Validated HPLC Method for Determination of Caffeine Level in Human Plasma using Synthetic Plasma: Application to Bioavailability Studies*

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Abstract

Several high-performance liquid chromatography (HPLC) methods have been described for the determination of caffeine in human plasma. However, none have been cross validated using synthetic plasma. The present study describes a simple and reliable HPLC method for the determination of the caffeine level in human plasma. Synthetic plasma was used to construct calibration curves and quality control samples to avoid interference by caffeine commonly present in donor's human plasma. After deproteination of plasma samples with perchloric acid, caffeine and antipyrine (internal standard, IS) were separated on a Waters Atlantis C18 column using a mobile phase of 15 mM potassium phosphate (pH 3.5) and acetonitrile (83:17, v/v), and monitored by photodiode array detector, with the wavelength set at 274 nm. The relationship between caffeine concentrations and peak area ratio (caffeine–IS) was linear over the range of 0.05–20 µg/mL. Inter-run coefficient of variation was ≤ 5.4% and ≤ 6.0% and bias was ≤ 3% and ≤ 7% using human and synthetic plasma, respectively. Mean extraction recovery from human plasma of caffeine and the IS was 91% and 86%, respectively. Caffeine in human plasma was stable for at least 24 h at room temperature or 12 weeks at –20°C, and after three freeze-thaw cycles. The method was successfully applied to monitor caffeine levels in healthy volunteers with correction of caffeine levels using the mean ratio of the slopes of the calibration's curves constructed using human and synthetic plasma.

Introduction

Caffeine (1, 3, 7-trimethylxanthine) is consumed in food and beverages throughout the world and widely used as a stimulant in humans (1–2). Peak plasma concentration of 15.9–18.7 µg/mL usually occurs 0.5 h after the ingestion of 500 mg of caffeine (3–4).

Caffeine levels in human plasma have been determined mainly by high-performance liquid chromatography (HPLC) following a clean-up procedure using solid-phase or liquid–liquid extraction (5–15). Because sample clean-up procedures are usually time-consuming and rate-limiting, protein precipitation with zinc sulfate or perchloric acid has been used for fast sample preparation and disruption of protein-drug binding (16–19). For the purpose of method validation, it is necessary to obtain drug free plasma for the preparation of calibration curves and quality control samples, which is extremely difficult in the case of caffeine. Abstinence for at least four times the elimination half life (i.e., 16–20 h) is necessary (3). Synthetic plasma has been constructed to store red blood cells and platelets (20).

In the present study, a reversed-phase (RP) HPLC method was cross validated using synthetic plasma, to determine caffeine levels in the therapeutic range in human plasma. The method was successfully applied to monitor caffeine levels in healthy adult volunteers. Further, the stability of caffeine was determined under various conditions generally, encountered in the clinical laboratory.

Experimental

Apparatus

Chromatography was performed on a Waters Alliance HPLC 2695 (Waters Associates, Inc., Milford, MA) consisting of a quaternary pump, autosampler, column thermostat, and photodiode array detector. A RP Atlantis C18 (4.6 × 150 mm, 5-µm) column and a guard pak pre-column module with a Bondapak C18, 4-µm insert were used for the separation. The data were collected with a Pentium IV computer using Empower Chromatography Manager Software (21).

Material and reagents

All reagents were of analytical grade unless stated otherwise. Caffeine (laboratory grade), acetonitrile, potassium phosphate monobasic, and phosphoric acid (70%) were purchased from Fisher Scientific, Fairlawn, NJ. Antipyrine, urea, and glucose were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Sodium, potassium, magnesium, and calcium chloride were purchased from BDH Chemicals, Pool, England. HPLC grade water was prepared by reverse osmosis and further purified

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by passing through a Synergy Water Purification System (Millipore, Bedford, MA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital and Research Center (KFSHRC) Riyadh, Saudi Arabia.

Chromatographic conditions

The mobile phase consisted of 15 mM potassium phosphate (pH 3.50 ± 0.05, adjusted with phosphoric acid) and acetonitrile (83:17, v/v). It was filtered through a 0.45-µm polytetrafuloroethylene (PTFE) filter (Waters Corporation) and sonicated for 5 min. The analysis was carried out under isocratic conditions using a flow rate of 1.0 mL/min. Chromatograms were recorded at 274 nm with a run time of 10 min.

Synthetic plasma preparation

The synthetic plasma consisted of sodium chloride (145 mM), potassium chloride (4.5 mM), calcium chloride (32.5 mM), magnesium chloride (0.8 mM), urea (2.5 mM), and glucose (4.7 mM) (20). It was stored at 4°C and used within two weeks.

Standard solutions and quality control samples

Caffeine stock solution was prepared in water (100 µg/mL) and stored at –20°C. Working solutions were prepared in drug-free human plasma or synthetic plasma (20 µg/mL). Nine calibration standards were prepared in human plasma or synthetic plasma in the range of 0.05 to 20 µg/mL. Antipyrine (IS) working solution was prepared in 30% (v/v) perchloric acid (40 µg/mL). Three quality control samples were prepared: low (0.15), middle (10), and high (18) µg/mL. The solutions were vortexed for one min then 0.5 mL aliquots were transferred into 1.5 mL eppendrof microcentrifuge tubes and stored at –20°C until used.

Plasma samples collection

The method was developed and validated for a clinical trial comparing the bioavailability of 300 mg caffeine presented as such or as placebo (clinical trials, gov, number NCT 00426010). The study was conducted in accordance with the Declaration of Helsinki and approved by the Research Ethics Committee of King Faisal Specialist Hospital & Research Centre, Riyadh Saudi Arabia. Blood samples were collected from healthy volunteers after abstaining from caffeine for 16 h and after a single oral administration of 300 mg caffeine. They were collected in heparinized polypropylene tubes and centrifuged for 10 min at 3500 rpm under a controlled temperature of 20°C. The plasma supernatant was carefully collected in polypropylene tubes and frozen at –20°C until analyzed (within 6 weeks from collection).

Samples preparation

Two hundred microliters of the IS working solution were added to 500 µL of the plasma samples, calibration standards, or quality control samples in a 1.5 mL microcentrifuge tubes. The tube was vortexed for 20 s then centrifuged at 12000 rpm for 10 min. The supernatant clear layer was collected and 100 µL were injected into the chromatographic system using an autosampler.

Method validation

The method was validated in both human and synthetic plasma according to the FDA guidelines for industrial bioanalytical method validation (22). The method involved cross validation which is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. The predetermined validation criteria included: specificity, accuracy, precision, linearity, and stability.

Results and Discussion

Optimization of chromatographic condition

Under optimal experimental conditions, a mobile phase of 15 mM potassium phosphate (pH 3.50) and acetonitrile (83:17, v/v), and a flow rate of 1.0 mL/min, caffeine, antipyrine (IS), and plasma components exhibited a well defined chromatographic separation (resolution factor more than 1.2) within a run time of 10 min. The retention times of caffeine and the IS were around 4.7 and 8.8 min, respectively. Figure 1 depicts chromatograms of synthetic and human plasma spiked with caffeine and the IS.

Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Potential interfering substances in plasma samples include endogenous components, metabolites, and decomposition products. Fourteen batches of blank plasma were screened to assess potential interferences with caffeine and the IS. There was no interference with the IS peak. However, only one batch of plasma was found to be free from caffeine. This batch was used for preparation of calibration standards and quality control samples for the current study. Seven commonly used drugs (acetaminophen, diclofenac sodium, omeprazole, nicotinic acid, ascorbic acid, ibuprofen, and ranitidine) were also screened for potential interference; none co-eluted with caffeine or the IS.

Limit of quantitation and linearity

The caffeine free plasma was used to determine the limit of quantitation (LOQ). The LOQ was defined as the lowest concentration on the calibration curve that can be determined with acceptable precision and accuracy (i.e., coefficient of variation

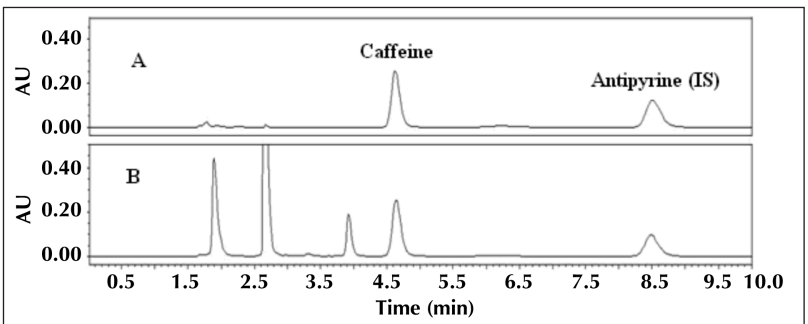

Figure 1. Representative chromatograms of synthetic (A) and human (B) plasma spiked with 10 µg/mL caffeine and 40 µg/mL antipyrine (IS).

and relative error < 20%). The LOQ of caffeine in human plasma was 0.05 µg/mL, whereas the lowest detection limit (LOD) was 0.02 µg/mL. The LOD and LOQ were also calculated using the equations LOD = N/B × 3 and LOQ = N/B × 5, where N is the noise [determined as SD of peak area ratio (n = 3) at caffeine concentration 0.05 µg/mL] and B is the slope of the calibration curve. The calculated LOD and LOQ ranged from 0.017 to 0.027 and 0.029 to 0.046, respectively.

Linearity of the analyte was evaluated by analyzing a series of standard concentrations over the range (0.05–20 µg/mL) in human and synthetic plasma. The peak area ratios were subjected to regression analysis. Data are given Table I. The mean equations were $y = 0.1516x - 0.0093$ and $y = 0.1712x + 0.004$, for human and synthetic plasma, respectively. The difference between the slopes (0.1516 in human plasma and 0.1712 in synthetic plasma) clearly indicates that LOQ in synthetic plasma is lower than in human plasma. We considered a value of 0.05 µg/mL as LOQ for both kinds of plasma, because the aim was to compare the responses under identical conditions. Figure 2 depicts an overlay of chromatograms of extracts of 0.5 mL human plasma spiked with the internal standard and one of nine concentrations of caffeine (in the range of 0.05–20.0 µg/mL).

Table I. Regression Analysis of 10 Caffeine Calibration Curves

Parameters / Matrix	Synthetic plasma	Human plasma
Number of samples per curve	9	9
Concentration range (µg/mL)	0.05–20	0.05–20
Regression equation*	$y = mx + c$	
Mean slope (SD)	0.1712 (0.004)	0.1516 (0.004)
Mean intercept (SD)	0.0041 (0.001)	–0.0093 (0.012)
Mean regression coefficient (SD)	0.9969 (0.003)	0.9987 (0.001)
Mean residual standard deviation (SD)	0.0410 (0.039)	0.0134 (0.004)
Limit of detection (µg/mL)	0.02	0.02
Limit of quantification (µg/mL)	0.05	0.05
Ratio of mean slope of calibration curves†	0.89	

* y = peak area ratio and x = concentration of caffeine.
† human/synthetic, 5 each.

Table II. Recovery of Caffeine and Antipyrine (IS) from 0.5 mL of Human Plasma

Concentration (µg/mL)	Human Plasma* (n = 5)	Mobile Phase* (n = 5)	Recovery (%)
Caffeine			
0.15	3.6 (0.75)	3.9 (0.06)	92
10	209.0 (2.48)	246.8 (0.77)	85
18	377.1 (4.71)	391.5 (1.97)	96
Internal Standard			
40	157.7 (1.49)	182.6 (0.82)	86

*Data are mean peak area divided by 10,000 (SD).

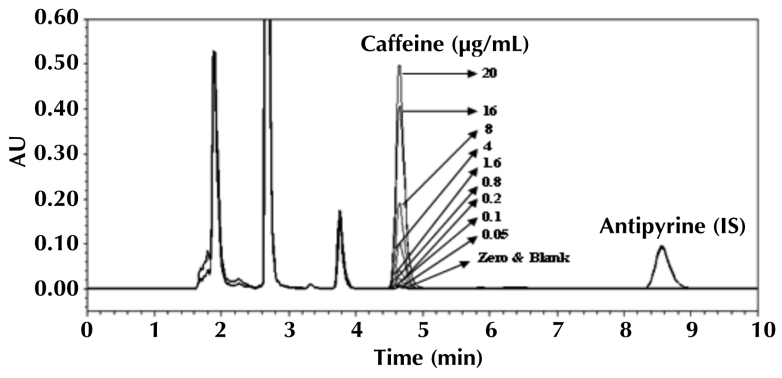

Figure 2. Overlay of chromatograms of extracts of 0.5 mL human plasma spiked with the internal standard (IS, 40 µg/mL) and one of nine concentrations of caffeine: 0.00, 0.05. 0.1, 0.2, 0.8, 1.6, 4.0, 8.0, 16.0, or 20.0 µg/mL.

Plasma sample preparation and recovery

Recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the plasma, compared to the detector response obtained for the true concentration. In order to study the recovery of caffeine and IS from plasma, we evaluated the effect of protein precipitation with different concentrations of perchloric acid (10–40%, v/v). The optimal extraction recovery was obtained with 30% (v/v) perchloric acid. To calculate absolute recovery of caffeine, 5 sets of samples (0.15, 10, and 18 µg/mL) were prepared in human plasma or in mobile phase and the peak areas were compared. Similarly, the recovery of the IS was determined by comparing the peak areas of the IS in 5 aliquots of human plasma spiked with 8 µg of the IS with the peak areas of equivalent samples prepared in mobile phase. The results are presented in Table II. Mean analytical recovery was 91% for caffeine and 86% for the IS. The relative recovery of caffeine from human plasma compared to synthetic plasma was calculated using the mean slopes of the curves constructed in human and synthetic plasma (23). A mean slope ratio of 0.89 was obtained.

Cross validation/intra-run and inter-run precision and accuracy

According to predetermined criteria (22), accuracy and precision were determined using five replicates for three concentrations in the expected range (0.15, 10, and 18 µg/mL). The results are presented in Table III. The maximum coefficients of variation and bias were 6.4% and 5.9%, and bias was 7% and 3%, in synthetic and human plasma, respectively. The results indicate that the method was reliable within the studied concentration range.

Stability studies

The stability of caffeine in plasma and processed samples, during the analysis and usual storage conditions were investigated. No significant decrease in the measured concentration or change in chromatographic pattern was observed. Stability data are summarized in Table IV.

Freeze and thaw stability

Stability of caffeine was determined over three freeze and thaw cycles. Fifteen aliquots of each of the two QC samples (0.15 and 18 µg/mL) were

stored at –20°C. After 24 h, all aliquots were left to thaw unassisted at room temperature. When completely thawed, 5 aliquots of each QC sample were analyzed. The other aliquots were returned to –20°C and kept for 24 h. The same procedure was repeated three times. The concentrations in the freeze-thaw samples were compared with the concentrations of freshly prepared and analyzed samples. The results showed that caffeine in plasma is resistant to at least three cycles of freeze and thaw (stability 95–102%).

Processed sample stability

Fifteen aliquots of each of the two QC samples (0.15 and 18 µg/mL) were processed. Five aliquots of each QC sample were analyzed immediately. The other aliquots were analyzed after being stored at room temperature for 24 h or at –20°C for 48 h. The results showed that caffeine is stable in processed samples for at least 24 h at room temperature (92–98%) and for at least 48 h at –20°C (92–103%).

Long term stability

Thirty five aliquots of each of the two QC samples (0.15 and 18 µg/mL) were prepared. Five aliquots of each QC sample were analyzed immediately. Five aliquots of each QC sample were allowed to stand on the bench-top for 24 h at room temperature before extraction. The results indicate that caffeine is stable in plasma for at least 24 h at room temperature (94–103%). Five aliquots of each QC sample were stored at –20°C for 1, 3, 6, 8, or 12 weeks before analysis. The results showed that caffeine in plasma is stable for at least 12 weeks at –20°C (98–102%). Caffeine in stock solution (0.1 mg/mL in water) was also stable for at least for 24 h at room temperature (102%) and for at least for 12 weeks at –20°C.

Table III. Intra- and Inter-run Bias and Precision of Caffeine Assay

Nominal Level*	Measured Level in Human Plasma*				Measured Level Synthetic Plasma*			
	Mean	SD	CV (%)	Bias (%)	Mean	SD	CV (%)	Bias (%)
Intra-run								
0.15	0.15	0.01	5.9	0	0.14	0.01	6.4	–7
10	10.14	0.39	3.9	1	10.07	0.39	3.7	1
18	18.69	0.59	3.2	3	18.58	0.59	3.2	3
Inter-run								
0.15	0.16	0.01	5.4	3	0.14	0.01	6.0	–7
10	10.22	0.36	3.5	2	10.15	0.36	3.6	2
18	18.48	0.62	3.4	3	18.38	0.62	3.4	2

* microgram/mL. SD, standard deviation; CV, standard deviation divided by mean measured concentration × 100; bias, measured level – nominal level divided by nominal level × 100.

Table IV. Stability of Caffeine Under Various Clinical Laboratory Conditions*

Nominal Level (µg/mL)	Stability (%)								
	Plasma samples†							Stock Solution‡	
	Unprocessed		Processed		Freeze -Thaw				
	24 h RT	12 weeks –20°C	24 h RT	48 h –20°C	Cycle 1	Cycle 2	Cycle 3	24 h RT	12 weeks –20°C
0.15	94	102	92	92	100	100	95	102	100
18.0	103	98	98	103	100	100	102		

* Stability (%) = mean measured concentration (n = 5) at the indicated time divided by mean measured concentration (n = 5) at baseline × 100.

† Spiked plasma samples were precipitated and analyzed immediately (baseline, data not shown), after 24 h at room temperature (24 h RT), after freezing at –20°C for 12 weeks (12 weeks –20°C), or after 1–3 cycles of freezing at –20°C and thawing at room temperature (freeze-thaw), or precipitated and then analyzed after storing for 24 h at room temperature (24 h RT) or 48 h at –20°C (48 h –20°C).

‡ Caffeine stock solution, 1 mg/mL in mobile phase.

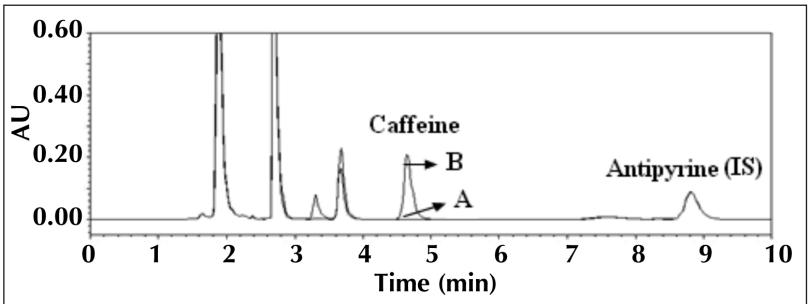

Figure 3. An overlay of chromatograms of plasma samples obtained from a healthy volunteer (who has abstained for 16 h from caffeine) before (A) and 3 h after (B) ingesting 300 mg caffeine.

Application to volunteer samples

Plasma samples from volunteers were processed according to the method and subjected to HPLC analysis. The concentration of caffeine was calculated from the synthetic plasma calibration curve using the standard equation: y = mx + c, where (y = peak area ratio; m = slope; and c = concentration) then corrected by multiplying it by the mean slopes ratio (human plasma/synthetic plasma) of 0.89. Mean (SD) caffeine level in 67 healthy volunteers was 0.28 (0.48) µg/mL after abstaining from caffeine containing food and beverages for 16 h, and increased to 10.58 (2.39) µg/mL, 3 h after the ingestion of a single dose of 300 mg caffeine. Figure 3 depicts an overlay chromatogram of samples collected before and 3 h after the intake of 300 mg caffeine by one volunteer.

Conclusion

The described method, combining the use of a simple plasma protein precipitation procedure and synthetic plasma, provides sensitive, accurate, and precise measurement of caffeine levels in human plasma in the therapeutic range.

Acknowledgements

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References

1. J.J. Barone, and H.R. Roberts. Caffeine consumption. *Food Chem. Toxicol.* **34:** 119–129 (1996).
2. B. Stavric, R. Klassen, B. Watkinson, J.K. Karpinski, R. Stapley, and P. Fried. Variability in caffeine consumption from coffee and tea: possible significance for epidemiological studies. *Food Chem. Toxicol.* **26:** 111–118 (1988).
3. G.B. Kaplan, D.J. Greenblatt, B.L. Ehrenberg, J.E. Goddard, M.M. Cotreau, J.S. Harmatz, and R.I. Shader. Dose-dependent pharmacokinetic and psychomotor effect of caffeine in humans. *J. Clin. Pharmacol.* **37:** 693–703 (1997).
4. J. Blanchard and S.J.A. Sawers. The absolute bioavailability of caffeine in man. *Euro. J. of Clin. Pharmacol.* **24:** 93–98 (1983).
5. I. Biaggioni, S. Paul, and D. Robertson, A simple liquid-chromatographic method applied to determine caffeine in plasma and tissues. *Clin. Chem.* **34:** 2345–2347 (1988).
6. K.D.R. Setchell, M.B. Welsh, M.J. Klooster, W.F. Balistreri, and C.K. Lim. Rapid high-performance liquid chromatography assay for salivary and serum caffeine following an oral load an indicator of liver function. *J. Chromatogr. A* **385:** 267–274 (1987).
7. A.H. Chalmers. HPLC determination of caffeine and theopyliine by direct serum injection. *Clinical Chemistry.* **39:** 1348–1349 (1993).
8. P. Fernandez, L. Morales, C. Vazquez, A.M. Bermejo, and M.J. Tabernero. HPLC-DAD determination of opioids, cocaine and their metabolites in plasma. *Forensic Science International.* **161:** 31–35 (2006).
9. A.W. Abu-Qare, and M.B. Abou-Donia. A validated HPLC method for the determination of pyridostigmine bromide, acetaminophen, acetylsalicylic acid and caffeine in rat plasma and urine. *J. Pharm. Biomed. Anal.* **26:** 939–947 (2001).
10. E. Tanaka. Simultaneous determination of caffeine and its primary demethylated metabolites in human plasma by high-performance liquid chromatography. *J. Chromatogr.* **575:** 311–314 (1992).
11. E.K. Bendriss, N. Markoglou, and I.W. Wainer. Liquid chromatographic method for the simultaneous determination of caffeine and fourteen caffeine metabolites. *J. Chromatogr. B Biomedical Sci. Appl.* **746:** 331–338 (2000).
12. T.E.B. Leakey. Simultaneous analysis of theophylline, caffeine and eight of their metabolic products in human plasma by gradient high-performance liquid chromatography. *J. Chromatogr.* **507:** 199–220 (1990).
13. A. Aldridge, J.V. Aranda, and A.H. Neims. Caffeine metabolism in the newborn. *Clin. Pharmacol. Ther.* **25:** 447 (1979).
14. P. Dobrocky, P.N. Benett, and L.J. Notaarianni. Rapid method for the routine determination of caffeine and its metabolites by high performance liquid chromatography. *J. Chromatogr. B* **652:** 104 (1994).
15. O.A. Ghosheh, D. Browne, T. Rogers, J. de Linda, L.P. Dwoskin, and P.A. Crooks. A simple high performance liquid chromatographic method for the quantification of total cotinine, total 3′-hydroxycotinine and caffeine in the plasma of smokers. *J. Pharm. Biomed. Anal.* **23:** 543–549 (2000).
16. H.N. Alkaysi, M.S. Salem, and Y.M. El-Sayed. High performance chromatographic analysis of caffeine concentration in plasma and saliva. *J. Clinical Pharm. Thera.* **13:** 109–115 (1988).
17. L. Richard, B. Leducq, C. Baty, and J. Jambou. Plasma determination of seven common drugs by high performance liquid chromatography. *Ann. Biol. Clin.* **47:** 79 (1989).
18. E. Schreiber-Deturmeny and B. Bruguerolle. Simultaneous high-performance liquid chromatographic determination of caffeine and theophylline for routine drug monitoring in human plasma. *J. Chromatogr. B* **677:** 305–312 (1996).
19. H.K. Gary, S.K. Chetan, R. Otterstetter, S. Cox Donna, J. B. Thomas, L.B. Gregory, and N.D. Eddington. The rate of absorption and relative bioavailability of caffeine administered in chewing gum versus capsules to normal healthy volunteers. *Int. J. Pharmaceutics* **234:** 159–167 (2002).
20. S. Holme, and W.A.L. Heaton. Synthetic, plasma-free, transfusible storage medium for red blood cells and platelets. *United State Patent* **5487971:** 1–25 (1996).
21. Empower Software, Waters Corporation, Milford, MA 01757, USA (2002).
22. Guidance for Industry "Bioanalytical Method Validation" US Department of Health Services, Food and Drug Administration, CDER, CVM, May (2001).
23. A. Laban-Djurdjevic, M. Jelikic-Stankov, and P. Djurdjevic. Optimization and validation of the direct HPLC method for the determination of moxifloxacin in plasma. *J. Chromatogr. B* **844:** 104–111 (2006).

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