EFFECT OF MORIN ON PHARMACOKINETICS OF FEBUXOSTAT /PIRACETAM

6.1 Introduction

Herbal medicines are becoming popular worldwide, despite their mechanisms of action being generally unknown, the lack of evidence of efficacy, and inadequate toxicological data. An estimated one third of adults in developed nations and more than 80% of the population in many developing countries use herbal medicines. To date, there are more than 11000 species of herbal plants that are in use medicinally and, of these; about 500 species are commonly used in Asian and other countries. These herbs are often co-administered with therapeutic drugs raising the potential of drug-herb interactions, which may have important clinical significance based on an increasing number of clinical reports of such interactions. The interaction of drugs with herbal medicines is a significant safety concern, especially for drugs with narrow therapeutic indices (e.g. warfarin and digoxin) [1-3]. Because the pharmacokinetics and/or pharmacodynamics of the drug may be altered by combination with herbal remedies, potentially severe and perhaps even life-threatening adverse reactions may occur. Because of the clinical significance of drug interactions with herbs, it is important to identify herb drug interaction [4].

Herbs in their crude form have long been and continue to be the basis of many traditional medicines while herbal products including herbal medicines, nutraceuticals and dietary supplements have increasingly become popular as alternative medicines worldwide [5-7]. Herbal medicines are, more than ever, receiving attention, both from
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the public and healthcare professionals alike, with many countries now undertaking registration schemes for traditional medicines. However, healthcare professionals still freely admit their lack of knowledge in this area, and surveys suggest that patients often rely on friends and family for advice about herbal medicines. Never has there been a more appropriate time to advise healthcare professionals so that they can provide balanced, helpful advice to patients wishing to take herbal medicines with their ‘conventional’ treatments [8-11]. The potential for herb–drug interactions has been highlighted by the recognition that St. John’s wort (Hypericum perforatum) may interact with certain prescription medicines, including HIV protease inhibitors, oral contraceptives, selective serotonin reuptake inhibitors (SSRIs), theophylline, cyclosporin and warfarin [12-16].

Many of these herbal preparations contain flavonoids as the major constituents that play a vital role in pharmacokinetic interactions leading to changes in drug efficacy or toxicity. Flavonoids are part of a number of dietary supplements, nutraceuticals and herbal medicines for example Red Clover, Soybean, St John’s wort and Grape fruit juice [4, 6, 9, 17, 18]. In recent years, there has been a resurgence of scientific interest in flavonoids with more than 5000 publications containing “flavonoids” as a key word upon making a search in Medline/NCBI, National Library of Medicine, USA (PubMed). This is due to the association of these compounds with a wide range of health promoting effects and general belief among the public that herbal and dietary preparations are “good for humans” as they are “all natural”. Numerous studies have indicated that flavonoids have anti-oxidant, anti-carcinogenic, anti-viral, anti-inflammatory and anti-estrogenic or estrogenic activities. Dietary intake of flavonoids has been linked with reduced risk of cancer, osteoporosis, cardiovascular diseases, and other age-related degenerative diseases [19-21].

Pharmacodynamic herb-drug interactions occur when herbal and/or dietary components and drugs share a common pharmacologic mechanism of action or when a pharmacokinetic interaction leads to an altered pharmacologic profile. The pharmacokinetic herb-drug interaction may take place in any and/or multiple places in the body e.g. in gastrointestinal tract mediating through absorption (e.g. modulation of efflux and uptake transporters, complex formation, GI motility and pH), in liver mediating through metabolism (e.g. induction or inhibition of metabolizing
enzymes/transporters) and in kidney mediating through renal clearance mechanisms. These herb-drug pharmacokinetic interactions mostly occur due to modulatory effect of herbal components on drug metabolizing enzymes and transporters [17-23]. Till date several significant herb-drug interactions have been reported in the literature [18-37]. The chemical structure of morin, a flavonoid along with the drugs and internal standard used in the study is depicting in the Figure 6.1.

Figure 6.1. Chemical structures of (a) febuxostat, (b) febuxostat D7 and (c) morin (d) piracetam (e) levetiracetam

6.2 Review of the literature

Pharmacodynamic herb-drug interactions occur when herbal and/or dietary components and drugs share a common pharmacologic mechanism of action or when a pharmacokinetic interaction leads to an altered pharmacologic profile [18, 24, 25]. The pharmacokinetic herb-drug interaction may take place in any and/or multiple place in the body e.g. in gastrointestinal tract mediating through absorption (e.g. modulation of efflux and uptake transporters, complex formation, GI motility and pH), in liver mediating through metabolism (e.g. induction or inhibition of metabolizing enzymes/transporters) and in kidney mediating through renal clearance mechanisms.
These herb-drug pharmacokinetic interactions mostly occur due to modulatory effect of herbal components on drug metabolizing enzymes and transporters. Till date several significant herb-drug interactions have been reported in the literature (Table 6.1)[23, 25, 26].

In general, adverse reactions are generally associated with drugs/herbs able to inhibit or induce drug metabolizing enzymes or transporters [38-40]. These mainly include Cytochrome P450s (CYPs), uridine diphosphate-glucuronosyltransferase (UGTs), efflux (P-Glycoprotein, P-gp; MRP-2; BCRP) and uptake transporters (organic anion-transporting polypeptides, OATPs). Cytochrome P450 monooxygenases are heme containing mixed function oxidases playing a key role in metabolism of hydrophobic endogenic substrates (sterols, prostaglandins, fatty acids) and xenobiotics (e.g. drugs, carcinogens, food components, pollutants). The Cytochrome P450 enzyme system consists of several isoforms like CYP1A2, CYP2D6, CYP2B6, CYP2C9, CYP2C19 and CYP3A4 etc. and each of these can metabolize multiple substrates [22]. Flavonoids may interact with other compounds by inducing biosynthesis of CYPs or modulation of activity of these enzymes. These are also found to be substrate of several CYPs and therefore competitive inhibition may be the cause of interaction [6, 9, 18, 20].

Therefore, the modulation of these transporters may have significant pharmacokinetic consequences. On the other hand, the interaction of flavonoids with these enzymes and transporters may be exploited as a way to improve pharmacokinetic properties of the coadministered drug. Since the pharmacokinetics and/or pharmacodynamics of the drug may be altered by combination with herbs/flavonoids, such interactions can have serious effects to the patients on co-administration; may lead to adverse side effects or even life-threatening conditions due to increased drug levels above the toxicity threshold or can be ineffective due to decreased drug levels below the effective concentration [3, 4, 9, 22]. Despite of the clinical significance of drug interactions with flavonoids present in different herbal and dietary preparations there are only few scientific reports describing the quantification of these compounds in biological matrices, and also their potential involvement in drug interactions is largely unknown [4, 6, 7, 22].
6.2.1 Mechanisms for drug interactions with herbal medicines

Alterations in absorption, metabolism, distribution or excretion of drugs are the cause of pharmacokinetic interactions. Altered drug metabolism by herbal medicines is often a result of cytochrome P450 (CYP) induction and/or inhibition [1, 4, 6, 7, 15, 22]. The most well studied and understood example of this is the induction of CYP3A4 and CYP2B6 by St John’s wort in humans [1, 15]. Of the components of St. John’s wort, hyperforin is purported to be the active constituent and it is the most potent agonist for pregnane X receptor (PXR) with a Ki of 27 nM [6, 9, 15]. Because of the important role of P-glycoprotein (P-gp) in drug transport and excretion, modulation of P-gp by herbal medicines may have significant pharmacokinetic consequences. St John’s wort induces intestinal P-gp in vitro and in vivo. Oral administration of St John’s wort for 14 days in healthy volunteers resulted in a 1.4-fold increase in P-gp expression [6, 9, 15, 22]. The substrates of P-gp, fexofenadine and digoxin, which are often used as probes for examining Pgp activity in vivo, were found to have increased clearance in healthy subjects treated with St John’s wort. However, there is rare clinical evidence for altered protein binding of drugs by herbal medicines. Given that many herbal components are highly bound by plasma proteins, they may displace the drugs from the binding sites. Herbal medicines are often administered orally and they can attain moderate to high concentrations in the gut lumen (the primary site of absorption for most orally-administered drugs) and liver, and may exert a significant effect on enterocytes and hepatocytes. Both P-gp and CYP3A4 are abundantly expressed in the villus tip of enterocytes and hepatocytes [4, 15].

By contrast, some herbal remedies may contain compounds with antagonistic properties, which are likely to reduce drug efficacy and produce therapeutic failure. The synergistic or antagonistic effects between herbs and drugs often result from the competitive or complementary effect of the drug and the coadministered herbal constituents at the same drug targets [1, 4, 7-9].

6.2.2 Herbal constituents are substrates for drug metabolizing enzymes

Conventionally, drug metabolism is broadly divided into phase I and phase II processes [26]. Phase I processes include oxidation, reduction, hydrolysis and hydration resulting in the formation of functional groups (OH, SH, NH₂ or COOH) that impart the
metabolite with increased polarity compared to the parent compound. Of the phase I processes, the CYP super family is responsible for the metabolism of a variety of xenobiotics and endobiotics [26]. Human CYP isoforms that are involved in the biotransformation of xenobiotics include CYP1A1/2, CYP2B6, CYP2C8/9/19, CYP2D6, CYP2E1, CYP3A4/5 and CYP4A [26, 27]. Phase II processes include sulphonation, methylation, acetylation, glutathione conjugation, fatty acid conjugation and glucuronidation [26]. The latter is catalyzed by uridine diphosphoglucuronosyltransferases (UGTs) and involves the transfer of the glucuronic acid residue from uridine diphosphoglucuronic acid to a hydroxy, either phenolic or alcoholic, or a carboxylic acid group on the compound. In humans, 16 different UGT isoforms have been classified into either 1A or 2B subfamilies. They metabolize a broad range of endogenous and exogenous substances with significant overlap in substrate specificity between isozymes [1, 3, 4, 6-9]. Among the UGT1A family, UGT1A1 is most notably involved in the glucuronidation of bilirubin but also metabolizes estradiol, acetaminophen and the active metabolite of irinotecan, SN-38. UGT1A6 and UGT1A9 metabolize short planar phenols including catechols, acetaminophen, and 4-methylumbelliferone [27]. Hydroxylation mediated by CYP3A and CYP2B enzymes is the primary pathway of metabolism of hyperforin, the major component in St. John's wort extract. Silybin is primarily glucuronidated in the liver at the 7 and 20 position with the latter preferred over the former. The precise UGT isoform responsible for the metabolism of silybin is currently unknown. The primary route of elimination is hepatic with both the parent and conjugate excreted into the bile accounting for 2–12% of the ingested dose [3, 6]. CYP enzymes play a minor role in the metabolism of silybin. Valerenic acid (valerian extract) is also metabolized by glucuronidation. The exact UGT isoform that is responsible for the glucuronidation of valerian is not known at this time [4, 27, 28].

6.2.3 Approaches (in vitro and in vivo) to evaluate herb drug interactions

Most research on drug–herb interactions has focused on the in vitro evaluation of herbal constituents in microsomal systems, supersomes, cytosols, expressed enzymes or cell culture systems such as transfected cell lines, primary cultures of human hepatocytes and tumor derived cells. In addition, studies have also been carried out in vivo in animals (normal, transgenic, humanized) and in humans (primarily healthy individuals).
Most of the studies have used the commercially available products or a crude extracts of the herbal product or isolated purified individual components. These studies so far have paid particular attention to the effect of herbal components on CYP enzymes.

6.2.4 In vitro studies using Microsomes

The interaction of silybin with CYPs in microsomal system and the possibility of drug–herb interactions have only recently been explored [2]. Silybin noncompetitively inhibited CYP3A4 activity ($IC_{50}$=29 $\mu$M; $K_i$=9 $\mu$M) and CYP2C9 activity ($IC_{50}$=44 $\mu$M; $K_i$=19 $\mu$M) in liver microsomes [50, 51]. Interestingly, it has recently been shown that this inhibition may result from irreversible binding of a reactive intermediate to the heme moiety of both CYP3A4 and CYP2C9 in human liver microsomes [52]. Silymarin also inhibits certain hepatic enzymes such as aminopyrine demethylase, benzopyrene hydroxylase, hexobarbital hydroxylase, and ethoxy coumarin O-deethylase in rats [53]. Using expressed liver enzymes, it was shown that silybin inhibited UGT1A1 ($IC_{50}$=1.4 $\mu$M), UGT1A6 ($IC_{50}$=28 $\mu$M), UGT1A9 ($IC_{50}$=20 $\mu$M), UGT2B7 ($IC_{50}$=92 $\mu$M) and UGT2B15 ($IC_{50}$=75 $\mu$M) [52]. This suggests that the effect of silybin on glucuronidation will be isoenzyme specific and is most likely to occur with UGT1A substrates. Studies in microsomes while providing information on the potential of a chemical to alter enzyme activity are limited in that they are useful only to evaluate acute inhibition of metabolism and not induction of metabolism as they are not intact cell systems. Furthermore, it is not possible to evaluate the effect of herbal components on transporters using microsomes. Since excess co-substrate is added in the system it is not possible to evaluate co-substrate depletion as a potential mechanism of any interactions. Microsomal studies also do not provide complete mechanistic information of any interactions (effects on m-RNA or protein and the potential role of any metabolite formed).

6.2.5 In vivo studies

In vivo studies in humans have been carried out with various experimental designs. Typically subjects receive a single dose of a test drug or a cocktail of drugs that are markers for various enzymes. This is followed by multiple daily dose treatment with the herbal product (typically one week) and on the last day of treatment, administration of the test drug or the cocktail of drugs. A comparison of the various pharmacokinetic
parameters or phenotypic measures is used as a method to evaluate the effect of herbal products on the pharmacokinetics of test drug or activity of various drug metabolizing enzymes. Several reports have documented decreased blood/plasma levels of CYP3A4 substrates, such as digoxin, indinavir, cyclosporine A and imatinib, in patients concomitantly taking St. John's wort [58-61]. Additional in vivo evidence has demonstrated that St. John's wort increased CYP3A4 and P-gp protein levels in rats [62]. St. John's wort extract containing low content of hyperforin appear not to alter the activity of CYP1A2, CYP2C9, CYP3A and MDR1 [63].

6.2.6 Morin (a flavonoid)

Morin is an antioxidant flavonol which is distributed in plant foods and herbs. It is a yellow color substance that can be usually isolated from Maclura pomifera (Osage orange), Maclura tinctoria (old fustic) and from leaves of Psidium guajava (common guava). In a preclinical study it was found to be the most potent flavonoid inhibitor of fatty acid synthase tested with an IC$_{50}$ of 2.33±0.09µM [29]. Morin (3,5,7,2’,4’-pentahydroxyflavone) is a flavonoid constituent of many herbs and fruits. Morin inhibits P-gp-mediated cellular efflux of P-gp substrates [22] and could modulate the activity of metabolic enzymes, including CYP [17, 18]. Morin significantly increased the bioavailability of diltiazem in rats, which may be due to the inhibition of CYP3A-mediated metabolism of diltiazem [30]. Morin, an inhibitor of CYPs and P-gp, may improve the bioavailability of orally and intravenously administered nicardipine [31]. Three doses (1.5, 7.5 and 15 mg/kg) of morin were selected based on previous reports [32-34]. The bioavailability of orally and intravenously administered nicardipine is mainly affected by CYP3A4 and P-gp during first-pass metabolism. When morin is administered with nicardipine, it may influence the bioavailability of nicardipine. However, the effects of morin on the pharmacokinetics of febuxostat/piracetam have not been reported in rats. The purpose of this study was to investigate the effects of morin on the pharmacokinetics and bioavailability of orally and intravenously administered nicardipine in rats. The interaction of various drugs with morin is shown in Table 6.1.
Therefore, it is imperative to gain thorough knowledge on absorption, metabolism and pharmacokinetics of morin, a flavonoids; & their interaction with clinically used drugs. In the current work, we propose to study the pharmacokinetics drug-flavonoid interaction of commonly consumed morin, a flavonoid.

### 6.3 Materials and methods

#### 6.3.1 Chemicals and reagents

**6.3.1.1 Febuxostat**

Febuxostat was obtained as a gratis sample from Micro Labs Ltd (Bangalore, India) and febuxostat 7 deuterated (7D) (Febuxostat D7, internal standard [IS]) was purchased from Eurasian Chemicals Pvt. Ltd (Mumbai, India). Morin was purchased from Sigma Aldrich Ltd (St Louis, USA). High performance liquid chromatography grade acetonitrile and methanol were purchased from E-Merck Ltd (Mumbai, India). Ammonium acetate and glacial acetic acid (analytical reagent grade) were purchased from E-Merck Ltd (Mumbai, India). Milli-Q pure water was obtained from a Millipore Elix water purification system purchased from Millipore India Pvt. Ltd (New Delhi, India). Carboxymethyl cellulose and Tween 80 were purchased from Sigma Aldrich Ltd (St Louis, USA). Heparin sodium for intraperitoneal injection (1000 IU/ml) was
purchased from Gland Pharma (Hyderabad, India). Blank, drug-free plasma samples 
were collected from healthy adult male Wistar rats at the Central Animal House Facility 
(CAHF) of the Jamia Hamdard (New Delhi, India). All other chemicals and reagents 
were of analytical grade.

6.3.1.2  Piracetam

Piracetam and levetiracetam were obtained as a gratis sample from Micro Labs Ltd 
(Banglore, India). Morin, midozolam and 6-Hydroxy midozolam were purchased from 
Sigma-Aldrich Co. (St Louis, MO, USA). HPLC-grade methanol and acetonitrile were 
obtained from the Merck Co. (Darmstadt, Germany). Disodium hydrogen phosphate, 
potassium dihydrogen phosphate, ammonium acetate, magnesium chloride and ethyl 
acetate were all obtained from Merck (Germany). All other chemicals for this study 
were of analytical grade and used without further purification. Prior approval from the 
Institutional Animal Ethics Committee (IAEC), Jamia Hamdard was sought for care 
and experimental studies with animals. All experiments, euthanasia, and disposal of 
carcasses were performed in accordance with the guidelines laid by IAEC for animal 
experimentation.

6.3.2 Animal studies for febuxostat and piracetam

Young adult male Wistar rats, weighing 210-230 g, were procured from the CAHF, 
Jamia Hamdard (New Delhi, India). Rats were housed in well-ventilated cages at room 
temperature (25 ± 2 °C) with 40-60% relative humidity, while on a regular 12 h light-
dark cycle. The animals were acclimatized for a minimum period of 1 week prior to the 
experiment. Prior approval from the Institutional Animal Ethics Committee, Jamia 
Hamdard (Approval no. 899 for febuxostat and 857 for piracetam) was sought, and the 
study protocols were approved before the commencement of the studies.

6.3.3 Pharmacokinetic study

6.3.3.1  Febuxostat

The rats were fasted overnight (12 - 14 h) prior to the experiment and had free access to 
water. Rats were divided into three groups (n = 5, each): control group (febuxostat 5 
mg/kg, oral, dissolved in a 0.9% NaCl-injectable solution and Tween 80, 9:1, v/v),
coadministered group (10 mg/kg of oral morin as suspension - suspended in distilled water) and pretreatment group (5 mg/kg of febuxostat was administered orally after 1 week pretreatment with morin 10 mg/kg). In the coadministered group, morin was administered 10 min prior to oral administration of febuxostat. The dose of morin (10 mg/kg) was selected based on the previously reported study by Choi et al. [11]. Blood samples (~150 µl) were collected from the retro-orbital plexus into heparinized microfuge tubes at 0, 0.5, 1, 2, 3, 4, 5, 8, 10, 12 and 24 h post-dosing and plasma was harvested by centrifuging the blood at 4000 rpm for 10 min and stored frozen at -70 ± 10 °C until bioanalysis.

### 6.3.3.2 Piracetam

Wistar rats were divided into three groups of each of five: the control group (Piracetam 50mg/kg, oral), co-administration group (10mg/kg of Morin orally co-administered with 50mg/kg of Piracetam), and pretreatment group (50mg/kg of Piracetam was administered orally after one-week pretreatment with Morin 10mg/kg). Blood samples (approx. 150 µl) from retro-orbital plexus were collected into heparinised microfuge tubes at 0.5, 1, 1.5, 2, 4, 8, 12, and 24 h post-dosing and plasma was harvested by centrifuging the blood at 14 000 rpm for 10min and stored frozen at -20°C until bioanalysis.

### 6.3.4 Instrumentation and chromatographic conditions for UPLC

#### 6.3.4.1 Febuxostat

UPLC was executed with a binary solvent delivery pump, an auto sampler and photodiode array (PDA) detector, Acquity UPLC system, manufactured by Waters Corporation, Milford, Massachusetts, USA; data were acquired and processed using Empower software. The chromatographic separation was carried out using a Waters Acquity bridged ethylene hybrid (BEH) 150 mm X 2.1 mm, 1.7 µm, C18 column. The mobile phase containing a mixture of acetonitrile and ammonium acetate buffer (pH 5.0) in the ratio of 60:40 (v/v) at a flow rate of 0.2 ml/min was applied. The detection was obtained at a wavelength of 220 nm. The injection volume was 5 µl; mobile phase was employed as a diluent, while the column was maintained at 30 °C. These studies were carried out with a PDA detector for checking purity of peaks. The retention times
at a flow rate of 0.2 ml/min are as follows: IS for 2.74 min, febuxostat for 2.89 and morin for 3.62 min.

6.3.4.2 Piracetam (PCM)

Piracetam was estimated in plasma samples using UPLC method. The chromatographic separation was performed using a Waters Acquity BEH, 10 mm X 2.1mm, 1.7 µm, C18 column. The mobile phase containing a mixture of acetonitrile and water in the ratio of 25:75 (v/v) at a flow rate of 0.3 ml/min was used. The detection was obtained at a wavelength of 209 nm. The injection volume was 5 µl; the mobile phase was used as a diluent while the column was maintained at 30 °C. These studies were carried out with a photo diode array detector for checking purity of peaks.

6.3.5 Working solutions, calibration curve and sample preparation

6.3.5.1 Febuxostat

The plasma concentrations of febuxostat were determined by the modified UPLC method reported by Sahu et al. [6]. The primary stock solutions of the analyte (febuxostat; 5 mg/ml) and 10 µl of febuxostat-D7 (200 µg/ml), as the IS, were used. Appropriate dilutions were made in acetonitrile for febuxostat to produce working stock solution of 200, 100, 50, 20, 10, 5, 1 and 0.5 µg/ml. On the day of analysis, this set of stocks was used to prepare standards for the calibration curve. Calibration plots were constructed in the range of 0.05- 20 µg/ml for febuxostat in rat plasma (concentrations 0.05, 0.1, 0.5, 1, 5, 10 and 20 µg/ml). Calibration standards were prepared by spiking 90 µl of control pooled rat plasma with the appropriate working solution of febuxostat (10 µl) and IS (10 µl) on the day of analysis. The calibration curve was developed by plotting the ratio to the peak area of febuxostat to that of IS versus the nominal concentration of calibration standards. The response for febuxostat was strictly linear in the investigated concentration range of a correlation coefficient ($r^2$) of 0.999. A simple liquid-liquid extraction method was followed for extraction of febuxostat from rat plasma. To 100 µl of plasma in a tube, 10 µl of IS solution (febuxostat D7 at 200 µg/ml in acetonitrile), was vortexed for 2 min on a cyclomixer (Spinix Tarsons, Kolkata, India). Next a 3 ml aliquot of extraction solvent, methanol, was added. The mixture was then vortexed for 5 min, followed by centrifugation for 10 min at 4000 rpm at 20 °C on Sigma 3-16K (Frankfurt, Germany). The organic layer (2 ml) was separated and
evaporated to dryness under vacuum in SpeedVac concentrator (Savant Instrument, Farmingdale, USA). The residue was reconstituted in 100 µl of the mobile phase and 5 µl was injected into the UPLC system for analysis.

6.3.5.2 Piracetam (PCM)

The primary stock solutions of the analyte (Piracetam; 5 mg/ml) and IS (levetiracetam; 200 µg/ml) were prepared in water and stored at 4°C. Appropriate dilutions were made in water for piracetam to produce working stock solution of 0.01, 0.02, 0.1, 0.5, 1 and 2 µg/ml and on the day of analysis this set of stock was used to prepare standards for the calibration curve. Another set of working stock solutions of piracetam was made in triple distilled water (TDW) at 0.2, 2, and 15 µg/ml for preparation of QC samples. Individually QC and CC working stock solutions of Piracetam were spiked into blank plasma for QC and CC samples. Calibration plots were constructed in the range 0.1–20 µg/ml for piracetam in rat plasma (concentrations 0.1, 0.2, 1, 5, 10 and 20 µg/ml). Calibration standards were prepared by spiking 90 µl of control pooled rat plasma with the appropriate working solution of piracetam (10 µl) and IS (10 µl) on the day of analysis. Samples for the determination of precision and accuracy were prepared by spiking control rat plasma in bulk at four concentration levels [40 ng/ml (lower limit of quantitation, LLOQ), 0.2 µg/ml (QC low), 2 µg/ml (QC medium) and 15 µg/ml (QC high)] and 100 µl volumes were aliquoted into different tubes and stored at -20°C until analysis.

A simple liquid-liquid extraction method was followed for extraction of piracetam from rat plasma. To 100 µl of plasma in a tube, 10µl of IS solution (levetiracetam at 200 µg/ml in water) was added; vortex was mixed for 30 s on a cyclomixer (Spinix Tarsons, Kolkata, India). Next a 3ml aliquot of extraction solvent, ethyl acetate was added. The mixture was then vortexed for 5min, followed by centrifugation for 5 min at 2000g at 20 °C on Sigma 3-16 K (Frankfurt, Germany). The organic layer (2ml) was separated and evaporated to dryness under vacuum in speedvac concentrator (Savant Instrument, Farmingdale, New York, USA). The residue was reconstituted in 100 µl of the mobile phase and 5 µl was injected into the UPLC system for analysis.
6.3.6 Pharmacokinetic analysis for febuxostat and piracetam

Plasma data were subjected to non-compartmental pharmacokinetic analysis using WinNonlin (version 5.1, Pharsight Corporation, Mountain View, USA). The observed maximum plasma concentration ($C_{\text{max}}$) and the time to reach the maximum plasma concentration ($T_{\text{max}}$) were obtained by visual inspection of the experimental data. Area under the plasma concentration-time curve from time zero to the last quantifiable concentration ($\text{AUC}_{0-t}$) was calculated using linear trapezoidal rule. The total area under the plasma concentration-time curve from time zero to time infinity ($\text{AUC}_{0-\infty}$) was calculated as the sum of $\text{AUC}_{0-t}$ and $C_{\text{last}}/k_{\text{el}}$, where $C_{\text{last}}$ represents the last quantifiable concentration and $k_{\text{el}}$ represents the terminal phase rate constant. The apparent elimination half-life ($t_{1/2}$) was calculated as $0.693/k_{\text{el}}$ and the $k_{\text{el}}$ was estimated by linear regression of the plasma concentrations in the log-linear terminal phase. The relative bioavailability of febuxostat was calculated as follows:

$$\text{Relative bioavailability (RB %)} = \frac{\text{AUC}_{\text{co-admin/pretreatment}}}{\text{AUC}_{\text{control}}} \times 100$$

6.3.7 Statistical analysis for febuxostat and piracetam

The data are presented as mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey’s test using GraphPad prism version 5 (GraphPad software, San Diego California, USA). The pharmacokinetic parameters were compared using unpaired Student’s t-test. A p value of < 0.05 was considered significant.

6.4 Results and discussion

6.4.1 Development and optimization of the UPLC method

6.4.1.1 Febuxostat

The best separation was achieved on the BEH column at 30 °C using the mobile phase acetonitrile:ammonium acetate buffer (60:40) in an isocratic mode. The flow rate was kept at 0.2 ml/min at constant injection volume of 5 µl, and the detection wavelength was 220 nm. The standard chromatogram of febuxostat containing the peak of morin and IS obtained from the analysis is shown in Figure 6.2.
6.4.1.2  Piracetam

Initially, this drug was analyzed on a BEH C18 column using acetonitrile:water (50:50) as mobile phase at a flow rate of 0.15ml/min. and a column temperature of 25 °C. Under these conditions, the shape of this drug peak was not good. Subsequent trials were made on plasma samples using different amounts of acetonitrile, pH, and temperature. The peaks for this drug and the degradation products were not well separated or did not have an acceptable shape at column temperatures <30 °C and acidic pH. The best separation was achieved on the same column at 30 °C using the mobile phase acetonitrile:water (25:75) in an isocratic mode. The flow rate was kept at 0.3ml/min at constant volume 5 µl and the detection wavelength was 209 nm. The standard chromatogram of Piracetam contains the peak of Morin and levetiracetam obtained from the analysis is shown in Figure 6.3.
6.4.2 Effect of morin on pharmacokinetic of febuxostat (drug-flavonoid interaction)

6.4.2.1 Febuxostat

The plasma concentration–time profiles of febuxostat after the oral administration of the febuxostat (5 mg/kg) in the control group, coadministered group and pretreated group were characterized in rats and illustrated in Figure 6.4. The mean pharmacokinetic parameters of febuxostat are also summarized in Table 6.2. However, no significant change was observed in plasma concentration of febuxostat in morin coadministered group at all the studied time points in comparison with that of the control group except $t_{1/2}$. 

Figure 6.3 Chromatogram showing separation of piracetam, levetiracetam (IS) and morin in a mixture of rat plasma samples by ultra-performance liquid chromatography
It was reported that febuxostat is metabolized mainly via CYP1A1, CYP1A2 and to a lesser degree via CYP 3A4, 2C8 and 2C9 [5]. It is hypothesized that febuxostat may be involved in the P-gp-mediated transporter/efflux. The morin was known to CYP1A1, CYP1A2 and CYP3A4 inhibitors [14]. As shown in Table 6.2, the pretreatment with the morin prior to the oral administration of febuxostat significantly altered the pharmacokinetic parameters of febuxostat in comparison with control group given febuxostat alone. The $C_{\text{max}}$ and AUC of oral febuxostat increased by 18-20 and 47-50%, respectively, in the pretreated group of morin, whereas there was no significant change in the $T_{\text{max}}$; however, terminal plasma half-life ($t_{1/2}$) was increased by 2.5-fold. Consequently, the relative bioavailability values of febuxostat in the rats pretreated with morin were significantly higher ($p < 0.05$) than those from the control. The pharmacokinetic profiles of febuxostat of the coadministered group were also evaluated in rats. The minor variations were also seen in the coadministered group in comparison with control as shown in Figure 6.4 and Table 6.2. However, the parameters are not significantly altered. The coadministration of morin with febuxostat suggested that there would be time-dependent mechanism. Based on the assumptions, morin inhibits...
the CYPs responsible for the metabolism of febuxostat; its exposure levels increase after multiple dosing as compared to those after single dosing owing to an actual decrease in the amount of metabolizing enzymes with time. Therefore, the coadministration of febuxostat was not affected much by the concurrent use of morin when compared to the control group of febuxostat. The variation in the half-life of pretreated as well as coadministered group suggested the involvement of hepatic metabolism of febuxostat. Higher plasma concentrations of febuxostat were obtained by the inhibition of above mentioned CYPs and/or P-gp. Morin significantly increased the AUC0-∞ of febuxostat in rats, which might be due to the inhibition of CYP-mediated metabolism of febuxostat [14]. In addition, morin is also an inhibitor of P-gp [5]. The proposed hypotheses suggest that the increase in the oral exposure of febuxostat may be attributed to the enhanced absorption of febuxostat in the gastrointestinal tract via the inhibition of P-gp efflux and intestinal metabolism by morin. Therefore, it could be expected that morin, an inhibitor of CYPs and P-gp, would improve the bioavailability. The nonclinical toxicology studies revealed that the use of febuxostat at higher dose causes transitional cell papilloma and carcinoma of urinary bladder. The possible side effects of febuxostat are heart problems, liver problems, gout flares, nausea, joint pain and rashes [17]. Due to the wide consumption of flavonoids, in the diet and as dietary supplements, and the important role of transporters in drug disposition, there is an increasing scientific interest in flavonoid--transporter interactions. Based on the broad overlap in the substrate specificities as well as co-localization of CYPs and P-gp in the liver and small intestine [18-20], CYPs and P-gp have been recognized as a concerted barrier to the drug metabolism and absorption, respectively [19]. Previous reports available from the esteemed scientific database on the effect of febuxostat on P-gp-mediated cellular efflux are less voluminous. Consequently, we proposed a hypothesis based on literatures that if the effect of P-gp-mediated cellular efflux on febuxostat, must be inhibited by morin [10]. Therefore, dual inhibitors against both CYPs and P-gp could have a great impact on the bioavailability of many drugs where CYP metabolism as well as P-gp-mediated efflux is the major barrier to systemic availability. The present study suggested that the presence of morin may increase the bioavailability of febuxostat. Therefore, concomitant use of morin or morin-containing dietary supplements with febuxostat will postulate close monitoring of potential drug interactions.
Table 6.2  Mean pharmacokinetic parameters of febuxostat after an oral (5 mg/kg) administration of febuxostat to rats in the control, co-administered and pre-treated groups (mean±S.D., n=5)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Mean ± SD</th>
<th>Febuxostat+ Morin (co-administered) Mean ± SD</th>
<th>Febuxostat+ Morin (pretreatment) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</td>
<td>7.9±0.854</td>
<td>8.2±0.932</td>
<td>9.4±1.235</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-t&lt;/sub&gt; (µg h/ml)</td>
<td>38.1±1.445</td>
<td>40.3±1.566</td>
<td>56.1±1.875*</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (µg h/ml)</td>
<td>38.2±1.549</td>
<td>40.4±1.644</td>
<td>57.9±1.851*</td>
</tr>
<tr>
<td>CL/F (L/h/kg)</td>
<td>0.131±0.022</td>
<td>0.123±0.021</td>
<td>0.086±0.019*</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>1.9±0.112</td>
<td>2.4±0.179*</td>
<td>4.8±0.213*</td>
</tr>
<tr>
<td>MRT&lt;sub&gt;t&lt;/sub&gt;</td>
<td>4.8±0.564</td>
<td>4.9±0.723</td>
<td>5.9±0.874*</td>
</tr>
<tr>
<td>RB (%)</td>
<td>100</td>
<td>106</td>
<td>152</td>
</tr>
</tbody>
</table>

RB: relative bioavailability compared to the control group. * p < 0.05, significant difference compared to the control using one-way ANOVA followed by Tukey’s test.

6.4.2.2  Piracetam

The results are shown in Figure 6.5 and the pharmacokinetic parameters are summarized in Table 6.3. The statistical comparison of mean plasma concentration of Piracetam in three groups by one-way ANOVA followed by Tukey’s test revealed significantly higher (P<0.01) plasma Piracetam level from 2 to 24 h in Morin-pretreated group in comparison with the control and Morin co-administered groups. However, no significant change (P>0.05) was observed in plasma concentration of piracetam in Morin co-administered group at all the studied time points in comparison with that of control group. Further, analysis of pharmacokinetic parameters revealed that Morin pretreatment caused a significant elevation in C<sub>max</sub> (1.5 fold) of Piracetam in comparison with the control group and PCM co-administered groups at 1 h. As the results shown in Table 6.3, the elimination half-life (t<sub>1/2</sub>), AUC<sub>0-t</sub>, clearance (CL), mean residential time (MRT) also affected with respect to time in comparison at different groups (control, coadministered and pretreated).
Figure 6.5 Mean plasma concentration time profile after oral administration of piracetam (control), co-administered (piracetam+morin) & pretreatment group on six rats.

Table 6.3  Pharmacokinetic parameters after oral administration of single dosage 50 mg/kg piracetam to rats (n = 3)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Mean ± SD</th>
<th>Morin (co-administered) + PCM</th>
<th>Morin (pretreatment) +PCM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean of observed concentration N=3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µg/ml)</td>
<td>14.442±0.973</td>
<td>14.828±1.173</td>
<td>17.606±0.988*</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>01.000</td>
<td>01.000</td>
<td>01.000</td>
</tr>
<tr>
<td>$AUC_{0-t}$ (µg h/ml)</td>
<td>86.490±1.430</td>
<td>91.095±0.973*</td>
<td>115.033±0.997*</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$ (µg h/ml)</td>
<td>88.656±1.422</td>
<td>93.127±1.374*</td>
<td>125.104±1.536*</td>
</tr>
<tr>
<td>CL (L/h/kg)</td>
<td>00.563±0.092</td>
<td>00.536±0.083</td>
<td>00.399±0.079</td>
</tr>
<tr>
<td>$AUC%\text{Extrap}$</td>
<td>02.442±0.916</td>
<td>02.182±1.093</td>
<td>08.050±1.278</td>
</tr>
<tr>
<td>$t_{\frac{1}{2}}$ (h)</td>
<td>04.451±1.333</td>
<td>04.759±1.279</td>
<td>06.904±1.473*</td>
</tr>
<tr>
<td>MRT$_t$</td>
<td>05.664±1.245</td>
<td>05.726±1.475</td>
<td>06.320±1.547</td>
</tr>
<tr>
<td>RB (%)</td>
<td>100.000</td>
<td>105.043</td>
<td>141.111</td>
</tr>
</tbody>
</table>

RB: relative bioavailability compared to the control group. * p < 0.05, significant difference compared to the control (given piracetam alone orally)
6.5 Conclusion

The pretreatment of naturally occurring dietary supplement morin significantly increased the relative bioavailability of febuxostat/piracetam in rats.

6.5.1 Febuxostat

The increase in the oral bioavailability of febuxostat might be mainly attributed to the inhibition of CYP (mainly via CYP1A1, CYP1A2 and to a lesser degree via CYP3A4)-mediated metabolism through the liver as well as a small intestine and/or in the small intestine inhibition of P-gp by morin. Therefore, the dose of febuxostat should be adjusted for potential drug interactions when febuxostat is used with morin or the morin-containing dietary supplements for a rational dosage regimen.

6.5.2 Piracetam

The present study suggests that there might be a potential interaction between piracetam and morin and therefore, quantitative evaluation of piracetam–morin interaction in humans needs to be verified to avoid food–drug interactions. The in vitro enzyme kinetics and metabolic studies methods may support for further interaction and inhibition studies of piracetam with other flavonoids.

6.6 References


[36] H. Zhang, J. Cao, Y. Wang, Spectroscopic and molecular modeling studies of the interaction between morin and polyamidoamine dendrimer, Luminescence.


