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# A VERY PRECISE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE DETERMINATION OF CEFMENOXIME, A NEW CEPHALOSPORIN ANTIBIOTIC, IN PLASMA

G. RICHARD GRANNEMAN and LAWRENCE T. SENNELLO*

*Drug Metabolism Department, Division of Drug Safety Evaluation, Abbott Laboratories, North Chicago, IL 60064 (U.S.A.)*

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## SUMMARY

A simple and very precise high-performance liquid chromatographic procedure has been developed for the determination of cefmenoxime, a new broad spectrum cephalosporin antibiotic, in plasma. The workup procedure involves ultrafiltration of samples which have been treated with sodium dodecyl sulfate to displace the drug from its binding sites on plasma proteins. The ultrafiltrates are then directly injected into a high-performance liquid chromatographic system utilizing a reversed-phase analytical column, and an ultraviolet spectrophotometric detector. The mean assay coefficient of variation over a concentration range of 0.5—200 μg/ml is slightly greater than 1% when either *p*-nitrobenzoic or *p*-anisic acid is used as the internal standard. Recoveries of drug are essentially quantitative at all levels investigated; hence the calibration curves are rectilinear from the limit of quantification (about 0.05 μg/ml) to at least 200 μg/ml.

## INTRODUCTION

Cefmenoxime, 7β-[2-(2-aminothiazol-4-yl)-(*z*)-2-methoxyiminoacetamido]-3-[(1-methyl-1H-tetrazol-5-yl)thiomethyl]ceph-3-em-4-carboxylic acid, a semisynthetic cephalosporin derivative (Fig. 1) developed by Takeda Chemical Industries (Osaka, Japan), has a broad spectrum of activity against gram-positive and gram-negative organisms, including *H. influenzae*, *C. freundii*, *E. cloacae*, indole-positive *Proteus*, and *S. marcessens* [1—3]. The hemihydrochloride salt of this cephalosporin is currently under clinical evaluation by Abbott Laboratories.

Because of poor specificity, slow turn around time, and relatively poor precision, microbiological analysis was not felt to be the method of choice for determination of cefmenoxime in plasma. High-performance liquid chromatography (HPLC) is ideally suited for the analysis of these relatively polar,

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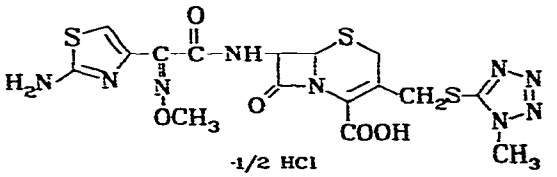

Fig. 1. Chemical structure of cefmenoxime hemihydrochloride.

nonvolatile compounds. Since the therapeutic concentrations of cephalosporins are usually in the μg/ml range, concentration techniques are usually not required; however, the majority of the high-molecular-weight proteins and fibrin must be removed from plasma samples to prevent column filter and packing bed damage. Several HPLC procedures for cephalosporins, employing classical deproteination reagents such as trichloroacetic acid [4, 5], or organic solvents [6—9], have been reported recently. The HPLC procedure described herein, involving removal of plasma proteins by ultrafiltration, offers the advantages of simplicity and high reproducibility, obviating the problems associated with the precipitation procedures (e.g. sample dilution, incomplete protein precipitation, drug co-precipitation, and acid catalyzed degradation of labile drugs). Furthermore, the ultrafiltration procedure allows the determination of the free and total drug concentrations in plasma. Cefmenoxime, as well as other protein-bound antibiotics, can be quantitatively displaced from the plasma proteins by addition of sodium dodecyl sulfate (SDS) or other highly protein-bound reagents.

## EXPERIMENTAL

### *Chromatography*

HPLC analyses were conducted using a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump in conjunction with a spectrophotometer operated at an analytical wavelength of 254 nm. The mobile phase, consisting of approximately 13% (v/v) acetonitrile in 0.2 *M* aqueous acetate buffer at pH 5.30, was pumped at a flow-rate of 2.0 ml/min through a Waters μBondapak $C_{18}$ analytical column (30 cm × 4 mm I.D., 10 μm particle size). Minor manipulations in the acetonitrile content and/or the pH of the mobile phase were occasionally required to accommodate column efficiency loss, or interference from atypical plasma samples.

### *Ultrafiltration apparatus*

Plasma ultrafiltrates are prepared using the Amicon (Lexington, MA, U.S.A.) Centriflow system consisting of conical centrifuge tubes (Model CT1), conical supports (Model CS1A) and membrane cones (either Model CF25 or CF50A, with respective molecular weight cutoff values of 25,000 and 50,000). Use of the CF-25 cones was slightly favored due to their higher apparent flux and protein retentivity. The two types of membrane cones were not mixed within an analytical run.

*Procedure*

Calibration curves for the analysis of clinical specimens were typically prepared by supplementing blank pooled plasma with a freshly prepared aqueous solution of cefmenoxime, followed by serial dilution with pooled plasma.

Sample processing entailed ultrafiltration of an accurately measured mixture of 5 volumes of plasma (usually 1.0 ml) and 1 volume of a solution containing the internal standard and 4% (w/v) SDS. Both *p*-nitrobenzoic acid (PNBA) and *p*-anisic acid (PAA), at final concentrations greater than 1.3 μg/ml, were found to be acceptable as internal standards. All samples were centrifuged at the same relative centrifugal force (typically 450 *g*) for at least 20 min. After transfer to clean test tubes, the ultrafiltrates were refrigerated until all samples were processed. Subsequently, the ultrafiltrates could be stored frozen for several days prior to analysis. Injection of 90 μl of ultrafiltrate from a plasma sample originally containing 0.05 μg/ml of cefmenoxime was found to produce a response 2—3 times greater than background noise at a detector attenuation of 0.02 a.u.f.s.

Since cefmenoxime was found to degrade slowly in the ultrafiltrates, it was deemed advisable to allow them to stand at room temperature for no more than 2—3 h prior to analysis. For enhanced room temperature stability (e.g. overnight automatic injection), a small volume of buffer could be added to the ultrafiltrates to reduce the pH to approximately 6.

## RESULTS AND DISCUSSION

*Method development*

*Cleanup procedures.* Initial attempts to prepare protein-free filtrates were largely unsuccessful. Deproteinization by addition of organic solvents (e.g. methanol. acetonitrile, dimethylformamide) caused chromatographic aberrations and apparent co-precipitation of cefmenoxime. Column chromatographic techniques (e.g. anion and cation exchange, Waters $C_{18}$ Sep-Pak™, alumina, and silica absorption) also showed little promise.

Ultrafiltration techniques are ideally suited for the determination of the free fraction of drugs in plasma; however, pressurized ultrafiltration techniques using cells or membrane tubing are slow and cumbersome. Alternatively, ultrafiltration by centrifugation with the Centriflow apparatus is simple, fast, and highly reproducible. The apparent plasma protein binding of cefmenoxime was approximately 77% as determined by this procedure. Addition of SDS, a highly protein-bound displacing agent, to plasma samples resulted in quantitative recoveries of cefmenoxime in the filtrates. The results of the competitive binding studies of SDS with cefmenoxime, PNBA, and PAA are shown in Fig. 2. Although recoveries of cefmenoxime and of both internal standards were quantitative at or above a final SDS concentration of 0.35% (w/v), slightly higher final concentrations were employed in routine analyses to ensure quantitative recovery from samples containing high concentrations of drug or albumin.

*Chromatographic conditions.* With the chromatographic conditions described earlier, the following order of increasing retention was observed for

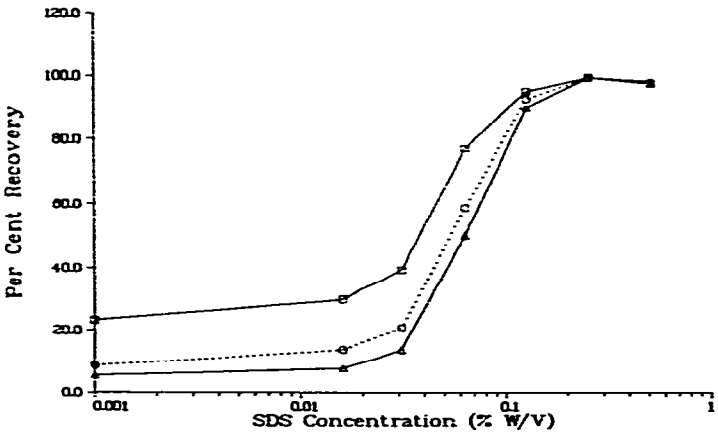


Fig. 2. Displacement of cefmenoxime (□), *p*-anisic acid (○), and *p*-nitrobenzoic acid (△) from plasma proteins by SDS.

typical plasma samples (see Fig. 3): unretained plasma constituents < PNBA < two minor plasma peaks < cefmenoxime < PAA. The relative retention volumes of these compounds varied slightly from column to column, and their resolution was moderately sensitive to pH, ionic strength, and acetonitrile content of the mobile phase.

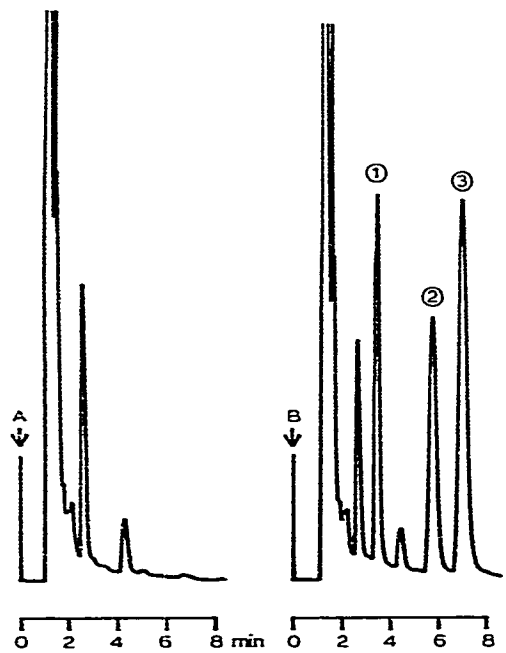


Fig. 3. Chromatograms of ultrafiltrates of (A) control plasma and (B) plasma supplemented with cefmenoxime and the proposed internal standards. Peaks: (1) *p*-nitrobenzoic acid; (2) cefmenoxime; (3) *p*-anisic acid. HPLC conditions are described in the text; mobile phase pH was 5.2. More than 2000 plasma ultrafiltrates had been injected onto this column prior to these chromatograms.

Increasing the pH of the mobile phase decreased the relative retention of both internal standards; however, near pH 5, PAA was more sensitive to pH changes, since its $pK_a$ is higher than that of PNBA (4.5 vs. 3.4). At a mobile phase pH greater than 5.5, PAA eluted prior to cefmenoxime. Conversely, reduction of the pH to below 4.5 increases the retention of both internal standards so that the elution order is cefmenoxime < PNBA < PAA. Thus, great flexibility in the relative retentions of cefmenoxime and the internal standards was realized by pH manipulation. Generally, the pH of the mobile phase should not be reduced below 4.0, since ion-pairing interactions between SDS and cefmenoxime will occur. Manipulation of the ionic strength had less dramatic effects on the resolution of the various compounds, although better resolution was generally observed with increasing ionic strength.

*Recovery*

The recoveries of cefmenoxime, PNBA, and PAA in the first 50—100 μl of ultrafiltrate were slightly lower than in subsequent fractions; however, the peak height ratios were essentially independent of ultrafiltrate volume. Nevertheless, when ultrafiltrate volumes approaching or exceeding 0.5 ml were collected (e.g. 20 min centrifugation at 450 *g*), recoveries were quantitative for cefmenoxime and internal standards, providing that sufficient (>0.25%) SDS was present.

Intersubject variability in the recovery of cefmenoxime and the internal standards was assessed using blank plasma from six subjects. The chromatographic interference from compounds endogenous to plasma was negligible. The recoveries of the compounds of interest were essentially quantitative, with coefficients of variation averaging approximately 1%.

The recoveries of cefmenoxime, PNBA, and PAA were also compared using Amicon membrane filters with molecular weight cutoff values of 25,000 (CF25) and 50,000 (CF50A). The recovery of cefmenoxime was roughly 2—3% lower with the CF50A cones. Thus, it is recommended that the two types of cones not be mixed within the same assay run, if such an error is considered significant.

*Precision and linearity*

The precision and linearity of the procedure was assessed by quadruplicate analyses of plasma samples supplemented with cefmenoxime in the concentration range of 0.50—200 μg/ml. Aliquots of these standards (1.0 ml) were mixed with equal volumes of 1% SDS containing PNBA and PAA, and the samples were then processed by the method described above. The results of the analyses are summarized in Table I.

With no corrections made for the internal standard response, the mean assay coefficient of variation (C.V.) was 2.5 ± 3.3%. Correction of the data with the internal standard response resulted in a mean C.V. of 1.2 ± 1.0% with PNBA, and 1.3 ± 0.8% with PAA. These extremely low errors were due to the high ultrafiltration recovery and the simplicity of the workup. The use of PNBA was slightly favored over PAA because it eluted prior to cefmenoxime, this allowing a higher sample analysis rate (roughly 7 to 8 injections per hour for PNBA, compared to 6 to 7 for PAA).

TABLE I

PRECISION AND LINEARITY OF THE ANALYTICAL PROCEDURE

Samples were assayed in quadruplicate, using PNBA as the internal standard. Results for PAA as the internal standard are given in parentheses

| Actual | Concentration calculated (μg/ml) | Coefficient of variation (%) |
|---|---|---|
| 0.50 | 0.50 (0.50) | 2.5 (2.8) |
| 1.00 | 0.98 (0.99) | 2.9 (2.6) |
| 2.00 | 2.01 (2.01) | 1.7 (1.2) |
| 5.00 | 5.10 (5.15) | 0.6 (1.0) |
| 10.00 | 10.22 (10.22) | 0.4 (0.2) |
| 20.00 | 19.85 (19.92) | 0.6 (1.3) |
| 50.00 | 49.78 (49.37) | 0.4 (0.9) |
| 100.00 | 99.77 (100.46) | 0.4 (0.9) |
| 200.00 | 195.66 (193.22) | 1.1 (1.0) |

The mean data of Table I were treated by linear regression analyses using reciprocal analytical variances as the weights, thus assuring that the contribution of each value to the sum of squared deviations was proportional to its precision rather than its magnitude [10]. Since it is generally impractical to determine assay variances for each of the standards during routine analyses, the data were then refitted using 1.0, 1/concentration ($1/c$), and 1/concentration-squared ($1/c^2$) as the weighting schemes. The regression correlation coefficients, ranging from 0.9996 to 1.0000, demonstrated that analytical response was rectilinearly dependent on concentration, whereas recovery was independent of concentration. In general, the regression $Y$-intercepts were negligibly small and statistically insignificant. For the uncorrected data, the $1/c$ weighting scheme gave regression results most representative of the reciprocal variance weighted fit. For the data corected through the use of either PNBA or PAA as internal standard, the $1/c$ and $1/c^2$ weighting schemes both gave regression results very similar to those obtained with reciprocal variance weights. Use of either scheme would be entirely satisfactory; nonetheless, the $1/c^2$ scheme appears to be marginally more appropriate.

*Stability*

The stability of cefmenoxime in plasma was first assessed in room temperature incubation studies. Three sets of spiked plasma samples were studied: (1) untreated plasma, (2) plasma mixed with an equal volume of 1% SDS, and (3) plasma mixed with an equal volume of 1.0 *M* phosphate buffer, pH 6. After approximately two days at room temperature, the loss of cefmenoxime in all samples was less than 10%. After one week, drug loss was 41% in the untreated plasma, 30% in the SDS-treated plasma, and was not demonstrable in the phosphate-buffered plasma. Additionally, drug loss was not evident in the untreated or treated plasma samples which were stored frozen for one week.

Subsequently, long-term stability studies were initiated with frozen plasma

samples. Freshly collected normal human plasma was supplemented with cefmenoxime at a concentration of 10 μg/ml, and aliquants of this standard were analyzed periodically for two months. The mean recovery of drug in this experiment was 103.3 ± 1.6%. Linear regression analyses of the data failed to demonstrate a statistically significant loss of drug during the two-month period; hence, drug degradation in frozen plasma would be expected to be minimal for considerably longer periods.

The stability of cefmenoxime was also evaluated in plasma ultrafiltrates at room temperature. Plasma was supplemented with the compound, and an aliquot was ultrafiltered using the normal procedure. Another aliquant was treated with an equal volume of 0.5 *M* phosphate buffer, pH 6, containing 1% SDS. The results of the analyses of these samples are given in Table II.

TABLE II

STABILITY OF CEFMENOXIME IN PLASMA ULTRAFILTRATES AT ROOM TEMPERATURE

| Time (h) | Percent remaining | |
|---|---|---|
| | unbuffered | buffered |
| 0.5 | 97.8 | 101.1 |
| 1.0 | 98.2 | 98.5 |
| 2.0 | 98.5 | 98.9 |
| 3.0 | 98.3 | 100.0 |
| 4.0 | 99.2 | 99.9 |
| 5.0 | 98.6 | 100.5 |
| 6.0 | 97.5 | 99.3 |
| 7.0 | 99.2 | 100.1 |
| 22.5 | 89.4 | 98.2 |
| 24.0 | 89.5 | 98.5 |
| 27.0 | 86.2 | 97.3 |
| 30.0 | 84.9 | 97.7 |

Linear regression analyses of the peak height ratios vs. time data failed to show statistically significant changes during the first 7 h for either the buffered or the unbuffered ultrafiltrates; however, at the end of the 30-h incubation, the unbuffered ultrafiltrates showed a statistically significant net rate of decrease in the peak height ratios of about 0.48% per hour. The rate of decrease in the peak height ratios for buffered ultrafiltrates in the same period averaged only 0.08% per hour. It appeared that the rate of cefmenoxime loss in the unbuffered ultrafiltrate increased slightly with time or with repeated exposure to the air. This phenomenon was consistent with the general observation that plasma becomes more alkaline upon standing, and that cefmenoxime degrades more rapidly with increasing pH. Nonetheless, it would appear from these data that ultrafiltrates could be allowed to stand at room temperature for at least 7 h with no significant compromise of analytical accuracy (e.g. roughly 50 injections by an unattended automatic sample injector). Additionally, if long (>7 h) automated injection runs were desired, and maximum sensitivity were not required, 1.0 *M* phosphate buffer, pH 6, may be added

to each ultrafiltrate to improve drug stability. The internal standard contained in the ultrafiltrate would allow compensation for the dilutions. For maximum assay accuracy, particularly with manual or attended automatic sample injection, the thawed ultrafiltrates of the standards and unknowns should be treated identically. This is easily accomplished by refrigeration of the ultrafiltrates before and after injection, leaving only small groups (5—10) at room temperature (immediately prior and subsequent to injection). With this precaution, the samples may be reinjected, if necessary, at a later time for verification of any anomalous results.

*Validation*

During the course of routine analyses, 110 plasma samples from a clinical pharmacokinetic study were also assayed by an agar diffusion microbiological technique employing *Proteus mirabilis* (strain 13300, Takeda Chemical Industries) as the test organism. The resultant data from samples having levels quantifiable by both procedures were submitted to linear regression analysis (see Fig. 4). Differences between the calculated slope (1.023) and *Y*-intercept (0.0631) and the respective theoretical values of 1.000 and 0.000 were not statistically significant ($p = 0.05$). The correlation coefficient of the regression was 0.995.

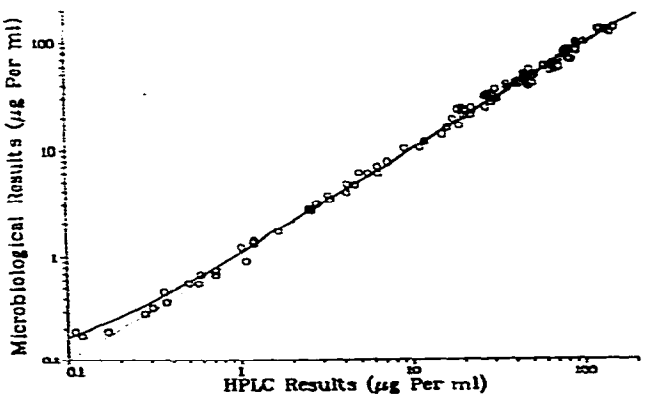


Fig. 4. Correlation of microbiological and HPLC results. Regression equation (solid line): $Y = 1.02\ (\pm 0.01)\ X + 0.06\ (\pm 0.08)$; $r = 0.995$. Dotted line = theoretical curve.

Significant chromatographic interference has not been observed in clinical studies conducted to date with cefmenoxime. Typical plasma level curves from a recent clinical study are shown in Fig. 5.

*Versatility*

The centrifugal ultrafiltration technique described above is an excellent alternative to classical deproteinization procedures because it is extremely simple and does not require sample adulteration. The adjunctive technique of displacement of protein-bound drugs with SDS increases the applicability range of the procedure. The ultrafiltration procedure has been successfully adapted in our laboratories for the determination of other antibiotics. Development of procedures for other similar compounds only requires selection of

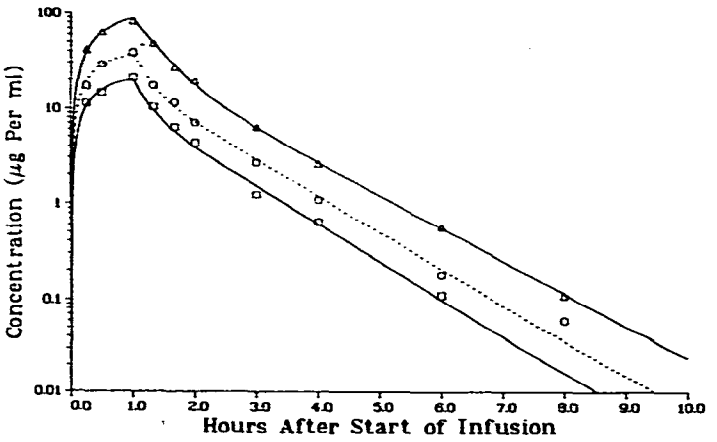


Fig. 5. Plasma level profiles, as determined by the proposed HPLC procedure for a subject receiving 500 (□), 1000 (○), and 2000 (△) mg of cefmenoxime as a 1-h intravenous infusion.

the proper chromatographic conditions to allow resolution of the drug and internal standard from compounds endogenous to plasma. Substituted aromatic acids serve well as internal standards because of their versatility. A wide range in retentivity can be realized by control of the mobile phase pH, and by choice of the substituent.

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