Analysis of caffeine and paraxanthine in human saliva with ultra-high-performance liquid chromatography for CYP1A2 phenotyping

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ARTICLE INFO

ABSTRACT

Cytochrome P450 1A2 (CYP1A2) plays an important role in drug metabolism. Caffeine (CAF) is converted into paraxanthine (PX) by this enzyme and is used as a xenobiotic substrate to determine the CYP1A2 phenotype in humans. A method for the quantification of CAF and PX in saliva was developed using liquid–liquid extraction with ethyl acetate and analysis with ultra-high-performance liquid chromatography. Peaks from CAF, PX and internal standard were resolved within 6 min. The method was validated from 0.05 to 5 μg mL^{-1} CAF and 0.025–2.5 μg mL^{-1} PX. Inter- and intra-day accuracies ranged from 91.2 to 107.2% with precisions <13.5%. The limits of detection were 0.16 and 0.63 ng mL^{-1} for PX and CAF, respectively. PX/CAF concentration ratios from volunteers were 0.26–1.09 with mean ratios of 0.78 ± 0.26 and 0.38 ± 0.10 for regular and light/non-coffee drinkers, respectively.

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1. Introduction

The Cytochrome P450 1A2 (CYP1A2) enzyme is important in the metabolism of many drugs, such as the psychotropic drugs olanzapine, clozapine and haloperidol and the antidepressants amitriptyline, imipramine and clomipramine, as well as other compounds such as caffeine (CAF) [1,2]. Inter-individual variability in CYP1A2 activity can lead to alterations in pharmacokinetics and could influence the response to therapy [2,3]. Herein, CYP1A2 genotyping or phenotyping could assist in dosage optimization. The latter is preferred to predict enzyme activity, as this is prevailingly dictated by external influences on enzyme induction and inhibition, such as cigarette smoking, consumption of coffee, grilled meat and cruciferous vegetables and the intake of certain drugs [4].

CAF is metabolized by multiple enzymes but the major metabolite paraxanthine (PX; 81.5%) is obtained solely through the CYP1A2 pathway. Other metabolites of CAF include theobromine (TB; 10.8%) and theophylline (TP; 5.4%), which are metabolized through both the CYP1A2 and the CYP2E1 pathways [5]. Studying the

PX/CAF concentration or molar ratio is widely accepted as a good way to phenotype CYP1A2 [6]. CAF and various metabolites have been identified and quantified using high-performance liquid chromatography (HPLC) in different matrices, such as urine, plasma and saliva [2,5,7–10]. Of these, the use of saliva has the advantage of being non-invasive and easily acquired. Besides, different studies show that the PX/CAF ratio in saliva correlates well with CYP1A2 activity [7–9].

Optimization of the separation of CAF and PX from other metabolites, in particular from TP, has not been studied so far. PX and TP often co-elute on reversed-phase (RP) HPLC systems [11–13] and therefore high TP concentrations could lead to inaccurate PX concentrations and PX/CAF ratios. In this study an accurate, effective and selective ultra-high- performance liquid chromatography (UHPLC) method is presented for the simultaneous analysis of CAF and PX in human saliva for CYP1A2 phenotyping.

2. Materials and methods

2.1. Chemicals and reagents

CAF, PX, TP, TB, acetaminophen (APAP), perchloric acid (PCA), acetic acid (HAc; LC–MS grade), tetrahydrofuran (THF), chloroform (CHCl$_3$), 2-propanol (IPA) and ethyl acetate (EtOAc) were obtained

from Sigma–Aldrich (St. Louis, MO, USA). The internal standard (IS) dioxypropyl theophylline was obtained from OPG (Utrecht, The Netherlands). Methanol (MeOH; LC–MS Chromasolv) and acetonitrile (ACN; LC–MS Chromasolv) were purchased from Biosolve BV (Valkenswaard, The Netherlands).

Stock solutions of 0.25 mg mL^{-1} TB and of 1 mg mL^{-1} CAF, PX, TP, APAP and IS were prepared in ultra-pure water. All stock solutions were kept at 4 °C throughout the study, for a maximum of 4 weeks.

2.2. Chromatography

A 1290 Infinity UHPLC system (Agilent Technologies, Waldbronn, BW, Germany) was used for UHPLC measurements. The system consisted of a binary pump, an autosampler, and a thermostatted column compartment at 25 °C and a variable wavelength detector at 273 nm. High-performance liquid chromatography (HPLC) was performed on a Shimadzu system consisting of a SCL-10Avp controller, a SIL-10ADvp autoinjector, a SPD-M10Avp diode array detector and a LC-10ADvp pump.

Several types of columns and eluents were tested to obtain separation between CAF, PX, other metabolites and endogenous compounds. The optimal separation was performed on a Waters (Milford, MA, USA) Acquity UPLC CSH C$_{18}$ column (2.1 × 100 mm, 1.7 μm particles) with an eluent consisting of MeOH: 10 mM PCA (pH 2.0) 11:89 v/v (%) at a flow rate of 0.5 mL min^{-1}. A 20-μL volume of extracted samples was injected onto the column.

2.3. Saliva

Blank saliva was obtained from volunteers who abstained from all CAF-containing foods and drinks for 72 h. Saliva samples for in vivo experiments were collected from 9 volunteers who abstained from all CAF-containing foods and drinks 24 h prior and during the experiment. All saliva samples were stored in plastic tubes at 4 °C until analysis.

2.4. Sample preparation

Calibration curves and quality control (QC) samples were prepared by spiking blank saliva with known amounts of CAF and PX, after which they were pretreated as described below.

A 300-μL volume of saliva sample was pipetted into a clean glass tube, 75 μL of a 1 mg mL^{-1} IS solution was added and the solution was vortex-mixed for 1 min. A 4-mL volume of EtOAc was added and the sample was vortex-mixed for 5 min and subsequently centrifuged for 10 min at 2000 g. A 3.5-mL volume of the organic layer was transferred to a clean glass tube and dried under a nitrogen stream at 45 °C. The residue was reconstituted in 300 μL of ultra-pure water and 20 μL was injected into the UHPLC system.

2.5. Validation

The validation of this method was performed with saliva samples ranging in concentration between the lower (LLQ) and higher limit of quantification (HLQ). Calibration curves consisted of standards with PX concentrations of 0.025, 0.12, 0.25, 0.50, 1.2 and 2.5 μg mL^{-1} and CAF concentrations of 0.050, 0.25, 0.50, 1.0, 2.5 and 5.0 μg mL^{-1}. Peak area ratios for CAF/IS and PX/IS were plotted against the CAF and PX concentration, respectively. Weighted linear regression (1/x^2) was used and linearity was assessed by performing an F-test for regression and a t-test of the slope on the regression lines. Furthermore, the Pearson product-moment correlation coefficient (r) was calculated for each calibration curve and accepted at r > 0.95. Intraday and interday accuracy and precision were evaluated by analyzing 6 QC samples per concentration on one day or on three different days, respectively at 0.05, 0.5 and 5 μg mL^{-1} CAF

and 0.025, 0.25 and 2.5 μg mL^{-1} PX. Accuracy was expressed as percentage of determined QC sample concentration with respect to the nominal concentration and was accepted at 85–115%. Precision was expressed as the relative standard deviation of the QC sample concentrations and was accepted at ≤15%. Selectivity was tested by analyzing blank saliva from six volunteers and by testing the interference of CAF metabolites and commonly used drugs (TP, TB and APAP). The limit of detection (LOD) was determined by analyzing saliva samples with CAF and PX at concentrations that were estimated to give a signal to noise ratio (S/N) ≥3.

2.6. Application

The application of the method was demonstrated by the analysis of saliva samples from a phenotyping experiment. Nine volunteers abstained from CAF-containing foods and drinks for at least 24 h. Each participant took 100 mg of CAF at t = 0. Saliva samples were taken at t = 0, 3, 6, 8 and 24 h after CAF intake and analyzed according to the described method.

3. Results and discussion

3.1. Method development

The UHPLC system and sample pretreatment were optimized to obtain good selectivity between CAF, PX, IS, endogenous saliva compounds and other CAF metabolites and commonly used drugs. Different extraction solvents were used to extract the compounds of interest from saliva. Clean extracts with clear CAF, PX and IS peaks were obtained using CHCl$_3$/IPA and EtOAc extraction solvents, although highest recoveries were acquired with EtOAc (>68% and >72% for PX and CAF, respectively for all QC sample concentrations; n = 5). Optimization of the chromatographic separation was achieved by testing several LC columns and adjusting the organic modifier and other additives of the eluent. Good separation was achieved between CAF, PX and IS on a 4.0 × 125 mm C$_8$ RP column with 5 μm particles using eluents consisting of ACN:water or MeOH:water 10:90 v/v (%). However, PX and TP eluted simultaneously on these system, and were just separated from the IS peak that eluted before. The addition of 10 mM PCA to the eluent resulted in a retention time shift of the IS peak, that now eluted after the PX and TP peak. The application of longer columns and other types of RP column materials improved separation between PX and TP, although still no baseline separation could be obtained. The addition of THF to the eluent changed selectivity for PX and TP and improved resolution. Good separation between CAF, PX, TP and IS was obtained with a Zorbax Eclipse XDB-C18 column (3.0 × 250 mm, 5 μm particles; Agilent Technologies, Waldbronn, BW, Germany) using a mobile phase consisting of ACN:THF:10 mM HAc 2:2:96 v/v/v (%). The usage of THF however, is not preferable because of PEEK tube softening caused by THF. The application of UHPLC columns packed with smaller particles allowed for more efficiency and a similar separation of CAF, PX and TP as with the optimized HPLC system, but without THF in the eluent. The selectivity between PX-IS and TP-IS peaks could be further adjusted by the addition of PCA to the eluent. With an Acquity UPLC CSH C$_{18}$ column (2.1 × 100 mm, 1.7 μm particles), an eluent composition of MeOH:10 mM PCA (pH 2.0) 11:89 v/v (%) and a column temperature of 25 °C CAF, PX, IS and TP peaks were baseline resolved within 6 min (Fig. 1). This system allows for selective and accurate PX, CAF and IS peak area determination in the presence of TP.

Table 1
Intraday and interday accuracy and precision results for the analysis of CAF and PX in saliva at LLQ, HLQ and intermediate concentrations.

Compound	Concentration ($\mu g\,mL^{-1}$)	Intraday ($n=6$)		Interday ($n=3$)	
		Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
CAF	0.050	99.4	9.8	102.6	10.2
	0.50	101.5	10.2	96.4	13.5
	5.0	95.4	5.0	96.7	2.8
PX	0.025	100.2	9.7	99.6	1.7
	0.25	108.7	9.3	104.8	2.8
	2.5	91.2	6.6	107.2	7.0
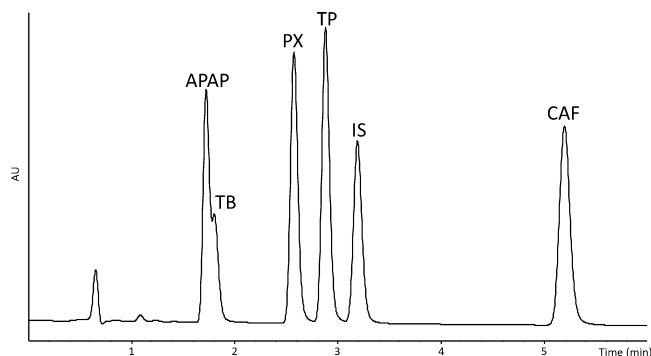
Fig. 1. Chromatogram obtained with the optimized method of a standard solution consisting of 2.5 $\mu g\,mL^{-1}$ APAP, TB, PX, TP, IS and CAF in water. Baseline resolved PX IS and CAF peaks were obtained with the optimal eluent (MeOH: 10 mM PCA (pH 2.0) 11:89 v/v (%)).
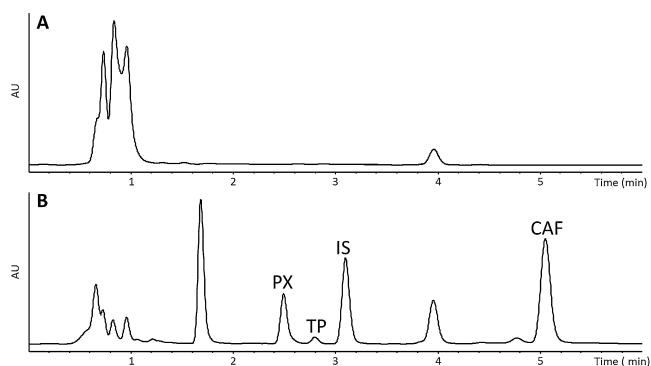
Fig. 2. Chromatograms obtained with the optimized method of (A) a blanc saliva sample, obtained from a volunteer who abstained from CAF for 72 h and (B) a chromatogram of an analyzed saliva sample from a subject 6 h after taking 100 mg of CAF, containing 0.49 $\mu g\,mL^{-1}$ PX and 1.24 $\mu g\,mL^{-1}$ CAF (B).

3.2. Method validation

QC samples and standards were prepared and analyzed with the optimized method as described in the method section. All pretreated samples were stable (>95%) for at least 50 h at room temperature and analyzed within this period of time. Blank saliva samples and several exogenous compounds were analyzed and tested for interference with CAF, PX or IS signals. No co-eluting peaks from endogenous compounds (Fig. 2A), CAF metabolites or commonly used drugs were found (Fig. 1). Linearity of the calibration curves ($r > 0.993$, $n = 8$) was assessed by an F-test for regression that showed a relation ($p < 0.05$) between peak area ratios of PAX/IS or CAF/IS and the PAX or CAF concentration, respectively. A t-test for the slope of the regression lines revealed a significant linear relationship ($p < 0.05$) between peak area ratios and concentration.

Inter- and intra-day accuracies of 91.2–107.2% and precisions <13.5% were found for the QC samples (Table 1). Limits of detection (S/N > 3) were 0.16 and 0.63 ng mL^{-1} ($n \geq 3$) for PX and CAF,

respectively, which is lower compared to other methods (15 ng mL^{-1} [7] and 3 ng mL^{-1} [10] for both PX and CAF).

All validation results were within the pre-set limits, demonstrating the suitability of the method for accurate and precise CYP1A2 phenotyping.

3.3. Application

Saliva samples from 9 volunteers, consisting of regular coffee drinkers (>2 cups of coffee per day; $n = 4$) and light or non-coffee drinkers ($n = 5$), were analyzed with the validated method. The volunteers took a single oral dose of 100 mg of CAF and saliva samples were collected before and 3, 6, 8 and 24 h after CAF intake. Fig. 2 shows representative chromatograms of a blank saliva sample and a sample taken 6 h after CAF intake.

There was a linear increase of the PX/CAF concentration ratios over time ($r > 0.97$) for all tested subjects. PX/CAF concentration ratios at 6 h post-dose ranged from 0.26 to 1.09, which is comparable to values found in literature [14]. Mean PX/CAF concentration ratios of regular coffee drinkers and light/non-coffee drinkers ($n = 5$) were 0.78 ± 0.26 and 0.38 ± 0.10, respectively, suggesting that habitual coffee consumption indeed significantly ($p = 0.014$) increases CYP1A2 activity [15].

4. Conclusions

In this study an optimized UHPLC method for the analysis of CAF and PX in saliva is presented. CAF and PX were accurately and selectively analyzed in the presence of TP and other CAF metabolites and endogenous compounds with a total run time of 6 min. The validation results show that the method is suited for the accurate and precise analysis of CAF and PX for CYP1A2 phenotyping. With the validated method 9 volunteers were phenotyped for their CYP1A2 enzyme, and using the PX/CAFconcentration ratios at 6 h post-dose it was confirmed that frequent caffeine consumption increases CYP1A2 activity.

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