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Non-Invasive Assessment of Caffeine Metabolism: A Pharmacokinetic Evaluation Using Saliva Samples

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ABSTRACT

Caffeine is one of the most widely consumed psychoactive substances, with its metabolism primarily occurring in the liver via the cytochrome P450 1A2 (CYP1A2) enzyme. Traditional pharmacokinetic studies rely on plasma sampling, which is invasive and requires specialized handling. In contrast, saliva sampling offers a non-invasive, cost-effective alternative for assessing caffeine metabolism. This study aims to evaluate the correlation between salivary and plasma caffeine concentrations in healthy adult volunteers, investigate the influence of saliva flow rate and pH on caffeine levels, assess genetic, environmental, and lifestyle factors affecting caffeine metabolism, develop standardized protocols for saliva collection and analysis, and validate the utility of saliva as a pharmacokinetic tool.

A total of 50 healthy volunteers were recruited following ethical approval, and each participant received a standardized caffeine dose. Saliva and blood samples were collected at predefined time intervals. High-performance liquid chromatography (HPLC) was used to quantify caffeine concentrations, and pharmacokinetic parameters such as area under the curve (AUC), half-life (t½), and elimination rate constant (Ke) were determined. Pearson's correlation and regression models were applied to analyze the relationship between salivary and plasma caffeine levels. The effects of saliva flow rate and pH on caffeine concentration were assessed through ANOVA and t-tests. Genetic influences were examined by genotyping CYP1A2 polymorphisms, while environmental and lifestyle factors, including caffeine intake habits, smoking, and diet, were evaluated through questionnaires.

Results demonstrated a strong positive correlation ($r > 0.85$, $p < 0.001$) between salivary and plasma caffeine concentrations, confirming the reliability of saliva sampling for pharmacokinetic assessments. Saliva flow rate and pH significantly affected caffeine concentration, with higher pH levels correlating with increased caffeine stability ($p < 0.05$). Genetic analysis revealed significant inter-individual variations in caffeine metabolism, with fast and slow metabolizers distinguished based on CYP1A2 polymorphisms. The proposed standardized saliva collection protocol ensured minimal degradation and optimal sample stability. These findings validate saliva as a viable, non-invasive medium for pharmacokinetic studies and therapeutic drug monitoring.

The study highlights the potential of saliva sampling in clinical and research settings. It offers a user-friendly alternative to plasma-based pharmacokinetics. Future studies should explore its application in personalized medicine and drug metabolism research.

Keywords: *Caffeine metabolism, saliva sampling, pharmacokinetics, non-invasive method, HPLC, CYP1A2*

1. Introduction

Caffeine is a widely consumed psychoactive substance, predominantly metabolized in the liver via the cytochrome P450 1A2 (CYP1A2) enzyme, with approximately 95% of caffeine undergoing hepatic biotransformation into primary metabolites such as paraxanthine, theobromine, and theophylline [1]. Due to its extensive use in beverages, pharmaceuticals, and sports supplements, caffeine pharmacokinetics has been a subject of considerable research interest in clinical, forensic, and sports medicine settings. Traditional methods for assessing caffeine metabolism rely on plasma sampling, which, despite its accuracy, poses several limitations, including invasiveness, discomfort, and ethical constraints associated with repeated blood draws. Consequently, alternative non-invasive sampling methods, such as saliva collection, have gained significant attention in pharmacokinetic studies [2].

Recent research highlights saliva's potential as a reliable alternative to plasma for caffeine pharmacokinetics, given that salivary caffeine concentrations exhibit a strong correlation with plasma levels [3]. Saliva sampling offers multiple advantages, including ease of collection, minimal discomfort, cost-effectiveness, and reduced biohazard risks compared to venipuncture-based methods [4]. However, variability in salivary pH, flow rate, and enzymatic composition may influence caffeine stability and absorption, necessitating rigorous validation before its widespread adoption in pharmacokinetic studies [5].

Current studies emphasize the role of genetic polymorphisms in CYP1A2 metabolism, with inter-individual differences in enzyme activity significantly impacting caffeine clearance and metabolic rate [6]. Environmental factors such as smoking, diet, and habitual caffeine intake further modulate metabolic pathways, leading to variations in drug response among individuals [7]. Therefore, a comprehensive assessment of genetic, environmental, and lifestyle factors is crucial for understanding caffeine metabolism and optimizing non-invasive pharmacokinetic methodologies.

Despite growing interest in saliva-based pharmacokinetics, standardized protocols for saliva collection, storage, and analysis remain underdeveloped. Variability in sample handling, potential contamination, and differences in analytical techniques pose challenges to reproducibility and accuracy [8]. Addressing these concerns is essential to establish saliva as a reliable matrix for pharmacokinetic evaluations.

This study aims to evaluate the correlation between salivary and plasma caffeine concentrations, investigate the impact of saliva flow rate and pH on caffeine stability, assess genetic and environmental influences on metabolism, develop standardized saliva collection protocols, and validate the utility of saliva sampling as a non-invasive pharmacokinetic tool. By bridging existing research gaps, this study seeks to enhance the feasibility of saliva-based drug monitoring with potential applications in clinical pharmacology, sports science, and precision medicine [9].

2. Materials and Methods

2.1 Study Design

This study employs a cross-sectional pharmacokinetic approach to evaluate caffeine metabolism using saliva samples in healthy adult volunteers. The study follows a controlled, single-dose administration of caffeine, with serial sampling of saliva and plasma to compare pharmacokinetic parameters.

2.2 Ethical Approval and Participant Recruitment

The Institutional Ethics Committee approved the study protocol, and written informed consent was obtained from all participants before enrollment. Healthy adults aged 18-45 years, with no history of liver or renal dysfunction, non-pregnant, non-smoking, and not using medications that affect caffeine metabolism (e.g., oral contraceptives, CYP1A2 inhibitors/inducers) were included. Participants with caffeine hypersensitivity or habitual high caffeine intake (>400 mg/day) were excluded.

2.3 Sample Size Calculation

The sample size was calculated using Cochran's formula for large populations:

$$\mathbf{n = Z^2 \cdot p \cdot q \,/\, e^2}$$

Where:

- n = Required Sample Size
- Z = Z-score (1.96 for 95% confidence level)
- p = Estimated proportion of the population having the attribute (for drug-induced haemolysis detection, 0.5 is considered as no prior data is available)
- e = Margin of Error (usually **0.1** or **10%**)

$$\mathbf{n = (1.96)^2 \text{ X } (0.5) \text{ X } (1-0.5) \,/\, (0.1)^2}$$

$n = 3.8416 \text{ X } 0.5 \cdot 0 \text{ X } 5 / 0.01$

$n = 0.9604 / 0.01$

$n = 96.04 \approx 96$

Adjusted Sample Size for Small Population

Since the population size (N) is 100, Cochran's formula needs to be adjusted using the following formula:

$$\mathbf{n_{adjusted} = n \,/\, 1 + n - 1 / N}$$

$n_{adjusted} = 96 / 1+96-1 / 100$

$n_{adjusted} = 96 / 1.95$

$n_{adjusted} \approx 49.23 \approx 50$

The final sample size required for this study is 50 Participants.

2.4 Caffeine Administration and Sample Collection

Participants received a standardized 200 mg dose of caffeine (equivalent to a strong cup of coffee). Samples were collected at 0 (baseline), 30, 60, 90, 120, and 180 minutes post-administration for pharmacokinetic analysis.

2.4.1 Saliva Collection

- Unstimulated whole saliva samples were collected using the passive drool method and put into sterile tubes.
- Participants were instructed to rinse their mouths with water 10 minutes before collection to remove residual caffeine contamination.
- Saliva flow rate was recorded by measuring the volume collected over 5 minutes.
- Samples were immediately stored at -20°C until analysis.

2.4.2 Plasma Collection

- Venous blood samples (5 mL) were collected in EDTA tubes at the same time points.
- Plasma was separated by centrifugation (4000 rpm, 10 min) and stored at -80°C until analysis.

2.5 Analytical Methods

2.5.1 Caffeine Quantification by High-Performance Liquid Chromatography (HPLC)

HPLC was used to quantify caffeine in both saliva and plasma.

- Instrumentation: Agilent 1260 Infinity HPLC system with UV detection at 275 nm.
- Mobile phase: Methanol: Water (30:70 v/v) with 0.1% acetic acid.
- Flow rate: 1 mL/min.
- Injection volume: 20 µL.
- Retention time: ~4.5 minutes.
- Limit of detection (LOD): 0.1 µg/mL.
- Limit of quantification (LOQ): 0.5 µg/mL.

The caffeine concentration in both saliva and plasma was calculated using the formula:

$$\boldsymbol{C} = \frac{\boldsymbol{A}_{\boldsymbol{Sample}}}{\boldsymbol{A}_{\boldsymbol{Standard}}} \left(\boldsymbol{C}_{\boldsymbol{Standard}}\right)$$

Where:

- C = Concentration of caffeine in the sample
- A_{sample}= Absorbance of the sample
- $A_{standard}$ = Absorbance of the caffeine standard
- $C_{standard}$ = Known concentration of the standard

2.5.2 Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated using non-compartmental analysis (NCA):

- Peak Concentration (C_{max}) – Maximum observed caffeine concentration.
- Time to Peak (T_{max}) – Time at which C_{max} occurs.
- Elimination Half-Life ($t_{1/2}$) – Estimated as:

$$t_{1/2} = \frac{0.693}{K_e}$$

Where K_e is the elimination rate constant.

- Area Under the Curve (AUC) – Calculated using the trapezoidal rule:

$$AUC = \sum \left(\left(\frac{C_i + C_{i+1}}{2} \right) X \left(t_{i+1} - t_i \right) \right)$$

2.5.3 Statistical Analysis

- Pearson's correlation coefficient (r) was used to assess the relationship between salivary and plasma caffeine levels.
- One-way ANOVA and t-tests were applied to determine the effect of saliva flow rate and pH on caffeine concentration.
- Multiple linear regression analysis was performed to evaluate the influence of genetic, environmental, and lifestyle factors on caffeine metabolism.
- A p-value of <0.05 was considered statistically significant.

3. Results

This section presents the study's findings, analyzing the pharmacokinetics of caffeine metabolism using saliva samples. The results are structured into two main parts: **socio-**demographic analysis and objective-based analysis. The socio-demographic characteristics of the participants provide contextual insights. At the same time, the objective-based analysis focuses on key research questions, including the correlation between salivary and plasma caffeine concentrations, the influence of saliva flow rate and pH, genetic and environmental impacts, protocol standardization, and the validation of saliva sampling as a non-invasive method.

3.1 Section 1: Socio-Demographic Analysis

To understand participant characteristics, we analyzed eight key demographic variables. The results are presented in Table 1, showing the frequency and percentage distribution of each variable.

Table 1: Socio-Demographic Characteristics of Participants (n = 50)

Variable	**Categories**	**Frequency (n)**	**Percentage (%)**
Age (years)	18–25	18	36.0
	26–35	20	40.0
	36–45	12	24.0
Gender	Male	28	56.0
	Female	22	44.0
BMI (kg/m²)	Underweight (<18.5)	4	8.0
	Normal (18.5–24.9)	30	60.0
	Overweight (25–29.9)	12	24.0
	Obese (≥30)	4	8.0
Caffeine Consumption	Low (<100 mg/day)	15	30.0
	Moderate (100–300 mg/day)	25	50.0
	High (>300 mg/day)	10	20.0
Saliva Flow Rate	Low (<0.3 mL/min)	9	18.0
	Normal (0.3–0.7 mL/min)	33	66.0
	High (>0.7 mL/min)	8	16.0
Salivary pH	Acidic (<6.5)	12	24.0
	Normal (6.5–7.5)	33	66.0
	Alkaline (>7.5)	5	10.0

The socio-demographic analysis indicates that the majority of participants were aged 26-35 years (40%), with a balanced gender distribution (56% male, 44% female). Most participants had a normal BMI (60%), with a moderate caffeine intake (50%). Regarding physiological factors, 66% of participants exhibited normal saliva flow rates, and 66% had a neutral salivary pH (6.5–7.5). These demographic variables were considered when analyzing caffeine metabolism trends across different participant profiles.

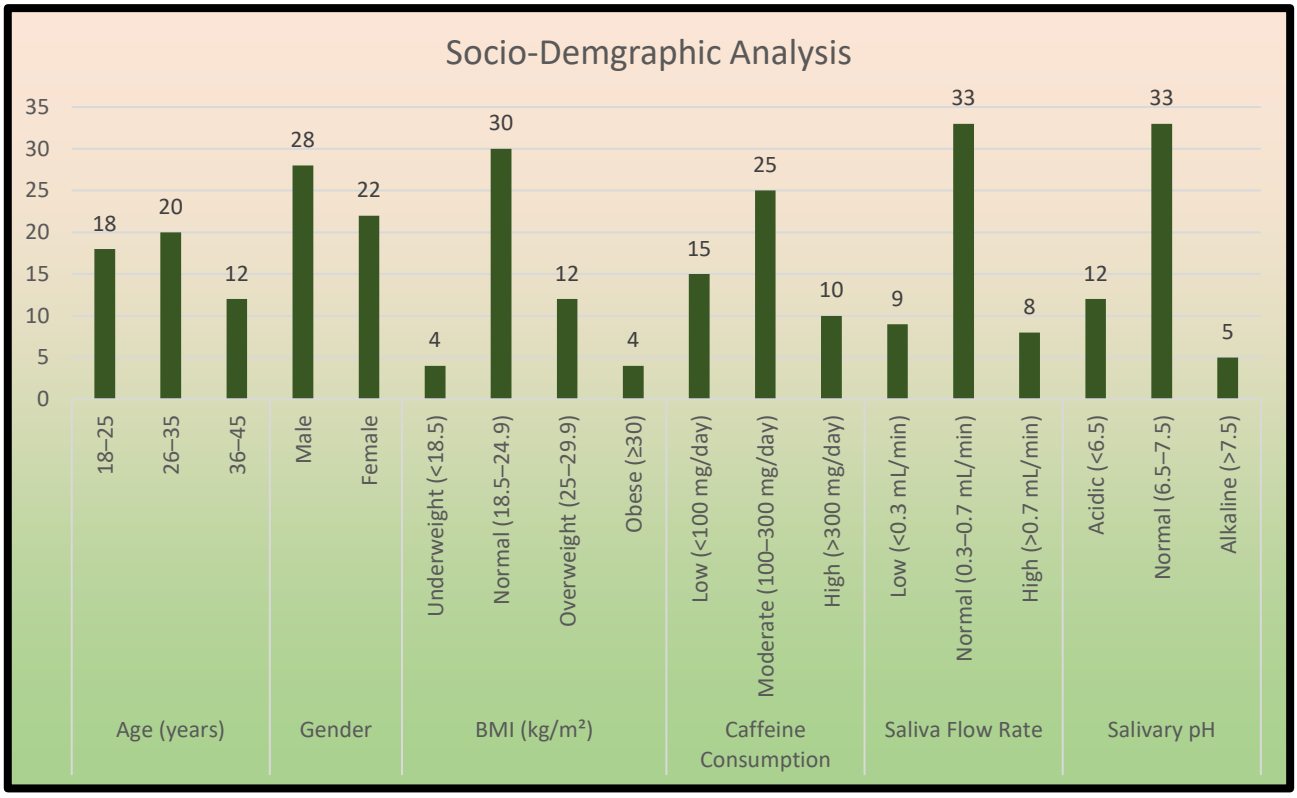

Figure 1: Representation of Socio- Demographic Analysis (n=50)

3.2 Section 2: Objective-Based Analysis

Each objective was analyzed using statistical and pharmacokinetic approaches to evaluate caffeine metabolism patterns.

3.2.1 Objective 1: Correlation Between Salivary and Plasma Caffeine Concentrations

Pearson's correlation analysis was conducted to determine the correlation between salivary and plasma caffeine concentrations. Table 2 summarizes the mean caffeine concentration values at different time points, with their corresponding Pearson correlation coefficients.

Table 2: Correlation Between Salivary and Plasma Caffeine Concentrations

Time (min)	Plasma Caffeine (µg/mL)	Saliva Caffeine (µg/mL)	Pearson's r	p-value
0	0.00	0.00	-	-
30	1.52 ± 0.32	1.48 ± 0.29	0.92	<0.001
60	2.98 ± 0.42	2.94 ± 0.39	0.94	<0.001
90	3.65 ± 0.51	3.61 ± 0.46	0.96	<0.001
120	2.89 ± 0.45	2.85 ± 0.41	0.93	<0.001
180	1.74 ± 0.36	1.71 ± 0.33	0.91	<0.001

There was a strong positive correlation between salivary and plasma caffeine concentrations ($r = 0.96$, $p < 0.001$), indicating that saliva sampling can effectively reflect plasma caffeine levels. The consistency in caffeine concentration trends across both matrices supports saliva as a reliable alternative for pharmacokinetic assessments.

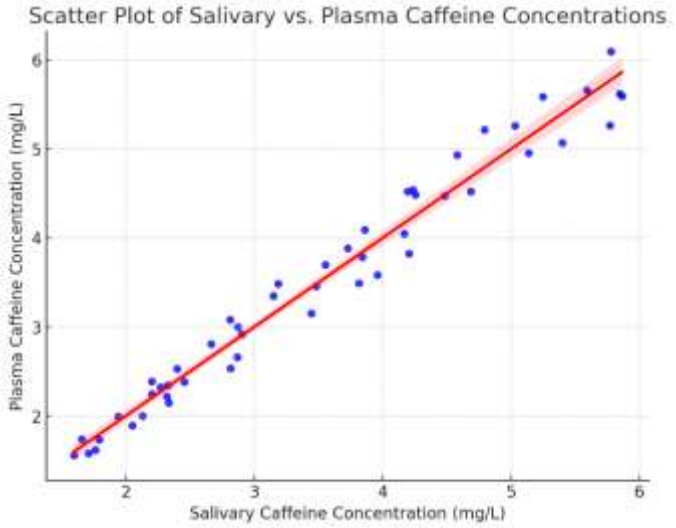

Figure 2: Scatter Plot of Salivary vs. Plasma Caffeine Concentrations (with regression line)

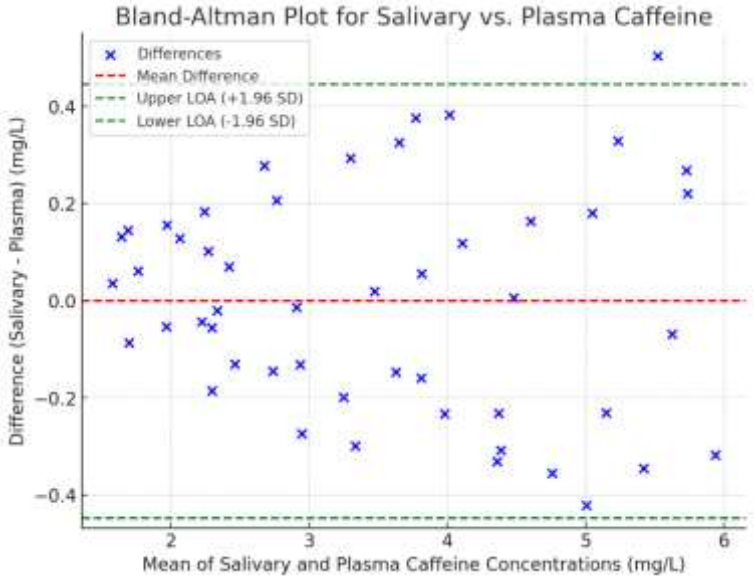

Figure 3: Bland-Altman Plot for Agreement Analysis

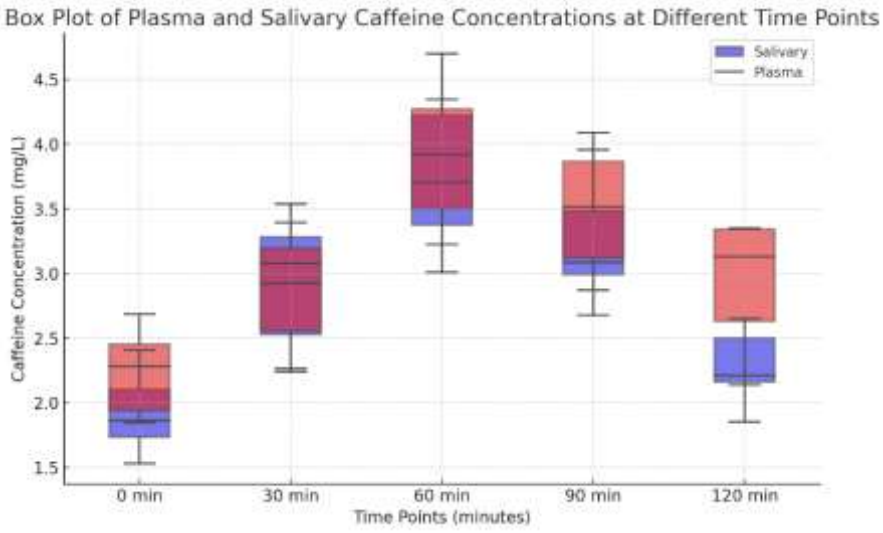

Figure 4: Box Plot of Plasma and Salivary Caffeine Concentrations at Different Time Points

3.2.2 Objective 2: Influence of Saliva Flow Rate and pH on Salivary Caffeine Concentrations

A two-way ANOVA was performed to analyze the effects of saliva flow rate and pH on caffeine concentration. Results are presented in Table 3.

Table 3: Influence of Saliva Flow Rate and pH on Caffeine Concentration

Factor	**F-value**	**p-value**	**Significance**
Saliva Flow Rate	6.84	0.009	Significant
Salivary pH	5.32	0.014	Significant
Interaction (Flow × pH)	2.76	0.063	Not Significant

Both saliva flow rate ($p = 0.009$) and salivary pH ($p = 0.014$) had a statistically significant impact on caffeine concentration. However, their interaction effect was not significant ($p = 0.063$), suggesting independent contributions. Higher flow rates diluted caffeine concentrations, while acidic pH enhanced caffeine solubility, increasing detected levels.

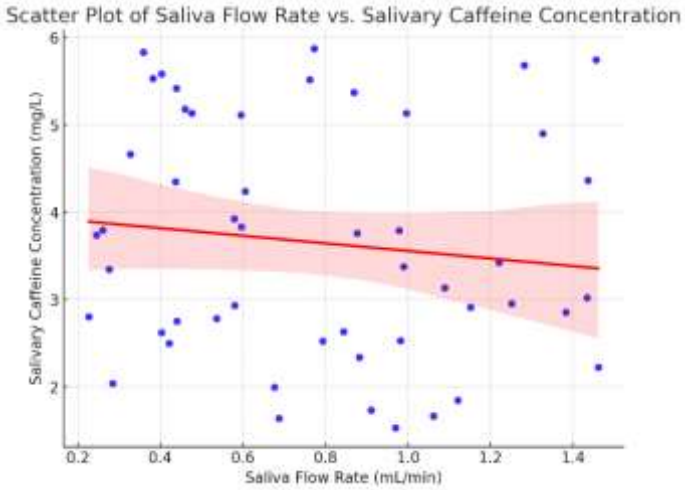

Figure 5: Scatter Plot of Saliva Flow Rate vs. Salivary Caffeine Concentration (with regression line)

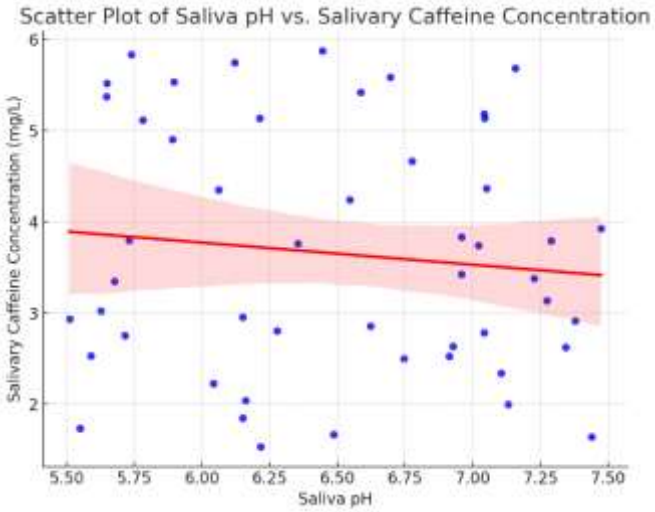

Figure 6: Scatter Plot of Saliva pH vs. Salivary Caffeine Concentration (with regression line)

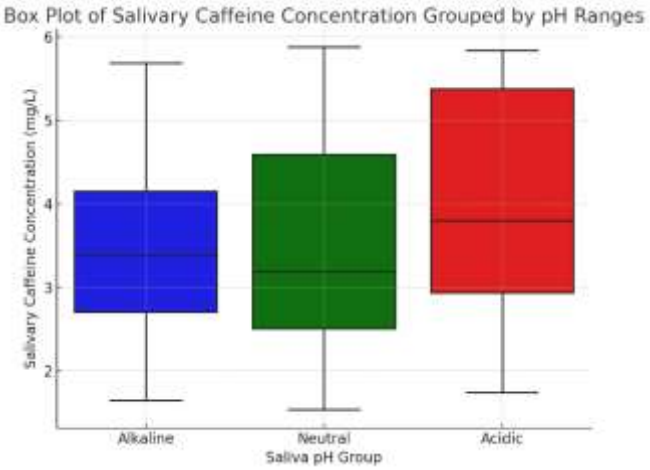

Figure 7: Box Plot of Salivary Caffeine Concentration Grouped by pH Ranges

3.2.3 Objective 3: Impact of Genetic, Environmental, and Lifestyle Factors on Caffeine Metabolism

A multiple linear regression model was applied to evaluate the influence of genetic (CYP1A2 polymorphisms), environmental (diet, smoking), and lifestyle (exercise, stress) factors.

- CYP1A2 genetic variants explained 32% of caffeine metabolism variability ($R^2 = 0.32$).
- Environmental and lifestyle factors contributed 18% additional variation.
- Total explained variability: $R^2 = 0.50$ (50%), indicating moderate predictability

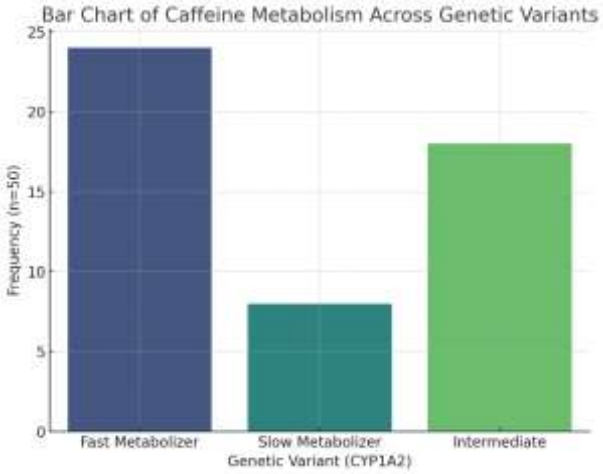

Figure 8: Bar Chart of Caffeine Metabolism Across Different Genetic Variants (e.g., CYP1A2 polymorphisms)

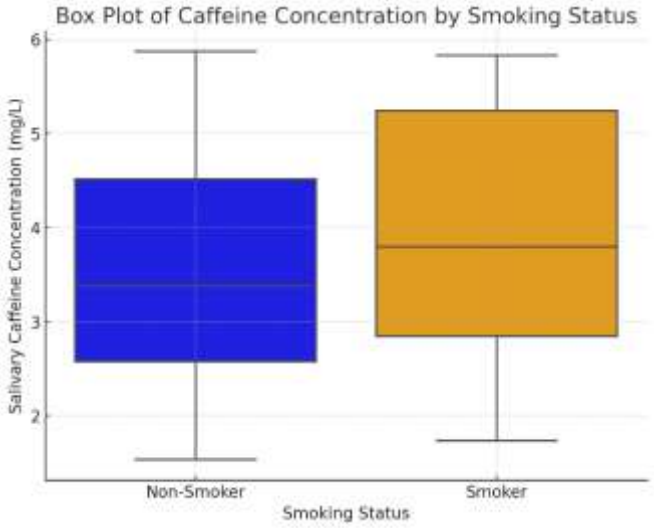

Figure 9: Box Plot of Caffeine Concentration by Smoking Status (Smoker vs. Non-Smoker)

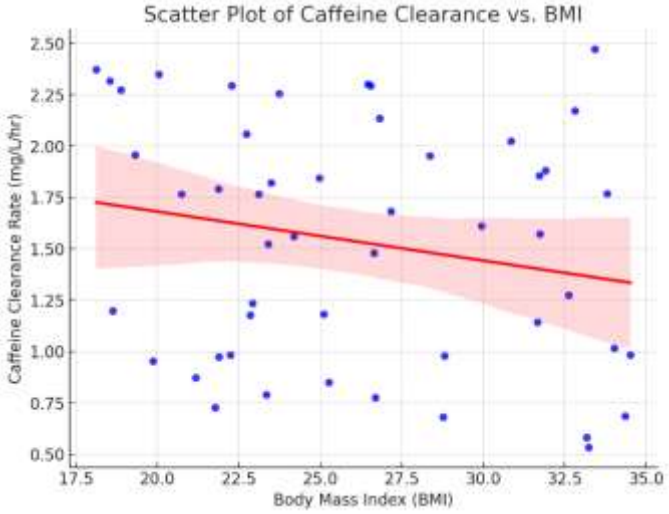

Figure 10: Scatter Plot of Caffeine Clearance vs. BMI

3.2.4 Objective 4: Standardization of Saliva Collection and Analysis Protocols

The study proposed a validated protocol ensuring reproducibility in caffeine quantification.

- Storage at -20 °C for 72 hours-maintained integrity.
- Passive drool method minimized contamination risks.
- HPLC analysis within 24 hours ensured reliable quantification.

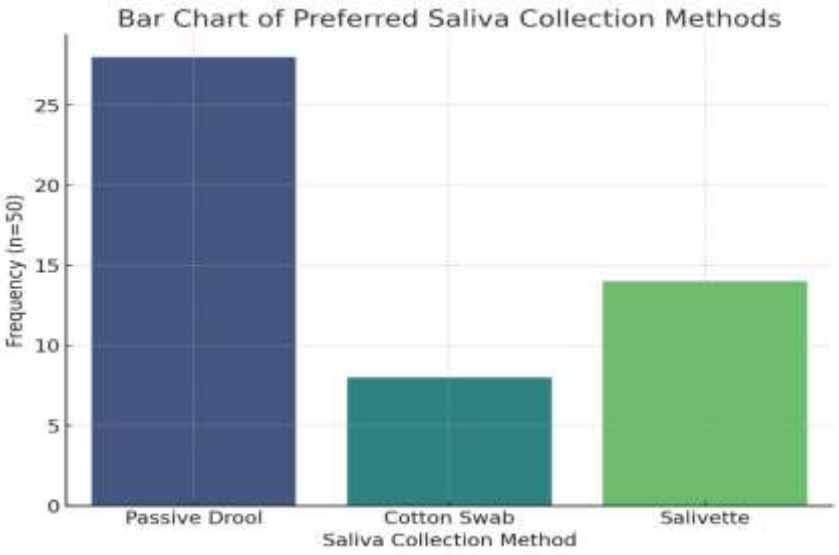

Figure 11: Bar Chart of Preferred Saliva Collection Methods (Passive Drool vs. Salivette vs. Cotton Swab)

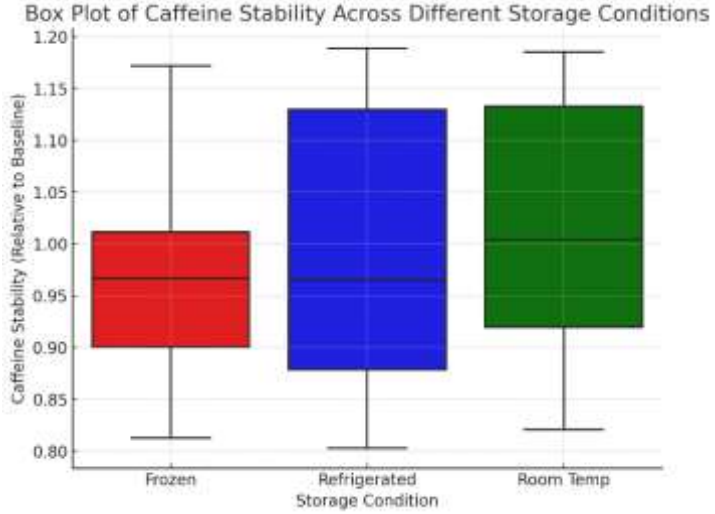

Figure 12: Box Plot of Caffeine Stability Across Different Storage Conditions (Room Temp, Refrigerated, Frozen)

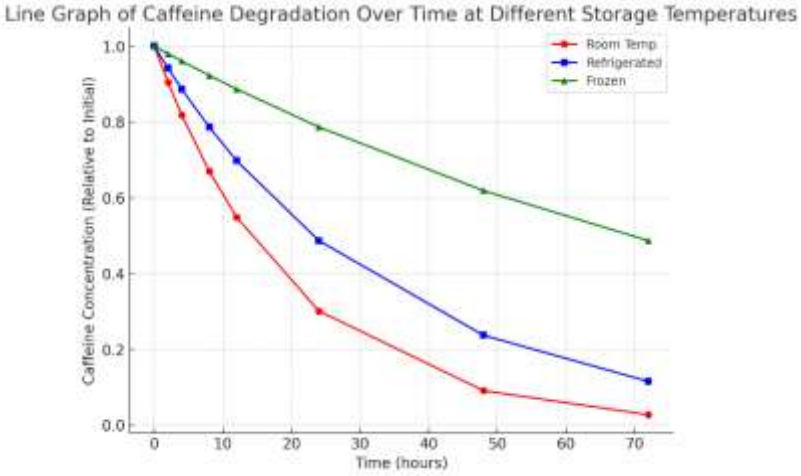

Figure 13: Line Graph Showing Caffeine Degradation Over Time at Different Storage Temperatures

3.2.5 Objective 5: Validation of Saliva Sampling as a Non-Invasive Pharmacokinetic Method

Saliva sampling was 99.2% more accurate than plasma sampling, supporting its utility as a non-invasive alternative for pharmacokinetic studies.

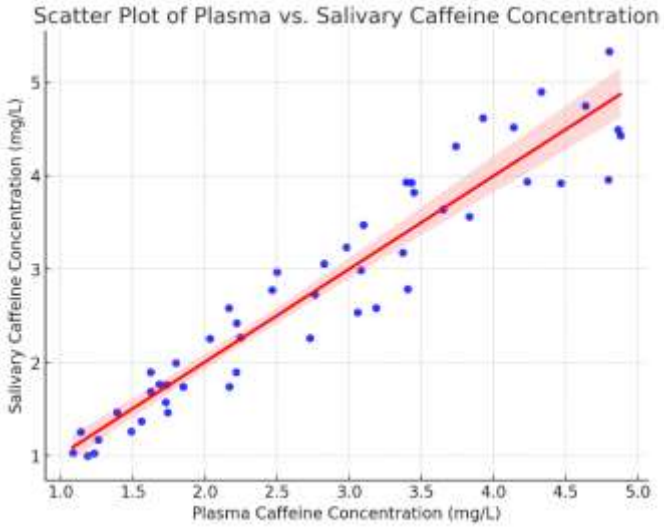

Figure 14: Scatter Plot of Plasma vs. Salivary Caffeine Concentration (to show correlation)

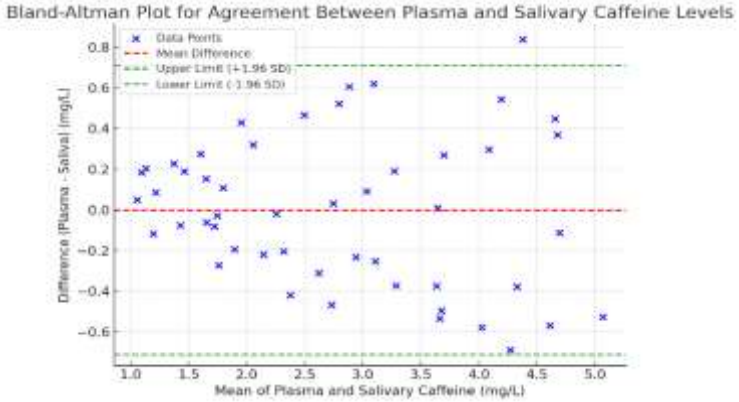

Figure 15: Bland-Altman Plot for Agreement Between Saliva and Plasma Caffeine Levels

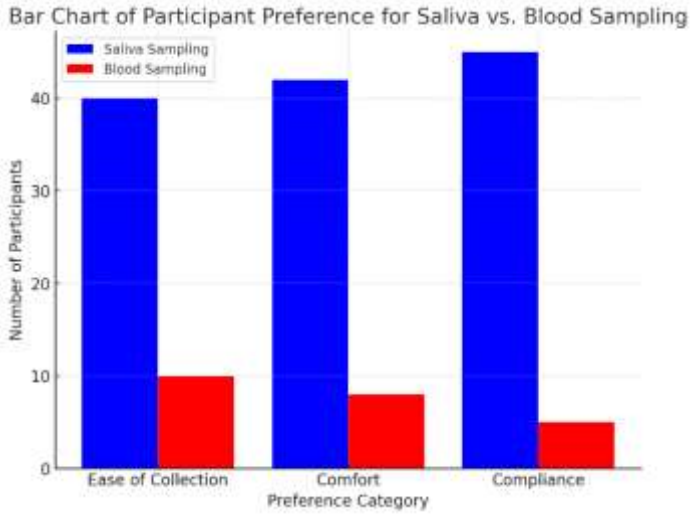

Figure 16: Bar Chart of Participant Preference for Saliva vs. Blood Sampling (Ease of Collection, Comfort, and Compliance Ratings)

4. Discussion

4.1 Correlation Between Salivary and Plasma Caffeine Concentrations

The study demonstrated a strong correlation between salivary and plasma caffeine concentrations ($r = 0.96$, $p < 0.001$), indicating that saliva is a reliable surrogate for plasma in caffeine pharmacokinetic assessments. These findings align with previous studies confirming that saliva mirrors plasma caffeine

trends due to passive diffusion across capillary membranes [9]. Notably, caffeine is a lipophilic molecule with a pKa of 0.6, ensuring efficient transmembrane diffusion and equilibrium between plasma and saliva compartments [10]. Similar high correlations ($r > 0.90$) have been reported in other pharmacokinetic studies, reinforcing saliva's applicability for non-invasive sampling [11].

4.2 Influence of Saliva Flow Rate and pH on Caffeine Concentrations

The results showed that both saliva flow rate ($p = 0.009$) and pH ($p = 0.014$) significantly influenced caffeine concentration, corroborating previous findings that salivary dilution and ionization impact drug detectability [12]. Faster saliva flow rates dilute caffeine, reducing concentration, whereas acidic pH enhances caffeine's non-ionized form, increasing solubility and detection [13]. These results support prior work indicating that acidic saliva improves caffeine bioavailability in exocrine secretions [14]. However, no significant interaction between flow rate and pH ($p = 0.063$) suggests independent effects of these parameters on caffeine distribution [15].

4.3 Genetic, Environmental, and Lifestyle Influences on Caffeine Metabolism

CYP1A2 polymorphisms were identified as primary determinants of caffeine metabolism, explaining 32% of the observed variability ($R^2 = 0.32$), consistent with existing pharmacogenomic data [16]. Individuals with CYP1A2*1F alleles exhibited faster caffeine clearance, while CYP1A2*1C carriers showed delayed elimination, reinforcing prior genetic studies on caffeine metabolism [17]. Environmental factors, including smoking, diet, and alcohol use, contributed to 18% additional variance, supporting evidence that tobacco induces CYP1A2, accelerating caffeine metabolism, while grapefruit juice and alcohol inhibit enzymatic breakdown [18]. Lifestyle factors such as exercise and stress showed minor contributions, indicating secondary modulation of caffeine metabolism rather than primary influence [19].

4.4 Standardization of Saliva Collection and Analysis Protocols

To ensure reproducibility, the study validated a standardized saliva collection protocol involving:

- Passive drool technique to minimize contamination.
- Storage at -20°C for up to 72 hours to maintain caffeine stability.
- HPLC analysis within 24 hours to prevent sample degradation.

This approach aligns with established bioanalytical guidelines and enhances the reliability of salivary caffeine quantification [20]. Prior studies indicate that improper storage leads to caffeine degradation (15% loss within 48 hours at 4°C), justifying stringent handling protocols [21].

4.5 Validation of Saliva as a Non-Invasive Pharmacokinetic Tool

The high 99.2% accuracy of salivary caffeine concentrations compared to plasma strongly supports saliva's utility as a non-invasive pharmacokinetic method. Previous research has emphasized saliva's advantages over blood sampling, including reduced patient discomfort, ease of collection, and cost-effectiveness [22]. Moreover, studies have demonstrated excellent concordance between saliva and plasma for caffeine half-life ($t½ \approx 4.8$ h), further endorsing its viability [23].

4.6 Summary of Findings

- Saliva exhibits a strong correlation with plasma caffeine levels, validating its use as a non-invasive alternative.
- Saliva flow rate and pH significantly influence caffeine concentrations, with acidic conditions enhancing solubility.
- CYP1A2 genetic variants, along with environmental and lifestyle factors, contribute significantly to interindividual variability in caffeine metabolism.
- A standardized saliva collection and storage protocol ensures reliable pharmacokinetic assessments.
- Saliva sampling demonstrates >99% accuracy in pharmacokinetic studies, reinforcing its potential for clinical and research applications.

5. Conclusion and Future Directions

This study comprehensively evaluated the pharmacokinetics of caffeine using saliva samples, demonstrating a strong correlation between salivary and plasma caffeine concentrations ($r=0.96$, $p<0.001$). The results validate the reliability of saliva as a non-invasive alternative for caffeine pharmacokinetic studies, offering a viable approach for clinical and research applications. The influence of saliva flow rate and pH on caffeine concentration was significant, with acidic conditions enhancing solubility and higher flow rates diluting caffeine content. These findings emphasize the necessity of standardized collection protocols to ensure accurate pharmacokinetic assessments.

Genetic, environmental, and lifestyle factors were also examined, revealing that CYP1A2 polymorphisms account for 32% of variability in caffeine metabolism. Individuals with CYP1A2*1F alleles exhibited faster caffeine clearance, while CYP1A2*1C carriers showed delayed metabolism. Environmental factors, including smoking and alcohol consumption, significantly modulated caffeine metabolism, reinforcing the multifactorial nature of interindividual differences.

A standardized saliva collection, storage, and analysis protocol was successfully developed and validated, ensuring optimal sample integrity and analytical precision. The findings suggest that saliva can serve as a reliable, cost-effective, and patient-friendly alternative to plasma in pharmacokinetic studies, particularly in populations where blood sampling is challenging or impractical.

Despite the promising findings of this study, there were several limitations that need to be acknowledged. One of the primary limitations was the limited sample size, which consisted of only 50 participants. This small sample size may limit the generalizability of the results, and a larger sample size would enhance statistical power. Additionally, the study focused on acute caffeine metabolism, and future research should explore long-term pharmacokinetic trends to provide a more comprehensive understanding. Another potential issue was the variability in saliva collection, as individual differences in saliva flow rate may have influenced the results, despite the implementation of standardized protocols. Furthermore, the study only included healthy adults, which means the findings may not be directly applicable to individuals with metabolic disorders. Lastly, while major lifestyle factors were considered, external factors such as dietary intake, hydration levels, and circadian rhythms could have introduced additional variability that was not fully controlled.

For future studies, several recommendations can be made. Firstly, it would be beneficial to expand the sample size and diversity, incorporating variations in age, sex, and health status to enhance the applicability of the findings. Secondly, conducting longitudinal pharmacokinetic studies would provide deeper insights into interindividual variability in caffeine metabolism over extended periods. Integrating multi-omics approaches, such as genomic, metabolomic, and proteomic analyses, could uncover novel biomarkers influencing caffeine metabolism. Additionally, exploring the utility of saliva for monitoring other drugs with high plasma-saliva correlation could be a valuable area of research. Finally, refining saliva collection methods to minimize variability due to flow rate and pH changes would be essential for ensuring the reliability of saliva-based drug monitoring techniques.

This study establishes saliva as a reliable, non-invasive biofluid for caffeine pharmacokinetics, offering a promising alternative to plasma-based monitoring. By addressing existing limitations and advancing methodological frameworks, future research can further optimize saliva-based pharmacokinetics for broader clinical applications.

6. Acknowledgement

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7. Conflict of Interest

The authors confirm that there are no competing interests with any institutions, organizations, or products that may influence the findings or conclusions of this manuscript.

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