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# Simultaneous Estimation of Epicatechin, Trigonelline, Stigmasterol, and Gymnemagenin in Polyherbal Products: A Bioanalytical RP-HPLC Approach

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**KEYWORDS**
Epicatechin, Trigonelline, Stigmasterol, Gymnamagenin, RP-HPLC

**ABSTRACT**

The present study is about the development of bioanalytical method for the simultaneous estimation of Epicatechin (EPI), Trigonelline (TRG), Stigmasterol (STIG) and Gymnemagenin (GYM) in the polyherbal formulation using HPLC.

The polyherbal formulation were prepared by extracting *Pterocarpus marsupium, Gymnema sylvestre, Trigonella foenumgrecum, Momordica charantia* herbs which has wide acceptance in the treatment of diabetes and other chronic disorders. To investigate the pharmacokinetic of this developed polyherbal formulation, current work have devised a simple reverse phase high-performance liquid chromatography (RP-HPLC) method for the estimation of EPI, TRG, STIG and GYM in rat plasma. In doing this the plasma samples were spiked with the marker compounds and then extracted by solid phase extraction with Phenomenex Strata-X 33µm cartridges. Further, chromatographic separation was performed on C18 column using isocratic acetonitrile:water composition. The developed method was validated for accuracy, precision, linearity and recovery. Linearity studies were found to be acceptable over the range of 0.1–6 lg/ml. The method was successfully applied for the analysis of rat plasma sample for the application in pharmacokinetic study, drug interaction, bioavailability and bioequivalence. The mean % recovery from plasma of EPI were 92.8, 91.2 & 94.7, for TRG 91.5, 92.8 & 93.4, STIG 91.8, 92.5 & 91.8 and GYM 92.8, 93.1 & 92.5 % for LQC, MQC & HQC respectively. The recovery of EPI, TRG, STIG and GYM were found to be within the limit of acceptance.

## 1. INTRODUCTION

Traditional systems of medicine like 'Ayurveda' has mentioned many plant drugs for the treatment of diabetes and many of these have also been studied scientifically. Based on the reported literature, some herbs like *Gymnema sylvestre, Trigonella foenumgrecum, Momordica charantia and Pterocarpus marsupium* were found to be very common plant drugs utilized in controlling blood glucose levels. Many polyherbal formulations containing these drugs are marketed; however the labels do not reveal the content of active constituents or marker compounds.Bioavailability of maximum drugs are attained by administered them via intravenous route, whereas drugs administered orally are poorly bioavailable as they readily undergo first pass metabolism and incomplete absorption. Herbal formulations mostly available in oral dosage form. Many studies have been published related to the evaluation of polyherbal formulation for their efficacy and chemical standardization; however none of the study shown bioanalysis of antidiabetic polyherbal formulation

## 2. METHODS

2.1. Instrumentation

HPLC: The HPLC instrument consisted of Water's gradient system, equipped with 600-pump having Inline degasser. UV detector autosampler with rheodyne 9725 injector with 20 µl loop. All the data was processed using EMPOWER- Pro 6.1 software. Separation was achieved using a Water's C18 EION stationary phase (250 x 4.6

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mm i.d. 5 µm particle size) and the analytical column was protected by a Phenomenex C18 guard column (4mm × 2.0 mm, i.d.) A cooling centrifuge having the model No. Remi CM-12. The maximum limit of speed is 16,000 rpm with digital temperature controller having the limit upto – 200C.

2.2 Materials and reagents

The marker compound viz Gymnamagenin were purchased from Yucca enterprises private ltd, epicatechin and stigmasterol from Natural remedies private ltd, Trigonelline from Sigma Aldrich. All the reagents and chemicals used were of AR analytical and HPLC grade. Methanol (Spectrochem) and water (Lobachem) used were of HPLC grade.

2.3. Chromatographic conditions

All determinations were carried out at room temperature. The isocratic separations of compounds were carried out by using mobile phase consisting of Acetonitrile : water (65:35 v/v). The flow rate was maintained at 1 ml/min and monitoring of analytes were carried out at λmax 215 nM, using a UV detector and Water's 515-autosampler. The mobile phase was filtered through 0.45 µM membrane filter and degassed by ultrasonification before using it for chromatographic analysis.

2.4. Preparation of standard solutions

2.4.1 Preparation of stock and standard solutions

The stock solution of Epicatechin (EPI), Trigonelline (TRG), Stigmasterol (STIG) and Gymnemagenin (GYM) combination (1.0 mg/mL) was prepared

and diluted with methanol to give standard solutions ranging from 0.1 µg/mL to 6 µg/mL. Selection of concentration of standard solution was done by trial and error. Standard calibration samples were prepared by spiking 100 µL of drug-free plasma with 100 µL of appropriate Epicatechin (EPI), Trigonelline (TRG), Stigmasterol (STIG) and Gymnemagenin (GYM) standard solutions to achieve the final concentrations of 0.1–6 µg/mL for plasma.

2.4.2 Extraction procedure of EPI, TRG, STIG and GYM markers from plasma.

Human blood samples were collected from blood bank, of Yavatmal (MS), registration no: BD/269 and were transferred into tubes. A 180 µL aliquot of blank rat plasma was mixed with a 20 µL aliquot of selected analytes EPI, TRG, STIG and GYM working solutions of respective concentration levels (LQC, MQC, HQC and linearity dilutions). Effective solid phase extraction (SPE) extraction was achieved on Phenomenex Strata-X 33µ cartridges containing Polymeric reverse phase of capacity 30 mg/mL, the overall process was supported by a vacuum manifold which assisted in suction of samples. The cartridges were mounted on the SPE vacuum manifold assembly. The polymeric reverse phase was initially conditioned by passing 1ml of methanol followed with 1ml of HPLC grade water after which the cartridges were loaded with 180µl of plasma sample followed by vacuum. After the suction of plasma the cartridges were added with 100µl of HPLC grade water to wash off the remaining traces of plasma, the drug bonded to polymeric material in the cartridges was recovered by passing 1ml of methanol which was collected and subjected to analyses by HPLC in developed chromatographic method.

2.4.2 Calibration curve

The calibration curve of an analytical method is its ability to elicit test results that are directly or by a well defined mathematical transformation, proportional to the concentration of analyst in sample within the given range [1]Calibration curve were performed on the different concentration like 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6 µg/mL which were run on HPLC.

At least three runs were performed for each concentration. Peak area was measured according to their concentration. Graph of concentration vs. peak area was plotted and the R2 value was calculated from the graph

2.4.3 Method Validation:[2,3,4]

The developed HPLC method was validated as per European guideline (European medical agency, 2011). Three different concentrations of quality control samples were selected for further validation of the developed method.

They were 0.1 µg/mL low quality control sample (LQC), 0.8 µg/mL medium quality control sample (MQC) and 4.0µg/mL high quality control sample (HQC) of EPI, TRG, STIG and GYM

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## 1. RESULTS AND DISCUSSION

The HPLC method was developed for the simultaneous estimation of Epicatechin (EPI), Trigonelline (TRG), Stigmasterol (STIG) and Gymnemagenin (GYM) using reference standards. To obtain peak of good characters i.e. proper retention time number of trials performed has been shown in Table 1.The final method was optimized comprising mobile phase Acetonitrile: water (pH modified upto 3.9 by orthophosparic acid) in the ratio of 65:35 % v/v at 1 ml/min, detection was carried out at 215 nm

**Table 1. Trials for method development of simultaneous estimation of EPI, TRG, STIG and GYM HPLC**

| Sr. No. | Chromatographic condition | Ph | Result |
|---|---|---|---|
| 1 | Mobile Phase:80% water: and 20 % water, $\lambda_{max}$ : 215 nm, Flow rate: 1 mL/min | Not Modified | Improper peaks with no symmetry and retention time. The analytes hold the stationary phase strongly. |
| 2 | Mobile Phase: Water 70 % and ACN 30%, $\lambda_{max}$: 215 nm, Flow rate: 1 mL/min | Upto 4.5 by OPA | Peaks with poor shape and symmetry |
| 3 | Mobile Phase Water 65% and ACN 35%, $\lambda_{max}$: 215nm, Flow rate: 1.2mL/min | Upto 4.0 by OPA | Poor peak symmetry |
| 4. | Mobile Phase Water 50% and ACN 50%, $\lambda_{max}$: 215nm, Flow rate: 1.0mL/min | Upto 3.5 by OPA | Sharp Peaks with some of poor symmetry were obtained. |
| 5. | Mobile Phase Water 20% and ACN 80%, $\lambda_{max}$: 215nm, Flow rate: 1.0mL/min | Upto 3.5 by OPA | Poor pattern of separation |
| 6. | Mobile Phase Water 60% and ACN 40%, $\lambda_{max}$: 215nm, Flow rate: 1.0mL/min | Upto 4.5 by OPA | Good peaks with higher total run time. |
| 7. | Mobile Phase Water 40% and ACN 60%, $\lambda_{max}$: 215nm, Flow rate: 1.0mL/min | Upto 4.0 by OPA | Good peaks with short run time. |
| 8. | Mobile Phase Water 35 % and ACN 65 %, $\lambda_{max}$: 215nm, Flow rate: 1mL/min | Upto 3.9 by OPA | Good sharp peaks having good retention time and symmetry |

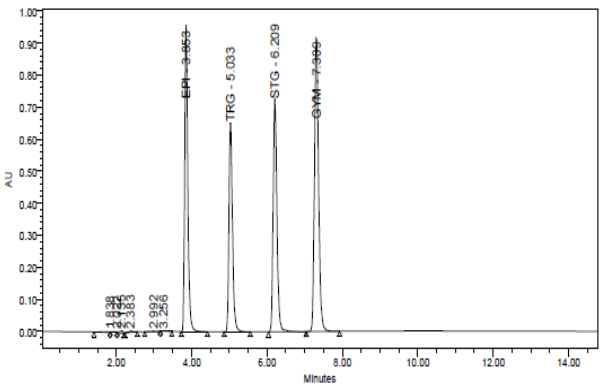


**Figure 1. Chromatogram of EPI, TRG, STIG and GYM in final chromatographic conditions HPLC**

3.1. Method validation[5-11.]

### 3.6.1 System suitability

System suitability test is an integral part of gas and liquid chromatographic method (European medical agency, 2011). They are used to verify whether the resolution and reproducibility of the chromatographic system. It is the verification of the system that ensures system performance before or during analysis.

The system suitability study for EPI, TRG, STIG and GYM (8 µg/ml) were performed by determining three factors like mean retention time, mean peak area and peak symmetry. The results were within the boundary of acceptance criteria and presented in the table 2.

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**.6.2 Linearity study**

Linearity study of developed method was done for concentration ranging from 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0 and 6.0 µg/mL for EPI, TRG, STIG and GYM respectively. A graph was plotted between concentration on X-axis and mean peak area on the Y-axis. The $R^2$ values were found to be 0.999 for EPI, TRG, STIG and GYM.

The results showed that an excellent correction exists between concentration and mean peak areas within the concentration range of 0.1 – 6.0 µg/mL which has been tabulated in Table 3 and Fig.2. Average area ratios of the above concentrations, gave the following equation Y = 65753x+10558 for EPI, Y = 56312x-22247 for TRG, Y=61729x+10233 for STG and Y=88489-6328 for GYM.

**3.6.3 LOD and LOQ**

Determination of LOD and LOQ is based on comparison of the SD of the peak area and the slope of the calibration curve. LOD for EPI, TRG, STIG and GYM were found to be 0.8, 0.6, 0.82 and 0.75 µg/ml respectively, whereas, LOQ were 2.64, 1.98, 2.7 and 2.47 µg/ml respectively (Table 4)

**3.6.4 Recovery studies**

Extraction recovery of EPI, TRG, STIG and GYM were determined by comparing peak areas obtained from extracted plasma samples with those found by extracting blank matrices through the extraction procedure and spiking with known amount of EPI, TRG, STIG and GYM.

The outcomes indicated that the mean extraction recoveries of EPI, TRG, STIG and GYM were >85% at concentrations of Quality control samples (QC Samples) that are Low quality control (LQC, 0.1 µg/mL) , Mid quality control (MQC, 0.8 µg/mL) and High quality control (HQC, 6 µg/mL) (n=6). Different organic extraction solvents were evaluated in the experiment, including methanol, acetonitrile, chloroform and diethylether.

Diethyl ether and acetonitrile combination proved to be the most efficient in extracting EPI, TRG, STIG and GYM from plasma and had a small variation in extraction recoveries over the concentration range.

The results of recovery study of EPI, TRG, STIG and GYM in the plasma sample were shown in Table 5.5. The % recovery of EPI were 92.8, 91.2 & 94.7, for TRG 91.5, 92.8 & 93.4, STIG 91.8, 92.5 & 91.8 and GYM 92.8, 93.1 & 92.5 % for LQC, MQC & HQC respectively. The recovery of EPI, TRG, STIG and GYM was found to be within the limit of acceptance.

**3.6.5 Accuracy**

Accuracy is the closeness of mean test results obtained by a method to the true value (concentration) of the analyte (European medical agency, 2011). It measures the exactness of the method. Accuracy was determined by replicate analysis of sample containing known amounts of the analyte.

Accuracy studies were performed for EPI, TRG, STIG and GYM in terms of recovery. For this quality control samples LQC, MQC & HQC of both the drugs in plasma were injected (n=6) and % accuracy was calculated.

The % accuracy of EPI was found to be 92.1, 91.6, and 92.5 %, for TRG was 93.1, 93.4 and 91.5 %, for STIG 91.8, 91.4 and 91.7 and GYM was91.4, 91.7 and 91.2 % for LQC, MQC & HQC levels respectively. Accuracy study results were found to be within the acceptance range as shown in Table 8.

**3.6.6 Precision**

Precision is the degree of agreement or closeness amongst individual test results when a method is applied to multiple aliquots of a single homogeneous volume of biological matrix. It is a measure of either the degree of reproducibility (agreement under different conditions) or of repeatability (agreement under same conditions) of the method.

Precision studies were performed for inter day and intraday variation in developed method by injecting quality control samples in developed chromatographic conditions (n=6). The % RSD value of EPI, TRG, STIG and GYM were found within the limit as shown in the Table 9 and 10.

**3.6.7 Stability**

Drug stability in a biological matrix is a function of the storage conditions, chemical properties of the drug, the matrix, and container system. Stability was evaluated for the duration of sample collection and handling.

**A. Stock solution stability**

The stability stock solutions of the EPI, TRG, STIG and GYM were made in 100% ACN and kept on bench as

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well as in refrigerator. % stability for EPI, TRG, STIG and GYM stock solution after 70 h at room temperature was found to be 93.1, 94.4, 93.8 and 95.30 % respectively. Percentage stability of stock solution of EPI, TRG, STIG and GYM after 70 hr refrigeration was found to be 93.0%, 94.7, 95.9 and 94.9 % respectively

The stability of the stock solution of EPI, TRG, STIG and GYM (refrigerated) were evaluated after 11 days by performing assay against freshly prepared stock solution. The percentage stability of EPI, TRG, STIG and GYM under refrigeration were 92.5, 93.4, 94.1 and 93.1 % respectively. The stability of stock solution was found to be in limit of acceptance criteria table 11.

**B. Post preparative stability**

*I.Post preparative stability at room temperature*

The stability of low and high quality control samples of EPI, TRG, STIG and GYM were evaluated after storing them at room temperature for a period of 76 hrs.

After 76 hrs, the stability was determined as percentage stability of samples (seven each of LQC and HQC) and was compared with their respective nominal concentrations.

The mean percentage values for EPI were 94.1 and 93.2, for TRG 93.7 and 94.3 % for STIG and 93.9 and 93.10 %, for GYM 94.5 and 93.2 % respectively for LQC and HQC. The results are shown in Table 12. The results obtained were within the acceptance criteria**.**

*II. Post preparative stability in refrigerator*

The stability of low and high quality control samples of EPI, TRG, STIG and GYM were evaluated by storing in refrigerator maintained at 2° to 8°C at an interval of 77 hr for the analyte.

After 77 hr, the stability was determined as percentage stability samples (seven each of LQC and HQC) and were compared with respective nominal concentration. For EPI the % stability was found to be 93.1 and 94.1%, for TRG 94.5 and 94.4 % for STIG 93.2 and 94.1 %, and for GYM 92.8 and 94.1 % respectively for LQC and HQC levels.

The results are shown in Table 13. The results obtained were within the acceptance criteria.

**C. Freeze-thaw Stability**

Stability of EPI, TRG, STIG and GYM were determined by the percentage stability samples compared with nominal concentrations of respective LQC and HQC samples after the second freeze and thaw cycle and was found to be 94.1 and 94.7 % for EPI, for TRG it was 94.2 and 93.1 %, for STIG it was 93.5 and 94.3 % whereas for GYM it was 93.4 and 93.2 % respectively.

The stability after three freeze thaw cycles LQC and HQC samples the stability was found to be 92.2 and 93.2 % for EPI, 94.2 and 92.5 % for TRG, 93.6 and 94.4, % for STIG and 93.1 and 93.3 % for GYM respectively as shown in Table 14 and 15.

**D. Short term stability**

The short-term stability of LQC and HQC were determined by comparing the mean concentrations of analyte in stability samples kept on bench for 6 hrs in ambient condition (Room Temperature) with respective nominal concentration.

The stability, determined by the % stability of samples with nominal concentration of respective low and high quality control samples. The percentage recoveries for LQC and HQC were 94.5 and 95.1 % for EPI, 93.1 and 93.5 % for TRG, 94.5 and 95.4 % for STIG and 94.1 and 95.3 % for GYM respectively. The results (Table 16 ) obtained are found to be within the acceptance criteria.

**E. Long term stability**

Long-term stability of analytes EPI, TRG, STIG and GYM in plasma, were analyzed after storing the quality control samples for 30 days at the temperature of -20°C, these samples analysis showed that the long term stability values were within the limit of acceptance criteria.

After 30 days, the stability was determined as percentage ratio of mean of concentrations of the stability samples (seven each of LQC and HQC) and were compared with respective nominal concentration

The percentage recoveries for LQC and HQC were 93.1 and 92.5 for EPI, 92.2 and 92.9 % for TRG, 93.1 and 93.1 % for STIG and 93.1 and 93.3% for GYM respectively. The results are presented in Table 17.

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**Table 2. System suitability for EPI, TRG, STIG and GYM (0.8 µg/mL, MQC dilution)**

| Sr. No. | Retention Time(min) | | | | Peak Area | | | | Peak Symmetry | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPI | TRG | STG | GYM | EPI | TRG | STG | GYM | EPI | TRG | STG | GYM |
| 1. | 3.83 | 5.03 | 6.20 | 7.35 | 547168 | 425313 | 506631 | 698983 | 1.1 | 1.5 | 1.5 | 1.45 |
| 2. | 3.80 | 5.05 | 6.25 | 7.30 | 548900 | 426900 | 507200 | 699980 | 1.15 | 1.55 | .5 | 1.45 |
| 3. | 3.98 | 5.06 | 6.22 | 7.38 | 547900 | 425820 | 508100 | 700010 | 1.1 | 1.5 | 1.45 | 1.5 |
| 4. | 3.95 | 5.05 | 6.25 | 7.35 | 545400 | 424108 | 506640 | 698710 | 1.12 | 1.45 | 1.55 | 1.5 |
| 5. | 3.90 | 5.04 | 6.28 | 7.30 | 543200 | 425895 | 505900 | 700120 | 1.2 | 1.5 | 1.55 | 1.5 |
| 6. | 3.88 | 5.06 | 6.25 | 7.32 | 543299 | 424825 | 506620 | 698970 | 1.2 | 1.5 | 1.5 | 1.4 |
| Mean±SD | 3.89 ±0.06 | 5.04 ±0.01 | 6.2± 0.02 | 7.33± 0.03 | 545977.8± 2403.19 | 425476.8±963.6 | 506848.5± 739.24 | 699462.2±638.52 | 1.14 ±0.04 | 1.5± 0.03 | 1.34 ±0.41 | 1.46± 0.04 |

The results were expressed as Mean ± SD (n= 6)

**Table 3. Linearity concentration for EPI, TRG, STIG and GYM**

| Sr. no. | Concentration (µg/mL) | Peak Area | | | |
|---|---|---|---|---|---|
| | | EPI | TRG | STIG | GYM |
| 1 | 0.1 | 67200± 403.1 | 55750 ± 610 | 64200 ± 425.1 | 88200 ± 352.5 |
| 2 | 0.2 | 145750 ± 874.1 | 108050 ± 980.5 | 126750 ± 1050.1 | 174800 ± 1223.4 |
| 3 | 0.4 | 263500 ± 1844 | 210001 ± 1710 | 255250 ± 3320.1 | 353800 ± 3180.2 |
| 4 | 0.6 | 415250 ± 2080 | 320107 ± 2570 | 381150 ± 3090.1 | 526210 ± 3160 |
| 5 | 0.8 | 541977 ± 2750 | 425313 ± 3402 | 506848 ± 7095.1 | 699462 ± 5510 |
| 6 | 1 | 682010 ±6138 | 542500 ± 4350 | 635600 ± 5090 | 871450 ± 7010.1 |
| 7 | 2 | 1351000 ± 9450 | 1055200 ± 10552 | 1267190 ± 10150 | 1773100 ± 14150.5 |
| 8 | 4 | 2558040 ± 23022 | 2184500 ± 17476 | 2554090 ± 20432.1 | 3485800 ± 41800 |
| 9 | 6 | 3999040 ± 40010 | 3401500 ± 23850 | 3822100 ± 26750 | 5332100 ± 47950 |

The results were expressed as Mean ± SD (n= 6),

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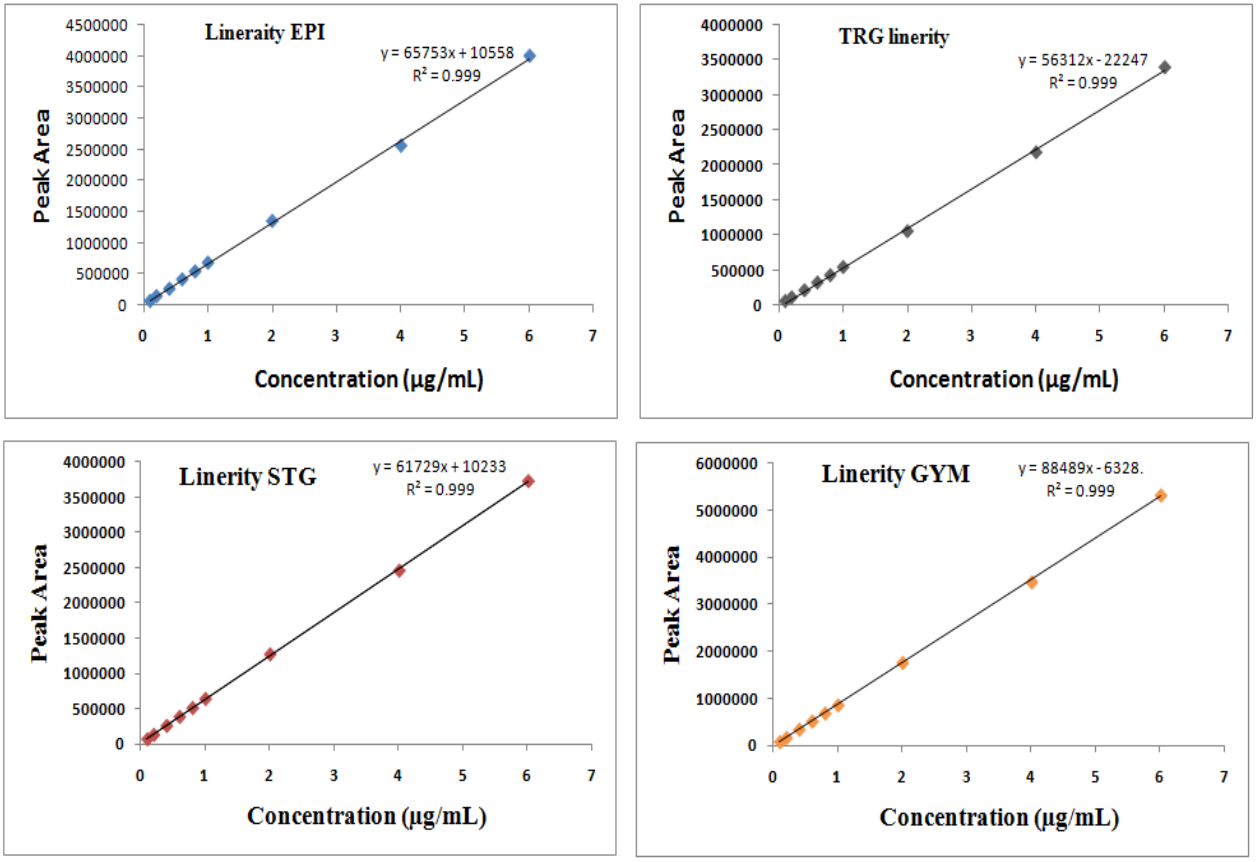


**Figure 2. Linearity graph for EPI, TRG, STIG and GYM in plasma.**

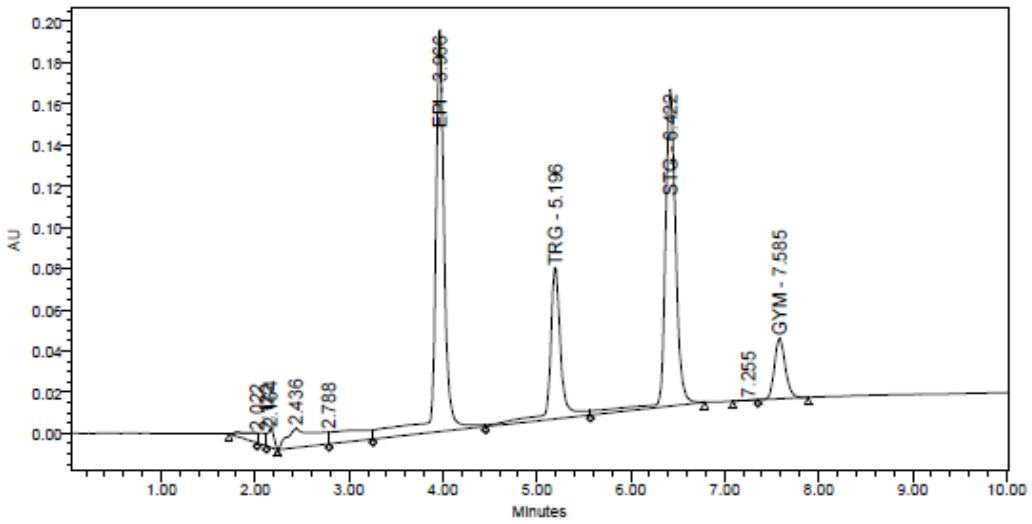


**Figure 3. Chromatogram of for EPI, TRG, STIG and GYM spiked in human plasma at LQC (0.1 µg/mL).**

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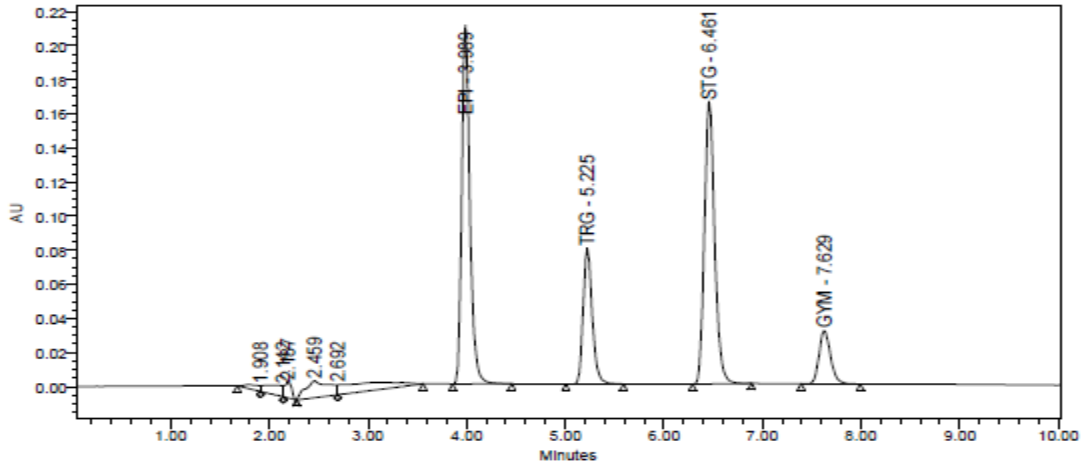


**Figure 4. Chromatogram of for EPI, TRG, STIG and GYM spiked in human plasma at MQC (0.8 µg/mL).**

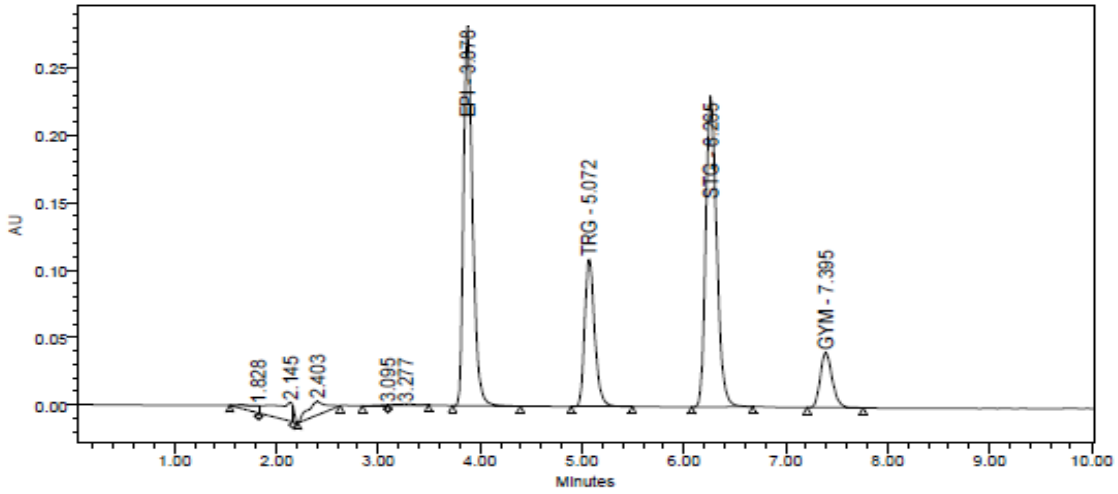


**Figure 5. Chromatogram of for EPI, TRG, STIG and GYM spiked in human plasma at HQC (6 µg/mL).**

**Table 4. LOD and LOQ of developed method for the estimation of EPI, TRG, STIG and GYM**

| Sr. No. | Drugs | LOD (µg/mL) | LOQ (µg/mL) |
|---|---|---|---|
| 1 | EPI | 0.8±0.008 | 2.64±0.02 |
| 2 | TRG | 0.6±0.005 | 1.98±0.02 |
| 3 | STG | 0.82±0.007 | 2.70±0.03 |
| 4 | GYM | 0.75±0.007 | 2.47±0.02 |

LOD- Limit of detection, LOQ- Limit of quantification

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**Table 6. The percentage extraction recovery of measurement of EPI and TRG, from spiked human plasma**

| Sr. No. | Sample | EPI | | | TRG | | |
|---|---|---|---|---|---|---|---|
| | | Un-extracted Area | Extracted Area | % Recovery | Un-extracted Area | Extracted Area | % Recovery |
| 1. | LQC | 66500±452 | 61712±326 | 92.8 | 55620±510 | 50892±368 | 91.5 |
| 2. | MQC | 540100±2610 | 492571±2230 | 91.2 | 425010±3240 | 393134±4110 | 92.5 |
| 3. | HQC | 3997030±39210 | 3785187±38620 | 94.7 | 3402100±24010 | 3177561±458 | 93.4 |

The results were expressed as Mean ± SD (n= 6),

**Table 7. The percentage extraction recovery of measurement of STIG and GYM from spiked human plasma**

| Sr. No. | Sample | STIG | | | GYM | | |
|---|---|---|---|---|---|---|---|
| | | Un-extracted Area | Extracted Area | % Recovery | Un-extracted Area | Extracted Area | % Recovery |
| 1. | LQC | 64105±425 | 58848±312 | 91.8 | 88210±325 | 818158±710 | 92.8 |
| 2. | MQC | 506050±7250 | 468096±6980 | 92.5 | 689950±4500 | 642343±3852 | 93.1 |
| 3. | HQC | 3821150± 24100 | 3507815±23110 | 91.8 | 5331150±47250 | 4931313±45560 | 92.5 |

LQC- Lower Concentration Quality Control Sample, MQC- Medium Concentration Quality Control Sample, HQC- Higher Concentration Quality Control Sample

**Table 8. Accuracy study for EPI, TRG, STIG and GYM in terms of recovery study**

| Sr. No. | Quality Control Samples | Recovered amount (µg/mL) | | | | Accuracy (%) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | EPI | TRG | STIG | GYM | EPI | TRG | STIG | GYM |
| 01 | LQC | 0.092±0.01 | 0.093±0.01 | 0.091±0 .01 | 0.091±0.01 | 92.1 | 93.1 | 91.8 | 91.4 |
| 02 | MQC | 0.74±0.1 | 0.75.±0.02 | 0.73± 0.01 | 0.73±0.01 | 91.6 | 93.4 | 91.4 | 91.7 |
| 03 | HQC | 5.55±0.2 | 5.49±0.15 | 5.47±0.10 | 5.76±0.1 | 92.5 | 91.5 | 91.7 | 91.2 |

The results were expressed as Mean ± SD (n= 6),

LQC- Lower Concentration Quality Control Sample, MQC- Medium Concentration Quality Control Sample, HQC- Higher Concentration Quality Control Sample

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**Table 9. Precision studies for developed method for estimation of EPI, TRG, STIG and GYM (Inter-day).**

| Sr. No. | Quality Control Samples | Inter-day Precision (Peak area ± S.D, %RSD) | | | |
|---|---|---|---|---|---|
| | | **EPI** | **TRG** | **STIG** | **GYM** |
| 1 | LQC | 62100± 1801, 2.9% | 50890±1323, 2.6% | 58712±1232, 2.1% | 817250±18796, 2.3% |
| 2 | MQC | 490010±16660, 3.4% | 394020±12214, 3.1% | 462020±8778, 1.9% | 632445±13913, 2.2% |
| 3 | HQC | 3798100±94952, 2.5 % | 3104210±90022, 2.9% | 3501000±63018, 1.8% | 4920210±103324, 2.1% |

The results were expressed as Mean ± SD (n= 6),

LQC- Lower Concentration Quality Control Sample, MQC- Medium Concentration Quality Control Sample, HQC- Higher Concentration Quality Control Sample, %RSD- % Relative Standard Deviation

**Table 10. Precision studies for developed method for estimation of EPI, TRG, STIG and GYM (Intra-day)**

| Sr. No. | Quality Control Samples | Intra-day Precision (Peak area ± S.D, %RSD) | | | |
|---|---|---|---|---|---|
| | | **EPI** | **TRG** | **STIG** | **GYM** |
| 1 | LQC | 63150± 1957, 3.1% | 51110±1737, 3.4% | 57910±1911, 3.3% | 818110±35361, 3.1% |
| 2 | MQC | 491220±16210, 3.3% | 393905±10635, 2.7% | 463110±11114, 2.4% | 634155±20292, 3.2% |
| 3 | HQC | 3789200±79573, 2.1% | 3113110±74714, 2.4% | 3502900±91075, 2.6% | 4921322±152560, 3.1% |

The results were expressed as Mean ± SD (n= 6),

LQC- Lower Concentration Quality Control Sample, MQC- Medium Concentration Quality Control Sample, HQC- Higher Concentration Quality Control Sample, % RSD- % Relative Standard Deviation

**Table 11. Stock solution stability for EPI, TRG, STIG and GYM**

| Time point | Solution kept for Stability | % Stability | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **EPI** | | **TRG** | | **STIG** | | **GYM** | |
| | | **Rt** | **Ref** | **Rt** | **Ref** | **Rt** | **Ref** | **Rt** | **Ref** |
| **70 hrs** | **Stock Solution** | 93.1 | 93.0 | 94.4 | 94.7 | 93.8 | 95.9 | 95.3 | 94.9 |
| **11 days** | **Stock Solution** | **NA** | 92.5 | NA | 93.4 | **NA** | **94.1** | NA | 93.1 |

Rt: Room temperature, Ref: Refrigerator

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**Table 12. Post preparative stability for EPI, TRG, STIG and GYM after 76 hr of storage at room temperature on bench-top**

| Samples | LQC | | HQC | |
|---|---|---|---|---|
| | NC (µg/mL) | After 76 hr (µg/mL) (%stability) (n=7) | NC (µg/mL) | After 76 hr (µg/mL) (%stability) (n=7) |
| EPI | 0.1 | 94.1 | 6.0 | 93.2 |
| TRG | | 93.7 | | 94.3 |
| STIG | | 93.9 | | 93.1 |
| GYM | | 94.5 | | 93.2 |

The results were expressed as % stability (n= 7),

LQC- Lower Concentration Quality Control Sample, HQC- Higher Concentration Quality Control Sample, NC- Normal Condition.

**Table 13. Post preparative stability for EPI, TRG, STIG and GYM after 77 hr of storage in refrigerator at 2° to 8 °C**

| Samples | LQC | | HQC | |
|---|---|---|---|---|
| | NC (µg/mL) | After 77 hr (µg/mL) (%stability) (n=7) | NC (µg/mL) | After 77 hr (µg/mL) (%stability) (n=7) |
| EPI | 0.1 | 93.1 | 6.0 | 94.1 |
| TRG | | 94.5 | | 94.4 |
| STIG | | 93.2 | | 94.1 |
| GYM | | 92.8 | | 94.1 |

The results were expressed as % stability (n= 7),

LQC- Lower Concentration Quality Control Sample, HQC- Higher Concentration Quality Control Sample, NC- Normal Condition.

**Table 14. Freeze thaw stability for EPI, TRG, STIG and GYM solution after 2 cycles**

| Samples | LQC | | HQC | |
|---|---|---|---|---|
| | NC (µg/mL) | After 2 cycles (µg/mL) (%stability) (n=7) | NC (µg/mL) | After 2 cycles (µg/mL) (%stability) (n=7) |
| EPI | 0.1 | 94.1 | 6.09 | 94.7 |
| TRG | | 94.2 | | 93.1 |
| STIG | | 93.5 | | 94.3 |
| GYM | | 93.4 | | 93.2 |

The results were expressed as % stability (n= 7),

LQC- Lower Concentration Quality Control Sample, HQC- Higher Concentration Quality Control Sample, NC- Normal Condition.

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**Table 15. Freeze thaw stability for EPI, TRG, STIG and GYM after 3 cycles**

| Samples | LQC | | HQC | |
|---|---|---|---|---|
| | NC (µg/mL) | After 3 cycles (µg/mL) (%stability) (n=7) | NC (µg/mL) | After 3 cycles (µg/mL) (%stability) (n=7) |
| EPI | 0.1 | 92.2 | 6.0 | 93.2 |
| TRG | | 94.2 | | 92.5 |
| STIG | | 93.6 | | 94.4 |
| GYM | | 93.1 | | 93.3 |

The results were expressed as % stability (n= 7),

LQC- Lower Concentration Quality Control Sample, HQC- Higher Concentration Quality Control Sample, NC- Normal Condition.

**Table 16. Short term stability for EPI, TRG, STIG and GYM after 6 hr at room temperature on bench top**

| Samples | LQC | | HQC | |
|---|---|---|---|---|
| | NC ( µg/mL) | After 6 hrs (µg/mL) (% stability) (n=7) | NC (µg/mL) | After 6 hrs (µg/mL) (% stability) (n=7) |
| EPI | 0.1 | 94.5 | 6.0 | 95.1 |
| TRG | | 93.1 | | 93.5 |
| STIG | | 94.5 | | 95.4 |
| GYM | | 94.1 | | 95.3 |

The results were expressed as % stability (n= 7),

LQC- Lower Concentration Quality Control Sample, HQC- Higher Concentration Quality Control Sample, NC- Normal Condition.

**Table 17. Long Term Stability for EPI, TRG, STIG and GYM after 30 days of storage in deep frizzed condition**

| Samples | LQC | | HQC | |
|---|---|---|---|---|
| | NC (µg/mL) | After 30 days (µg/mL) (%stability) (n=7) | NC (µg/mL) | After 30 days (µg/mL) (%stability) (n=7) |
| EPI | 0.1 | 93.1 | 6.0 | 92.5 |
| TRG | | 92.2 | | 92.9 |
| STIG | | 93.1 | | 93.1 |
| GYM | | 93.1 | | 93.3 |

The results were expressed as % stability (n= 7),

LQC- Lower Concentration Quality Control Sample, HQC- Higher Concentration Quality Control Sample, NC- Normal Condition.

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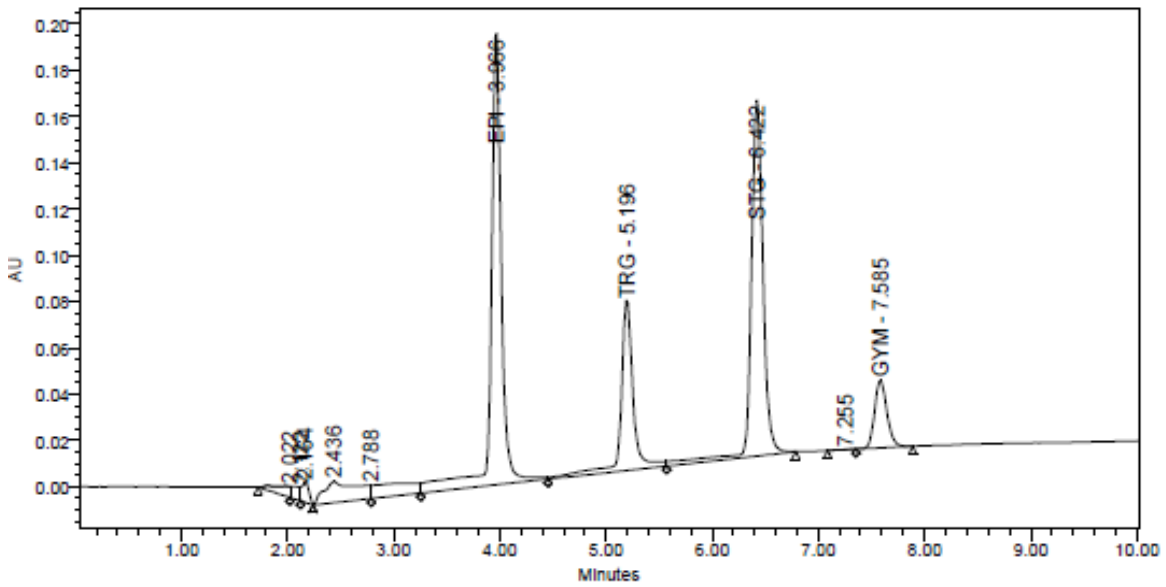


**Figure 6. a. Application of developed method for the estimation of EPI, TRG, STG and GYM in spiked human plasma followed by HPLC estimation. B. Placebo sample in developed HPLC method.**

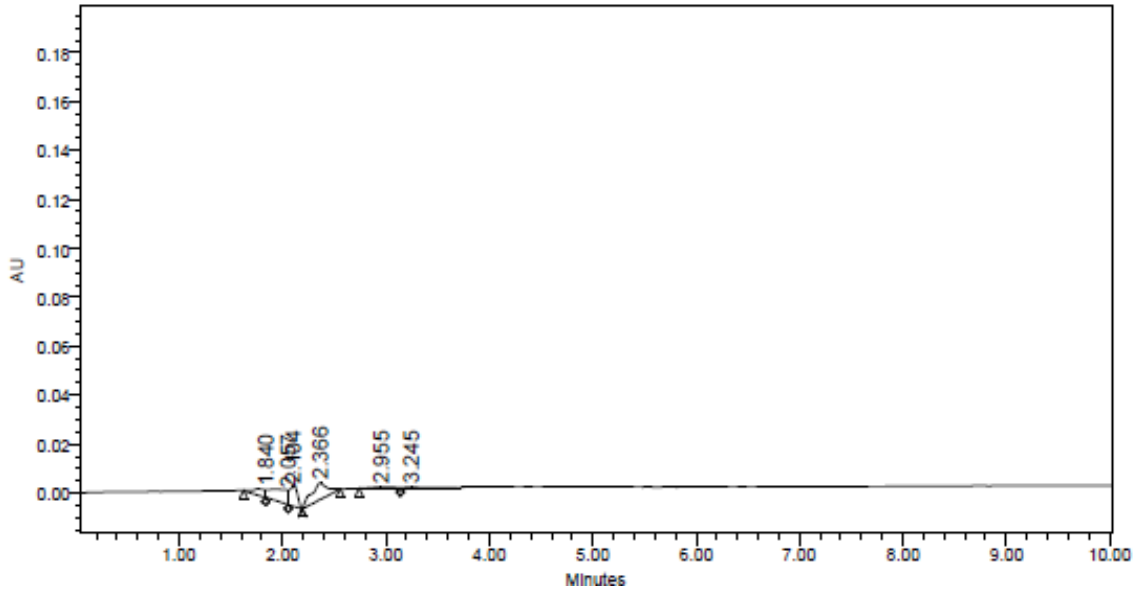


**Figure 6 b. Placebo chromatogram**

## 4. CONCLUSION

The work has demonstrated new bio-analytical HPLC method for the estimation of active components of polyherbal formulation. The method qualifies the validation process and criteria for the analysis and quantification of EPI, TRG, STIG and GYM . The bio-analytical method is Sensitive, accurate and precise for the estimation of these marker compounds in polyherbal . The mean % recovery of EPI, TRG, STIG and GYM from plasma was found to be within the acceptance limit of validation.The applicability of method suggests its

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further application for bioequivalence, bioavailability and drug interaction studies.

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