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Sensitive determination of gentamicin in plasma using ion-exchange solid-phase extraction followed by UHPLC-MS/MS analysis

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ABSTRACT

Background: Therapeutic drug monitoring (TDM) of gentamicin sulfate (GEN) is usually recommended, particularly in critical patients. Only a few reports had described the determination of GEN in plasma or plasma using LC-MS/MS.

Objective: This study aimed to develop and validate a sensitive ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) assay for the quantification of GEN in small volumes of human plasma.

Results: The use of a very low concentration of the ion-pairing agent HFBA allowed significant retention of the very polar GEN forms in a reversed phase UHPLC column. The solid-phase extraction (SPE) procedure allowed clean extracts, with no interferences detected in blank samples, and high sensitivity. The assay was linear on the range of 0.2–40 mg L^{-1} of GEN complex. The combined GEN complex had inter-assay CV of 8.8–10.0%, intra-assay CV of 10.2–11.0%, and accuracy of 96.8–104.0%. The assay was applied to 17 clinical samples obtained from neonate patients. Measured concentrations were in the range of 0.15–3.57 mg L^{-1} for GEN C1, 0.12–3.55 mg L^{-1} for GEN C1a, 0.20–5.77 mg L^{-1} for GEN C2, and 0.47–12.88 mg L^{-1} for the GEN complex, all within the linear range of the assay.

Conclusion: A sensitive assay for the quantification of gentamicin in plasma using anion-exchange SPE and UHPLC-MS/MS was validated. The assay can be used for TDM of gentamicin, particularly in centers with access to proper instrumentation and with a low demand for gentamicin measurements, where immunoassays are not cost-effective.

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1. Introduction

Therapeutic drug monitoring (TDM) of gentamicin sulfate (GEN) is usually recommended, particularly in critical patients [1]. Pharmaceutical preparations of GEN contain a complex mixture of 5 different structural isoforms, named GEN C1, GEN C1a, GEN C2, GEN C2a e GEN C2b, at variable proportions [2]. GEN C2a and C2b are minor components of the GEN complex, having the same molecular mass and chromatographic behavior of GEN C2 [3]. GEN plasma levels are usually measured using immunochemical methods [4]. The use of liquid chromatographic (LC) methods for GEN measurement in biofluids pose some challenges, particularly due to its high hydrophilicity and lack of chromophores [5,6]. More recently, the use of LC associated to mass spectrometry (LC-MS/MS) has become a method of choice for drug measurements in the context of TDM. Particularly, LC-MS/MS assay can be a valuable alternative to immunoassays for laboratories processing a small number of tests of particular assays, also allowing multiplexed analyses. To date, only a few reports described the determination of GEN in plasma or plasma using LC-MS/MS [3,7]. These reported assays had some limitations such as the use of a large volume of specimen, long chromatographic runs, use of highly acidic extracts, and use of high concentrations of ion-pairing reagents in mobile phases.

This study aimed to develop and validate a sensitive ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) assay for the quantification of GEN in small volumes of human plasma, for use in TDM of neonates.

2. Experimental

2.1. Reagents and materials

GEN (item#G4918, batch 028m4827v), acetonitrile, formic acid, heptafluorobutyric acid (HFBA), and methanol were purchased from Sigma Aldrich (Saint Louis, USA). GEN had 30.3% of GEN C1, 20.6% of GEN C1a, and 49.1% of GEN C2. Kanamycin B was from European Pharmacopeia (Strasbourg, France). Solid-phase extraction (SPE) cartridges Oasis MCX 30 mg (1 mL) were obtained from Waters (Milford, USA). Blank plasma was obtained from venous blood collected from healthy volunteers in EDTA containing tubes.

2.2. Solutions

The stock solution of GEN complex, at the concentration of 4 mg L^{-1}, was prepared by dissolution of the powder in a mixture of water:methanol:formic acid (49.5:49.5:1, v/v/v). Independent stock solutions were used for the preparation of calibration and quality control (QC) samples. Working solutions were obtained by dilution of the stock with the same solvent mixture and had concentrations of 4, 7, 10, 20, 30, 50, 100, 150, 200, 400, 600, and 800 mg L^{-1}. Calibration and QC samples were prepared by dilution the working solution with blank plasma (1:20, v/v). The internal standard (IS) working solution was KAN 5 mg L^{-1} in water containing 5% formic acid.

2.3. Sample preparation

GEN was separated from plasma using a SPE method, based on Chan et al. (2020), with several modifications [8]. Aliquots of 50 μL of plasma were added with 50 μL of IS working solution and 100 μL of formic acid 5%, followed by vortex mixing for 30 s. This mixture was submitted to SPE using Oasis MCX 30 mg (1 mL) cartridges. Liquid flow throughout the cartridge was about 1 mL min^{-1}. SPE cartridges were conditioned sequentially with 1 mL of methanol and 1 mL of water. After conditioning, the diluted sample was added to the cartridge, and the sorbent was washed with 1 mL of formic acid 5%, followed by 1 mL of methanol:water (3:1, v/v). The cartridge was then vacuum-dried for 10 min. Elution was performed with 1 mL of a mixture of methanol:water:isopropanol:ammonium hydroxide (1:1:1:1, v/v/v/v). The eluate was dried under a gentle air stream, at 60 °C. The dried extract was recovered with 100 μL of the initial mobile phase, and 1 μL was injected into the UHPLC-MS/MS system.

2.4. Chromatographic and mass spectrometry conditions

Analysis were performed using an Acquity I-Class UHPLC system coupled to a Xevo TQD triple-quadrupole mass spectrometer (Waters, Milford, EUA). UHPLC-MS/MS, with conditions based on da Silva et al. (2019), with minor modifications, using 0.01% of HFBA as ion-pairing agent [9]. Quantification MRM transitions were 478.3 → 322.2 for GEN C1, 450.3 → 322.2 for GEN C1a, 464.3 → 322.2 for GEN C2 and 484.3 → 163.2 for KAN. Collision energies were 14 and 25 V for GEN and KAN, respectively. Detailed instrumental conditions are presented on supplementary material.

2.5. Selectivity

The presence of interfering peaks in 6 blank plasma specimens obtained from healthy volunteers was evaluated [10].

2.6. Linearity

Linearity was evaluated at 8 concentration levels, in sextuplicate. The concentrations of the calibrators were 0.2, 0.5, 1, 2.5, 5, 10, 20, and 40 mg L^{-1} of the GEN complex. This calibration range was from 0.06; 0.04, and 0.10 mg L^{-1} of GEN C1, GEN C1a, and GEN C2,

respectively, to 12.12, 8.24, and 19.64 mg L^{-1} of GEN C1, GEN C1a, and GEN C2, respectively. GEN complex concentrations were obtained by adding the concentration of each individual form. Calibration curves were obtained relating the GEN to IS peak area ratio to the concentration of the calibrator, with 1/x as weighing factor. Acceptance criteria of linearity were correlation coefficients higher than 0.99 and back-calculated calibrator concentrations within ±15% of nominal values [11].

2.7. Precision and accuracy

Precision and accuracy were evaluated by analyzing QC concentrations in triplicate at five different days. The evaluated QC levels of the GEN complex were 0.2 mg L^{-1} for the QC at the lower limit of quantification (QCLLQ), 0.35 mg L^{-1} (0.10, 0.07, and 0.17 mg L^{-1} of GEN C1, GEN C1a, and GEN C2, respectively) for the QC at low concentration (QCL), 7.5 mg L^{-1} (2.27, 1.54, and 3.68 mg L^{-1} of GEN C1, GEN C1a, and GEN C2, respectively) for the QC at medium concentration (QCM), and 30 mg L^{-1} (9.09, 6.18, and 14.73 mg L^{-1} of GEN C1, GEN C1a, and GEN C2, respectively) for the QC at high concentration (QCH). Precision was calculated as CV%, using ANOVA. Accuracy was calculated as the percentage of the nominal concentration of the QC. Precision of the GEN complex was calculated using the error propagation formula [3]. Accuracy of the assay was considered acceptable if values were in the range of 85–115%, and precision was acceptable if CV values were below 15% [10].

2.8. Stability

Plasma extracts containing GEN at QCL and QCH concentration levels were kept at the chromatograph autosampler and injected at 1 h intervals for 12 h, to evaluate autosampler stability. Freeze-thaw stability was evaluated using QC samples subjected to three freeze-thaw cycles prior to analysis.

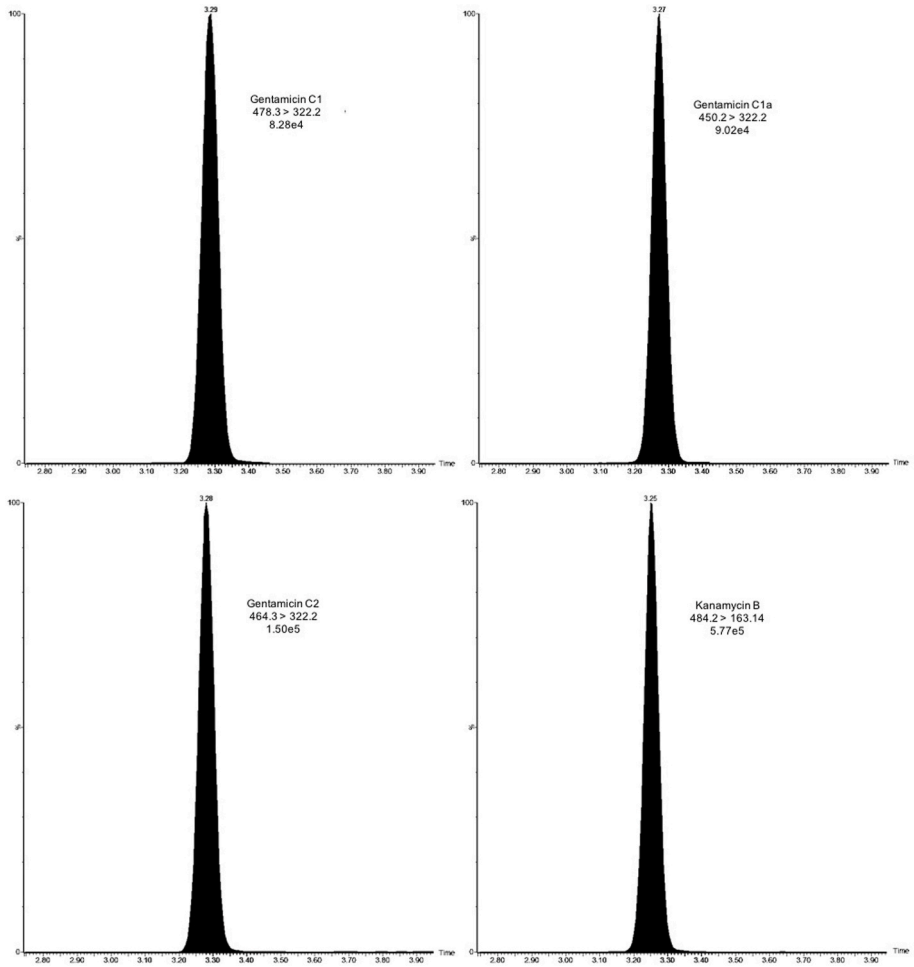

Fig. 1. Ion chromatogram obtained from a patient sample, with total GEN concentrations of 0.81 mg L^{-1} (GEN C1 0.20 mg L^{-1}; GEN C1a 0.22 mg L^{-1}; GEN C2 0.39 mg L^{-1}, and internal standard).

2.9. Matrix effect and extraction yield

Matrix effect (ME) was calculated for each GEN form using the post-extraction spike method, at QCL and QCH levels, using blank plasma of 5 volunteers, tested in triplicate [12]. Extraction yield (EY) were calculated comparing peak areas of QCL and QCH samples with solutions containing GEN and KAN in initial mobile phase, in concentrations equivalent to complete recovery, and expressed as percentage.

2.10. Clinical application

The assay was applied to 17 plasma specimens obtained from 11 infant patients. Blood samples were collected into EDTA containing tubes and plasma was separated by centrifugation. Patients had between 26 and 40 weeks of age and received GEN doses from 5.0 to 19.0 mg kg^{-1}. Dosing intervals from 20 to 36 h were used in these patients, with blood collections between 0.3 and 34.5 h after the end of the infusion. The study was approved by the Institutional Review Board.

3. Results and discussion

The used chromatographic conditions were the same that our group recently described for the determination of amikacin and vancomycin [9], allowing the addition of GEN to this antibiotic measurement method. The use of a very low concentration of the ion-pairing agent HFBA allowed significant retention of the very polar GEN forms in a reversed phase column, with retention times of about 3.25 min for GEN and KAN (Fig. 1).

Sample preparation employed anion-exchange SPE, differently of previous LC-MS/MS reports, which used protein precipitation procedures. The SPE procedure allowed clean extracts, with no interferences detected in blank samples, and high sensitivity. In addition, the SPE extracts were recovered with mobile phase, avoiding the injection of crude or very acidic extracts on the column.

The assay was linear on the range of 0.2–40 mg L^{-1} of GEN complex, with calibration curves presenting correlation coefficients higher than 0.99, with calibrators presenting back-calculated concentrations within ±15% of nominal values. The linear range of the assay covered the clinically significant range of concentrations [10,11]. Validation assay data is summarized on Table 1. Precision and accuracy of the assay fulfilled the acceptance criteria. Inter-assay CV was 3.3–7.4% and inter-assay CV was 3.8–7.7% for the different GEN forms. Accuracy was in the range of 95.9–104.3% for the different GEN forms. The combined GEN complex had inter-assay CV of 8.8–10.0%, intra-assay CV of 10.2–11.0%, and accuracy of 96.8–104.0%. The QCLLOQ had inter-assay CV of 9.0–11.8%, intra-assay CV of 9.9–11.9%, and accuracy of 104.2–109.7% for the GEN forms. The lower limit of quantification was 0.2 mg L^{-1} of the GEN complex, with inter-assay precision of 18.5%, intra-assay precision of 18.7%, and accuracy of 107.7%. The combination of selective sample preparation and efficient chromatography allow low matrix effects, which were in the range of −4.5 to −7.0%. Extraction yield of the different GEN forms was in the range of 70.3–76.8%. Recovered extracts had no indication of instability on autosampler conditions and after three freeze-thaw cycles.

The assay was applied to 17 clinical samples obtained from infant patients, being 8 though and 9 peak levels. Measured concentrations were in the range of 0.15–3.57 mg L^{-1} for GEN C1, 0.12–3.55 mg L^{-1} for GEN C1a, 0.20–5.77 mg L^{-1} for GEN C2, and 0.47–12.88 mg L^{-1} for the GEN complex, all within the linear range of the assay.

Table 1
Method validation figures of merit for the determination of gentamicin in serum by UHPLC-MS/MS.

Target compound	QC sample	Nominal concentrations (mg L^{-1})	Precision (CV %)		Accuracy (%)	Matrix effect (%)	Extraction yield (%)	Freeze-thaw stability (% change)[a]	12 h autosampler stability (% change)
			Intra-assay	Inter-assay					
Gentamicin C1	QCLLOQ	0.06	10.6	9.0	109.1	-	-	-	-
	QCL	0.11	7.7	7.4	97.4	−6.5	70.3	−3.5	2.5
	QCM	2.27	6.2	6.6	97.5	-	-	-	-
	CQH	9.09	6.5	5.4	104.3	−5.1	72.1	−2.2	4.0
Gentamicin C1a	QCLLOQ	0.04	9.9	11.8	109.7	-	-	-	-
	QCL	0.07	6.8	5.7	95.9	−4.5	76.8	−4.0	−3.5
	QCM	1.55	5.9	5.5	98.6	-	-	-	-
	CQH	6.18	5.7	5.1	103.6	−6.0	75.1	−5.3	−4.2
Gentamicin C2	QCLLOQ	0.10	11.9	11.0	104.2	-	-	-	-
	QCL	0.17	3.8	3.3	97.0	−7.0	72.5	−2.1	−5.1
	QCM	3.7	5.9	5.0	99.2	-	-	-	-
	CQH	14.7	5.3	4.7	104.0	−6.2	74.4	2.6	−3.7
Gentamicin complex	QCLLOQ	0.2	18.7	18.5	107.7	-	-	-	-
	QCL	0.35	11.0	9.9	96.8	-	-	-	-
	QCM	7.5	10.5	10.0	98.4	-	-	-	-
	CQH	30.0	10.2	8.8	104.0	-	-	-	-

[a] After three freeze-thaw cycles.

4. Conclusion

A sensitive assay for the quantification of gentamicin in plasma using anion-exchange SPE and UHPLC-MS/MS was validated. The assay was applied to plasma obtained from neonates, with peak and though concentrations within the linear range of the assay. The assay can be used for TDM of gentamicin, particularly in centers with access to proper instrumentation and with a low demand for gentamicin measurements, where immunoassays are not cost-effective.

Authors contribution declaration

The authors of the manuscript entitled "Sensitive determination of gentamicin in plasma using ion-exchange solid-phase extraction followed by UHPLC-MS/MS analysis" declare the following contributions for the manuscript:

Ana Laura Anibaletto dos Santos: Conceptualization, Methodology, Writing - original draft.
Anne Caroline Cezimbra da Silva: Conceptualization, Methodology.
Lilian de Lima Feltraco Lizot: Conceptualization, Methodology.
Anelise Schneider: Conceptualization, Methodology.
Roberta Zilles Hahn: Conceptualization, Methodology.
Yasmin Fazenda Meireles: Conceptualization, Methodology.
Lidiane Riva Pagnussat: Conceptualization, Methodology.
Julia Livia Nonnenmacher: Conceptualization, Methodology.
Siomara Regina Hahn: Supervision, Validation, Writing - review & editing.
Rafael Linden: Conceptualization, Methodology, Supervision, Validation, Writing - review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2021.e00246.

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