isotope Ratio; **RSTD:** Reference Standard; **R_T:** Retention time.

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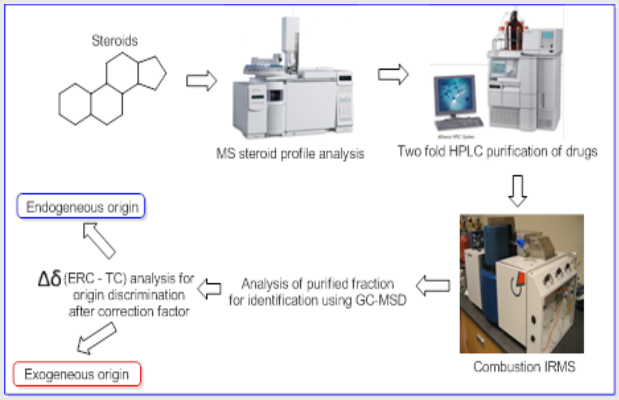
PICTORIAL ABSTRACT



SUMMARY

The present review describes an improved method for the discrimination of endogenous and exogenous origin of steroids and the same was also found suitable for confirmation analysis. The sample preparation and purification procedure was employed successfully in sport events of international fame and various athletes were caught using endogenous steroids. The method successfully cleared various proficiency testing rounds by WADA, WAADS and other proficiency testing agencies and thus found fit for purpose.

Cite this article: Shrivastava A, Jain S, Sahu PL, Shukla S, Sahu K. Improved Method for Differentiation of Synthetic and Natural Endogenous Anabolic Steroids using Gas Chromatography Isotope Ratio Mass Spectrometry (GC/C/IRMS) Followed by Two-Fold High Performance Liquid Chromatography (HPLC) Cleanup Method: A Perspective. Indian J of Pharmaceutical Education and Research. 2019;53(4s):s460-s465.