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International Journal of Pharmaceutical Research & Allied Sciences, 2016, 5(3):124-131

Research Article

ISSN : 2277-3657
CODEN(USA) : IJPRPM

Validated HPTLC method for estimation of γ-oryzanol in rat plasma and its application to pharmacokinetic study

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ABSTRACT

The current research describes the validated HPTLC method for the estimation of γ-oryzanolin rat plasma. The protein precipitation method was applied for extracting γ-oryzanol from rat plasma, and a desired amount of the extracted drug was spotted on precoated silica gel 60F_{254} plates using a Camag Linomat V sample applicator. γ-oryzanol was quantified using Camag TLC scanner III. Linearity range was observed to be 400-1400ng band^{-1}.Limit of detection and limit quantification was found to be 56.03 and 169.81 ng band^{-1} respectively. Accuracy was calculated for percentage recovery, and was found in the range of 97.95 to 100.06% for the matrices studied for γ-oryzanolin plasma. The method provides a straight estimate of the concentration of drug present in plasma after oral administration to rat. The C_{max} was found to be 1380ng band^{-1} whereas T_{max} was 300min. The method was successfully applied in the Pharmacokinetic study for the determination of plasma levels of γ-oryzanol.

Keywords: Pharmacokinetic, γ-oryzanol, HPTLC, rat plasma.

INTRODUCTION

More than half of the world population consumes rice as a main constituent in their diet. Rice provides energy in the form of carbohydrate and also has medicinal properties. Rice is utilized in conventional medicines as a medication for inflammation, gastrointestinal ailments, hypercholesterolemia, diabetes, and skin diseases. Experimental and clinical data demonstrate that rice and bran oil reduce hypercholesterolemia and cardiovascular risk, it also has an anti-inflammatory and immunostimulatory. This medicinal effect of the rice and rice bran oil is attributed to chemical constituents present in it [1-3].

The kernel contains largely of starch (about 70%), other polysaccharides, in addition to simple sugars such as glucose, fructose, and saccharose. Lipids like oleic and linoleic acids, whereas main proteins are prolamins, glutelins, globulins, and albumins. Phenols comprise of flavonoids, as tricin plus tricinin, and various phenolic acids, like ferulic, coumaric, sinapic, protocathecuic, chlorogenic, hydroxybenzoic, vanillic, syringic, caffeic, and gallic acids [2, 4-7]. Other secondary metabolites consist of tocopherols and tocotrienols (vitamin E), the sterols; β-sitosterol, γ- sitosterol, and campesterol, the diterpenes momilactone A and B, along with B-group vitamins, as thiamin, riboflavin, along with niacin [3, 8-11]. Bran dry extract composed of 18% protein, 24% lipids, and 38% sugars. Secondary metabolites and bioactive molecules are also found in bran extracts as aliphatic alcohols, steroids, triterpenes, plus their glycosylated derivatives, anthracene derivatives, along with terpenoids [4]. Rice bran oil was extracted from rice bran with the help of petroleum ether or n-hexane contains about 47% monounsaturated, 33% polyunsaturated and 20% saturated fats. Main fatty acids present in rice bran oil are

oleic (about 42%), linoleic (40%), palmitic (15%), stearic (2%), linolenic (1%), arachidonic (0.5%), and behenic (0.2%) acids [5, 12, 13]. The unsaponifiable fraction contains 4% of the total amount, and itholds phytosterols, like campesterol, stigmasterol.Also, the same fraction contains 13-sytosterol, triterpenic alcohols, like cycloartenol ferulic acid and 24-methylcycloartenol ferulic acid (components of γ-oryzanol), aliphatic alcohols, and hydrocarbons [5, 14-17].Antioxidants like tocopherols, tocotrienols, and γ-oryzanolare also found in rice bran oil extracted with petroleum ether or n-hexane. γ-oryzanolcomposed of trans-ferulic acid esters of triterpene alcohols and sterols, of which cycloartenol ferulic acid (CAF), 24- methylcycloartenol ferulic acid (24-mCAF) are present in high concentration [6-8, 18-20]. γ-oryzanolwas discovered in 1950s, since then numerous studies have been executed exploring the pharmacological potentials of it. In Asia, it has been widely used as a therapeutic agent for the remedy of various clinical disorders. Spectral data shows that the ferulic/caffeic acid part plays an important role to elucidate the biological activity while triterpine unit adds lipophilicity. Ferulic acid and its analogs are extremely studied for various biological activities too. However,only one method is reported for bioanalytical studies of γ-oryzanol.Fujiwara*et al.*, in 1983 performed pharmacokinetic study of γ-oryzanol by using ^{14}C-labled compounds of ferulic acid esters, this method has drawbacks of less sensitivity and use of radioactive substance [9, 21, 22]. The aim of the present study was to develop, validate, simple, accurate, precise and robust bioanalytical method for quantitative determination of γ-oryzanolin rat plasma by high perfomance thin layer chromatography (HPTLC) as per US FDA guidelines [10, 23, 24].

MATERIALS AND METHODS

Solvents and chemicals

Tinidazole used as aninternal standard was gifted by Aarti Drugs, Mumbai, India. γ-oryzanol (i.e. mixture of 24-mCAF and CAF) was purchased from Oryza Oil and Fat Chemicals Co. Ltd. (Lot no. S1412), Numata Kitagata-cho Ichinomiya City, Japan. Analytical grade reagents and chemicals were used in the study and purchased from Merck Specialties Private Limited, Mumbai, India. Double distilled water filtered through 0.45 µ filter paper was utilized in the research work.

Animals

Male Wistar rats weighing in the range of 200 –250 gm were purchased from National Institute of Biosciences, Pune, India.All the animals were housed in solid bottom polypropylene cages at an ambient temperature of 24 ±1°C, with relative humidity of 45– 55% and 12-12 h dark/light cycle. The animals were acclimatized for a period of 2 weeks and were kept under pathogen-free conditions. The animals had free access to standard pellet chow (Chakan Oil Mills, Sangli) and water throughout the experimental protocol, with the exception of overnight fasting before pharmacokinetic experiment and allowed free access to water during the experiment. The work was conducted as per CPCSEA (CPCSEA/PCL/09/2014-15) guidelines with prior approval by Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Pune

HPTLC Instrumentation and chromatographic conditions

The HPTLC analysis was done on pre-washed and activated precoated silica gel aluminum plate 60F$_{254}$ (20 cm × 10 cm with 250 µM thickness; E. Merck, Darmstadt, Germany) as a stationary phase for γ-oryzanol. The samples were applied in the form of bands of width 6 mm wide and 10 mm apart by using Hamilton syringe (100 µL) using a *Camag Linomat V* (Switzerland) sample applicator. The constant application rateof 150 nL s^{-1}, slit dimension of 5 mm × 0.45 mm with scanning speed of 10 mm s^{-1}. The source of radiation: deuterium lamp. The mobile phase composed of toluene: ethyl acetate: methanol (15:1.7:3.3, v/v/v) for 20 mL. Linear ascending development was carried out in 20 cm x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 15 min at room temperature (25 ± 2°C) at 60 ± 5% relative humidity. The length of the chromatogram run was 8 cm. Densitometric scanning was performed within 10 min after chromatographic development by using *CAMAG*TLC scanner III with *win* CATS software version 1.4.4 in the reflectance mode at 317 nm.

Preparation of standard stock solutions

Standard stock solutions of tinidazole and γ-oryzanol were prepared separately by dissolving 10 mg each in 10 mL methanol and 10 mL ethanol respectively to get thefinal concentration of 1000 µgmL^{-1}. The stock solutions were prepared freshly and were used for further studies.

Calibration standards (CS) and quality control (QC) samples of plasma

The stock solutions of the standard compounds were further diluted with ethanol to concentrations in the range of 30µg to 300µg/1000µL to establish calibration curve. The 100 µL of each working solution of γ-oryzanol spiked to plasma (400µL) in order to obtain calibration standards ranging from 2 to 20 ng µL^{-1}. QC samples were prepared at concentrations of 6, 10, 14 and 18 ng µL^{-1}.

Sample preparation

Protien precipitation method was used to isolate the drug from rat plasma. Prior to analysis, plasma samples were thawed at room temperature for 15 min. The500 µL of plasma calibration standards and QC samples were transferred to 2 mL eppendorf tubes and mixed with 1 mL of acidified acetonitrile solution. Acidified acetonitrile was prepared by mixing 0.1 mL formic acid in 100 mL acetonitrile. Acetonitrile formic acid solution showed highest extraction of analyte. Acetonitrile-formic acid solution provides increased purity and stability of extracted analyte. After vigorous mixing for 3 min, the samples were centrifuged for 10 min at 15000 rpm at 4°C. The supernatant was transferred to another empty 2 mL eppendorf tube and the solvent was evaporated under a stream of nitrogen. The plasma samples were reconstituted with 1500 µL of ethanol. The 50 µL of each extracted sample was spotted on TLC plate to obtain the final calibration range was of 400 to 1400 ng band^{-1}. Each concentration was spotted seven times on aplate. Internal standard, tinidazole was also spotted on the plate and its concentration was 300 ng band^{-1}.

Specificity

The specificity of the proposed bioanalytical method was estimated by analyzing three different batches of blank plasma samples. Peaks for γ-oryzanol were confirmed by comparing UV spectrum of the spot at the peak start, peak apex and peak end positions i.e., r (start, middle) and r (middle, end). Other constituents present in the plasma did not interfere with the peaks of γ-oryzanol

Precision and accuracy

Precision and accuracy were assessed by executing replicate analysis of QC samples at three levels (400, 800 and 1200 ngband^{-1}) in plasma. Intra-day repeatability was assessed by treating spiked plasma samples on the same day. Inter-day repeatability was performed by comparing the results of the assay executed on different days on same spiked plasma samples. For each kind of samples study was repeated in six replicates.

Accuracy studies were achieved to study the suitability and reliability of the method. Accuracy studies were performed in triplicate by standard addition method. Accuracy was determined during the percentage recoveries of known amount of γ-oryzanoladded to samples. The samples were spiked with 80, 100 and 120 % of γ-oryzanol(600 ng band^{-1}). The percent ratios between the recovered and expected concentrations were determined.

Robustness

The effect of small, deliberate variation of the analytical conditions on the peak areas of the γ-oryzanolin plasma was studied. The robustness of the proposed chromatographic method was carried out at a concentration of 600 ng band^{-1} for γ-oryzanol. The standard deviation of peak areas and % Relative Standard Deviation (% RSD) were studied for each variable parameter.

Stability

The stability of γ-oryzanolin plasma was studied.Two sets of QC samples at low concentration 400ng band^{-1} and at higher concentration1000ng band^{-1}were prepared and were stored at –80°C until use. Another set of QC samples were freshly prepared and used for analysis immediately at zero time (baseline) to know about accuracy and precision of the method. Short term stability also known as Bench-top stability was evaluated for low and high concentrations [10] (QC standards) by standing on the bench-top for 4 h at room temperature (25 ± 2°C). Long term stability study was doneby storing the QC samples at –80°C for 7 weeks before being analyzed. Freeze-thaw stability was studied at the two QC standards after freezing it at –80°C for 24 h and thawing completely at room temperature for three cycles. All the QC samples were analyzed for three times and the results obtained were compared with results obtained from freshly prepared samples.

Application to Pharmacokinetic study

The rats were administered with an oral suspension of 1600 mg/kg γ-oryzanolas 1% suspension in Tween® 80. Blood samples were collected at predetermined intervals into heparinized tubes at 0 min (predose) 15, 30, 45, 60, 120, 180, 240, 300, 360, 420, 480, 540, 600, 660, 720,1440and 2880 min (post dose). The tubes were centrifuged at 7000 rpm for 10 min at 4 °C. The total blood volume collected from each rat was approximately 0.75 to 1 mL which does not exceed the maximal recommended blood volume of 20% (2.0 mL for a 200 g body weight rat). Plasma was obtained by centrifuging blood samples at 7,000 rpm at 4° C for 10 min. The obtained plasma samples were transferred into pre-labeled microcentrifuge tubes and stored at −80°C. All the samples were analyzed by the developed method.

RESULTS

Optimization of chromatographic conditions Mobile phase

The separation γ-oryzanol could be accustomed by varying the composition of mobile phase. Initially solvents like

toluene, ethyl acetate, ethanol, methanol, dichloromethane, acetone and chloroform as a single component and in varying compositions mobile phase were tested. The results showed that γ-oryzanol peak was obatined with toluene, ethyl acetate and methanol; however with unacceptable Rf value (lower than 0.2). Various ratios of toluene, ethyl acetate and methanol were explored. The results shows that the best Rfvalue was obtained with toluene: ethyl acetate: methanol in the ratio 15.0:1.7:3.3(v/v/v) (total 20 mL) with proper separation with sharp and well defined symmetrical peaks of tinidazole at Rf = 0.28 ± 0.02 and γ-oryzanol at 0.72 ± 0.02 using the selected mobile phase system.

Selection of detection wavelength

Following chromatographic development bands were scanned over the range of 400-700 nm and the spectra were observed. It was recognized that tinidazole and γ-oryzanol exhibit considerable absorbance at 317 nm and hence, wavelength 317 nm was selected for analysis.

Internal standard

Different 5-Nitromidazoles were tested as internal standards in order to check its fidelity. The result showed that the tinidazole was found to be an excellentone because its excitation and emission wavelengths are close to those of γ-oryzanol. Also, excellent resolution between tinidazole (0.28 ± 0.02) and γ-oryzanol(0.72 ± 0.02) was achieved under the applied chromatographic conditions. Hence, tinidazole was finalized for all the subsequent experiments as an internal standard (Figure 2B).

Analytical method validation

The US FDA guidelineswere followed for validation of the bioanalytical method. All results were exhibited in percentages, whereas 'n' defines the number of replicates. Statistical analysis was carried out by using Microsoft Office Excel 2007. Level of significance was 5%. Following parameters were validated by the developed HPTLC method.For the pharmacokinetic study of γ-oryzanol'Kineticav5.0' software was used.

Calibration curve and linearity

The linear calibration curve was obtained for γ-oryzanol in rat plasma (**Error! Reference source not found.**A). The peak area *vs.* concentration fitted well to straight line with equation y =1.643X -55.19. The correlation coefficient for calibration curve was 0.998 (**Error! Reference source not found.**B) (Table 1).

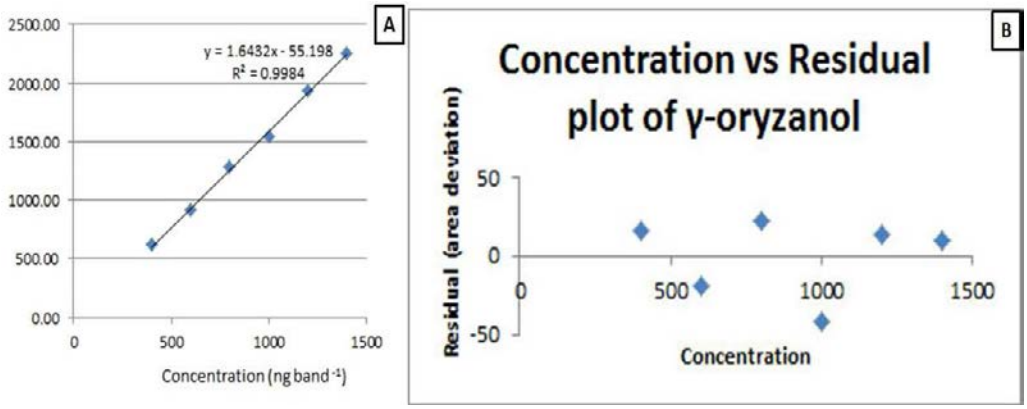

Figure 1. Linearity (A) and Concentration versus residual plot of γ-oryzanol (B)

Table 1. Linear regression data for the calibration curves (n=6)

Parameters	γ-oryzanol
Linearity range (ng band $^{-1}$)	400-1400
r^2	0.998
Slope	1.643
Intercept	-55.19
Confidence limit of slope[a]	1.55-1.73
Confidence limit of intercept[a]	-144.159-33.762
S_{y_x}[b]	27.84
LOD	56.03 ng band $^{-1}$
LOQ	169.81 ng band $^{-1}$

[a] 95% confidence limit; [b] Standard deviation of residuals from line

Limits of detection and quantitation

Sensitivity of the method is given by the values of limits of detection (LOD) and limits of quantitation (LOQ). The values achieved for LOD and LOQ for γ-oryzanol were 56.03 and 169.81 ng band $^{-1}$respectively.

Precision and accuracy

The intra and inter-day precision and accuracy of the assay are given in Table 2. The values found for intra-day precision study mentioned in terms of percentage relative standard deviation (% RSD) for γ-oryzanol was found to be 1.33 to 1.68whereas the inter-day precision study showed 1.03 to 1.26%RSD of γ-oryzanolin plasma. Accuracy was calculated for % recovery, and it was ranged from 97.95 to 100.06% for the matrices studied for γ-oryzanolin plasma. The values of % RSD was less than two (< 2%), with no significant difference in values for intra-day and inter-day precision, indicate the method's reproducibility with high precision.

Table 2. Intra- and interday precision of the HPTLC method, Recovery and Robustness study for γ-oryzanol (n=6)

Standard drug	Nominal concentration[a]	Concentration obtained [a]		Precision obtained [b]	
		Intra day	Inter day	Intra day	Inter day
γ-oryzanol	400.00	420.04	414.56	1.68	1.12
	800.00	793.29	807.08	1.33	1.03
	1200.00	1217.26	1212.59	1.24	1.26

Amount Taken[a] (%) γ-oryzanol	Amount added [a]	Total[a]	Amount found [a]	SD	% Recovery	% RSD
600 (80)	480	1080	1064.85	58.36	98.59	3.44
600(100)	600	1200	1175.45	71.04	97.95	3.78
600(120)	720	1320	1320.89	83.61	100.06	3.95

Parameter	γ-oryzanol concentration found	% RSD
Mobile phase (Ethyal Acetate) composition (± 0.1 mL)	15.02	1.64
Amount of mobile phase (± 5 %)	13.05	1.41
Time from band application to chromatography(+ 10 min)	17.45	1.91
Time from chromatography to scanning (+ 15 min)	10.47	1.13

[a]Concentration in ng band^{-1}; [b]% RSD; RSD is relative standard deviation, SD is a standard deviation

Robustness

The lower value of (% RSD < 2) was obtained after changes in mobile phase composition time from band application to chromatography and time from chromatography to scanning (Table 2)indicated the robustness of the method.

Selectivity

Selectivity of the method was assessed by analyzing blank, spiked and rat plasma samples containing γ-oryzanol in presence of tinidazole as an internal standard. The densitogramswere visually screened for any interfering peaks from the endogenous matrices. It was noticed that there were no interfering peaks from the biological matrix. Moreover, the Rf values of both, γ-oryzanol and tinidazole remained unaffected bypresence of plasma indicating selectivity of the densitometric method (Figure 2A).

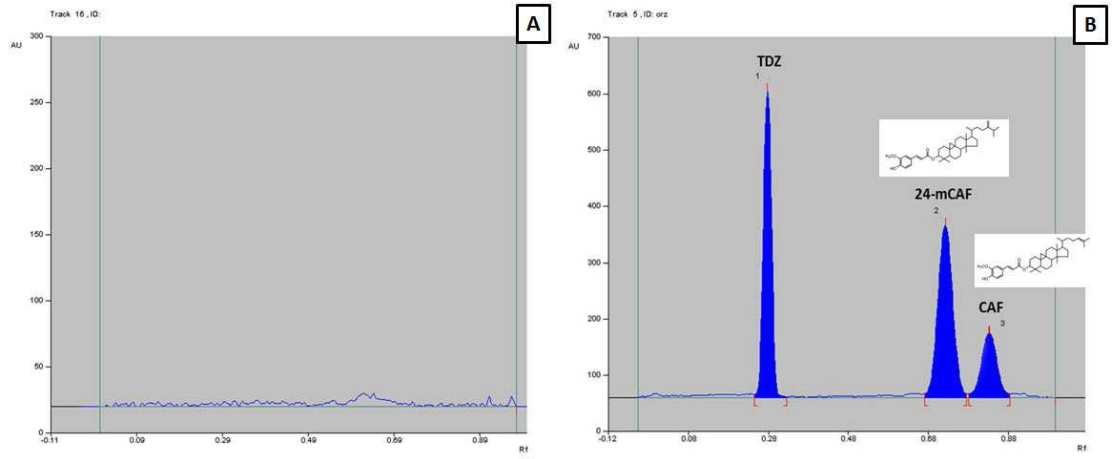

Figure 2. Chromatograms of drug-free plasma (A) and Chromatograms of plasma samples (collected at 2 h) of rat having received γ- oryzanol 1600mg/kg orally (B)

Stability

Plasma QC samples of γ-oryzanol at two concentrations (400 and 1000ng band^{-1}) were used for the stability experiments. The results shown in Table 3 signify that no significant degradation was seen under these conditions. Hence, it is inferential that the plasma samples containing γ-oryzanol can be managed under normal laboratory

conditions without any significant loss of compound. Stock solution of γ-oryzanol which was stored at room temperature (25ºC ± 2) for 48 h and at -80ºC for 7 weeks and were found to be stable with no significant changes in the peak area of drug.

Table 3. Solution Stability of γ-oryzanol under various stage conditions

Concentration ng band^{-1}	% Stability ± SD				
	Plasma sample			Stock solution	
	Short term (48 h)	Long Term (7 weeks)	freeze thaw		
400	95.66± 16	94.51 ± 8.14	95.64 ±17.98	97.92 ±1.53	97.83 ± 1.50
1000	97.88 ± 14.59	96.42 ± 2.53	95.43 ± 9.44	98.17 ±18.50	99.16 ±12.47

Stability % represents the recovery %, and equals mean measured concentrations (n=3) at the indicated time divided by mean measured concentration (n=3) at baseline x 100.

Pharmacokinetic study

The method was applied for analysis of plasma samples collected after oral administration of single γ-oryzanol (1600 mg/kg). The maximum plasma concentration (C_{max}) was observed to be 1380ng band^{-1}. The result indicates that higher plasma levels were obtained after oral administration of γ-oryzanol. The t_{max} for γ-oryzanol was found to be 300 min whereas Fujiwara (1983) reported for γ-oryzanol was 240min after oral administration in rats. Experimental C_{max} values after oral administration were comparable with reported data showing that this method is applicable successfully tostudy the preclinical pharmacokinetics of γ-oryzanol (Figure 3) (Table 4).

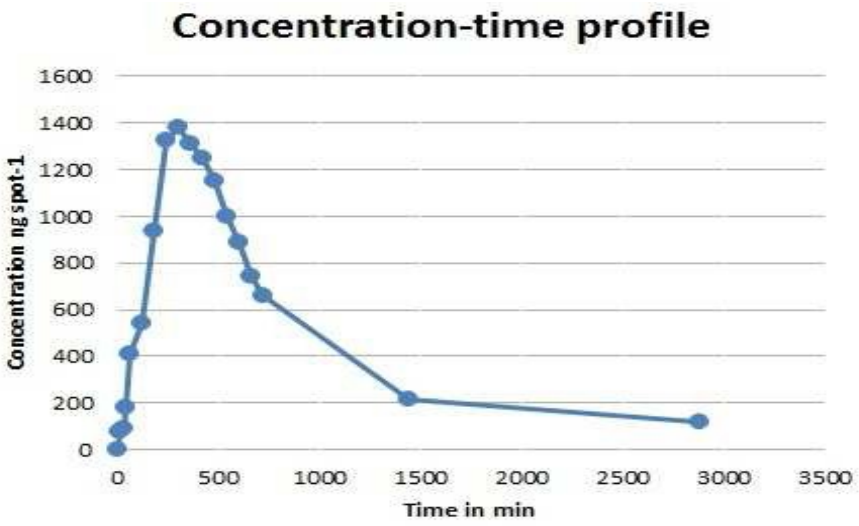

Figure 3. Concentration-time profile for γ-oryzanol in rat plasma

Table 4. Pharmacokinetic parameters of γ-oryzanol (1600mg/kg) in rat plasma

Parameter	γ-oryzanol
C_{max}(ng band $^{-1}$)	1380
t_{max} (min)	300
$t_{1/2}$ (min)	690.29
AUC$_{extra}$(ng.min band $^{-1}$)	90132
AUC$_{extra}$(%)	7.045
Lz	0.001004
MRT (min)	1083.07

AUC is an area under curve; AUMC is area under the first moment curve, Lz is elimination rate constant, MRT is mean residence time.

DISCUSSION

In the present study pharmacokinetic profile of γ-oryzanol was studies. The pharmacokinetic profile of any drug gives good correlation of concentrations and its effect in either humans or experimental animals[11].Many

nutritional and herbal components are biologically effective but lacks standardization[12, 13]. Hence there is need to develop a suitable bioanalytical method. The pharmacokinetic profile of γ-oryzanolis not well established. We are the first group to develop the HPTLC method for quantitative determination of γ-oryzanol from rat plasma. In case of natural product analysis HPTLC is most widely used than other chromatographic methods [12]. In the present work attempt has been made to develop and validate rapid, accurate, robust, and precise HPTLC method to quantify γ-oryzanolin rat plasma. The results indicate the reliability of the proposed densitometric method.

However, the recovery and relative standard deviation are increased by our proposed HPTLC method. The limit of detection is in the range of nanogram as compared to reported HPLC method. The R_f value of the γ-oryzanol was found 0.72±0.02; eluted samples were free of any metabolites or degraded products. The optimized method was free of interferences in the drug free blood plasma the spectra measured on the spots showed maximum absorbance at about 317nm. Thus this method improved detection sensitivity, specificity, robustness, minimized the interferences from the serum samples. The solvents used for the extraction of the drug were optimized to minimize the degradation of the product. The mobile phase ratio was optimized to get optimum R_f value, and clean densitogram. We also observed that stock solution of γ-oryzanol was stable in ethanol without any measurable degradation. The pharmacokinetic investigations were carried out using HPTLC validated method to quantify γ-oryzanol from rat plasma. We found that disposition process of the γ-oryzanol in rats could be described by two compartment open model. The T_{max} was achieved at 300 min whereas; C_{max} was 1380ngband^{-1}is higher as compared to other reported methods. As the method has low detection limits, high recovery rate and good reproducibility in biomatrices; therefore it can be employed for routine pharmacokinetic analysis.

CONCLUSION

In the present study the HPTLC method was developed and validated for γ-oryzanol in rat plasma. The high sensitivity achieved by this HPTLC method confirmed the determination of γ-oryzanolin rat plasma. It can measure the plasma concentration of γ-oryzanol at a dose of 1600 mg/kg. Pharmacokinetic study showed that, the plasma concentration of γ-oryzanol reached maximum 300 min after administration. After 48h also the quantity was detected in plasma. This HPTLC method for quantification of γ-oryzanol in rat plasma was found to be accurate, rapid, sensitive, selective, precise in the biomatrices. The advantage of this method is that it is suitable and sensitive to study pharmacokinetic profile in biological matrix and would be applied to study the bioavailability of the novel formulations.

Acknowledgements
The authors are thankful to University Grants Commission (UGC), New Delhi, India, for financial assistance for the research under the scheme of Major Research Project (MRP). The authors acknowledge Dr. S. S. Kadam, Vice Chancellor, Bharati Vidyapeeth Deemed University and Dr. K. R. Mahadik, Principal, Poona College of Pharmacy for keen interest and providing the necessary facilities to carry out this study. The authors wish to thank Amit Kandhare and Pravin Pawar for carrying out this research work.

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