Highly Sensitive LC-MS/MS-ESI method Development for the Determination of 5,7-dihydroxyflavone in Mouse Plasma and Pharmacokinetic Study in Lean and Diet Induced Obese Mice

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ABSTRACT

Diet-induced obese (DIO) mice have been commonly used extensively as an animal model of obesity and diabetes in the efficacy assessment for new drug candidates. Physiological and biochemical alterations are reported in DIO mice due to modulations of drug-metabolizing enzymes and high calorie intake. Limited studies have been reported regarding the effect of obesity/diabetes on pharmacokinetics (PK) in animals. A simple, specific and sensitive rapid LC-ESI-MS/MS method has been developed and validated for the quantification of chrysine (5,7-dihydroxyflavone) using tolbutamide as an internal standard (IS) as per regulatory guidelines. Sample preparation was accomplished through a simple protein precipitation. Chromatographic separation of 5,7-dihydroxyflavone and IS was performed on Atlantis C-18 column using an isocratic mobile phase comprising 0.2% formic acid in water and acetonitrile (20:80, v/v) at a flow rate of 0.9 mL/min. Elution of 5,7-dihydroxyflavone and IS occurred at ~2.49 and 2.34 min, respectively. The total chromatographic run time was 3.2 min. A linear response function was established in the concentration range of 4.50-4500 ng/mL. This novel method has been applied to a pharmacokinetic study in lean and DIO mice.

Keywords: 5,7-dihydroxyflavone; LC-MS/MS; DIO; Mice plasma; Pharmacokinetics; Retention time.
INTRODUCTION

Obesity is one of the increasing threats to human health. It significantly increases the risk of Type II diabetes as well as other metabolic and vascular disorders, such as hepatic steatosis, hypertension, and high cholesterol levels [1,2]. Changes in physiological and/or pathophysiological conditions resulting from obesity and other metabolic diseases often affect expression of cytochrome P-450 enzymes [3,4]. Since obese and diabetic patients generally have higher need for pharmacotherapy, it is important to understand the adverse drug-drug interactions in these patients. Several animal models, including diet-induced obese (DIO) mice, genetically diabetic db/db or ob/ob mice and genetically obese Zucker rats, are routinely used to evaluate the pharmacology of anti-diabetic and anti-obesity agents [2,5]. It has been previously reported that the expression of phase I and II enzymes are altered in the liver of these obese/diabetic model animals [3]. Recent studies have shown that of chrysin (5,7-dihydroxyflavone) has many biological activities and pharmacological effects including antioxidation, anti-inflammation, antiaging, anticancer, antidiabetic, antiestrogenic, and so forth [6-10]. It is also available as a dietary supplement because of its reported anticancer activities. However, its bioavailability is very poor due to extensive phase II metabolism [11,12] and demonstration of these claimed biological effects in vivo (especially in humans) remains a challenge.

There are limited studies reported on quantification of 5,7-dihydroxyflavone and its metabolites in vitro and in vivo using HPLC [11,12]. However, to perform disposition and pharmacokinetic studies especially in disease animal, a more sensitive and reliable method is required. To date there are few LC-MS/MS method reported for quantification of 5,7-dihydroxyflavone in biological matrix. In this paper, we reported the development and validation of 5,7-dihydroxyflavone in mouse plasma by LC-MS/MS method. The method was successfully applied to quantitate the level of 5,7-dihydroxyflavone in mice pharmacokinetic studies. To our knowledge, limited studies were reported in literature to systemically evaluate the effect of high fat diet in the pharmacokinetics of DIO mice. The objective of this study (i) To develop and validate the method of quantification of 5,7-dihydroxyflavone in mouse plasma. (ii) To investigate and compare the PK profiles of 5,7-dihydroxyflavone in DIO and lean mice.

MATERIAL AND METHODS

Chemicals and materials

Chrysin (5,7-dihydroxyflavone, purity >99%) and Tolbutamide (purity >99%) was purchased from Sigma-Aldrich, St. Louis, USA. High performance liquid chromatography (HPLC) grade Acetonitrile and methanol were purchased from Rankem, Ranbaxy Fine Chemicals Limited, New Delhi, India. Analytical grade formic acid was purchased from S.D Fine Chemicals, Mumbai, India. All other chemicals and reagents were of analytical grade and used without further purification.

Instrumentation and Chromatographic conditions

To determine the plasma concentration of 5,7-dihydroxyflavone, a liquid chromatography coupled with mass spectrometer was employed. A Agilent (Agilent, Germany) LC system equipped with quaternary pump along with degasser and auto-sampler was employed to inject 5 µL aliquots of the cleaned samples on a Atlantis C-18 column (50 × 4.6 mm), which was conserved at 50°C. The mobile phase solvents used for chromatography were strained through a 0.45 µm membrane filter (Millipore, USA) and
degassed before use. Gradient mobile phase containing acetonitrile and 0.2% formic acid in water at a flow rate of 0.9 mL/min was employed.

Quantitation of 5,7-dihydroxyflavone was achieved by MS/MS detection in negative ion mode for analyte and IS (tolbutamide) using a MDS Sciex (Foster City, CA, USA) API-4000 mass spectrometer, equipped with electrospray ionization interface at 500°C temperature and 4000 V ion spray voltage. The source parameters viz., curtain gas; GS1, GS2 and CAD were set at 25, 60, 65 and 15 psi. The compound parameters viz., declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) were -80, -10, -35, and -18 V for 5,7-dihydroxyflavone and -56, -10, -40, and -15 V for IS (Tolbutamide). Detection of the ions was performed in the multiple reaction monitoring (MRM) modes, monitoring the transition of the m/z 253 precursor ions to the m/z 142.9 product ion for 5,7-dihydroxyflavone and m/z 269 to 169 for IS. Quadruples Q1 and Q3 were set on unit resolution. The dwell time was 200 m sec. The analytical data were processed by Analyst software (version 1.6.3).

**Preparation of stock and standard solutions**

The primary stock solutions of 5,7-dihydroxyflavone were prepared in methanol (800 µg/mL). The IS stock solution of 2000 µg/mL was prepared in methanol. The primary stock of analyte was diluted in 80% methanol to prepare secondary stocks and working solutions for preparing calibration curve (CC) of 5,7-dihydroxyflavone. Working stocks were used to prepare plasma calibration standards. Blank mice plasma was screened first before spiking to ensure that plasma is free from any endogenous interference at retention times of 5,7-dihydroxyflavone and IS. Eight point calibration standards samples (4.50-4500 ng/mL) were prepared by spiking the blank mice plasma with appropriate concentration of 5,7-dihydroxyflavone. To determine the precision and accuracy, samples were prepared by spiking control mice plasma along with 5,7-dihydroxyflavone at appropriate concentrations 4.5 ng/mL (LLOQ, lower limit of quantitation), 13.5 ng/mL (LQC, low quality control), 1858 ng/mL (MQC, medium quality control) and 3600 ng/mL (HQC, high quality control). All the samples were stored at -80 ± 10°C.

**Sample preparation**

A protein precipitation method was followed for extraction of 5,7-dihydroxyflavone from mouse plasma. An aliquot (50 µL) of plasma was precipitated with 300 µL of acetonitrile containing IS (200 ng/mL). The mixture was centrifuged for 5 min at 14,000 rpm in a refrigerated centrifuge (Eppendorf 5424R) maintained at 5°C. Clear supernatant (150 µL) was transferred into vials and 5 µL was injected onto LC-MS/MS system for analysis.

**Bioanalytical method validation**

As per US FDA guidelines (US DHHS, 2001), a full method validation was performed for the assay in mouse plasma. The method validation parameters tested was: system suitability, carryover test, selectivity, matrix effect, linearity, precision and accuracy, recovery, dilution integrity and stability analysis. Analyte at MQC concentration level with the internal standard was injected for system suitability experiment. System suitability test was performed every day before start of the analysis or as and when required. Carry over test was evaluated by injecting blank plasma sample → LLOQ sample → blank plasma sample → ULOQ sample → blank plasma sample. Method for selectivity was evaluated by analyzing six different K2, EDTA plasma lots. The sensitivity was evaluated by analyzing six sets of spiked lowest limit of reliable quantification (LLOQ) samples. The
enhancement of matrix ions in negative electrospray ionization (ESI) made was assessed by comparing the mean area response of post extraction spiked samples with mean area of aqueous samples (neat samples) prepared in mobile phase solutions at LQC and HQC levels. For linearity assay, a total of three calibration curves containing eight non-zero concentrations were generated. Each CC was analyzed individually by least square weighted ($1/x^2$) linear regression. LLOQ QC, LQC, MQC and HQC sample were analyzed for intra-day precision and accuracy (six replicates) each and for inter-day accuracy and precision; three batches of samples were assessed by analyzing on three consecutive days. The precision (% CV) at each concentration level should not be greater than 15% and the accuracy (%) must be within 15% at each QC level except LLOQ QC where it must be within 20%. Stability of 5,7-dihydroxyflavone in the biomatrix during 6h (bench-top) was determined at two concentrations (13.5 and 3600 ng/mL)in six replicates at ambient temperature (25 ± 2°C). Freezer stability of 5,7-dihydroxyflavone was assessed by analyzing QC samples (13.5 and 3600 ng/mL) stored at -80°C for at least 30 days. The stability of 5,7-dihydroxyflavone in mouse plasma following three freeze-thaw cycle was analyzed using QC samples spiked with 5,7-dihydroxyflavone. Samples were considered stable if assay values were within acceptable limits of accuracy (85-110% of nominal value) and precision (±15% RSD) as compared with freshly spiked samples. Upper concentration limit of the 5,7-dihydroxyflavone can be extended by performing the dilution integrity experiment. Six replicates each at a concentration of about 10 times of the uppermost calibration standard were diluted 5-fold and 10-fold with screened blank plasma. The diluted samples were processed and analyzed with undiluted calibration curve standards. The recovery of 5,7-dihydroxyflavone determined at LQC and HQC levels. It was calculated by comparing the mean peak response of pre-extraction spiked samples (spiked before extraction; n=6) to that of non-extracted samples (neat samples; n=6) at each QC level.

Pharmacokinetic study design

The Pharmacokinetic study was approved by the Institutional Animal’s Ethics Committee Vipragen Bioscience Pvt ltd, Mysore India. 5-6 weeks old male C57bl/6 mice were procured from Vivo biotech, Hyderabad. Animals were quarantined for 1 week. Mice were housed, 5-6 animals per cage, under standard laboratory conditions, air conditioned with adequate fresh air supply and with 12-15 air cycles per hour. Environment with temperature 22° to 25°C, relative humidity 60-70%, with 12 h light and 12 h dark cycle. The temperature and relative humidity were recorded daily morning between at 9.00 AM to 10.00 AM. Water ad libitum: Water from Aquaguard cum purifier manufactured by Eureka Forbes Ltd., Mumbai, India was provided. DIO mice weighed around 48-50 g and age matched lean mice weighed around 28-30 g were used for acute pharmacokinetic study. All were Animals were fasted for 4hrs with free access of water on the day of experiment. An oral gavage of 5,7-dihydroxyflavone in 1% CMC as suspension was given to mice at a dose of 60 mg/kg. Blood samples were collected from three mice at each time points into the tubes containing K$_2$-EDTA solution as an anti-coagulant at 0, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 hr post compound dosing in both in lean and DIO C57BL/6 mice. Post collection of blood samples, plasma was harvested by centrifuging the blood at 4000 rpm for 8 min and stored frozen at -80 ± 10°C until analysis.

Pharmacokinetic data analysis

Plasma concentration of 5,7-dihydroxyflavone and pharmacokinetic parameters ($C_{max}$, $T_{max}$, half- life and AUC) were analyzed by non-compartmental method using Phoenix WinNonlin Version 7.0 (Pharsight Corporation, Mountain View, CA). Data were presented as means ± S.D.
RESULT AND DISCUSSION

Liquid chromatography

Various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid along with altered flow-rates (in the range of 0.8-1.4 mL/min) were performed to optimize for an effective chromatographic resolution of 5,7-dihydroxyflavone and IS (data not shown). A variety of analytical columns (Inertsil, Atlantis, Kromasil, Hypersil, Chomolith etc.) were evaluated to obtain good and reproducible response with short run time. The resolution of peaks was best achieved with an isocratic mobile phase comprising 0.2% formic acid: acetonitrile (20:80, v/v) at a flow rate of 0.9 mL/min. Atlantis C-18 column (50 × 4.6 mm) was found to be suitable with sharp and symmetric peak shapes in the method optimization process. 5,7-dihydroxyflavone and IS eluted at ~2.49 and 2.34 min, respectively are shown in Fig-2b and 2c.

Mass spectroscopy

To optimize the ionization mode for 5,7-dihydroxyflavone and IS, electro-spray ionization (ESI) full scans were carried out both in positive and negative ion detection mode. It was observed that signal intensity in ESI negative ion mode was better. During a direct infusion experiment, the mass spectra for 5,7-dihydroxyflavone and IS revealed peaks at m/z 253 and 269. Following detailed optimization of mass spectrometry conditions, MRM reaction pair of m/z 253 precursor ion to the m/z 142.9 was used for quantification for 5,7-dihydroxyflavone. Similarly, for IS MRM reaction pair of m/z 269 precursor ion to the m/z 169 was used for quantification purpose. The chemical structure of 5,7-dihydroxyflavone (Figure 1) and MRM chromatograms of 5,7-dihydroxyflavone and IS are shown in Figures 2a- 2c.

Figure 1: Structural representation of 5,7-dihydroxyflavone.
Figure 2(a): Typical MRM chromatograms of 5,7-dihydroxyflavone and IS in (a) mice blank plasma.

Figure 2(b): Typical MRM chromatograms of 5,7-dihydroxyflavone and IS in (b) mice blank plasma spiked with 5,7-dihydroxyflavone at LLOQ (4.5 ng/mL)

Figure 2(c): Typical MRM chromatograms of 5,7-dihydroxyflavone and IS in (c) IS.
Optimization of sample preparation and recovery

The results of the comparison of plasma-extracted standards versus the neat solution spiked into post extracted blank sample at equivalent concentration were estimated for 5,7-dihydroxyflavone and IS. The mean percent recovery of 5,7-dihydroxyflavone was at LQC and HQC was found to be 85.6 ± 0.60 and 92.7 ± 5.25, respectively. The recovery of IS was 92.0 ± 10.0%. Protein precipitation with acetonitrile found to be devoid of matrix effect and interference from endogenous plasma components.

Matrix effect

Mean matrix factor for 5,7-dihydroxyflavone in control mice plasma was 0.93 ± 0.02 and 0.96 ± 0.06 at QC low (13.5 ng/mL) and QC high (3600 ng/mL) concentrations, respectively. No significant signal suppression was observed in the region of elution of 5,7-dihydroxyflavone and IS.

Specificity and selectivity

Blank mice plasma chromatogram shown in Figure 2a (free of analyte and IS) and blank mice plasma spiked with 5,7-dihydroxyflavone at LLOQ and IS shown in Figure 2b. No interfering peaks from endogenous compounds were observed at the retention times of 5,7-dihydroxyflavone and IS in the matrix. The retention time of 5,7-dihydroxyflavone and IS was ~2.49 and 2.34 min, respectively. The total chromatographic run time was 3.2 min. For specificity, mice plasma samples from six different lots were evaluated to investigate the potential interferences at the LC peak region for analyte and IS. Six replicates of LLOQ samples were prepared from the cleanest blank samples and analyzed samples were acceptable with precision (% CV) is less than 5%.

Calibration curve

Eight point plasma calibration curves was constructed in the linear range with calibration standards viz., 4.50, 9.0, 124, 450, 1239, 2478, 3938 and 4500 ng/mL. The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The average regression (n=4) for 5,7-dihydroxyflavone was found ≥ 0.996. The lowest concentration with the RSD <20% was taken as LLOQ and was found to be 4.50 ng/mL. The % accuracy observed for the mean of back-calculated concentrations for four calibration curves for 5,7-dihydroxyflavone was within 93.4-105; while the precision (% CV) values ranged from 4.56-9.67.

Accuracy and precision

Accuracy and precision data for intra- and inter-day plasma samples for 5,7-dihydroxyflavone are presented in Table 1. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.
### Table 1: Intra- and inter-day precision and accuracy determination of 5,7-dihydroxyflavone quality controls in mice plasma.

**Stability**

The predicted concentrations for 5,7-dihydroxyflavone at 13.5 and 3600 ng/mL samples deviated within ±15% of the fresh sample concentrations in a battery of stability tests viz., bench-top (6 h), in-injector (12 h), repeated three freeze/thaw cycles and repeated three freeze/thaw cycles and...
freezer stability at -80 ± 10°C for at least for 30 days (Table 2). The results were found to be within the assay variability limits during the entire process.

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Stability</th>
<th>Mean ± S.D. <em>(n = 6)</em></th>
<th>Accuracy (%) <em>b</em></th>
<th>Precision (% CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.5</td>
<td>0 h (for all)</td>
<td>13.87 ± 0.54</td>
<td>102.7</td>
<td>3.88</td>
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<tr>
<td></td>
<td>6 h (bench-top)</td>
<td>12.5 ± 1.03</td>
<td>92.6</td>
<td>8.22</td>
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<tr>
<td></td>
<td>12 h (in-injector)</td>
<td>12.5 ± 1.03</td>
<td>92.6</td>
<td>8.22</td>
</tr>
<tr>
<td></td>
<td>3rd F/T cycle</td>
<td>14.1 ± 0.40</td>
<td>104.5</td>
<td>2.82</td>
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<tr>
<td></td>
<td>30 days (-80°C)</td>
<td>13.8 ± 1.01</td>
<td>102.5</td>
<td>7.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.7 ± 1.57</td>
<td>109</td>
<td>10.7</td>
</tr>
<tr>
<td>3600</td>
<td>0 h (for all)</td>
<td>3373 ± 200</td>
<td>93.7</td>
<td>5.94</td>
</tr>
<tr>
<td></td>
<td>6 h (bench-top)</td>
<td>3101 ± 263</td>
<td>86.1</td>
<td>8.49</td>
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<tr>
<td></td>
<td>12 h (in-injector)</td>
<td>3188 ± 281</td>
<td>88.5</td>
<td>8.81</td>
</tr>
<tr>
<td></td>
<td>3rd F/T cycle</td>
<td>3696 ± 144</td>
<td>102.7</td>
<td>3.91</td>
</tr>
<tr>
<td></td>
<td>30 days (-80°C)</td>
<td>3188 ± 281</td>
<td>88.5</td>
<td>8.81</td>
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<tr>
<td></td>
<td></td>
<td>3482 ± 242</td>
<td>97</td>
<td>6.95</td>
</tr>
</tbody>
</table>

*Note:* aBack-calculated plasma concentrations; b(Mean assayed concentration/mean assayed concentration at 0 h) x 100; F/T: freeze-thaw

**Table 2:** Stability data of 5,7-dihydroxyflavone quality controls in mice plasma.

**Dilution effect**

The dilution integrity was confirmed for QC samples. Standard curve can be extended up to 3600 ng/mL without affecting the final concentrations. The precision (% CV) values for dilution integrity were between 5.30 and 4.52 for both (5- and 10-fold) dilutions.

**Pharmacokinetic Study of 5,7-dihydroxyflavone in lean and DIO mice**

The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of 5,7-dihydroxyflavone in lean and DIO mice. The validated analytical method was employed to study the pharmacokinetic
profile of 5,7-dihydroxyflavone in lean and DIO mice. Pharmacokinetic profile of 5,7-dihydroxyflavone was found altered in DIO mice as compared to lean mice. The mean plasma concentration–time curve is shown in Figure 3 post oral administration (60 mg/kg dose) in lean and DIO mice.

Figure 3: Mean plasma concentration-time profiles of 5,7-dihydroxyflavone in mice plasma following oral administration of 5,7-dihydroxyflavone.

5,7-dihydroxyflavone reached a maximum concentration (C_{max}) of 108 ng/mL in DIO mice and 71 ng/mL in lean mice at approximately 0.25h. The t_{1/2} of 5,7-dihydroxyflavone was 1.16 h and 4.68h in lean and DIO mice, respectively. The AUC_{0-24h} and Vdss were summarized in Table 3.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>5,7-dihydroxyflavone 60 mg/kg, PO, Suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DIO mice</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>4.68</td>
</tr>
<tr>
<td>AUC_{(0-t)} (ng.h/ml)</td>
<td>973</td>
</tr>
<tr>
<td>AUC_{(0-∞)} (ng.h/ml)</td>
<td>1272</td>
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<tr>
<td>C_{max} (ng/mL)</td>
<td>108</td>
</tr>
<tr>
<td>t_{max} (h)</td>
<td>0.25</td>
</tr>
<tr>
<td>Tlast (h)</td>
<td>24</td>
</tr>
<tr>
<td>Vdss (L/kg)</td>
<td>318</td>
</tr>
</tbody>
</table>

Table 3: Pharmacokinetic parameters of following oral administration of 5,7-dihydroxyflavone at 60 mg/kg to lean and DIO male C57BL/6 mice (n=3).

The altered pharmacokinetic profile could be due to high fat diet which is reported to down regulate the cytochrome P450 enzymes and liver proteins [3]. It also increases the intestinal permeability through unknown mechanism [13] leading to changes in the pharmacokinetics (lower \textit{in vivo} clearance) and pharmacodynamics of therapeutic agents. In drug discovery research...
program, PK properties of novel therapeutic agents are often studied in healthy mice and pharmacodynamic (PD) effects or efficacy studies are evaluated in disease models assuming that the PK properties of disease model are similar to those of healthy lean mice. If the PK profile is not similar then, the PK/PD model obtained would over- or under-estimate the efficacious exposure required in the disease model.

Dietary flavonoids or 5,7-dihydroxyflavone and their conjugated metabolites are also recognized as breast-cancer related protein BCRP substrates [14,15]. BCRP, which is located at apical membranes of hepatocytes and enterocytes, is known as one of the most important efflux transporter. The high fat diet down regulated the BCRP expression by 67% and increases the uptake of drugs into the enterocytes [16]. We also observed 1.6 fold higher AUC and 1.5 fold higher C\text{max} values of 5,7-dihydroxyflavone in DIO mice. Fig-3 confirmed a two compartmental pharmacokinetic model and shows that 5,7-dihydroxyflavone exhibited bimodal phenomenon in their plasma concentration and time profiles, which is also reported by several workers [16,17]. Evidences suggested that the first absorption sites of 5,7-dihydroxyflavone can be attributed to direct absorption, whereas their glucuronidation, enteric and enterohepatic circulation contributed to second peak [17,18].

Interestingly, Vdss values also changed in DIO mice. Vdss for 5,7-dihydroxyflavone had about 2 fold increase in DIO mice compared to lean mice. Multiple factors including lipophilicity, permeability as well as protein binding govern the Vdss. In DIO mice, fat content in body and tissues are quite higher than in lean mice. In this aspect, plasma protein binding assuming to be similar however tissue protein binding can be quite different, this could be one of the reasons for higher Vdss.

CONCLUSION

In conclusion, a sensitive and reliable LC-MS/MS method for determination of 5,7-dihydroxyflavone in mouse plasma was developed and validated. This method was successfully applied for pharmacokinetic study in mice. Additionally, it demonstrated good accuracy and precision and is fully validated according to FDA guidelines. In this study, 5,7-dihydroxyflavone demonstrated that pharmacokinetic differences existed between high-fat diet-induced obese and lean mice, Alterations in the expression of drug metabolizing and efflux enzymes in high-fat diet-induced obese animal model underscore the importance of PK study in diseased animal models. So care to be taken while extrapolating PK and exposure data from healthy animals to diseased animals in designing pharmacological studies.

REFERENCES


