



RESEARCH ARTICLE

Phenotyping determination of CYP1A2 enzyme activity using caffeine in sheep

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Özet

**Üney K, Traş B.** Koyunda kafein kullanılarak CYP1A2 enzim aktivitesinin fenotipik olarak belirlenmesi. *Eurasian J Vet Sci*, 2011, 27, 1, 27 - 32

**Amaç:** Prob ilaç olarak kafein (KF) kullanılarak Morkaraman (MK), Akkaraman (AK) ve Anadolu Merinosu (AM) koyun ırklarında *in vivo* CYP1A2 enzim aktivitesini karşılaştırmak ve pratikte CYP1A2 aktivitesini belirlemede plazma metabolik oranlarının (MO) geçerliliğini ortaya koymaktır.

**Gereç ve Yöntem:** Kafein tüm koyunlara 5 mg/kg dozda damar içi yolla uygulandı. Plazma KF ve paraxanthin (PX) düzeyleri yüksek performanslı sıvı kromatografi kullanılarak ölçüldü. CYP1A2 fenotipi PK ve KF'nin plazma konsantrasyon-zaman eğrisinin altındaki alanlar (EAA) arasındaki oran  $[(PK/KF)_{EAA}]$  ve KF uygulamasından sonra 3-16. saatlerde PK ve KF'nin plazma konsantrasyonları arasındaki oranlar  $[(PK/KF)_{MO \rightarrow 3-16 \text{ saat}}]$  kullanılarak ölçüldü. Pratikte tek bir kan örneği üzerinden CYP1A2 fenotipini araştırmak için  $(PK/KF)_{MO \rightarrow 3-16 \text{ saat}}$  ve  $(PK/KF)_{EAA}$  oranları arasındaki ilişkiler belirlendi.

**Bulgular:** CYP1A2 fenotipinin belirlenmesinde  $(PK/KF)_{MO \rightarrow 3-16 \text{ saat}}$  oranlarının içinde en güvenilir örnekleme zamanının KF uygulamasından sonraki 10. saat olduğu tespit edildi.  $(PK/KF)_{MO \rightarrow 10 \text{ h}}$  ve  $(PK/KF)_{EAA}$  oranları koyun ırkları arasında benzerdi ( $p > 0.05$ ).

**Öneri:** Koyunda plazma  $(PK/KF)_{MO \rightarrow 10 \text{ h}}$  oranı CYP1A2 fenotipinin belirlenmesinde hızlı ve basit bir test olarak kullanılabilir. Ancak, bu çalışmanın sonuçlarına göre CYP1A2 enziminin MK, AK ve AM koyun ırkları arasında substratı durumundaki ilaç ve çevresel bileşiklerin etkilerinde görülen farklılıklarda klinik yönden öneminin olmayabileceği anlaşılmaktadır.

Abstract

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**Aim:** The aims of this study were to determine the validity of the plasma metabolic ratios (MR) to investigate the CYP1A2 activity in practice and to compare *in vivo* CYP1A2 enzyme activity using caffeine (CF) as a probe in Morkaraman (MK), Akkaraman (AK) and Anatolia Merino (AM) sheep breeds.

**Materials and Methods:** Caffeine was administered as a single dose of 5 mg/kg b.w. by the intravenous in MK, AK and AM sheep breeds. The plasma levels of CF and paraxanthine (PX) were measured using high-performance liquid chromatography. CYP1A2 phenotyping was measured using the ratio  $[(PX/CF)_{AUC}]$  between areas under the plasma concentration-time curve (AUCs) of PX and CF and the ratios  $[(PX/CF)_{MR \rightarrow 3-16 \text{ h}}]$  between plasma concentrations of PX and CF at 3 to 16 h after CF administration. Correlations between the plasma  $(PX/CF)_{MR \rightarrow 3-16 \text{ h}}$  and  $(PX/CF)_{AUC}$  ratios were determined to investigate of the CYP1A2 phenotyping by single blood sampling in practice.

**Results:** It was determined that the more reliable sampling time within the plasma  $(PX/CF)_{MR \rightarrow 3-16 \text{ h}}$  ratios in the determination of the CYP1A2 phenotyping was 10 h after CF administration.  $(PX/CF)_{MR \rightarrow 10 \text{ h}}$  and  $(PX/CF)_{AUC}$  ratios were similar ( $p > 0.05$ ) among sheep breeds.

**Conclusion:** The plasma  $(PX/CF)_{MR \rightarrow 10 \text{ h}}$  ratio might be used as a rapid and simple screening test for CYP1A2 phenotyping in sheep. CYP1A2 enzyme may not be clinically important in the observed differences to the effects of drugs and environmental chemicals with its substrates among MK, AK and AM sheep breeds.

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## ► Introduction

The cytochrome P450 (CYP) enzymes are a family of mixed function oxidases, which are highly expressed in liver, and are responsible for metabolism of numerous xenobiotics, including drugs and environmental chemicals, as well as endogenous compounds (Guenengerich 1992, Streetman et al 2000). The expression of CYP enzymes is influenced by both internal factors (species, breed, sex, age, physiopathological conditions, etc.) and external factors (diet, environment, etc.) (Nebbia 2001). Therefore, phenotyping provides the most clinically relevant information because it is a reflection of the combined effects of these factors (Rostami-Hodjegan et al 1996, Streetman et al 2000). *In vivo* activity (phenotyping) of CYP enzymes can be measured by the administration of the probe substrate, specifically metabolized by a CYP enzyme, and the metabolic rate is determined (Fuhr and Rost 1994, Fuhr et al 2007). Currently, various probe substrates are widely used to determine genetic, ethnic, race/breed and environmental differences in the *in vivo* metabolism of drugs and environmental chemicals (Rostami-Hodjegan et al 1996, Faber et al 2005, Kot and Daniel 2008).

Cytochrome P450 1A2 (CYP1A2) account for approximately 13-15% of the total P450 content in the liver (Zhou et al 2010). It is documented that CYP1A2 contributes to the metabolism of clinically important drugs (albendazole, thiabendazole, lidocaine, caffeine, theophylline, etc.), endogenous substrates (melatonin, estradiol, etc), procarcinogens and environmental compounds (polycyclic aromatic hydrocarbons, aflatoxin B1, etc.) and some natural flavonoids (Faber et al 2005, Zhou et al 2010). Therefore, it is important the definition of the individual's enzyme activity to avoid therapeutic failure or toxicity (Ghotbi et al 2007). However, the determination of individual's enzyme activity in food producing animals may not be easy like humans. The determination according to breed populations of reference ranges of enzyme activities may practically allow to predict more precisely the optimal doses of the enzyme substrates, and to avoid adverse drug reactions and toxicity.

CYP1A2 phenotyping may be used in therapeutic drug monitoring, in finding the cause of adverse drug reactions or nonresponse, and to identify more specifically subjects with abnormal enzyme activity (Zaigler et al 2000, Faber et al 2005). Caffeine can be used as a "gold standard" probe for measuring CYP1A2 phenotyping in mouse (Casley et al 1997), human (Kallow and Tang 1993, Carrillo et al 2000) and rat (Kot and Daniel 2008). The primary metabolism of CF is by N-3, N-1, and N-7 demethylations to form paraxanthine (PX), theobromine (TB), and theophylline (TP), respectively. CYP1A2 is the enzyme principally responsible for the N-3 demethylation of CF to PX (Kallow and Tang 1993). It has been reported that the ratio  $[(PX/CF)_{AUC}]$  between areas under the plasma

concentration-time curves (AUC) of PX and CF based on metabolism CF is a reliable metric for assessment of CYP1A2 activity (Rostami-Hodjegan et al 1996, Bruce et al 2001). However, since the use of this metric requires intensive sampling and is not suitable for use in large groups the PX/CF metabolic ratio (MR) which calculated from the plasma concentrations of CF and PX was proposed and validated for clinical monitoring in practice (Zaigler et al 2000).

No data with the contribution of CYP1A2 enzyme on the metabolism of CF in sheep are available. However, 7-ethoxyresorufin O-dealkylation (EROD) and 7-methoxyresorufin O-dealkylation (MROD) activities are usually ascribed to CYP1A1 and CYP1A2 in different species, respectively (Burke et al 1994, Machala et al 2003). Although CYP1A1 and CYP1A2 are different, substrate specificities can overlap due to similarities between the active sites of CYP1A1 and CYP1A2 (Tasaneeyakul et al 1993). EROD and MROD activities in Texel sheep breed (Szotakova et al 2004) and mouflon rams (Machala et al 2003) have been studied. Additionally, N-3 demethylation of CF to PX has been applied as a specific indicator of CYP1A2 phenotyping in Suffolk ewes (Danielson and Golsteyn 1996).

Breed-related differences can influence the effectiveness and toxicological responses to drugs and environmental chemicals according as enzyme activities. Several studies have reported the presence of inter-ethnic and race variations in CYP1A2 activity in human (Relling et al 1992, Shimada et al 1994, Bartoli et al 1996, Ghotbi et al 2007) and dog (Scherr et al 2010, Aretz and Geyer 2010). To our knowledge, no phenotyping comparisons of CYP1A2 activity have yet been carried out in sheep breeds. The aims of our study were to determine the validity of the plasma PX/CF ratios to investigate the CYP1A2 phenotyping in sheep and to compare *in vivo* CYP1A2 enzyme activity in sheep breeds using CF as a probe.

## ► Material and methods

### • Chemicals and reagents

PX, CF and  $\beta$ -hydroxyethyl-theophylline were obtained from Sigma (Steinheim-Germany). High performance liquid chromatographic method (HPLC)-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Analytical grade glacial acetic acid (100%) and sodium acetate were purchased from Merck (Darmstadt, Germany). The water was obtained using a Milli-Q system from ELGA.

### • Animals and study design

Morkaraman (MK, 34.70 $\pm$ 1.16 kg, b.w.), Akkaraman (AK, 41.70 $\pm$ 2.54 kg, b.w.) and Anatolia Merino (AM, 36.00 $\pm$ 1.70 kg, b.w.) sheep breeds including ten animals in each breed (10-12 months, female) were used in the study. The health status of animals was evaluated by physical examination and serum biochemi-

cal analysis. They were housed in individual pens and were fed on barley grains, stalks, and dry grass. Drinking water was available ad libitum. They were received no pharmacological substances within 2 months of beginning the study. The Ethics Committee of the Faculty of Veterinary Medicine (University of Selcuk, Konya, Turkey, report no: 2007/065) approved the study protocol. Caffeine was given as a bolus intravenous (IV) injection (into the jugular vein) at a dose of 5 mg/kg body weight. Blood samples were collected via a jugular catheter into tubes with EDTA before and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 10, 12, 16, 24, 32 and 48 h after CF injection. Plasma was separated immediately by centrifugation within one hour after collection and stored at -20 °C until analysis.

#### • Chromatographic analysis

Plasma concentrations of CF and PX were measured by a HPLC method as described by Christensen et al (2003), with minor modifications. In brief, 25 µl of 10 µg/ml internal standard β-hydroxyethyl-theophylline was added to 250 µl of plasma. To all samples, 750 µl of acetonitrile to precipitate the plasma proteins were added and mixed for 30 seconds and followed by centrifugation at 19.000 g for 5 minutes. The supernatant was evaporated to dryness at 40°C under a gentle stream of nitrogen gas and reconstituted in 200 µl of mobile phase. The sample of 15 µl was injected into the HPLC.

Chromatography was carried out using a Phenomenex Gemini C-18 column (250 mm × 4.60 mm I.D., 5 µm particle size, Phenomenex, USA), which was maintained at 25 °C. The analytical wavelength was set at 273 nm. The mobile phase consisted of 30% methanol in 25 mM sodium acetate buffer, pH 4.0. The mobile phase was pumped through the system at a rate of 1.0 ml/min.

This method was validated prior to the start of analysis. Baseline separation and simultaneous quantification was obtained for CF, PX, and the internal standard within a single run without any interference from blank plasma samples. Calibration curves of CF and PX were linear over the concentration range investigated (0.01–10 µg/ml). Correlation coefficients (*r*) were greater than 0.9951 for extracted samples and 0.9998 for standard solutions. The mean percentage recoveries of PX, and CF at the different concentrations (0.01–10 µg/ml) tested were 99.7±3.4% and 97.8±2.8%, respectively. The limit of detection was determined to be 0.020 µg/ml for CF or 0.010 µg/ml for PX. The limit of quantification was determined to be 0.025 µg/ml for CF or 0.015 µg/ml for PX. The precision were established by using plasma quality control samples (*n* = 3) at low, medium and high concentrations of CF and PX in 0.025, 0.100 and 0.800 µg/ml, respectively. At all levels, intra- and inter-assay precision was lower than 4.5 and 8%, respectively.

#### • Data analysis

Areas under the plasma concentration-time curves of CF and PX were determined with the help of a specialized computer program (WinNonlin® Professional Version 4.1, Pharsight Corporation, Scientific Consulting Inc., North Carolina, USA). AUC from time zero to last time with a measurable concentration was calculated by trapezoidal rule. For CYP1A2 phenotyping, the following ratios were calculated; (1) the ratio [(PX/CF)<sub>AUC</sub>] between AUCs of PX and CF and (2) the ratios [(PX/CF)<sub>MR→3-16 h</sub>] between plasma concentrations of PX and CF at 3 to 16 h after CF administration.

All data were expressed as mean ± SD. (PX/CF)<sub>AUC</sub> and plasma (PX/CF)<sub>MR</sub> ratios were analyzed using the one way ANOVA, followed by the Duncan test. Statistical significance was assigned at *p*<0.05. Correlations between plasma (PX/CF)<sub>MR→3-16 h</sub> and (PX/CF)<sub>AUC</sub> ratios were assessed using Pearson Correlation test.

#### ► Results

To determine the validity of the plasma (PX/CF)<sub>MR</sub> in the investigation of the CYP1A2 phenotyping in sheep, correlations between plasma (PX/CF)<sub>MR→3-16 h</sub> and (PX/CF)<sub>AUC</sub> ratios were examined. There were significant correlations (3 h; *r* = 0.514, 4 h; *r* = 0.507, 5 h; *r* = 0.563, 6 h; *r* = 0.612, 7 h; *r* = 0.709, 8 h; *r* = 0.791, 10 h; *r* = 0.855, 12 h; *r* = 0.817 and 16 h; *r* = 0.808, *p*<0.01) between the plasma (PX/CF)<sub>MR→3-16 h</sub> ratios and (PX/CF)<sub>AUC</sub>. These data indicate that the best sampling time within the plasma (PX/CF)<sub>MR→3-16 h</sub> ratios in the determination of the CYP1A2 phenotyping was 10 h after CF administration. Correlation between (PX/CF)<sub>MR→10 h</sub> and (PX/CF)<sub>AUC</sub> ratio in in MK, AK and AM sheep breeds is presented in Figure 1.

Mean±SD plasma (PX/CF)<sub>MR→10 h</sub> and (PX/CF)<sub>AUC</sub> ratios used in the phenotyping evaluation of CYP1A2 activity in MK, AK and AM sheep breeds are presented in Table 1. These data indicate that (PX/CF)<sub>MR→10 h</sub> and (PX/CF)<sub>AUC</sub> ratios were similar among breeds.

#### ► Discussion

In this study, (PX/CF)<sub>AUC</sub> and (PX/CF)<sub>MR→10 h</sub> ratios were used in determination of CYP1A2 activity in sheep. No statistically significant difference was found among breeds (Table 1). In MK, AK and AM breeds, the plasma (PX/CF)<sub>MR→10 h</sub> ratio was relatively higher than the ratio (0.03) determined by Danielson and Golsteyn (1996) in Suffolk sheep at the 5 h after CF administration. This difference may be due to PX concentration being higher at 10 h compared to 5 h, and CF concentration being lower. In human, it was reported that there were large ethnic, racial and individual differences in CYP1A2 activity (Campbell et al 1987, Relling et al 1992, Shimada et al 1994). CYP1A2 activity varies up to 60-fold because of genetic or non-genetic factors as sex, diet, disease, drug and smoking (Guengerich 1995, Zaigler et al 2000, Hamdy et al

Table 1. Mean±SD of the plasma concentration ratio of paraxanthine to caffeine at 10 h after CF administration and the area under the curve ratio of paraxanthine to caffeine in Morkaraman, Akkaraman and Anatolia Merino sheep breeds (n = 10 in each breed) after IV administration of caffeine at a dosage of 5 mg/kg of body weight.

Ratio	MK	AK	AM	P value
$(PX/CF)_{MR \rightarrow 10 h}$	0.042±0.019	0.053±0.020	0.048±0.010	0.335
$(PX/CF)_{AUC}$	0.041±0.019	0.043±0.020	0.048±0.012	0.662

MK; Morkaraman, AK; Akkaraman, AM; Anatolia Merino, PX; paraxanthine, CF; caffeine, AUC; area under the curve, MR; metabolic ratio.

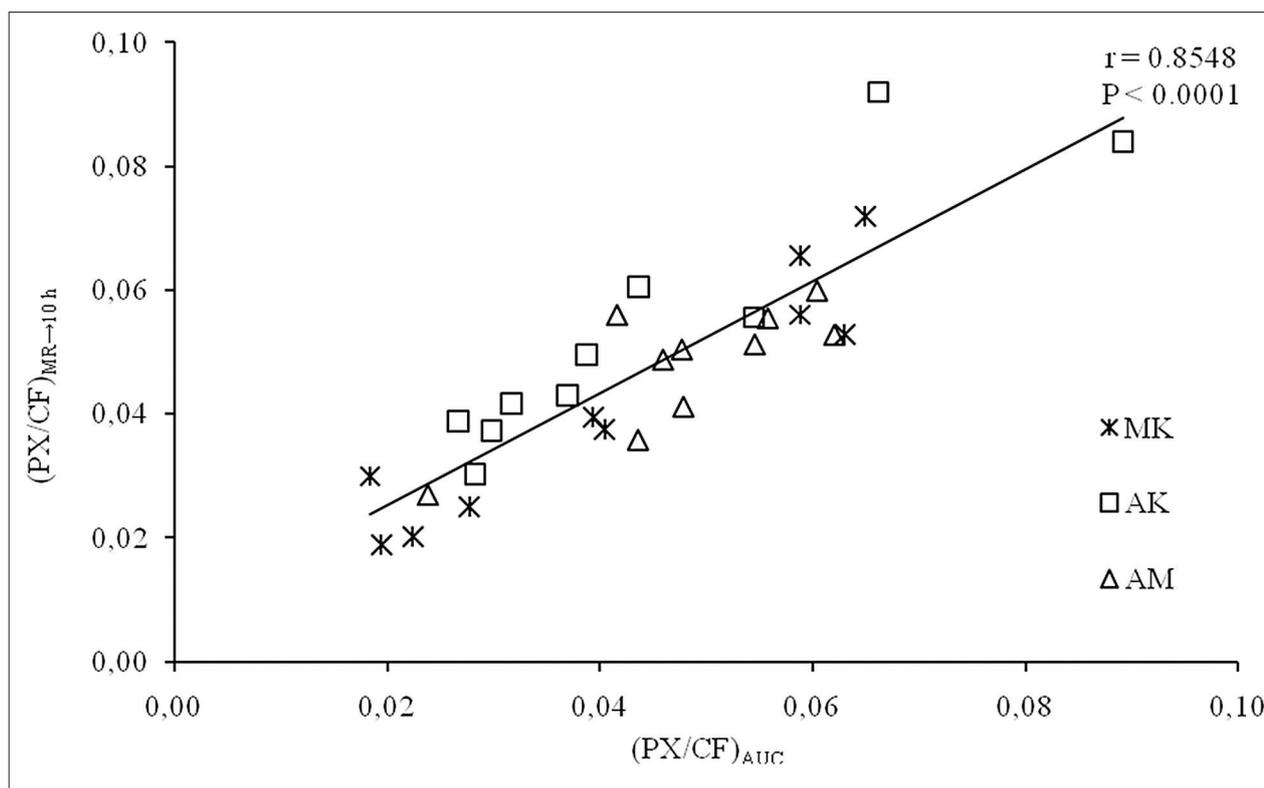


Figure 1. Correlation between the plasma concentration ratio of paraxanthine (PX) to caffeine (CF) at 10 h after CF administration and area under the curve (AUC) ratio of paraxanthine to caffeine in Morkaraman (MK), Akkaraman (AK) and Anatolia Merino (AM) sheep breeds (n = 10 in each breed). MR; metabolic ratio.

2003). The finding that there are no large differences among breeds in the study may be attributed to the fact that sheep are less subject to factors as smoking, dietary components and charcoal-broiled beef that affect CYP1A2 activity compared to human. In addition to this, it may be due to sheep population being more homogeneous than human population. However, in a study on some mice breeds under controlled cultivation (Casley et al 1997), it was determined that PX/CF ratios at the 2 h after CF administration ranged between 0.12 and 2.92 in male mouse breeds, and ranged between 0.12 and 1.69 in female mouse breeds and these differences were attributed to genetic variation.

Studies aiming of the determination of the validity of the parameters used in the assessment of CYP1A2 activity were especially carried out on human (Butler et al 1989, Fuhr et al 1996, Streetman et al 2000). In the literature, there was not found any study inclu-

ding the correlation between plasma PX/CF ratio after CF administration and genotypic or *in vitro* phenotypic methods in sheep. However, Van't Klooster et al (1993) reported that EROD activity in noninduced hepatocytes is higher in cattle and goats compared to sheep. In parallel with this, Szotakova et al (2004) determined that EROD activity was significantly lower in ewes (51 pmol /min/mg) compared to cattle (522 pmol/min/mg). Danielson and Golsteyn (1996) determined PX/CF ratio in relation with CYP1A2 activity at the 300 min after CF administration as 0.03 in sheep, and as 0.23 in cattle. In the assessment of CYP1A2 activity, the conformity of these values determined as *in vitro* and *in vivo* may support the reliability of the results obtained as *in vivo* in sheep.

In the study, plasma  $(PX/CF)_{MR \rightarrow 3-16 h}$  ratios were evaluated as they completely reflect the plasma PX formation from CF. The timing of sample collection has

critical importance because plasma PX concentrations according as the time after CF administration are more likely to reflect PX degradation (Streetman et al 2000). Investigations have suggested that plasma PX/CF ratio obtained 3 to 12 after CF administration is a reliable measure of CYP1A2 activity (Fuhr et al 1996, Spigset et al 1999, Akinyinka et al 2000). However, it was determined that in a comprehensive computer simulation of 10 000 patients, plasma PX/CF ratio obtained at 5-7 h after CF administration is robust measures of CYP1A2 activity (Rostami-Hodjegan et al 1996). In this study on thirty sheep, the more reliable sampling time within the plasma (PX/CF)<sub>MR→3-16 h</sub> ratios in the determination of the CYP1A2 phenotyping was 10 h after CF administration.

In human, the systemic clearance of CF is also a gold standard for the determination of CYP1A2 phenotyping as approximately 90% of CF clearance is mediated by CYP1A2 (Kalow and Tang, 1993). However, the theophylline formation from CF, which is catalyzed by CYP1A2, CYP2E1 (Tassaneeyakul et al 1992) and flavin-containing monooxygenases (Chung and Cha 1997), is the important metabolic pathway of CF metabolism in sheep (Danielson and Golsteyn 1996, Uney and Traş 2011). Therefore, the systemic clearance of CF was not evaluated in this study.

### ► Conclusion

CYP1A2 activities were similar in MK, AK and AM sheep breeds. The plasma PX/CF ratio at 10 h after CF administration may be used as a rapid and simple screening test to evaluate overall CYP1A2 phenotyping by single blood sampling in sheep. CYP1A2 enzyme may not be clinically important in the observed differences to the effects of drugs and environmental chemicals among MK, AK and AM sheep breeds. Researches on differences of the drug-metabolizing enzyme activities that can cause the varieties in the inter-breeds may improve sheep population predictions to drugs and toxins.

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