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Original Article

AZITHROMYCIN AND OSELTAMIVIR QUANTIFICATION METHOD DEVELOPED AND VALIDATED USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY IN DRIED BLOOD SPOT

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ABSTRACT

Objective: The development and use of bio-sampling techniques for the analysis of COVID-19 drugs oseltamivir and azithromycin using the Dried Blood Spot technique simultaneously using LC-MS/MS aims to obtain optimal conditions and validated analytical methods using LC-MS/MS according to Food and Drug Administration 2018 recommendations.

Methods: Azithromycin and oseltamivir analyses were performed using LC-MS/MS with C18 Acquity® Bridged Ethylene Hybrid (BEH) column 1.7 m, 100 x 2.1 mm. The matrix sample used is Dried Blood Spot (DBS) with azithromycin and Oseltamivir as the raw material and acyclovir as the internal standard. Optimum analytical conditions were obtained on a gradient mobile phase using 0.1% formic acid-methanol solution with a flow rate of 0.2 ml/minute. The quantification of the analysis was carried out using triple quadrupole mass spectrometry with positive electrospray ionization (ESI) mode.

Results: The calibration curve ranged from 0.5 to 160 g/ml, and the Lower Limit of Quantification (LLOQ) achieved was 25.31 and 25.37 ng/ml. Sensitivity, selectivity, linearity, precision, carry-over, accuracy, stability, and recovery were found to be within the suitable limits and fully validated by the guidelines from the Food and Drug Administration 2018.

Conclusion: The method developed successfully passed all of the FDA's 2018 full validation guidelines, with the LLOQ achieved for azithromycin and Oseltamivir was 25.31 and 25.37 ng/ml.

Keywords: Azithromicyn, Oseltamivir, Acyclovir, Dried blood spot, LC-MS/MS, COVID-19

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INTRODUCTION

According to WHO [1], Coronavirus Disease, or COVID-19 is an infection caused by the SARS-CoV-2 virus, which first appeared in Wuhan, China in December 2019. Based on data obtained from the Directorate General of Disease Prevention and Control [1] in the official document entitled "COVID-19 Prevention and Control Guidelines", acute respiratory diseases like fever and cough are clinical indications and symptoms of COVID-19 infection, and X-ray results reveal severe pneumonia infiltrates in both lungs.

Oseltamivir is a drug that is usually given to patients who have tested positive for COVID-19. Oseltamivir is marketed as oseltamivir phosphate; this compound is a prodrug that is metabolized by plasma and liver esterases to the active form oseltamivir carboxylate [2]. So far, Oseltamivir is used for treat and prevention of influenza types A and B and is available only as an oral dosage form. This drug has several side effects that will result if it is not used properly, namely nausea, vomiting, epilepsy, and arrhythmias [3]. Azithromycin is a class of antibacterial drugs that have a broad spectrum, working by inhibiting protein synthesis in bacteria [4]. This drug also has activity to inhibit various viruses such as Ebola, Zika, viruses in the respiratory tract, H1N1, enterovirus, and rhinovirus. In the case of COVID-19, azithromycin is used to reduce the entry of the virus into cells. Azithromycin plays a role in regulating the production of interferon types I and III and genes involved in virus recognition, such as MDA5 and RIG-1 [5]. The use of azithromycin as a single treatment or in combination with other antiviral drugs for COVID-19 patients still requires some evaluation because, according to the study, found that no significant difference for patients receiving hydroxychloroquine with azithromycin (51.1%), hydroxychloroquine alone (18.8%), and treated azithromycin alone (14.7) and 15.4% treated with other drugs [6]. Therefore, a validated analytical method is needed for the use of Oseltamivir and azithromycin for monitoring blood drug levels in hospitals in COVID-19 patients.

The method of analysis of oseltamivir and oseltamivir carboxylate has been carried out by [7] using human blood samples with the Dried Blood Spot technique using UHPLC-MS/MS and analysis of azithromycin doses has been carried out by [8] using UV and HPLC spectroscopy. The development and use of bio-sampling techniques for the analysis of COVID-19 drugs oseltamivir and azithromycin using the Dried Blood Spot technique simultaneously using LC-MS/MS has never been carried out and studied. Therefore, this study aims to obtain optimal conditions and validated analytical methods using LC-MS/MS according to Food and Drug Administration 2018 recommendations.

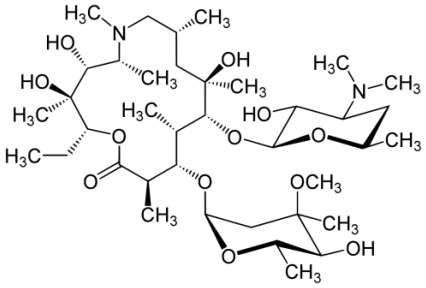

Fig. 1: Azithromycin chemical structure [9]

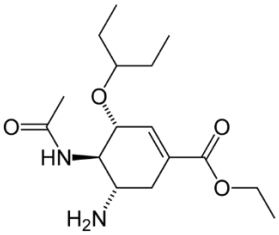

Fig. 2: Oseltamivir chemical structure [10]

MATERIALS AND METHODS

Material

Azithromycin and Oseltamivir was purchased from BPOM Indonesia, internal standard (Acyclovir) was bought from Cayman Chemical (USA), formic acid (Merck), acetonitrile (Merck), ethanol (Merck), methanol (Merck), sodium dihydrogen phosphate (Merck), Dried Blood Spot was purchased from Whatman 903 (Sigma Aldrich, Singapura), Aquabidestilata was purchased from Ikapharmindo in Indonesia, and whole blood was obtained from the Indonesian Red Cross in Jakarta, Indonesia.

Chromatography condition

Optimum conditions for analysis of azithromycin and Oseltamivir in DBS using Liquid Chromatography-Tandem Mass Spectrometry/ Mass Spectrometry with coloumn Chromatography with Acquity® UPLC BEH C18 column (1.7 m, 2.1 x 100 mm) achieved with 0.1% formic acid-methanol as the mobile phase, 0.2 ml/min flow rate, gradient elution, and 10 L of injection volume. Mass spectrometry detection using positive ESI ionization mode.

Methods

Sample preparation

Preparation of stock solution

Stock Solutions of azithromycin, Oseltamivir, and acyclovir were prepared by diluting them in methanol: water (1:1) to obtain azithromycin, oseltamivir and acyclovir concentration of 1000 µg/ml respectively.

Optimization of azithromycin and Oseltamivir analytical condition

50 microliters of a mixture containing 20 g/ml each of azithromycin, Oseltamivir, and acyclovir were injected into the LC-MS/MS apparatus. The best mobile phase consisted of a mixture of 0.2% formic acid in methanol and 0.2% formic acid in acetonitrile. Gradient elution was used to optimize the elution profile. The ideal column temperature ranges were 30, 40, and 50 °C. The optimal flow rates for the mobile phase were 0.1, 0.15, and 0.2 ml/min. Subsequently, a suitability assessment of the system was employed to determine the optimal analytical configuration [13].

System suitability test

To confirm that the instrument performance and analytical condition are appropriate for analysis, the outcomes of the optimization of the analysis condition must be checked. 50 microliters of a mixture solution containing 20 g/ml each of the internal standards (20 g/ml of Oseltamivir, 20 g/ml of azithromycin, and 20 g/ml of acyclovir) were injected into the LC-MS/MS apparatus six times. Peak area ratio (PAR) coefficients of variation (%CV) and retention time should both be under 2.0%. Other elements included resolution, theoretical plates, HETP value, and tailing factor [13].

Optimization of dried blood spot (DBS) sample preparation

Based on the extraction process, the DBS sample preparation was improved. By drying DBS devices for 30, 60, and 120 min after absorbing spiked blood, DBS drying time was optimized. Protein precipitation was selected as the extraction method due to the procedure's ease of use and time-saving capabilities. Half of the methanol was used in the extraction solution. The optimum sonication times were 10, 20, and 30 min. Following the optimization of analytical conditions and sample preparation, the optimized method was verified by Food and Drug Administration 2018 standards [16].

Method validation

The validation of the analytical method was completed in compliance with the Food and Drug Administration's 2018 requirement for full validation. The parameters of thorough validation that were assessed were recovery, sensitivity, selectivity, linearity, carry-over, accuracy, precision, and stability [11]. Fundamental parameters, including selectivity, specificity, sensitivity, accuracy, precision, linearity, carryover, recovery and stability, were assessed to ensure the acceptability of the method performance.

RESULTS AND DISCUSSION

In order to achieve better sensitivity of this method, we further optimized the chromatographic condition by evaluation the mobile phase, flow rate, column temperature and also the method of elution.

The condition of the sample's analytical optimization

The methanol-0.2% formic acid mobile phase mixture was discovered to have the highest peak area and shortest retention duration. Gradient elution analysis was used to create a high-quality chromatogram. The flow rate of 0.2 ml/min produced the highest retention time since the retention time reduces as the flow rate increases [12]. Finally, a column temperature of 50 °C was chosen since the retention duration was slightly shortened and it offered a superior theoretical plate.

System suitability test

System suitability testing was used to assess the analytical condition that optimization produced. Analysis of five injections revealed a %CV of 2.0% for the mix solution retention duration and peak area. Azithromycin, Oseltamivir, and acyclovir had retention times of 1.79, 3.45, and 1.13 min, respectively. In fig. 3, a chromatogram of the combination of azithromycin, Oseltamivir, and acyclovir was shown.

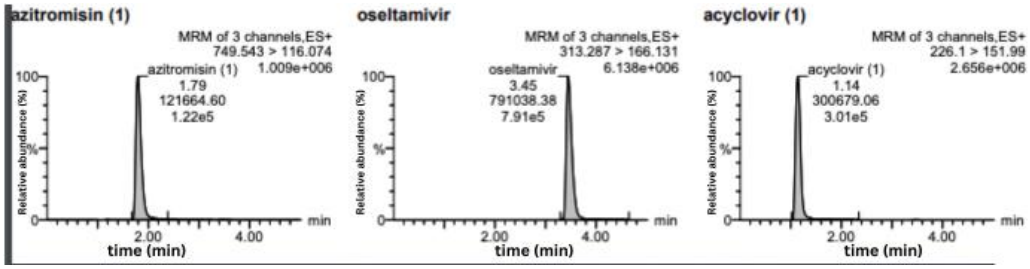

Fig. 3: Chromatograms from system suitability test

The retention time of azithromycin shows that it was faster compared to previous studies [17]. This is due to the increase in temperature in the column used. Temperature can affect the solubility of the contents, which can alter the retention time [19].

Optimization of sample preparation

The sample preparation optimization method started with the optimization of DBS drying time. The ideal drying time for DBS samples containing azithromycin and Oseltamivir was 120 min at room temperature. Using the protein precipitation method, azithromycin and Oseltamivir were extracted. It was found that 100 ml of methanol extracted more azithromycin and Oseltamivir than another extracting solution. The best amount of time for sonication to extract azithromycin and Oseltamivir was 30 min.

Method validation

Sensitivity

Analysis using five replicates of DBS containing 25.31 ng/ml azithromycin and Oseltamivir showed that %diff obtained was–

16.78 to 17.32% and-17.84 to 14.30, %CV obtained was 14.14% and 13.76%. The Lower Limit of Quantification (LLOQ) for azithromycin and Oseltamivir were determined to meet FDA standards at 25.31 ng/ml. When compared to the LLOQ (Lower Limit of Quantification) of azithromycin in plasma measured using LC-MS/MS in other studies, a result of 0.5 ng/ml was obtained [14]. As for the LLOQ of Oseltamivir in other research analyzed using HPLC-MS/MS in human plasma, an LLOQ of 0.30 ng/ml was achieved [15]. However, in our study, there is an innovation in the form of simultaneous analysis of azithromycin and Oseltamivir, even though the obtained LLOQ values are higher. LLOQ chromatograms are displayed in fig. 4.

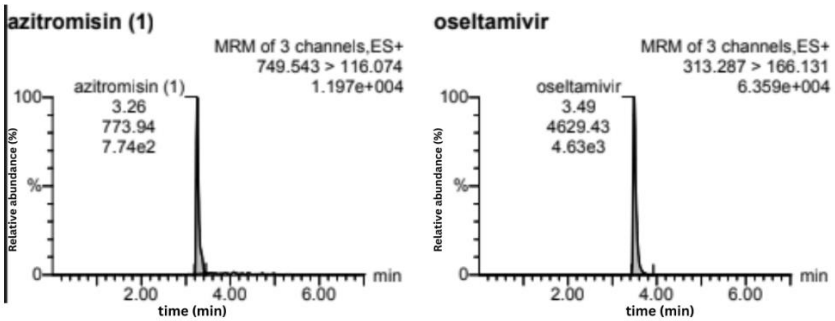

Fig. 4 Chromatogram of azithromycin and Oseltamivir at LLOQ concentration (25.31 ng/ml)

Linearity

According to the above-described sample preparation process, calibration samples were created using DBS at seven different concentration levels (25.59, 51.19, 102.38, 255.88, 511.88, 1023.75, and 2047.50 ng/ml), zero, and blank. The calibration curves were linear, and the correlation coefficients for azithromycin and doxycycline were both more than 0.99 (Tables 1 and 2).

Table 1: Results of calibration curve oseltamivir in three consecutive days

Replicate	Slope	Intercept	R
1	0.0073	1.1032	0.9936
2	0.0075	1.0796	0.9930
3	0.0074	1.1045	0.9932

Table 2: Results of calibration curve azithromycin in three consecutive days

Replicate	Slope	Intercept	R
1	0.0043	0.1116	0.9978
2	0.0043	0.1099	0.9962
3	0.0042	0.1023	0.9972

Selectivity

The investigation of 6 different matrix sources revealed no interferences with azithromycin's retention time. As seen by the 5% interference recorded in the retention time of Oseltamivir, the analysis method also complied with the FDA's (2018) standards for bioanalytical method validation.

Carry-over

The carry-over test was conducted by injecting the blank samples after the upper limit of quantification (ULOQ); the results on the blank samples should not exceed 20% of LLOQ. According to the results of the present experiment, no carry-over effect was found (<20%) in the blank samples after the injection of ULOQ concentration.

Accuracy, precision, and recovery

Analysis of five replicates in four different concentration levels-LLOQ (25.31 ng/ml), QCL (76.11 ng/ml), QCM (1014.80 ng/ml), and QCH (1522.20 ng/ml)-was done as part of an accuracy and precision test during a period of three days. The chromatograms of oseltamivir and azithromycin at concentrations of QCL, QCM, and QCH are displayed in fig. 5, 6, and 7, respectively. Accuracy and precision within and across runs are presented in table 3.

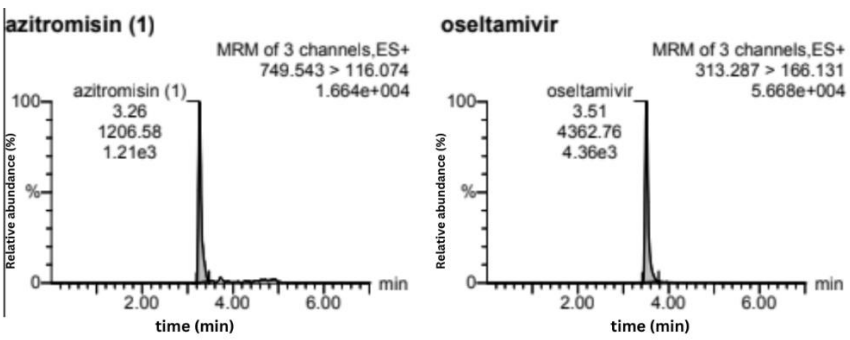

Fig. 5: Chromatogram at QCL concentration (76.11 ng/ml)

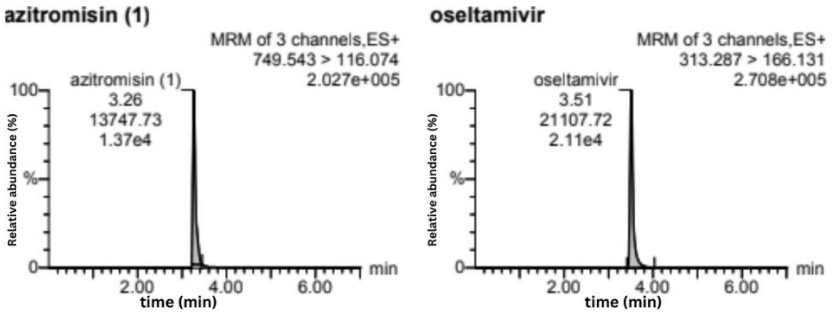

Fig. 6: Chromatogram QCM concentration (1014.80 ng/ml)

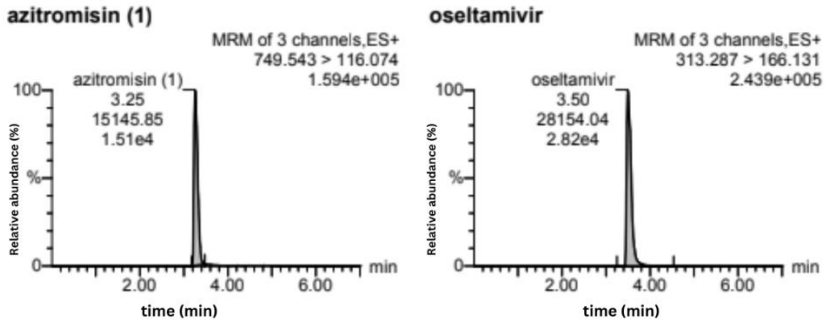

Fig. 7: Chromatogram QCH concentration (1522.20 ng/ml)

For the recovery test, the concentration levels of QCL, QCM, and QCH were established. The efficiency of the extraction was assessed by contrasting pre-and post-extraction samples. Pre-extraction samples were created by preparing DBS following the aforementioned process and adding various concentration of whole blood to an azithromycin and oseltamivir standard solution. After extracting the control samples, the post-extraction samples were made by adding the same amounts of azithromycin and Oseltamivir standard solution to the supernatant. The results of the recovery test are shown in tables 5 and 6.

Table 3: Results of accuracy and precision within-run and between-run oseltamivir

Actual conc.	Within-run*		Between-run*	
(ng/ml)	CV (%)	%diff	CV (%)	%diff
25.37	9.49	-15.03 to 3.76%	9.45	-15.03 to 19.03%
76.11	8.33	-6.35 to 14.9%	7.66	-12.82 to 14.9%
1014.80	1.65	-14.81 to-10.97%	4.72	-14.81to 7.70%
1522.20	6.62	-9.74 to 5.76 %	6.29	-14.72 to 11.95%

*Measured concentration (Average±SD; ng/ml), n=5 Within run: 23.50±2.23; 77.21±6.43; 879.85±14.55; 1477.16±97.82. Between run: 24.97±2.38; 73.91±5.69; 928.24±48.39; 1482,70±92,89

Table 4: Results of accuracy and precision within-run and between-run azithromycin

Actual conc.	Within-run*		Between-run *	
(ng/ml)	CV (%)	%diff	CV (%)	%diff
25.31	10.83	-12.55 to 12.54%	10.53	-15.22 to 15.31%
75.94	7.60	-6.95 to 14.45%	6.23	-6.95 to 14.45%
1012.60	5.64	0.21 to-14.77%	6.40	-12.78 to 14.77%
1518.90	12.13	-14.63 to 12.26 %	9.16	-14.63 to 12.86%

*Measured concentration (Average±SD; ng/ml), n=5 Within run: 23.94±2.59; 78.19±5.95; 1114.41±62.81; 1502.17±182.21. Between run: 25.35±2.64; 79.33±4.93; 1059.92±67.48; 1541.73±140.96

Table 5: Recovery of oseltamivir in DBS

Actual concentration (ng/ml)	Replicate	CV (%)
76.11	3	6.02
1014.80	3	4.54
1522.20	3	1.82

*Recovery (Average±SD; %), n=5: 84.62±5.09; 88.66±4.03; 88.89±1.61.

Table 6: Recovery of azithromycin in DBS

Actual concentration (ng/ml)	Replicate	CV (%)
76.11	3	2.63
1014.80	3	4.17
1522.20	3	1.75

*Recovery (Average±SD; %), n=5: 89.98±2.37; 85.21±3.55; 93.66±1.64.

Stability

Stock solution stability, short-term DBS stability, long-term DBS stability, autosampler stability, and heat stability are all included in the stability evaluation process. Oseltamivir in stock solution remained stable for 24 h at ambient temperature and 29 d at-20 °C in the refrigerator. At ambient temperature, the azithromycin stock solution demonstrated stability for a duration of 24 h; at-20 °C, it was found to be stable for 29 d. DBS was kept at room temperature for 0, 6, and 24 h to conduct a test on its short-term stability. Azithromycin and Oseltamivir in DBS were stable over 24 h at room temperature, according to the data, which was supported by the fact that the %diff and %CV obtained were both below 15.0%. (See Tables 7 and 8). To assess the long-term stability, a-20 °C freezer was used for 0 to 30 d. According to the findings (Tables 9 and 10), Oseltamivir and azithromycin in DBS were sufficiently stable at a-20 °C freezer for 30 d. Extracted samples were kept in the autosampler for 24 h to evaluate their stability. According to the findings, azithromycin and Oseltamivir were stable in the autosampler for 24 h (Tables 11 and 12).

Table 7: Short-term stability of Oseltamivir in DBS at room temperature

Hours	QCL (76.11 ng/ml)*		QCH (1522.20 ng/ml)*	
	CV (%)	%diff	CV (%)	%diff
0	9.91	-6.35 to 14.29%	7.41	-8.83 to 5.76%
6	0.06	-3.55 to-8.53%	0.02	-8.72 to–5.34%
24	0.16	-13.57 to-13.44%	0.04	-9.47 to-1.57%

*Measured concentration (Average±SD; ng/ml), n=5 QCL: 79.54±7.88; 77.93±4.60; 73.08±11.50. QCH: 1505.16±111.60; 1413.27±25.98; 1425.90±63.75.

Table 8: Short-term stability of azithromycin in DBS at room temperature

Hours	QCL (75.94 ng/ml)*		QCH (1518.90 ng/ml)*	
	CV (%)	%diff	CV (%)	%diff
0	10.30	-6.94 to 14.46%	4.02	4.11 to 12.26%
6	5.63	0.18 to 7.88%	1.51	-14.32 to-11.70%
24	9.06	-7.07 to-8.80%	3.15	-0.34 to 5.79%

*Measured concentration (Average±SD; ng/ml), n=5 QCL: 78.95±8.13; 81.02±4.56; 78.27±7.09. QCH: 1630.69±65.55; 1320.42±19.94; 1569.77±49.41.

Table 9: Long-term stability of Oseltamivir in DBS at-20 °C freezer

Days	QCL (76.11 ng/ml)		QCH (1522.20 ng/ml)	
	CV (%)	%diff	CV (%)	%diff
0	4.67	-2.88 to 5.98%	3.18	-14.72 to-5.98%
30	14.14	-13.99 to 9.28%	7.48	-10.62 to 2.84%

*Measured concentration (Average±SD; ng/ml), n=5 QCL: 78.09±3.65; 71.50±10.11. QCH: 1336.02±42.53; 1443.30±108.02.

Table 10: Long-term stability of azithromycin in DBS at-20 °C freezer

Days	QCL (75.94 ng/ml)		QCH (1518.90 ng/ml)	
	CV (%)	%diff	CV (%)	%diff
0	2.96	-4.54 to 1.25%	10.49	-12.47 to-7.23%
30	4.15	-4.11 to 3.72%	10.37	-7.18 to 14.33%

*Measured concentration (Average±SD; ng/ml), n=5 QCL: 74.84±2.21; 75.24±3.12. QCH: 1508.82±158.20; 1579.32±163.74.

Table 11: Autosampler stability of Oseltamivir for 24 h

Hours	QCL		QCH	
	CV (%)	%diff	CV (%)	%diff
0	2.01	5.03 to 8.95%	1.81	-5.91 to-2.51%
24	12.72	-6.33 to-2.93%	10.09	-4.47 to 14.33%

*Measured concentration (Average±SD; ng/ml), n=5 QCL: 1.61±0.032; 1.51±0.191. QCH: 114.75±2.075; 122.93±12.401.

Table 12: Autosampler stability of azithromycin for 24 h

Hours	QCL (75.94 ng/ml)		QCH (1518.90 ng/ml)	
	CV (%)	%diff	CV (%)	%diff
0	10.30	-6.94 to 14.46%	4.02	4.11 to 12.26%
24	3.10	6.05 to-12.30%	2.27	8.11 to 13.04%

*Measured concentration (Average±SD; ng/ml), n=5 QCL: 78.95±8.13; 83.50±2.59. QCH: 1630.69±65.55; 1675.66±38.04.

CONCLUSION

In the fast-growing field of pharmaceutical DBS analysis, we demonstrated with the current work that DBS are feasible for analysis of azithromycin and oseltamivir concentration measurements in humans. The Food and Drug Administration's full validation requirements were met by the technique created for quantifying azithromycin and Oseltamivir in 2018. The measured LLOQ was 25.31 and 25.37 ng/ml. The procedure created can be used to measure the concentrations of azithromycin and Oseltamivir in human blood using less intrusive and uncomfortable biosampling methods.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

All authors are contributed equally.

CONFLICT OF INTERESTS

All of the authors declare that there is no conflict of interest in this research.

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