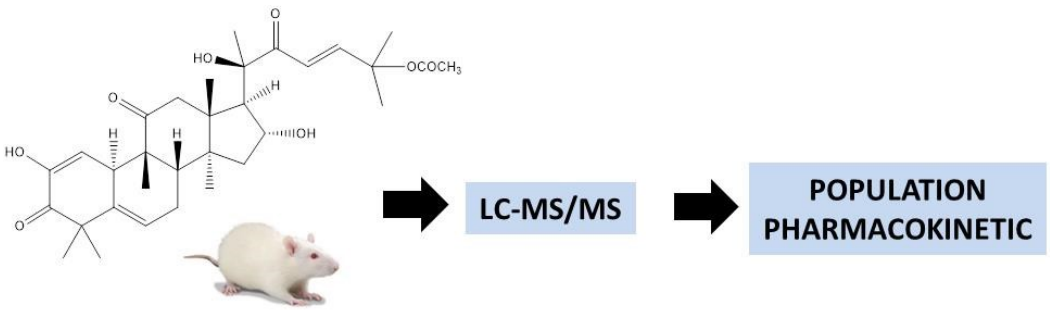
GRAPHICAL ABSTRACT



Development and validation of a quantification method for cucurbitacins E and I in rat plasma: Application to population pharmacokinetic studies

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ABSTRACT

Cucurbitacin E is a potential drug candidate due to its anticancer activity, recognition of its molecular targets, and synergism with other drugs used for cancer treatment. However, the use of cucurbitacin E in clinical practice is not possible because of important knowledge gaps in its preclinical and clinical pharmacokinetic characteristics. Cucurbitacin E is hydrolyzed to cucurbitacin I in plasma and in human liver microsomes. The aim of this study was to evaluate the population pharmacokinetics of cucurbitacin E and of its metabolite cucurbitacin I in rats. The method for the sequential analysis of cucurbitacins E and I in rat plasma was developed using LC-MS/MS. Plasma aliquots of 50 μL were deproteinized with acetonitrile and clobazam was added as internal standard. The extracts were injected into an RP-18 column and eluted with a mobile phase consisting of a mixture of acetonitrile:water:methanol (32:35:33, v/v/v). The method was precise and accurate, showing linearity in the range of 1-100 ng cucurbitacin E/mL plasma and of 0.4-200 ng cucurbitacin I/mL plasma. The method was applied to the pharmacokinetic evaluation of cucurbitacin E administered intravenously to male Wistar rats (1 mg/kg). Serial blood samples were collected up to 24 h after administration. The plasma concentrations of cucurbitacin E were quantified up to 16 h, while the plasma concentrations of cucurbitacin I remained below the limit of quantification. A population pharmacokinetic model was developed for cucurbitacin E using the NONMEM program, with adequate goodness of fit and predictive performance. The following pharmacokinetic parameters were obtained: release time of 0.45 h, volume of distribution of 27.22 L, clearance of 4.13 L/h, and elimination half-life of 4.57 h.

Keywords: cucurbitacin E, cucurbitacin I, rats, LC-MS/MS, population pharmacokinetics

1. INTRODUCTION

Cucurbitacins found mainly in the family Cucurbitaceae are oxygenated tetracyclic triterpenes [1]. These compounds have a great pharmacological potential because of their broad spectrum of biological activities such as antimicrobial [2], anti-inflammatory [2,3], anti-HIV [4], antioxidant [5], cytotoxic and antitumor properties [6,7].

The current interest in cucurbitacins focuses on their antiproliferative and cytotoxic potential against a large number of human cancer cell lines, including colon, breast, liver, skin, lung, central nervous system, prostate, and nasopharyngeal cancer [6-11]. In this respect, promising targets of cucurbitacins in cancer therapy are being identified, including inhibition of the JAK2/STAT3 signaling pathway [12-14], rupture of the cytoskeletal actin and vimentin networks [12-13].

Although neglected for decades, cucurbitacin E (Figure 1) has gained special attention of many research groups due to its promising anticancer activity [8, 10, 12-13]. Its ability to disrupt the cytoskeleton and to inhibit the JAK2-STAT3 pathway, among other mechanisms, and the synergisms with other drugs used in cancer therapy have rendered cucurbitacin E a candidate for clinical evaluation [6, 9,15].

Preliminary studies on the metabolism of cucurbitacins have shown that cucurbitacin E is hydrolyzed to cucurbitacin I (Figure 1) in human plasma by paraoxonase [16] and in human liver microsomes by carboxylesterases [17]. The analytical methods for cucurbitacins described in the literature generally use plant extracts [11,16,18], buffer solutions and organic solvents [16,19] and HPLC with ultraviolet detection. The analysis of cucurbitacins in biological fluids is only described for cucurbitacin B in rat plasma by UPLC-MS/MS [20] and for cucurbitacin I in rat plasma using LC-MS [21]. No data are available regarding the development and validation of analytical methods for cucurbitacin E in biological fluids.

The present study reports for the first time the development and validation of a sequential analytical method for cucurbitacin E and its metabolite cucurbitacin I in rat plasma using LC-MS/MS. The analytical method was applied to pharmacokinetic studies of cucurbitacins E and I in rats. We also describe for the first time the development of a population pharmacokinetic model of cucurbitacin E in rats.

2. MATERIAL AND METHODS

2.1. Development and validation of a sequential analytical method for cucurbitacin E and cucurbitacin I in rat plasma

2.1.1 Chemicals and reagents

The cucurbitacin E (≥ 95%) and cucurbitacin I (≥ 95%) standards, clobazam (internal standard) (Figure 2), and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). All solvents used were of HPLC grade. Methanol, acetonitrile, sodium chloride, monobasic sodium phosphate, and dibasic sodium phosphate were purchased from Merck (Darmstadt, Germany). Isopropanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). The water used in the experiments was purified with the Synergy® UV water purification system (Millipore, Belford, MA, USA).

2.1.2 Chromatographic analysis

Cucurbitacin E and cucurbitacin I were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using the Acquity UPLC H-Class System®, which consists of a quaternary pump, column oven and automatic injector, coupled to a Xevo® TQ-S triple quadrupole detector (Waters, Milford, MA, USA). Cucurbitacin E, cucurbitacin I and clobazam (internal standard) were separated on a Chromolit® RP-18 column (100 x 4.6 mm, 4-µm particle size) maintained at 24ºC. The mobile phase consisted of a mixture of acetonitrile:water:methanol (32:35:33; v/v/v) eluted at a flow rate of 0.8 mL/min.

The tandem mass spectrometer with electrospray ionization operated under the following conditions: nitrogen as nebulizer gas at a flow rate of 150 L/h and argon as collision gas at a flow rate of 0.18 mL/min, capillary voltage of 2.5 kV, temperature of the ionization source of 150ºC, and desolvation temperature of 600ºC. The analyses were performed by multiple reactions monitoring (MRM) in the negative mode for cucurbitacin E (555.2>537.3 *m/z*) and cucurbitacin I (513.4>495.3 *m/z*) and in the positive mode for clobazam (301.0>256.0 *m/z*). The MassLynx 4.1 program (Waters, Milford, MA, USA) was used for data acquisition and samples quantification.

2.1.3 Preparation of the standards and quality controls

Stock solutions of cucurbitacin E and cucurbitacin I were prepared at a concentration of 100 µg/mL methanol and diluted to obtain working solutions of 2, 4, 8, 20, 40, 80, 160 and 200 ng cucurbitacin E/mL methanol and of 0.8, 1.6, 4, 10, 20, 40,

100, 160, 320 and 400 ng cucurbitacin I/mL methanol. The clobazam solution (internal standard) was prepared at a concentration of 1000 μg/mL methanol and diluted to 500 ng/mL methanol.

The calibration curves were constructed using 50-μL blank rat plasma spiked with 25 μL of each standard solution of cucurbitacin E and cucurbitacin I, resulting in concentrations of 1, 2, 4, 10, 20, 40, 80 and 100 ng cucurbitacin E/mL plasma and of 0.4, 0.8, 2, 5, 10, 20, 50, 80, 160 and 200 ng cucurbitacin I/mL plasma.

The quality controls (QC) were prepared using aliquots of blank plasm spiked with the standard solutions of cucurbitacin E and cucurbitacin I. The following QC samples were prepared: lower limit of quantification (LLOQ; 1.0 ng/mL for cucurbitacin E and 0.4 ng/mL for cucurbitacin I), low concentration (LQC; 2.0 ng/mL for cucurbitacin E and 0.8 ng/mL for cucurbitacin I), medium concentration (MQC; 40 ng/mL for cucurbitacin E and 80 ng/mL for cucurbitacin I), and high concentration (HQC; 80.0 ng/mL for cucurbitacin E and 160 ng/mL for cucurbitacin I).

2.1.4 Sample preparation

Aliquots (50 μL) of rat plasma were added to 100 μL acetonitrile for protein precipitation. The tubes were shaken for 30 s in a shaker and centrifuged at 6,000 rpm for 2 min at 5^{o}C. Next, 100 μL aliquots of the supernatants were transferred to vials of the automatic injector of the HPLC system and 100 μL of a mixture of acetonitrile:water (1:1, v/v) and 25 μL of the internal standard solution were added. The vials were shaken in a shaker for 30 s and 50 μL aliquots were injected into the chromatographic system for analysis.

2.1.5 Validation of the analytical method

The analytical method for cucurbitacin E and cucurbitacin I in rat plasma was validated according to the guidelines of the European Medicines Agency (EMEA) [22] for bioanalytical methods. The following parameters were evaluated: lower limit of quantitation, selectivity, carry-over, matrix effect, linearity, precision, accuracy and stability.

The method was validated using aliquots of blank plasma derived from blood samples of male Wistar rats not submitted to the experimental protocol as biological matrix (blank sample). The blood samples were collected using heparin as anticoagulant.

Calibration curves (n= 6) were obtained by spiking aliquots of 50 µL blank plasma samples with cucurbitacin E and I standard solutions. The calibration curve was constructed by plotting the cucurbitacin E and I/IS peak area versus cucurbitacin E and I concentrations. The correlation coefficient (r) and linear regression equation were calculated using the linear regression method ($1/x^2$). The lower limit of quantitation (LLOQ) was defined as the lowest concentration of cucurbitacin E and I in plasma quantified with acceptable accuracy and precision (coefficient of variation and percent inaccuracy of less than 20%).

Carry-over was assessed by directly injecting an extracted blank after both replicates of the upper limit of quantification of cucurbitacin E and I.

The matrix effect was assessed by direct comparison of the peak areas of cucurbitacin E and I and the internal standard injected directly into the mobile phase, and spiked into extracts originating from six diferente sources of rat plasma. The IS normalized matrix factor was calculated for each matrix lot by dividing the ratio of the analyte/IS response in the presence of matrix by the ratio of the analyte/IS response in the absence of matrix. The coefficient of variation for the IS normalized matrix factor should be less than 15%.

Precision and accuracy were evaluated by intra and inter-assay studies. Six replicates of quality control samples were evaluated in a single analytical run (intra-assay) and in three different runs on different days (inter-assay). Precision is reported as the coefficient of variation (CV), that must be equal to or less than 15%, except for LLOQ, for which it should be up to 20%. Accuracy is determined by percent inaccuracy, excluding values higher than 15% of the nominal value, except for LLOQ, for which values higher than 20% of the nominal concentration should be excluded.

The stability of the cucurbitacin E and I was guaranteed by two freeze (-70 °C) and thaw (25 °C) cycles lasting 12 h each and by the evaluation of short-term stability (4 h at room temperature, 25 °C) and postprocessing stability (12 °C for 12 h). For this purpose, blank plasma samples spiked with cucurbitacin E and I concentrations of LQC and HQC ng/mL plasma were analyzed in six replicates. The results of the stability tests are reported as accuracy in relation to freshly prepared samples. The samples were considered stable when the relative error (RER, %) from the nominal concentration was within ±15% and when the CV was below 15%.

2.2. Population pharmacokinetics of cucurbitacin E in rat plasma

2.2.1. Experimental protocol

Male Wistar rats weighing 200-300 g, obtained from the Animal House of the Faculty of Pharmaceutical Sciences of Ribeirão Preto, were used. The procedures were approved by the Ethics Committee on Animal Use of the University of São Paulo (Protocol CEUA USP 12.1.1382.53.9).

The cucurbitacin E solution administered to the animals was prepared at a concentration of 0.4 mg/mL in a mixture (5:95, v/v) of DMSO and phosphate-buffer saline (67 mM, pH 7.4). The animals (n = 6 per sampling time) were treated with a single intravenous dose (1 mg/kg) of cucurbitacin E. Serial blood samples of 200 μL (3-4 samples per animal) were collected of the tail vein of each animal at time zero, 5, 15, 30 and 45 min and 1, 2, 3, 4, 6, 8, 12, 16 and 24 h after the cucurbitacin administration. In this study were used 36 animals. The blood samples were transferred to tubes containing heparin and immediately centrifuged (2 min, 6000 rpm, 5^{o}C) for the separation of plasma. The plasma aliquots were immediately analyzed using the method described in item 2.1.4.

2.2.2. Development of the population pharmacokinetic model

For evaluation of population pharmacokinetics, a nonlinear model of mixed effects was developed with the NONMEM v.7.3 program (ICON Development Solutions, Ellicott City, MD, USA) in the first-order conditional estimation mode with the interaction option (FOCE-I) [23] using a GNU Fortran 4.6 compiler (Free Software Foundation, Inc.) and PsN interface, version 4.4.0 (Perl-speaks-NONMEM, University of Uppsala, Sweden) [24].

Model building criteria included successful minimization without termination of the covariance step, standard error of estimates and absence of correlation between parameter estimates. Comparison of hierarchical models was based on the objective function (OF) value. A parameter was considered statistically relevant and included in the model if it decreased the OF more than 3.84 (p=0.05), following the assumption that the change in the OF after the addition of a parameter approximate a χ^2 distribution with one degree of freedom. Goodness of fit was assessed by graphical methods, including population and individual predicted vs. observed concentrations, conditional weighted residuals vs. population predicted concentrations and time.

The samples below the limit of quantification (BQL) were retained for model building purposes. These samples were analyzed by modelling the probability that they are actually below the limit of quantification [25].

The model was evaluated for its predictive performance using graphical criteria, as assessed by visual predictive check based on 1,000 individual simulations of plasma concentrations vs. time. The confidence intervals around the median 5% and 95% intervals of the simulated concentrations were plotted together with the observed data to visually evaluate the fit of the model to the data, its precision, and predictive performance.

3. RESULTS AND DISCUSSION

3.1. Development and validation of the sequential analytical method for cucurbitacin E and cucurbitacin I in rat plasma

The present study describes for the first time the development and validation of a method using LC-MS/MS for the analysis of cucurbitacin E in rat plasma and the first sequential analytical method for cucurbitacin and its metabolite using LC-MS/MS.

The analytical method for cucurbitacin E and cucurbitacin I in rat plasma permitted the sequential analysis of both cucurbitacins, with a chromatographic run time of 6 min. The lowers limits of quantification (LLOQ) were 0.4 ng cucurbitacin I and 1.0 ng cucurbitacin E/mL plasma using plasma aliquots of only 50 μL (Figure 3). It should be noted that the limit of quantification obtained for cucurbitacin I was 25 times lower than that reported in the literature (10 ng/mL plasma) by Molavi et al., 2006 [21].

Tables 1 and 2 show the results of linearity, LLOQ, precision and accuracy obtained in the validation of the sequential analytical method for cucurbitacin E and cucurbitacin I, respectively, in rat plasma. The validated method showed no matrix effect, considering that the coefficients of variation obtained for all matriz factor (MF) values were less than 15%. Analysis of different blank plasma samples revealed the absence of interference of endogenous compounds with cucurbitacin E, cucurbitacin I and internal standard, indicating adequate selectivity. The two blank plasma samples analyzed immediately after injection of the upper limit of quantification (ULOQ) sample exhibited no residual effect. Coefficients of variation and standard errors of less than 15% were obtained in the precision (CV=9.6%) and accuracy (RSE=10.3%) studies (Table 1 and Table 2), indicating that the method is accurate and precise.

Stability tests showed that cucurbitacin E and cucurbitacin I are stable for 30 days when prepared in methanol (stability testing in solution). With respect to the stability of cucurbitacin E and cucurbitacin I in rat plasma, the samples were stable after two freeze-thaw cycles, at room temperature for up to 4 h, after processing for 12 h at 12ºC, and during storage at -70ºC for a period of 30 days.

The validated sequential analytical method for cucurbitacin E and cucurbitacin I in rat plasma was applied to investigate the population pharmacokinetics of cucurbitacin E and its metabolite cucurbitacin I in rats.

3.2. Plasma concentrations of cucurbitacin E and its metabolite cucurbitacin I in rat plasma samples

The administration of a single intravenous dose of 1 mg/kg cucurbitacin E to rats resulted in plasma concentrations of cucurbitacin E above the LLOQ up to 16 h after administration. Figure 4 shows the chromatograms obtained for the analysis of a plasma sample collected 3 h after intravenous administration of 1 mg/kg cucurbitacin E to a rat. However, the plasma concentrations of cucurbitacin I remained below the LLOQ (0.4 ng/mL plasma) in all samples collected up to 24 h after the administration of cucurbitacin E.

Studies on the metabolism of cucurbitacin E in human liver microsomes and in human plasma have shown the formation of cucurbitacin I [17,16]. Saade et al. (2009) [16] suggested the participation of paraoxonase in the hydrolysis of cucurbitacin E to cucurbitacin I in human plasma. However, the concentrations of esterases in human plasma differ from those found in rat plasma; for example, the concentration of paraoxonase is 2-fold higher in human plasma than in rat plasma [26]. Thus, it is possible that rat plasma is unable to hydrolyze cucurbitacin E to cucurbitacin I, a fact that would explain the plasma concentrations of cucurbitacin I below the LLOQ in the study of intravenous administration of cucurbitacin E to rats.

3.3. Development and validation of the population pharmacokinetic model of cucurbitacin E in rat plasma after intravenous administration

This is the first study describing a population pharmacokinetic model for the class of cucurbitacins, particularly cucurbitacin E administered intravenously. The plasma concentration-time profile of cucurbitacin E after a single intravenous dose administration to rats (Figure 5) and the release time (D) of 0.45 h estimated with the

population pharmacokinetic model (Table 3) show that the pharmacokinetic behavior of cucurbitacin E resembles more a controlled release or infusion profile rather than the bolus intravenous administration used in the experimental study. Based on this observation, the population pharmacokinetic model of cucurbitacin E was developed assuming administration as an intravenous infusion. An overview of the goodness-of-fit is shown in the Figure 6. The plots show that individually predicted concentrations are unbiased and that residual errors are randomly distributed around mean zero.

Cucurbitacin E is not soluble in polar solvents and therefore requires prior solubilization in DMSO followed by dilution in phosphate-buffered saline. When diluted in aqueous buffer, analytes soluble in DMSO form a homogenous solution, a homogenous suspension, or a heterogenous suspension with the observation of precipitation [27]. The behavior of cucurbitacin E in the phosphate buffer-DMSO mixture (95:5, v/v) at the concentration used in the pharmacokinetic study (0.4 mg cucurbitacin E/mL) resembles a homogenous suspension, a fact explaining the release time of 0.45 h after intravenous administration. Concentrations higher than 0.4 mg cucurbitacin E/mL were not used because of the change from a homogenous suspension to a heterogenous suspension.

An overview of the goodness-of-fit is shown in the Figure 6. The plots show that individually predicted concentrations are unbiased and that residual errors are randomly distributed around mean zero. The high conditional weighted residuals shown are a consequence the high inter-individual and experimental variability of the data in 6 subjects. This variability led the predicted concentrations to deviate considerably from the observed concentrations. However, these deviations have not affected the estimation of the pharmacokinetic parameters. In fact, the model parameter estimates proved to be robust. During the analysis, the exclusion of the outliers resulted in estimates values similar to the ones obtained with the full data set.

Figure 7 illustrates the visual predictive check of the population pharmacokinetic model of cucurbitacin E in rat plasma after intravenous administration. Additionally, the figure indicates the percentage of BQL samples for each sample time, in case they are present, to highlight their influence on the prediction of the drug concentrations. The pharmacokinetic profile of the median plasma concentrations of cucurbitacin E was described by a two-compartment model with first-order distribution and elimination. The pharmacokinetic parameter estimates, interindividual variability, residual variability of the model and relative standard deviation (RSD%) of these estimates are

shown in Table 3 for release time (D), volume of distribution from the central compartment (Vc) and from the peripheral compartment (Vp), clearance (Cl), and intercompartmental clearance (Q).

The population pharmacokinetic profile of cucurbitacin E administered intravenously to rats (Table 3) suggests the need to develop formulations that result in a solubility compatible with the requirements of preclinical pharmacokinetic studies. Additionally, studies on the metabolism of cucurbitacin E and *in vitro* membrane transporters using human cells are necessary.

4. CONCLUSION

The sequential analytical method for cucurbitacin E and cucurbitacin I in rat plasma using LC-MS/MS is precise, accurate and sensitive, permitting its application to preclinical pharmacokinetic studies of single intravenous dose. The cucurbitacin I plasma concentrations remained below the LLOQ in all samples collected after intravenous administration of cucurbitacin E. It is possible that rat plasma is unable to hydrolyze cucurbitacin E to cucurbitacin I because of differences in esterase concentrations between human and rat plasma.

The pharmacokinetic profile of cucurbitacin E administered intravenously to rats was described by a two-compartment model with first-order distribution and elimination and the following pharmacokinetic parameters were obtained: volume of distribution of 27.22 L, clearance of 4.13 L/h, and elimination half-life of 4.57 h. In addition, the pharmacokinetic behavior of cucurbitacin E administered intravenously to rats resembled an infusion, with a release time of 0.45 h due to the low solubility of cucurbitacin E prepared in a mixture of phosphate-buffered saline (pH 7.4) and DMSO (95:5, v/v).

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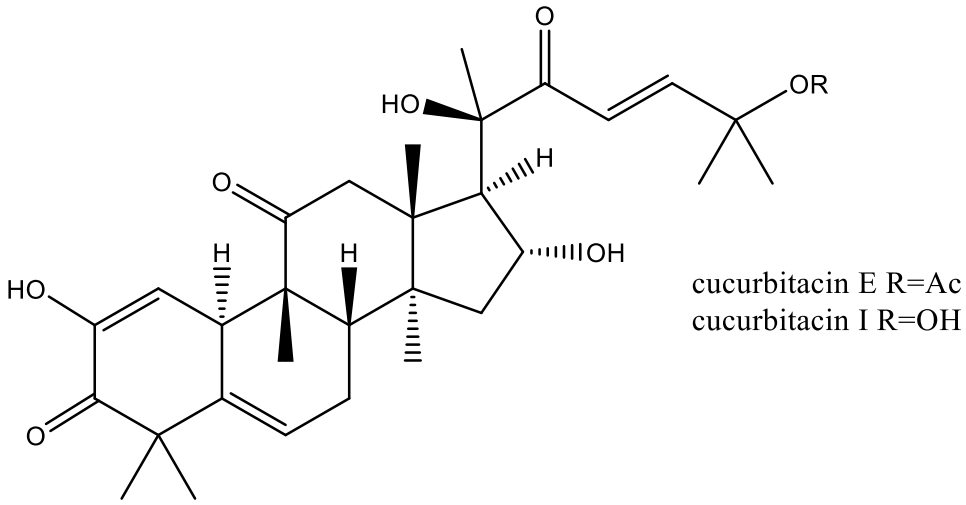

Figure 1. Chemical structure of cucurbitacin E and cucurbitacin I.

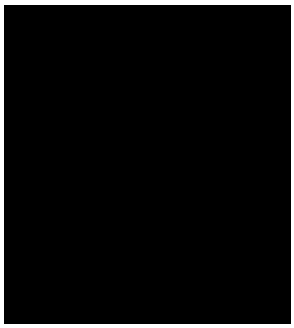

Figure 2. Chemical structure of clobazam (internal standard).

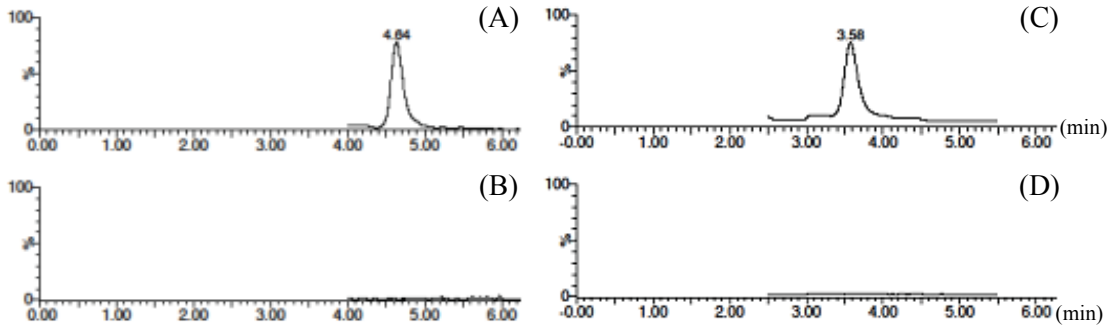

Figure 3. Representative MRM chromatograms for cucurbitacin E and cucurbitacin I. (A) Chromatogram of rat plasma spiked with 1.0 ng/mL (LLOQ) of cucurbitacin E; (B) Chromatogram of blank plasma monitored in the transition of cucurbitacin E (555.2>537.3); (C) Chromatogram of rat plasma spiked with 0.4 ng/mL (LLOQ) of cucurbitacin I; (D) Chromatogram of blank plasma monitored in the transition of cucurbitacin I (513.4>495.3).

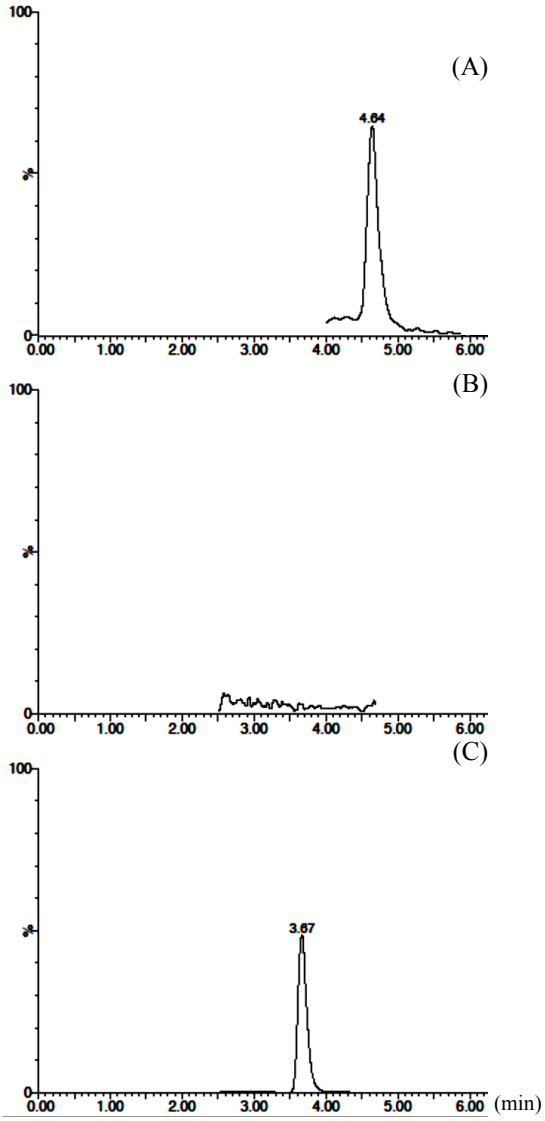

Figure 4. Chromatograms obtained for the analysis of a plasma sample collected 3 h after intravenous administration of cucurbitacin E (1 mg/kg) to a Wistar rat. (A) Chromatogram showing the transition of cucurbitacin E (555.2>537.3). (B) Chromatogram showing the transition of cucurbitacin I (513.4>495.3). (C) Chromatogram showing the transition of clobazam (internal standard) (301.0>224.0).

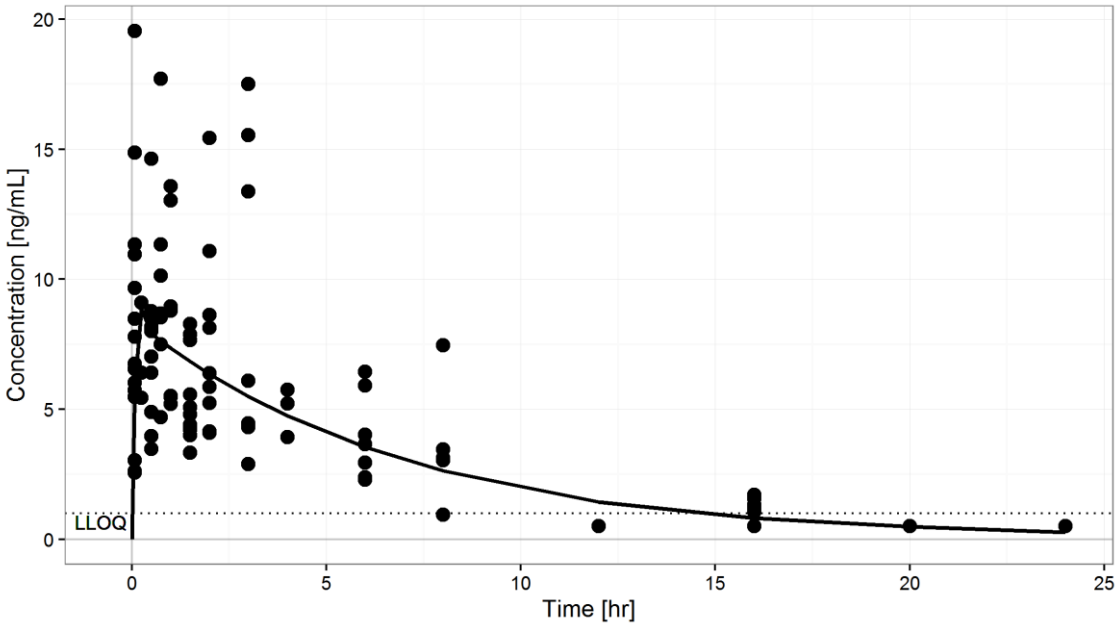

Figure 5. The plasma concentration-time profile of cucurbitacin E after a single intravenous dose administration to rats.

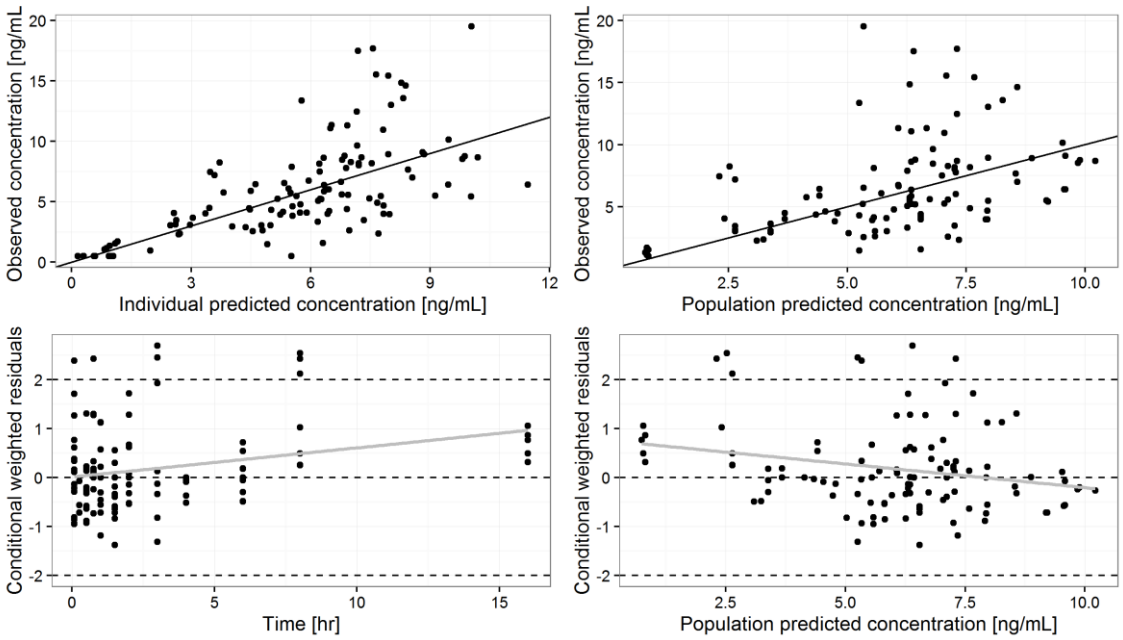

Figure 6. Goodness-of-fit. The plots show that individually predicted concentrations are unbiased and that residual errors are randomly distributed around mean zero.

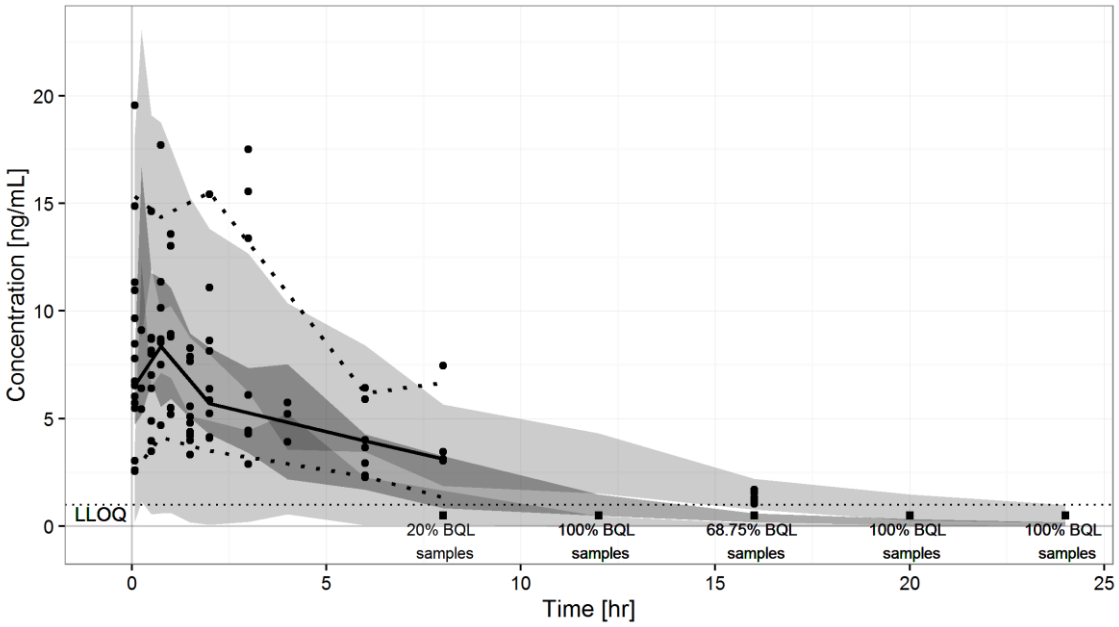

Figure 7. Visual predictive check. The observed plasma concentrations are shown as dots. The solid line represents the median of the observed plasma concentrations. The dotted lines indicate the 5th and 95th percentiles of the observed plasma concentrations. The dark grey shaded area represents the confidence interval around the median of the predicted plasma concentrations. The light grey shaded areas show the confidence interval around the 5th and 95th percentiles of the predicted plasma concentrations.

Table 1. Validation parameters of the analytical method for cucurbitacin E in rat plasma.

Parameter			
Linearity (ng/mL)	1.0 - 100.0		
Straight-line equation	y=0.0277031x+0.0352023		
Linear correlation coefficient	r=0.991606		
(ng/mL)	**Measured concentration (ng/mL) ± S.D**	**Between-run precision (n=18)** (CV %)	**Within-run precision (n=6)** (CV %)
1.0	1.12 ± 0.13	12.78	7.26
2.0	2.17 ± 0.20	9.59	10.06
40.0	39.10 ± 1.11	8.71	12.2
80.0	82.34 ± 3.24	13.85	13.43
	Measured concentration (ng/mL) ± S.D	**Between-run accuracy (n=18)** (RSE %)	**Within-run accuracy (n=6)** (RSE %)
1.0	1.01 ± 0.07	10.66	1.6
2.0	2.04 ± 0.05	7.87	3.0
40.0	40.24 ± 0.68	4.72	4.47
80.0	79.32 ± 1.56	-5.8	2.1

CV: coefficient of variation [(standard deviation/mean) x 100]; RSE: relative standard error [(mean - nominal value)/nominal value x 100]; r: linear correlation coefficient.

Table 2. Validation parameters of the analytical method for cucurbitacin I in rat plasma.

Parameter			
Linearity (ng/mL)	0.4 - 200.0		
Straight-line equation	y=0.00202823x+0.00490914		
Linear correlation coefficient	r=0.993945		
(ng/mL)	**Measured concentration (ng/mL) ± S.D**	**Between-run precision (n=18)** (CV %)	**Within-run precision (n=6)** (CV %)
0.4	0.48 ± 0.12	9.32	12.45
0.8	0.76 ± 0.22	11.2	9.15
80.0	83.12 ± 3.18	7.9	11.3
160.0	164.73 ± 6.43	10.34	8.64
	Measured concentration (ng/mL) ± S.D	**Between-run accuracy (n=18)** (RSE %)	**Within-run accuracy (n=6)** (RSE %)
0.4	0.35 ± 0.09	8.65	8.45
0.8	0.87 ± 0.56	7.6	9.5
80.0	81.12 ± 4.03	10.15	6.8
160.0	162.17 ± 9.56	11.25	-5.4

CV: coefficient of variation [(standard deviation/mean) x 100]; RSE: relative standard error [(mean - nominal value)/nominal value x 100]; r: linear correlation coefficient.

Table 3. Estimates of the population pharmacokinetic model of cucurbitacin E (1 mg/kg) administered intravenously to rats.

	Typical value (θ)	**RSD%**	**IIV (CV%)**	**RSD%**
Clearance (L/h)	4.13	5.1%	28.8%	6.6%
Vc (L)	0.815	14.2%	--	--
Q (L/h)	110	9.7%	48.7%	9.8%
Vp (L)	26.4	6.2%	--	--
D (h)	0.452	8.6%	--	--
	σ^2			
	0.562	9.2%	--	--

Vc: central volume of distribution; Q: intercompartmental clearance; Vp: peripheral volume of distribution; D: release time; IIV: interindividual variability (expressed as the coefficient of variation: $CV(\%) = \sqrt{\omega^2} \cdot 100$); χ^2: variance of arwhere $Y = F + F \cdot \varepsilon$); RSD%: relative standard deviation.