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Dedicated to Professor Costel Sârbu on the Occasion of His 65th Anniversary

VALIDATED LC-MS/MS METHOD FOR THE CONCOMITANT DETERMINATION OF AMOXICILLIN AND CLAVULANIC ACID FROM HUMAN PLASMA

JÓZSEF BALÁZSI[a], CSABA PAIZS[b], FLORIN-DAN IRIMIE[b], MONICA IOANA TOŞA[b], LÁSZLÓ CSABA BENCZE[b], RÓBERT TŐTŐS[b*]

ABSTRACT. The purpose of this study was the development and validation of an LC-MS/MS method, for the concomitant and rapid determination amoxicillin and clavulanic acid from human plasma. The sample workup involved a simple protein precipitation procedure. A core/shell type analytical column (50×2,1 mm, 2.6 Å) was used with PFP stationary phase. A mobile phase with high aqueous composition provided satisfactory separation with good accuracy and precision (stable ionization). The mass spectrometer was operated in positive electrospray ionization mode for both analytes and internal standard. The following parameters were evaluated for validation purpose: Selectivity, sensitivity, matrix effect, anticoagulant effect, linearity, precision and accuracy, recovery, analyte/IS stability in solvent/matrix and carryover. The validated calibration range was 190-22222 ng/ml for amoxicillin, and 147-4908 ng/ml for clavulanic acid. The correlation coefficient R^2 was at least 0.99 for both analytes. The validated method has been successfully used for the evaluation bioequivalence of generic amoxicillin/potassium clavulanate formulations.

Keywords: *amoxicillin, clavulanic acid, method validation, bioequivalence trial, LC-MS/MS*

[a] *S.C. Kynetyx HT SRL, 34 Frunzișului street, RO-400664 Cluj-Napoca, Romania*
[b] *Babeş-Bolyai University, Faculty of Chemistry and Chemical Engineering, Biocatalysis and Biotransformation Research Centre,11 Arany János str., RO-400028, Cluj-Napoca, Romania*
** Corresponding author: totosr@chem.ubbcluj.ro*

INTRODUCTION

Amoxicillin (2*S*,5*R*,6*R*)-6-[(*R*)-(-)-2-Amino-2-(p-hydroxyphenyl) acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0]-heptane-2-carboxylic acid trihydrate ($C_{16}H_{19}N_3O_5S•3H_2O$) is a semisynthetic antibiotic, an analog of ampicillin derived from 6-aminopenicillanic acid is shown in Figure 1, with a broad spectrum of bactericidal activity against many gram-positive and gram-negative microorganisms [1].

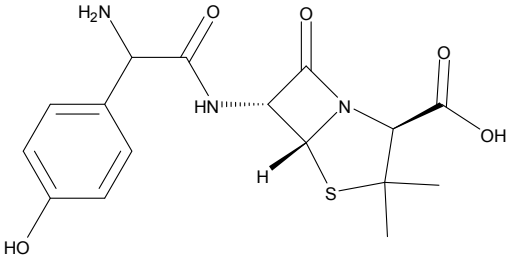

Figure 1. Structure of amoxicillin

Amoxicillin is susceptible to degradation by β-lactamases, and therefore, the activity spectrum does not include organisms which produce these enzymes [1].

Clavulanic acid (*Z*)-(2*R*,5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]-heptane-2-carboxylic acid ($C_8H_8KNO_5$ – potassium salt) is a β-lactam (Figure 2), produced by the fermentation of *Streptomyces clavuligerus* structurally related to the penicillins, which possesses the ability to inactivate a wide range of β-lactamase enzymes commonly found in microorganisms resistant to penicillins. In particular, it has good activity against the clinically important plasmid-mediated β-lactamases frequently responsible for transferred drug resistance [15].

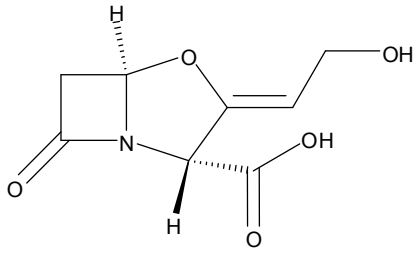

Figure 2. Structure of clavulanic acid

The formulation of amoxicillin and clavulanic acid (as potassium salt) protects amoxicillin from β-lactamase enzymes degradation and effectively extends the antibiotic spectrum of amoxicillin to many bacteria normally resistant to amoxicillin and other β-lactam antibiotics. Thus amoxicillin/ clavulanic acid possesses the distinctive properties of a broad-spectrum antibiotic and a β-lactamase inhibitor [5].

RESULTS AND DISCUSSION

Determination of acquisition parameters

There are several methods known in the literature for the individual and/or simultaneous determination of amoxicillin and clavulanic acid in human plasma and other biological matrix using UV [2,6,11,13] and MS/MS methods [3-5,7-9,12]. All MS/MS methods involve polarity switching, for monitoring the clavulanic acid in negative electrospray ionization mode. The method developed in this study uses positive ionization mode for the detection of all entities. Even if the clavulanic acid prefers a negative ionization mode, the abundance in positive ionization mode was high enough to achieve a suitable LLOQ (Lower Limit of Quantitation).

The m/z transitions used for multiple reaction monitoring (MRM) were chosen based on the spectra from Figures 3-5. The monitored transitions should not interfere in their m/z value, specific for a given analyte. Their intensity should be as high as possible for the qualifiers, and the qualifier/ quantifier ratio should remain stable over the time. Taking into account the considerations above the following transitions were chosen for the quantitative assay method:

Amoxicillin: m/z 366.2→349.2, (366.2→208.1 qualifier ion) CE 5V,

Clavulanic acid: m/z 200.1→96.0 (200.1→112.1 qualifier ion) CE 7V,

Ampicillin (IS): m/z 350.2→106.2 (350.2→160.0 qualifier ion) CE 10V. (CE – Collision Energy)

For each analyte/IS (Internal Standard) the single charged molecular ions were used as precursors.

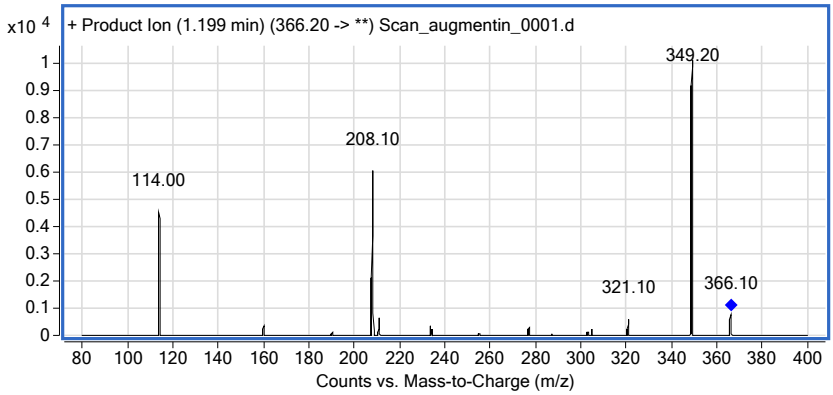

Figure 3. ESI (+) Spectrum of Amoxicillin

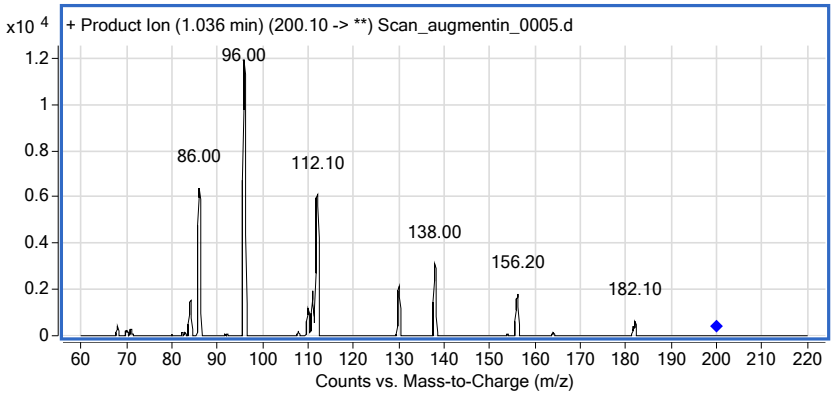

Figure 4. ESI (+) Spectrum of – Clavulanic acid

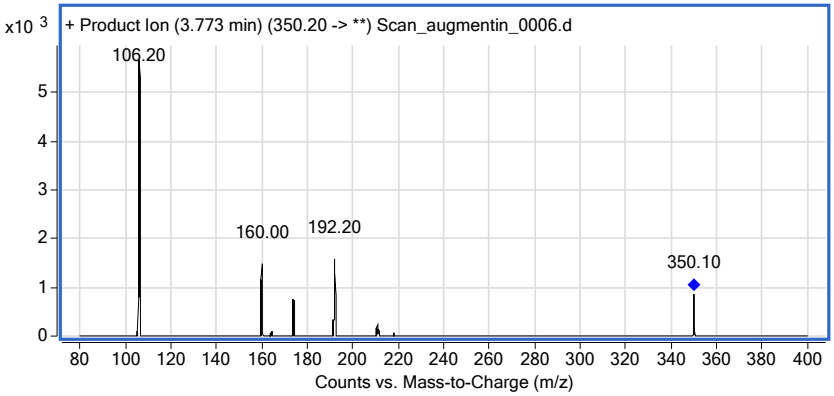

Figure 5. ESI (+) Spectrum Ampicillin (IS)

Figure 6 shows a typical MRM total ion chromatogram for an ULOQ (upper limit of calibration) sample. The elution order is: clavulanic acid, amoxicillin, ampicillin (IS) Values are back calculated concentrations for each analyte.

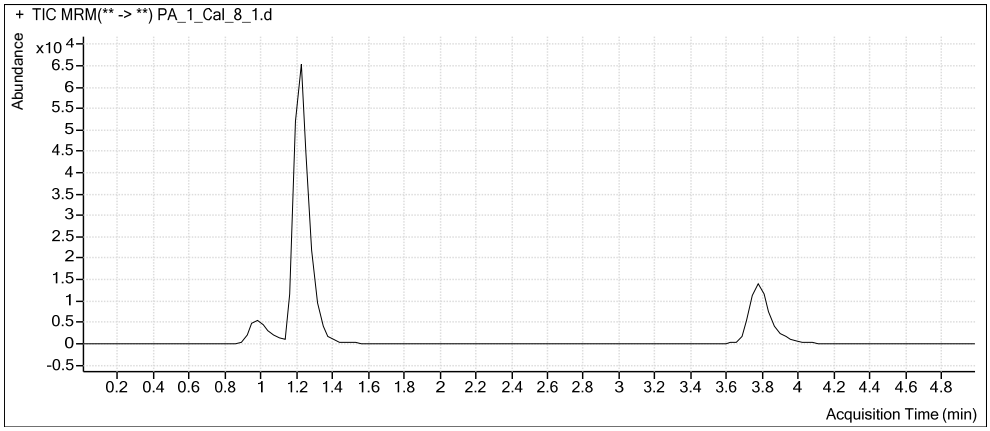

Figure 6. MRM chromatogram of Cal8 (clavulanic acid 4822.58 ng/ml, amoxicillin 22146.87 ng/ml, ampicillin 598.82 ng/ml)

It's noticeable, that no significant spectral response has been observed at the retention time of the analytes/IS in matrix blank samples (Figure 7).

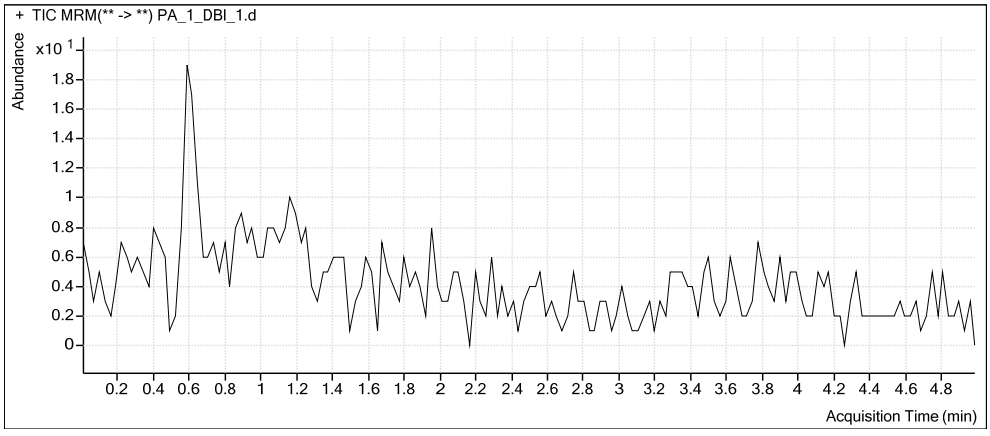

Figure 7. MRM chromatogram of DBl1 (matrix blank 0 ng/ml each analyte)

Bioanalytical method validation

The analytical method was validated according to the EMEA/CHMP/EWP/192217/2009 Guideline on validation of bioanalytical methods [14].

The tested parameters were: selectivity, sensitivity, matrix effect, anticoagulant effect, intra/interbatch precision and accuracy, recovery, short/long term stability of stock solutions of analytes, short term stability of working solutions of analytes, bench top stability in biological matrix, freeze thaw stability in biological matrix, injector/autosampler stability of the processed samples, stability during delayed processing, dilution integrity, carryover. All tests were performed using 6 replicates at the mentioned QC (Quality Control) levels.

The validated calibration range was 190-22222 ng/ml for amoxicillin, and 147-4908 ng/ml for clavulanic acid. The calibration curves were obtained using a quadratic weighted ($1/x^2$) for Amoxicillin and quadratic weighted ($1/x^2$) for Clavulanic Acid regression analysis of the peak area ratio (analyte/internal standard) versus the nominal concentration of the calibration standards. A summary of main results of validation batches is presented in Tables 1 and 2.

Summary of method validation

Table 1. Bioanalytical method validation summary for amoxicillin

Calibration concentrations (Units)	190.48, 476.20, 1190.50, 2380.99, 4761.99, 9523.98, 15873.30, 22222.61 (ng/ml)
Lower limit of quantification (Units)	LLOQ, 190.48 ng/ml, Accuracy 113.22 %, RSD 0.86
QC Concentrations (Units)	LLOQ-QC, LQC, MQC, HQC 190.48, 476.20, 4761.99, 15873.30 (ng/ml)
Between-run accuracy (%)	LLOQ-QC, LQC, MQC, HQC 111.08, 94.84, 99.46, 104.46
Between-run precision (RSD)	LLOQ-QC, LQC, MQC, HQC 2.78, 2.38, 1.76, 1.42
Matrix factor (MF) RSD	LQC 0.8385 3.97
Recovery (%)	LQC MQC HQC 73.64 75.94 77.34
Long term stability of stock solution and working solutions (Observed change %)	Confirmed up to 30 days at 4 °C LQC Stab. 95.21, change –4.79% HQC Stab. 93.81 change –7.19%
Short term stability in biological matrix at room temperature or at sample processing temperature. (Observed change %)	Confirmed up to 48.71(6) h LQC Stab. 100.85, change + 0.85% HQC Stab. 100.28 change +0.28%
Long term stability in biological matrix (Observed change %)	Confirmed up to 38 days at -50 °C LQC Stab. 107.60, change +7.60% HQC Stab. 105.00 change +5.00%

Autosampler storage stability (Observed change %)	Confirmed up to 72 h LQC Stab. 97.85, change -2.15% HQC Stab. 97.39 change -2.61%
Freeze and thaw stability (Observed change %)	-50 °C , 3 cycles LQC Stab. 100.23, change +0.23% HQC Stab. 98.99, change –1.01%
Dilution integrity	Concentration diluted (2-fold) 99.67 %; RSD 1.46 % Concentration diluted (4-fold) 99.55%; RSD 3.39 %

Table 2. Bioanalytical method validation summary for clavulanic acid

Analyte – Clavulanic acid	
Calibration concentrations (Units)	147.25, 267.73, 501.99, 1003.97, 1673.29, 2454.15, 3569.68, 4908.31 (ng/ml)
Lower limit of quantification (Units)	LLOQ, 147.25 ng/ml, Accuracy 105.98 %, RSD 5.22
QC Concentrations (Units)	LLOQ-QC, LQC, MQC, HQC 147.25, 267.73, 1673.29, 3569.68 (ng/ml)
Between-run accuracy (%)	LLOQ-QC, LQC, MQC, HQC 101.91, 100.73, 100.26, 101.74
Between-run precision (RSD)	LLOQ-QC, LQC, MQC, HQC 4.99, 3.09, 2.24, 1.79
Matrix factor (MF) RSD	LQC 0.7038 3.68
Recovery (%)	LQC MQC HQC 72.04 75.16 75.37
Long term stability of stock solutions (Observed change %)	Confirmed up to 14 days at 4 °C LQC Stab. 90.52, change –9.48% HQC Stab. 89.57 change –10.43%
Short term stability in biological matrix at room temperature or at sample processing temperature. (Observed change %)	Confirmed up to 48.71(6) h LQC Stab. 97.33 change -2.67% HQC Stab. 96.41 change –3.59%
Long term stability in biological matrix (Observed change %)	Confirmed up to 38 days at -50 °C LQC Stab. 102.05, change +2.05% HQC Stab. 107.86 change +7.86%
Autosampler storage stability (Observed change %)	Confirmed up to 72 h LQC Stab. 96.33, change –3.67% HQC Stab. 96.05 change - 3.95%
Freeze and thaw stability (Observed change %)	-50 °C , 3 cycles LQC Stab. 103.66, change +3.66% HQC Stab. 99.12, change –0.88%
Dilution integrity	Concentration diluted (2-fold) 97.24 %; RSD 0.99% Concentration diluted (4-fold) 93.65 %; RSD 2.42 %

PA – Precision and Accuracy batch
LQC/MQC/HQC – Low/Medium/High Quality Control sample

Table 3. Linearity summary results for amoxicillin

Calibration level	Nominal conc. (ng/ml)	Mean conc.±S.D (ng/ml)	RSD %	Accuracy %
Cal_1_1	190.48	213.34±7.77	3.64	112.00
Cal_1_2	190.48	215.05±2.07	0.96	112.90
Cal_2	476.20	439.69±3.47	0.79	92.33
Cal_3	1190.50	1037.28±41.79	4.03	87.13
Cal_4	2380.99	2215.72±69.24	3.12	93.06
Cal_5	4761.99	4569.95±126.44	2.77	95.97
Cal_6	9523.98	10229.15±303.30	2.97	107.40
Cal_7	15873.30	15943.79±209.70	1.32	100.44
Cal_8_1	22222.61	22094.46±185.96	0.84	99.42
Cal_8_2	22222.61	22073.03±240.97	1.09	99.33

Table 4. Linearity summary results for clavulanic acid

Calibration level	Nominal conc. (ng/ml)	Mean conc.±S.D (ng/ml)	RSD %	Accuracy %
Cal_1_1	147.25	152.20±3.62	2.38	103.36
Cal_1_2	147.25	145.84±3.17	2.18	99.04
Cal_2	267.73	266.28±4.17	1.57	99.46
Cal_3	501.99	486.25±10.79	2.22	96.86
Cal_4	1003.97	1022.20±13.02	1.27	101.82
Cal_5	1673.29	1632.69±34.29	2.10	97.57
Cal_6	2454.15	2526.76±91.56	3.62	102.96
Cal_7	3569.68	3526.70±120.87	3.43	98.80
Cal_8_1	4908.31	4893.25±70.37	1.44	99.69
Cal_8_2	4908.31	4929.98±43.00	0.87	100.44

CONCLUSIONS

A rapid and robust method has been developed and validated for the simultaneous determination of amoxicillin and clavulanic acid in human plasma. The quantitation was performed on an Agilent 1200 series HPLC system, coupled to an Agilent 6410 triple quadrupole mass spectrometer, using electrospray ionization technique. All components were detected in positive ionization mode. The method was successfully used for the evaluation of bioequivalence of a generic formulation of amoxicillin/ clavulanic acid in human subjects.

EXPERIMENTAL SECTION

Solvents and reference materials used

All used solvents are of HPLC grade. Dichloromethane (stabilized with amylene) was purchased from Riedel (Sigma), formic acid and acetonitrile from Merck KGaA, HPLC water was obtained using a Millipore Simplicity UV water purification system. Certified reference materials of amoxicillin trihydrate, potassium clavulanate and ampicillin trihydrate (internal standard-IS) were obtained from Sigma-Aldrich and are of analytical standard grade (Vetranal). Blank human plasma was obtained from the regional blood transfusion center (CRTS) Cluj.

Instrumentation and working parameters

An Agilent 1200 series HPLC system with a Phenomenex Kynetex PFP column (50 × 2.10 mm) equipped with Phenomenex Security Guard (4 ×2.0 mm) was used for separation. The used mobile phase was an isocratic mixture of 8:92 acetonitrile:water (containing 0.25% formic acid). The used flow rate was 0.3 ml/min, the column temperature was set to 38 ºC. An Agilent 6410 triple Quadrupole Mass Spectrometer (Agilent Technologies, USA), equipped with electrospray ion source was used for the LC-MS/MS analyses. The runtime was 5 min/sample. The data acquisition and processing was carried out using MassHunter software. The whole system (software and hardware) was validated. The mass spectrometer was operated in positive ionization mode for both analytes and IS. Nitrogen was used as nebulizing gas and collision cell gas. The temperature of the ESI source was set to 350 ºC, and the needle voltage to 4000V.

The quantitation was performed using MRM (multiple reaction monitoring) of the transitions: m/z 366.2→349.2, (366.2→208.1 qualifier ion) collision energy 5V, for amoxicillin, 200.1→96.0 (200.1→112.1 qualifier ion) collision energy 7V, for clavulanic acid, and 350.2→106.2 (350.2→160.0 qualifier ion) collision energy 10V for ampicillin (IS).

The mass spectrometer was operated at unit resolution with a dwell time of 300 ms per transition.

Stock and working solutions preparation

Stock solutions were prepared in ultrapure water dissolving accurately weighed amounts of reference materials, at 1.7 mg/ml – amoxiclillin and 1 mg/ml K clavulanate and ampicillin. They were stored between 2-8 °C.

Working solutions of analytes and internal standard were prepared freshly before use by successive dilutions from stock solutions to appropriate levels, using water as solvent. They were used for spiking in human plasma used for calibrators and QC samples preparation.

Calibrators and QC samples preparation

400 µl of blank human plasma, 50 µl of spiking solution of analyte and 50 µl of spiking solution of internal standard were added in polypropylene tubes, to yield final concentrations of 190.48, 476.20, 1190.50, 2380.99, 4761.99, 9523.98, 15873.30, 22222.61 ng/ml for amoxicillin and 147.25, 267.73, 501.99, 1003.97, 1673.29, 2454.15, 3569.68, 4908.31 ng/ml for clavulanic acid.

Sample preparation (workup)

To precipitate proteins, 1500 µl of acetonitrile was added to the spiked samples, then vortexed for 20 minutes at 1500 rpm. Further the samples were centrifuged for 10 minutes at 4000 rpm. 1750 µl of supernatant was transferred into a new test tube. 1500 µl of ultrapure water and 5000 µl of dichloromethane were added and the samples were vortexed for 5 minutes at 1500 rpm. To accelerate phase separation samples were centrifuged for 10 minutes at 4000 rpm. Finally 800 µl of the resulting supernatant were transferred to HPLC autosampler vials and injected into the analytical system (15 µl/sample).

The novelty of the method is the extraction of acetonitrile from the aqueous mixture with dicloromethane which was meant to reduce the organic content of the samples as much as possible without evaporation. In this way the organic content of the samples was close to the composition of the mobile phase, giving a better peakshape of the chromatograms. Furthermore, the presence of high amounts of acetonitrile in the samples acts inhibitory on the ionization of clavulanic acid in positive acquisition mode. The used doclorometha ne was stabilized with amylene (ethanol stabilized dichloromethane should not be used, because alcohol promotes the decomposition/hydrolysis [10] of the β-lactam ring in penicillin class antibiotics).

Calibration curve parameters

The linearity of the method was evaluated using spiked plasma samples in the concentration range mentioned above using the method of least squares. Three linearity curves were analyzed.

Each calibration batch (curve) consisted of: blank samples in duplicate, zero samples (blank with IS) in duplicate and eight non-zero concentration levels, of which the lower and upper limit of quantitation samples were in duplicate. The calibration curves were obtained by using a quadratic weighted ($1/x^2$) for Amoxicillin and quadratic weighted ($1/x^2$) for Clavulanic Acid regression analysis of the peak area ratio (analyte/internal standard) versus the nominal concentration of the calibration standards. Study samples concentrations were obtained by interpolation from the calibration curve.

The linearity results are summarized in Tables 3 and 4 in the 'Results and Discussion' section.

ACKNOWLEDGMENTS

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