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Highly sensitive UPLC-MS/MS method for the quantification of paromomycin in human plasma

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ABSTRACT

A highly sensitive method was developed to quantitate the antileishmanial agent paromomycin in human plasma, with a lower limit of quantification of 5 ng/mL. Separation was achieved using an isocratic ion-pair ultra-high performance liquid chromatographic (UPLC) method with a minimal concentration of heptafluorobutyric acid, which was coupled through an electrospray ionization interface to a triple quadrupole - linear ion trap mass spectrometer for detection. The method was validated over a linear calibration range of 5 to 1000 ng/mL ($r^2 \geq 0.997$) with inter-assay accuracies and precisions within the internationally accepted criteria. Volumes of 50 μL of human K_2EDTA plasma were processed by using a simple protein precipitation method with 40 μL 20 % trichloroacetic acid. A good performance was shown in terms of recovery (100 %), matrix effect (C.V. ≤ 12.0 %) and carry-over (≤17.5 % of the lower limit of quantitation). Paromomycin spiked to human plasma samples was stable for at least 24 h at room temperature, 6 h at 35 °C, and 104 days at −20 °C. Paromomycin adsorbs to glass containers at low concentrations, and therefore acidic conditions were used throughout the assay, in combination with polypropylene tubes and autosampler vials. The assay was successfully applied in a pharmacokinetic study in visceral leishmaniasis patients from Eastern Africa.

1. Introduction

Paromomycin (aminosidine) is an antimicrobial drug from the aminoglycoside branch. It is the only aminoglycoside with a clinically important anti-leishmanial activity and was discovered in 1963 in the USSR [1]. Paromomycin was primarily used as an antibiotic against gram-negative bacteria in humans before modern-day antibiotics were discovered and is still being used as a veterinary drug [2]. Since 2006, intramuscular paromomycin has been licensed for the treatment of the neglected tropical disease visceral leishmaniasis in India. High efficacy rates in a series of clinical trials in India and Eastern Africa, also in combination with sodium stibogluconate, combined with a good cost-effectiveness led to widespread implementation of this drug to treat visceral leishmaniasis [3–5].

Chemical characteristics of aminoglycosides include a high polarity, absence of hydrophobic side chains and poly-ionic charge in aqueous environments, which lack interaction with traditional reversed-phase (RP) liquid chromatography (LC) [6]. Hydrophilic interaction liquid chromatography (HILIC) [7,8], zwitterionic-HILIC (ZIC-HILIC) [9–11], derivatization of paromomycin [12] or ion-pairing (IP) combined with an RP stationary phase chromatographic system [13–16] have been reported in the analysis of paromomycin. Internal standards used in these bioanalytical assays were synthesized permethylated aminoglycosides (spectinomycin, dihydrostreptomycin and kanamycin A) or chemical analogues (various other aminoglycosides).

Published bioanalytical assays of paromomycin in human plasma have relatively high lower limits of quantification (LLOQ) of 50 [17], 100 [9] and 500 ng/mL [12], require larger sample volumes and involve labour-intensive analyte extraction (e.g. solid phase extraction (SPE) and liquid-liquid extraction (LLE)). Using these existing methods, trough concentrations and the terminal elimination rate could not be quantified previously due to a lack of sensitivity. A sensitive method to quantitate paromomycin

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in human plasma is therefore required to adequately perform pharmacokinetic studies to improve paromomycin-based dosing regimens, particularly in combination therapies. Other liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) bioanalytical assays quantifying paromomycin have been reported for use in residue analysis in food such as meat [7,11,13–16,18,19], milk [7,10,11,14,19], and honey [10,19] samples or pre-clinical in mice plasma [8].

The aim of the current investigation was to develop a sensitive and simple method for the quantification of paromomycin in human plasma using a small volume of human plasma and validate it according to current EMA and FDA guidelines [20,21]. To our knowledge, this is the first paromomycin bioanalytical assay fulfilling these requirements and using a stable isotopically labelled internal standard (IS) instead of a chemical analogue. The LLOQ of the assay is 5 ng/mL using only 50 μL of human plasma.

2. Materials and methods

2.1. Chemicals

Paromomycin sulfate and internal standard (multiple deuterated paromomycin acetic acid) were both purchased from Toronto Research Chemicals (North York, Ontario, Canada). The deuterium-label in the deuterated paromomycin IS varied between 0 (D_0) and 7 (D_7) deuterium atoms, with the following distribution: D_0 0.13 %; D_1 0.53 %; D_2 2.47 %; D_3 8.77 %; D_4 18.13 %; D_5 22.23 %; D_6 20.60 %; D_7 13.62 % according to the certificate of analysis. Methanol, formic acid, acetonitrile (ACN) and water (ULC grade) were bought from Biosolve Ltd (Valkenswaard, The Netherlands). Trichloroacetic acid (TCA) (99.5 %) was supplied by Merck Chemicals B.V. (Amsterdam, the Netherlands), heptafluorobutyric acid (HFBA) solution (0.5 M) was from Sigma-Aldrich (Zwijndrecht, the Netherlands). Distilled water used for sample preparation came from B. Braun Medical (Melsungen, Germany). Blank human dipotassium ethylenediaminetetraacetic acid (K_2EDTA) plasma was obtained from BioIVT (West Sussex, United Kingdom).

2.2. Stock solutions and working solutions

Paromomycin stock solutions with a concentration of 0.1 mg/mL (free base) were made in water. Paromomycin stock solutions were separately made for calibration standards and quality control (QC) samples. The stock solutions were diluted in water to obtain working solutions. A stock solution of internal standard was prepared in water with a concentration of 1 mg/mL for the sum of all deuterated paromomycin. The working solution of IS (WIS) was made from dilution of IS stock solution in water to a concentration of 111 ng/mL D_5-paromomycin. The certificate of analysis of deuterated paromomycin estimated the amount of D_5-paromomycin in the sum of total deuterated paromomycin at 22.23 %. D_5-paromomycin is the most abundant isotope form in the deuterated paromomycin mixture. Stock and working solutions were stored at −20 °C.

2.3. Calibration standards, quality control samples

Calibration samples were prepared in a batch prior to validation. The stability of the samples was determined afterwards. Forty μL of working solution is spiked to 760 μL blank human plasma and aliquots of 50 μL were made. QC samples were prepared in batches before storing at −20 °C in aliquots of 50 μL. The stability of these QC samples was tested afterwards with freshly prepared QC samples. Seven non-zero calibration standards were used in the development of this assay. Calibration standards were prepared in concentrations of 5, 10, 25, 50, 100, 500 and 1000 ng/mL with QC samples at concentrations of 5, 15, 300 and 800 ng/mL for QC-LLOQ, QC-LOW, QC-MID, and QC-HIGH, respectively.

2.4. Sample preparation

Human plasma samples (calibration, QC or unknown) were thawed prior to preparing the samples for analysis, aliquots (50 μL) were transferred into 1.5 mL polypropylene reaction tubes. Ten μL of IS working solution was spiked to the aliquots except to the double blank before vortex mixing. Plasma proteins were precipitated by the addition of 40 μL of 20 % (w/v) TCA in water. The samples were vortex mixed and centrifuged at 23,100*g* for 5 min in a cooled down environment set at 5 °C. Fifty μL of clear supernatant was then transferred to a 1.5 mL polypropylene reaction tube and 50 μL distilled water was added to the samples to dilute 1:1 (v/v) yielding a TCA concentration of 4 % (w/v) in the final extract. The final extracts were vortex mixed and transferred to polypropylene autosampler vials prior to analysis.

2.5. LC equipment and conditions

The chromatographic system used was a UPLC LC-30AD pump with an inline degasser connected to a UPLC LC-30AMCP autosampler, set at 4 °C and CTO-20AC column oven (Nexera X2 series, Shimadzu Corporation, Kyoto, Japan). Chromatographic separation was achieved using an Acquity UPLC HSS T3 analytical column (Waters, Milford, MA, USA; 150 mm x 2.1 mm ID, 1.8 μm particles). The column temperature was kept at 40 °C. The purge and strong wash solvent used was 0.1 % formic acid in water/methanol (50:50, v/v). The eluent consisted of 5 mM HFBA in water/ACN (7:3, v/v) mixture (3.5 mM HFBA in the mixture), at an isocratic flow rate of 0.4 mL/min.

2.6. MS equipment and conditions

Detection of paromomycin was performed using a QTRAP 6500 (Sciex, Framingham, MA, USA) quadrupole - linear ion trap MS equipped with a turbo ionspray interface operating in positive ion mode. The mass spectrometer and ionization conditions were optimized to obtain maximal sensitivity for the analyte. The following settings were used: ion source voltage at 5500 V, ion source temperature at 500 °C; ion source gas 1 at 60 psi (4.1 bar); ion source gas 2 at 40 psi (2.8 bar), curtain gas at 25 psi (1.7 bar) and collision gas at 10 psi (0.69 bar). Multiple reaction monitoring (MRM) mode was performed to quantify paromomycin $[M+H]^+$ using the transition of *m/z* 616.6 → 163.1 and the deuterated paromomycin (IS) with *m/z* 621.6 → 165.1 (Fig. 1). Data acquisition and processing were performed with Analyst™ software (Sciex, version 1.6.2).

2.7. Validation procedures

The validation of the assay was performed using the current EMA and FDA guidelines for the validation of bioanalytical assays in plasma [20,21]. The assay was validated for linearity, LLOQ, accuracy and precision, dilution integrity, carry-over, selectivity, matrix effect, recovery and stability under various conditions.

2.7.1. Calibration model and lower limit of quantitation

Seven non-zero calibration standards ranging from 5 to 1000 ng/mL were prepared in duplicate for each run in three separate validation runs. Linear regression was performed on the analyte peak area/IS peak area ratio versus nominal analyte concentration (x), weighted by a weighting factor of $1/x^2$. The deviations from the mean for each non-zero calibration standard should be within ±15 % (±20 % for the LLOQ) for a minimum of 75 % of the non-zero standards.

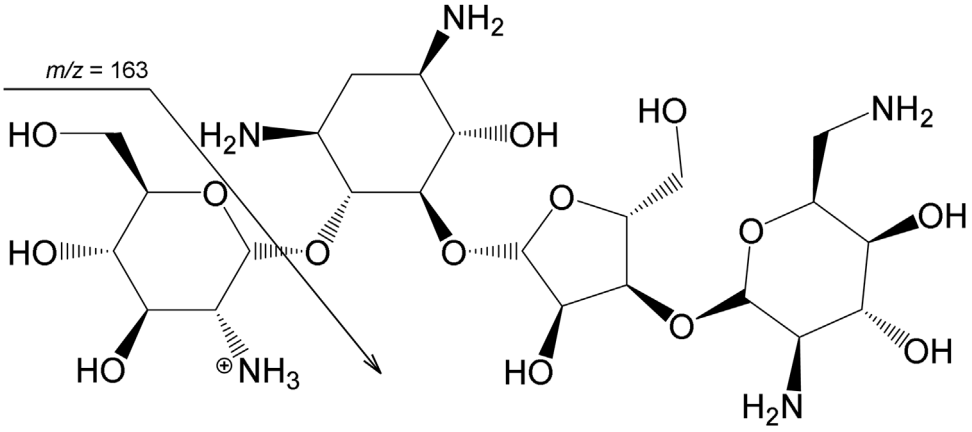

Fig. 1. The chemical structure of paromomycin, including the proposed fragmentation pattern and the mass-over-charge (*m/z*) of the monitored product ion. The fragmentation pattern is indicated by an arrow.

2.7.2. Accuracy and precision

Intra-and inter-assay accuracy and precision were determined by analyzing five replicates of QC samples in three separate validation runs at the LLOQ (5 ng/mL), low (15 ng/mL), mid (300 ng/mL) and high (800 ng/mL) levels. The concentration of each sample was determined using the calibration standards prepared and analyzed in the same batch. The accuracies were expressed as the bias from the nominal concentration and precision was calculated as the coefficient of variation (CV %). The intra-assay bias (%) is the bias of the mean measured concentration per analytical run as compared to the nominal concentration. The inter-assay bias (%) is the bias of the mean measured concentration in three analytical runs compared to the nominal concentration. The inter-run precision was calculated by performing a one-way ANOVA. The accuracy values should be within ±15 % (±20 % at the LLOQ) and precision ≤15 % (≤20 % for the LLOQ).

2.7.3. Carry-over

Carry-over was assessed by injecting two double blank samples after the upper limit of quantification (ULOQ, 1000 ng/mL) of the calibration standards. The peak areas at the retention times of the analyte and its internal standard detected in the double blank samples were compared to the mean area of the analyte and the IS in five QC-LLOQ samples. The carry-over was assessed in three separate validation runs. The peak areas in the double blank samples compared to the QC-LLOQ samples should be ≤20 % for paromomycin and ≤5 % for the IS.

2.7.4. Specificity and selectivity

Specificity and selectivity were evaluated in blank K_2EDTA human plasma batches from six different individuals. Co-eluting peaks at the retention time of the analyte and IS from endogenous interferences were assessed in double blanks and compared to the LLOQ samples from each individual batch. The peak areas in the double blank samples should be ≤20 % compared to the peak areas of the LLOQ samples in each batch and ≤5 % for the IS. The bias of the LLOQ samples should be ±20 % in at least 4 of the 6 human plasma batches. Cross analyte/IS interferences were determined by spiking the analyte at the ULOQ concentration and separately the IS at the IS level. Interference of paromomycin with the IS should be ≤5 % (peak area) and the interference of the IS with paromomycin should be ≤20 % of the analyte peak area compared to the LLOQ samples.

2.7.5. Matrix effect and recovery

Matrix effect and recovery of the assay were determined using blank K_2EDTA human plasma batches from six individuals at two QC levels (QC-LOW and QC-HIGH). The absolute matrix effect factor was calculated as the analyte or IS peak area ratio between these QC samples (matrix present) and matrix absent samples at similar concentration levels. Furthermore, the IS-normalized matrix effect factor was calculated as the ratio between the absolute matrix effect factor of paromomycin and IS. The IS-normalized matrix factor value is accepted ≤15 %. Matrix present samples were prepared by spiking QC working solutions to extracted blank plasma samples. The sample preparation recovery was calculated as the ratio between peak areas of the processed QC samples and spiked matrix samples.

2.7.6. Dilution integrity

Dilution integrity was determined in five-fold by applying a ten-fold, fifty-fold, and hundred-fold dilution of spiked human plasma samples at a concentration of 5000 ng/mL (5 times the ULOQ). Dilution was performed using blank human K_2EDTA plasma. The accuracy and precision were determined with acceptance criteria of ±15 % bias and ≤15 % CV %, respectively.

2.7.7. Stability

Short-term stability in human plasma at QC-LOW and QC-HIGH concentrations was evaluated for 6 and 24 h at 2–8 °C, room temperature and 35 °C (simulated room temperature in tropical regions, in view of future pharmacokinetic studies). Additionally, stability was evaluated in final extracts at 2–8 °C, stock solution and working solutions at −20 °C. Long-term stability in human plasma at QC-LOW and QC-HIGH concentrations stored at −20 °C was evaluated for at least 104 days. The stability after 4 freeze/thaw cycles in human plasma was determined. One freeze/thaw cycle consisted of unassisted thawing at room temperature and subsequently freezing at −20 °C for at least 12 h straight. The acceptance criteria for the precision and accuracy for the human plasma QC samples and final extracts were ≤15 % CV and ±15 % bias, respectively, while for stock and working solutions these were ≤5 % CV and ±5 % bias, respectively.

2.8. Clinical application

This bioanalytical assay was developed to support paromomycin pharmacokinetic studies in visceral leishmaniasis patients. Human plasma K_2EDTA samples from visceral leishmaniasis patients were collected in a clinical trial conducted in Kacheliba Sub County

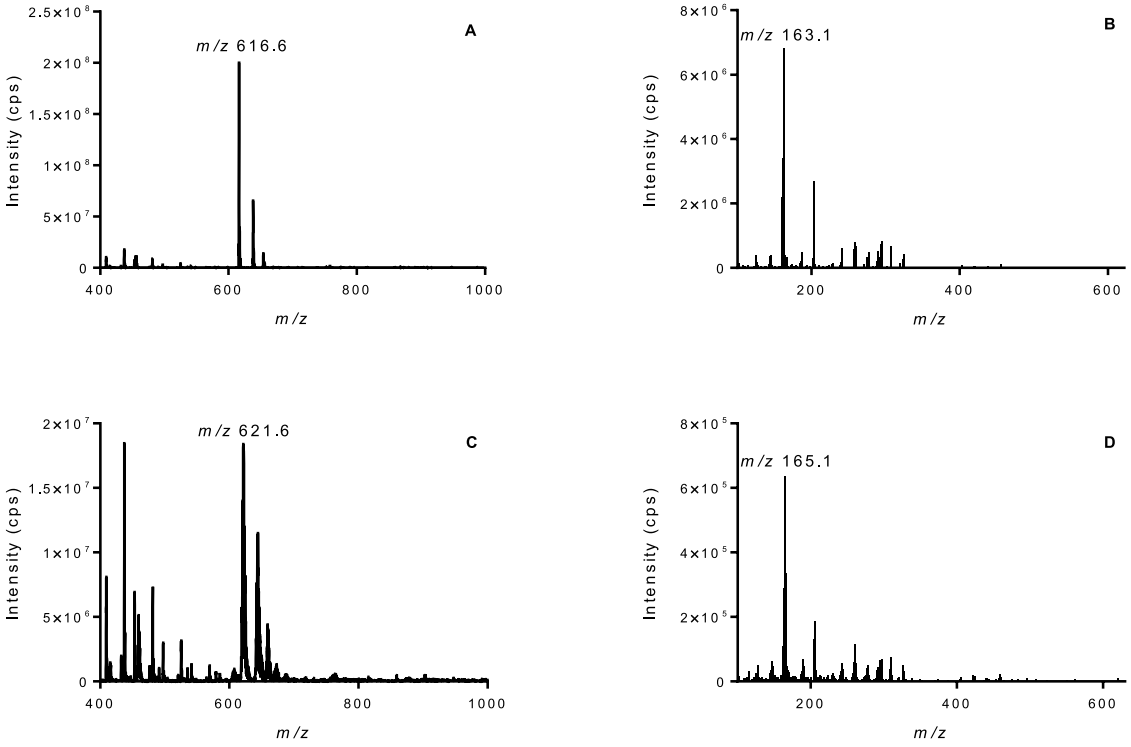

Fig. 2. MS spectrum of paromomycin (A) and its product-ion spectrum (*m/z* 616.6), MS spectrum of D_5-paromomycin C) and its product-ion spectrum (*m/z* 621.6).

Hospital, Kenya. Ethical approval was granted by all relevant institutional and national ethical review committees. Patients were treated with intramuscular injections of 20 mg/kg paromomycin q.d. for 14 days. Plasma samples were taken on day 1 and 14 prior to paromomycin treatment, and after 1, 2, 4/8 and 24 h after administration. Written informed consent was obtained. At scheduled time points, a 2 mL blood sample was obtained via venipuncture. Blood samples were anticoagulated in K_2EDTA containing tubes and plasma was obtained by centrifugation at approximately 2000*g* at room temperature. Separated plasma was immediately (within 60 min of collection) stored at −20 °C. The human plasma samples were eventually transported on dry-ice to the bioanalytical laboratory of The Netherlands Cancer Institute. The human plasma samples were further processed as described in section 2.4.

3. Results and discussion

3.1. Development

3.1.1. Sample preparation

Various sample preparation methods, including protein precipitation, were tested during the development of the bioanalytical assay. Protein precipitation is often performed using organic solvents e.g. ACN and methanol in combination with LC–MS/MS analysis. Paromomycin showed poor solubility in various organic solvent mixtures, therefore a polar protein precipitant agent was desired. Protein precipitation using TCA, a highly acidic and ionic chemical, showed an increase in recovery and sensitivity, as well as a low background noise compared to other precipitants. Given the substantial improvements, TCA was chosen as protein precipitant. Systematically increasing the concentration of TCA in water, starting from 10 % (w/v) to 20 % (w/v), showed the highest precipitation potential around a concentration of 20 % TCA in water (w/v). Dilution of the final extract resulted in an increase of retention through HFBA ion-pair mechanisms on the stationary phase of the column. Dilution of the final extract with water (1:1, v/v) was implemented to gain a constant retention time and to avoid solvent effects, yielding a TCA concentration of 4 % (w/v) in the final extract.

3.1.2. Chromatography and mass spectrometry

Previously reported methods for the quantification of paromomycin and other aminoglycosides have already indicated challenges regarding the LC-system. Aminoglycosides are highly polar, do not contain any hydrophobic groups, and exhibit a multiple ionic state, which results in poor retention using conventional reversed-phase methods. Ion-pair or HILIC are the most commonly applied methods to solve retention problems for aminoglycosides. We evaluated the use of a zwitterion-HILIC column, but paromomycin did not elute well resulting in broad peaks and poor peak shapes and thus poor sensitivity. Ion-pair chromatography using HFBA combined with the use of an aqueous C18 UPLC column improved analyte retention and provided an acceptable peak shape. Subsequently, the modifier percentage (ACN) in the eluent was optimized and the minimal required amount of ion-pairing agent HFBA (3.5 mM) was established to obtain stable retention of the analyte with minimum matrix effects of the ion pairing reagent. Isocratic elution was desirable in order to prevent the conditioning of the column with the ion-pair reagent after each injection, which reduced the required run time substantially. After analysis of a batch of samples, the LC–MS/MS system was flushed using 0.1 % formic acid in water-methanol (50:50, v/v) to prevent accumulation of HFBA. The LC system was equipped with an ESI interface operating in positive ion mode connected to an ultra-sensitive quadrupole linear ion trap (QTRAP 6500, Sciex) mass spectrometer. Single charged paromomycin $[M+H]^+$ with a Q3 mass transition at *m/z* 616.6 → 163.1 for paromomycin and *m/z* 621.6 → 165.1 for the D_5-paromomycin IS (Fig. 2) were monitored. Considering the aim to develop a method with improved sensitivity, the bioanalytical range was determined by the lowest quantifiable concentration with an acceptable signal to noise ratio (5 ng/mL) and the corresponding ULOQ at which linearity of the calibration

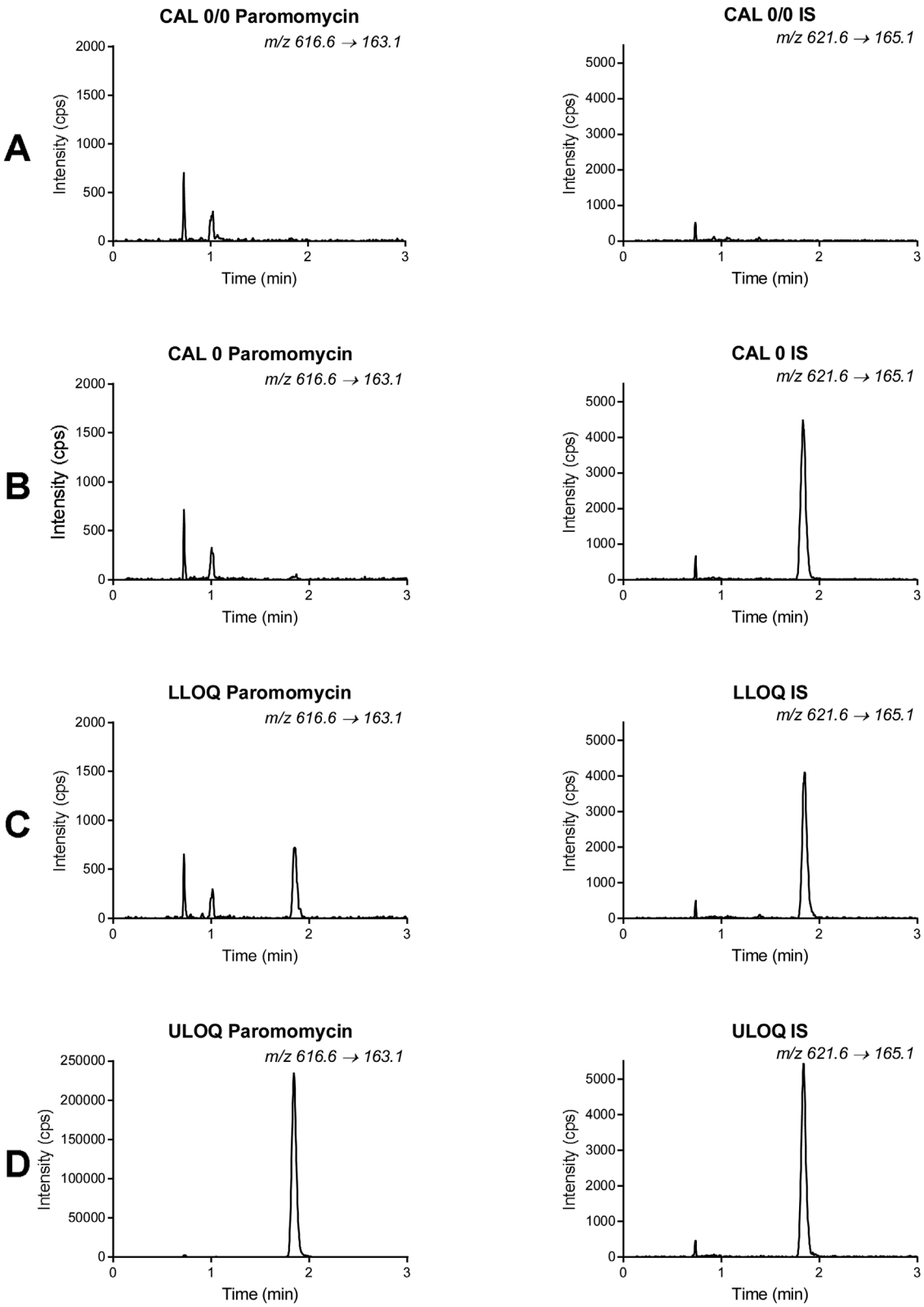

Fig. 3. Representative MRM chromatograms of processed human plasma samples. A: double blank (CAL 0/0), B: blank (CAL 0, containing 111 ng/mL internal standard), C: LLOQ (5 ng/mL paromomycin and 111 ng/mL internal standard) and D: ULOQ (1000 ng/mL paromomycin and 111 ng/mL internal standard).

curve was not compromised (1000 ng/mL). Above 1000 ng/mL the linearity of the bioanalytical range was highly compromised. Representative chromatograms of double blank, blank, LLOQ and ULOQ samples are shown in Fig. 3 for paromomycin and its internal standard.

3.2. Validation procedures

3.2.1. Calibration model

Calibration standards were analyzed in duplicate in three separate analytical runs. The reciprocal of the squared concentrations

Table 1
Assay performance data for paromomycin. Accuracy and precisions were established in 3 analytical runs and each run contained 5 replicates per tested concentration.

Nominal paromomycin concentration (ng/mL)	Intra-run bias (%)	Inter-assay bias (%)	Intra-run precision (%)	Inter-assay precision (%)
5	12.5-16.6	15.1	2.3-3.6	1.4
15	0.7-4.0	2.0	1.4-4.0	1.1
300	3.5-5.6	4.6	1.3-4.3	[a]
800	3.1-8.3	6.1	2.0-2.8	2.3

[a] No significant additional variation was found due to the performance of the assay in different batches.

Table 2
Matrix factor and sample preparation recovery data for paromomycin.

Nominal paromomycin concentration (ng/mL)	Matrix Factor Analyte	Matrix Factor IS	IS-normalized Matrix Factor	Sample Preparation Recovery
15	1.02	0.91	1.12	109.3 %
800	0.89	0.79	1.10	100.1 %

$(1/x^2)$ was used as a weighting factor to obtain a constant bias over the validated concentration range. A linear fit with correlation coefficient (r^2) of ≥0.997 was obtained in three individual runs. The bioanalytical assay was linear over the range (5−1000 ng/mL) with accuracies of ≤5.7 % for all calibration standards. The LLOQ was established at 5 ng/mL, exhibiting a minimum signal to noise ratio of 8:1.

3.2.2. Accuracy and precision

The assay performance data for paromomycin are summarized in Table 1. The performance at the QC-LOW, QC-MID and QC-HIGH levels were ±6.1 % and ±8.3 % for inter- and intra-assay accuracy, respectively, and ≤2.3 % and ≤4.3 % for inter- and intra-assay precision, respectively. At the LLOQ level the inter- and intra-assay accuracies were 15.1 % and ±16.6 %, respectively, while the inter- and intra-assay precisions were 1.4 % and ≤3.6 % respectively. The accuracy and precision data met the specified acceptance criteria.

3.2.3. Carry-over

Carry-over was determined by comparing the peak area present in the first double blank sample following an ULOQ sample to the mean peak area of LLOQ samples in two-fold. The carry-over in three validation runs was ≤17.5 %, which is within the acceptance criteria of ≤20 %.

3.2.4. Specificity and selectivity

Selectivity in 6 individual human plasma batches was determined at the blank and LLOQ levels. The maximum interference co-eluting with paromomycin at the LLOQ level was 3.2 % for paromomycin and there was no interference present at the retention time of the IS. The accuracy of paromomycin at the LLOQ level was also established for all six human plasma batches with a deviation of ≤13.2 % compared to the nominal concentration. Cross analyte/IS interference was determined. Paromomycin spiked at ULOQ level to human plasma did not show any interferences in the IS. The IS interference at the paromomycin mass transition shows 7.1–13.5 % interference. These interferences were well within the acceptance criteria.

3.2.5. Matrix effect and recovery

Absolute matrix effect factors for both analyte and IS were calculated based on QC-LOW and QC-HIGH concentrations in matrix present and matrix absent samples. The IS-normalized matrix effect factor were 12.0 % and 10.3 % for QC-LOW and QC-HIGH levels, respectively (Table 2). The sample preparation recovery at QC-LOW and QC-HIGH concentrations varied between 100.1 % and 109.3 %, reflecting the recovery of paromomycin after the sample pretreatment procedure.

3.2.6. Dilution integrity

Concentrations above the ULOQ were diluted 10, 50 and 100 times in a two-step dilution in five-fold. The intra-assay bias and precision for the 10 times dilution were 7.7 % and 3.2 % respectively, 0.5 % and 5.1 % respectively for the 50 times dilution, and -13.4 % and 6.1 % respectively for the 100 times dilution. Therefore, it can be concluded that samples with a concentration above the ULOQ can be diluted up to 100 times.

3.2.7. Stability

The stability of paromomycin in human plasma was investigated under various conditions (Table 3). Stability was determined as a function of accuracy and precision for both QC-LOW and QC-HIGH concentrations. Paromomycin in human plasma stored for 6 and 24 h at 2−8 °C and room temperature were all within the criteria (±15 %) and were considered stable (Table 3). Paromomycin in human plasma kept at 35 °C was stable for a period of 6 h, but not for 24 h (bias QC-LOW -17.6 %). The stability of final extracts was guaranteed for at least 44 days at 2−8 °C. The stock solution was stable for at least 431 days and the working solutions for at least 60 days, both stored at −20 °C in water.

3.3. Paromomycin glass adsorption

Only a few publications on the subject of aminoglycoside quantification address avoiding glassware due to possible adsorption of this class of compounds [10,22–25]. The adsorption issue was first assessed during the quantification of aminoglycosides using sodium pentanesulfonate ion-pair HPLC and fluorescent detection after post-column derivatization using o-phthalaldehyde reagent in 1978, mentioning the potential adsorption of aminoglycosides to glass [26]. This was described more extensively in radioimmunoassays for gentamycin and tobramycin, warning kit manufacturers of the possible impact to assay characteristics due to adsorption [27]. The proposed solution was to lower the pH of the environment, occupying the negatively charged silanol groups on the walls of the glass, avoiding positively charged aminoglycosides to bind the silanol groups. We confirmed that a final concentration of 4 % TCA in water (v/v) prevented any adsorption issues to glass and allowed quantification of the LLOQ level. Alternatively, the use of polypropylene labware throughout the sample preparation and analysis is recommended.

3.4. Clinical application

This analytical assay was used to determine paromomycin plasma concentration in pharmacokinetic samples from visceral leishmaniasis patients treated with intramuscular paromomycin (20 mg/kg q.d. for 14 days). Paromomycin plasma concentration-time curves for 3 typical patients are shown in Fig. 4. All trough

Table 3
Stability data for paromomycin (n = 3 per quality control level) expressed in accuracy (bias %) and precision (coefficient of variation).

Conditions	Matrix	Nominal concentration (ng/mL)	Bias (%)	C.V. (%)
−20 °C, 431d	Stock	100,000	2.5	4.9
−20 °C, F/T 4 cycles	Human plasma	15.0	11.1	5.4
		800	12.2	3.1
35 °C, 6h	Human plasma	15.0	−6.0	3.1
		800	1.0	5.8
35 °C, 24h	Human plasma	15.0	−17.6	4.0
		800	−10.6	2.6
−20 °C, 104 days	Human plasma	15.0	14.9	3.9
		800	0.7	3.2
RT, 6h	Human plasma	15.0	−2.7	4.3
		800	3.9	0.4
RT, 24h	Human plasma	15.0	1.8	6.4
		800	3.6	4.6
2–8 °C, 24h	Human plasma	15.0	12.9	2.7
		800	7.4	1.4
2–8 °C, 44d	Final extract	15.0	11.6	1.4
		800	5.0	3.6

Abbreviations: C.V. = coefficient of variation; d = days; F/T = freeze/thaw cycles; h = hours; RT = room temperature between 20 and 25 °C.

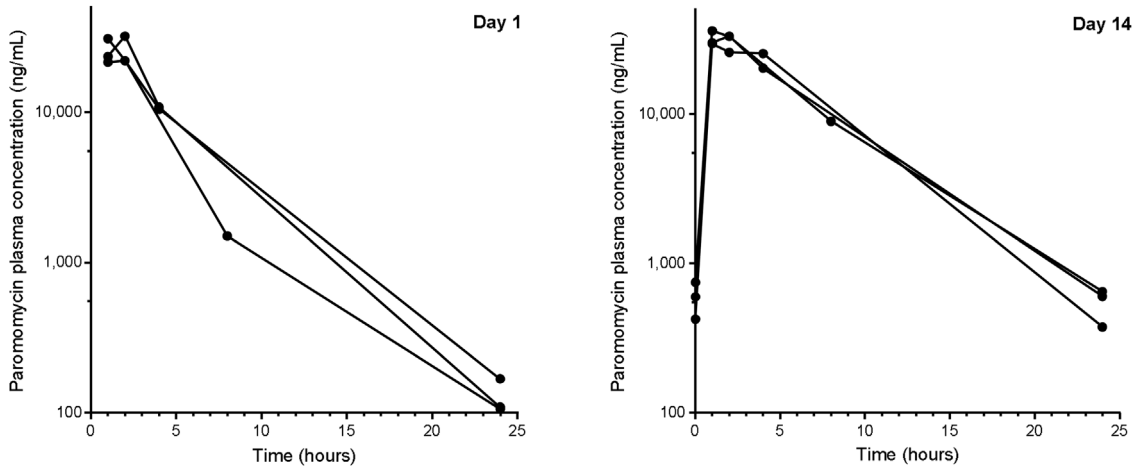

Fig. 4. Concentration of paromomycin in human plasma versus time after paromomycin administration on day 1 and day 14 of the treatment in 3 patients.

samples after the first drug administration (t = 0 h and t = 24 h) were quantifiable and above 100 ng/mL, which is well above the LLOQ level. Previous pharmacokinetic studies failed to quantify these trough concentrations due to a lack of sensitivity of the bioanalytical assay [28,29]. Aminoglycoside pharmacokinetics are characterized by large variability between regions and populations; moreover, lower paromomycin mg/kg regimens have been licensed for the treatment of visceral leishmaniasis in India. The here presented assay enables the quantification of trough concentrations, also in patients that have been lower exposed than the patients in the current clinical application. Taking into account the demonstrated dilution integrity for concentrations above the ULOQ, this method is shown to be applicable for the quantification of paromomycin in clinical pharmacokinetic studies.

4. Conclusion

A fast and simple highly sensitive bioanalytical assay for the quantification of paromomycin in human K_2-EDTA plasma was developed and validated. The validated range of the assay is 5–1000 ng/mL using a stable isotope as the internal standard. This bioanalytical assay for paromomycin has a ten-fold lower limit of quantification compared to previously reported assays for paromomycin, while using an ion-pair LC system with simplified sample preparation. The assay setup consists of TCA protein precipitation as sample preparation, an isocratic ion-pair LC system connected to a QTRAP 6500 equipped with ESI in positive ion mode. Furthermore, the method maintained a high assay performance for the validated ranges in terms of linearity ($r^2 \geq 0.997$), accuracy and precision. Paromomycin was adsorbed by glass, lowering the sensitivity of the assay. Using a high concentration of TCA and/or the replacement of glass with polypropylene material solved the adsorption issue.

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CRediT authorship contribution statement

Ignace C. Roseboom: Methodology, Investigation, Writing - original draft, Writing - review & editing. **Bas Thijssen:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision. **Hilde Rosing:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision. **Jane Mbui:** Resources. **Jos H. Beijnen:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision. **Thomas P.C.**

Dorlo: Conceptualization, Methodology, Validation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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