HIGH SPEED LIQUID CHROMATOGRAPHIC DETERMINATION OF CEPHALEXIN IN HUMAN PLASMA AND URINE

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A rapid and accurate high speed liquid chromatographic method has been developed for the determination of cephalexin in human plasma and urine. The method involves micropore filtration of urine specimens and methanol extraction of plasma samples followed by HSLC separation on a bonded reverse phase column utilizing a mobile phase of methanol-water containing acetic acid. The quantitativity of UV response at 254 nm covered a wide range of cephalexin concentrations down to 0.5 μg/ml, and no metabolite peaks were detected. The time courses of plasma level and urinary excretion were determined until 6 hours after oral administration of cephalexin capsules to healthy volunteers. The pharmacokinetic parameters were estimated using a two compartment open model.

Cephalexin, a semisynthetic derivative of cephalosporin C, is known to have a broad spectrum of anti-Gram-positive and Gram-negative activities, weak bondability to blood protein, no metabolites, low toxicity, and to be rapidly absorbed following oral administration to give a high serum levels and urine concentrations.[1~8] The drug, therefore, is widely used for clinical chemotherapy. The analytical methods so far employed in extensive investigations of this drug have been based on microbioassay, chemical assay, spectrophotometry, and chromatography. The details can be found in review articles.[4~6] High speed liquid chromatography (HSLC), above all, provides the advantages of high specificity, accuracy, sensitivity, and reproducibility with least pretreatments, prompting the analysis of this drug as well as other β-lactam antibiotics present in biological fluids. WHITE *et al.*[7,8] reported HSLC methods for the separation and detection of various antibiotics, but their limit of detection is too high to determine plasma level and low concentration region of urinary excretion of cephalexin administered to humans in therapeutic doses. The routine analyses of cephalexin in biological samples as often encountered in pharmacokinetic studies have generally been achieved by microbiological method. The methods, however, sometimes give erroneous results in that the total recovery exceeds 100%. From an analytical view point, especially for the purpose of pharmacokinetic investigations, it is desired to develop more reliable methods. This paper, following our previous report,[9] presents a rapid and accurate reverse phase HSLC method for the separation and determination of cephalexin in human plasma and urine and the estimation of pharmacokinetic parameters from the data obtained thereby.

Materials and Method

Chromatography

A high speed liquid chromatograph (Waters Assoc., Model ALG GPC) equipped with UV detector (254 nm) was used in a reverse phase with a μ-Bondapack C_{18}® column (30 cm × 4 mm i.d.) and the mobile phase of methanol - water (1 : 8, v/v) for urine specimens and methanol - water (1 : 5) for plasma samples, both containing 0.5% acetic acid. The flow rates were maintained at 1.8 ml/min

(1,800 p.s.i.) and 0.9 ml/min (1,100 p.s.i.) for urine and plasma samples, respectively.

Reagents and Materials

The mobile phase was prepared by microfiltration (0.45 μm) and degassing of a mixture of distilled water and methanol. Acetic acid of analytical grade was used as supplied. The monohydrate form of cephalexin (945 μg/mg as potency) used as standard and cephalexin capsule (Keflex® 250 mg as potency) received by volunteers were provided by Shionogi Seiyaku Co. (Osaka, Japan). The potency of the standard cephalexin was regarded as chemical purity, because no impurity peaks were detected on a chromatogram and any substance undetected at 254 nm, if present, could have no potency. Hence the results given below are those corrected to this purity factor (0.945).

Administration and Sampling

The cephalexin capsules were orally administered to three healthy male volunteers (each 500 mg as potency) who were 28~37 years of age and 67~79 kg of body weight. The volunteers were fasted for 12 hours before receiving the capsules. Urine and blood samples were taken just before and after the administration according to the planned time schedule (see Table 1). After measuring the volume of urine, a 1- to 2-ml aliquot was filtered through 0.45 μm pore size triacetylcellulose membrane (Fuji Photo Film, Tokyo, Japan) and kept frozen until analysis. Venous blood samples drawn into 2 ml heparinized syringe were immediately transferred to a heavy duty glass tube and centrifuged at 3,600 r.p.m. for 10 minutes to separate the plasma layer. To 0.5 ml of the plasma was added 0.5 ml methanol, and the mixture was shaken vigorously for 5 minutes and centrifuged at 3,600 r.p.m. for 10 minutes. The supernate was filtered through the 0.45 μm filter and exactly 10~40 μl of the filtrate was used for injection. The volume injected was based on a rough estimation of plasma concentration.

Calibration Graph

Calibration graphs were prepared for the determinations of plasma and urine concentrations, respectively. With urine, known amounts of standard cephalexin were dissolved in the control urine to make nine different concentrations between 50 μg/ml and 5 mg/ml. The peak heights were plotted against the concentration ranging from 50 μg/ml to 1 mg/ml and 500 μg/ml to 5 mg/ml. The linear regression analysis of the plots in the two regions gave the same calibration equation with correlation coefficient 0.9997 and standard deviation 0.06. The control plasma was spiked with standard cephalexin to six different levels between 0.5 μg/ml and 20 μg/ml. After the spiked plasma had been equilibrated by shaking for 10 minutes, a 0.5 ml portion was treated in the same manner as described above. The relationship between peak height and concentration showed good linearity through the origin which also agreed with the calibration graph for the urine samples. Therefore, the calibration graph is common to both plasma and urine samples.

Results and Discussion

It is known that cephalexin is stable in acidic media and its degradation is independent of pH even in strong acidic solution.[10] The stability of cephalexin in the media used in this study (pH value of mobile phase 3.0~3.1 and urine 5.5~6.5) was examined prior to *in vivo* experiment. No peaks due to degradation products were found on the chromatograms of ordinary urine and plasma solutions of standard cephalexin, and the intensity of UV response for a given amount of the standard cephalexin was reproducible, when the solutions were stored frozen or even kept at room temperature overnight. As shown in Figs. 1 and 2, separation of the cephalexin peak from background peaks of control plasma and urine was almost complete within the concentration range to be determined. A minor interfering peak was observed on a chromatogram of plasma concentration below 0.2 μg/ml which, therefore, was the limit of accurate determination. The limit of detection, however, could be as low as 0.1 μg/ml at maximum sensitivity (AUFS 0.005) with 40 μl injection of plasma sample. The increasing water content in the mobile phase produced more increase in the retention time of cephalexin than in those

Fig. 1. Separation of cephalexin from normal plasma components.

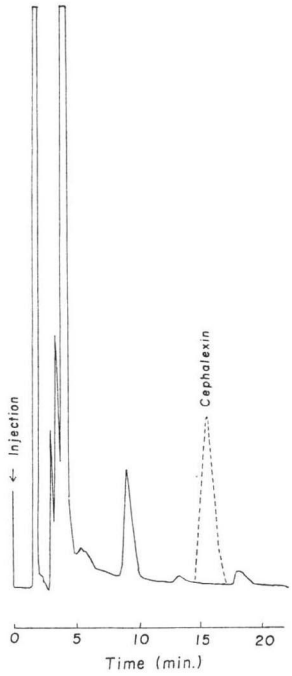
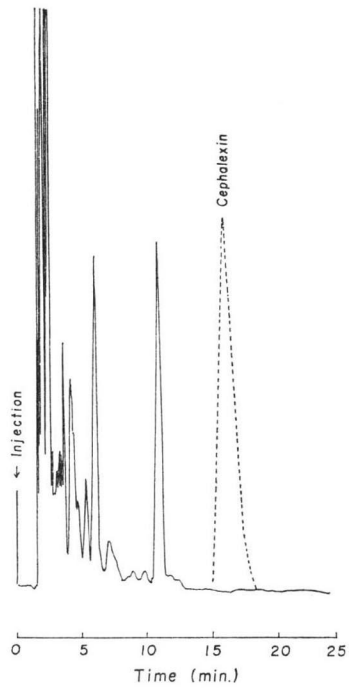


Fig. 2. Separation of cephalexin from normal urine components.



of urinary background peaks. Thus, the water content and flow rate of the mobile phase were adjusted to give complete separation in as short a retention time (16 minutes) as possible. Since the plasma background peaks, as seen in Fig. 1, had more scattered retention times than the urinary ones, the water content and flow rate were chosen so that the cephalexin peak had the retention time in between those of two minor background peaks. The difference in the retention times and intensities of background peaks due to individual volunteers did not interfere with the separation of the cephalexin peak, and no peaks were assignable to metabolites. The calibration graph obtained from aqueous solutions of standard cephalexin was found to have the same slope as that from plasma solution. The change in the equilibration time from 10 to 90 minutes in preparing the spiked plasma and the change in the volume of methanol added to plasma from 0.5 to 1.5 ml did not affect the recovery of cephalexin. Therefore, the present method quantitates the total amount of cephalexin bound and unbound to plasma protein with almost 100% recovery. One of the advantages of the present method is a simple pretreatment of urine and plasma samples. The ultrafiltration using the membrane-filter can remove the visible particles from urine and macromolecules of plasma protein as well. The filtrate, however, may partly coagulate when introduced into a flow of mobile phase, which may cause a column-filter to be plugged and the packing materials to be stained. Repeated injections of urine and plasma samples actually gave rise to a slight increase in inlet pressure of the mobile phase. Therefore, the column was eluted overnight with large volume of distilled water and methanol after every fifty injections or so, and the column-filter was also cleaned in an ultra-sonic bath. The column efficiency (HETP 0.3 mm for cephalexin) was thus conserved.

Table 1 shows the results for the urinary excretion of cephalexin for each of three subjects and

Table 1. Urinary excretion amount (mg) of cephalexin following single oral administration of two 250 mg-capsules (500 mg) to healthy volunteers.

Subject	K.Y.	M.M.	T.N.	Mean and S.D.
Age (year)	28	28	37	
Body weight (kg)	68	68	79	
0 ~0.25 hours	0.42	2.13	1.02	1.19± 0.71
0.25~0.75	77.81	52.71	59.37	63.30±10.62
0.75~1.25	136.08	148.23	126.41	136.91± 8.93
1.25~1.75	88.64	100.43	110.70	99.92± 9.01
1.75~2.25	46.74	50.69	63.01	53.48± 6.93
2.25~2.75	28.79	29.88	30.91	29.86± 0.87
2.75~3.25	24.50	18.90	21.33	21.58± 2.29
3.25~4.50	22.45	17.00	36.21	25.22± 8.08
4.50~5.50	9.01	6.67	11.71	9.13± 2.06
5.50~6.50	3.36	2.93	9.68	5.33± 3.09
Total	437.80	429.57	470.35	445.91

their average values. The urine specimens were collected every 30 minutes for first 4 hours (except for first 15 minutes) and 60 minutes for the following 3 hours by asking the volunteers not to urinate in the interim time. Fig. 3 illustrates the average urinary excretion rate-time curve. It is found that all the volunteers show similar excretion profile, the average maximum rate being 273.8 mg/hour at 1.25 hours after the administration and the concentration thereat ranging 4.32~8.47 mg/ml. The excretion rate thereafter slowed down to 5.33 mg/hour at 6.5 hours when the final sample was taken. The time course of average cumulative excretion amount is graduated by % dose of 500 mg cephalexin as potency (Fig. 4). The average cumulative excretion accounts for 42.8% of the dose at 1.25 hours where the concentration reaches maximum, and 89.2%

Fig. 3. Urinary excretion rate profile of cephalexin after oral administration of 500 mg capsule (average of three volunteers).

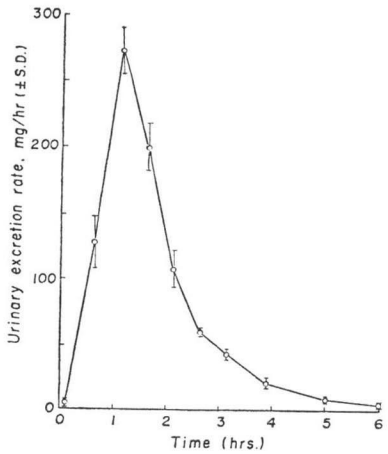
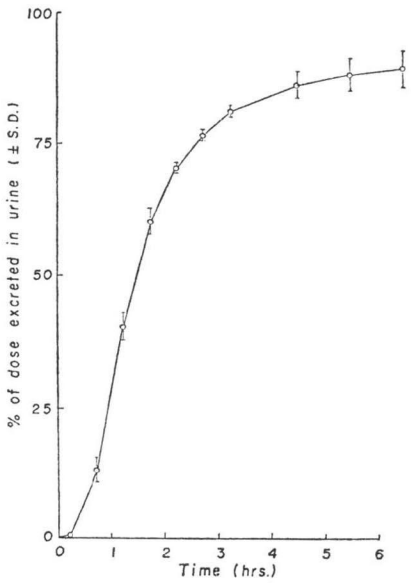


Fig. 4. Cumulative urinary excretion ratio (%) of cephalexin after oral administration of 500 mg capsule (average of three volunteers).



at 6.5 hours where the excretion is almost completed. It has been reported that, on average, over 90% of the dose was excreted in human urine in 6 hours after oral administration of cephalexin.[1~3] The investigation of the individual data, however, reveals that microbioassay often gave the urinary recovery considerably exceeding 100%, probably owing to the error in the assay method. The present method, we believe, offers more reliable results than microbioassay, although the general profile of excretion is similar to those earlier reported.

The plasma data obtained are shown in Table 2 where the concentration are given in μg/ml for each of the volunteers and their mean values along with standard deviations. Fig. 5 illustrates the average plasma level-time curve where the time interval was 30 minutes for the first 3 hours and 1 hour thereafter, that is, the blood samples and urine specimens were taken alternatively every 15 minutes for the first 3.25 hours and 30 minutes for the subsequent 3 hours. It is found that the peak plasma

Table 2. Plasma concentration (μg/ml) of cephalexin following single oral administration of two 250 mg-capsules (500 mg) to healthy volunteers.

Subject	K.Y.	M.M.	T.N.	
Age (year)	28	28	37	Mean and S.D.
Body Weight (kg)	68	68	79	
0.5 hour	13.26	4.95	8.15	8.79±3.42
1.0	17.85	16.91	29.10	21.29±5.54
1.5	9.76	9.35	13.11	10.74±1.68
2.0	7.00	4.90	7.49	6.46±1.12
2.5	3.39	2.66	5.12	3.72±1.03
3.0	2.82	1.78	2.82	2.47±0.49
4.0	1.11	0.92	1.49	1.17±0.24
5.0	0.55	0.53	0.85	0.64±0.15
6.0	0.33	0.26	0.63	0.41±0.16

Fig. 5. Plasma concentration-time curve of cephalexin (average of three volunteers).

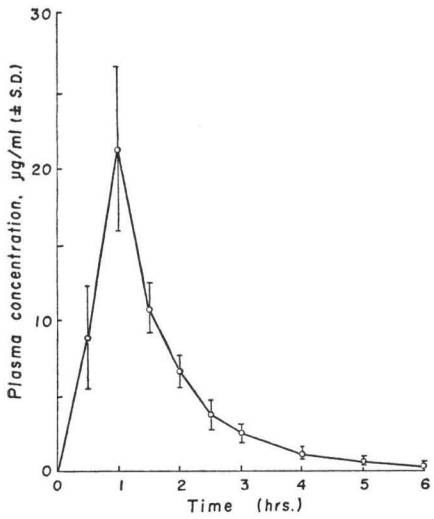


Fig. 6. Urinary excretion rate *vs.* plasma concentration plots of cephalexin.

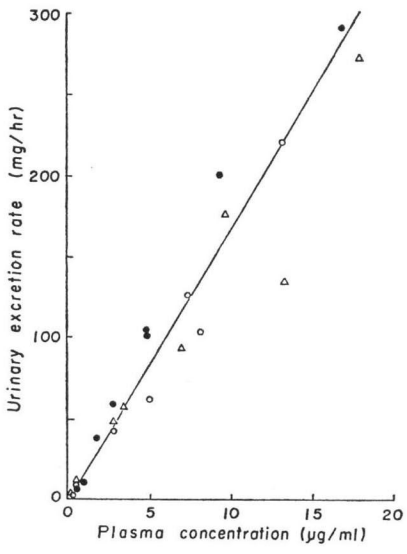


Table 3. Pharmacokinetic parameters for cephalexin administered orally, two compartment model analysis.

Parameter		subject			mean±S.D.
		T.N.	K.Y.	M.M.	
α	(hr^{-1})	1.441	1.233	1.024	1.233±0.170
β	(hr^{-1})	0.401	0.665	0.511	0.526±0.108
k_a	(hr^{-1})	2.481	6.094	4.660	4.412±1.485
k_{12}	(hr^{-1})	0.253	0.086	0.023	0.121±0.097
k_{21}	(hr^{-1})	0.564	0.873	0.532	0.656±0.154
k_e	(hr^{-1})	1.052	0.939	0.979	0.981±0.057
lag time	(hr)	0.113	0.121	0.086	0.107±0.015
$T_{1/2\alpha}$	(hr)	0.481	0.562	0.677	0.573±0.080
$T_{1/2\beta}$	(hr)	1.728	1.042	1.356	1.375±0.280
V_c	(liters)	15.993	15.228	18.378	16.533±1.341
V_p	(liters)	7.187	1.508	0.807	3.167±2.857

level of 21.29 μg/ml at 1 hour is followed by the monotonic decrease to 0.41 μg/ml at 6 hours after administration. Taking the urinary excretion data into account, these results suggets that the accumulation of cephalexin in the human body is negligible and the dosing interval of 6 hours which is usually employed clinically may cause a problem in the light of therapeutic effect. The uncorrected renal clearance was estimated as a slope of urinary excretion rate-plasma concentration plots,[11] since the plasma samples were taken, as mentioned above, at the midpoint of the urinary collection interval. Fig. 6 shows the plots thus obtained, where the keys specify the subjects. The regression analysis of all the data points gave a line (correlation coefficient, 0.9712) with a slope 16.38±1.54 (liters/hour). This value is close to those which have appeared in the literature.[12,13] NIGHTINGALE *et al.*[14] stated that the use of a one compartment open model for the kinetic analysis of cephalexin may lead to error in the calculation of the volume of distribution and the elimination rate constants, and showed the reevaluation of some selected literature data[2,15,16] using a two compartment open model. Hence, the kinetic parameters were estimated from the present data by the least squares method using a two compartment open model, where it is postulated that absorption (with rate constant k_a) and elimination (with k_e) occur in the central compartment whose apparent volume is V_c and the first order rate constants for transfer from central to peripheral and from peripheral to central compartments are denoted by k_{12} and k_{21}, respectively. The volume of the peripheral compartment, V_p, was calculated by $V_c k_{12}/k_{21}$. The results obtained are summarized in Table 3, where it is found that the values except for k_{12} and k_{21} are comparable with those reported in the literature.[17]

References

1) GOWER, P. E. & C. H. DASH: Cephalexin: Human studies of absorption and excretion of a new cephalosporin antibiotic. Brit. J. Pharmacol. 37: 738~747, 1969
2) PERKINS, R. L.; H. N. CARLISLE & S. SASLOW: Cephalexin: *In vitro* bacterial susceptibility, absorption in volunteers and antibacterial activity of sera and urine. Amer. J. Med. Sci. 256: 122~129, 1968
3) GRIFFITH, R. S. & H. R. BLACK: Blood, urine and tissue concentrations of the cephalosporin antibiotics in normal subjects. Postgrad. Med. J. 47S: 32~40, 1971
4) HUGHES, D. W.; A. VILIN & W. L. WILSON: Chemical and physical analysis of antibiotics. II. Penicillins and cephalosporins. Canad. J. Pharm. Sci. 11: 97~108, 1976
5) FLYNN, E. H.: Cephalosporins and penicillins: Chemistry and biology. p. 609, Academic Press, New

York, 1972
6) WHEALS, B. B. & I. JANE: Analysis of drugs and their metabolites by high performance liquid chromatography. Analyst 102: 625~644, 1977
7) WHITE, E. R.; M. A. CARROLL, J. E. ZAREMBO & A. D. BENDER: Reverse phase high-speed liquid chromatography of antibiotics. J. Antibiotics 28: 205~214, 1975
8) CARROLL, M. A.; E. R. WHITE, Z. JANCSIK & J. E. ZAREMBO: The determination of cepharadine and cephalexin by reverse phase high performance liquid chromatography. J. Antibiotics 30: 397~403, 1977
9) NAKAGAWA, T.; J. HAGINAKA, K. YAMAOKA & T. UNO: Direct high speed liquid chromatographic determination of cephalexin in urine. J. Chromatogr. 147: 509~512, 1978
10) YAMANA, T. & A. TSUJI: Comparative stability of cephalosporins in aqueous solution: Kinetics and mechanisms of degradation. J. Pharm. Sci. 65: 1563~1574, 1976
11) WAGNER, J. G.: Biopharmaceutics and relevant pharmacokinetics. p. 247, Drug Intelligence Publications, Hamilton, 1971
12) KIRBY, W. M. M.; J. B. DEMAINE & W. S. SERRILL: Pharmacokinetics of the cephalosporins in healthy volunteers and uremic patients. Postgrad. Med. J. 47S: 41~46, 1971
13) KIRBY, W. M. M. & C. REGAMEY: Pharmacokinetics of cephazolin compared with four other cephalosporins. J. Infec. Dis. 128: S341~S346, 1973
14) NIGHTINGALE, C. H.; D. S. GREENE & R. QUINTILIANI: Pharmacokinetics and clinical use of cephalosporin antibiotics. J. Pharm. Sci. 64: 1899~1927, 1975
15) NICHOLAS, P.; B. R. MEYERS & S. Z. HIRSHMAN: Cephalexin: Pharmacologic evaluation following oral and parenteral administration. J. Clin. Pharmacol. 13: 463~468, 1973
16) O'CALLAGHAN, C. H.; J. P. R. TOOTILL & W. D. ROBINSON: A new approach to the study of serum concentration of orally administered cephalexin. J. Pharm. Sci. 23: 50~57, 1971
17) GREENE, D. S.; D. R. FLANAGAN, R. QUINTILIANI & C. H. NIGHTINGALE: Pharmacokinetics of cephalexin: An evaluation of one- and two-compartment model pharmacokinetics. J. Clin. Pharmacol. 16: 257~264, 1976