Rapid high performance liquid chromatographic assay for antifungal agents in human sera


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Serum concentration of seven antifungal agents, amphotericin B, 5-flucytosine, ketoconazole, fluconazole, itraconazole, miconazole and econazole were assayed using a single step sample preparation and an isocratic High Performance Liquid Chromatography (HPLC) procedure based on three mobile phases of similar components. Our method was simple, flexible and rapid, the assays being completed within half an hour. The method showed high reproducibility, good sensitivity with detection limits of 0.078 to 0.625 mg/L except for miconazole and econazole, and high recovery rates of 86-105%. Out of 24 therapeutic agents tested only aztreonam and trimethoprim were found to interfere with the assay of 5-flucytosine and fluconazole respectively, using this protocol. HPLC assay should be useful in the clinical laboratory for monitoring patients on antifungal therapy.

Introduction

Antifungal agents are frequently used for the treatment of invasive fungal infections, systemic mycoses and cryptococcal meningitis, especially in immunocompromised patients. Amphotericin B, despite its nephrotoxicity and other serious side effects, is still used for primary treatment of these infections (Graybill & Craven, 1983; Drouhet & Dupont, 1987; Gallis, Drew & Pickard, 1990; Hay, 1991) while newer agents like fluconazole and itraconazole are now widely used as well (Hay, 1991). Although it is generally not required to measure the level of these agents, clinical situations arise when therapeutic monitoring becomes necessary for dosage adjustment, such as in patients with renal malfunction, in cases when poor absorption is suspected, or when treatment failure occurs (Working Party, 1991).

High performance liquid chromatography (HPLC) has been used for assay of various individual antifungal agents in biological fluids (Diasio et al., 1978; Turner, Turner & Warnock, 1986; Hardin et al., 1988; Hosotsubo et al., 1988, 1990). In this report we describe a method which utilizes a single step of sample preparation and an isocratic HPLC procedure based on three mobile phases of similar components for the assay of amphotericin B, 5-flucytosine, ketoconazole, fluconazole, econazole, itraconazole and miconazole.

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Materials and methods

Standards and reagents

Seven antifungal agents in pure powder forms were used as standards for the HPLC assays; amphotericin B (Sigma, St. Louis, USA), fluconazole (Pfizer Inc, NY), itraconazole (Janssen, Belgium), ketoconazole (Janssen), 5-flucytosine (Sigma), miconazole (Sigma) and econazole (Sigma). The agents were solubilised initially in dimethyl-sulphoxide with further dilutions being made in water for the construction of calibration curves and for the determination of absorption maxima using a Uvikon 860 spectrophotometer (Kontron Instruments, Switzerland). For 5-flucytosine, the initial solubilization and subsequent dilutions were made in water. Ampicillin, aztreonam, benzylpenicillin, carbenicillin, cefamandole, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, cyclosporin A, erythromycin, fusidic acid, ganciclovir, imipenem, nalidixic acid, ofloxacin, pefloxacin, piperacillin, tetracycline, teicoplanin, trimethoprim, vancomycin, hydrocortisone and phenytoin were used to assess interference by other therapeutic agents in the assay of these antifungal drugs. Acetonitrile and diethylamine (both HPLC grade) were obtained from Merck, Darmstadt, West Germany. Ammonium acetate (molecular biology reagent grade) was obtained from Sigma, St. Louis, USA. Reagent grade water (Milli-Q, Millipor Corp.) was used throughout.

Mobile phase

Three mobile phases of similar components were used. Mobile phase I, 10 mM ammonium acetate, was used for assaying 5-flucytosine. Mobile phase II, which was composed of acetonitrile-10 mM:ammonium acetate (30:70; v/v) with 0.5% diethylamine, was used for the assay of amphotericin B and fluconazole. Mobile phase III consisted of acetonitrile-10 mM:ammonium acetate (65:35; v/v) with 0.5% diethylamine and was used for assay of ketoconazole, itraconazole, miconazole and econazole. All the mobile phases were filtered and degassed prior to use.

HPLC apparatus

The reversed-phase HPLC used consisted of an HP1050 Autosampler (Hewlett Packard, USA), a 2150 HPLC pump (LKB Products, Bromma, Sweden), a Nova-Pak RP-18 column (100 x 8 mm, 4 μm) (Waters, Division of Millipore, USA), a 490E programmable multiwavelength detector (Waters). The chromatographic output was recorded on a one-channel chart recorder (LKB Products). The pump was set at 1 mL/min and the chart recorder at a chart speed of 2 mm/min. Monitor wavelength was set at 260 nm except in the analysis of amphotericin B where 405 nm was used.

Assay procedures

Specimens of serum (minimal volume = 100 μL) were simultaneously deproteinated and spiked with a known concentration of an internal standard by adding an equal volume of an internal standard-acetonitrile solution. The internal standard used was an antifungal agent other than the one being assayed. The precipitated mixture was shaken for 20 sec on a Vortex Shaker at maximum speed followed by centrifugation at 10,000 g
for 1 min at room temperature. Ten to twenty microlitres of the supernatant was then injected into the HPLC system for analysis. To construct the calibration curves and determine assay parameters of linearity, precision, reproducibility, detection limits and mean recovery rates of each of these antifungal agents, 10 μL of each agent was spiked into 990 μL of drug-free human plasma, and serial dilutions made, using the same human plasma, down to concentrations which approximated the achievable plasma concentrations. These spiked specimens were then processed using the aforementioned procedures and analysed. Serial dilutions of corresponding aqueous standards were also prepared using water instead of plasma for determination of mean recovery rates.

For stability assessment, portions of the spiked samples before and after deproteination were stored at −20°C and 4°C, and reassayed by the same protocol at 1, 7, 14 and 28 days after spiking. Interference studies were also performed by mixing the various drugs listed previously with each of the antifungal agents, and processing for HPLC analysis using the same assay procedure.

Calculations

Standard statistical methods were used for analyses of results, using peak height measurements on recorded chromatograms. Coefficient of variation, mean percentage recovery and correlation coefficient for linearity of calibration curves were determined as previously described (Chan, Chan & French, 1986). The lowest concentration of the antifungal agents giving a peak height equal to or greater than three times the height of the chromatographic baseline was determined as the detection limit for each antifungal agent (Signs, File & Tan, 1984; Chan et al. 1986).

Results

The UV-absorption maxima of the antifungal agents tested in this study were found to range between 258 and 291 nm, hence the monitor wavelength used was 260 nm except for amphotericin B which has an absorption maxima of 408 nm, for which the monitor wavelength used was 405 nm (Table). All the antifungal agents were eluted by the respective mobile phases at retention times ranging from 3.8 to 21.0 min (Table) thus allowing the whole procedure of sample preparation and analysis to be completed within 30 min. Under the different mobile phases, the assayed agents 5-flucytosine (Figure 1(a)), fluconazole and amphotericin B (Figure 1(b)), ketoconazole, itraconazole, econazole and miconazole (Figure 1(c)) were baseline resolved from one another as well as from the solvent peak and the UV-absorbing endogenous materials, giving symmetrical peaks with minimal tailing. Detection limits for the antifungals ranged from 0.07 mg/L for 5-flucytosine to 6.25 mg/L for miconazole, a topical antifungal agent, while the recovery rates were 86–105%. These results were reproducible for all agents except itraconazole where the coefficient of variation was 13.0% (Table). The antifungal agents in the serum, whether deproteinated or not, were found to be stable for at least 28 days when stored at 4°C or −20°C. Of the 24 agents tested for interference with these antifungals, only aztreonam and trimethoprim were found to interfere with 5-flucytosine and fluconazole respectively.

This protocol was employed in the antifungal assay of a blood specimen from a
<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>Absorption maximum (nm)</th>
<th>Mobile phase (see text)</th>
<th>Monitor wavelength (nm)</th>
<th>Retention time (min (SD))</th>
<th>Coefficient of variation (%) for precision</th>
<th>Correlation coefficient for linearity of calibration curves</th>
<th>Mean recovery (%)</th>
<th>Detection limit (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Flucytosine</td>
<td>270</td>
<td>I</td>
<td>260</td>
<td>3.76 (0.09)</td>
<td>3.97</td>
<td>0.998</td>
<td>96.2</td>
<td>0.078</td>
</tr>
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<td>Amphotericin B</td>
<td>408</td>
<td>II</td>
<td>405</td>
<td>10.21 (0.10)</td>
<td>6.36</td>
<td>0.999</td>
<td>91.9</td>
<td>0.313</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>260</td>
<td>II</td>
<td>260</td>
<td>3.92 (0.06)</td>
<td>9.58</td>
<td>0.999</td>
<td>86.6</td>
<td>0.625</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>291</td>
<td>III</td>
<td>260</td>
<td>4.73 (0.08)</td>
<td>5.70</td>
<td>0.999</td>
<td>97.4</td>
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</tr>
<tr>
<td>Itraconazole</td>
<td>258</td>
<td>III</td>
<td>260</td>
<td>9.70 (0.08)</td>
<td>12.96</td>
<td>0.999</td>
<td>105.0</td>
<td>0.313</td>
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<tr>
<td>Econazole</td>
<td>272</td>
<td>III</td>
<td>260</td>
<td>12.97 (0.05)</td>
<td>5.01</td>
<td>0.999</td>
<td>99.5</td>
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<td>Miconazole</td>
<td>273</td>
<td>III</td>
<td>260</td>
<td>20.97 (0.05)</td>
<td>6.50</td>
<td>0.999</td>
<td>101.9</td>
<td>6.250</td>
</tr>
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</table>
HPLC assay for antifungals

Figure 1. Chromatograms showing the separation of (a) 5-flucytosine, (b) fluconazole (F) and amphotericin B (A), and (c) ketoconazole (K), itraconazole (I), econazole (E) and miconazole (M) in spiked serum. —, OD_{260}; ---, OD_{465}.

...patient receiving fluconazole therapy concurrently with other anti-infective drugs, ganciclovir, fosfarnet and ceftazidime (Figure 2). The peak of fluconazole was found to be baseline-resolved from other agents administered, with complete separation from the solvent peak and the endogenous materials.

Discussion

Increasing numbers of reports have pointed out the rising prevalence and importance of serious fungal infections especially in patients who are immunocompromised by their underlying disease or as a consequence of therapy (Denning, 1991). In addition to amphotericin B, newer triazole antifungal agents such as fluconazole and itraconazole have come to be used with more frequency (Hay, 1991). Clinical laboratories may be required to assay the level of various antifungal drugs from selected patients where there is concern about renal function impairment and other drug toxicities, or there is inappropriate clinical response to therapy caused by poor absorption or inadequate bioavailability at the infection site (Working Party, 1991). Although, based on the current knowledge of the pharmacodynamics and kinetics of antifungal agents, assays are not generally indicated...
in patient management, research on new drugs will prompt a need for drug assay, for example as in patients receiving lipid-complexed formulations of amphotericin B (Proffitt et al., 1991). Additionally, a minimal plasma concentration of itraconazole (250 μg/L) has been shown to be required for effective prophylaxis against fatal Aspergillus infection in prolonged neutropenia (Boogaerts et al., 1989) indicating the need for assay of its plasma level in special clinical situations. Lack of a standard method for antifungal susceptibility testing and failure of correlation between in-vitro
sensitivities and the outcome of antifungal treatment have been reported by previous workers (Drutz, 1987; Galgiani, 1987). Ongoing efforts to develop a clinically applicable antifungal susceptibility test procedure must take account of the pharmacokinetics and bioavailability of various antifungals which in turn requires a reliable system for their assay.

HPLC assay of antifungal agents in human plasma has been shown to compare favourably with biological methods (St.-Germain, Lapierre & Tessier, 1989; Rex et al., 1991) and its application in monitoring drug levels either for individual therapy (Petersen et al., 1994) or for determining treatment regimen in immunocompromised patients (Persat et al., 1992) have been documented. However, these methods involved variations in sample preparation and analytical procedures which were applicable to individual agents only. To overcome these limitations, an assay system which is simple, reliable and can be applied to multiple antifungal agents would be desirable. The isocratic HPLC assay protocol described in this study meets these requirements in addition to being reasonably sensitive and reproducible (Table). The protocol allowed the analysis of most of the antifungal agents commonly used for treating systemic fungal infections. It detected the agents at concentrations much lower than those achievable following administration of standard therapeutic doses. Miconazole had the highest detection limit of 6.25 mg/L which is of little relevance to the overall sensitivity of our method since this is a topical agent which will not require serum assay. The higher coefficient of variation of 12.96% for itraconazole obtained by our technique is comparable to the finding of Warnock, Turner & Burke (1988). Trimethoprim showed the same retention time as fluconazole while aztreonam and 5-flucytosine are eluted at the same time. This interference makes assay of these antifungal agents unreliable in the presence of trimethoprim or aztreonam.

Our procedure involved only one step of sample preparation for all the antifungal agents assayed, and the mobile phases were of similar components which could easily be changed from one to another by addition of reagent components. A gradient mixer for setting up stepwise gradient of the three mobile phases, together with a dual-array UV-monitor, will add further to the simplicity and flexibility of the present system. The ability of the method described to assay more than one agent by a single-step sample preparation offers opportunity for antifungal assays in patients who may be on combination therapy for severe fungal infections whenever the need arises. Evidently there is urgent need for more work on standardization of all aspects of antifungal therapy including in-vitro sensitivity testing, assessment of therapeutic response and prevention of toxicity in patients at increased risk of morbidity and mortality from fungal infections. In this regard, a versatile, sensitive and specific assay method as described in this study is ideal for both routine clinical service and research purposes.

References


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