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RESEARCH ARTICLE

Preparation of Formulation Containing Standardized Isoflavone Rich Extract of Glycine Max Seeds and its Bioavailability Studies

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Abstract

Background: Formulations containing soy extracts containing genistein, daidzein and glycitein are widely available in market and have been utilized for treatment of hyper lipidemia, postmenopausal problems and cancer. **Aim:** The present study focuses on preparation of capsule formulation containing soy isoflavone rich extract and determination of bioavailability using validated bioanalytical HPTLC method. **Methods:** The standardized extract containing 40% w/w total isoflavones (genistein 5.74% w/w) was formulated into capsule formulation containing 20 mg genistein per capsule. The bioanalytical HPTLC method was developed wherein protein precipitation of plasma sample was carried out using methanol followed by liquid liquid extraction using diethyl ether. The HPTLC method involved use of precoated silica gel $^{60}GF_{254}$ plates as stationary phase with mobile phase of Ethyl Acetate: Pet ether: Toluene [4:4:2 (v/v)] and detection at 262 nm. The absolute bioavailability of genistein was determined by administering 2 mg/kg *i.v.* and 10 mg /kg per oral to albino rats. The bioavailability of the formulation was determined at the dose of 20 mg/kg per oral. **Results:** The HPTLC method yielded maximum recovery of 72.77 % w/w of genistein from plasma, with accuracy of 101.69-115.22 % w/w and linearity range was found to be 20-140 ng/band. The absolute bioavailability of genistein was observed to be 14.78%. The C_{max} and t_{max} for formulation containing genistein rich extract (equivalent to 20 mg/kg bw po) were found to be 1.004 ug/ml and 8 h respectively. Relative bioavailability as calculated by comparing AUCs of pure genistein (10 mg/kg bw po) with the formulation (20 mg/kg bw po genistein) was found to be 77.4%. **Conclusion:** The validated bioanalytical HPTLC method was found to be selective, accurate, reproducible and cost effective. The method was successfully applied for pharmacokinetic study and confirmed that formulation containing genistein showed better bioavailability as compared to pure genistein.

Keywords: Bioanalytical Method Validation, Bioavailability, Genistein, High Performance Thin Layer Chromatography, Standardization

1. Introduction

Seeds of *Glycine max* (*Leguminosae*) commonly called as soya beans form one of the common food ingredients in Asian countries. The seeds are known for presence of isoflavones. The three major isoflavones found in soybeans are genistin (I), daidzin, and glycitin. Isoflavones has attracted major interest in last few decades as several studies proved that intake of soy rich diet lowers incidences of breast and prostate cancer and are very beneficial in the treatment of breast and prostate cancer[1-5]. The isoflavones are also reported to possess estrogenic and antioxidant activity[6]. The soy isoflavones are also reported to have beneficial effects in lowering cholesterol inhibiting atherosclerosis especially in postmenopausal conditions[7-9].

Genistin (I) is one of the major isoflavones glycosides present in soy meal and the aglycon of which is called as genistein (II) (Figure 1). Genistin being a glycoside has good solubility in water and gets absorbed followed

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by hydrolysis to release genistein. The bioavailability of genistein is very less due to poor solubility in water[10]. Studies carried out on bioavailability and pharmacokinetic profiles on pure genistein and genistin have been reviewed[6]. The review reveals that clinical trials have also been conducted on patients having cancers of breast and prostate. However, these studies were carried out on the pure marker compounds or on the soy meals and the products of soy such as milk, fermented soy products, flour *etc*[11-15].

In light of the widespread uses of these dietary isoflavones and their impact on human health, it has become necessary to develop specific, sensitive, accurate and precise analytical and bioanalytical methods for separation, identification and quantifications of these phytoestrogens in food products as well as biological matrices. Several HPLC, HPLC-MS methods have been developed for quantification of isoflavones in food supplement and in various biological matrices *eg*. plasma, serum and urine. However, HPLC-MS continues to hold its prime position in pharmaceutical industry.

One of the drawbacks of LCMSMS method is it requires enormous sample preparation before analysis which is very complicated and time consuming; indeed, previous studies using LC-MS/MS techniques for the quantification of total daidzein, genistein, and equol in human biological fluids required prolonged hydrolysis and cleanup by SPE which makes cost of analysis tremendous high.

HPTLC is a method of choice for analysis of phytoconstituents as it helps for authentication of various species and assures quality of their preparations. Few analytical HPTLC methods are available in literature but there is no method reported in literature for bioanalytical and pharmacokinetic estimation using HPTLC method.

Therefore, the present study was focused on preparation of the capsule formulation containing the standardized soy extract, development and validation of HPTLC method followed by its application for bioavailability studies of formulation.

2. Materials and Methods

2.1 Procurement of Materials and Chemicals

The soy seed extract containing 40%w/w of isoflavone was procured from Kshipra Biotech Pvt Ltd, Mumbai, India. The standard genistein was procured from the Sigma-Aldrich, Darmstadt, Germany. All the solvents and chemicals utilized were of analytical grade. Ethyl acetate, pet ether, toluene are obtained from Molychem and AR grade methanol is obtained from Loba Chemie Pvt Ltd, Mumbai India.

2.2 Analytical Method Development and Validation

Weighed accurately about 5mg of reference standard of genistein and was dissolved in 5ml of methanol to prepare a stock solution of 1000 µg/ml (solution A). Solution A was further diluted 100 times with methanol to get a concentration of 10 µg/ml (Solution B).

Precoated TLC silica gel Plates 60 GF254(20 cm × 10 cm, 250 µm thickness, Merck, Darmstadt, Germany) were utilized as stationary phase and were pre-washed with methanol followed by activation at 110°C for 5 mins prior to the sample application. Different volumes of working standards of the above-mentioned marker compounds were loaded in the form of bands (6 mm width) with a Hamilton microliter syringe (100 µl)

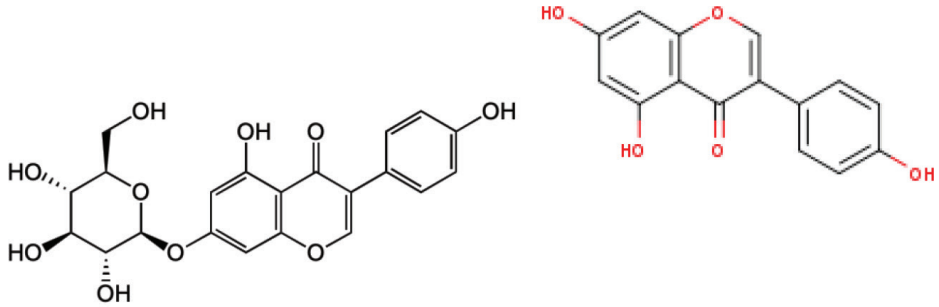


Figure 1. Structure of phytoconstituents genistin and genistein.

under a controlled nitrogen stream using a Camag Linomat V sample applicator (Switzerland) to get concentration in range of 20-140 ng/band. The slit dimensions were 5 mm × 0.45 mm with the scanning speed of 10 mm/s. Method development was executed in a Camag twin trough a glass chamber (20 cm × 20 cm) saturated with the mobile phase (Ethyl Acetate: Pet Ether: Toluene (4:4:2) v/v). Plate was scanned at 262 nm using CAMAG HPTLC scanner using wincats software 1.2.2. Linearity study was determined by analyzing series of calibration standards in the range of 20-140 ng/band using developed HPTLC method in triplicates. Calibration curve was constructed and correlation coefficient was calculated. The method was used for analysis of drug from the inhouse formulation.

2.3 Preparation and Evaluation of Formulation

Spray dried extract containing 40% isoflavone of *G. max* was purchased from Kshipra Biotech Pvt. Ltd., was utilized for development of formulation. The formula for the capsule formulation is given in Table 1.

The dough was prepared using microcrystalline cellulose (Avicel) and 90% v/v aqueous ethanol followed by passing of the dough through sieve number 10. The granules were gently spread and dried at 45°C in oven for 2 hours followed by sieving through sieve number 20 supported on 40 and mixed with 10% of the fines and 2 % talc. The granules were evaluated for flow properties and filled into a hard Gelatin capsule of size "0" using manual capsule filling machine. The flow properties of the granules were evaluated by determining tap density, bulk density, angle of repose and Hausner's ratio. The capsule formulation was evaluated for disintegration, weight variation and content uniformity[16-18].

Table 1. Composition of formulation containing standardized glycine max extract

Sr. No.	Ingredients	Composition Per Capsule
1	Glycine max extract (equivalent to 20 mg of genistein)	355 mg
2	Microcrystalline cellulose	50 mg
3	talc	2%
4	90% Aqueous ethanol solution	0.11 ml

2.3.1 Determination of Content of Genistein in the Formulation

2.3.1.1 Sample Preparation

Contents from three capsules were mixed and weighed the powder equivalent to 20 mg of genistein and hydrolyzed by heating with 10ml concentrated HCl for 60 mins in a heating mantle under a reflux condenser. The solution was then transferred to 25 ml volumetric flask. About 10 ml of methanol was added and sonicated for 15 min or till dissolution of extract. The volume was then made to the mark using methanol (stock I) and it was filtered through Whatmann paper. 0.5 ml of stock I was further diluted to 10 ml using methanol (solution II). 2 µl of solution II was loaded on HPTLC plates and analyzed for the content of genistein using the method described in section 2.2.

2.4 Bio-analytical Method Development

2.4.1 Preparation of Standard Solution

Weighed accurately about 5mg of reference standard of genistein and was dissolved in 5ml of methanol to prepare a stock solution of 1000 µg/ml (solution A). Solution A was further diluted 100 times with methanol to get a concentration of 10 µg/ml (Solution B).

2.4.2 Bioanalytical Sample Preparation and Extraction Technique

0.5ml of genistein working standard solution (10µg/ml) was spiked with 0.5ml of blank human plasma and the mixture was vortex mixed for 5 min. Further 0.5ml diethyl ether was added to it and again vortex mixed for 5 min followed by separation of layers by centrifugation. Organic layer was collected and the aqueous layer was striped with 0.5 ml of diethyl ether. Both the diethyl ether layers were pooled and evaporated. The residue was reconstituted with 0.5ml methanol.

2.4.3 Bioanalytical Method Development

Suitable amounts of reconstituted solution obtained in 2.4.2 were loaded on HPTLC plate (silica gel GF 254) using CAMAG LINOMAT V using 100ul Hamilton syringe, where band size of each track was 6mm to get concentrations *viz.* 20 ,40,60,80,100,120 and 140 ng/band. The HPTLC plate was developed using Ethyl Acetate: Pet ether: Toluene [4:4:2 (v/v)] as a mobile phase. The developed HPTLC plate was

then scanned by CAMAG TLC scanner III at 262 nm in absorbance mode and integrated by WIN CATS V 1.2.2. The experiment was carried out in triplicate. The calibration curve was constructed using amount loaded (ng) against area of the peak.

2.4.4 Determination of Extraction Efficiency

The % recovery method was used to determine extraction efficiency of genistein from biological matrix. Recovery of drug using liquid-liquid extraction technique was determined at LQC, MQC and HQC levels. % recovery was calculated by comparing the analytical results for extracted samples at with unextracted standards that represent 100% recovery.

2.5 Bioanalytical Method Validation

The developed bioanalytical method was validated in terms of specificity, matrix effect, linearity, precision, accuracy, limit of detection, limit of quantification and stability studies[19].

2.5.1 Specificity and Matrix Effect

Specificity of the method was ascertained by analyzing the standard genistein extracted from plasma and compared with chromatogram of blank plasma. To check matrix effect, LQC(40ng/spot) and HQC (120 ng/spot) were spiked in three different blank plasma and analyzed in triplicates using HPTLC method.

2.5.2 Linearity, LOD and LOQ

Linearity was determined by analyzing series of calibration standards in the range of 20-140 ng/band using developed HPTLC method in triplicates. Calibration curve was constructed and correlation coefficient was calculated. LOD and LOQ was calculated using formula LOD=3.3(SD/S) and LOQ=10(SD/S), respectively based on Standard Deviation (SD) of the response and Slope(S) of the calibration curve.

2.5.3 Precision and Accuracy Studies

Precision was calculated by intraday and interday studies. Both precisions were determined at three different QC levels (low, medium, high) *viz*. 40, 80, 120 ng/band. Six samples of each concentration were prepared to study intraday and inter-day precision and the results are presented as % RSD.

Accuracy study was performed for genistein reference standard in terms of % recovery. The stock solution was spiked in the formulation having same concentration of genistein at different levels *viz*. 50% (40ng/band), 100% (80 ng/band) and 150% (120ng/band) of the linear range concentrations. The results are presented as % recovery.

2.5.4 Stability

Stability of genistein reference standard in human plasma was determined at different storage conditions *viz*. Bench top stability, post preparative stability and freeze thaw stability (three cycles). Stability of analyte in biomatrix after exposure for four hours on bench top at LQC (40 ng/band) and HQC level (120ng/band) was determined and analyzed against standard curve prepared by freshly spiked QC samples. The post preparative stability was evaluated by analyzing LQC and HQC four hours later the processing using the developed HPTLC bioanalytical method as described section 2.4.3. The freeze-thaw stability experiments were conducted by analyzing content of genistein at LQC (40 ng/band) and HQC level (120ng/band) which were frozen followed by thawing samples three times. The concentration of genistein in the plasma was determined and compared with freshly spiked QC samples.

2.6 Bioavailability and Pharmacokinetic Studies

The bioavailability and pharmacokinetic studies were carried out in female wistar rats (150-180 gm). Initially the absolute bioavailability of standard genistein was determined, followed by determination of bioavailability of the formulations with respect to genistein. The protocols of the animal studies were approved by Institutional Animal Ethics Committee (IAEC) [SVBCP/IAEC/M.Pharm/19-20/69]. The experimental animals were maintained at 22 ±2°C and 55 ±5 % relative humidity with the light and dark cycles of 12 hours each. The animals were given full access to food and drinking water throughout the experiment period.

2.6.1 Determination of Absolute Bioavailability of Genistein

Absolute bioavailability of genistein was determined by administering the solution of genistein (polyethylene

glycol 300: Tween 80: benzyl alcohol: ethanol (65:8:3:24 % v/v) through *i.v. via* tale vein (2mg/kg b.w.) and by oral routes (10 mg/kg b.w.). The rats were anesthetized using ether and blood samples were collected through puncturing the retro-orbital plexus at 0.5, 1, 2, 3, 4, 5, 6, 7 and 8hours time-points later *i.v*, administration and at 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 hours after oral administration of pure genistein[20-21]. The proteins were precipitated by addition of 0.5ml of methanol to 0.5ml of plasma sample followed by extraction of genistein using diethyl ether.

2.6.2 Determination of Bioavailability of Formulation

The content of capsule formulation (2.3) was suspended in water and kept in ultrasonicator for 15 min. The solution was then administered orally with animal equivalent dose of 20 mg/kg b.w. The rats were anesthetized using ether and blood samples were collected through puncturing the retro-orbital plexus 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 hours time points. Blood samples were centrifuged to separate plasma and stored at -20°C until analysis for determination of content of genistein. The proteins were precipitated by addition of 0.5ml of methanol to 0.5ml of plasma sample followed by extraction of genistein using diethyl ether.

2.6.3 Determination of Pharmacokinetic Parameters

The pharmacokinetic parameters were calculated for the pure marker compound as well as the formulation. Non compartmental analysis of data was performed using statistical moment theory. The peak plasma concentration (Cmax) and the corresponding time (Tmax) were directly obtained from raw data. The area under the plasma concentration versus time curve upto the last quantifiable point, AUC (0-t) was calculated by the linear and log-linear trapezoidal rule summation;

$$AUC(0-t) = \Sigma\left(\frac{Co+Ct}{2}\right)\times Tt - To$$

C= Plasma drug concentration at the time t.

T= Time required to attend C

The AUC (0-t) was extrapolated to infinity (that is AUC(0-t)) by adding the quotient of Clast/Kel,

$$AUC(0-t) = \frac{\text{C Last}}{\text{Kel}}$$

Where C last represents the last measurable time concentration and Kel represents the elimination rate constant.

Kel was calculated by the linear regression of the log-transformed concentrations of the last three data points in the terminal phase.

Kel= -slope × 2.303

Slope was obtained from log-transformed concentrations of the last three data points in terminal

phase. The half-life of the elimination phase was obtained from using the relationship,

$$t_{1/2} = \frac{0.693}{\text{K}}$$

Clearance was calculated using the relationship,

CL = Kel ×Vd

Vd= Apparent volume of distribution

Kel = Elimination rate constant

Apparent volume of distribution was calculated using relationship,

Vd = Dose Administered / serum concentration

Absolute Oral bioavailability (F) was calculated using relationship,

$$F = \left\{\left[\frac{\text{AUC}(\text{oral})}{\text{AUC}(\text{iv})}\right]\times\left[\frac{\text{Dose}(\text{iv})}{\text{Dose}(\text{oral})}\right]\right\}\times 100$$

$$\textit{Relative bioavailability} = \left\{\left[\frac{\text{AUC}(\text{oral formulation})}{\text{AUC}(\text{oral GENISTEIN})}\right]\times\left[\frac{\text{Dose}(\text{GENISTEIN})}{\text{Dose}(\text{oral formulation})}\right]\right\}\times 100$$

3. Results

3.1 Composition of Standardized Extract

The extract of *G. max* Merr was purchased from Kshipra Biotech Pvt. Ltd. Certificate of analysis indicated that the extract was prepared using ethanol as solvent. The resulting extract was light yellow in colour having bulk density of 0.3-0.5 gm/ml and moisture content 3.06%. The content of total soy isoflavones was found to be 40.65%w/w comprising of diazin (24.16%), diadzin

(0.61%), glycitin (10.69%), glycitein (0.28%), genistin (5.62%) and genistein (0.12%).

3.2 Analytical Method Development

Solvents like ethyl acetate, benzene, methanol, toluene, diethyl ether and dichloromethane were tried in different proportions for development of optimum mobile phase. Ethyl Acetate: Pet Ether: Toluene (4:4:2v/v) showed good separation for genistein with Rf value 0.6, hence it was finalized as analytical method for further studies. The calibration curve demonstrated good linearity over the concentration range of 20 to 140 ng/band, with the correlation coefficient of 0.997 (R^2). The observed regression equation for genistein was y=365.0x +463.7.

3.3 Evaluation of Formulation

The granules to be filled in capsules were evaluated for flow properties. The results presented in Table 2 indicate that the parameters like angle of repose, Hausner's ratio and Carr's index were found to be in acceptable range indicating good flow properties of the granules. The flow properties of granules form an important aspect in capsule formulation to qualify the weight variation of capsules in the acceptable range (Table 2). The mean content of genistein in capsule formulation was found to be 18.8 mg.

Table 2. Evaluation of granules and capsule formulation prepared from glycine max seeds

Evaluation of Granules		
Parameters	**Results per Capsule**	**Acceptable Range**
Bulk density (gm/ml)	41 gm/ml	1 to 50 gm/ml
Tapped density (gm/ml)	44 gm/ml	1 to 50 gm/ml
Carr's index (%)	6.81	<10 to 26%
Hausner's ratio	1.07	1 to 1.46
Angle of repose	28.64^0	<30 to 56^0
Evaluation of Formulation		
Parameters	**Results**	**Remark**
Weight variation	±0.27	±7.5% as per IP (passes)
Disintegration test	26 minutes 15 second ±1 minutes	30 minutes as per IP (passes)
Drug content of genistein in formulation (mg/ capsule) mean s.d.	18.8 ±0.31	Label claim : 20 mg/ capsule

3.4 Bio-analytical Method Development

Different solvent systems in various compositions were tried to get separation of genistein without interference of biological matrix. The mobile phase of Ethyl Acetate: Pet ether: Toluene [4:4:2 (v/v)] indicated appropriate separation of genistein with Rf values of 0.6 (Figure 2) in HPTLC bioanalytical method, hence it was optimized and validated for determination of concentration of genistein in plasma.

Sample preparation was done by liquid-liquid extraction method. The sample preparation involve precipitation of proteins as an initial step for which the technique of precipitation with solvents such as methanol and acetone was tried. The methanol precipitation was finalized as it indicated good recovery of genistein. Genistin is a isoflavone glycoside, when administered orally undergoes acid hydrolysis and the aglycone genistein appears in blood. Hence genistein was utilized as marker compound for determination of bioavailability. The genistein is soluble in organic solvent like diethyl ether therefore the deprotonated plasma was extracted using diethyl ether for further analysis. The genistein recovery by the developed method was found to be 72.77 % w/w of the spiked standard, qualifying requirement of recovery of the genistein from plasma as per ICH guidelines for bioanalytical method.

3.5 Validation of Bioanalytical Method

3.5.1 Specificity and Matrix Effect

The HPTLC chromatograms (Figure 3a and b) indicate that there is no interference of peaks from blank plasma at the Rf values (0.6) of genistein. Also no matrix effect

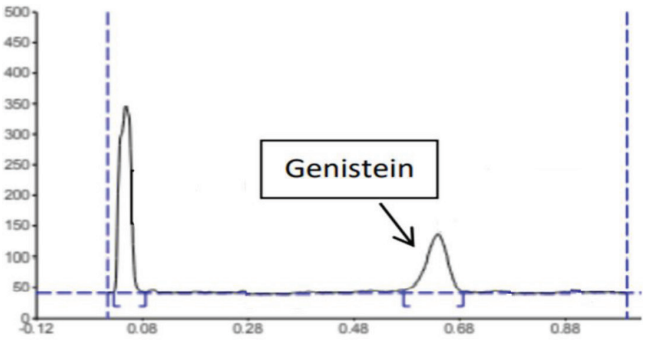


Figure 2. HPTLC Chromatogram for separation of Genistein (0.60) and other isoflavones (Stationary phase: Silica gel GF 254, Mobile Phase-Ethyl Acetate: Pet Ether: Toluene (4:4:2v/v), detection at 262nm.

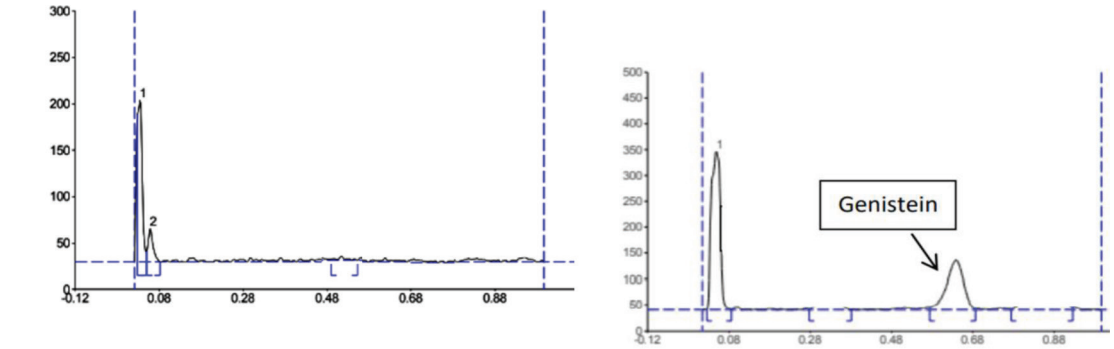


Figure 3. Specificity study- HPTLC chromatograms of (a) blank rat plasma (b) rat plasma spiked with genistein [Mobile phase: Ethyl acetate: Pet Ether: Toluene (4:4:2), detection at 262 nm].

was observed when three human plasma samples were analyzed hence it can be concluded that HPTLC method is specific and there is no significant matrix effect found in different sources of human plasma tested for genistein.

3.5.2 Linearity

The calibration curve demonstrated good linearity over the concentration range of 20 to 140 ng/band, with good value of correlation coefficient (R^2). The observed regression equation for genistein was y=22.331x-92.143 (Figure 4).

3.5.3 Accuracy and Precision

The accuracy of the bioanalytical method by % recovery method indicated accuracy of genistein in the range of 101.69-115.22 % W/W. The intraday and inter-day precision studies on the developed bio-analytical method indicated that the % RSD values at LQC, MQC, and HQC levels for genistein were within the specifications (Table 3).

3.5.4 Stability

The stability of the analyte was evaluated using quality control samples at LQC, MQC and HQC for post preparative (4 hours), bench-top (4 hours) and three repeated freeze-thaw cycles.

The bench top stability revealed that the unprocessed samples remain stable for 4 hours at room temperature as content of genistein was found to be in the range of 95.81 to 97.38 %. The post preparative study indicated solution remains stable for 4 hours after processing of samples as content of genistein was found to be in the range of 88.31 to 99.32%. The content of genistein was in the range of 98.97 to 98.55 % w/w, after completion of second freeze thaw cycle, which falls in the acceptable range. The content of genistein was found to be reduced significantly (78.99%) at the end of third freeze thaw cycle indicating the solution upto two freeze thaw cycles (Table 3).

3.6 Pharmacokinetic Study

3.6.1 Determination of Absolute Bioavailability of Marker Compounds

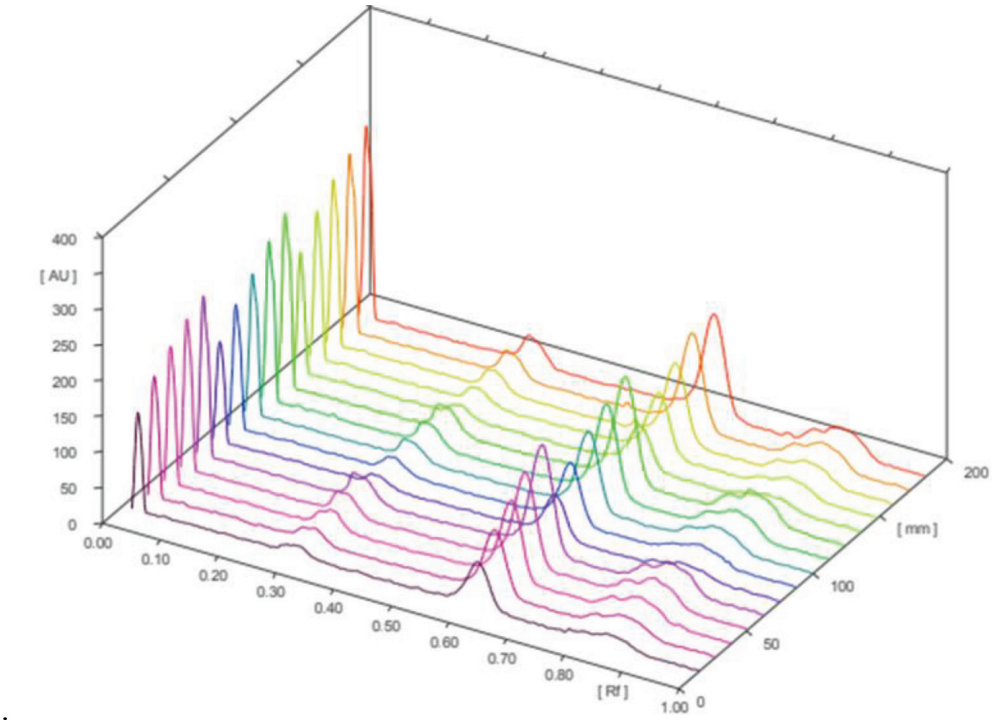
The developed bio-analytical method was utilized for determination of concentration of genistein from plasma of female wister rats. The absolute bioavailability of genistein was observed to be 14.78 % as determined by dose normalization method. Administration of pure genistein (10 mg/kg.b.w.p.o.) indicated Cmax of 1.16 µg/ml (Figure 5).

3.6.2 Bioavailability of Formulation

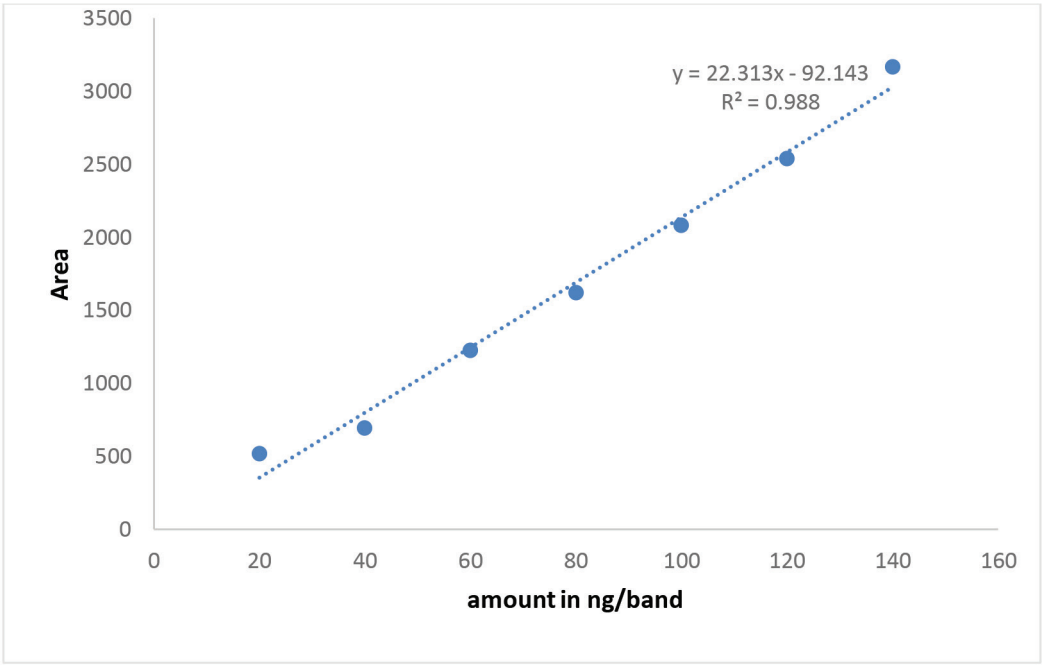
The relative bioavailability of the formulation was determined with respect genistein by administering formulation equivalent to 20mg/ kg b.w.p.o. of (genistin). Cmax values for genistein after administration of the formulation was observed to be 1.004µg/ml. The relative bioavailability of genistein after administration of the formulation as determined by dose normalization method involving comparison of AUCs which was found to be 77.4 %.

3.6.3 Pharmacokinetic Parameters

Pharmacokinetic parameters for administration of pure genistein by I.V (2 mg/kg) and oral (10 mg/kg) indicated t max values of 0.05 hours to 2 hour respectively with $t_{1/2}$ of 4.42 and 3.96 hours respectively.



.



2b

Figure 4. Linearity graph of genistein; **(a).** 3D graph of different concentrations of genistein; **(b).** Calibration curve of Genistein

The other parameters involved $AUC_{0-\infty}$ of 3236.2 and 2392.1 ng/ml with clearance of 0.2572 and 2.49 L/hr after administration of pure genistein by *i.v* (2 mg/kg) and oral (10 mg/kg) respectively.

Administration of formulation by oral route (equivalent to 20 mg /kg of genistin) indicated t max and $t_{1/2}$ of 8 hours, 11.13 hrs, $AUC_{0-\infty}$ of 2554ng/ml with clearance of 0.1714 L/hr (Table 4).

Table 3. Regression equation parameters and validation report of the HPTLC-densitometric method for determination of genistein in plasma

Parameters	Results		
Linearity	20-140 ng/band		
Slope	22.331		
Y intercept	92.143		
Correlation coefficient	0.997		
Accuracy	Levels		
	50% %	100%	150%
(% mean recovery)	101.69	101.49	115.22
Precision	Quality control standards		
(±%RSD)	LQC 40 ng/band	MQC 80 ng/band	HQC 120 ng/band
Intra day	4.21	2.90	2.97
Interday	4.72	4.87	3.11
LOD and LOQ	7 ng/band and 20 ng/band		
Stability studies (%)	LQC 40 ng/band	MQC 80 ng/band	HQC 120 ng/band
Bench top(%)	97.38	-	95.81
Post preparative stability(%)	99.32	-	88.31
Freeze thaw stability	LQC 40 ng/band	MQC 80 ng/band	HQC 120 ng/band
Cycle 1 (%)	99.99 %	-	99.40 %
Cycle 2(%)	98.95 %	-	98.55 %
Cycle 3(%)	75.14 %	-	78.99 %

4. Discussion

Soya bean is utilized as food in various forms and it also possesses various health benefits[6]. Soya bean contains isoflavones namely genistein, 4,5,7-trihydroxy isoflavones, diazein, glycitein. These isoflavones are also called as phytoestrogens and utilized for treatment of breast and prostate cancer[22]. Genistein is a main isoflavone and is reported to have chemoprevention action by down regulating genes related to self-proliferation and cell cycle, also induces apoptosis[23].

The present study involved bioanalytical method development using HPTLC. This method was found to have linearity range from 20 to 140 ng/band which is found to be more sensitive as compared to HPLC method reported by Suk Hyung Kwon[24]. Also recovery of the HPLC method is not reported. However developed HPTLC method is simple, rapid, cost effective along with good sensitivity of 20ng/band (LOQ).

The pharmacokinetic studies were carried out by Leslie Fischer *et al.*, included administration of pure isoflavones from soya bean in patients suffering from prostate cancer for 84 days. Yang Zen 2012 had reviewed studies carried out on bioavailability and pharmacokinetic of genistein in which soyabean products especially food products were found to be studied for the said aspects. The review reveals that very

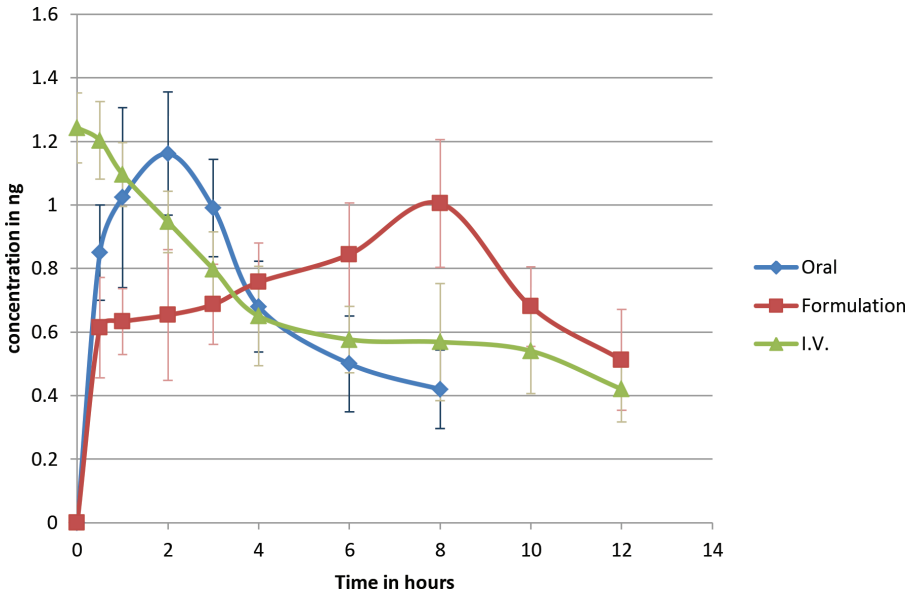


Figure 5. Mean concentration–time profile of Genistein I.V. (2 mg/kg i.v.), pure Genistein oral (10 mg/kg) and formulation of Glycine max extract oral (20 mg/kg genistein) in rat plasma (n=6, Mean ± s.d.).

Table 4. Pharmacokinetic parameters of pure marker compound genestein (2 mg/kg b.w.i.v), Greenstein (10 mg/kg b.w.p.o.) and formulation prepared from standardized soy extract equivalent to 20 mg of genistein mg/kg bw po in the rats after single dose administration

Parameters	Genistein		
	IV Single dose administration (2 mg/kg i.v) (µg/ml)	Oral single dose administration (10 mg/kg oral) (µg/ml)	Soy formulation (20 mg/kg oral) (µg/ml)
Cmax (ug/ml)	1.3065±0.054	1.1646±0.010	1.0042±0.0007
Tmax (hr)	0.05	2	8
T ½ (hr)	4.4242±0.403	3.964±0.078	11.1318±0.185
AUC0-∞ (ng/ml)	3236.2+ 33.20	2392.1+415.3	2554.1±181.7
Cl (L/hr)	0.2572±0.011	2.49±0.0450	0.1714±0.0053
Kel (h-1)	0.1708±0.002	0.1663±0.001	0.143±0.0085
Vd (L /kg)	1.553±0.045	-----	-----
Relative bioavailability (%)	-	77.4	
Absolute bioavailability (%)	14.78 + 0.260		-

less studies are available on formulations containing standardized soy extract. The present study focuses on formulation of total isoflavones rich extract (equivalent followed by bioavailability and pharmacokinetic parameters. Bioavailability of formulation was estimated with respect to genistein as it is one of the important isoflavones responsible for therapeutic action, also the extract contains lowest concentration of genistein *viz* 5.73% w/w.

Capsule formulation containing standardized soy extract (total isoflavones 40% and total genistein 5.73%w/w) was granulated by wet granulation method and evaluated for flow properties. The flow properties are essential for getting uniform content of the marker compound in capsule formulation. The results of content uniformity and weight variation were found to be complying with the specifications for capsule formulations.

The results presented in Table 4 and Figure 5 reveal that T_{max} of genistein and genestin from the formulation are 2 and 8 hours respectively owing to the faster rate of absorption of genistein, an aglycon as compared to the glycoside genestin, as the aglycon is more hydrophobic and has lower molecular weight as compared to the glycoside[25]. Hydrolysis of the glycoside genestin by β-glucosidase in jejunum and ileum along with enterobacteria present in colon, leads to delay in the absorption of the aglycon genistein. The $AUC_{(0-\infty)}$ of the marker compound genistein followed by oral administration of pure genistein (10 mg /kg) is almost similar to the formulation containing genestin (20 mg /kg p.o.), indicating that about 53 % of the genistein is appearing in blood after conversion of the glycoside genestin. These results are in line with the reported results for pharmacokinetics of pure genistein[26-28].

The concentration- time plot (Figure 45 of the oral administration of the formulation containing soy extract reveals two maximal peaks, and the similar results have been reported in papers[29-32]. The second maximal peak is as a result of enterohepatic circulation and this would increase the duration of exposure in body, in turn can provide prolonged pharmacological action[33].

The greater bioavailability of the glycoside genestin (77.4 %) as compared to the aglycon genistein (14.78 %) may be attributed to cleavage of the glycoside and transportation of the metabolites (genistein-glucuronide and genistein-sulfate) into blood stream.

Administration of capsule formulation containing standardized extract in the dose (equivalent to 20 mg/kg b.w. of genistin) yielded T max of 8 hours. This delay in the T max value is due to the delay in absorption of genistin which is a glycoside and its further conversion to genistein. Teylor *et al*., reported that the conversion of genistin to genistein takes place gradually and it is also reported that the genistein is transported across small intestine through passive diffusion and enters the systemic circulation *via* sodium dependent glucose transporter located in small intestine brush border membrane[27]. Therefore, there is delay in T max value

four times due to administration of formulation containing genistin as compared to oral administration of pure genistein.

The Ke was found to be similar and very less which indicates longer duration of stay of the drug. This can be justified by the enterohepatic recirculation and appearance of secondary peak. Plasma half-life (t 1/2) indicates the time required for reducing the concentration of drug to the half of its concentration and it also indicates whether the accumulation of the drug occurs under a multiple dosage regimen and it is essential to decide on the appropriate dosing intervals. No significant difference was observed in the t 1/2 values in all the three cases *viz*. administration of pure genistein (2mg/kg *i.v* and 10 mg/kg oral) and formulation containing genistin (equivalent to 20mg/kg). Hence, it can be concluded that the kinetics followed for elimination of genistein may be first order.

5. Conclusion

The study demonstrates that the developed validated bioanalytical HPTLC method is simple, precise, accurate and comparable to the existing HPLC methods. The HPTLC method can be utilized for determination of bioavailability of the formulations containing standardized extract of soy as the results of bioavailability and the pharmacokinetics are also observed to be at comparable with the reported literature.

6. Author Contribution

Archana N. guided the analytical method development work, Chhaya G. designed and directed the project. Harshala B. developed the bioanalytical method for the estimation of secoisolariciresinol from plasma. Siddhi B. carried out work on extraction, isolation, formulation, and animal studies. All of the authors have read and agreed to the publication of the submitted manuscript.

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