

Rapid Analysis of Cefazolin in Serum by High-Pressure Liquid Chromatography

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A high-pressure liquid chromatography (HPLC) method has been developed for the analysis of cefazolin in serum. Serum was deproteinized by the addition of 6% trichloroacetic acid and injected onto a reverse-phase column with a mobile phase of 10 to 15% methanol in 1% aqueous acetic acid. Cefazolin chromatographed without interference from ultraviolet-absorbing components of serum, with a retention time of 3.1 min. Standard curves comparing peak area with concentration prepared from dog or human sera were linear over a range of 1.6 to 200 $\mu\text{g/ml}$. Results from the HPLC assay were compared with microbiological assays (cylinder plate method) on both standard serum samples and sera from dogs and human subjects receiving intramuscular cefazolin. The HPLC method was somewhat more accurate in comparison with the microbiological assay performed on serum samples of known concentration. The comparison of results from an analysis of serum levels of dogs or human subjects receiving cefazolin indicated that the two methods would lead to identical conclusions concerning pharmacokinetics or the achievement of therapeutic serum levels. The HPLC assay method presents an alternative to conventional microbiological assays, with marked improvement in speed (30 min) and considerable potential for future development.

The antibiotics as a class are unique among drugs or xenobiotics in that the analytical methods used in the routine determination of serum levels have involved bioassay rather than chemical methodology.

Cephalosporins are a widely used group of drugs that have traditionally been assayed in serum by microbiological assays. Sera from patients, clinical pharmacology test subjects, or experimental animals receiving cephalosporins are usually assayed by the disk plate, cylinder plate, or agar well diffusion methods, which depend on the inhibition of growth of *Bacillus subtilis* or *Sarcina lutea*.

The conventional methods routinely used for drug assay in serum, such as gas chromatographic, photometric, or fluorometric methods, have not been applied to the cephalosporins, except to a very limited extent. Tune (5) determined cephaloridine concentrations in serum by a method involving the fluorometric determination of pyridine released by acid hydrolysis. Mullen et al. (3) reported an indirect method for the determination of cephaloridine, involving the gas chromatographic determination of pyridine released by alkaline hydrolysis.

Polarographic methods for the determination of cephaloridine, cephalothin, and cephalixin in an ultrafiltrate of serum have been reported

by Benner (1). The chemical methods described above, although satisfactory under the conditions used, do not appear to have widespread utility.

The cephalosporins as a group do not lend themselves to simple gas chromatographic or photometric assays because of their low volatility and poor extraction from serum into organic solvents.

High-pressure liquid chromatography (HPLC) seems to offer the advantages of gas chromatography, such as simultaneous separation and quantitation, in a system that does not necessarily require either extraction or volatilization of the antibiotics.

An HPLC technique for the analysis of tetracycline after extraction from serum has recently been reported by Nilsson-Ehle et al. (4). Cooper et al. (2) reported on the use of HPLC, employing anion exchange chromatography of cephalothin and deacetylcephalothin after ion pair extraction from human serum.

This report describes the development of a method for determining cefazolin in serum by a direct injection of deproteinized serum onto a reverse-phase liquid chromatographic column. The advantages of this approach are simplicity and speed, with accurate determination of serum concentrations available in a properly pre-

pared laboratory within 30 min after the receipt of a serum sample.

MATERIALS AND METHODS

Antibiotics. Cefazolin sodium (Kefzol) was obtained from Eli Lilly & Co., Indianapolis, Inc.

HPLC. Chromatography was carried out on a model ALC 202 liquid chromatograph (Waters Associates, Milford, Mass.). A stainless-steel column (57 cm by 2 mm) was packed with Phenyl Corasil (37 to 50 μ m, reverse phase; Waters Associates, Milford, Mass.).

The mobile phase consisted of 10 to 15% methanol in 1% aqueous acetic acid. The concentration of methanol in the mobile phase was adjusted daily for optimum separation and peak shape. The flow rate was maintained at 2.0 ml/min at pressures of 1,500 to 2,000 lb/in. The detector monitored absorption at 254 nm, recorded at 0.02 to 0.04 absorbance units (full scale).

Standard solutions were prepared by dissolving weighed amounts of cefazolin sodium in dog or human sera at various concentrations. The cefazolin sodium standard had been previously assayed by conventional methodology and was shown to contain 815 mg of cefazolin activity per g. Concentrations of cefazolin sodium were obtained from the HPLC analysis by a comparison with standard curves prepared daily, relating the peak area to cefazolin sodium concentrations. The resultant data were then corrected by the above factor to allow comparison with microbiological assay results expressed as cefazolin activity.

Sample preparation. Frozen serum samples obtained from dogs receiving 10 mg of cefazolin sodium per kg intramuscularly or human volunteers receiving a 1-g intramuscular dose were thawed just prior to analysis. A 0.5- to 1-ml portion of serum was removed and an equal volume of cold 6% trichloroacetic acid was added. The samples were kept at 0°C for 5 min and centrifuged at 1,800 rpm for 10 min (model K laboratory centrifuge, International Equipment Co., Needham Heights, Mass.). The supernatant solution was removed and kept at 0°C until a 90- μ l portion was injected into the liquid chromatograph. Standards in serum were prepared in an identical manner.

RESULTS

Chromatography. Representative chromatographic tracings resulting from the injection of samples prepared from blank dog serum or from dog serum containing 20 μ g of cefazolin sodium per ml are shown in Fig. 1. The chromatographic conditions provided adequate separation of the cefazolin peak from ultraviolet-absorbing components of the serum sample. Control serum contained no interfering ultraviolet-absorbing components. Chromatography of blank and cefazolin-containing samples of human serum was identical to the results described for dog serum.

Standard curves. The reproducibility and linearity of a standard curve generated from

dog or human serum containing various amounts of cefazolin sodium are shown in Fig. 2. The standard curve represents triplicate analyses. A standard curve was prepared daily from single analyses at each concentration. When higher serum concentrations were expected, standard curves were prepared over a concentration range of 10 to 200 μ g/ml, with a full-scale absorbance at an optical density of 0.04 with similar reproducibility and linearity.

The reproducibility of the analytical method was demonstrated by the analysis of 12 portions of blank human serum containing cefazolin sodium at a concentration of 25 μ g/ml assayed against a standard curve prepared from single samples containing 5, 10, 20, 30, and 50 μ g of cefazolin sodium per ml. The resultant data gave a mean of 24.3 μ g/ml, with a standard deviation of 1.2 and a coefficient of variation of 4.9%. The range of values was from 21.9 to 26.6 μ g/ml.

Comparison with microbiological assay. (i) **Standards.** Two sets of standard solutions were prepared in dog serum. One set was prepared by the HPLC laboratory and the other by the laboratory performing the microbiological as-

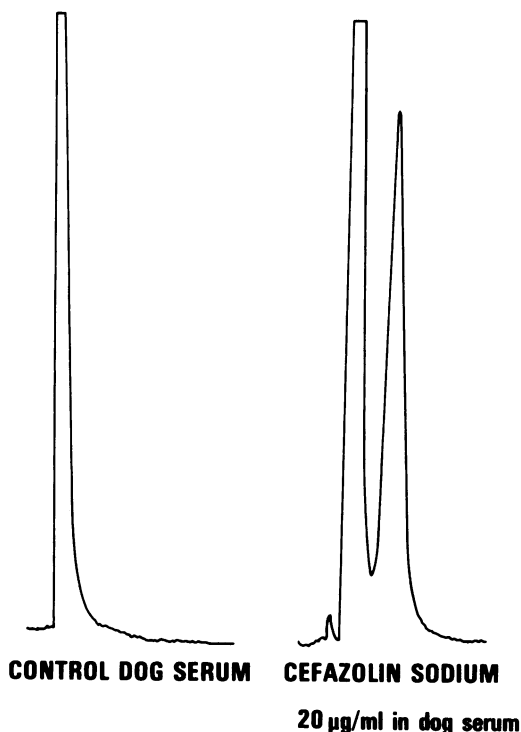


FIG. 1. HPLC tracings of control dog serum and dog serum containing 20 μ g of cefazolin sodium per ml. The retention time of the cefazolin peak is 3.1 min (optical density, 0.02, full scale).

says. Standards were then assayed by both laboratories. The results are shown in Table 1.

The HPLC assay gave results ranging from 85 to 113% (mean, 104%) of the actual cefazolin concentration, and the relative deviation from the actual value was similar over the complete range of concentrations studied (1.6 to 100 $\mu\text{g/ml}$). The microbiological assay gave results that ranged from 72 to 106% of the actual values (mean, 87%). The values determined by the microbiological assay were consistently lower than the actual value, and the deviation be-

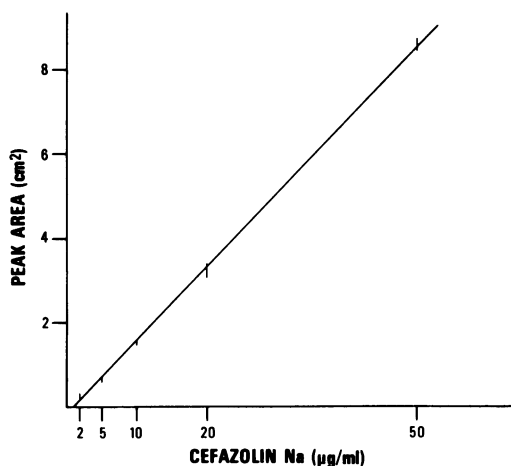


FIG. 2. Typical standard curve relating the area of the HPLC peak to the standard concentration of cefazolin sodium in dog serum. Vertical bars represent the range of triplicate analyses at each concentration. The line is the least-squares regression line with slope = 0.1743, intercept = 0.2037, correlation coefficient = 0.999.

came more exaggerated at higher serum concentrations.

(ii) Serum levels. Serum samples from dogs receiving 10 mg of cefazolin per kg intramuscularly and human subjects receiving 1-g intramuscular doses were analyzed by both HPLC and microbiological methods. The resultant serum level-time curves are presented in Fig. 3 and 4.

There is generally good agreement between the two assay methods. The correlation coefficient between the HPLC and microbiological assay in dog and human sera over all time points was 0.96. The HPLC technique gave slightly higher values at most time points although the agreement between the two methods was better at the lower serum levels observed in dogs (Fig. 3).

In cases in which there was a significant discrepancy between the two methods, as shown in Fig. 4 (subject F, 3 h), the HPLC assay appeared to give a more reasonable value since there is no pharmacokinetically logical explanation for a rise in the serum level from 3 to 4 h, as indicated by the microbiological assay.

DISCUSSION

The liquid chromatographic analysis of cefazolin in serum offers an alternative to the conventional microbiological assay. The HPLC method is accurate and reproducible and appears to give results that are at least comparable to or perhaps slightly better than the microbiological assay. The differences between the results obtained by the two methods are not of a great enough magnitude, however, to alter any

TABLE 1. Comparison of HPLC and microbiological assays of standard cefazolin solutions in dog serum

Source of standard sera	Actual concn of cefazolin ($\mu\text{g/ml}$)	HPLC assay ^a concn of cefazolin ($\mu\text{g/ml}$)	%	Microbiological assay ^b concn of cefazolin ($\mu\text{g/ml}$)	%
HPLC laboratory	1.6	1.9	119	1.7	106
	4.1	3.5	85	3.9	95
	8.2	8.8	107	6.7	82
	16.3	15.7	96	11.7	72
	40.8	40.8	100	30.9	76
Microbiological assay laboratory	5	5.1	102	4.7	94
	10	9.7	97	8.8	88
	25	26.4	106	21.5	86
	50	55.6	111	46.3	93
	100	113	113	81.7	82
Mean			104		87
Standard error			3.1		3.2
Standard deviation			9.8		10.0

^a HPLC assay values are the result of single determinations.

^b Microbiological assay values are the result of one assay (two plates) against *B. subtilis*, using the cylinder plate method.

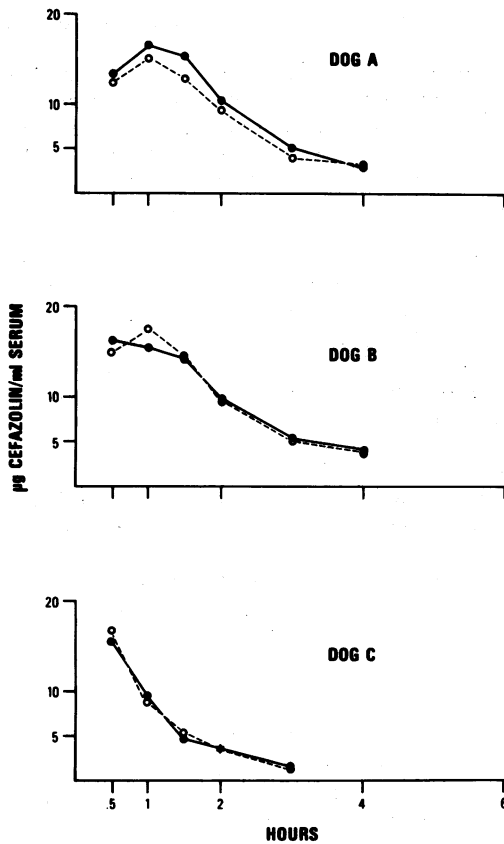


FIG. 3. Serum levels of cefazolin in dog serum after the administration of 10 mg/kg intramuscularly: comparison of HPLC and microbiological assay. Symbols: ●, single HPLC assay corrected to cefazolin activity; ○, microbiological assay against *B. subtilis*, using the cylinder plate method (single assay, two plates); *, coincident points.

conclusions about pharmacokinetics or the attainment of therapeutic serum levels. The most significant advantage of the HPLC method is the speed with which results can be obtained. The concentration of cefazolin in serum can be determined within 30 min of venipuncture through use of the HPLC assay as compared with the 16- to 18-h overnight incubation used in conventional microbiological assays.

With continued importance being placed on the monitoring of antibiotic serum levels in special clinical situations, such as impaired renal function, the advantage of rapid analytical methods will become increasingly important.

This report describes an assay for cefazolin. Similar assays have been developed in this laboratory for cephaloridine and cefamandole, using different mobile phases (unpublished data),

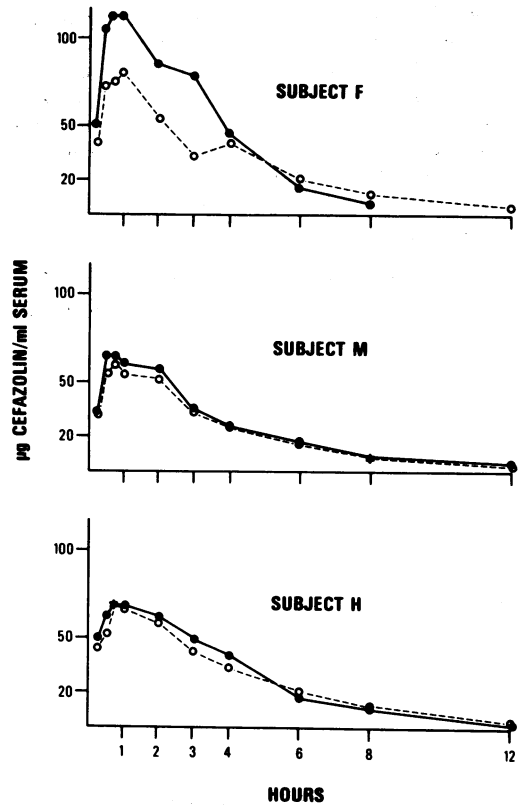


FIG. 4. Serum levels of cefazolin in human subjects after the administration of 1 g intramuscularly: comparison of HPLC and microbiological assay. Symbols: ●, single HPLC assay corrected to cefazolin activity; ○, microbiological assay against *B. subtilis*, using the cylinder plate method (single assay, four plates, two zones per plate); *, coincident points.

and the chromatographic method should be applicable to most cephalosporins.

Another potential advantage of the HPLC assay technique is in the simultaneous determination of two antibiotics in serum resulting from combination therapy. This approach has not yet been investigated.

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