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Short Communication

High-performance liquid chromatographic assay of cefazolin in rat tissues

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Abstract

A rapid, sensitive and reproducible high-performance liquid chromatographic (HPLC) assay for cefazolin in rat tissues was developed. Tissue samples were homogenized in distilled water, acidified with 8.5% phosphoric acid, and centrifuged. Cefazolin was isolated from the supernatant by solid-phase extraction on C_{18} cartridges. The eluate containing cefazolin and internal standard, cephalexin, was injected onto a reversed-phase C_{18} column and eluted with a mobile phase of 23% methanol in 0.02 *M* sodium phosphate monobasic (pH 5.0) and detected with UV absorbance at 270 nm. Recoveries of cefazolin were 33.7 ± 2.5%, 45.4 ± 2.1%, and 42.9 ± 1.0% from liver, spleen and lung, respectively. The calibration curves for cefazolin were established at 0.5–1500 μg/g in spleen, 0.1–250 μg/g in liver and 0.1–75 μg/g in lung. The assay was reproducible with within-day and between-day variations of 1–2 and 1–4%, respectively. Application of the assay for tissue distribution of cefazolin in liposomal targeting study was demonstrated.

1. Introduction

Cefazolin is the most widely used broad-spectrum parenteral cephalosporin with an antibacterial spectrum similar to cephalothin. It is active against *Escherichia coli*, *Klebsiella* species and staphylococcal infections such as endocarditis, *etc.* [1]. Like most cephalosporin antibiotics, the use of cefazolin is limited by its poor intracellular penetration due to its high water solubility [2]. Liposomal encapsulation of antibiotics is one of the logical approaches to improve the antimicrobial activity of these antibiotics in the treatment of intracellular bacterial infections [3]. In addition, there is evidence suggesting that liposomal encapsulation also improves the efficacy of antibiotics in the treatment of extracellular infections. A rapid, selective and specific HPLC assay to analyze the tissue level of the drug is a prerequisite to evaluate the *in vivo* targeting characteristics in terms of organ distribution and pharmacokinetics of any developed liposomal cefazolin.

Reports on the HPLC assays of cefazolin in human plasma [4–6], peritoneal dialysis fluids [7], and human polymorphonuclear leukocytes [8] have been published. However, only two assays have been reported for cefazolin, one in rat tissues, including heart, skin, gut, bone, and lung [9], and another in muscle from a surgery

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patient [10]. Terasaki *et al.* [9] and Conner *et al.* [10] employed a C_{18} cartridge to isolate cefazolin in their procedures, but no recovery data nor validation of the assay was reported. At present, no published assay is available for cefazolin in liver and spleen tissues. These tissues are of prime interest in evaluating liposomal cefazolin since they contain residence macrophages which harbor facultative bacteria and phagocytize liposomes [3].

In this paper, a rapid, sensitive, and reproducible HPLC assay of cefazolin in rat liver, spleen, and lungs is presented. The assay was applied to characterize cefazolin tissue levels in rats after a treatment with liposomal formulation.

2. Experimental

2.1. Chemical and reagents

Cefazolin sodium, cephalexin (I.S.), and sodium phosphate monobasic were purchased from Sigma (St. Louis, MO, USA). All reagents were of analytical grade. HPLC-grade methanol was from Mallinckrodt (Paris, KY, USA). Phosphoric acid (85%) was from Fisher Scientific (Fair Lawn, NJ, USA). Sep-Pak C_{18} cartridge (100 mg) was purchased from Waters (Milford, MA, USA).

2.2. Chromatographic conditions

The liquid chromatograph used (Consta-Metric III, LDC, Riviera Beach, FL, USA) was equipped with a 100-μl sample loop (Valco, Houston, TX, USA), a variable-wavelength UV detector set at 270 nm (Spectro-Monitor III, LDC), a Nova-Pak C_{18} guard column (Waters, Milford, MA, USA), a Econosphere C_{18} column (5 μm, 250 mm $\times$ 4.6 mm I.D., Alltec, Deerfield, IL, USA) and a Shimadzu C-R6A Chromatopac integrator (Shimadzu, Kyoto, Japan). The mobile phase was 0.02 *M* sodium phosphate monobasic–methanol (77:23, v/v, pH 5.0). The flow-rate was 1 ml/min. The concentration of cefazolin in tissues was determined from calibration curves of peak-height ratios *vs.* cefazolin concentrations in spiked tissue samples.

2.3. Calibration curve

Blank liver, spleen and lung homogenates were prepared from untreated animals by homogenization with four (for liver and lung) or twenty nine (for spleen) volumes of water (w/v) in a Thomas tissue grinder (Series 3431-D70, Swedesboro, NJ, USA). Standard liver, spleen, and lung homogenates were prepared by spiking 1 ml of the homogenates with 20 μl of I.S. stock solution (500 μg/ml) and a serial dilution of cefazolin stock solution (1 mg/ml), to obtain cefazolin standards of 0.02, 0.1, 0.2, 0.5, 1, 3, 5, 10, 30, 50 μg/ml and I.S. concentration of 10 μg/ml. The spiked tissues were then extracted as described below. Calibration standards were prepared on the day of analysis. The stock solutions of cefazolin and I.S. were stored at −20°C and stable for at least two weeks [11].

2.4. Extraction procedure

To 1 ml of tissue homogenate, 50 μl of 8.5% phosphoric acid was added. The tubes were vortex-mixed for 30 s, then centrifuged for 5 min at 2000 *g*. The supernatant was loaded onto a C_{18} cartridge pre-conditioned with 5 ml each of methanol and water. The column was then washed with 3 ml of water. Cefazolin and I.S. were eluted from the column by 2 ml of 60% methanol. Aliquots of 10 μl of the eluate were injected onto the HPLC system.

2.5. Assay validation

Five sets of tissue standards were prepared and analyzed on the same day to establish the within-day variation. The assay was repeated over an eight-month period to establish the between-day variation. Accuracy of the analytical methods was determined by comparing actual concentrations to predicted concentrations calculated from equations obtained by linear regression of calibration curves. Recoveries of cefazolin from the tissue homogenates were

evaluated by spiking a series of known amounts of cefazolin into the blank samples and extraction as previously described. The recovery was determined by comparing the slopes of the regression curves from the extracted tissue standards with those from the non-extracted standards.

2.6. Organ distribution kinetics

Male Sprague–Dawley rats (Sasco, Omaha, NE, USA), weighing 200–250 g, received an intravenous dose of 50 mg/kg body weight of free (cefazolin saline solution) or liposomal encapsulated cefazolin. Animals were sacrificed at 1 and 2 h postdose for free cefazolin, and at 1, 6, and 48 h postdose for liposomal encapsulated cefazolin. Tissue samples were collected and weighed. All tissue samples were frozen and thawed before assay, a procedure known to disrupt liposomal membranes and allow leakage of entrapped drug. Tissue samples were homogenized with water in a 1:4 ratio (g/ml) for liver and lung, and a 1:29 ratio (g/ml) for spleen at 4°C. Tissue homogenates were kept at −20°C and assayed within a week.

3. Results and discussion

Baseline separation of cefazolin and cephalexin was achieved with retention times of 9.5 and 11.5 min, respectively. No interfering peaks were found in the drug-free tissue extract (Fig. 1). The assay was found to be linear over the concentration range 0.1–250 μg, 0.5–1500 μg, and 0.1–75 μg per g for liver, spleen and lung, respectively (Table 1). The slopes of the calibration curves for the various tissue sample types did not change significantly over the eight-month period. The within-day variability (C.V.) was less than 2% and the between-day C.V. less than 5%. The quantitation limits were low enough to characterize tissue cefazolin levels in our preclinical formulation studies.

Accuracy and precision of the assay are presented in Table 2. Accuracy, estimated as the deviation from the theoretical values was less

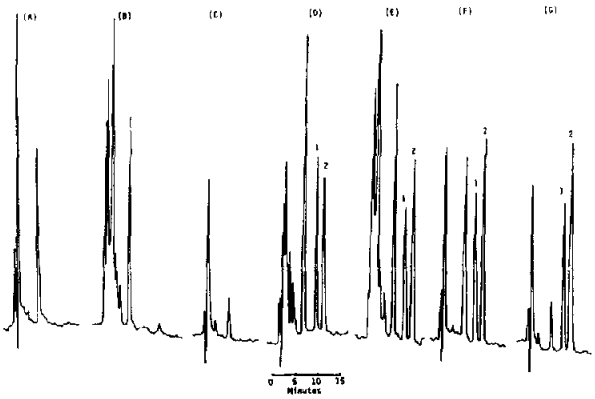

Fig. 1. Chromatograms of extracts obtained from tissue homogenates of drug-free rat lung (A), liver (B), and spleen (C); rat liver spiked with 5 μg/ml cefazolin (D); and the rat liver (E), lung (F) and spleen (G) obtained at 9 h after dosing of 50 mg/kg liposome encapsulated cefazolin. Peaks: 1 = cefazolin; 2 = I.S. (cephalexin). All monitored at 270 nm and 0.01 AUFS.

than 4% at all concentration levels assayed for each sample type. Precision of the assay was estimated by triplicate analyses of spiked tissue samples at cefazolin concentrations of 0.5, 5.0, and 45.0 or 10 μg/ml. The intra-assay and inter-assay coefficients of variation at each concentration for all sample types ranged from 1 to 6%.

Recoveries were 33.7 ± 2.5, 45.4 ± 2.1, and 42.9 ± 1.0% from liver, spleen, and lung, respectively (Table 1). Many factors might influence the recovery of cefazolin from tissue samples, including tissue protein binding [12], the selectivity of the solid-phase cartridge used, and the choice of eluent for the solid-phase extraction (SPE) [13]. Ligandin, a water-soluble intracellular basic protein isolated from rat liver and kidney, is one of the major tissue proteins binding to anionic drugs, such as β-lactam antibiotics, both *in vitro* and *in vivo* [14,15]. This might partially explain the low recoveries of cefazolin found in these tissues. In this study, a known amount of cefazolin was spiked to a blank tissue homogenate containing ligandin. Thus, the *in vitro* binding situation mimicked that of the *in vivo* condition. The recoveries found for the spiked and the pure samples were comparable. SPE was chosen as the sample clean-up procedure since SPE has been previously applied to

Table 1
Calibration curves of cefazolin in tissue samples

Parameter	Liver	Spleen	Lung
Concentration range (μg/g tissue)	0.1–250	0.5–1500	0.1–75
Slope	0.1498 (0.008)	0.1457 (0.003)	0.1393 (0.001)
Between-day variation (%)	4.26	1.94	1.02
Within-day variation (%)	1.55	1.67	0.86
Intercept	0.052 (0.041)	0.020 (0.017)	0.013 (0.012)
Coefficient of correlation	0.9993	0.9997	0.9984
Recovery (%)	33.7 (2.5)	45.4 (2.1)	42.9 (1.0)
Limit of quantitation (μg/g tissue)	0.1	0.5	0.1

Numbers in parentheses are standard deviations ($n = 5$).

extract chemicals from various type of tissues [16]. Cefazolin has also been extracted from muscle [10] and skin, heart, bone, gut and lung [9] by SPE and determined by HPLC, but no recovery data have been reported. In our preliminary study, attempts to employ the published extraction procedures [4,10] to determine cefazolin concentrations in liver, spleen and lung were unsuccessful, due to endogenous interferences, low extraction efficiency, unsatisfactory reproducibility, and the inability of tissue homogenates to pass through tightly packed bonded-phase cartridges. Therefore, the procedure was modified. Tissue proteins were precipitated with phosphoric acid, which enabled the tissue homogenates to pass easily through the cartridges. In addition, acidification of the tissue sample (pH 2.5) maximized the retention of antibiotics on the C_{18} cartridge after sample loading, because cefazolin ($pK_{a1} = 2.5$) and the I.S. ($pK_{a1} = 5.2$) are both in their un-ionized form at this pH. Our study demonstrated that

Table 2
Accuracy and precision of the assay for determining cefazolin concentrations in rat tissue samples

Tissue	Added concentration (μg/ml)	Evaluated[a] concentration (μg/ml)	C.V. (%)[b]		Bias[c] (%)
			Within-day	Between-day	
Liver	0.5	0.48 ± 0.02	4.05	4.17	4.0
	5.0	4.98 ± 0.16	1.30	3.55	0.5
	45.0	43.84 ± 0.88	0.92	2.02	2.6
Spleen	0.5	0.50 ± 0.03	2.59	5.39	0.0
	5.0	4.99 ± 0.13	2.62	2.59	0.2
	45.0	43.60 ± 0.98	1.63	2.24	3.1
Lung	0.5	0.50 ± 0.02	4.65	3.83	0.0
	5.0	4.86 ± 0.10	2.03	5.05	2.8
	10.0	10.30 ± 0.48	5.94	4.64	3.0

[a] $n = 3$.
[b] C.V.% = (standard deviation/mean) · 100%.
[c] Bias = {(evaluated concentration − added concentration)/added concentration} · 100%.

Table 3
Mean cefazolin concentrations (μg/g) in tissues after i.v. doses of the antibiotic in rats determined by HPLC

Tissue	Free cefazolin		Liposomal cefazolin		
	1 h	2 h	1 h	6 h	48 h
Liver	0.8	ND	57	75	10
Spleen	ND[a]	ND	760	786	105
Lung	5.4	1.3	38	25	ND

Male Sprague–Dawley rats received 50 mg/kg body weight of cefazolin intravenously in either free (cefazolin saline solution) or liposome-encapsulated form.
Values are mean cefazolin concentrations in tissues (microgram per gram organ) of six rats for every time point. The average tissue weights were 11.8, 0.85, and 1.19 g for liver, spleen, and lung, respectively.
[a] ND: Not detectable.

SPE is a potential tool for the reproducible recovery of cefazolin from tissue samples.

The developed assay was applied to evaluate the liposomal targeting of cefazolin in rats after intravenous administration of 50 mg/kg of cefazolin as a saline solution or as a liposome preparation. No interfering peaks were found in the blank tissue samples with respect to cefazolin and cephalexin (Fig. 1). Tissue levels of cefazolin are summarized in Table 3 and the distribution profiles are constructed in Fig. 2. Liposome encapsulation significantly modified the tissue distribution pattern of cefazolin (Table 3), and targeted the drug to the reticuloendothelial system. The cefazolin levels found in the liver and spleen are in the bactericidal range for many strains of bacteria.

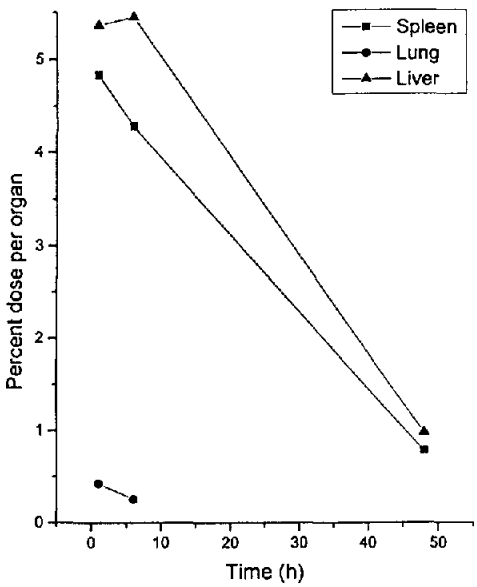

Fig. 2. Tissue distribution kinetics of cefazolin as percentage of the dose per organ after intravenous injection of a 50 mg/kg liposomal preparation in rats. Each point represents mean values from six rats.

This work is the first study of a specific HPLC assay allowing the monitoring of cefazolin in rat liver and spleen. The assay is sensitive and reproducible, and can be applied for tissue distribution studies to evaluate various liposomal formulations, and to correlate the effects of formulation on drug disposition.

4. Acknowledgements

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5. References

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