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The development of a bioanalytical method for the simultaneous analysis of gentamicin and tacrolimus in Rat whole blood using UHPLC–MS/MS

Shrooq Altaweel[1,2], Ann Van Schepdael[2], Erwin Adams[2]✉ & Aliyah Almomen[1]✉

Tacrolimus (TAC) is commonly administered to patients who have undergone organ transplantation to prevent the immune system from rejecting the transplanted organ. Multidrug-resistant bacterial infections are the most frequent complications during the first-month post-transplantation. Old antimicrobial agents such as gentamicin (GEN) are widely used to treat opportunistic nosocomial infections in immunosuppressed TAC patients. Nephrotoxicity is a significant side effect of GEN and TAC, but some studies indicated their concurrent administration. However, there is no information on whether the combination of the two drugs may result in a more significant impairment of kidney function than either drug used separately. To investigate this, both drugs should be monitored in blood. Sample preparation was carried out using protein precipitation, requiring only 50 µL of WB sample with an extraction recovery of not less than 95.2% (GEN) and 93.2% (TAC). Analytes and internal standard (IS) were monitored using mass spectrometry (MS) in positive ion mode by multiple reaction monitoring (MRM). Chromatographic analysis was performed on an Acquity UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 µm), kept at 50 °C and using gradient elution. Mobile phase A contained 2 mmol/L ammonium formate acidified with 0.1% formic acid in water, and mobile phase B was a mixture of 2 mmol/L ammonium formate and 0.1% formic acid in methanol, pumped at a flow rate of 0.25 mL/min. The analysis time was only 6 min. The method was verified according to the European Medicines Agency (EMA) guidelines over a concentration range of 19.5–2500 ng/mL for GEN and 1.95–250 ng/mL for TAC. Determination coefficients for the calibration curves were found to be ≥ 0.999. Within- and between-run precision and accuracy were evaluated for both drugs with relative standard deviations (RSD) ≤ 6.5% and inaccuracy ≤ 6.6%. The proposed method was successfully applied to analyze the WB samples at different time points after the co-administration of GEN and TAC to Wistar rats. In this work, a new bioanalytical UHPLC–MS/MS method was developed and validated for simultaneous quantification of total GEN congeners (C_1, C_{1a}, and C_2/C_{2a}) and TAC in Wistar rats whole blood (WB). The protein precipitation method has been chosen to extract the drug from the WB sample. The assay method has been successfully used to estimate the concentration of TAC and GEN after co-administration in rats.

Keywords UHPLC–MS/MS, Gentamicin, Tacrolimus, Rats whole blood, Ion pairing reagent

Solid-organ transplantation (SOT) is a therapeutic option that provides a new life for many patients at the end stage of organ failure. The ultimate goal of post-organ transplantation is to minimize the recognition of the allograft by the immune system, preventing its destruction[1]. To maximize the chances of long-term survival of the transplanted organ, immunosuppressives are utilized to induce tolerance. However, the chronic use of immunosuppressives was found to be associated with the risk of infections and other complications due to the weakened immune system. This could eventually lead to graft loss and recipient death[2].

[1]Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, P.O. Box 22452, 11495 Riyadh, Saudi Arabia. [2]Department of Pharmaceutical and Pharmacological Sciences, Pharmaceutical Analysis, KU Leuven, Herestraat 49, O&N2, PB, 923, 3000 Leuven, Belgium. ✉email: erwin.adams@kuleuven.be; alalmomen@ksu.edu.sa

Tacrolimus (TAC), a macrolide calcineurin inhibitor, is a highly effective immunosuppressant drug used after organ transplantation, preventing organ rejection and graft loss[1]. It binds to an immunophilin-binding protein and inhibits calcineurin phosphatase, inhibiting interleukin-2 transcription and reducing T-lymphocyte activation[3]. TAC has been proven to prolong liver, kidney, heart, lung, pancreas, and intestinal allograft survival. However, it can cause degenerative changes in the proximal tubular epithelium, leading to acute renal failure. Therefore, regular therapeutic drug monitoring (TDM) is a valuable tool for adjusting TAC levels and minimizing the risk of toxic effects[4].

Bacterial infection is the most common type of post-transplantation infection, followed by fungal, viral, and protozoal infections[5]. The different risk factors associated with bacterial infections depend on the duration of the transplant. Many pathogens can infect the recipient during the first three months following transplantation, but the most frequent nosocomial infections and leading causes of death are Gram-positive and Gram-negative bacteria[6]. It has been reported that most surgical site infections, like pneumonia, bacteremia, and urinary tract infections, are caused by multidrug-resistant strains of pathogens[6]. A significant concern for the successful transplantation of organs is the emergence of multidrug-resistant Gram-negative bacteria (MDR GNB) infections in transplant patients, which represent one of the highest risk groups for acquisition and infection, particularly carbapenenm-resistant Enterobacterales (CRE)[7]. Current therapeutic options with CRE include using older agents like polymyxins, fosfomycin, tigecycline, and aminoglycosides, such as gentamicin (GEN), tobramycin, and amikacin[8]. GEN constitutes some of the most effective choices for treating severe Gram-negative infections[9]. It has been reported that using GEN as a prophylactic antibiotic regimen before and after SOT surgery for liver transplant patients can significantly lower bacterial infections, the length of Intensive Care Unit and hospital stays, and the mortality rate compared to ceftizoxime[10]. Another study found that adding GEN to the antibiotic prophylaxis regimen for patients undergoing renal transplantation significantly reduced urinary tract infections and nosocomial infections during the post-intervention period[11].

GEN is a mixture of the main components gentamicin C_1 and C_{1a}, as well as two stereoisomers C_2 and C_{2a}, along with several minor components, including C_{2b} as shown in Fig. 1. GEN mechanism of action depends on blocking the initiation step of protein synthesis in bacteria by binding to the aminoacyl-transfer RNA on the (A) acceptor site of 16 S ribosomal RNA within the 30 S ribosomal subunit. This results in a misreading of the genetic code and the blocking of ribosomal translocation[12]. There is a similar binding site for all GEN C components, but each binds with different affinity to the 30 S ribosomal subunit. GEN C_{1a} has a slightly higher affinity than C_2, while C_1 binds with the lowest affinity[13].

GEN can induce tubular cytotoxicity in the kidney, causing a small amount to accumulate on proximal tubular epithelial cell lysosomes after glomerular filtration. This increase in GEN accumulation will cause acute tubular necrosis and cell death[14]. Fortunately, GEN-induced nephrotoxicity is often reversible or partly reversible[15]. Monitoring peak concentrations is crucial for GEN's effectiveness as an antimicrobial agent and minimizing toxicity, especially when combined with other nephrotoxic drugs[16]

Undoubtedly, GEN and TAC are both nephrotoxic agents, but aminoglycoside still holds significant value as an effective agent in treating severe pseudomonal infections in transplant recipients[17]. A recent study, however, showed the possibility of the concurrent administration of TAC and GEN post-transplantation, even in the early post-transplantation period[18]. As of right now, no information is available regarding whether the simultaneous use of TAC and GEN would cause more severe renal impairment compared to taking either drug alone. Furthermore, pharmacokinetic (PK) parameters can be altered in patients with kidney injury[19]. Thus, it is

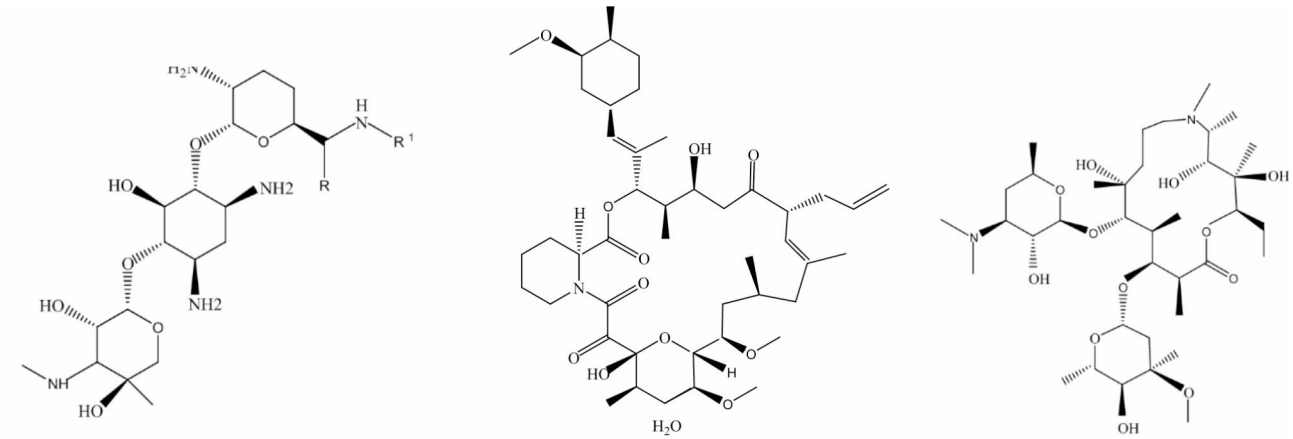


Fig. 1. Chemical structure of the studied compounds.

crucial to perform TDM in cases of concurrent use. Biological samples, such as plasma, serum, and whole blood (WB), are widely used to evaluate PK parameters. Bioanalytical applications typically involve plasma samples, but for specific compounds that mainly distribute in erythrocytes, measuring drug exposure in WB is preferred over measuring it in plasma or serum. Unlike plasma and serum, WB contains blood cells and more proteins, which generate more precipitates during protein precipitation. However, developing and validating methods for WB samples poses unique challenges due to their dense nature and complex composition[20].

Since both hematocrit and protein concentrations strongly influence the distribution of TAC in blood, and because of deficient levels of TAC found in plasma, WB is the preferred specimen of choice commonly used for TDM[21]. Two standard techniques have been reported in the literature for estimating TAC separately or combined with other drugs in WB using immunoassay[22–24] and LC–MS[25–29]. It has been noted that the LC–MS method was sensitive for detecting TAC concentrations[24]. Despite the clinical relevance of GEN, it is interesting to note that no reliable method was available for assessing its levels in WB, neither in humans nor animals, and that GEN concentration in blood was only measured after serum and plasma extraction by using immunochemical methods[30–32], LC[33–35], and more recently LC coupled to mass spectrometry (LC–MS/MS)[32,36–42]. Quantifying the drug in plasma may not be entirely accurate since it does not consider drug partitioning to the blood cell compartment, which can underestimate the amount of drug that reaches the bloodstream. An assay method for GEN in WB using LC–MS/MS could be a helpful option for clinical laboratories as it enables the use of a tiny amount of biological sample and achieves low limits of detection and quantification, together with sufficient specificity, which is highly important for TDM[36]. Compared to conventional LC-UV, routine analysis of GEN is inconvenient since it usually involves a derivatization process to enhance the chromatographic characteristics of the analyte. This is because GEN lacks a UV-absorbing chromophore, and it is highly polar[43].

So, in this work, a rapid and simple UHPLC–MS/MS method was developed to simultaneously determine GEN and TAC in the WB of Wistar rats. The method was fully validated following the European Medicines Agency (EMA) guideline for bioanalytical method validation in terms of specificity, linearity, accuracy, precision, carry-over, matrix effects, extraction recovery, and stability[44].

Results and discussion

Optimization of chromatographic conditions

The chromatographic conditions were carefully optimized to determine all compounds well in a single chromatographic run. Attention was paid to achieving sharp, symmetrical peaks with high response and sufficient resolution within a reasonable runtime.

Effect of organic modifiers

The study tested different organic modifiers, using either acetonitrile or methanol as mobile phase B. The results showed that 100% methanol produced up to two times higher peak areas for GEN and TAC than acetonitrile. In addition, GEN can also be retained more effectively by using methanol than acetonitrile. This is because methanol has a lower elution strength. Therefore, methanol was chosen as mobile phase B based on the higher retention and higher sensitivity obtained.

This study examined multiple elution programs and various water/methanol ratios to assess the target analytes' mobile phase efficiency. The mobile phase's initial composition was established as water-methanol (97:3, v/v) to enhance the retention of GEN. The gradient time was adapted to obtain a decent separation between GEN, TAC, and IS within a reasonable analysis time.

Effect of ammonium formate concentration

The use of ammonium formate in the mobile phase was essential for TAC to create ammonium adduct ions, which have higher abundance and can be easily fragmented in MS-MS, compared to the protonated ions $[M+H]^+$, which show low abundance in the positive ESI scan[45]. The effect of four concentrations of ammonium formate (1, 2, 3, and 5 mM) added to the mobile phase was studied. Although a low concentration of 2 mM ammonium formate was found to improve the detection of TAC, its response decreased when the concentration was increased to 5 mM. That is because increasing the concentration of ammonium formate leads to an increase in the ionic strength of the mobile phase, which can cause discharges and poor spray performance of ionization by ESI, decreasing the response of the drugs. The response of GEN was not affected by increasing the concentration of ammonium formate to 5 mM.

Effect of different percentages of formic acid

Formic acid played an essential role in enhancing ionization in the positive ESI mode by producing $[M+H]^+$ ions, ultimately increasing the signal response of GEN, but it also reduced the sensitivity of the ammonium adduct. The effect of various concentrations of formic acid was examined, ranging from 0.05 to 0.15%. The results indicated that increasing the formic acid concentration above 0.1% increased the width of GEN, while some peak tailing was observed with TAC. The highest response for GEN was associated with 0.1% formic acid. TAC demonstrated its highest response at 0.05%, while GEN displayed the lowest response at that percentage. It was concluded that the best results for both drugs were achieved by adding 0.1% formic acid in the mobile phase.

Effect of flow rate

Different flow rates were tested to achieve satisfactory retention, peak shape, and sensitivity for both analytes. Flow rates were evaluated from 200 to 500 µL/min. The 500 µL/min flow rate did not produce satisfactory sensitivity for GEN and TAC, and the back pressure produced was too high. The 200 µL/min flow rate showed the highest response, but the width of the GEN peak was increased. Therefore, the flow rate was adjusted to 250 µL/min to achieve a sharp and symmetrical peak with satisfactory sensitivity.

Effect of column temperature

It was observed that increasing the column temperature from 35 °C to 55 °C resulted in a slight reduction (approximately 5%) in the retention times of both analytes. The TAC peak became narrower and higher until the temperature reached 50 °C, after which no significant change was observed in peak width or height. For GEN, increasing the temperature did not affect much the peak shape. So, a column temperature of 50 °C was selected.

Optimization of ion-pairing reagent

Two major separation modes (followed by tandem MS) can be used to analyze insufficiently retained polar compounds. One involves adding a volatile ion-pairing reagent to the mobile phase. This method is known as ion-pairing liquid chromatography (IPLC). The other is hydrophilic interaction liquid chromatography (HILIC). A problem with HILIC separations is that slight variations in the composition of the mobile phase, buffer concentration, pH, or temperature can significantly affect the selectivity and retention of compounds[43]. Therefore, we will rely on IPLC for our method.

With no ion-pairing reagent, GEN was eluted close to the column dead volume. GEN possesses multiple amino groups, exhibiting pKa values between 6.4 and 9.8 [46]. At an acidic pH (2.6) below the pKa values of GEN, the molecule becomes fully protonated, resulting in a maximum positive charge. The charged state of GEN significantly enhances its ability to effectively interact with negatively charged ion-pairing reagents. The protonated amino groups of GEN make it highly hydrophilic. The addition of a negatively charged ion-pairing reagent like HFBA reduced this hydrophilic character and enhanced the affinity of GEN to the hydrophobic reversed phase stationary phase[47]. In our experiment, HFBA was added to the injection vial rather than the mobile phase to prevent contamination and frequent ion source maintenance so that GEN could be detected with sufficient sensitivity using ESI-MS[48]. Based on experimental optimization, different concentrations of HFBA were evaluated by mixing 200 µL of the final extract solution with 200 µL of ion-pairing reagent solution at 0, 30, 50, 60, and 70 mM (prepared in ultra-pure water) in the injection vial. The retention of GEN using 70 mM HFBA was close to 60 mM. Incorporating 60 mM of HFBA in the sample vial yielded the best results, achieving a good peak shape and retention of GEN. Moreover, HFBA had no impact on the analysis of TAC in the positive ion mode.

Selection of internal standard for accurate quantification

AZM was selected as IS for several reasons, including the estimation of GEN and TAC in the sample matrix. Firstly, the extraction of GEN, TAC, and IS was successfully performed from WB using the same protein precipitation method, with optimum recovery percentages for GEN (100.9 ± 1.3%), TAC (98.7 ± 1.5%), and IS (99.2 ± 0.9%). Secondly, the retention times of AZM (4.1 min), GEN (1.2 min), and TAC (5.0 min) were near to each other and all eluted within 6 min with baseline separation, indicating a rapid methodology that saves time and consumes less mobile phase. Lastly, it is rare to prescribe AZM for patients receiving GEN and TAC simultaneously. Hence, the proposed UHPLC–MS/MS method can be applied after validating the results on human WB samples to understand any harmful effects of GEN and TAC on the kidneys.

Optimization of extraction conditions

Releasing the drugs from their binding proteins during sample processing is essential to guarantee an accurate measurement of the total drug concentrations. Proteins are quickly and efficiently removed by adding an organic crash solvent, such as acetonitrile or methanol. It has been reported that adding a mixture of cold methanol-acetonitrile to WB samples can effectively rupture red blood cells and release their contents into the surrounding blood plasma, resulting in powdery precipitates. To optimize the extraction procedure, freeze-cold methanol-acetonitrile (15:85, v/v) was used based on a previous study with other drugs[49]. However, GEN showed higher recoveries by modifying the solvent to 20:80 (v/v). Evaluation of various volumes of the crashing solvent from 100 to 200 µL resulted in the selection of 175 µL as the optimal solvent volume for extraction. When further increasing the amount of methanol in the mixture, no considerable improvements in the recoveries of the analytes were observed. However, it was found that using only methanol - acetonitrile was insufficient for the complete extraction of the analytes from the erythrocyte membranes. Next, the influence of NH_4OH and $CuSO_4$ was tested, as they may influence one another in the extraction procedure. The use of 5% NH_4OH is compatible with MS and has a favorable impact on the deprotonation of essential compounds from WB[50]. So, a diluted NH_4OH was selected as a pretreatment of the GEN, the solution can shift the GEN balance toward its undissociated form, resulting in a higher extraction yield of[50,51]. Adding divalent metal ions (such as zinc and copper) with methanol or acetonitrile is mandatory in WB assays of TAC, as it involves hemolysis and deproteination of TAC based on a reference method[25,27,28]. It was discovered that TAC was extracted efficiently when the sample was treated with a low concentration of divalent metal ion MeOH – 3 mM $CuSO_4$ (4:1, v/v) compared with the previous methods that utilized a high concentration of divalent metal ion. Since $CuSO_4$ is nonvolatile, a lower concentration will decrease the instrument maintenance and thus increase its lifespan.

Finally, the extraction step from WB involved the addition of an RBC lysis buffer (ammonium chloride, potassium carbonate, and EDTA). Different volumes of lysis buffer, including 0, 5, 10, 20, and 30 µL, were tested to find the optimal amount that enhanced the drug extraction. It was found that the extraction recovery improved by adding 20 µL lysis buffer. This is logical since an increase in the lysis of RBCs will enhance the drug extraction.

The optimum extraction recovery of not less than 95.2% (GEN) and 93.2% (TAC) was achieved when the mixture was pretreated with 80 µL of freezer-cold methanol – 3 mM $CuSO_4$ (4:1, v/v) solution containing 5% NH_4OH to induce cell lysis. Next, 175 µL of a freeze-cold mixture of acetonitrile - methanol (4:1, v/v) was added for protein precipitation, followed by 20 µL of RBC lysis buffer.

Method validation

Selectivity

Based on the comparison between total ion chromatograms (TIC) of blank samples and spiked WB samples, no interfering peaks were observed from the endogenous WB substances at the retention times of GEN (1.2 min), TAC (5.0 min), and IS (4.1 min) in all six WB blank samples Fig. 2a,b shows that the LLOQ signals for the analytes were at least five times higher than the blank signals. Additionally, the signal of the IS was at least twenty times higher than the blanks. Thus, the selectivity of the method was confirmed..

Lower limit of quantification (LLOQ)

The study found that the LLOQ of GEN and TAC were 19.5 and 1.95 ng/mL, respectively. These concentrations were evaluated practically. The proposed method was found to have lower LLOQ values, which is crucial for TDM, compared with previously published methods for the determination of GEN[36,39,40,52] or TAC[25,53–56]. Although some of the previous reports showed even lower LLOQ values for GEN[41,57,58] and TAC[26,27,29], our method was considered to be sensitive enough for its purpose.

Linearity

Linearity assessment involved eight matrix-based calibration solutions, each analyzed in triplicate. To plot the calibration curves, the ratio of the peak area of each analyte to the IS was used against the concentration of the analyte. Table 1 displays the linearity data of the three independent calibration curves for determining the two analytes simultaneously using the proposed method. The relationship was found to be linear according to the regression equations over a concentration range of 19.5–2500 ng/mL for GEN and 1.95–250 ng/mL for TAC, with determination coefficients not less than 0.9994 for GEN and 0.9995 for TAC. The 95% confidence intervals of the intercepts included all zero, so they were insignificant.

Precision and accuracy

Results for the intra- and inter-day precision and accuracy of the four QC concentration levels were summarized in Table 2. The intra-day accuracy of GEN and TAC was 103.4 and 104.1%, respectively, for the LLOQ and 95.3–99.4% for all other concentrations of the two drugs. The intra-day precision (%RSD) was ≤ 3.6% for all concentrations of both drugs. For inter-day accuracy and precision, it was found that the inaccuracy was within ± 6.6%, and RSD was ≤ 6.5% for all concentrations of both GEN and TAC, including their LLOQ. These results indicated that the intra- and inter-day precision and accuracy were well below 15% for a wide concentration range of GEN and TAC, including LLOQ, further demonstrating the proposed method's high accuracy and precision Fig. 2. c shows a representative chromatogram of the medium QC of GEN and TAC in WB.

Extraction recovery and matrix effect

The mean extraction recovery values were consistent and repeatable for the two analytes. The results (Table 3) ranged from 96.4 to 104.2% for GEN and 95.5–101.5% for TAC. It was found that AZM (IS) could also provide an acceptable extraction recovery (ER) ranging from 98.7 to 102.4%, which was evaluated by using the concentration utilized in the analysis. The mean IS-normalized ER % values were within acceptable limits at each level. Table 4 presents the results of the matrix effect on the determination of GEN, TAC, and IS. The mean IS-normalized values for GEN and TAC were close to 100%. So, no substantial matrix effect was observed for WB samples. This enables trace analysis of the analytes in actual samples.

Carry-over

The present study indicated that carry-over of GEN and TAC in blank samples injected directly after the elution of highly concentrated WB samples at the corresponding retention times was less than 14.1% ±2.6 and 19.2% ±1.7 of their recorded values at LLOQ, respectively. In comparison, the carry-over of IS was less than 1.1%. This indicates that the proposed method has an acceptable carry-over effect, as illustrated in Fig. 2a.

Dilution integrity

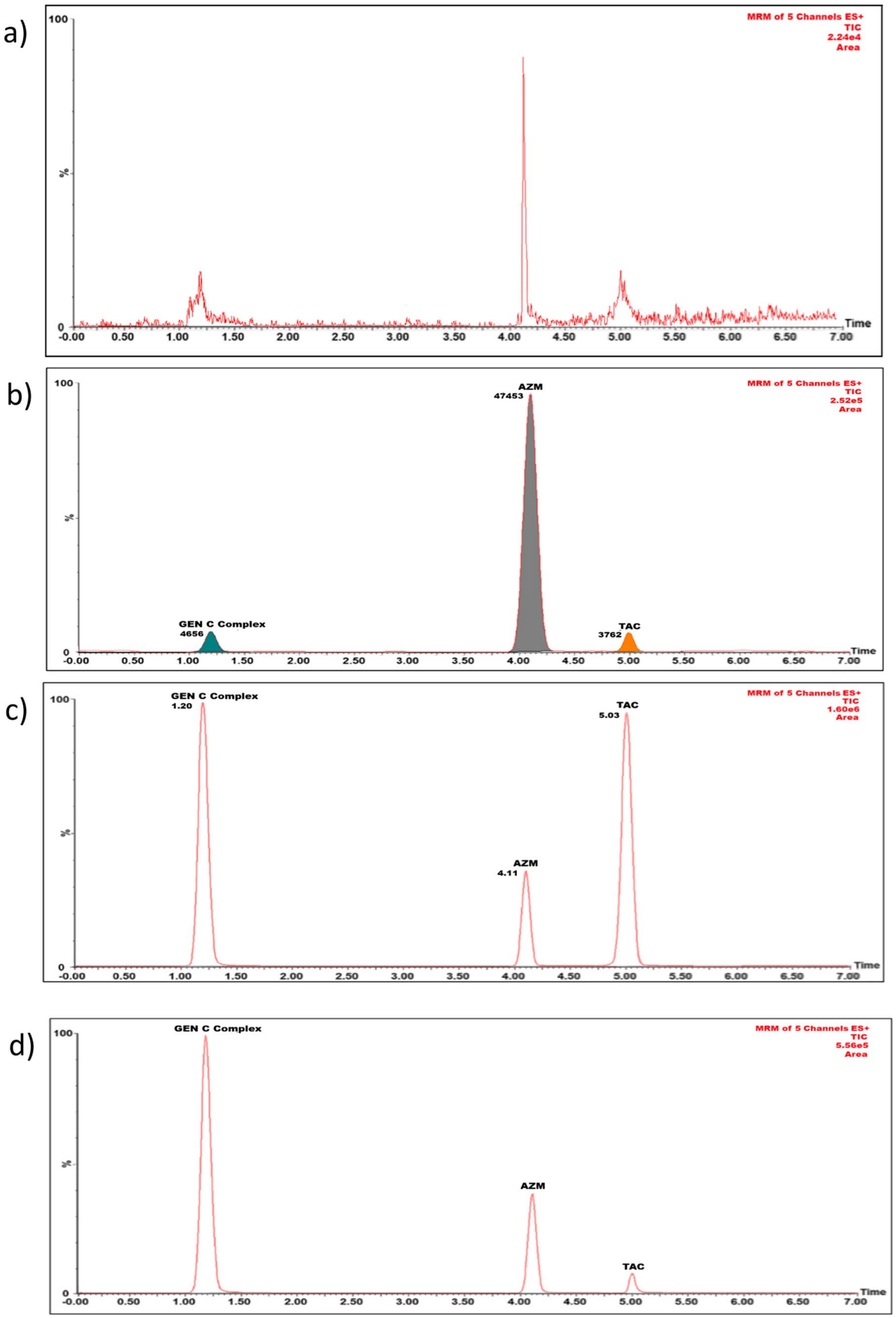
The dilution integrity of the two analytes was assessed by diluting samples ($n = 6$) prepared at a concentration up to 4 times the ULOQ, followed by 4-fold and 8-fold dilutions. According to Table 5, the diluted samples had acceptable recovery and deviation values, with all values falling within the acceptance limits of ± 15%.

Stability studies

The stability of GEN and TAC was assessed under various storage and treatment conditions, as outlined in the validation section. According to Table 6, the mean and RSD of the average concentration at each QC level fell within the acceptance range of ± 15% compared with the freshly prepared QC samples at zero time. This indicates that no significant degradation occurred, and all analytes remained stable throughout the studied conditions. Additionally, the stock solutions of the analytes remained stable when refrigerated at 4 °C for at least two months.

Application of the method to rat WB samples

The developed LC–MS/MS method's applicability was effectively evaluated by analyzing WB samples from 3 rats treated once daily with a TAC dose of 3.2 mg/kg every 24 h for six days through an oral gavage needle (from TAC capsules, weight equivalent to 32 mg dissolved in 50 mL of water). On the sixth day, the three rats were treated once with GEN at a dose of 20 mg/kg body weight through subcutaneous injection (from 100 mg/ml GEN drug solution, a concentration of 20 mg/5 mL was prepared in 25 mL 0.9% NaCl (normal saline)). Both agents were administered at a volume of 5 mL/kg per day. Blood samples (0.2 mL) were drawn immediately from each rat



through the tail vein before dosing (time 0) and at different time intervals (0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8 h). After collection, the samples were stored in the freezer at −80 °C and thawed at RT for 10 min before analysis. Each sample was then vortexed for 10 s. Next, 46 µL of the sample was pipetted and treated as specified under sample preparation. The sample was freshly prepared before injection. Figure 2d shows a representative chromatogram

◀**Fig. 2**. Total ion chromatogram (TIC) of (**a**) blank WB sample, (**b**) WB sample spiked with GEN and TAC at their LLOQ level, together with AZM (IS), (**c**) WB sample spiked with a standard mixture of 950 ng/mL of GEN and 95 ng/mL of TAC, together with AZM (IS), (**d**) and WB sample of treated rats collected 30 min after a co-administration of multiple oral doses of TAC (3.2 mg/kg) and one dose of subcutaneous injection of GEN (20 mg/kg).

Analyte	Intercept	Slope	Determination coefficient (r^2)	Recoveries by back calculation (%)
Gentamicin	0.0041	0.0129	0.9994	97.5–107.5
	0.0040	0.0125	0.9997	94.2–106.4
	0.0041	0.0127	0.9995	97.6–105.2
Tacrolimus	0.0357	0.027	0.9997	94.2–105.5
	0.0353	0.029	0.9996	95.2–103.7
	0.0351	0.026	0.9995	93.7–102.0

Table 1. Results of 3 independent calibration curves for GEN and TAC in rat WB.

Analyte	Concentration spiked (ng/mL)	Intra-day accuracy (%)	Intra-day precision (%)	Inter-day accuracy (%)	Inter-day precision (%)
		n = 6		*n* = 18	
Gentamicin	19.5 (LLOQ)	103.4	2.6	104.3	5.2
	60 (low QC)	97.2	2.3	94.5	4.5
	950 (medium QC)	96.8	3.5	96.3	3.2
	1900 (high QC)	99.4	3.6	99.7	2.4
Tacrolimus	1.95 (LLOQ)	104.1	1.7	106.6	6.5
	6 (low QC)	97.8	2.1	99.3	4.3
	95 (medium QC)	95.3	3.4	97.7	2.4
	190 (high QC)	96.8	2.7	97.3	2.6

Table 2. Intra- and inter-day precision and accuracy for the UHPLC–MS/MS analysis of GEN and TAC with AZM (IS).

Analyte	Concentration spiked (ng/mL)	Extraction recovery (mean, *n* = 6)			
		ER analyte%	ER IS%	IS-normalized ER %	%RSD
Gentamicin	LLOQ	104.2	102.4	101.8	4.9
	Low QC	96.8	101.7	95.2	2.3
	Medium QC	96.4	99.0	97.4	5.1
	High QC	97.5	98.7	98.8	3.9
Tacrolimus	LLOQ	95.5	102.4	93.2	7.3
	Low QC	101.5	101.7	99.8	4.9
	Medium QC	96.0	99.0	96.9	4.5
	High QC	100.8	98.7	102.1	2.4

Table 3. Extraction recovery (ER) of GEN, TAC, and IS in rat WB.

Analyte	Concentration spiked (ng/mL)	Matrix effect (mean, *n* = 6)			
		ME analyte%	ME IS%	IS-normalized ME %	%RSD
Gentamicin	Low QC	106.7	107.0	99.7	7.1
	High QC	108.9	110.4	98.6	3.2
Tacrolimus	Low QC	108.5	107.0	101.4	6.5
	High QC	112.8	110.4	102.2	4.1

Table 4. Matrix effect (ME) of GEN, TAC, and IS in rat WB.

Dilution	Gentamicin		Tacrolimus	
	Mean recovery (%)	%RSD	Mean recovery (%)	%RSD
1:8	101.4	2.7	103.2	2.2
1:4	98.7	3.5	103.0	3.7

Table 5. Accuracy and precision data for Dilution integrity for GEN (starting from 10,000 ng/mL) and TAC (starting from 1000 ng/mL) in rat WB: mean recovery (%) and % RSD of six determinations.

Storage conditions	Concentration level	Gentamicin		Tacrolimus	
		% mean	% RSD	% mean	% RSD
Room temperature (25 °C, 24 h)	low QC	99.5	2.2	93.46	2.6
	high QC	101.3	1.3	104.1	3.7
Refrigerator (4 °C, 24 h)	low QC	99.5	3.7	94.4	3.5
	high QC	98.6	2.7	100.7	3.3
Long term stability (– 20 °C, 30 days)	low QC	96.1	6.1	95.3	6.6
	high QC	96.9	5.6	102.2	6.4
Long term stability (– 80 °C, 30 days)	low QC	100.9	1.7	95.5	1.4
	high QC	101.7	1.5	102.9	1.5
Freeze thaw stability (– 80 °C, 3 cycles)	low QC	95.6	7.2	108.2	4.3
	high QC	96.5	6.3	92.6	2.1
Autosampler stability (5 °C, 72 h)	low QC	104.1	3.2	94.7	1.5
	high QC	103.8	3.1	102.5	2.8

Table 6. Stability results of GEN and TAC in rat WB.

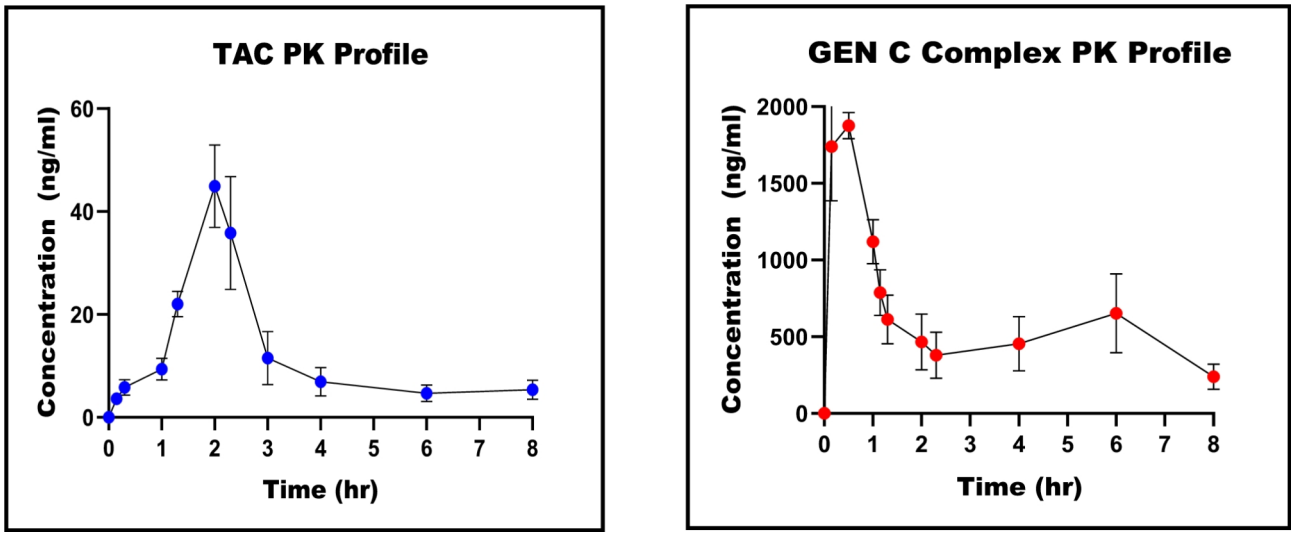


Fig. 3 . The concentration-time profile of the studied drugs in rats after co-administration of multiple oral doses of TAC (3.2 mg/kg) and one dose of subcutaneous injection of GEN (20 mg/kg).

of WB samples collected 30 min after co-administration of GEN and TAC. No potentially interfering peaks at GEN and TAC retention times were observed in the analyzed samples. Ten-time points for each rat were tested. All the concentrations obtained from the rat sample were above the LLOQ and within the calibration range. The concentrations of GEN and TAC in rats' WB at different sampling time points were illustrated graphically, as shown in Fig. 3. The highest WB concentration for GEN was 1876.5 ng/mL at 30 min, while for TAC, the highest concentration was 45 ng/mL at about 2.00 h of administration.

Conclusion

We have developed the first simple UHPLC–MS/MS method for bioanalytical quantitation of GEN and TAC simultaneously in rat WB. The analysis time was only 6 min. This is also the first reported method for

quantitatively determining both GEN and TAC in rat WB using LC–MS. The sample preparation involved protein precipitation, requiring only 50 µL of WB sample. The proposed method was fully validated following the EMA guideline. The UHPLC method's applicability for GEN and TAC in rat WB was demonstrated. In the future, it can be applied to study the pharmacokinetic (PK) parameters that can be altered due to the combined use of GEN and TAC, compared to their single administration.

Experimental

Materials and reagents

TAC was purchased from Sandoz Lek (Ljubljana, Slovenia), azithromycin (AZM) from Sigma-Aldrich (St. Louis, MO, USA), and GEN from Haoyuan ChemExpress (Shanghai, China). Acetonitrile and methanol, both UHPLC grade, were from AppliChem Panreac (Darmstadt, Germany). Ammonium hydroxide, for HPLC, 35% solution in water, was from Fisher Scientific (Oslo, Norway), and copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) was purchased from Merck Life Science (Merck KGaA, Darmstadt, Germany). Heptafluorobutyric acid (HFBA) was purchased from Rhawn (Beijing, China). Formic acid and ammonium formate were purchased from Sigma-Aldrich. The red blood cell (RBC) lysis solution was from QIAGEN Sciences (Germantown, MD, USA). Ultrapure water for LC analysis was produced with a Millipore Milli-Q Plus 185 purification system (Milford, MA, USA). Argon gas of 99.99% purity was obtained from Alkhafrah holding company (Riyadh, SA).

Instrumentation

Analysis was performed using a Waters® Acquity H-Class UPLC® tandem triple-quadrupole mass spectrometer (TQD) (Milford, MA, USA). The instrument was coupled with a quaternary solvent manager (Acquity H-UPLC class) and sample manager-FTN (Acquity UPLC). The TQD detector was equipped with an electrospray ionization (ESI) probe. The system was controlled with Masslynx Version 4.2 software to process the data. The Targetlynx application manager further automated the processing, acquisition, and data collection. IntelliStart® assisted the mass tuning, and the tuning parameters were optimized manually in both LC and fluidics modes to optimize the peak parameters. The instrumentation included a rotary pump (Sogevac, Paris, France) to aid the vacuum and a nitrogen generator (Peak Scientific, Scotland, UK) to supply desolvation gas.

Chromatographic conditions

Chromatographic analysis was carried out using a binary mobile phase. Eluent A contained 2 mmol/L ammonium formate acidified with 0.1% formic acid in water, and eluent B was a mixture containing 2 mmol/L ammonium formate and 0.1% formic acid in methanol. The gradient program is shown in Table 7.

The column applied for the analysis was an Acquity UPLC BEH™ C18 column (50 mm × 2.1 mm i.d., 1.7 µm particle size) (Waters, Kildare, Ireland) and was maintained at 50 °C in a column oven. The analytical column was protected with an Acquity UPLC®BEH VanGuard Pre-column (5 mm × 2.1 mm, 1.7 µm). The auto-sampler temperature was maintained at 4 °C. Volumes of 20 µL were injected into the system using partial loop mode. The flow rate was 0.25 mL/min.

Mass spectrometric conditions

The positive electrospray ionization (ESI^+) mode was used. GEN and internal standard (IS) were quantified by choosing a protonated molecule $[M+H]^+$ using MS/MS in the multiple reaction monitoring (MRM) mode to detect product ions by fragmentation mass analysis at ion transitions *m/z* 478 → 157 for GEN C_1, 450 → 322 for GEN C_{1a}, 464 → 160 for GEN C_2/C_{2a}, and 749 → 592 for IS (AZM). It seemed that the GEN components can be identified by the appearance of product ions at *m/z* 160, 205, and 322. Additionally, characteristic product ions for GEN C_1, C_{1a}, and C_2/C_{2a} were observed at *m/z* 157, 129, and 143, respectively. TAC was quantified with the ammonium adduct ion $[M+NH_4]^+$ generated from ESI^+ and MRM mode at ion transition *m/z* 821 → 768. The product ion with the highest intensity was chosen for quantification. The different mass spectra are shown Fig. 4.

Different detection parameters were chosen to optimize ionization efficiency, fragmentation pattern, resolution, and peak shape, thus achieving the highest protonated precursor and product ions response. Key parameters include source temperature, desolvation and cone gas flow rates, desolvation temperature, capillary voltage, cone voltage, and collision energy. Higher ion source temperatures improved electrospray ionization (ESI) efficiency, producing more abundant ion signals and sharper peaks. Elevating the desolvation temperature effectively minimizes solvent and matrix interferences, which enhances signal detection for low-concentration analytes and thereby increases the sensitivity of the compounds. Cone voltage allows precise control of fragmentation in MS/MS experiments, leading to specific fragment ions essential for compound identification and structural elucidation. The capillary voltage was properly optimized to enhance analyte ionization, producing more abundant ions and better sensitivity. Adjusting collision energy was crucial in determining the degree of

Time (min)	Mobile phase (A)	Mobile phase (B)
Initial	97	3
2	97	3
3	3	97
5	3	97
7	97	3

Table 7. Gradient scheme of the mobile phases.

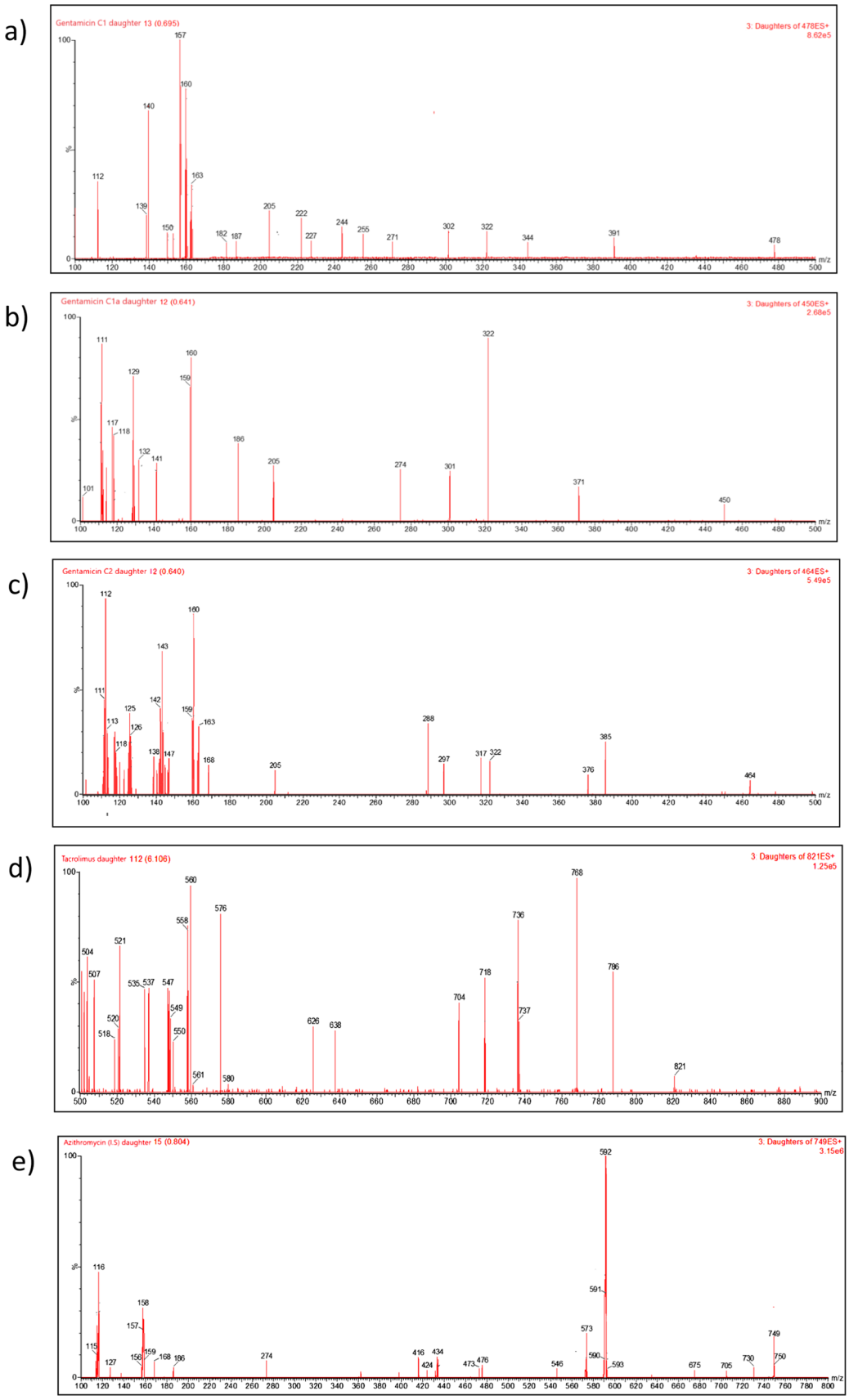


Fig. 4. Product ion spectra of (**a**) GEN C1, (**b**) GEN C1a, (**c**) GEN C2, (**d**) TAC, and (**e**) AZM.

MS/MS Condition	GEN C1	GEN C1a	GEN C2	TAC	IS
Precursor ion (m/z)	478	450	464	821	749
Product ion (m/z)	157	322	160	768	592
Cone voltage (V)	24	20	20	35	30
Collision energy (eV)	24	14	26	22	25

Table 8. MS parameters of GEN, TAC, and IS.

fragmentation in mass spectrometry. Optimizing this energy allows sufficient fragmentation while preserving the molecular ion for accurate identification. An ion source temperature of 150 °C was applied. The desolvation and cone gas flow rates were 900 and 20 L/h, respectively. The desolvation temperature was 450 °C. The capillary and cone voltages were 3.05 kV and 50 V, respectively. The MS analyzer was set for ion energy 1 and 2 at the following resolutions: low mass resolution (LMR) of 10 and high mass resolution (HMR) of 15. Ion energy 1 (IE_1) was 0.5, and IE_2 was 3.0. The dwell time of all compounds was set as 0.05 s, according to each compound's retention time and peak width, to improve the analyte's response. The optimum values of MS parameters for each compound, including cone voltage (V) and collision energy (eV), were illustrated in Table 8.

Preparation of standard solutions and construction of calibration curves

Stock solutions

A stock solution of GEN (2,000 µg/mL) was prepared by dissolving the accurately weighted powder in water. Stock solutions of TAC (1,000 µg/mL) and IS (1,000 µg/mL) were prepared separately by dissolving an appropriate amount in methanol. All solutions were stored at 4 °C and were stable for at least two months.

Intermediate solution

Individual solutions of GEN (200 µg/mL) and TAC (20 µg/mL) were prepared by diluting the stock solutions with water and acetonitrile, respectively. The stock solution of IS was diluted with methanol to give a concentration of 50 µg/mL. A mixed solution of GEN and TAC was prepared weekly from the respective intermediate solutions by pipetting 2 mL from each intermediate solution into a 5 mL volumetric flask to obtain concentrations of 80 µg/mL and 8 µg/mL of GEN and TAC, respectively. The volume was made up using a diluent consisting of water - acetonitrile (50:50, v/v).

Working solutions

Those were obtained from the mixed solution, which was serially diluted using water - acetonitrile (50:50, v/v) as a diluent to attain different concentrations: 40, 20, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15 µg/mL of GEN and 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0.015 µg/mL of TAC. The IS working solution (10 µg/ml) was prepared by pipetting 1 mL of IS intermediate solution into a 5 mL volumetric flask and diluting to volume with acetonitrile. The solution was kept in a freezer at -20 °C for 10 min before use. Using this ice-cold organic solvent can be advantageous as it has the potential to enhance the efficiency of protein removal. Working solutions were used to prepare GEN and TAC's calibration and quality control (QC) samples together.

Calibration and quality control (QC) samples

Calibration solutions were obtained by spiking water or blank WB samples with suitable amounts of working solutions to obtain eight different concentration levels of 19.5, 39, 78, 156.3, 312.5, 625, 1250, 2500 ng/mL for GEN, and 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250 ng/mL for TAC. For GEN, the final concentration was determined as the sum of all individual congeners (gentamicin C_1, $C_{1a,}$ and C_2/C_{2a}). QC samples were run for accuracy and precision at four different concentration levels for GEN: the lower limit of quantification (LLOQ) (19.5 ng/mL), low (60 ng/mL), medium (950 ng/mL), and high (1900 ng/mL) level, whereas QC samples for TAC were prepared to achieve the following four concentration levels: LLOQ (1.95 ng/ml), low (6 ng/ml), medium (95 ng/ml), and high (190 ng/ml).

Blood sample preparation

WB was withdrawn from male healthy Wistar rats weighing 200 ± 30 g (rats obtained from the animal house, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia) and collected in K_2EDTA. These blank blood samples were immediately stored at − 80 °C after withdrawal. The animals were housed at room temperature (25°C) and an average relative humidity of 50%. Animals were euthanized through asphyxiation in a controlled saturated CO2 chamber. All methods were carried out in accordance with relevant guidelines and regulations and was approved by the Research Ethics Committee (REC) at the King Saud University (authorization number: KSU-SE-23-46) and the Ethical Committee of KU Leuven M-number (M019/2023). All methods are reported in accordance with ARRIVE guidelines."

The frozen blood (in K_2EDTA) was thawed at room temperature. The calibration curve of the drug mixture was constructed by spiking an aliquot of 50 µL of WB samples with 46 µL of the previously prepared working solutions. Next, the mixture was pretreated with 80 µL of freezer-cold methanol – 3 mM copper sulfate (4:1, v/v) solution containing 5% NH_4OH to induce cell lysis, followed by vortex for 10 s. Later, protein precipitation was performed by adding 175 µL of − 20 °C acetonitrile—methanol (4:1, v/v) containing IS (IS working solution), followed by vortex for 10 s. Finally, 20 µL of RBC lysis buffer was added. Then, each sample was vortexed for 10 s and sonicated in ice-cold water for 20 s. Sonication was an essential step in ensuring the proper recovery of the

drugs by breaking down sample components. After centrifugation at 1500 g for 15 min, at a temperature of 4 °C, a 200 µL aliquot of the supernatant layer was separated and transferred to a new Eppendorf tube and diluted with 200 µL of 60 mM HFBA solution to obtain a final concentration after extraction of 19.5–2500 ng/mL for GEN, 1.95–250 ng/mL for TAC and 2350 ng/mL for IS.

Method validation

The sample pretreatment method was validated according to the EMA guideline for bioanalytical method validation[44]. As part of the validation process, blank WB samples were spiked with the analytes and IS. The method validation included several aspects such as selectivity, linearity, accuracy, precision, carry-over, matrix effect, extraction recovery, and stability to ensure its reliability.

Selectivity

To test the method's selectivity, WB blanks of six rats were analyzed individually to ensure no endogenous components in the matrix or any other components in the sample interfered with the analytes and IS results. It is commonly accepted that there are no interfering components when the response is less than 20% of the lower limit of quantification for the analyte and 5% for IS.

Calibration curve

Evaluating the instrument's response based on the concentration of the analytes within a specific range is essential. To check this, calibration standards at eight concentration levels were created by adding known concentrations of GEN and TAC to the sample matrix. For bioanalytical method validation, it is recommended to calculate at least three calibration curves. This will help to ensure that the instrument provides reliable and consistent results. The linearity curve determines the relationship between the peak area ratios of each drug to IS and the corresponding spiked concentration. The calibration graphs determination coefficient (R^2), slope, and intercept values have been determined. Additionally, the back-calculated concentration of the three calibration curves will be presented, and the deviation should be at most 15% of the nominal value, except for the LLOQ, for which it should be at most 20%.

Lower limit of quantification (LLOQ)

LLOQ values were selected for the two analytes based on the baseline noise method. It was ensured that the concentrations at LLOQ would generate analytical signals of at least five times as much as blank signals. Moreover, the accuracy and precision of the quantification were also taken into account, with error values ≤ 20% for accuracy and relative standard deviation (RSD) values ≤ 20% for precision.

Accuracy and precision

The accuracy and precision of intraday measurements were assessed by analyzing four different QC concentration levels within the calibration curve range (LLOQ, low QC, medium QC, and high QC) on the same day. Each drug's measured QC concentration value was calculated using freshly prepared solutions. Inter-day experiments of accuracy and precision were evaluated after repeated analysis of freshly prepared QC samples at the same concentration levels, and each level was analyzed in six runs on three different days. The accuracy was derived based on the deviation from the nominal value and expressed as % mean recovery. Precision was evaluated by the RSD values obtained from within- and between-day performance. Based on the EMA guideline[44], it is recommended that the % mean recovery of the low, medium, and high QC samples is between 85% and 115%, while the LLOQ must be within 80–120%. The RSD of all QC samples, except for LLOQ, should not exceed ± 15%. For LLOQ, RSD should not exceed ± 20%.

Carry-over effect

Testing for carry-over during method validation is essential to ensure accurate results. This can be done by injecting the upper limit of quantification (ULOQ) sample, followed by a blank WB sample, and checking for analyte peaks in the blank chromatogram. The carry-over should be at most 20% of the LLOQ and 5% of the IS.

Extraction recovery and matrix effect

The extraction recovery was determined for six lots of blank samples, each spiked with four QC concentration levels, by comparing the mean area response of the extracted analytes (which were spiked before extraction) to those obtained from blank WB spiked with the analytes post-extraction at the same concentration level. The recovery of the analytes should be consistent and repeatable. Furthermore, IS extraction recovery was also calculated at the specified concentration. Evaluating the matrix of biological samples is extremely important since the matrix may suppress or enhance the ionization of the analyzed drugs. The biological matrix effect was evaluated by obtaining blank rat blood samples from six animals. To determine the matrix factor (MF) for GEN, TAC, and IS, the peak area in the presence of the matrix was calculated. The blank matrix was first pretreated. Next, a specific amount of IS and low/high QC samples were spiked to the supernatant. Then, the ratio of this peak area to the peak area in the absence of a matrix (i.e., pure solution of GEN, TAC, and IS) will be calculated. Additionally, the IS normalized MF was calculated by dividing each MF of GEN and TAC by the MF of IS. The RSD should not exceed 15% for the IS-normalized MF for both concentration levels.

Dilution integrity

Dilution integrity ensures that the analyte is accurately measurable after dilution to bring its concentration within a range of valid values for analysis when samples contain a concentration more than the ULOQ of the analytes of interest. Spiked WB samples were prepared at 10,000 and 1000 ng/mL concentrations for GEN and

TAC, respectively. The dilution integrity of the analytes was assessed by a 4-fold and an 8-fold dilution with blank WB. Samples were analyzed six times ($n = 6$), and the % mean recovery and RSD % were then calculated.

Stability studies
A stability test should confirm that sample preparation and storage at various temperatures do not affect the analyte concentration. This experiment tested the stability of GEN and TAC's low and high QC concentrations under different storage conditions, including freezing and thawing, short-term, long-term, and autosampler stability. Recovery of low and high QC samples should be within 85–115%, based on the EMA guideline[44].

Freeze and thaw stability
Low and high QC WB samples were frozen at − 80 °C without performing the extraction process for 12 h. After the first cycle, samples were thawed at room temperature (25 °C) for 45 min and frozen again. Two more freeze-thaw cycles were performed in the same conditions. After the third cycle, the thawed samples were extracted and immediately analyzed. The results were compared against freshly spiked QC samples (zero cycles).

Short-term stability
Short-term stability was investigated by testing low and high QC samples after leaving part on a laboratory bench for 24 h at room temperature (25 °C) and part in the refrigerator (4 °C) for 24 h. The recoveries of the samples were checked against freshly prepared QC samples.

Long-term stability
Long-term stability was assessed over one month. Low and high-QC WB samples were initially extracted and analyzed before storage to determine zero-time values. Next, the remaining spiked samples were placed in Eppendorf tubes and stored in the freezer for 30 days at − 20 °C and − 80 °C. Finally, samples were thawed and extracted to be analyzed. Comparisons were made between the resultant and recorded concentrations at zero time.

Autosampler stability
The stability of extracted low and high QC samples was assessed when they were left in the autosampler (4 °C) for a period that was expected to be the maximum waiting time before injection (~72 h). The results were compared to the nominal values from the extracted QC samples at zero time.

Data availability
The authors declare that the data supporting the findings of this study are available within the paper. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

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Author contributions

Erwin Adams and Aliyah Almomen designed and supervised the whole work. Shrooq Altaweel did the animal work, carried out the practical experiments, analyzed the data, and wrote the manuscript. Erwin Adams, Aliyah Almomen, and Ann Van Schepdael revised the manuscript and gave critical comments.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to E.A. or A.A.