Cumhuriyet Sci. J., 44(3) (2023) 485-490
DOI: https://doi.org/10.17776/csj.1319590

Cumhuriyet Science Journal

| csj.cumhuriyet.edu.tr | Founded: 2002 ISSN: 2587-2680 e-ISSN: 2587-246X Publisher: Sivas Cumhuriyet University

Determination of Caffeine in Human Plasma by Using Liquid Chromatography-Tandem Mass Spectrometry

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Research Article

History
Received: 24/06/2023
Accepted: 28/08/2023

ABSTRACT

A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated to determine caffeine (CAF) in human plasma. The plasma samples were extracted by protein precipitation using CAF-D3 as an internal standard (IS). The chromatographic separation was performed on GL Sciences InertSustain C18 Column (4.6 x 50 mm, 5 µm) maintained at 40 °C with a mobile phase consisting of formic acid, water, and methanol at a 1 mL/min of flow rate using two separate lines. CAF was detected and identified by mass spectrometry with electrospray ionization (ESI) in positive ions and multiple-reaction monitoring (MRM) mode. The MRM transitions of m/z 195.10 > 138.00 for CAF and 198.10 > 141.10 for IS were used for quantification. The standard curve was linear in the range of 10 - 10000 ng/mL for CAF. The within-batch precision and accuracy were evaluated by analyzing QC samples at five different concentration levels with six replicates in a batch. The between-batch precision and accuracy were determined by analyzing three different batches. The within-batch accuracy and precision was -8.76% - 9.61% and 0.95% - 7.22%, respectively. The between-batch accuracy and precision was -7.47% -1.42% and 1.83% - 8.66%, respectively. The results of the intra- and inter-day precision and accuracy studies were within the limits. The validated method applied to a pharmacokinetic study and the test product containing 60 mg CAF administered to total of 12 subjects. The mean ± SD of maximum plasma concentration (Cmax) was found to be 147.94 ± 139.39 ng/mL and the mean ± SD of area under the plasma concentration-time curve from zero to last measurable concentration (AUC0-tlast) was found to be 1119.59 ± 1468.30 h.ng/mL for the fasting conditions. The median time to reach peak plasma concentration (Tmax) was found to be 12.00 (6.50 - 12.00). The developed and validated method can be used for bioavailability and bioequivalence studies in human plasma samples..

Keywords: Caffeine, Caffeine-D3, human plasma, LC-MS/MS

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Introduction

CAF is a naturally occurring stimulant found in coffee, tea, and various other plants. It is a central nervous system stimulant that can increase alertness and reduce fatigue. CAF works by blocking the action of adenosine, a neurotransmitter that normally slows down brain activity and causes drowsiness. By blocking adenosine, CAF allows other neurotransmitters like dopamine and norepinephrine to become more active, leading to increased alertness and a feeling of wakefulness. It is also found in some medications, such as pain relievers and cold and allergy medications. The amount of CAF in different products can vary widely, with coffee and tea generally containing higher amounts than soft drinks and energy drinks. While CAF can have some benefits, such as increasing alertness and improving cognitive performance, it can also have negative effects, such as interfering with sleep, causing anxiety or jitters, and leading to addiction or dependence. It is important to consume CAF in moderation and to be aware of its potential risks and benefits [1-2].

The chemical formula of CAF is 1,3,7-Trimethylpurine-2,6-dione. The empirical formula is $C_8H_{10}N_4O_2$ and its molecular weight is 194.19 [3]. Due to its high selectivity and sensitivity, a bioanalytical method based on liquid chromatography and tandem mass spectrometry (LC-MS/MS) was used to determine the amount of CAF in human plasma. In several published reports, CAF has been successfully separated using diode array detection HPLC combined with electrospray tandem mass spectrometry (HPLC-DAD/ESI-MS/MS) as well as HPLC and UFLC-Q-TOF-MS/MS [4-8]. However, HPLC techniques have certain limitations such as lower specificity, selectivity, and longer analysis times. Alvi et al. (2011) described a method for determining CAF levels in human plasma, but the chromatographic analysis time was quite long (10 min), making it unsuitable for high-throughput analysis. Thus, our aim was to develop a sensitive and specific LC-MS/MS method that employed simple protein precipitation and a short 3-min run time for the quantitative determination of CAF in human

plasma. The validated method was successfully applied to CAF pharmacokinetic studies.

Experimental

Chemicals and Materials

CAF (purity 99.8%) was supplied by Siegfried Pharma Chemikalien (Minden, Germany). CAF -D3, the internal standard (IS), was obtained from Clearsynth (Mumbai, India). Formic acid, methanol, and ethanol were purchased from Merck (Darmstadt, Germany). The ethylene diamine tetra acetic acid (K_2EDTA) blank human plasma was obtained from Bioivt Laboratories International Ltd. (UK). The ultrapure water was produced in-house using Millipore's (USA) Milli-Q water purification system.

Stock Solutions, Calibration Standards and QCs

Stock solutions of CAF (1 mg/mL) in methanol were diluted to make working solutions (0.2 - 200 µg/mL).The internal standard working solution was prepared at a concentration of 1 µg/mL. Stock solutions of CAF and IS were stored at -20 °C. Calibration standards (10 – 10000 ng/mL) and QC samples (10, 30, 300, 4000, and 8000 ng/mL) were prepared in human blank plasma. All calibration standards and QC samples were stored at -70 °C until analysis.

Instrument

The system of LC-MS/MS (Shimadzu, Japan) consists of LC-20AD XR solvent pumps, SIL-20AC XR autosampler, CTO-10AS VP column oven, and Shimadzu 8040 Tandem Mass Spectrometer. Lab Solutions Version 5.93 was used to acquire and evaluate chromatographic data. Separations were carried out on GL Sciences InertSustain C18 Column (50 x 4.6 mm, 5 µm) at 40 °C. The mobile phase consisted of methanol, water and formic acid (30:70:0.1 *v/v/v*) with a flow rate of 1 mL/min. The run time was 3.0 minutes. A 20 µL sample was injected into the LC-MS/MS system for analysis.

The multiple reaction monitoring (MRM) transitions were carried out at *m/z* 195.10 > 138.0 for CAF and *m/z* 198.10 → 141.10 for IS. Mass spectrometric detection was performed using an ESI ion source operating in the positive ionization mode. The nebulizing gas flow rate, drying gas flow rate, and ESI voltage were set to 2.5 L/min, 15 L/min, and 4500 V, respectively. The nebulizing and drying gases used were high-purity nitrogen generated by the Peak Scientific NL-60 system. MS data acquisition was conducted in the MRM mode in order to quantify and identify the target analytes. MS parameters are presented in Table 1.

Table 1. MS parameters for CAF and CAF-D3.

Compound	Precursor	Product	Dwell Time	Q1 pre-bias (V)	Collision energy (V)	Q3 pre-bias (V)
CAF	195.1	138.0	100.0	-13.0	-20.0	-22.0
IS	198.1	141.1	100.0	-13.0	-28.0	-21.0

Sample Preparation

Prior to analysis, the plasma sample was allowed to thaw to room temperature. Next, aliquots of 100 µL plasma samples and 50 µL of IS (1 µg/mL) was added into a 10 mL centrifuge tube and vortexed for 5 s. 300 µL of methanol was added to the tube to precipitate proteins and then vortex for 30 s. The samples were centrifuged at 5500 rpm for 10 min. After centrifugation, 20 µL of the supernatant was injected into the LC-MS/MS system.

Result and Discussion

Method Validation

The method was validated according to US-FDA Guidance for Industry: Bioanalytical Method Validation [9] with respect to selectivity, linearity, accuracy and precision, matrix effect, recovery, carry over, dilution integrity, batch size, and stabilities. Method validation was conducted using K_2EDTA human plasma as the sample matrix.

Selectivity

Eight different sources of human blank plasma (including haemolysed and hyperlipidaemic plasma) were subjected to a selectivity test. The selectivity assessment revealed that the presence of an interfering peak accounted for less than 20% of the response at the same retention time as the analyte's lower limit of quantification (LLOQ). Figure 1 shows chromatograms of (a) blank plasma spiked with IS and analytes at LLOQ, and (b) drug-free human plasma.

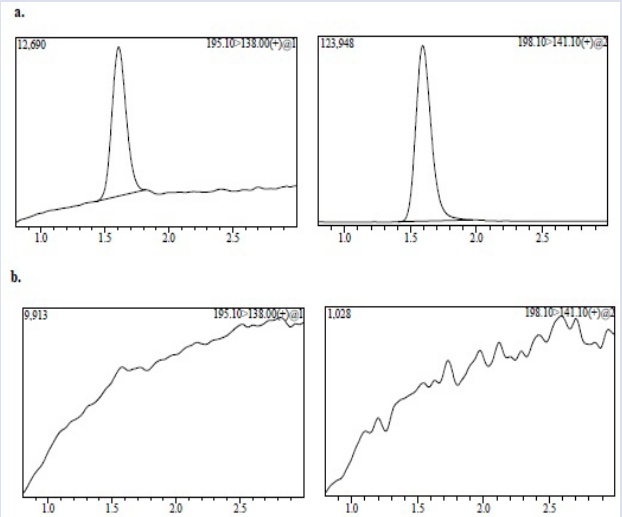

Figure 1. MRM chromatogram of 10 ng/mL (LLOQ) of CAF spiked with internal standard (a), blank human plasma (b).

Linearity

In the concentration range of 10 to 10000 ng / mL, including the LLOQ, the method's linearity was demonstrated for CAF. Eight freshly prepared calibration standards for CAF (10, 20, 100, 500, 2500, 5000, 9000, and 10000 ng/mL) were assayed in each of the three validation batches. The best fit based on accuracy was found using a linear equation with $1/C^2$ weighting. The analyte's average determination coefficients (r^2) were 0.9987 or higher (x = 0.00398556a + 0.000103298). The standard curves covered the quantitation range and were made up of eight non-zero samples, a blank sample, and an IS sample that had been spiked with zero. RE of calculated concentrations from the nominal values should be within ± 15% (LLOQ: ± 20%). At least 75% of the calculated concentrations of the calibration curve should fulfil the acceptance criteria (at least 6 non-zero samples, including the LLOQ and the ULOQ) [9, 10].

Accuracy and precision

The within-batch and between-batch precision and accuracy were assessed by analysing QC samples at five different concentration levels: 10 ng/mL (LLOQ), 30 ng/mL (QC Low), 300 ng/mL (QC Medium), 4000 ng/mL (QC High), 8000 ng/mL (ULLOQ), with six replicates in three consecutive validation runs. The acceptance criteria were defined for within batch and between batch precision as CV ≤ 15 % (20% for LLOQ), for within-batch accuracy as RE of calculated concentrations from the nominal values must be within ±15% (±20% for LLOQ), and two third of the QC samples at each concentration must fulfil the acceptance criteria. Additionally, RE of the mean concentration from the nominal value must be within the ±15% range (±20% for LLOQ). The within-batch and between-batch values did not exceed 15% for QC samples, as expected for LLOQ which did not exceed 20%. Table 2 and Table 3 provide a summary of the method's within- and between-batch precision and accuracy data. Based on the analyte to IS peak-area ratios, the regression algorithm was $1/C^2$ weighting linear regression. Shimadzu LabSolutions version 5.93 Software program was used for data acquisition and evaluation of chromatographic data. Results in detail are given in Table 2 - 3 and the results demonstrate that the acceptance criteria were met.

Table 2. Within-batch precision and accuracy of the method for determining CAF in plasma samples.

Nominal Concentration (ng/mL)	Batch No. 1 (n = 6)			Batch No. 2 (n=6)			Batch No. 3 (n=6)		
	Actual Concentration (mean ± SD; ng/mL)	RD (%)	CV (%)	Actual Concentration (mean ± SD; ng/mL)	RD (%)	CV (%)	Actual Concentration (mean ± SD; ng/mL)	RD (%)	CV (%)
10	9.65 ± 0.70	-3.45	7.22	9.81 ± 0.67	-1.89	6.84	10.96 ± 0.67	9.61	6.19
30	28.24 ± 0.37	-5.87	1.30	28.11 ± 0.70	-6.31	2.49	29.05 ± 0.28	-3.17	0.95
300	277.24 ± 6.05	-7.59	2.18	281.84 ± 6.17	-6.05	2.19	273.72 ± 4.69	-8.76	1.71
4000	3770.95 ± 59.66	-5.73	1.58	3932.88 ± 76.32	-1.68	1.94	3791.00 ± 77.59	-5.23	2.05
8000	7832.89 ± 186.67	-2.09	2.38	7960.40 ± 87.25	-0.50	1.10	7783.05 ± 86.20	-2.71	1.11

Table 3. Between-batch precision and accuracy of the method for determining CAF in plasma samples.

Nominal Concentration (ng/mL)	Batch No. 1-3 (n = 18)		
	Actual Concentration (mean ± SD; ng/mL)	RD (%)	CV (%)
10	10.14 ± 0.88	1.42	8.66
30	28.47 ± 0.62	-5.12	2.20
300	277.60 ± 6.34	-7.47	2.28
4000	3831.61 ± 100.16	-4.21	2.61
8000	7858.78 ± 143.45	-1.77	1.82

For between-batch accuracy, calculated mean concentration from three different runs must be in the ±15% range (±20% for LLOQ) of the nominal value.

Matrix Effect

For the matrix effect, blank plasma samples were taken from six different human plasma sources, including one lipemic and one haemolytic plasma. For both the analyte and the internal standard, the matrix factor (MF) in each matrix was calculated by determining the ratio of the peak area in the presence of the matrix (measured by analysis of the matrix blank spiked with the analyte at the concentration of QC low and QC high after extraction) to the peak area in absence of matrix (pure solution of the analyte). The normalized IS MF was calculated by dividing the analytes MF IS by the IS MF. The precision (CV%) of QC Low and QC High were 1.02% and 4.09% observed for CAF. The matrix effect result was summarized in Table 4. The accuracy should be within ±15% of the nominal concentration and the precision should not be greater than 15% in all individual matrix sources/lots [10]. Our findings show that, at both low and high concentrations of the analyte in all six lots of human plasma, there was no matrix effect. The matrix effect result was summarized in Table 4.

Table 4. Results of matrix effects for CAF (n = 6).

Sample	Plasma QC Low					Plasma QC High				
	Mean Peak Area (n = 6)	Matrix Factor	Mean Peak Area IS (n = 6)	IS Matrix Factor	IS Normalised MF	Mean Peak Area (n = 6)	Matrix Factor	Mean Peak Area IS (n = 6)	IS Matrix Factor	IS Normalised MF
Solution	99220.00	-	1081952.67	-	-	24168940.00	-	1081952.67	-	-
Matrix 1	108246.50	1.09	1180754.33	1.09	1.00	25915277.50	1.07	1180754.33	1.09	0.98
Matrix 2	105866.50	1.07	1188919.17	1.10	0.97	27440756.33	1.14	1188919.17	1.10	1.03
Matrix 3	106676.50	1.08	1179014.83	1.09	0.99	27974463.83	1.16	1179014.83	1.09	1.06
Matrix 4	103945.67	1.05	1152197.00	1.07	0.98	27981808.33	1.16	1152197.00	1.07	1.09
Matrix 5	103981.33	1.05	1149130.67	1.06	0.99	25555420.83	1.06	1149130.67	1.06	1.00
Matrix 6	100725.33	1.02	1102840.67	1.02	1.00	24540301.50	1.02	1102840.67	1.02	1.00

Recovery

The recovery of CAF was assessed by comparing the analyte responses of six extracted samples of low, medium, and high QC concentrations (30, 4,000, 8,000 ng/mL) with those of six appropriately diluted standard solutions. The mean total recovery of CAF was determined to be 93.99 %.

For IS, the mean responses of six internal diluted standard solutions and six extracted samples with medium QC concentrations (4000 ng/ mL) were compared. Internal standard recovery was found to be 99.44% on average.

Results are given in Table 5 and the results demonstrate that the acceptance criteria were met.

Acceptance criteria: The CV of the recovery results of the QC levels should be lower than ±15%.

Table 5. Recovery rates and calculated CV (3 concentrations: QC2, QC32, QC4) for precision (n = 6).

Concentration (ng/mL)	Mean (Ext/Unext) %	Recovery Rate (%)	SD	CV (%)
30.000	82.030332			
4000.000	100.373935	93.990340	10.365531	11.028294
8000.000	99.566754			
Internal Standard	99.440756			

Carryover

A high-quality control concentration sample (QC) and a blank sample after calibration at the upper limit of quantification (ULOQ) were used to evaluate carry-over in validation batches. Figure 2 shows that no carry-over was observed.

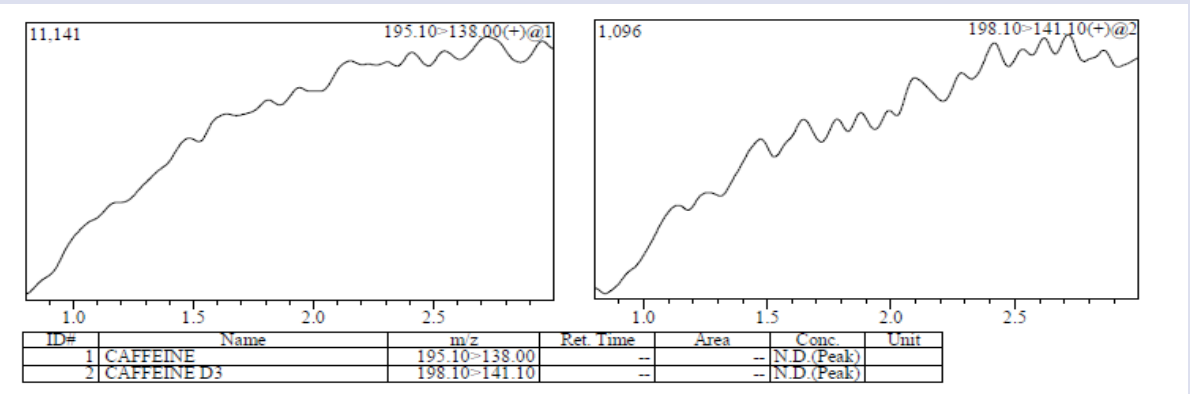

Figure 2. Visual presentation for the absence of carry-over effect for CAF.

Dilution integrity

The integrity of the dilution was confirmed by producing 1.7 times the ULOQ. This dilution sample was then diluted 1/2 and 1/20 with blank human plasma and tested with freshly spiked calibration standards. After accounting for dilution, the results were compared to the concentration. The mean values of diluted samples for the analyte CAF were within 15.0% of nominal value (0.758% for 1/2 dilution and -0.083% for 1/20 sample), and CV at each level was within ± 15.0% (1.464% for 1/2 and 1.320 % for 1/20 sample). Accordingly, the samples can be diluted 1/2 or 1/20 times to fit within the

calibration curve when their calculated concentration exceeds ULOQ.

Batch size

To determine the maximum batch size, the accuracy and precision of QC samples were tested over a run in a size equivalent to the expected analytical run. To mimic the actual size of a study run, blank samples were injected into the system between two validation batches. The QC samples were evaluated against the calibration curve obtained from the first validation batch. Based on this analysis, it was concluded that the maximum injection capacity of the instrument, which is 103, could be used as the batch size.

Stability

Stability evaluation in the matrix were made using freshly spiked calibration standards. The stability of QC samples at both low and high levels (n = 6, each) was evaluated by subjecting them to a freeze-thaw cycle. The samples were initially stored and frozen at -70 °C for 24 h, followed by unassisted thawing at room temperature. Once thawed, the samples were refrozen at -70 °C for a minimum of 12 h. This freeze-thaw cycle was repeated 4 times. After the 4th freeze-thaw cycle, the stability samples were processed with freshly prepared calibration standards and freshly prepared low and high QC samples. All samples were analysed in a single run. The results of the analysis indicated that CAF remains stable in human plasma for up to 4 freeze-thaw cycles when stored at -70 °C and thawed to room temperature.

The benchtop stability was assessed by maintaining CAF QC plasma samples at room temperature for 5 h at low and high QC values (n = 6 each). The QC samples were left on the bench at room temperature before extraction for 5 h. After 5 h, the stability samples were processed with freshly prepared calibration standards. All samples were analysed in a single run. Benchtop stability test results indicated that CAF in plasma remains stable for 5 hours at room temperature.

The processed sample stability was assessed by leaving sets of low and high QC samples in the autosampler for a longer period of time than required for processing a run of study samples. The samples were stored at autosampler conditions (10 °C) for 27 h and then injected with freshly prepared calibration standards and QC samples. CAF was stable in extracts.

During method validation, long-term plasma stabilities at both -20 °C and -70 °C was assessed for 7 days. The summarized stability results were presented in Table 6.

Table 6. Results of stability of CAF in human plasma under different storage conditions (n = 6).

Storage Condition	Nominal Concentration (ng/mL)	Actual Concentration (mean ± SD; ng/mL)	CV (%)	RD (%)
Autosampler stability a	30	29.40 ± 0.72	2.45	-1.98
	8000	7771.33 ± 94.20	1.21	-2.86
Short-term plasma stability b	30	29.04 ± 0.44	1.52	-3.19
	8000	7867.11 ± 130.81	1.66	-1.66
Validation long-term plasma stability c	30	27.53 ± 0.77	2.79	-8.23
	8000	7867.80 ± 109.22	1.38	-1.65
Validation long-term plasma stability d	30	29.09 ± 0.40	1.39	-3.05
	8000	7832.86 ± 58.23	0.74	-2.09
Freeze-thaw stability d	30	29.54 ± 0.82	2.76	-1.54
	8000	7765.21 ± 70.84	0.91	-2.94

RD: Relative Deviation (Accuracy), CV: Coefficient of Variation (Precision), SD: Standard Deviation

[a] Kept at autosampler temperature, 10 °C. [b] Stored at room temperature. [c] Stored at -20 °C. [d] Stored at -70 °C.

Application to a Pharmacokinetic Study

The successfully validated method was applied to a pharmacokinetic study. A total of 12 subjects completed the clinical phase of the study. Serial blood samples were collected throughout 16 h. The plasma samples obtained from 12 subjects following oral administration of a single dose of CAF 60 mg capsules under fasting conditions (the volunteers were fasted at least 2 hours) were analysed. The mean ± SD of maximum plasma concentration (C_{max}) for the fasting conditions was found to be 147.94 ± 139.39 ng/mL. The median (min-max) times to reach peak plasma concentration (T_{max}) for the fasting was found to be 12.00 (6.50 - 12.00). The mean ± SD of area under the plasma concentration-time curve from zero to last measurable concentration ($AUC_{0\text{-tlast}}$) for the fasting conditions was found to be 1119.59 ± 1468.30 h.ng/mL. When the clinical studies in the literature are evaluated, different results are striking both among other studies and with this study. The root cause of this is considered to be differences in study design, research products, and inter-subject variations [11].

Conclusion

We created and tested a positive ion mode LC-MS/MS method for simultaneously determining CAF in human plasma. Using deuterated internal standards ensured the success of the assay by eliminating the matrix effects. The parameters (selectivity, linearity,

lower limit of quantification, accuracy, precision, dilution integrity, carry-over, and recovery) were successfully validated. Further, the stability of the analyte was evaluated, as was the matrix effect. The developed method's adequate sensitivity, satisfactory selectivity, and good reproducibility were all confirmed. In order to achieve good extraction recovery without any obvious matrix effects, the protein precipitation method was developed. After an oral administration of CAF capsules, the validated method could be used to assess their pharmacokinetics and ascertain their bioavailability.

Conflicts of Interest

There are no conflicts of interest in this work.

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