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Pharmacokinetics of Fluconazole in Cerebrospinal Fluid and Serum of Rabbits: Validation of an Animal Model Used To Measure Drug Concentrations in Cerebrospinal Fluid

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Complete concentration-time data describing the pharmacokinetics of fluconazole in the cerebrospinal fluid (CSF) following a single dose are not available for humans or animals. We studied the pharmacokinetics of fluconazole with an indwelling intracisternal needle as described by R. G. Dacey and M. A. Sande (Antimicrob. Agents Chemother. 6:437–441, 1974). To determine whether the presence of an intracisternal needle alters pharmacokinetics in the CSF, we validated this model with uninfected rabbits by measuring pharmacokinetic constants following direct intracisternal and intravenous administration of fluconazole. Following direct injection, there was no alteration of elimination rates in the CSF with increasing sample number or time. Following intravenous administration, the penetration and kinetic constants were the same in individual animals from which multiple CSF samples were obtained as in a composite subject constructed by pooling virgin samples from different animals. The presence of the intracisternal needle did not alter CSF chemistry or leukocyte counts, and erythrocyte contamination was <0.001%. While drug concentrations were measured by a microbiological assay, we also compared the sensitivity and reproducibility of a high-performance liquid chromatography (HPLC) assay with those of the microbiological assay. Following a single intravenous dose, the maximum concentration of the drug in serum, the time to maximum concentration of the drug in serum, the terminal elimination half-life in the CSF, and the percent penetration by fluconazole were 6.12 μg/ml, 1 h, 9.0 h, and 84.3%, respectively. We conclude that the sampling of CSF via an indwelling needle does not alter fluconazole pharmacokinetics, cause inflammation, or alter chemical parameters; that the microbiological assay is at least equivalent in sensitivity and reproducibility to the HPLC assay; and that robust parameters describing the pharmacokinetics of fluconazole are possible with this model.

Animal studies of the pharmacokinetics of antibiotics in which an intracisternal needle is used to obtain cerebrospinal fluid (CSF) have been helpful in predicting the efficacies of different regimens in the treatment of bacterial meningitis in humans (4, 17, 18, 20). There are no reports describing the pharmacokinetics of azole antifungal agents in CSF in which complete concentration-time data for rabbits or humans are presented. Moreover, it has never been established whether serial sampling with an indwelling cisternal needle affects pharmacokinetics in the CSF or CSF physiology. The minimal trauma associated with paracenteses in other organ systems is known to cause disruption of tight vascular endothelial junctions and a breakdown of barrier function (2, 12, 13). Existing data describing the pharmacokinetics of azoles in the CSF are from concentration-time points pooled from different animal and human subjects (5, 14), which results in limited pharmacokinetic parameter reliability due in part to intersubject variability.

The relative penetration of azoles and amphotericin B into the CSF does not correlate with clinical efficacy (6, 7, 15, 21). This lack of correlation could be due either to the absence of a sufficiently sensitive assay to measure drug concentrations in the CSF, which would permit a comparison of concentration in CSF/MIC ratios for different drugs, or to the lack of an association between CSF penetration and efficacy. The primary objectives of our studies were to determine the pharmacokinetics of fluconazole in the serum and CSF following intracisternal and intravenous administration and to validate the animal model in which serial samples of CSF are obtained via an indwelling intracisternal needle (4). As part of our preparation, we additionally sought to optimize and compare the sensitivities and reproducibilities of two commonly employed assays (the microbiological assay and the high-performance liquid chromatography [HPLC] assay).

(Part of this research was presented at the 1991 Interscience Conference on Antimicrobial Agents and Chemotherapy.)

MATERIALS AND METHODS

Animal model. Adult male New Zealand White rabbits (Hare Marland, Nutley, N.J.) weighing 2 to 3 kg were used. Five animals were used for the direct intracisternal injection; 5 were used for the determination of the effects on cell count and protein, glucose, and lactate concentrations; and 10 were used for systemic administration studies. The anesthetic consisted of 15 to 20 ml of 25% urethane given subcutaneously and 15 mg of pentobarbital per kg of body weight given intravenously. Following anesthesia, a 24-gauge angiocatheter was inserted into a marginal ear vein for drug administration. A second angiocatheter was placed in the central artery of the contralateral ear for serum sampling. For systemic administration, 10

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mg of fluconazole per kg was infused over a period of 10 min. For direct injection, 200 μg of fluconazole in 100 μl of phosphate-buffered saline was injected intracisternally. CSF sampling and direct intrathecal drug administration were performed with a 25-gauge spinal needle in animals immobilized in a stereotactic frame by a modification of the technique described by Dacey and Sande (4). Samples of CSF (100 μl each) and blood (0.5 ml each) were obtained. For the systemic administration studies, the animals were divided into two groups. In group 1, the intracisternal needle was put in place prior to drug administration, and serial samples were taken at 0.25, 1.25, 2.25, 3.25, 4.25, 5.25, and 6.25 h. In group 2, the needle was placed in subsequent animals at sequential time points so that each sample gave virgin data. For group 1 animals, pharmacokinetics was determined by the standard two-step technique (19), whereas for group 2 animals, single virgin concentration-time datum points from different animals were used to generate a single-subject estimate. Following the designated sampling periods, animals were sacrificed by intravenous administration of pentobarbital sodium (125 mg/kg) followed by bilateral pneumothoraces.

Microbiological assay. Following drug administration, blood and CSF samples were obtained at designated time points. After clotting, the blood was immediately centrifuged at 500 × *g* for 10 min. Blood samples were decanted, and serum and CSF were stored at −70°C. Drug concentrations in the serum and CSF were measured by a modification of the well diffusion microbiological assay described by Jorgensen et al. (9). Several modifications aimed at improving the sensitivity and reproducibility of this assay were examined: varying the inoculum, the agar well depth, and the temperature and duration of incubation. Twenty-two yeast isolates were compared by agar dilution MIC determinations to identify the most susceptible isolate. *Candida pseudotropicalis* (ATCC 46764) was used as the assay organism. The organism was maintained on Sabouraud dextrose slants at 25°C. Prior to use, the organism was inoculated into Trypticase soy broth. The broth was incubated overnight at 35°C in ambient air. The next day, the cells were diluted to an optical density of 0.18 at A_{600}, giving an inoculum of 5.7×10^6 CFU/ml. Twenty milliliters of a 1:10 dilution in 1.5% Synthetic Amino Acid Medium Fungal (American Biorganics, Niagara Falls, N.Y.) molten agar at 50°C was poured into plates (150 by 15 mm). After the agar solidified at room temperature, 8-mm-diameter wells were made. Ten-microliter aliquots of serum, CSF, or standards were then pipetted into the wells, incubated at 4°C for 1 h, and then incubated overnight (14 to 16 h) at 28°C in an ambient air incubator. Standards were diluted in normal rabbit serum for serum assays and in balanced salt solution for CSF assays. Zone sizes in balanced salt solution and CSF were equivalent. Zones of inhibition were read to 0.1 mm, using a vernier caliper under direct magnification. A standard curve was constructed by plotting zone sizes versus $\log_{10}$ fluconazole concentrations. Unknowns were calculated from the curves derived from the standards contained on the same plate. The accuracy and reproducibility of this method were analyzed by using least-squares analysis and inter- and intraday coefficients of variation.

HPLC assay. The biological assay was compared with an HPLC method that was a modification of that previously described by Harris et al. (8). Fluconazole powder and the internal standard UK54373 were gifts from Pfizer Pharmaceuticals. All reagents were HPLC grade. Double-distilled water was used, and all solvents were passed through a 0.45-μm-pore-size filter prior to use. The assay was performed with a Hewlett-Packard HP1090 HPLC device with an autosampler, a diode array detector, and an HP3390A recording integrator. The mobile phase consisted of methanol with 0.025 M sodium phosphate (pH 7.0). Aliquots of samples and standards (fluconazole-spiked rabbit plasma or balanced salt solution for CSF) were diluted with 0.4 ml of water and 0.1 ml of an internal standard (100 μg/ml). The sample was made basic with 1 ml of 1 M NaOH, and 4 ml of ethyl acetate was added in a 15-ml conical test tube. All samples at each step were mixed with a vortex mixer for 2 min and then were centrifuged at 1,000 × *g* for 5 min to separate the layers. The organic layers were extracted into acid with 1 M hydrochloric acid, then realkalinized with 5 M NaOH, and reextracted into 4 ml of ethyl acetate. The final extract was evaporated to dryness under nitrogen at 37°C. The extract was resuspended in 200 μl of mobile phase and vortexed for 5 min. Samples (75 μl each) were injected onto an Adsorbosphere C_{18} 5-μm HPLC column with a guard column. Fluconazole was detected by UV A_{260}. The flow rate was 1.0 ml/min. The retention times for fluconazole and the internal standard were 5.0 and 6.2 min, respectively. A standard curve was constructed by plotting the area under the sample peak versus the fluconazole concentration. Fluconazole concentrations were calculated by comparison with the standard curve after correction for recovery of the internal standard.

Measurement of cells and chemical parameters in CSF. Leukocyte density was measured with a counter (Coulter Electronics, Hialeah, Fla.). CSF samples were centrifuged at 10,000 × *g* for 5 min, and the supernatant was stored at −70°C until assays were run. The protein concentration was measured by the bicinchoninic acid method (BCA kit; Pierce Chemical Company, Rockford, Ill.). The CSF lactate and glucose concentrations were measured calorimetrically following enzymatic conversion to pyruvate and H_2O_2 and by the quinoneimine dye method, respectively, with assay kits from Sigma Diagnostics (St. Louis, Mo.).

Pharmacokinetic analysis. Pharmacokinetic analyses of the plasma and CSF fluconazole concentration-time relationships after systemic administration and direct intracisternal administration were performed with a nonlinear least-square regression program, RSTRIP (Micromath Scientific Software, Salt Lake City, Utah). The most appropriate pharmacokinetic models were determined by using model selection criteria based upon a modified form of Akaike's information criterion (1). To determine the area under the concentration-time curve, the trapezoidal method was used from time zero to the last observed time point.

Statistical analysis. Overall differences in pharmacokinetic parameters among rabbits were evaluated by analysis of variance. The two-tailed paired *t* test was used to determine if there were any statistically significant differences in pharmacokinetic parameters in the serum and CSF. The mean and the standard deviation of each kinetic variable were calculated. In all tests performed, a *P* value of <0.05 was considered statistically significant. All statistical tests were performed with MINITAB (W. W. Norton, New York, N.Y.).

RESULTS

Comparison of the microbiological assay with the HPLC assay. Data for the sensitivity and reproducibility of the HPLC and microbiological assays are shown in Table 1. The bioassay had well-demarcated zones of inhibition, with a log-linear correlation between zone sizes and concentrations for standards ranging from 2 to 32 μg/ml ($r > 0.99$). The sensitivity of the assay was 2 μg/ml. The coefficients of variation for the lower and upper limits of sensitivity were 5.51% and 1.22%,

TABLE 1. Comparison of microbiological assay and HPLC assay data

Assay	Fluconazole concn (μg/ml)	Coefficient of variation (%)[a]
Microbiological[b]	32	1.22
	16	4.59
	8	6.3
	4	7.0
	2	5.51
HPLC[c]	20	3.01
	10	2.92
	5	3.64
	2	10.75
	1	4.54

[a] Average coefficients of variation for the microbiological and HPLC assays are 4.92 and 4.97%, respectively.
[b] Sensitivity, 2 μg/ml; sensitivity considering sample size, 0.02 ng.
[c] Sensitivity, 0.1 μg/ml; sensitivity considering sample size, 0.1 ng.

respectively, with a mean of 4.92%. The HPLC assay also showed an excellent correlation between peak height or area under the curve and standard drug concentrations, with an r of ≥ 0.99 and a sensitivity of 0.1 μg/ml. The interday coefficients of variation for upper and lower limits of sensitivity were 4.54% and 3.01%, respectively, with a mean of 4.97%.

Pharmacokinetics following direct intracisternal injection. Figure 1 shows the relationship between concentration in CSF and time following direct fluconazole injection into the cisterna magna. The rationale behind these studies is as follows: if serial sampling caused an alteration in the kinetics of efflux, then the slope of the elimination curve would vary as a function of the volume of CSF removed and/or the time the intracisternal spinal needle was in situ. The rate of drug elimination did not vary ($r > 0.999$), indicating that the indwelling intracisternal needle did not alter the kinetics of fluconazole in the CSF following direct intracisternal injection. Drug elimination from the CSF following direct injection followed a one-compartment model, with an elimination half-life of 0.43 h. The elimination half-life for direct injection was significantly shorter than that for systemic injection (0.43 versus 9.00 h, $P < 0.05$).

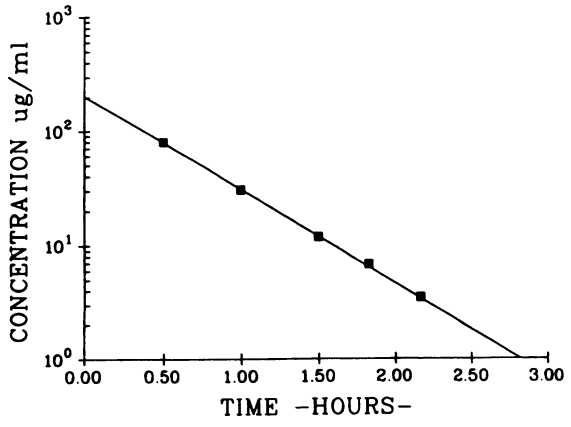

FIG. 1. Fluconazole pharmacokinetics following direct injection. Semilogarithmic plot showing the mean fluconazole concentrations in five rabbits receiving 200 μg of fluconazole which was directly injected into the cisterna magna. One hundred microliters of CSF was sequentially removed from each rabbit via a stereotactically placed indwelling intracisternal spinal needle.

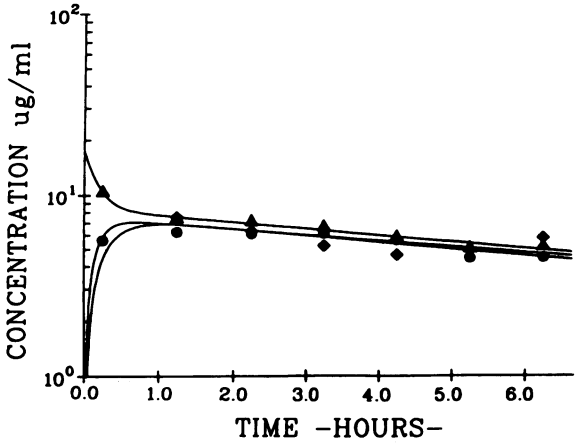

FIG. 2. Concentration-time curves for the serum and CSF following intravenous fluconazole administration. Semilogarithmic plot of mean concentrations in CSF and serum in 10 rabbits receiving 125 mg of fluconazole per kg via intravenous administration over a period of 10 min. Concentrations in serum for all animals are shown. Serum, triangles; group 1 CSF, circles; group 2 CSF, diamonds.

Pharmacokinetics following systemic drug administration. Figure 2 shows concentration-time data from a 6-h period for the serum and CSF following systemic drug administration. The serum data for both group 1 (serially sampled) and group 2 (naive pooled data [NPD]) animals followed a two-compartmental model, with a beta elimination half-life of 8.228 h. In group 1 animals, the beta elimination rate constant was 0.076 h^{-1} and the half-life was 9.008 h. The penetration to the last time point for group 1 was 84.3%; the maximum concentration of the drug in serum was 6.12 μg/ml. In group 2 animals, the beta elimination rate constant was 0.075 h^{-1} and the half-life was 9.138 h. The penetration to the last time point for group 2 animals was 86%; the maximum concentration of the drug in serum was 7.49 μg/ml. The time to the maximum concentration of the drug in serum was 1 h for both group 1 and 2 animals. There were no significant differences in pharmacokinetic parameters for group 1 or 2 animals, indicating that the intercisternal needle did not alter the percent penetration of or the elimination rate constant from the CSF.

Effects of serial sampling on CSF chemistry and leukocyte and erythrocyte numbers. Figure 3 shows data on the effect of an indwelling needle on cell counts and chemical parameters in the CSF. There was no increase in the number of leukocytes or in levels of protein noted over the 7 h of the study. The lactic acid and glucose levels also did not change over time. The initial mean erythrocyte count was 550; this fell over the course of the experiment. However, even at the earliest time point, the blood contamination of the CSF was less than 0.001%.

DISCUSSION

To examine the effect of repeated cisternal paracenteses on the rate of drug elimination, we first examined pharmacokinetics in CSF following direct intracisternal injection. There was no effect on the elimination rate constant ($r > 0.999$), indicating that the indwelling needle did not affect drug elimination from the CSF. To further validate the animal model, we examined fluconazole pharmacokinetics following systemic administration. The percent penetration was deter-

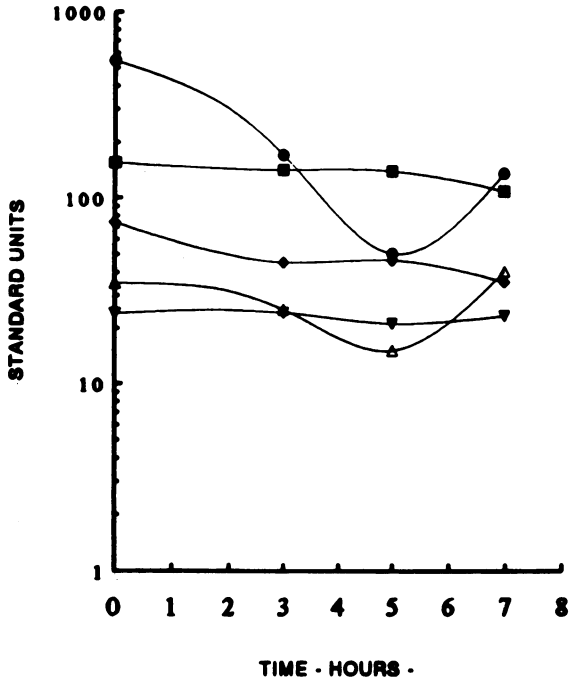

FIG. 3. Effect of a stereotactically placed indwelling intracisternal spinal needle on selected CSF parameters. Plot showing the effects of the intracisternal needle in five rabbits over 7 h. Data are means for erythrocytes (circles), leukocytes (open triangles), protein (diamonds), glucose (squares), and lactate (closed triangles). Units for cells are cell numbers per microliter. Units for chemical parameters are milligrams per 100 ml.

mined by comparing the area under the curve for the CSF and the area under the curve for serum AUC following systemic administration in two groups of animals. Group 1 pharmacokinetic data were determined by obtaining serial datum points, whereas for group 2 animals, virgin datum points from different animals were used to construct a hypothetical single-subject estimate by the NPD approach. As expected, on the basis of the model selection criteria, the NPD approach was not as reliable as the approach using kinetic parameters derived from complete concentration-time data sets because of wide inter- and intrasubject variations (19). Nevertheless, the elimination half-lives in the CSF were similar in group 1 and 2 animals, and they were also similar to the elimination half-lives in the serum. Elimination half-lives in the CSF were also similar to those in unanesthetized animals (8.85 h^{-1}) as determined by Walsh et al. (22). The percent penetration into the CSF in group 1 and 2 animals were similar not only to each other but also to those described by others using the NPD approach with animals (82.4%) (15) and humans (73.8 to 88.7%) (21). While in group 2 animals the NPD approach was less robust than that derived with complete CSF datum points, there was no difference in the percent penetration in group 1 and 2 animals.

Direct intracisternal injection was associated with more rapid elimination from the CSF than that observed with the systemically administered drug. Slower elimination following systemic drug administration occurs since the rate-limiting step is the terminal elimination rate in the serum. As we have shown for other body compartments (12), pharmacokinetics in CSF behaves like pharmacokinetics in the peripheral compartment in a two-compartment model in which elimination in the central compartment and that in the peripheral compartment are identical (12).

To determine if the indwelling needle altered CSF chemistry or caused inflammation or bleeding, we measured the numbers of erythrocytes and leukocytes and the concentrations of glucose, lactate, and protein. Except for erythrocyte number, there was no effect of the intracisternal needle on any parameter. While initially there were 550 erythrocytes per μl, this number fell with time to <50 erythrocytes per μl. Moreover, even at the initial time point, the degree of CSF contamination was less than 0.001%. As a result, CSF contamination with blood did not affect the results of analysis of pharmacokinetics in the CSF.

Both the microbiological assay and the HPLC assay were equally reproducible, with mean interday coefficients of variation of 4.92 and 4.97%, respectively. While the sensitivity of the HPLC assay (0.1 μg/ml) appeared greater than that of the microbiological assay (2 μg/ml), this comparison assumes that relatively large sample volumes of 500 to 1,000 μl are obtained, extracted, and reconstituted into smaller volumes for HPLC. However, in studies examining the relative levels of penetration of azoles into the eye and CSF, serially obtained sample volumes are limited to 5 to 100 μl (10, 11). Considering the volume limitation of our samples, the HPLC assay could detect a minimum of 0.1 ng, whereas the microbiological assay could detect a minimum of 0.02 ng. Therefore, depending upon the volume of the sample, the microbiological assay can be up to fivefold more sensitive than the HPLC assay. Recently, Rex and colleagues also compared a microbiological assay with an HPLC assay, and their data indicated sensitivities which were identical to ours, 0.1 and 2 μg/ml, respectively (16). While the between-day coefficient of correlation for the HPLC assay was similar to that described by us (<5 versus 4.92%), the reproducibility of their biological assay was less (<12 versus 4.92%). The reasons for the latter difference are unclear, but the difference may be due to assay modifications optimizing the microbiological assay.

Both itraconazole, which is more active than fluconazole (7–9), and amphotericin B are effective in the therapy of cryptococcal meningitis despite low or unmeasurable (i.e., below the level of detection of the assay) concentrations of the drugs in the CSF. The ability to measure low concentrations of azoles in the CSF is important in establishing why drugs with poor CSF penetration (i.e., itraconazole and amphotericin B) are as effective as drugs with good penetration (i.e., fluconazole) in treating cryptococcal meningitis in humans (3) and animals (15). The use of a more sensitive assay will help to establish if concentration in CSF/MIC ratios for these drugs account for their comparable efficacies. If not, other explanations must be sought. Itraconazole, unlike fluconazole, is a lipophilic drug, with much higher tissue-to-serum concentrations than those for fluconazole (6). As a result, penetration into the meninges rather than that into the CSF may explain efficacy. The present studies of azole pharmacokinetics in the CSF using a sensitive and reproducible assay were necessary in developing an animal model which may be capable of comparing azole concentrations in the meninges and CSF (10).

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