

## ORIGINAL RESEARCH

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# The effect of licorice drink on cytochrome P3A6 and P-glycoprotein gene transcription in rabbits

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## Abstract

Licorice is a widely used herb, and its components interact with a number of drugs. Rifampicin is an antibiotic known to induce some drug metabolizing enzymes and transporters and used in our study as a reference drug. The purpose of this research is to study the effect of licorice drink on the levels of cytochrome P3A6 (CYP3A6) and P-glycoprotein (P-gp) gene transcripts using rabbit as an animal model. Four groups of locally inbred male New Zealand rabbits were used, including two control groups, and two treated groups. One group was given a daily oral dose of licorice drink (4 ml/kg body weight) for one week, while the other group was given a daily oral dose of rifampicin (100 mg/kg body weight) for four days. Messenger RNAs were extracted from hepatic and the proximal (first 10-15 cm) intestinal tissues of the four groups and real-time polymerase chain reaction was conducted on them. The licorice treated group showed a significant induction of hepatic CYP3A6 expression (17.6-fold,  $p=0.004$ ), and inhibition of intestinal CYP3A6 expression (7.6-fold,  $p=0.0006$ ). Licorice administration did not affect P-gp expression neither in hepatic nor in intestinal tissues. Rifampicin administration significantly induced hepatic CYP3A6 expression with no significant changes in transcription of intestinal CYP3A6, hepatic and intestinal P-gp. It is concluded that licorice can affect the metabolism of drugs that are CYP3A6 substrates, but it might not affect the transport of drugs dependent on P-gp.

**Keywords:** Licorice, CYP3A4, CYP3A6, P-gp, rifampicin, transcription

## Introduction

The cytochrome P450 3A (CYP3A) subfamily metabolizes a large number of endogenous compounds including testosterone and cortisol, and approximately 40-50% of drugs like cyclosporin and simvastatin [1]. CYP3A4 is the most prominent CYP3A isozyme constituting about 30% of whole CYP content in adult liver [2]. It is also the principally expressed CYP enzyme in the small intestine, constituting about 80% of the entire intestinal enzymes [3]. The hepatic and intestinal CYP3A4 can be co-induced by CYP3A4 inducers such as rifampicin and inhibited by CYP3A4 inhibitors like ketoconazole [4,5]. Noteworthy, CYP3A4 in both tissues appear to be regulated independently [6]. The hepatic extraction of alprazolam and quinidine, CYP3A4 substrates, is significantly higher than the intestinal extraction [7,8]. Metabolic enzymes expression, especially CYP3A4, is not homogeneous within the small intestinal villi, with the highest expression found in mature enterocytes that line the villi tips [9]. The regional intestinal enzymes expression is more heterotropic than the hepatic, varying along the intestinal length, with

the maximum CYP3A4 levels being found in the duodenum, and then decrease along the intestines [10].

In addition to CYP3A-mediated metabolism, many drugs are subject to active efflux by drug-transporting proteins, of which P-glycoprotein (P-gp) is the most important [11]. The P-gp efflux transporter can functionally protect the body against toxic xenobiotics by excreting them into bile, urine and the gastrointestinal tract (GIT) lumen [12]. In addition, and like CYP3A, P-gp is significantly involved in many drug-drug interactions [13]. Many drugs can induce P-gp like carbamazepine, and others can inhibit it like verapamil [14]. The distribution pattern of P-gp mRNA is opposite to that of CYP3A4 mRNA in the GIT, in which there is a continuous increase in multi drug resistance mRNA expression along the GIT to distal descending colon [10]. This high expression of P-gp in the more distal part of GIT may be beneficial in expelling xenobiotics not yet metabolized by CYP3A [10,15]. In enterocytes, the apically (luminally) located P-gp reduces the exposure of the microsomal (intracellular) CYP3A to drugs that are given orally [16]. In contrast, in hepatocytes, orally administered drugs would first encounter intracellular CYP3A, before being transported into the bile by P-gp [16].

Licorice (*Glycyrrhiza glabra*) is one of the oldest widely used herbs [17]. This plant has been used in folk medicine since at least 500 BC, sometimes known as “the

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grandfather of herbs" [18]. The name *Glycyrrhiza* is derived from the Greek word "glykos" meaning sweet, and the word "rhiza" meaning root [19]. Its rhizomes and roots have been traditionally used to treat wounds, kidney stones lung ailments and peptic ulcers [18]. In addition, many studies showed that licorice constituents are effective in the treatment of many other diseases like eczema, postural hypotension, malaria and melasma [20]. However, large doses of licorice may lead to sodium and water retention, hypertension, hypokalemia, muscle weakness, metabolic alkalosis and cardiac arrhythmias [21,22]. Also, many studies found that licorice can interact with many drugs. A study performed on the kidney cortex microsomes of guinea pig demonstrated that the simultaneous intake of licorice with ethacrynic acid, naringenin (found in grapefruit juice), chenodeoxycholic acid or furosemide increased potassium excretion and mineralocorticoid activity [23]. Furthermore, it has been shown that licorice drink dramatically reduced area under the curve (AUC) and maximum serum concentration ( $C_{max}$ ) of verapamil in rabbits after a single and multiple daily doses for 7 and 14 days [24].

In The Middle East, licorice is a commonly used drink especially in Ramadan and summer to quench thirst during fasting. Therefore, the major objective of this study was to assess the effect of licorice drink on CYP3A6 and P-gp gene transcription in rabbits. Rabbits were used in our study because CYP3A6 of rabbits and the CYP3A4 of human share similarity in CYP predominance and substrate specificity and they are both inducible by rifampicin [25]. In addition, both rabbits and humans are found to express P-gp transporter and hence rabbits were used by many researchers as animal model to study the effects of drugs and herbs on P-gp activity and expression [26,27].

Rifampicin is widely used by many investigators as a prototypical inducer of some transporters and drug-metabolizing enzymes and therefore used in our experiment as a positive control [28].

## Materials and Methods

### Animals

Forty locally-inbred New Zealand male rabbits weighing 1.7-3 kg were used in this study and housed in metal cages, one per cage. Animals were left for one week before the experiments for acclimatization, given regular diet and provided tap water ad libitum. Four groups of rabbits, 10 rabbits each, were used in this study. The 1<sup>st</sup> group was given 4 ml/kg licorice once daily for 7 days (licorice treated group). The 2<sup>nd</sup> group was given 4 ml/kg tap water once daily for 7 days (licorice control group). The 3<sup>rd</sup> group was given 100 mg/kg rifampicin once daily for 4 days (one rabbit died after severe diarrhea overnight after rifampicin administration). The 4<sup>th</sup> group was given 4 ml/kg distilled water once daily for 4 days (Rifampicin control group) (one rabbit died after suffocation during oral

tube insertion). Treatments were given by oral gavage, followed by 2 ml of tap water for the 1<sup>st</sup> and 2<sup>nd</sup> group and 2 ml of distilled water for the 3<sup>rd</sup> and 4<sup>th</sup> groups to ensure the full administration of the dose.

All experimental animals were fasted overnight (16 hours) before each treatment, and allowed access to water one hour before the treatment. Rabbits of the 1<sup>st</sup> and 2<sup>nd</sup> groups were sacrificed on the 8<sup>th</sup> day of the experiment, while the 3<sup>rd</sup> and 4<sup>th</sup> groups were sacrificed on the 5<sup>th</sup> day of the experiment. Liver and intestine samples were obtained from the four groups. They were taken from the middle, right and left lobes of the livers, and from the first 10-15 cm of the upper intestines. All samples were flushed with isotonic normal saline, and immediately stored at -80° C. The first two groups were used to demonstrate the effect of licorice on both CYP3A6 and P-gp gene transcription. The 3<sup>rd</sup> and 4<sup>th</sup> groups were used to demonstrate the effect of rifampicin on both CYP3A6 and P-gp gene transcription as a positive control. The transcripts of  $\beta$ -actin, the housekeeping gene, were used as a reference gene for optimization of gene transcript experiments. The institutional and national guide for the care and use of laboratory animals was followed.

### Preparation of Licorice Drink

Dried licorice root was bought from a local market in Amman-Jordan, and authenticated by a botanist in the Faculty of Agriculture, at the University of Jordan. Grinded licorice root powder (325 g) was added to 250 ml of tap water and mixed until it became viscous. The mixture was placed in a white gauze cloth and tied. Then the gauze was immersed in 1.25 liters of water to give a total volume of 1.5 liters, and was reserved in fridge for 6 hours with stirring every half an hour. Then the gauze containing licorice was squeezed to give the juice. Licorice was administered as 4ml licorice juice/ kg (866.7 mg/kg).

### Rifampicin Preparation

Rifampicin suspension was freshly prepared for each experiment as 25mg rifampicin/ ml distilled water and was administered to rabbits as 4ml/ Kg (100 mg/ Kg).

### RNA Extraction and cDNA Synthesis

Total RNA was extracted from the livers and intestines of rabbits using one step RNA reagent® trizol (Bio Basic Inc., Canada) which was added to 50-100 mg homogenized tissue sample. The concentration and purity ( $A_{260/280}$  ratio) of RNA were determined spectrally. The Ultraviolet spectrophotometer Smart Spec™ (Bio-Rad Laboratories, Inc., USA) was used to measure the concentration of RNA, with an absorbance maximum at 260 nm ( $A_{260}$ ). One  $\mu$ g of single stranded complementary DNA (cDNA) was synthesized from 1  $\mu$ g mRNA using the RevertAid® First Strand Aid cDNA Synthesis Kit (Thermoscientific, USA). Oligonucleotide primers provided with this kit were used in cDNA synthesis. The volume of each 1  $\mu$ g cDNA was

determined after the optical density measurement by Smart Spec™.

### Real Time-Polymerase Chain Reaction (RT-PCR)

The gene expression level was measured using Chromo4™ CFB-3240G (Bio-Rad Laboratories, Inc., USA), with 2μl

of cDNA for β-actin, CYP3A6, and P-gp primers were used to amplify cDNA sequences with the use of GoTaq® qPCR (SYBR green) master mix (Promega, USA). These primers are listed in Table 1.

**Table 1.** Real Time Primers. P-gp: P-glycoprotein, bp: base pair

| Gene    | Forward Primer           | Reverse Primer          | Product Length (bp) | Reference |
|---------|--------------------------|-------------------------|---------------------|-----------|
| CYP3A6  | TCCTTCATTATGCATTTGTTGGCC | ACCACCATGTCCAGATATTCATC | 137                 | 29        |
| B-actin | TCCTTCCTGGGCATGGAGTC     | GGATGTCCACGTCGCACTTC    | 76                  | 29        |
| P-gp    | GCATCTCCAAATGTTGAAGCAT   | GCTCTCCACCTTCAGGTTGAGG  | 196                 | 30        |

The GoTaq® was used with a final reaction volume of 25μl. Duplicate trials were generally performed to measure gene expression level, with the exception of hepatic β-actin gene of rifampicin control groups which was measured by a single run. Hepatic CYP3A6 gene of both licorice and rifampicin treated and control groups was measured by triplicate runs.

For β-actin and CYP3A6 RT, the initiation step was at 94°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 15 seconds, annealing at 58°C for 15 seconds and extension at 72°C for 15 seconds. For P-gp RT, the initiation step was at 94°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 20 seconds, annealing at 55°C for 20 seconds and extension at 72°C for 20 seconds. The mRNA expression levels of CYP3A6 and P-gp were normalized to the mRNA levels of β-actin.

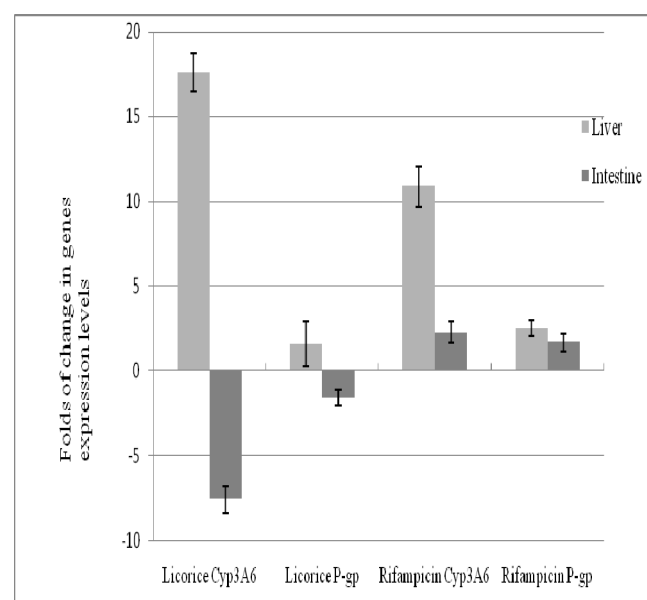
### Statistical Analysis

To study the effect of licorice drink on CYP3A6 and P-gp gene expression, RT was used. Beta-actin was used as the housekeeping gene. The significance of the differences between the treated and the control groups were determined by the Student's t-test and a p-value of < 0.05 was considered statistically significant.

### Results

The effects of licorice and rifampicin on CYP3A6 and P-gp gene expression levels in the liver and intestine of rabbits are shown in Figure 1 and Table 2. After 7 days of licorice administration, a significant 17.6-folds increase in hepatic CYP3A6 transcription and a significant 7.6-folds

decrease in intestinal CYP3A6 transcription were observed (p values=0.004 and 0.0006, respectively).



**Figure 1.** The effect of licorice and rifampicin on cytochrome 3A6 (Cyp3A6) and P-glycoprotein (P-gp) gene transcription in the liver and intestine of rabbits. The graph shows a comparison of relative mRNA transcription levels of the Cyp3A6 and P-glycoprotein genes. All values were calculated as mean ± standard error of the mean (SEM).

On the other hand, statistical analysis revealed insignificant induction in hepatic P-gp transcription (p=0.75) and insignificant inhibition in intestinal P-gp transcription (p=0.12) in licorice treated rabbits as compared to control group (Figure 1 and Table 2).

**Table 2.** Mean ± SEM folds of changes of hepatic and intestinal cytochrome 3A6 (CYP3A6) and P-glycoprotein (P-gp) gene expression levels in licorice and rifampicin treated rabbits.

| Tissue            | Mean ± SEM fold changes in licorice-treated rabbits (N=10) | P-value | Mean ± SEM fold changes in rifampicin-treated rabbits (N=9) | P-value |
|-------------------|------------------------------------------------------------|---------|-------------------------------------------------------------|---------|
| Hepatic CYP3A6    | 17.6 ± 1.14                                                | 0.004*  | 10.86 ± 1.21                                                | 0.031*  |
| Intestinal CYP3A6 | -7.6 ± 0.76                                                | 0.0006* | 2.26 ± 0.64                                                 | 0.1     |
| Hepatic P-gp      | 1.57 ± 1.33                                                | 0.75    | 2.5 ± 0.49                                                  | 0.08    |
| Intestinal P-gp   | -1.59 ± 0.47                                               | 0.12    | 1.67 ± 0.53                                                 | 0.2     |

\*p-value of <0.05 was considered statistically significant

After 4 days of rifampicin administration, rifampicin resulted in a significant increase in the transcription levels of hepatic CYP3A6 by 10.86-folds as compared to control group ( $p=0.031$ ). The transcription levels of the intestinal CYP3A6 in the rifampicin treated group showed a statistically insignificant increase ( $p=0.1$ ). Rifampicin administration also resulted in statistically insignificant increase in P-gp expression in the liver and the intestine ( $p$  values=0.08 and 0.2, respectively) (Figure1 and Table 2).

## Discussion

The major objective of this research was to study the effect of licorice on the transcription levels of CYP3A6 and P-gp in liver and intestine of rabbits. Rifampicin was used as a positive control because it induces both CYP3A6 and P-gp genetic transcriptions [28].

Most previous studies focused on the beneficial clinical effects of individual components of licorice and their effects on CYP3A6 and P-gp activities, but there are no available studies on the effect of licorice root as a whole on their transcription levels. For example, Glabridin and glabrene, two flavanoid components of licorice, were shown to have antiviral, antimicrobial, antioxidative, anti-inflammatory and antitumorigenic effects [31]. Another study on glabridin showed an antinephritis effect in mouse with glomerular disease [32]. Liquiritin, a licorice flavanoid, has shown beneficial effect in melasma, a common hypermelanotic disorder that affects the face [33]. Quercetin, another licorice flavonoid, has been reported to prevent ethanol-induced gastric damage in rats, which could be due to its antihistaminic and antioxidant effects [34].

A previous study on the effect of licorice root drink on plasma concentration of verapamil, a well known CYP3A4 substrate and P-gp inhibitor, using rabbit as animal model, reported that licorice root drink significantly decreased verapamil systemic exposure after single and daily doses of licorice. These results of multiple-dose treatment might be explained by induction of CYP3A4 or P-gp activities or gene expression levels or both [24].

In this study we used 4ml/ kg body weight of licorice drink, which was the same volume used in the study by Al Deeb et al. [24]. Furthermore, in this study licorice drink was administered for 7 days, because it was the same time of first period of multiple dose treatment study (7 days) by Al Deeb et al. that gave a significant reduction in mean maximum concentration ( $C_{max}$ ) and area under the curve (AUC) [24].

In addition, it has been shown that licorice when taken with certain drugs may lead to serious interactions. For example, licorice has been found to increase digoxin toxicity, increase blood pressure in women taking oral contraceptive pills, increase plasma concentration of prednisolone and many other interactions [23,24,35,36]. Such licorice drug interactions could be, at least in part,

due the effects of licorice on CYP3A and/or G-pg gene expression reported in this study.

This study reports that licorice affected CYP3A6 in both hepatic and intestinal rabbit tissues. In the licorice treated group and as compared to control group, hepatic CYP3A6 transcription was increased by 17.6-fold in the licorice treated group. Meanwhile, intestinal CYP3A6 transcription was inhibited by 7.6-fold.

These results of hepatic CYP3A6 induction are consistent with an in vitro study using human liver carcinoma cells in which glycyrrhizin, a major component of licorice, markedly increased the levels of CYP3A4 mRNA [37].

This effect of licorice on transcription of CYP3A may alter certain pharmacokinetic parameters like  $C_{max}$  and AUC of drugs that are substrates of CYP3A6 and CYP3A4 in patients taking licorice at the same time, and consequently can increase or decrease their blood concentration levels. Therefore, it is coherent that patients who are taking medications that are CYP3A4 substrates should avoid drinking licorice concurrently with these medications.

On the other hand, P-gp transcription in licorice administered group was not significantly affected neither in the liver nor in the intestine. Therefore, the precise effect of licorice on P-gp transcription could not be identified very well from this study. However, the effect of licorice on P-gp transcription may also alter the pharmacokinetics of P-gp substrates when they are co-administered with licorice, and therefore an increase or a decrease in their blood levels is possible.

In addition, our results are in agreement with a study performed by Yan et al. which revealed that continuous administration of glycyrrhizin did not induce the expression of P-gp using talinolol as a substrate drug for P-gp activity in humans [38].

With regard to rifampicin, it was administered orally as 100 mg/ kg body weight for 4 days in this study. The same dose and the same treatment period were used in many studies involving effects of rifampicin on body enzymes, like induction or toxicity studies [25,27]. For example, Weber et al. studied in vivo and ex vivo CYP3A6 induction by rifampicin in rabbits using an oral dose of 100 mg/kg for 4 days [25].

In the present study rifampicin administration induced hepatic and intestinal CYP3A6 transcription which is consistent with the study of Nakamura et al., who reported induction of hepatic and intestinal CYP3A transcription in rabbits that were pretreated with rifampicin [39].

In our study, the rifampicin treated groups showed statistically insignificant induction in hepatic and intestinal P-gp expression levels. In a study performed on healthy subjects, rifampicin was administered in a dose of 600 mg for 9 days that resulted in upregulation of intestinal P-gp

mRNA [40]. It appears that the duration of rifampicin treatment in our study might not have been enough to observe an effect on P-gp transcription levels. In addition, it has been reported that P-gp expression is found to be higher in the distal parts of the intestine than the proximal regions [10]. This may be another reason for the insignificant changes in the expression of P-gp in this study because we studied the changes only in the proximal part of intestine.

## Conclusions

Licorice administration to rabbits resulted in an induction of hepatic CYP3A6 expression and an inhibition of intestinal CYP3A6 expression. Licorice drink does not appear to significantly affect p-glycoprotein transcription. Rifampicin administration significantly induced hepatic CYP3A6 expression with no significant changes in transcription of intestinal CYP3A6, hepatic and intestinal P-gp. It is concluded that licorice can affect the metabolism of drugs that are CYP3A6 substrates, but it might not affect the transport of drugs dependent on P-gp.

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