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ORIGINAL RESEARCH

Validation of a high-performance liquid chromatography assay for quantification of caffeine and paraxanthine in human serum in the context of CYP1A2 phenotyping

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ABSTRACT: In this study the validation of a reversed-phase high-performance liquid chromatography (HPLC) method, with UV-detection, for both caffeine and paraxanthine in human serum is described. This method is feasible for cytochrome P450 1A2 (CYP1A2) phenotyping, according to the results of a pilot study. With this HPLC method caffeine and paraxanthine can be determined selectively and specifically. In the expected concentration range, caffeine recoveries were 98–108% (within-run variation 4.0–6.4%, between-run variation 6.4–8.8%), paraxanthine recoveries were 96.6–97.5% (within-run variation 5.0–7.2%, between-run variation 7.2–10.8%). The limits of detection for caffeine and paraxanthine using this HPLC system were 0.3 and 0.1 mg/L, respectively. Linear calibration curves for both caffeine and paraxanthine were obtained in the concentration range 0.5–30 mg/L ($r > 0.9999$. Serum samples were stable for a week, when stored at −20 and +4°C. Copyright © 1999 John Wiley & Sons, Ltd.

INTRODUCTION

Liver microsomes play an important role in both the metabolism and activation of many pharmaceuticals and other xenobiotics (Lindsay DeVane, 1994 Vermes *et al.*, 1997. A great inter- and intra-individual variability is observed in the activity of these enzymes, including some iso(en)zymes of the cytochrome P450 system. This variation in enzyme activity may be explained partly by the occurrence of genetic polymorphism of the cytochromal enzymes. Polymorphism of enzymes is caused by specific genetic mutations or deletions in the regulatory or the structural sequence of the genes coding for these enzymes (Vermes *et al.*, 1997). The prevalence of polymorphism in most studied enzymes of the cytochrome P450 system is approximately 1–5% (Lindsay DeVane, 1994; Nemeroff *et al.*, 1996). Genetic polymorphism can lead to different phenotypes: slow, extensive and fast metabolizing individuals. These population differences in the metabolism of pharmaceuticals may give rise to variability with respect to drug toxicity and efficacy (Lindsay DeVane 1994; Nemeroff *et al.*, 1996; Vermes *et al.*, 1997). The CYP1A2 (cytochrome P450 1A2) isoform is involved in the metabolism of tricyclic antidepressiva, theophyllin, caffeine, phenacetin and clozapine. Caffeine (1,3,7-trimethylxanthine) is mainly (80–90%) metabolized to paraxanthine (1,7-dimethylxanthine), specifically by CYP1A2.

Apart from genetic polymorphism, external factors, such as cigarette smoke, charcoal broiled food and pollution with polycyclic aromatic hydrocarbons (PAH), can induce cytochrome activity (Bock *et al.*, 1994).

CYP1A2 activity can be measured by phenotyping: a fixed dose of a substance, specifically metabolized by CYP1A2, is administered and the metabolic rate is determined. Caffeine can be used as a probe for CYP1A2 phenotyping. The specific catalysed metabolism of caffeine by CYP1A2 can be determined by measuring metabolic ratios in urine, saliva or serum (Fuhr and Rost, 1994. The paraxanthine/caffeine metabolic ratio has been recognized as a good estimate of CYP1A2 activity (Fuhr and Rost, 1994, Fuhr *et al.*, 1996). Caffeine serum clearance may also be used as an estimate of CYP1A2 activity, because caffeine is mainly cleared by this liver enzyme.

Another way of determining cytochrome polymorphism is by genotyping: the polymerase chain reaction (PCR) is used to detect mutations in the gene coding for

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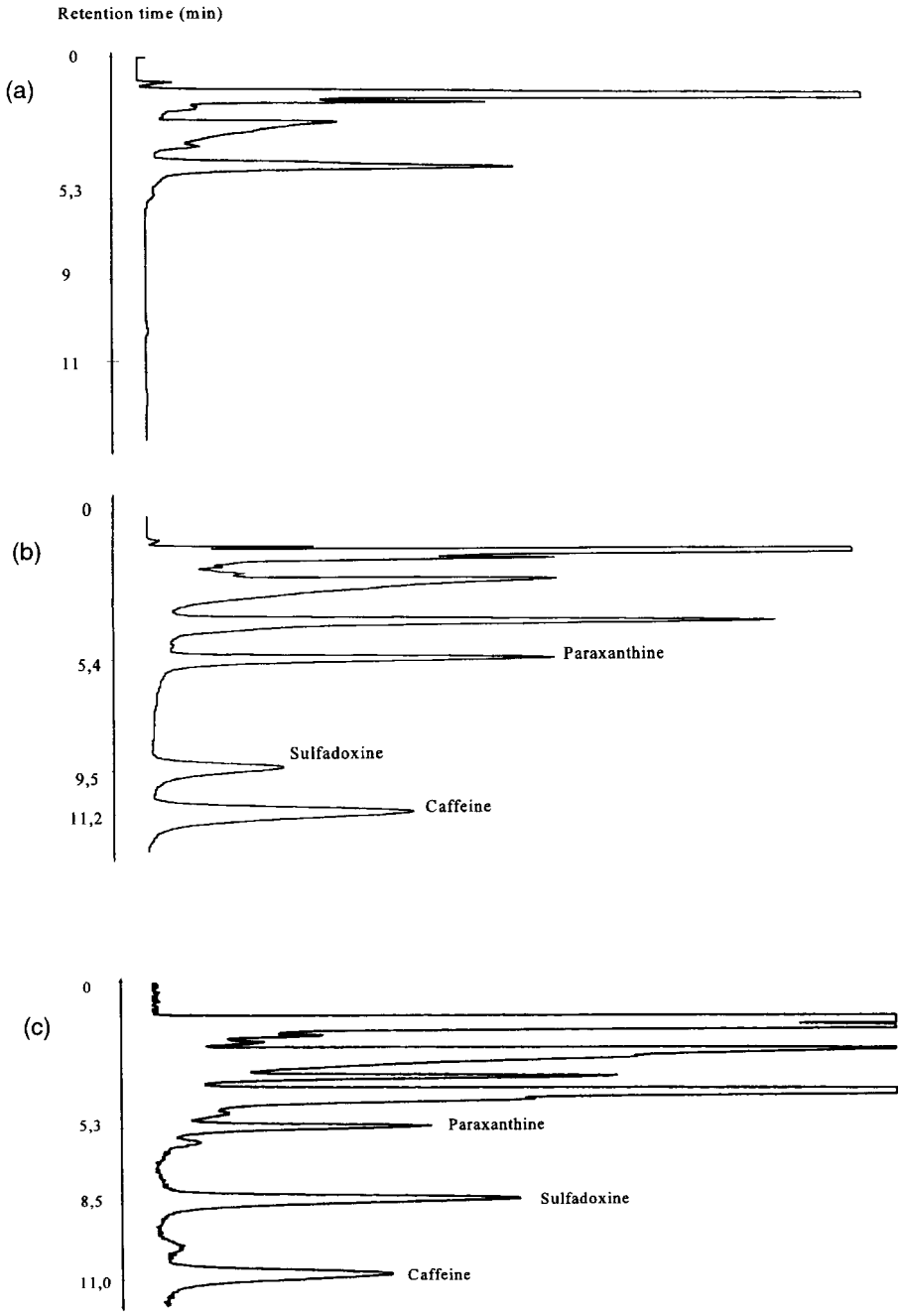

Figure 1. (a) HPLC chromatogram of human blank serum. (b) HPLC chromatogram of human serum spiked with caffeine and paraxanthine. (c) HPLC chromatogram of test subject's serum, 6 h after administration of caffeine.

the cytochrome enzyme. Transient changes in enzyme activity, for example caused by inducing agents, cannot be assessed with this method.

In our institute, the effects of dioxin exposure via mother's milk on CYP1A2 activity are studied in a cohort exposed children. Methods of analysis for simultaneous determination of paraxanthine and caffeine have been described earlier (Fuhr and Rost, 1994, Fuhr *et al.*, 1996; Holland *et al.*, 1998), however only in a single, recent publication is the necessary validation of the assay described (Holland *et al.*, 1998).

Therefore a high-performance liquid chromatography (HPLC) assay for determination of paraxanthine and caffeine was developed and validated before the above-mentioned study was performed. In a pilot study involving six volunteers the practical applicability of the assay was tested. In this report the results of the validation and the pilot study are described.

EXPERIMENTAL

Methods

Method of analysis. Caffeine and paraxanthine in serum can be quantitated with 'reversed-phase' HPLC using UV-detection. This system is a modification of the HPLC method used at our laboratory for determination of caffeine in serum.

Chemicals. The following chemicals were used for validation of the HPLC method: paraxanthine (Sigma, Zwijndrecht, batch no. 117H4066); caffeine (OPG, Utrecht, batch no. 92K10AL-WP04732); sulphadoxine, theophylline and methanol (Merck, Amsterdam); trichloro acetic acid and tri-ethylamine (Merck, Amsterdam, batch no. 40613917); and sodium dihydrogenphosphate monohydrate (Merck, Amsterdam, batch no. A858546. All chemicals used were of analytical grade.

Equipment/materials. The method of analysis was performed using the following HPLC system: pump, Waters M45; UV-detection, Applied Biosystems 757, the detection wavelength was set to 273 nm; integrator, Shimadzu-CR3A; column, Supelco LC-18-DB-087311AC; and injector, Rheodyne 7125. The pH of the mobile phase was adjusted using a Radiometer Copenhagen PHM83 Autocal and filtrated over a Millipore GVHP04700 filter (poresize 0.22 μm. During the monster preparation a Hettich Rotanta/P centrifuge was used.

Mobile phase. The mobile phase used in this assay was composed of 830 ml 0.05 M NaH_2PO_4, 300 μL triethylamine (pH 6.4) and 160 mL methanol. The eluent was filtrated over a Millipore filter before it was used in the assay. The flow of the eluent was 1.5 mL/min.

Preparation of standard and validation solutions. A stock solution of 15 mg caffeine and 15 mg paraxanthine in eluent was used for the preparation of standard solutions of caffeine and paraxanthine. All standard solutions were prepared by diluting this stock solution. The validation and calibration samples were prepared by spiking human blank serum with the above-mentioned stock solution.

Sample preparation. Serum proteins were precipitated before the sample could be injected onto the chromatographic system. Protein precipitation was achieved using the following method: 50 μL serum +20 μL internal standard (sulphadoxine 5 mg/L) +50 μL methanol +50 μL 10% trichloro acetic acid were mixed on a Vortex for 1 min and centrifuged at 4000 rpm for 10 min. 15 μL of supernatant were injected into the HPLC system.

Quantification. Quantification of caffeine and paraxanthine was achieved by comparing peak areas, using sulphadoxine as an internal standard.

Calculation of precision. Using SPSS 6.1, an analysis of variance (ANOVA) was carried out to calculate the precision (repeatability and reproducibility) of the assay. This software package calculates the within-run (repeatability) and between-run (reproducibility) variances. These variances were then converted to coefficients of variation using the formula $CV = (\sqrt{s^2}/TM)\cdot 100$, where CV is the coefficient of variation (%), s^2 is the variance and TM is the total mean of all runs.

Research

Validation of the method of analysis. Validation of the method of analysis was performed according to the procedure 'Validation of bioanalytical methods' (Manual of quality control, Department of Pharmacy, Academic Medical Center, Amsterdam), which is derived from the methods described by Van der Vaart (1992) and Shah *et al.* (1992).

Determination of the selectivity and specificity. It is of great importance that metabolites, co-medication and serum components co-elute separately from caffeine and paraxanthine. Therefore these potential disturbing factors should be separated from caffeine and paraxanthine signals. In this context caffeine, paraxanthine, theophyllin, sulphadoxine and blank calf serum were examined. As this study is carried out on a cohort of children, the following co-medication was examined: fenytoin, carbamazepine, clonazepam, valproic acid and acetaminophen. First, all substances mentioned above were dissolved in eluent and injected onto the HPLC system, then human serum was spiked with these drugs and injected again to trace possible matrix interactions.

Accuracy or recovery. Caffeine and paraxanthine recoveries (accuracy) in human serum were determined by preparing three different references of low, normal and high concentrations in eluent and quantification in duplicate. Then a series of five test samples was made for each of the three different concentrations of caffeine and paraxanthine. These samples were quantitated and compared to the appropriate reference. The mean concentration of each series of samples was divided by the nominal concentration and expressed as a percentage. The recovery of the internal standard was determined in a similar way at concentrations of $\frac{1}{2}$ (duplicate), 1 (quintuple) and 2 (duplicate) times the amount of sulphadoxine to be used. The accuracy of all tested concentrations should range between 85–115%.

Precision: repeatability and reproducibility. For each series of samples the repeatability and reproducibility coefficients of variation were determined. The repeatability coefficient of variation gives an indication of the intra-day or within-run variation ($n = 5$), the reproducibility coefficient of variation gives an indication of the inter-day or between-run variation ($n = 2$). The repeatability and reproducibility coefficients of variation are not allowed to exceed the 15% limit.

Determination of range, limits of detection and quantification. After injection of human serum the mean difference between minimal and maximal signal, amplitude or peak-to-peak-noise, was determined and expressed in concentration units. The limit of detection (LOD) is defined as three times this amplitude. The lower limit of quantification (LLQ) is defined as five times the peak-to-peak-noise; the higher limit of quantification (HLQ) is defined as twice the highest therapeutic concentration. The concentration range of the assay is defined as LLQ to HLQ. The determination of range, limits of detection and quantification was carried out for both caffeine and paraxanthine.

Table 1. Accuracy and precision

	Concentration (mg/L)	Recovery (%) ($n = 5$)	Repeatability coefficient (%, $n = 5$) (=within-run variation)	Reproducibility coefficient (%, $n = 2$) (=between-run variation)
Paraxanthine	1	97.5	5.0	10.8
	8	96.6	4.6	4.7
	16.0	97.0	7.2	7.2[b]
Caffeine	1	108	4.0	8.4
	8	98.5	4.2	8.8
	16.0	98.0	6.4	6.4[b]
Sulhadoxine	0.3	97.8[a]		
	0.6	96.6		
	1.2	93.9[a]		

[a] $n = 2$.
[b] No additional statistical variation observed: between-run variation is smaller than within-run variation.

Linearity. A calibration curve composed of five different concentrations, 0.5–1–7.5–15–30 mg/L, in blank human serum was prepared. These references were determined in duplicate. Linearity, expressed as $y = b \cdot x + a$ (y = response; b = slope = sensitivity; x = theoretical concentration and a = the intercept), and the coefficient of correlation were determined using linear regression.

Stability. The stability of spiked samples was examined in duplicate at different storage conditions. Spiked samples with a known concentration of caffeine and paraxanthine were stored at −20, +4 and +20°C. The concentrations of caffeine and paraxanthine in these samples were determined at day 0, 1, 2, 3, 8 and 10 after spiking. Samples stored at −20°C were thus submitted to five freeze–thaw cycles.

Experimental provocation test. Finally the above-described method of determining the paraxanthine/caffeine molar ratio in serum was tested for its practical feasibility. Six healthy volunteers were asked to refrain from caffeine for 48 h, whereupon a (blank) blood sample was taken. After this each volunteer was administered 200 mg of caffeine dissolved in a caffeine-free soft drink. Six hours after caffeine intake another blood sample was taken, in which the paraxanthine, caffeine concentrations and molar ratio were determined. The rate of caffeine elimination and clearance was estimated as well, using MW/PHARM (Proost and Meyer, 1992. This program fits the serum concentration found using a pharmacokinetic model, based on Bayesian forecasting (Denaro and Jacob, 1998), on population averages and calculates individual pharmacokinetic parameters such as clearance and volume of distribution.

RESULTS

Determination of the selectivity and specificity

Caffeine, paraxanthine and internal standard eluate well separated from the column (Fig. 1b and c). From comparing Fig. 1a and b it appears that serum peaks, all with retention times of less than 4 min, do not disturb the paraxanthine signal at approximately 5 min. Sulphadoxine has a retention time of approximately 9 min, but its chromatographic behaviour is sensitive to pH changes. Caffeine has a retention time of about 11 min. Theophyllin, a caffeine metabolite, gives a signal right after paraxanthine, but is *in vivo* quantitatively negligible (Fig. 1c). The examined co-medication did not disturb the determination of caffeine and paraxanthine, except for acetaminophen, which gave a signal at 3.9 min. However, this signal does not interfere with paraxanthine, caffeine or the internal standard.

Accuracy and precision

Repeatability and reproducibility results are presented together with accuracy data in Table 1. The accuracy, or recovery, at the concentrations studied was within the limits aimed for (85–115%. Within-run and between-run coefficients of variation at different concentrations also did not exceed the aimed criteria.

Determination of range and limits of detection and quantification

The limits of detection for the determination of paraxanthine and caffeine are 0.096 and 0.27 mg/L, respectively. The lower limits of quantification for paraxanthine and caffeine are 0.16 and 0.45 mg/L, respectively. The higher limits of quantification will be approximately 20 mg/L. Therefore the range for paraxanthine quantification is 0.16–20 mg/L and for caffeine 0.45–20 mg/L.

Linearity

Five-point calibration curves of both caffeine and paraxanthine were linear. Correlation coefficients for both curves were 0.99994. The calibration curves are shown in Fig. 2.

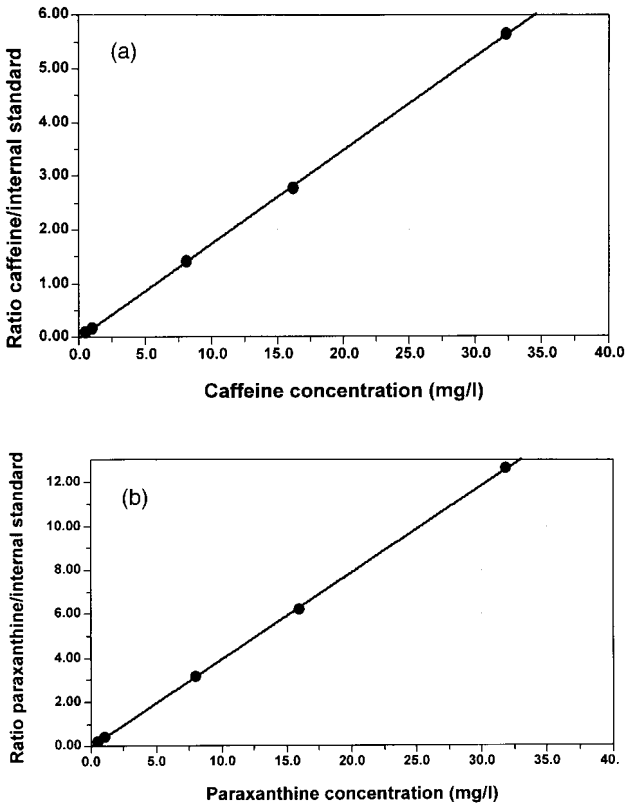

Figure 2. (a) Mean calibration curve of caffeine in human serum ($n = 2$). (b) Mean calibration curve of paraxanthine in human serum ($n = 2$).

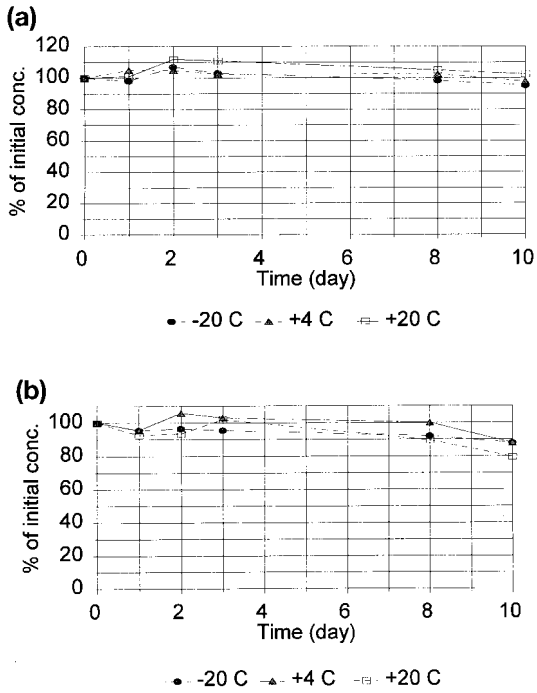

Figure 3. (a) Stability of caffeine spiked human serum. (b) Stability of paraxanthine spiked human serum.

Stability

Figure 3 shows the results of the stability experiments. Caffeine in serum (3 mg/L) is stable for 1 week at −20, +4 and +20°C, paraxanthine in serum (3 mg/L) is stable for 1 week at −20 and +4°C. At room temperature (+20°C) paraxanthine in serum is stable for only 1 day.

Experimental provocation test

In five out of six serum samples, taken before the administration of the test dose of caffeine, paraxanthine and caffeine were not detectable; one sample contained a low concentration (approximately 0.5 mg/L) of both caffeine and paraxanthine. Caffeine and paraxanthine serum concentrations were determined and paraxanthine/caffeine molar ratio (*P/C*), clearance (*Cl*) and volume of distribution (*V*) were calculated (Table 2).

DISCUSSION

Unexpected effects after administration of pharmaceuticals can sometimes be explained by different enzyme activities of the cytochrome P450 system, involved in metabolism or activation of these drugs. Characterization of enzyme activity can be performed by phenotyping: a probe, or marker substance, specifically metabolized by the studied enzyme, is administered as a single dose and the metabolic rate is determined by means of measuring serum concentrations of the probe and the metabolite(s) formed.

Caffeine is often used as a probe for CYP1A2 phenotyping. This method is frequently described in literature, however detailed information on the analytical chemical validation of the method is scarce. In this report the validation of the caffeine and paraxanthine method of analysis is described. Further, the results of a pilot study involving phenotyping CYP1A2 in six healthy volunteers are shown.

Caffeine and paraxanthine can be determined simultaneously using the described reversed-phase HPLC system with UV-detection. Co-medication, often prescribed for children (anti-epileptics, acetaminophen), did not disturb the assay. Caffeine and paraxanthine recovery was nearly complete. The assay is characterized by a low inter- and intra-day variation; in our laboratory both coefficients of variation are intended to be smaller than 15%; this target is easily met.

At caffeine doses usually applied in phenotyping experiments (3 mg/kg body weight), expected serum concentrations are 2 mg/L and 1.5 mg/L for caffeine and paraxanthine, respectively; these values are well above the lower limits of quantification. Calibration curves for quantification of caffeine and paraxanthine were linear over the range of the assay.

Table 2. Caffeine provocation test: test subjects and population records

Subject	Age (years)	Weight (kg)	*Cl* (L/h.kg)	*V* (L/kg)	k_{el} (L/h)	Ratio *P*/*C*[a]
1	30	55	0.088	0.550	0.160	0.96
2	27	66	0.066	0.430	0.153	0.21
3	24	73	0.129	0.670	0.193	1.2
4	24	92	0.090	0.530	0.169	0.49
5	36	74	0.094	0.570	0.165	0.59
6	40	70	0.101	0.590	0.171	0.99
Mean			0.095	0.557	0.169	0.74
SD			0.021	0.079	0.014	0.37
CV (%)			22	14	8.1	50
Population[b]			0.114	0.630		0.83
CV (%)			41	20		(0.16–1.7)

[a] Molar ratio paraxanthine/caffeine
[b] Molar ratio range.
[c] Denaro *et al.* (1998).

When phenotyping is performed in a large group of individuals it may be necessary to store serum samples for a longer period of time. It can be observed from the stability study that storage conditions investigated (-20 and +4°C) have a negligible influence on serum caffeine and paraxanthine concentration. When stored longer than 1 week serum concentrations of paraxanthine seem to be notably affected.

In most phenotyping studies using caffeine as a probe, test subjects refrain from caffeine (coffee, tea, chocolate) for 24–48 h before administration of a test dose of caffeine. In this pilot study a refraining period of 48 h was used, which seemed satisfactory. One test subject showed low caffeine and paraxanthine concentrations; on inquiry this subject appeared to have used some herbal tea, apparently containing some caffeine. Caffeine serum concentrations ranged between 1.3 and 3.7 mg/L, paraxanthine serum concentrations ranged between 0.8 and 2.9 mg/L; these serum levels are well within the range of the assay.

The paraxanthine/caffeine molar ratio can be used as an indicator of CYP1A2 activity. Normal values for this ratio range from 0.2 to 1.7; in several studies mean paraxanthine/caffeine molar ratio is approximately 0.8 (Fuhr *et al.*, 1996). Ratios of the test subjects varied between 0.2 and 1.2 (mean: 0.74). When caffeine clearance is used as an estimate of CYP1A2 activity, no significant differences between population (Denaro and Jacob, 1998) and test subject clearance were found (Table 2).

CONCLUSION

It can be concluded that the HPLC assay described is suitable for caffeine and paraxanthine quantification and CYP1A2 phenotyping.

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