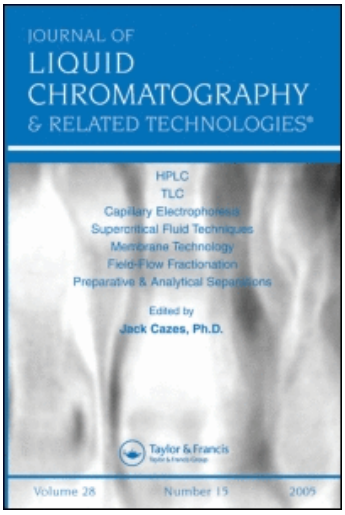

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Bompadre, S. , Leone, L. , Ferrante, L. , Alo, Fp. and Ioannidis, G.(1998) 'Determination of Cefazolin in Human Serum by High Performance Liquid Chromatography with On-Line Solid Phase Extraction', Journal of Liquid Chromatography & Related Technologies, 21: 3, 417 — 426
To link to this Article: DOI: 10.1080/10826079808000500
URL: http://dx.doi.org/10.1080/10826079808000500

J. LIQ. CHROM. & REL. TECHNOL., 21(3), 417–426 (1998)

DETERMINATION OF CEFAZOLIN IN HUMAN SERUM BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ON-LINE SOLID PHASE EXTRACTION

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ABSTRACT

A simple, sensitive, reproducible, and rapid high performance liquid chromatographic assay, with column switching, is described for the determination of cefazolin in human serum. Serum samples were directly injected, first, in a chromatographic column for sample clean-up and extraction, and then, analysed on a analytical column using ultraviolet absorption at 273 nm. The assay allows direct determination of cefazolin over the range of 1 - 250 μg/mL using 12.5 μL of serum.

Validation of the method demonstrated a good sensitivity, accuracy, and precision. It has been applied to study the pharmacokinetics of the drug in patients undergoing vascular surgery.

INTRODUCTION

Cefazolin is a first generation cephalosporin antibiotic and is used in the treatment of a variety of infections due to susceptible organisms. It is currently still the recommended antibiotic for the prophylaxis in patients undergoing vascular surgery.[1] Determination of this drug which raised a new interest due to its new formulations, may be required for experimental and clinical purposes.

Antibiotics can be quantitated by microbiological assays, but they lack specificity due to possible presence of other antibiotics or active metabolites. High performance liquid chromatography (HPLC) is finding an increasingly important role in the assay of antibiotics, especially cephalosporins, in clinical laboratory. Reports on the HPLC assay for cefazolin in biological fluids and tissues have been published[2] (and references cited therein). HPLC analysis of drugs in biological matrices usually involves treatment of the sample before injection into the chromatograph. Typical treatments include precipitation, liquid-liquid extraction and solid-phase extraction. Such conventional procedure may involve tedious, complex, and time-consuming steps. Because of the extensive sample handling, contamination and sample loss are not unusual. The direct sample injection may solve these problems. Therefore, extraction procedures which eliminate manual sample manipulation must be developed. On-line solid-phase extraction, separation, and quantification of drugs in biological samples is gaining widespread acceptance and application.[3] In column switching, a fraction of the effluent from a primary column which produces an on-line sample clean-up, is selectively transferred to an analytical column. An internal standard is not necessary.

Using a column-switching approach, a rapid, accurate, and precise method with on-line solid-phase extraction has been developed for the determination of cefazolin in serum. The method has been applied to study the kinetics of cefazolin in patients undergoing vascular surgery. A similar chromatographic system has previously been used to determine other antibiotics in biological fluids.[4,5]

EXPERIMENTAL

Chemicals

All reagents were of analytical grade. HPLC-grade methanol was purchased from E. Merck (Darmstadt, Germany). Sodium dihydrogen

orthophosphate was supplied by Carlo Erba Farmitalia (Milano,Italy); cefazolin was from Bristol Italiana Sud. Analytical grade, filtered water was obtained daily from an Elgastat UHQ PS apparatus (ELGA, High Wycombe, BUCKS, England).

Apparatus

A Varian (Walnut Creek, CA, USA) model Vista 5500 HPLC pump, a Spectra Physics model SP-8000B solvent-delivery system, and a Model 166 programmable ultraviolet detector from Beckman were used. Integration of chromatograms was performed by means of a System Gold laboratory data system (Beckman, Berkeley, CA, USA). The injector was a Rheodyne model 7725i manual injection valve equipped with a 50μL sample loop. The coupled-column system was operated by a pneumatic, six-port, automated switching valve (VALCO Schenkon, Switzerland), controlled by the HPLC system.

Chromatographic Conditions

All analysis were performed at room temperature. The analytical column was a HP ODS analytical column (20 cm x 0.4 cm I.D., particle size 5 μm) from Hewlett-Packard (Palo Alto, CA, U.S.A.). The extraction column was a 5 cm x 0.4 cm I.D., dry-filled with a C_8 40 μm silica. The mobile phase 1 (extraction eluent) consisted of 10 mM aqueous sodium dihydrogen orthophosphate and methanol (90:10, v/v), pH 5.0. The mobile phase 2 (analytical eluent) was 30% methanol in 10 mM aqueous sodium dihydrogen orthophosphate. The flow rate in both columns was set at 1 mL/min and the pressure in the analytical column was 150 atm. The effluent from the analytical column was monitored by UV at a wavelength of 273 nm.

Switching Procedure

50 μL of sample (serum + 10 mM aqueous sodium dihydrogen orthophosphate, 1:3, v/v) were injected, without any sample preparation, directly onto the extraction column. It was brought onto the column by the mobile phase 1 delivered by the pump 1, while the pump 2 delivered the same mobile phase 1 to the analytical column (Fig.1A). The mobile phase 1, which passed through the extraction column and was directed to waste, removed the polar components of the serum matrix whilst cefazolin was trapped in the pre-column. After 1 minute the analyte was transferred from the extraction column

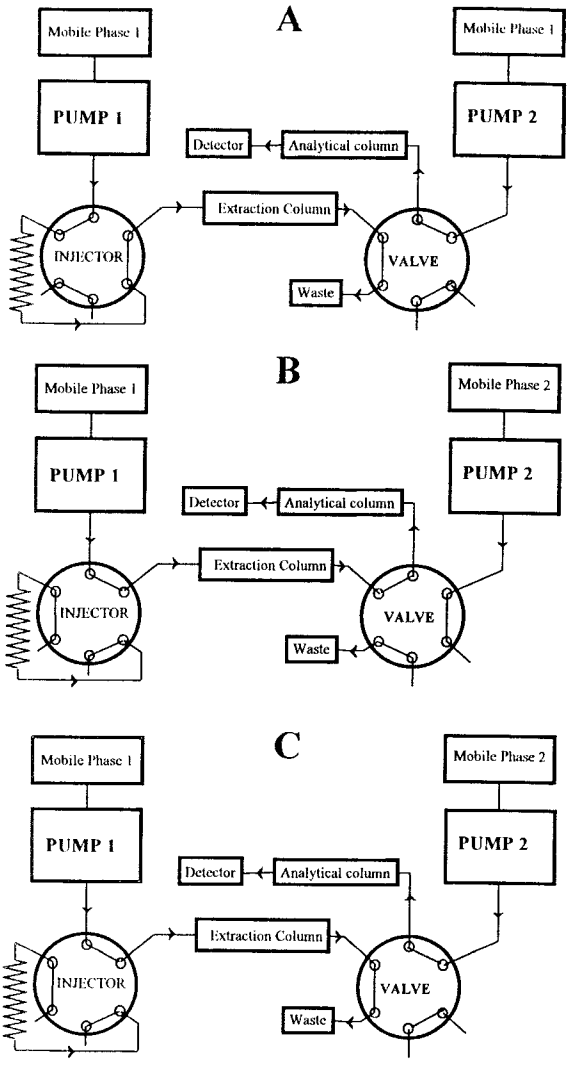

Figure 1. Schematic diagram of the column switching system developed and used: A: Injection and extraction of sample (Mobile phase 1 from pump 1 to extract cefazolin and from pump 2 to prepare analytical column). B: Elution of the analyte-containing fraction from the extraction column into the analytical column, with mobile phase 1 from pump 1. C: separation of the drug with the mobile phase 2 from pump 2 and conditioning of extraction column with mobile phase 1 from pump 1.

to the analytical column, by switching the valve, with the mobile phase 1 from the pump 1, and the mobile phase in pump 2 changed from mobile phase 1 to mobile phase 2 (Fig.1B). After 5 minutes the valve was switched and mobile phase 2 from pump 2 passed through the analytical column where cefazolin was separated and detected by UV, while the pump 1 was allowed to maintain the mobile phase 1 to flow through the extraction column to prepare it for the next analytical run (Fig.1C).

Sample Processing

The suitability of the method to study cefazolin pharmacokinetics was assessed by analysing serum samples from patients after i.v. administration of cefazolin (2 g). Blood samples were collected at appropriate time intervals post-dose, serum was stored at -20°C and analysed as described.

Quantitation

The samples were analysed by comparison with a calibration curve obtained by spiking drug-free serum with increasing amounts of cefazolin, leading to concentrations of 1, 5, 10, 25, 50, 100, and 250 μg/mL. Each spiked serum standard was injected eight times. Quantification was based on peak areas, as measured by integrator. The calibration data of peak-area against the concentration of the drug were fitted to a linear, unweighted, model. The resultant linear regression curves were used to calculate the drug concentrations in the samples. The recoveries of the extraction step were determined by comparing the peak areas of spiked samples with those obtained for an equivalent amount (dissolved in buffer) directly injected into the analytical column.

RESULTS AND DISCUSSION

The method described, which combines HPLC with column-switching for samples clean-up, overcomes the problems of complicated and time-consuming procedures of the conventional HPLC methods. No extraction or cleanup steps, and no internal standard are required. The assay allows the direct injection of serum, and high sensitivity can be achieved in a relatively short analytical time. The extraction column was connected before a switching valve and the analyte of interest was eluted into the analytical column.

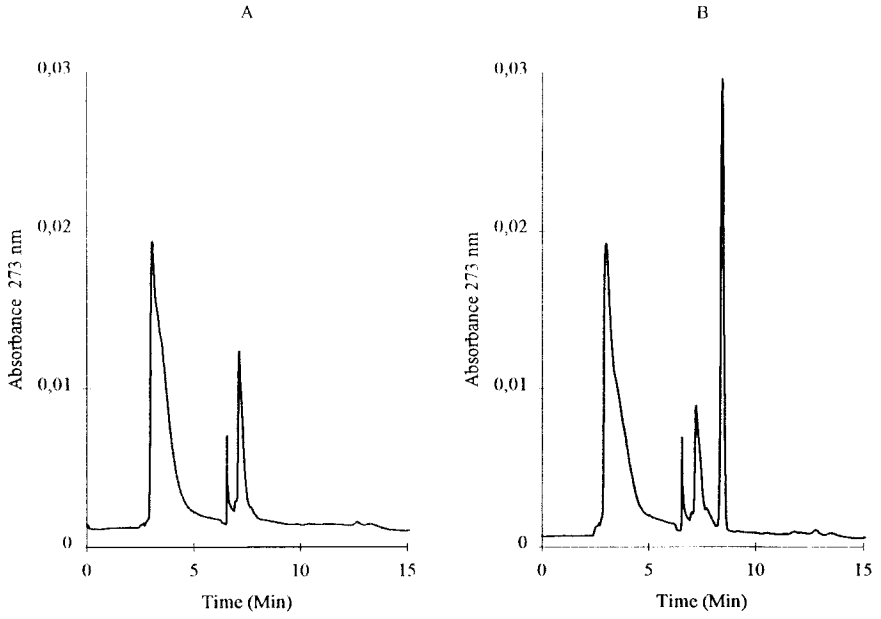

Figure 2. Chromatograms of drug-free serum (A) and serum spiked with 15 μg/mL of cefazolin.

The extraction process was optimized by testing columns dry packed with different phases. C_{18} (octadecil), C_8 (octyl), and CN (cyanopropyl) silica phases (particle size 40 μm) were tentatively used. A mobile phase weaker than the previously selected analytical eluent, which consisted of a mixture of 30% methanol in 10 mM of sodium dihydrogen orthophosphate, was chosen. Various methanol concentrations, from 5% to 25%, in the mobile phase were investigated using different stationary phases. The best results were obtained using C_8 packing material and 10% methanol. A good sample clean-up, a short retention time on the pre-column, and a good separation of the drug were achieved. When CN was used as packing material, low extraction efficiency was observed. C_{18} required higher concentrations of organic modifier in the mobile phase and gave larger peaks. The time required for a satisfactory retention of cefazolin in the column was chosen by analysing recoveries after different extraction times. The connection time of the pre-column to the analytical column was selected by stepwise reduction until the peak area of cefazolin started to decrease. An optimum connection time was found to be 4 min. The chromatograms of a drug-free serum sample and a spiked serum are

Table 1

Precision, Accuracy and Linearity for Cefazolin (Spiked Serum)*

Nominal (μg/mL)	Actual Value (Mean ± S.D., n=8) (μg/mL)	Precision (%)	Accuracy (%)
1	1.09 ± 0.05	4.40	9.00
5	4.82 ± 0.18	3.73	-3.60
10	9.77 ± 0.48	4.91	-2.30
25	25.06 ± 0.85	3.39	0.24
50	48.70 ± 1.54	3.16	-2.60
100	97.75 ± 2.34	2.39	-2.25
250	252.46 ± 3.56	1.41	0.98

* Standard regression line: $y = (3.05 \pm 0.08)x + (-8.36 \pm 5.67)$

shown in Figure 2. Cefazolin was found to have a retention time of 8.9 min and was well separated from the other detectable components in human serum at the selected wavelength. The absence of interferences demonstrates the clean-up efficiency.

Validation of the Assay

The analytical methodology was validated in terms of linearity, precision, accuracy, recovery, and sensitivity. Linearity was examined at concentrations within the range 1-250 μg/mL (Table 1). Standard curves were linear over the indicated concentration range, with the correlation coefficient $r^2 = 0.998$. The equation of the regression line of the cefazolin concentrations versus peak-areas ratio was: $y = (3.05 \pm 0.08)\ x + (-8.36 \pm 5.67)$. Precision and accuracy of the assay are shown in Table 1. The accuracy was +9.00% for 1 μg/mL and +0.98% for 250 μg/mL. Values for precision [coefficient of variation (CV)] at these concentrations were 4.40% and 1.41%, respectively. Recovery was determined by the ratio of the peak areas resulting from spiked sera and aqueous solutions, at the same drug concentration, passed through both the extraction and the analytical column to the peak areas of aqueous solutions injected directly onto the analytical column. The recovery of cefazolin was found to be 97% in serum samples.

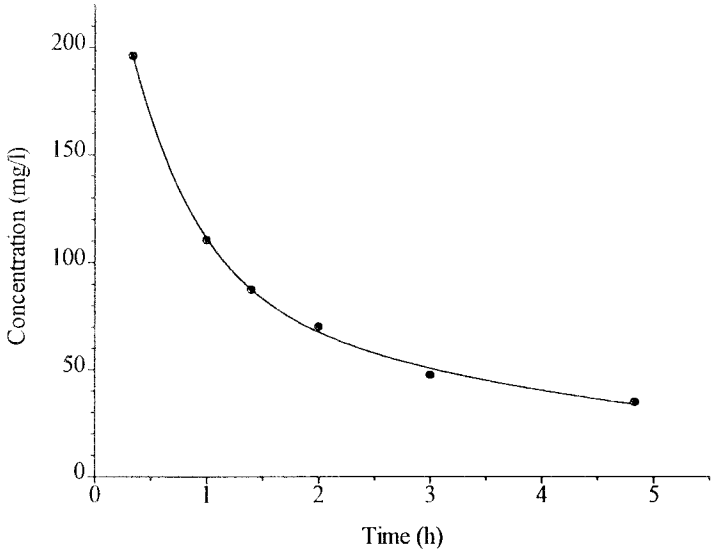

Figure 3. Serum concentration-time curve of cefazolin from a patient after 2 g i.v. administration.

Based on a 50 μL sample volume (serum + 10 mM aqueous sodium dihydrogen orthophosphate, 1:3, v/v), the detection limit (for a signal to noise ratio = 3) was of the order of 0.3 μg/mL. This limit is low enough to characterize serum cefazolin levels in clinical pharmacokinetic studies. However, it could be improved by injection of larger volumes of serum samples. The cycle time of one analysis was 12 minutes.

Clinical Pharmacokinetics

The assay described above was employed in a pharmacokinetic study of 8 patients undergoing vascular surgery, after administration of cefazolin for antimicrobial prophylaxis.

Figure 3 shows the concentration-time curve for cefazolin obtained from a patient after i.v. administration of 2 g dose. The regression analysis on individual data was achieved by assuming a two compartment model.

Table 2

Pharmacokinetic Parameters After Intravenous Administration of 2g Cefazolin*

$t_{1/2\beta}$ (h)	AUC (mg.h/L)	Cls (mL/min)	V_1 (L)	Vss (L)
3.65 ± 0.624	519.62 ± 97.34	64.15 ± 12.44	9.86 ± 1.42	16.30 ± 2.74

* means ± SD

Table 2 depicts the main pharmacokinetic parameters (means ± SD) of the patients: terminal half-life ($t_{1/2\beta}$), area under the serum concentration-time curve (AUC), volume of distribution of the first compartment (V_1), volume of distribution at steady state (Vss) and serum clearance (Cls). The results obtained are in agreement with those reported.[6]

CONCLUSIONS

The column-switching HPLC method described has advantages over conventional HPLC in that it allows the on-line determination of cefazolin without sample pre-treatment. The direct injection of biological sample eliminates time-consuming cleanup steps and increases analytical accuracy. Internal standard is not necessary. Validation of the assay demonstrated good sensitivity, accuracy, and precision. The system has the advantages of speed of analysis and high recovery It is relatively inexpensive and requires a very simple equipment. The method is sufficiently sensitive to analyse cefazolin in small sample volumes (e.g. from pediatric patients). The assay is suitable for human pharmacokinetic studies and clinical purposes. The analysis of concentrations in patients after a 2 g i.v. dose are reported to document the utility of this method in clinical pharmacokinetic studies.

ACKNOWLEDGMENT

This work was supported by grants from MURST 60% and CNR 8600312.04.

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Received January 12, 1996
Accepted February 5, 1996
Manuscript 4069