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Liquid chromatographic determination of CPZEN-45, a novel anti-tubercular drug, in biological samples

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Abstract

CPZEN-45 is a new drug candidate being considered for the treatment of tuberculosis (TB). The aim of this study was to develop and validate a reverse-phase high-performance liquid chromatographic (HPLC) method suitable to determine CPZEN-45 concentrations in biological samples. CPZEN-45 was extracted from biological fluids and tissues (plasma, lung and spleen from guinea pig) by sequential extraction with acetonitrile and quantified by a Waters HPLC Alliance System coupled with a ZORBAX Bonus-RP column, guard column and UV detection at 263 nm. The mobile phase was 20:80 acetonitrile:ultrapure-water with 0.05% TFA. The CPZEN-45 peak was eluted at 5.1 min with no interference from the inherent peaks of plasma, bronchoalveolar lavage (BAL), lung or spleen tissues. Recovery of CPZEN-45 from biological samples was >96% of the spiked amount. The limit of detection was 0.05 µg/ml and the limit of quantitation was 0.29 µg/ml which was more than 5 and 21 times lower than the reported minimal inhibitory concentration of CPZEN-45 (MIC = 1.56 µg/ml for *Mycobacterium tuberculosis* and 6.25 µg/ml for MDR-TB, respectively). Thus, HPLC method was deemed reliable, sensitive, reproducible and accurate for the determination of CPZEN-45 concentrations in plasma, BAL, lung and spleen tissues. Therefore, this method was used in subsequent studies in the guinea pig model to determine the disposition of CPZEN-45 after administration of solutions by the IV and SC routes.

Keywords

CPZEN-45; HPLC; Validation; Tuberculosis; Guinea pig

1. Introduction

Tuberculosis (TB) remains the single largest infectious killer of adults worldwide [1] and the leading cause of death among people living with the human immunodeficiency virus (HIV) [2]. The greatest threat to global TB control is the growing prevalence of highly resistant

strains: multi-drug resistant (MDR) and extensively drug resistant TB (XDR-TB). The conventional treatment of these patients consists of high doses of 4–6 drug combinations delivered orally and parentally for 18–24 months and is often associated with severe side effects. Therefore, there is a pressing need for new and more effective drugs that shorten the length of treatment, reduce the side effects and can be effective against MDR/XDR and safe for HIV infected TB patients [3].

In recent studies, caprazene (CPZEN), a core structure of the caprazamycins, proved to be a good precursor of anti-TB antibiotics. Structure–activity relationships studied on a range of CPZEN derivatives against *Mycobacterium tuberculosis* showed excellent activity, of which CPZEN-45 (Fig. 1) was the most promising [4]. Recently the path to ease of manufacture has also been described [5,6]. The compound exhibits a solubility of approximately 10 mg/ml in water and has low oral bioavailability as a result of poor absorption from the gastrointestinal (GI) tract [7]. CPZEN-45 appears to be a promising candidate for treatment of TB because of its activity against MTB strains *in vitro*. Its minimum inhibitory concentration (MIC) is 1.56 µg/ml for *M. tuberculosis* (H37Rv) and 6.25 µg/ml for MDR-TB [7]. CPZEN-45 may be a good candidate for treatment of MDR- and XDR-TB since it has never been used in therapy; thus, no bacterial strain resistance to this compound is expected. Despite its attractive features, development of an oral formulation for CPZEN-45 may not be feasible due to its poor solubility and potentially low bioavailability. Parenteral formulation of this compound may be possible but the daily injections for TB treatment are undesirable and would decrease the enthusiasm for its use by patients and clinicians. Therefore, alternative drug delivery strategies should be explored for this new drug.

Since the lungs are the primary site of TB infection [8], formulation of CPZEN-45 as powder for inhalation can potentially enhance the efficacy of TB treatment with this new agent. Thus, our overall goal is to demonstrate that CPZEN-45 is effective in eliminating TB infection when administered to the lungs as a respirable powder. The first step in assessing the potential of inhaled CPZEN-45 to treat TB is to evaluate its systemic disposition in a relevant animal model after pulmonary administration. In order to achieve this, a sensitive, reproducible and reliable analytical method should be developed and validated to determine CPZEN-45 in biological samples.

A high-performance liquid chromatographic method was initially developed by our collaborators at Eli Lilly and Company (Indianapolis, IN) to determine CPZEN-45 concentrations in solution (unpublished data), but there were no analytical methods to determine concentrations of this compound in biological samples. Therefore, the aim of this study was to develop and validate a reliable and reproducible method to extract CPZEN-45 from plasma, lung and spleen tissues and to develop and validate an HPLC method to quantify CPZEN-45 in plasma, BAL, lung and spleen tissues.

2. Materials and methods

2.1. Materials

Caprazamycin hydrochloride salt (CPZEN-45, Lot #: NC7-E07149-099) was obtained from the Infectious Disease Research Institute (IDRI, Seattle, WA) and was used as received.

Acetonitrile (ACN) (HPLC grade 99.5%) and trifluoroacetic acid (TFA) were obtained from Sigma, USA. Ultrapure-water was obtained from Pure Lab Ultrapure-Water System (ELGA, UK).

2.2. Animals and collection of biological fluids and tissue samples

Male Dunkin-Hartley guinea pigs (470 ± 33 g) were purchased from Charles River, USA. Blood was collected from guinea pigs and centrifuged (Eppendorf centrifuge 5424, USA) at $13,500 \times g$ for 10 min to separate plasma. Bronchoalveolar lavage (BAL) was performed as described previously [9]. The BAL fluid was sonicated for 15 s to disrupt cell contents, centrifuged at room temperature for 10 min at $13,500 \times g$ and supernatant was separated. After BAL was performed, all lung lobes and spleen were resected from the animal. The right lower lung lobe (0.95 ± 0.2 g) and the whole spleen (0.47 ± 0.4 g) were weighed and homogenized each separately in 4.5 ml saline using a Sonic Dismembrator probe (Fisher Scientific, USA).

2.3. HPLC equipment and chromatographic conditions

The chromatographic equipment employed was the Waters HPLC Alliance system, equipped with the e2695 separation module, quaternary, low pressure mixing pump, in-line vacuum degassing auto-sampler with capacity for 120 vials with programmable temperature control, heated column compartment and dual channel tunable UV detector (model 2489). All components of the HPLC system were controllable through the Empower 3 chromatography data software.

Chromatographic separation of CPZEN-45 was achieved on a ZORBAX Bonus-RP ($3.5 \mu\text{m}$, $75 \text{ mm} \times 4.6 \text{ mm ID}$) rapid resolution analytical column (Agilent Technologies, Santa Clara, CA) preceded by a $12.5 \text{ mm} \times 4.6 \text{ mm}$ guard column filled with the same packing material (Agilent Technologies, Santa Clara, CA) and a UV detector was set at $\lambda_{\text{max}} 263 \text{ nm}$ as indicated by a scan of the drug performed with a UV spectrophotometer. The optimal mobile phase composition (20:80 ACN:ultrapure-water with 0.05% TFA) was selected by screening varying ratios of ACN to water (10–30%) at 25°C and optimized flow rate of 0.75 ml/min. Different percentages of TFA (0.025, 0.05 and 0.1%) were used to evaluate the effect of TFA concentration on pH, as it pertains to the stability and durability of the analytical column, as well as the stability of the baseline and peak properties (Table 2). The injection volume for each sample was 2 μl .

2.4. Preparation of the standards in mobile phase

A stock solution was made by weighing 2.9 mg of CPZEN-45 accurately and then dissolving into 1 ml of 20:80 ACN:water without TFA. From the stock solution, concentrations of 0.29, 2.9, 7.25, 14.5, 29 $\mu\text{g/ml}$ CPZEN-45 were prepared each day that the assay was performed, using 20:80 ACN:water without TFA for dilution. All standards were prepared in triplicate.

2.5. Extraction of CPZEN-45 from biological samples

To optimize the extraction of CPZEN-45, blank plasma, lung homogenate or spleen homogenate were spiked with known concentrations of the drug. Briefly, 10 μl of 29 $\mu\text{g/ml}$

CPZEN-45 solution was added to 90 μ l of blank plasma and the vial vortexed to ensure thorough mixing. Four different methods with three replicates each were evaluated to maximize drug extraction from plasma as described in Table 1. For method 1, a single extraction was performed by adding 400 μ l of ACN to the plasma spiked with drug to precipitate proteins. Samples were then vortexed for 10 s and centrifuged at room temperature for 10 min at $13,500 \times g$, and the supernatant was collected into an eppendorf tube. For methods 2–4, two extractions were performed, in first extraction, 200–400 μ l of ACN was added to the plasma spiked with drug to precipitate proteins. Samples were then vortexed for 10 s and centrifuged at room temperature for 10 min at $13,500 \times g$, and the supernatant was collected into an eppendorf tubes. In second extraction, an extra volume of ACN (100–200 μ l, Table 1) was added to the tubes containing the remaining pellet. The pellet was physically disrupted by using pointed spatula and the contents vortexed and centrifuged at room temperature for 10 min at $13,500 \times g$. The supernatants from all the extractions were collected into a single eppendorf tube for each concentration and evaporated using the Savant DNA 120 speedvac concentrator (Thermo Electron Corporation, USA) at room temperature for approximately 2–3 h at maximum vacuum level (7 Torr). The residue remaining after solvent evaporation was then reconstituted with 50 μ l of mobile phase (20 ACN:80 water with 0.05% TFA) before injection into the HPLC. The volume injected for analysis was 2 μ l. The same procedure was followed to optimize the extraction of CPZEN-45 from liquid lung and spleen homogenates.

Extraction efficiency was determined by comparing the amount of the drug extracted from plasma, lung and spleen samples with the amount of drug extracted using the same procedure from CPZEN-45 in water. The 29 μ g/ml sample was prepared in triplicate, extracted and analyzed by HPLC as described above.

2.6. Stability of CPZEN-45 after extraction from biological samples

In order to assess possible changes in drug concentration during the period of the assay, the stability of the drug in mobile phase was evaluated after extraction from biological samples. HPLC vials ($n = 3$) containing the drug in mobile phase after extraction were stored at room temperature for 24 h and 18 days. After each of these time points, the solution from these vials was re-injected onto the HPLC and the resulting areas and peak shapes compared to the original freshly extracted drug.

2.7. Preparation of calibration standards in biological samples

After the drug extraction method for each biological sample was established, calibration standards were prepared in triplicate for each biological fluid or tissue. Aliquots (90 μ l) of plasma were placed in disposable polypropylene micro-centrifuge tubes and 10 μ l of each concentration of CPZEN-45 standard solution was added to prepare a linear standard curve with CPZEN-45 plasma concentration ranging between 0.29 and 29.0 μ g/ml as described in Section 2.3. After thorough mixing the drug was extracted and reconstituted in mobile phase as described in Section 2.4, prior to injection in to the HPLC. A similar procedure was employed to prepare standard curves for lung and spleen tissues.

2.8. Method validation

The HPLC method to determine CPZEN-45 in biological samples was validated according to the “FDA guidance for bioanalytical method validation” [10]. Briefly, the standards containing pure CPZEN-45, either in water, plasma, lung or spleen tissues ($n=3$, each) were used to evaluate the linearity of the assay in a range of concentrations from the limit of quantification (LOQ) to 20 times the MIC of CPZEN-45 for relevance to therapeutic concentration of the drug. After analysis, the resulting peak area was plotted against its corresponding CPZEN-45 concentration [11]. The best-fit line was obtained by the least square regression method and the regression coefficient (R^2) was calculated and used as an indicator of the linearity of the assay in the range of concentrations studied. Background levels were measured and the limit of detection (LOD) was determined by signal to background ratio of three. According to FDA guidelines, the smallest CPZEN-45 concentration in the standard curve was considered as the limit of quantification (LOQ) [12]. Specificity was evaluated by separation of the CPZEN-45 peak from the peaks corresponding to inherent plasma compounds. Intra-day precision and accuracy of the HPLC method were evaluated by assaying at least three replicates of standard plasma solutions at five concentrations (0.29, 2.9, 7.25, 14.5, 29 $\mu\text{g/ml}$, respectively) in 1 day. The inter-day variation was determined by assaying these five concentrations on each of three consecutive days. The accuracy expressed the closeness of agreement between the true value and the value found at each concentration [13]. The precision of the measurement was the coefficient of variation (CV%) which was calculated by the ratio of standard deviations of replicates ($n=3$) to the mean concentrations [11].

2.9. Preliminary CPZEN-45 disposition studies

Three healthy male Dunkin-Hartley guinea pigs weighing an average of 470 ± 33 g were employed in the study. All of the animal experiments were approved by OUHSC Institutional Animal Care and Use Committee (IACUC). Twenty-four hours before the study, each animal underwent cannulation of the right external jugular vein using silicone polymer tubing connected to polyethylene PE-50 tubing for continuous blood sampling. The cannula was routed subcutaneously, externalized at the neck, and secured to musculature [14,15].

CPZEN-45 solutions for intravenous (IV) and subcutaneous (SC) administration were prepared by dissolving a weighed amount of CPZEN-45 in water for injection (WFI) and filtering the solution through a $0.22 \mu\text{m}$ Millex[®]GS filter (Millipore, Cork, Ireland). Each guinea pig received either a dose of 5.5 mg/kg or 12.5 mg/kg by intravenous (IV) administration through the cannula implanted in the jugular vein or 12.5 mg/kg subcutaneously (SC). Blood samples were collected at various time points (0, 0.08, 0.25, 0.50, 1, 1.5, 2, 3, 4, 5 h) and drug concentrations were determined by the validated HPLC assay.

3. Results

3.1. Validation of the analytical method for CPZEN-45 in mobile phase

The optimization of the HPLC method to quantify CPZEN-45 was conducted using samples containing the pure drug dissolved in 20% ACN in water which was determined to be the ratio of ACN to water that yield the sharpest peak for the drug after screening varying ratios of acetonitrile to water (data not shown). The comparison of four parameters (pH, baseline, peak shape and retention time) indicated that by using 0.05% TFA with 20% ACN in water, the pH was above 2.0 (Table 2) as recommended by the manufacturer of the column, the baseline was stable and peak shape was narrow and sharp (Fig. 2A). With this optimized mobile phase, the retention time of CPZEN-45 dissolved in 20% ACN in water was 5.1 min at a flow rate of 0.75 ml/min as shown in Fig. 2A. Calibration curves prepared in mobile phase with concentrations of CPZEN-45 ranging from 0.29 to 29.0 µg/ml had a regression equation of peak area = 7304.9(conc.) – 1073.6 with correlation coefficients indicating linearity at the concentrations studied ($R^2 > 0.999$).

3.2. Efficiency of CPZEN-45 extraction from biological samples

The efficiency of CPZEN-45 recovery from plasma for each of the methods employed is shown in Table 1. Method 3, which consisted two extractions using 300 µl of ACN for the first extraction and 100 µl for the second extraction, was selected because of the highest efficiency of recovery (96%) with the smallest ACN volume. The efficiency of CPZEN-45 recovery from lung and spleen tissues was >96% with methods 3 (data not shown).

3.3. Stability of extracted CPZEN-45 in mobile phase

Similar peak areas ($36,399 \pm 192$) were obtained after injecting samples from freshly extracted CPZEN-45 and samples that were kept at room temperature for 24 h and 18 days. The HPLC analysis demonstrated that CPZEN-45 was stable at room temperature under the analytical conditions employed.

3.4. Validation of the analytical method for CPZEN-45 in biological samples

When the extract was reconstituted with mobile phase and injected into the HPLC, the CPZEN-45 peak (5.1 min) had sufficient resolution from the two inherent peaks of plasma (4.2 min) and (10.9 min) (Fig. 2B). The chromatogram of blank guinea pig plasma showed only the peaks corresponding to inherent plasma compounds (4.2 min) and (10.9 min) along with the solvent front peaks (Fig. 2C). Chromatograms after injection of the lung, spleen extract and BAL had single, well-defined CPZEN-45 peak (4.9 and 4.8 min, respectively) as shown in Fig. 3A–C. The LOQ was established at 0.29 µg/ml and the LOD was determined to be 0.05 µg/ml.

Standard curves were then constructed from CPZEN-45 extracted from plasma using the same concentrations described for those curves made with mobile phase (0.29–29 µg/ml). These curves had a regression equation of peak area = 5571.2 (conc.) + 1534.7 with correlation coefficients indicating linearity at the concentrations studied ($R^2 > 0.99$). Similarly, standard curves were then constructed from CPZEN-45 extracted from lung and spleen tissues using the same concentrations as plasma. The lung and spleen had a

regression equation of peak area = $4724.7(\text{conc.}) + 1726.3$ and $5589.0(\text{conc.}) + 144.25$ respectively with correlation coefficients indicating linearity at the concentrations studied ($R^2 > 0.999$). Statistical analysis of significance of the offset value (intercept) indicated that there were no matrix effects related to sample preparations. The parameters of validation for determining CPZEN-45 concentrations in plasma, lung and spleen tissues are shown in Table 3 respectively. The intra-day and inter-day precision of the method for all biological samples were below 20% with accuracy >90% for all concentrations in spleen and most concentrations in plasma and lung.

3.5. Preliminary CPZEN-45 disposition studies

CPZEN-45 plasma concentration *versus* time profiles is shown in Fig. 4. The range of CPZEN-45 concentrations validated by the HPLC methods in this study was sufficient to determine drug plasma concentrations at initial time points after IV administration of 5.5 mg/kg and SC administration of 12.5 mg/kg. However, plasma samples at initial time points after IV administration of 12.5 mg/kg required to be diluted 2–3 times prior to injection into the HPLC in order to be within the range of concentrations validated in the present method. CPZEN-45 plasma concentrations were below MIC 2 and 3 h after IV dosing of the 5.5 and 12.5 mg/kg doses, respectively, and after 4 h after SC administration (Fig. 4). CPZEN-45 plasma concentrations were below the LOD of the validated method 4 and 6 h after IV administration of 5.5 and 12.5 mg/kg respectively and 6 h after SC administration. Finally, CPZEN-4 plasma concentrations remained above the LOQ at 5 and 6 h (end of the study for 5.5 mg/kg (IV) and 12.5 mg/kg (IV, SC)), indicating that the validated HPLC method will be sufficient, appropriate and accurate to measure drug concentrations in future pharmacokinetics studies.

4. Discussion

The lengthy and complex treatment of TB, emergence of multidrug (MDR-) and extensively drug-resistant TB (XDR-TB) has increased the need for the development of new drugs that can be safer, less toxic, reduce the occurrence of MDR-TB, and enable shorter treatment periods [16].

In search of new drugs, preliminary *in vitro* studies suggested that CPZEN-45 can be a good drug candidate in the armamentarium of drugs to treat TB. The next step in order to establish the utility of CPZEN-45 in TB treatment is to evaluate its deposition in a relevant animal model and determine its key pharmacokinetics parameters to establish a dosing regimen. However, before *in vivo* studies can be performed, a reliable and accurate method to determine CPZEN-45 levels in plasma and tissues was required.

Even though a mobile phase was recommended for CPZEN-45 by our collaborators at Eli Lilly, this mobile phase did not yield a desirable peak shape for CPZEN-45 when extracted from biological samples. Furthermore, a rapid decline in the performance of the column was observed with this mobile phase, which was followed by the instability to stabilize the baseline of the chromatogram prior to quantification of drug in samples. The pH of this mobile phase was determined to be below 2.0, which is indicated as the minimum pH to use with the column. The manufacturer of the column (Agilent Technologies, Santa Clara, CA)

recommends that the pH of the mobile phase should be greater than 2 to avoid potential changes in the column composition and increase its life-span.

Therefore, in the present study, we first optimized the composition of the mobile phase with a ratio of organic solvent to water that would result in the sharpest peak possible. A mobile phase consisting of 20% ACN in water resulted in a rather broad peak, thus the addition of the ion-pairing agent, TFA, to the mobile phase was required to improve peak shape. In addition, mobile phase composition was optimized to have a pH greater than 2 and to stabilize baseline of the chromatogram. This was achieved by optimizing the percentage of TFA in the mobile phase. A 0.05% TFA in the mobile phase resulted in both, a stable baseline and a sharp peak shape besides the pH greater than 2.0 to preserve the life of the analytical column.

Despite the availability of more sophisticated extraction methods such as solid phase extraction; we opted to use a simple liquid-liquid extraction, because it had a high recovery of CPZEN-45 with small solvent volumes and was inexpensive. The efficacy of extraction was increased with the number of sequential extractions of drug from the plasma pellet. Method #3 (Table 1) was the extraction method that achieved the highest extraction efficiency (96%) with the smallest volume of acetonitrile.

The HPLC method developed to determine CPZEN-45 in biological samples was a simple procedure with a good peak resolution and short running time (12 min). Validation parameters (Table 3) indicated that the method was precise, accurate and robust with low limit of quantification as defined by the FDA guidance [10]. The LOD was 0.05 µg/ml and the LOQ was 0.29 µg/ml which was more than 5 and 21 times lower than the reported minimal inhibitory concentration of CPZEN-45 (MIC = 1.56 µg/ml for *M. tuberculosis* and 6.25 µg/ml for MDR-TB, respectively). Typically, when an analytical method is developed, one of the goals is that it can determine the drug concentrations as low as nanograms/ml. However, for the purpose of determining therapeutic concentrations of an antibiotic, the LOD and LOQ should be placed in the context of MIC. Even though detection of smallest concentrations of the antibiotics is desirable, if they are more than 10 times smaller the MIC, they would not be therapeutically relevant. Previously Wang et al. [11] reported LOQ 0.25 µg/ml for the HPLC determination of isoxyl, another anti-mycobacterial, which was 8 times lower than its reported MIC = 2 µg/ml. Similarly Song et al. [17] established that the lower LOD for isoniazid and ethambutol was 0.01 µg/ml and LOQ for isoniazid and ethambutol was 0.05 µg/ml, Since their MIC are 0.1 and 4 µg/ml respectively, they concluded that these values are sufficient for routine drug monitoring because these LOQs were much lower than the MIC of these drugs.

Determination of CPZEN-45 plasma concentrations obtained from the preliminary disposition study of CPZEN-45 was achieved successfully with the optimized method except for initial time points from the animal dosed with the highest dose, all plasma concentrations from 3 studied animals were within the concentrations of the calibration curve in the present study. Since there are no previous studies describing the disposition of CPZEN-45 in laboratory animals that have been published, the plasma concentrations in the

present preliminary disposition studies also served as a guide to determine the length of the sample period in future pharmacokinetic studies.

5. Conclusions

A reliable, reproducible and accurate reverse-phase HPLC method was developed for determination of CPZEN-45 concentrations in plasma, BAL, lung and spleen tissues. The suitability of this HPLC method was assessed by determining CPZEN-45 plasma concentrations in preliminary disposition studies after the drug was administered intravenously and subcutaneously. The newly validated HPLC method will facilitate the development of novel formulations targeting the lungs, the main site of TB infection, and determining its disposition after administration by different routes, which in turn will aid in designing CPZEN-45 dosage regimens for more effective treatment of TB.

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Fig. 1.
Structure of CPZEN-45.

CPZEN-45 (1.56, 500)

Fig. 2. HPLC chromatograms of (A) CPZEN-45 in 20% ACN; (B) CPZEN-45 extracted from plasma; and (C) blank plasma. Arrow shows the peak of the drug (29.0 $\mu\text{g/ml}$).

Fig. 3. HPLC chromatograms of CPZEN-45 extracted from lung (A), spleen (B) and sonicated BAL (C). Arrow shows the peak of the drug (29.0 µg/ml).

Fig. 4. CPZEN-45 plasma concentration *versus* time curves after IV or SC administration 12.5 or 5.5 mg/kg doses; MIC = 1.56 µg/ml.

Table 1

Methods employed to extract CPZEN-45 from plasma ($n = 3$ for each method) and efficiency of CPZEN-45 recovery from plasma samples extracted (mean $\pm$ stdev, $n = 3$).

Method #	Extraction #1	Extraction #2	Extraction efficiency
1	90 μ l plasma + 10 μ l of a 29 μ g/ml aqueous solution of drug + 400 μ l ACN	-	88.6 $\pm$ 1.6
2	90 μ l plasma + 10 μ l of a 29 μ g/ml aqueous solution of drug + 200 μ l ACN	Pellet + 200 μ l ACN	82.8 $\pm$ 1.1
3	90 μ l plasma + 10 μ l of a 29 μ g/ml aqueous solution of drug + 300 μ l ACN	Pellet + 100 μ l ACN	96.0 $\pm$ 3.2
4	90 μ l plasma + 10 μ l of a 29 μ g/ml aqueous solution of drug + 400 μ l ACN	Pellet + 100 μ l ACN	92.9 $\pm$ 2.4

Table 2

Effect of %TFA on optimization of assay conditions.

Parameters	0.025% TFA	0.05% TFA	0.1% TFA
pH	2.6	2.2	1.6
Baseline	Stable	Stable	Stable
Peak shape	Distorted	Sharp and narrow	Sharp and narrow
Retention time (RT)	3.2	5.0	8.0

Table 3

Parameters of validation for the HPLC assay of CPZEN-45 in plasma, lung and spleen ($n = 3$ for each parameter and concentration).

Spiked concentration ($\mu\text{g/ml}$)	Intra-day		Inter-day			
	Measured conc. ($\mu\text{g/ml}$)	Precision (%CV)	Accuracy (%recovery)	Measured conc. ($\mu\text{g/ml}$)	Precision (%CV)	Accuracy (%recovery)
Plasma						
0.29	0.2 $\pm$ 0.01	5	69	0.2 $\pm$ 0.01	5	69
2.9	2.6 $\pm$ 0.2	8	90	2.7 $\pm$ 0.1	4	93
7.25	7.1 $\pm$ 0.9	13	98	7.4 $\pm$ 0.7	9	102
14.5	14.1 $\pm$ 0.6	4	97	14.8 $\pm$ 0.6	4	102
29	29.3 $\pm$ 0.6	2	101	30.7 $\pm$ 1.7	6	106
Lung						
0.29	0.2 $\pm$ 0.03	15	69	0.2 $\pm$ 0.03	15	69
2.9	3.2 $\pm$ 0.6	19	110	2.9 $\pm$ 0.3	10	100
7.25	7.6 $\pm$ 0.5	7	105	7.9 $\pm$ 0.6	8	109
14.5	16.1 $\pm$ 1.9	12	111	13.1 $\pm$ 2.5	19	90
29	28.1 $\pm$ 0.8	3	97	29.5 $\pm$ 1.4	5	102
Spleen						
0.29	0.3 $\pm$ 0.01	3	103	0.3 $\pm$ 0.01	3	103
2.9	2.9 $\pm$ 0.1	3	100	2.9 $\pm$ 0.1	3	100
7.25	7.2 $\pm$ 0.2	3	99	7.6 $\pm$ 0.7	9	105
14.5	14.9 $\pm$ 1.6	11	102	15.1 $\pm$ 1.1	7	104
29	28.8 $\pm$ 0.8	3	99	29.8 $\pm$ 2.1	7	103