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Fluconazole Concentrations in Saliva from AIDS Patients with Oropharyngeal Candidosis Refractory to Treatment with Fluconazole

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Fluconazole (FCZ) has been extensively used as a primary therapy for oropharyngeal candidosis in AIDS patients. Clinical resistance to FCZ is now encountered, often related to decreased susceptibility of the isolate in vitro. We wondered if low levels in saliva play a role in the therapeutic failure, especially in patients complaining of dry mouth. Sixteen AIDS patients treated for oropharyngeal candidosis with FCZ were studied. MICs for the isolates were determined. Serum and saliva samples were collected to measure FCZ levels with a bioassay using paper disks loaded with the clinical specimens. We showed that (i) paper disks were convenient for collecting saliva in patients with dry mouth; (ii) levels in saliva depended on the FCZ dosage regimen but did not correlate with the response to therapy; (iii) correlation between concentrations in saliva and serum was poor and independent of clinical response to treatment, other therapies, or decreased salivation; and (iv) levels in saliva were always lower than MICs in patients who failed to respond to treatment. In conclusion, therapeutic failures are more likely to be related to in vitro resistance of the isolate to FCZ or insufficient dosage regimen than to decreased salivary secretion.

Oropharyngeal candidosis (OPC) is a common mucosal infection among patients infected by the human immunodeficiency virus, occurring in 11 to 95% of these patients according to the degree of the immune defect (6, 7). *Candida albicans* is the most frequent opportunistic pathogen, responsible for more than 90% of the infections in this group of patients. However, other *Candida* species such as *Candida tropicalis*, *Candida krusei*, and *Candida* (*Torulopsis*) *glabrata* have emerged as significant pathogens (10, 21).

The efficacy of fluconazole (FCZ) as a primary therapy for OPC in immunocompromised patients, especially AIDS patients (8), and even as prophylaxis of recurrent OPC (14), has been demonstrated. The rapid resolution of candidosis under FCZ therapy is probably related to the usual susceptibility of *C. albicans* to FCZ (3, 12). Furthermore, studies have shown that levels in saliva are similar to those in plasma in healthy volunteers (4, 12).

Despite their usual efficacy, some FCZ treatments are considered to be clinical failures (persistence of clinical lesions despite adequate therapy) or mycological failures (culture of a *Candida* sp. showing in vitro resistance to FCZ) (17, 18). The clinical failures usually correlate with decreased susceptibility of the isolate to FCZ in vitro (5, 18) and are often related to previous treatments with this drug (18, 24). Explanations for this phenomenon include the mutation (2, 22, 27) or the selection of a resistant population (1, 2, 11, 22, 27) or local factors (17). Among these factors, diminution of FCZ concentration in saliva could alter FCZ efficacy. Xerostomia (dry mouth and diminished salivation) can modify the course of OPC not only by augmenting the risk of traumatic lesions of the oral mucosa but also by decreasing salivary constituents important in local immunity and diminishing local diffusion of antifungal agents such as FCZ (15).

The aim of this work was to study one of the local factors by comparing FCZ levels in saliva from AIDS patients with OPC clinically susceptible or resistant to treatment with FCZ. A bioassay and a sampling technique that allowed FCZ measurement in saliva even from patients complaining of dry mouth were developed.

MATERIALS AND METHODS

Patient population. Patients with AIDS who were monitored for their human immunodeficiency virus infection at the Pasteur Institute Hospital in Paris (France) were sampled, and their informed consent was obtained. Patients were eligible for study either with or without clinical signs of oral candidosis at the time of sampling, but they had to have been under treatment with FCZ for a minimum of 7 days. To reduce expenses in the follow-up of human immunodeficiency virus-positive patients and to avoid unnecessary bothering of the patients, the diagnosis of OPC was made by an experienced physician when clinical lesions of thrush were observed. Clinical resistance was defined as persistence of the lesions (less than 50% improvement) despite treatment with at least 50 mg of FCZ per day for a minimum of 7 days. Fungal cultures were performed only when the episode was clinically resistant to antifungal therapy. Therefore, for some of the patients, no data on the FCZ susceptibility of the fungus responsible for the lesions seen when the treatment was started were available. The clinical data available for the 16 selected patients are given in Table 1.

Clinical samples. Biological fluids were obtained before drug intake (T_0) and 4 h afterwards (T_4) when possible. These fluids were obtained by the collection of 1 to 2 ml of saliva into sterile tubes (saliva sampling), the collection of saliva by introducing sterile paper disks (diameter, 6 mm [catalog number 66100]; Sanofi-Diagnostics Pasteur, Marnes-La-Coquette, France) under the patient's tongue (disk sampling), and the taking of a blood sample. Saliva was obtained before breakfast for T_0 and before lunch for T_4. For the disk sampling, two paper disks were introduced in the patient's mouth under the tongue near the frenulum. Excess fluid was removed by gently scraping both sides of the disk on the edge of a petri dish. When lesions of OPC were still observed, samples of buccal lesions were taken. The cotton swabs were discharged on Sabouraud agar plates in order to allow separate colony growth. Identification of a *Candida* sp. was done by the routine technique (chlamydospore formation on depleted medium or carbon compound assimilation by using ID32C strips from bioMérieux, Marcy-l'Etoile, France). Clinical specimens were always stored frozen at −20°C prior to testing.

FCZ bioassay. The High Resolution (HR) agar was used as recommended by Pfizer Central Research (Sandwich, United Kingdom (20). *Candida kefyr* 706 susceptible to FCZ (MIC of FCZ, 0.19 μg/ml) was maintained on Sabouraud agar slants. A 4-day-old subculture on Sabouraud agar was harvested, and yeasts were inoculated at a final concentration of approximately 10^4 CFU per ml in HR agar cooled at 45°C. A volume of 25 ml of the suspension was poured into sterile, level, square plastic plates (12 by 12 cm). The plates were allowed to dry at 25°C

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TABLE 1. Characteristics of AIDS patients treated with FCZ for oral candidosis

Patient no.	Age (yr)	Sex	Risk factor for HIV infection[a]	CD4 count (mm^{-3})	Other drugs prescribed[b]
1	36	M[c]	Homosexual	16	A
2	40	M	NA	3	Cli, Cla, TMP-SMX, P
3	32	M	Drug abuse	26	TMP-SMX, S, P
4	33	F	Drug abuse	201	TMP-SMX, INH,
5	27	M	Drug abuse	12	Cli, D
6	32	M	Homosexual	6	TMP-SMX, P, Cla, F
7	45	M	Homosexual	217	TMP-SMX
8	43	M	NA	36	TMP-SMX, Cy
9	31	M	Bisexual	60	TMP-SMX
10	28	M	Homosexual	10	TMP-SMX, A,
11	32	M	Drug abuse	126	R, INH, P, S, AZT
12	33	M	Homosexual	5	
13	27	F	Heterosexual	128	TMP-SMX
14	31	M	Drug abuse	5	DDI, TMP-SMX,
15	31	M	Drug abuse	6	D, P, Cla, P
16	48	M	Homosexual	11	TMP-SMX

[a] NA, not available; HIV, human immunodeficiency virus.
[b] A, acyclovir; AZT, zidovudine; C, clonazepam; Cla, clarithromycin; Cli, clindamycin; Cy, cycline; D, dapsone; DDI, didanosine; P, pyrimethamine; F, foscarnet; INH, isoniazide; R, rifampin; S, sulfadiazine; TMP-SMX, trimethoprim-sulfamethoxazole.
[c] M, male; F, female.

and used the same day, since yeast growth prevented the appearance of the inhibition zone after storage (even at 4°C).

FCZ powder (lot no. R54; Pfizer Central Research) was dissolved at 1 mg/ml in sterile distilled water. Dilutions were then made in normal human serum and saliva, giving FCZ concentrations of 2.5, 5, 10, 20, 30, and 40 μg/ml. The absence of antifungal activity in serum and saliva from subjects with no antifungal treatment was previously checked. Sterile paper disks were then immersed for various periods of time into each of the standard solutions or loaded with known volumes of the solutions (see Results).

Measurement of FCZ levels in the clinical samples (saliva and serum) required the previous preparation of paper disks. Each sample was tested twice (i.e., a pair of paper disks was used). The sample loading was done by using the technique used for the standard curves. The disks prepared as mentioned above (standards and clinical samples), as well as the disks passively loaded with saliva, directly in the oral cavity of the patients, were then placed on the surfaces of the agar plates with sterile forceps. The plates were incubated for 24 h at 27°C. The inhibition zones were measured with vernier calipers. Each diameter was measured twice, and the average of the four measurements (per pair of disks) was considered the zone diameter for that sample.

Results obtained with disks loaded with known concentrations of FCZ were plotted as inhibition zone diameter versus $\log_{10}$ FCZ concentration to obtain a standard curve. Unknown concentrations were calculated by interpolating from the standard curve constructed from the same plate. The ratio of the FCZ concentrations in the saliva specimen and that in the corresponding serum specimen was calculated for T_0 and T_4. Concentrations were considered similar when the ratio was in the range of 0.9 to 1.1.

FCZ susceptibility testing. The susceptibility to FCZ of *C. albicans* isolates that were cultured from some of the patients' mouths was assessed by a broth microdilution method, essentially as described previously (2). The medium was HR medium buffered with 0.3 M morpholinepropanesulfonic acid at pH 7. *C. albicans* isolates grown on Sabouraud-chloramphenicol agar slants for 48 h were subcultured in Sabouraud broth overnight at 30°C. A concentration of 10^5 CFU/ml was obtained by appropriately diluting the culture suspension into sterile water. A stock solution of FCZ at 1 mg/ml was diluted twofold in HR medium to give concentrations ranging from 0.18 to 100 μg/ml. Each well of a sterile microplate was then inoculated with HR medium (100 μl), a dilution of the FCZ solution (100 μl), and the yeast inoculum (10 μl). Final concentrations of FCZ thus ranged from 0.09 to 50 μg/ml. Control wells included medium alone (no growth) and each isolate in drug-free medium (maximum growth).

Optical densities were recorded at 492 nm with a spectrophotometer (Titertek II; Flow Laboratories, Rockville, Md.) after incubation for 24 h at 28°C. The MIC was the FCZ concentration preventing any growth of the organism compared with the growth in the corresponding drug-free well. Strains for which MICs were greater than 12.5 μg/ml were defined as resistant, whereas isolates for which MICs were lower than 3.12 μg/ml were considered to be susceptible. Intermediate susceptibility was associated with MICs at 3.12 and 6.25 μg/ml.

Statistical analysis. Peak and residual levels of FCZ were compared by using the Wilcoxon signed-rank test. The level of significance was 0.05. Correlation coefficients were calculated using StatView II software (Abacus Concepts Inc., Berkeley, Calif.).

RESULTS

Influence of disk loading on FCZ level measurement. In all cases, we used aliquots of standard solutions prepared in either human serum or saliva and stored at −20°C. This storage procedure has already been reported to yield reproducible and accurate results (23).

We first checked any possible variation in the inhibition zone diameters related to the technique used to load the disks. The three techniques assessed were (i) a brief immersion of 1 s repeated two to six times; (ii) a precise timing of 15, 25, 30, or 60 s with only one immersion; and (iii) a precise volume of 25, 30, or 35 μl pipetted and loaded on the disks (paper disks will not absorb more than 35 μl). Excess fluid was removed from the disks after the loading by gently scraping both sides on the edge of a petri dish, and the disks were then allowed to dry before being placed on the surfaces of the agar plates. Each factor was studied at least three times during independent experiments to check day-to-day variation. Within-run variability was also tested with two to three disks.

Less than 4% variability in inhibition zone diameter was found for standard curves obtained when using the first two techniques of loading. Less than 7% variation was seen when comparing diameters obtained with disks loaded with 25, 30, or 35 μl. Within-day variation was negligible. Day-to-day variation was greater (>20% with the brief immersion and with the loading of the disks with a precise volume), showing the necessity of a daily standard curve. In any case, removal of excess fluid was mandatory to prevent false interpretations. Whatever the procedure used and the day of the experiment, a concentration of FCZ lower than 2.5 μg/ml was undetectable.

We then decided, for convenience and accuracy, to prepare all standard curves using disks immersed for 1 min in the standard solutions. Under these conditions, the standard curves were linear between 2.5 and 40 μg/ml with $r^2 > 0.95$. The bioassay was sensitive down to 2.5 μg/ml. Below this value, the zones of inhibition were too close to the size of the disk for accurate measurement. The coefficients of variation between assays (multiple days) and within assays were ≤10% at high (40 μg/ml) and low (2.5 μg/ml) concentrations.

Influence of the sampling method on FCZ levels measured in saliva. Collection of saliva by the two methods (disk and saliva sampling) offered advantages and disadvantages. Collection of saliva was probably easier when the patient did not complain of dry mouth. When patients complained of dry mouth, the disk sampling was more appropriate since the small volume of saliva usually obtained often prevented testing. However, care was taken when removing the disks from the patient's mouth since small but painful ulcerations could be made at that time.

With the disk sampling technique, we first evaluated for two patients the variations in FCZ concentrations related to the duration of incubation in the mouth (10, 20, 30, 40, 50, and 60 s). Very few differences were observed, with less than 8% variation between the 10- and 60-s samples. Therefore, saliva samples were obtained either as fluid used to immerse the disks for 1 min before FCZ level determination or as disks moistened for 1 min under the patient's tongue.

We then compared results obtained with the two sampling methods (saliva and disk sampling) in regard to efficacy for measuring FCZ levels in human saliva (Fig. 1). All the samples (saliva and disk sampling at T_0 and T_4) were not available for

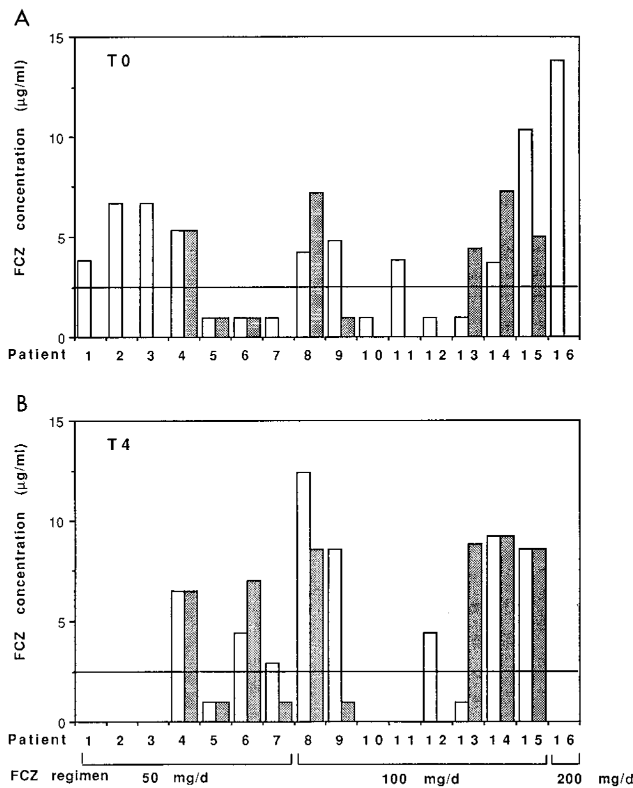

FIG. 1. FCZ concentration in saliva samples from 16 AIDS patients with oral candidosis treated with FCZ. Sampling of saliva was done before drug intake (A) and 4 h after drug intake (B). The FCZ dosage regimen is noted below panel B. Two methods of saliva collection were used: collection of saliva that was then loaded on a sterile paper disk for the assay (▨) and placement of a sterile paper disk under the patient's tongue for 1 min (□). FCZ levels were measured with a bioassay (limit of detection, 2.5 μg/ml). Therefore, samples that were tested but contained no detectable concentration of FCZ were arbitrarily assigned a value of 1 μg/ml to differentiate them from the unavailable samples (missing values).

each patient because of the absence of compliance usually related to other health problems. For three of the eight patients evaluable at T_0 and four of nine patients evaluable at T_4, similar concentrations of FCZ were obtained by both sampling methods, including cases where FCZ levels were undetectable (correlation coefficient, 0.481 at T_0 and 0.452 at T_4). Two discrepant results were observed (patients 9 and 13, Fig. 1). They had undetectable levels of FCZ by one sampling technique in contrast to measurable levels by the other technique as well as in serum.

There was no decrease in salivary FCZ levels for the five patients who complained of dry mouth compared with that for patients with normal salivation. Of the five patients, four had measurable levels of FCZ at T_0, whereas one (patient 6), who had undetectable levels at T_0, was found to have detectable concentrations at T_4.

Correlation between FCZ concentrations in saliva and serum specimens. At T_0, 8 of the 16 evaluable patients had similar FCZ concentrations in saliva and serum, including 5 patients with undetectable FCZ levels in both fluids (correlation coefficient, 0.471). FCZ concentrations were higher in saliva than in the corresponding serum sample for four patients and lower for four others (Table 2).

At T_4, only 2 of the 10 evaluable patients had similar levels of FCZ in saliva and serum, including one patient with undetectable FCZ levels in both fluids, a finding already noted for this patient at T_0 (correlation coefficient, 0.340). Salivary FCZ concentrations were higher in six patients and lower in two (Table 2).

Serum FCZ concentrations did not correlate better with salivary FCZ levels in patients with normal salivary secretion than in patients with xerostomia. The agreement between salivary and serum FCZ levels was also not related to the clinical susceptibility of OPC to treatment with FCZ (Table 2).

We did not find that other drugs prescribed for the patients (Table 1) influenced salivary and/or serum FCZ levels, but the data collected and the number of patients prevent precise analysis. Only one patient (patient 14) was treated with didanosine, a drug known to decrease salivary secretions.

TABLE 2. Treatment, outcome, and FCZ concentrations in saliva and serum samples from 16 AIDS patients with oral candidosis

Patient no.	Clinical lesion[a]	Xerostomia	FCZ regimen (mg/day)	Treatment duration (days)[a]	Culture[b]	MIC (μg/ml)	FCZ concn at: T_0		T_4	
							Saliva	Serum	Saliva	Serum
1	No	No	50	>20	ND[c]		3.8	3.4	ND	ND
2	Yes	Yes	50	12	ND		6.7	<2.5	ND	ND
3	Yes	Yes	50	8	+	50	6.7	7.8	ND	ND
4	No	No	50	10	−		5.3	3.4	6.5	4.3
5	No	No	50	7	−		<2.5	<2.5	<2.5	<2.5
6	No	Yes	50	8	+	6.25	<2.5	<2.5	4.4	3.5
7	No	No	50	8	+	0.78	<2.5	<2.5	2.9	<2.5
8	No	No	100	20	−		4.2	6.0	12.4	7.2
9	No	No	100	7	+	1.56	4.8	7.2	8.6	8.6
10	Yes	No	100	7	+	3.12	<2.5	<2.5	ND	ND
11	Yes	Yes	100	14	+	6.25	3.8	3.4	ND	ND
12	No	No	100	7	+	0.78	<2.5	<2.5	4.4	3.5
13	No	No	100	13	−		<2.5	4.3	<2.5	8.2
14	Yes	Yes	100	10	+	>50	3.7	11.0	9.2	14.1
15	Yes	No	100	12	+	50	10.4	4.2	8.6	6.0
16	Yes	No	200	>20	+	25	13.8	7.8	ND	ND

[a] At the time of sampling.
[b] +, positive culture; −, negative culture.
[c] ND, not done.

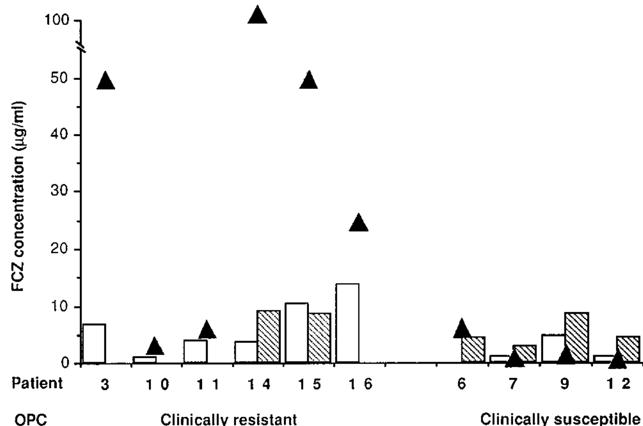

FIG. 2. Relationship between FCZ levels in saliva and in vitro susceptibility of *C. albicans* isolates to FCZ. Patients with OPC clinically resistant or susceptible to FCZ (more than 1 week of treatment) were evaluated. Saliva was obtained by disk sampling at T_0 (□) and T_4 (▧), and FCZ levels were estimated with a bioassay. MICs of FCZ (▲), expressed in micrograms per milliliter, were determined by a broth microdilution method.

We also compared FCZ concentrations obtained at T_0 and T_4 in serum and saliva specimens (disk sampling). Peak levels of FCZ were significantly higher than residual levels in both saliva and serum ($P < 0.0001$, Wilcoxon signed-rank test). When dosage regimens were compared, patients treated with 100 mg/day were more likely to have detectable residual FCZ levels in serum and saliva (six of eight and five of eight patients, respectively) than patients treated with 50 mg/day (three of seven and four of seven patients, respectively), although this difference was not significant ($P > 0.05$, chi-square test).

FCZ resistance. Candidosis was resistant to FCZ treatment in 7 of the 16 patients. Six of the corresponding isolates of *C. albicans* as well as four isolates cultured from the mouths of patients clinically cured by the antifungal treatment were tested for FCZ susceptibility (Fig. 2). *C. albicans* isolated from lesions resistant to FCZ were resistant in vitro, with MICs >25 µg/ml in four cases, and had intermediate susceptibility in two cases. All of these patients had salivary and serum FCZ concentrations lower than the MICs. Four of the six patients with persistent thrush complained of dry mouth or diminished salivation. In contrast, for none of the four *C. albicans* isolates cultured from patients cured by the FCZ treatment were MICs higher than 6.25 µg/ml. In these four cases, the salivary FCZ levels were higher than or close to the MIC.

DISCUSSION

The emergence of OPC clinically resistant to treatment with FCZ and of in vitro resistance to FCZ among *C. albicans* isolates responsible for these infections, especially in AIDS patients, explains the need for in vitro assays well correlated with the clinical course of fungal diseases and the efficacy of antifungal chemotherapy. Apart from reliable routine tests for antifungal susceptibility testing, controlling drug intake and good diffusion at the site of infection are necessary (17).

We developed a bioassay in order to evaluate FCZ levels during therapy. We wanted to test the hypothesis that a diminished FCZ diffusion in saliva correlates with clinical resistance, especially in AIDS patients complaining of dry mouth. During the evaluation of this method we noted advantages and disadvantages in relation to the well-being of the patient, the sampling technique, and the reliability of the bioassay itself.

Saliva sampling offers the advantage over serum sampling of being less invasive for the patients. However, dry mouth prevented collection of saliva, and the paper disk inserted under the tongue sometimes caused pain upon removal. In any case, compliance from the patient was mandatory to obtain good saliva samples. This compliance is often hard to achieve when dealing with AIDS patients. We recommend using it only with patients still in fair condition. From our point of view, disk sampling should be favored over saliva sampling, since samples can be obtained by the former method in most cases.

As noted before by Rex et al. (23), the bioassay has the advantage of simplicity and has enough sensitivity to measure the clinically relevant concentrations (2.5 to 40 µg/ml). Accurate measurement of inhibition zone diameter was sometimes liable to variation. The fact that fluids from which FCZ was measured were loaded on disks instead of being added to wells cut in the agar did not increase this problem. The differences noted between the results obtained with the saliva and the disk sampling techniques are unexplained. No factor such as clinical resistance, in vitro resistance, or dry mouth was associated with a diminished value obtained with one method compared with the other. The fact that the ducts of submandibular salivary glands are located under the tongue where the disks were placed might account for some of the discrepancies. It was, however, the only way to keep the disks in the same location for all patients and, therefore, to better standardize the sampling.

FCZ concentrations measured in saliva and serum samples from our patients were higher than what was expected by the FCZ dosage regimen (4, 12, 13, 26). Rifampin has been found to lower levels of FCZ by about 50% (25). Although not characterized, interferences due to other substances present in the clinical specimens could have modified the results. The various drugs prescribed for our patients were essentially antibiotics (erythromycin, aminoglycosides, penicillin, sulfamides, and fluoroquinolones) and antiviral drugs (zidovudine and didanosine). No clear relationship between a drug and FCZ level was found, but the small number of patients prevented precise analysis. Determination of FCZ concentrations by high-performance liquid chromatography (HPLC), a method often used to dose FCZ (13, 19), could have confirmed our findings. However, since there is no active metabolite of FCZ produced in humans (12), there is little reason why HPLC-determined levels should differ. This has already been proved by comparing results obtained with a bioassay and HPLC (23).

In our study, salivary FCZ levels were often higher than the corresponding levels in serum. Sputum FCZ levels were either higher or lower than levels in serum in one study (9). The higher salivary FCZ levels found in volunteers and in patients who underwent local radiotherapy in another study were attributed to probable binding to salivary constituents, as protein binding of FCZ is very low (19). The diminished salivation observed in five of the patients did not modify the trend. System hysteresis could also explain the relative concentrations in the two sites.

We then wondered if FCZ levels in saliva would predict clinical outcome. We thus determined the MICs of FCZ for 10 *C. albicans* isolates recovered from the mouths of six patients clinically resistant to FCZ and four patients cured by the treatment. Antifungal susceptibility testing was performed by using a method different from that proposed by the National Committee for Clinical Laboratory Standards (16). Because the National Committee for Clinical Laboratory Standards publishes the only available standard, we always referred to it. Results obtained by both methods were, in our hands, equiv-

alent (data not shown). Furthermore, by our method, MICs were usually well correlated with clinical outcome in AIDS patients, as demonstrated here. The isolates of the four patients who responded well to therapy required low MICs of FCZ, whereas the six patients whose therapy failed had isolates with intermediate (two cases) or low (four cases) susceptibility to FCZ. We did not find lower FCZ levels in cases of OPC that did not respond to FCZ treatment. However, salivary FCZ levels were always lower than the MIC for the corresponding *C. albicans* isolate. This includes two cases in which *C. albicans* isolates were still not resistant to FCZ in vitro. The FCZ concentrations in saliva achieved with 100 mg/day were slightly lower than the corresponding MICs. This could then account for the inefficacy of FCZ treatment and might have been corrected by increasing the dosage. Whether the FCZ-resistant isolates were the same genetically as the pretreatment isolates will remain unclear, since we neither stored nor tested the latter. However, results obtained in our laboratory (2) suggest that this is likely to be the case. The isolates with intermediate susceptibility to FCZ deserve further study, because they can be associated with clinical resistance as a result of insufficient dosage and also because they might be the first sign of acquisition of an in vitro resistance to FCZ.

In conclusion, clinical resistance to FCZ treatment seems more likely to be related to in vitro resistance of the isolate to this drug or insufficient levels of FCZ achieved by the dosage regimen than to diminished concentrations in saliva. From our point of view, the monitoring of salivary FCZ levels should not be used routinely unless antifungal susceptibility testing has clearly shown the in vitro susceptibility of the isolate to FCZ.

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