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Quantification of caffeine in human saliva by Nuclear Magnetic Resonance as an alternative method for cytochrome CYP1A2 phenotyping

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ABSTRACT

The first step in caffeine metabolism is mediated for over 95% by the CYP1A2 isoform of cytochrome P450. Therefore, CYP1A2 activity is most conveniently measured through the determination of caffeine clearance. The HPLC quantification of caffeine is fully validated and is the most widely used method. It can be performed on saliva, which is gaining importance as a diagnostic biofluid and permits easy and low invasive sampling.

Here, we present a quantitative ^{1}H nuclear magnetic resonance (NMR) method to determine caffeine in human saliva. The procedure is simple because it involves only an ultra-filtration step and a direct extraction in a deuterated solvent, yielding a matrix that is then analyzed. The reliability of this NMR method was demonstrated in terms of linearity, accuracy, recovery, and limits of detection (LoD). Good precision (relative standard deviation, RSD $<4\%$), a recovery of $>95\%$ and LoD of $6.8 \cdot 10^{-7}$ mol L^{-1} were obtained. The method was applied to samples collected from different volunteers over 24 h following a single oral dose of about 100 mg of caffeine administered with either coffee beverage or a capsule.

1. Introduction

The enzyme cytochrome P450 1A2 (CYP1A2) accounts for about 13% of the total cytochromes P450 (CYPs) in the human liver [1] and is involved in a number of metabolic pathways that process endogenous substrates, xenobiotics and environmental toxins and also in the activation of carcinogens, such as dietary heterocyclic amines or polycyclic aromatic hydrocarbons [2,3]. It is responsible for the metabolism of several drugs used in various therapeutic areas, including antidepressants, antipsychotics, antiarrhythmics, broncho-dilators and many others [2].

Constitutional and genetic factors can cause different levels of CYP1A2 activity and factors such as life style (smoking, physical exercise), diet (caffeine, cruciferous vegetables, grilled meat, alcohol) as well as many drugs are known to modulate enzyme induction or inhibition [2,3]. Depending on the analytical method used, *inter* individual differences up to 60-fold can be found, and 5- and 15-fold variations are common [3].

The study of CYP1A2 activity is important to better understand the great *intra* and *inter* individual variability in response to therapeutic treatments. Some drugs influence the metabolization rate of xenobiotics and even their own clearance leading to a higher or lower exposition to the corresponding metabolites. In such cases, unpredictable CYP1A2 activity may cause non optimal therapeutic response or a bigger probability to develop adverse effects.

Considering the important role of CYP1A2 in the elimination or in the metabolism of a wide range of xenobiotics including drugs, environmental compounds and (pro) carcinogens [2,3], phenotyping represents the way to identify sources of variations of enzyme activity and, in turn, an instrument to calibrate drug therapies in the clinical practice, discover drug interactions and understand the cause of possible adverse drug effects or non-response to a therapy [3–5].

Usually, enzymatic activity can be easily determined through *ex vivo* tests in tissues expressing the enzyme, but this is not feasible in the case of CYP1A2 because this enzyme is not present in blood cells or in another readily accessible tissue or fluid. Since CYP1A2 is mainly confined to liver, to avoid liver biopsies, *in vivo* tests are preferred [3].

Caffeine is the most commonly used probe for CYP1A2 phenotyping as over 95% of the first step in its metabolism is mediated by CYP1A2, and it is ideal for many reasons, allowing non-invasive epidemiological studies: it is safe and commonly present in the diet, it can be administered orally and its gastrointestinal absorption is rapid and complete; once absorbed, it is found in all body fluids, there is no long-term accumulation and it is extensively metabolized by the liver with minimal renal elimination [3,5,6].

For CYP1A2 phenotyping, caffeine is the only substrate for which a fully validated method is available [3,5,6], and the

Abbreviations: DMF, N,N-dimethylformamide; HPLC, High Performance Liquid Chromatography; NMR, Nuclear Magnetic Resonance; qNMR, quantitative Nuclear Magnetic Resonance

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systemic clearance of caffeine, following the administration of a known dose, is considered the 'gold-standard' in comparison to CYP1A2 activity in liver biopsies [3,5]. The effectiveness of other proposed metrics is evaluated against this one: one example is the method based on the determination of the paraxanthine to caffeine ratio between 4 and 6 h after caffeine intake, that showed a good correlation too [3,5,7]. Literature studies on caffeine determination in various matrices (plasma, saliva, urine) are mainly based on HPLC (*High Performance Liquid Chromatography*) and the most widely used detection techniques are ultraviolet absorption [4,7–11] and to a minor extent mass spectrometry [12–16]. In most cases, a calibration curve is required for quantitative analysis. Some GC–MS methods have also been proposed (see for example [17]). With MS detection, the use of expensive stable-isotope labeled standards is common.

Biological matrices such as saliva and plasma are complex, principally because of the high content of proteins, and considering the low analyte concentrations expected in this type of studies (C_{max} = 1.2–1.9 μg mL^{-1} or 6–10 μmol L^{-1}), most of the methods proposed require a liquid–liquid extraction [7,8,10,14,16] or solid-phase extraction [9,11,12] step during the sample pretreatment, with subsequent solvent removal and reconstitution in an appropriate solvent.

In the present study, we analyzed caffeine clearance in saliva, which is the biofluid showing the closest correlation with immunoreactive CYP1A2 liver intrinsic activity [3]. In addition, saliva is an interesting matrix for drug monitoring purposes, gaining importance over plasma/serum, as it offers the advantage to be more easily, more cheaply, and less invasively sampled, ideal in the case of multiple and long treatments, especially in diseased individuals, children or elderly people [5,18].

This work describes the quantitative determination of caffeine in saliva by Nuclear Magnetic Resonance (NMR). A powerful characteristic of NMR spectroscopy is that it is inherently quantitative and it provides a linear response; because of these features, the lengthy construction of a calibration curve is usually not required and a reference compound (internal or external) is sufficient to provide absolute concentrations [19,20].

The method we propose is rapid because a filtration step allows the extraction to be performed directly in a deuterated solvent, with no need for solvent evaporation and reconstitution in a different solvent, involving a reduction of time of analysis and volume of solvent needed. For absolute quantification, an external standard in a coaxial insert is used which simplifies the sample preparation step and reduces the risk of introducing errors in the procedure.

The first part of the work was finalized to optimize sampling, pretreatment, and NMR protocol and included validation of the method in terms of precision and accuracy. The method was then applied to samples collected from volunteers to compare it to previously published studies.

2. Materials and methods

2.1. Chemicals

Capsules of caffeine (100 mg caffeine per capsule) were purchased from Scitec Nutrition P.P. Box 431975, Miami, FL33243, USA, distributed by Scitec KFT, Hungary. Caffeine (≥ 99.0%) was purchased from Sigma-Aldrich. As standard compound for quantitative determination of caffeine, N,N-dimethylformamide (DMF, ≥ 99.99% (GC), Fluka-Sigma-Aldrich) was used. Deuterated chloroform (99.96%D) was purchased from Sigma-Aldrich, and deuterated water (≥ 99.96% D), from Eurisotop. Ultrafiltration was performed using Sartorius Vivaspin devices (2 kDa cutoff).

2.2. Preparation of the standard solutions

Caffeine and DMF standard solutions were prepared weighing accurately the compound and the chosen solvent. In the case of the caffeine solution in water, considering the low solubility of caffeine in water (15 mg mL^{-1}), the exact concentration was verified using a UV calibration curve (λ_{max}=273 nm, ε=(9633.9 ± 70.4) L mol^{-1} cm^{-1}).

2.3. Participants and sample collection

Three healthy subjects (two females, one male), non smokers and not under pharmacological treatment were asked to abstain from any caffeine or methylxanthines intake for at least 48 h. Participants were asked to expectorate into a graduated conical disposable centrifuge tube (15 mL) a volume of about 5 mL of saliva corresponding to a collection time of approximately 3–6 min. Salivation was stimulated by chewing a piece of Parafilm®. At the beginning of the sampling day, a blank sample prior to caffeine ingestion was collected to assure the absence of the analyte. Samples were collected at least 30 min after any meal. The samples were stored at −20 °C. Caffeine was administered with a capsule (100 mg), assuring no permanence of caffeine in the mouth, or with an espresso coffee regular beverage (~6.5 g roasted coffee, 25 mL), in which the caffeine content was measured by ^{1}H NMR (~77 mg).

2.4. Sample treatment

The frozen saliva was thawed and centrifuged at 6000 rpm and the supernatant was ultrafiltered. Low-molecular-mass ultrafiltrates were obtained using Sartorius Vivaspin devices (2 kDa cutoff) washed with water before use. To 4 mL of filtered saliva, 1 mL of deuterated chloroform was added, the sample was vortex mixed (15 min) and then centrifuged (7200 rpm, 10 min, T=20 °C). The organic layer (500 μL) was transferred into a 5 mm precision glass NMR tube (Wilmad 535-pp) and the coaxial insert with the standard solution was put in the same tube, which was then analyzed.

Saliva was also analyzed as such or after the ultrafiltration step. In these cases, 10% of D_2O was added to the saliva, which was then analyzed in the same way.

2.5. NMR spectroscopy

^{1}H NMR data were acquired using a Bruker Avance DMX600 instrument, operating at 599.90 MHz and equipped with a 5 mm TXI xyz-triple gradient probe.

Aqueous ultrafiltered samples were acquired with the DPFGSE [21] sequence to maximize the sensitivity of the experiment, using an adiabatic pulse; for the $CDCl_3$ extracts, a common one pulse sequence was used. Typically, spectral widths of 6000 Hz, 32,768 data points were used. A relaxation delay of 43 s was used when the aliphatic spectral region was to be integrated and of 50 s when the aromatic region was to be integrated. The number of scans varied between 32 (25 min) and 128 (1 h 40 min) depending on caffeine concentration, to reach at least a S/N of 10. In the case of very low caffeine concentrations in the extracts (~0.75 μmol L^{-1}, 0.15 μg mL^{-1}), corresponding to 20–24 h after caffeine ingestion, and in the ultrafiltered aqueous samples, 256 scans were used (3 h 20 min).

The ACD software (ACD 12 Labs) was used to process the spectra. Fourier transformation was performed after zero filling the free induction decay data by a factor of 2 and after exponential line-broadenings of 0.2 Hz. Integrations were manually obtained after careful manual phase and baseline correction.