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Clinically Achievable Plasma Deferoxamine Concentrations Are Therapeutic in a Rat Model of *Pneumocystis carinii* Pneumonia

SALIM MERALI,[1] KEVIN CHIN,[1] LAURA DEL ANGEL,[1] ROBERT W. GRADY,[2] MICHAEL ARMSTRONG,[3] AND ALLEN B. CLARKSON, JR.[1]*

Department of Medical and Molecular Parasitology, New York University School of Medicine, New York, New York 10016[1]; Department of Pediatrics, Cornell University Medical College, New York, New York 10021[2]; and 3813 N. Forrest Lane, Phoenix, Arizona 85323[3]

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The iron-chelating drug deferoxamine (DFO) has been shown to be active in animal models of *Pneumocystis carinii* pneumonia (PCP), with effective daily intraperitoneal bolus dosages being 400 and 1,000 mg of DFO mesylate kg of body weight^{-1} in mouse and rat models, respectively. Continuous infusion produced a moderately improved response in a rat model. The data reported here demonstrate that the response achieved by continuous infusion of 195 and 335 mg of DFO mesylate kg^{-1} day^{-1} in the rat model is associated with mean concentrations in plasma of 1.3 and 2.5 μg of DFO ml^{-1} and mean concentrations in lung tissue of 4.9 and 6.0 μg of DFO g of lung tissue^{-1}, respectively. Since current clinical use of DFO mesylate for the treatment of iron overload produces higher concentrations in the plasma of patients, DFO may prove to be a useful anti-PCP treatment. The 2.4- to 3.8-fold higher DFO concentration observed in lung tissue compared with that observed in plasma may be important in the response of PCP to DFO.

Deferoxamine (DFO), which is administered as the mesylate salt, was introduced more than 30 years ago to treat primary and secondary hemochromatoses and acute iron poisoning; in particular, it is used to treat the iron overload caused by the frequent blood transfusions required to treat β-thalassemia (15). This trihydroxamic acid, hexadentate iron chelator has also been used as an investigational drug in the treatment of malaria (7, 8, 16), rheumatoid diseases (6, 17), and ischemic reperfusion injury following ischemia (1), as well as an adjuvant in leukemia chemotherapy (2).

Recently, DFO has been shown to be effective against *Pneumocystis carinii* in culture (21) and in animal models (4, 20) of *P. carinii* pneumonia (PCP), one of the most important opportunistic infections associated with AIDS and other immunosuppressed conditions (10). While the standard drugs used to treat PCP, pentamidine and the combination of trimethoprim and sulfamethoxazole, are effective, they are associated with considerable toxicity, especially in patients with AIDS (5). As for any drug in widespread use, adverse side effects have been observed, but DFO is generally well tolerated and is used for life-long treatment of patients with β-thalassemia. Therefore, DFO could be useful in treating PCP if effective concentrations could be achieved safely. However, the daily intraperitoneal dosages found to be effective in animal models ranged from 400 mg kg of body weight^{-1} in a mouse model (20) to 1,000 mg kg^{-1} in a rat model (4), far in excess of those used in the clinical applications referred to above. A moderately improved response was achieved in the rat model when DFO mesylate was administered by continuous infusion at a dosage of 335 or 195 mg kg^{-1} day^{-1} (13).

Because drug pharmacokinetics can differ widely among species, it is important to know the plasma DFO concentrations associated with a response to PCP in animal models to determine if these are within the range that can be safely achieved in human patients. To address this issue, we measured the DFO concentrations in the plasma and lungs of *P. carinii*-infected rats treated for 22 days. We studied rats continuously infused with DFO because this necessarily produces a dynamic equilibrium between drug administration and drug metabolism and elimination. Thus, single measurements at the end of treatment could be expected to reflect the drug concentrations maintained for most of the treatment period. The concentrations in plasma were measured for comparison with published data for humans. The concentrations in lung tissue were measured because this is the primary site of *P. carinii* infection. For the work described here we developed and validated a modified method for detecting DFO in plasma and tissue samples.

MATERIALS AND METHODS

Materials. The DFO reference standard was USP deferoxamine mesylate Reference Standard lot H (U.S. Pharmacopeia Inc., Rockville, Md.). Adrenochrome semicarbazone sulfonate (ASS) was obtained from Sigma, St. Louis, Mo. Waters (Milford, Mass.) C_8 Novapak columns (3.9 by 150 mm) with a 4-μm packing size were used. High-pressure liquid chromatography (HPLC)-grade methanol (Fisher Scientific, Springfield, N.J.) and water produced by a Milli-Q water purification system (Millipore, Marlborough, Mass.) were used for the mobile phases. All other chemicals were of reagent grade or better.

Animal model and DFO administration. The plasma and tissue samples examined in the present study were from animals used in a previous study involving the response of PCP to the continuous infusion of DFO (13). Details of the animal model and drug delivery system are presented there.

Collection of samples. At the end of the treatment period, the rats were sacrificed and tissue samples were collected. Blood was collected in Vacutainer tubes containing EDTA (Becton-Dickinson, Franklin Lakes, N.J.), and the plasma was separated by centrifugation at 2,500 × *g* for 15 min. Lungs were removed, weighed, and homogenized in a motorized Potter-Elvehjem homogenizer with 10 ml of 25.6 mM disodium phosphate, 3.72 mM monosodium phosphate, 0.74 mM monopotassium phosphate, 31.0 mM sodium chloride, 1.34 mM potassium chloride, 0.025 mM $CaCl_2$, 0.025 mM $MgCl_2$, and 50 mM dithiothreitol (pH 7.4).

Preparation of samples for HPLC analysis. The methods for conversion of DFO to its iron complex, feroxamine (FO), and subsequent partial purification by solid-phase extraction were extensively modified from those described by Kruck and Teichert-Kuliszewska (11) and Singh et al. (18). At the time of collection, $FeCl_3$ (0.25 M) was added to 0.5-ml aliquots of plasma and lung homogenate to a final concentration of 5 mM. The samples were stored for 2 days at −85°C and were then transferred to liquid N_2 until they were thawed for analysis. Stability after conversion to FO was demonstrated by adding known

* Corresponding author. Mailing address: Department of Medical and Molecular Parasitology, New York University School of Medicine, 550 First Ave., New York NY 10016.

amounts of DFO to plasma and lung homogenate, converting to FO, freezing, and storing for 45 days. The means ± standard deviations of recoveries were 90% ± 4.28% and 101% ± 2.64%, respectively, in samples containing 1 and 20 μg of DFO ml^{-1}. The addition of $FeCl_3$ was essential for the prevention of degradation during storage, even at ultralow temperatures. At the time of analysis, 25 μl of a novel internal standard (ASS; 60 μg ml^{-1}) was added to each 0.5-ml aliquot of thawed plasma or lung homogenate (final concentration, 3.00 μg ml^{-1}). Proteins were precipitated by adding 0.5 ml of chloroform, centrifuging at 2,000 × *g* for 5 min, and collecting the aqueous upper layer. A 0.40-ml volume of the aqueous extract was applied to an octadecane silanol solid-phase extraction cartridge (Sep-Pak Plus; Waters). The Sep-Pak Plus columns had been preconditioned by passing 3 ml of methanol-acetic acid (80/20; vol/vol), 3 ml of methanol, and 9 ml of Milli-Q water through the cartridges. After the addition of the extract, the cartridges were rinsed with 5 ml of water and 0.5 ml of methanol prior to the elution of FO and ASS with 1.5 ml of methanol-acetic acid (80/20; vol/vol). The eluant was evaporated to dryness in a centrifugal vacuum concentrator and was then redissolved in 200 μl of PB (50 mM NaH_2PO_4 [pH 8.0]) with the help of ultrasonication. After removing nonredissolved material by centrifugation at 2,000 × *g* for 5 min, the samples were analyzed by HPLC. Multiple analyses of the same extract over 48 h showed that there was no loss of detectable FO over such an analytical period.

HPLC method. The HPLC system used was manufactured by Waters and included a model 625 pump, a model 600E system controller, a model 715 autosampler, and a model 996 photodiode array detector; the system was controlled by and the data were analyzed with Waters Millennium software. The mobile phase consisted of PB, methanol, and water. The injection volume for biological samples was 100 μl, and the injection volume for quality control samples was 160 μl. The A_{300} to A_{500} was recorded at 1.2-nm intervals, with the chromatograms being processed at the peak of FO absorbance, $A_{427.5}$. The absorbance data at wavelengths other than 427.5 nm were used for determining peak purity by using the algorithms included in the Millennium software and for three-dimensional displays which aided method development and subsequent troubleshooting. The peaks were eluted from the column at 1.2 ml min^{-1} by an 18-min linear gradient from 100% PB to 50% PB–25% methanol–25% water. The column was purged by a 4-min linear gradient ending at 40% methanol–60% water, with the flow decreased to 1.0 ml min^{-1} to avoid excessive backpressure at the point of 40% methanol. Starting conditions were regenerated by using a 3-min linear gradient ending at 100% PB, and the flow rate was restored to 1.2 ml min^{-1}.

Preparation of calibration curves. Calibration curves were prepared by adding known amounts of DFO, ranging from 0.33 to 84.84 μg ml^{-1}, to the appropriate biological matrix and assaying as described above. The internal standard was always present at a final concentration of 3.00 μg ml^{-1}.

RESULTS

The suitability of the analytical system was ensured by demonstrating that FO and ASS were resolved from all other peaks found in the extracts from plasma and lung tissue; resolution of FO and ASS remained within the range of 1.60 to 3.29. For quality control, six independently extracted aliquots of normal rat serum to which 10.61 μg of DFO mesylate standard ml^{-1} had been added were included in each set of assays. The relative standard deviation (standard deviation expressed as a percentage of the mean) of the control samples ranged from 10.74 to 16.26%. Instrument precision was monitored by making duplicate injections into the HPLC system from a single pooled standard after every six experimental sample analyses. Here the relative standard deviations ranged from 3.0 to 8.6%. To demonstrate linearity, a standard curve was generated by adding various amounts of DFO standard to plasma (Fig. 1) and lung homogenate (data not shown). The response was linear from 0.33 to 84.84 μg of DFO ml^{-1}, with *r* values of greater than 0.99 for both types of biological samples. Recovery rates were calculated by comparing the results obtained after DFO standards were added to six aliquots of plasma and three aliquots of lung homogenate with those obtained by direct analysis of the standards themselves. The means ± standard deviations for recoveries of FO from plasma were 98.7% ± 8.7%, 96.7% ± 18.2%, and 76.8% ± 25.5% when the concentrations of DFO standard were 84.84, 10.61, and 0.33 μg ml^{-1}, respectively. The corresponding recoveries from lung homogenates were 106% ± 16%, 88.2% ± 3.0%, and 95.8% ± 4.8%. The mean recovery of ASS added to the same standards was 103.6% ± 20.4%. Representative chromatograms of plasma and lung samples are presented in Fig. 2A and B, respectively. FO and ASS in both plasma and lung extracts routinely eluted at approximately 12 and 14 min, respectively.

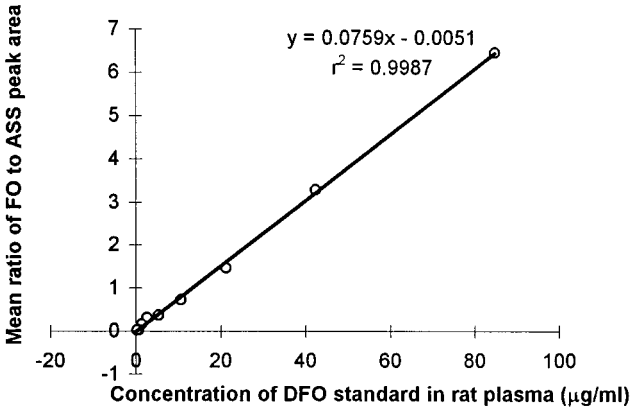

FIG. 1. Linear regression of peak area ratios versus DFO extracted from the plasma of rats.

Two groups of *P. carinii*-infected rats were sacrificed after 22 days of continuous DFO infusion, and samples were collected. Each group initially contained 20 rats. One animal from the group to receive a mean dosage of 335 mg of DFO mesylate kg^{-1} day^{-1} died during surgical implantation of the infusion pump, and another animal died early during the treatment period. Data for these animals were excluded, so that *n* was equal to 18 for the animals which survived the full treatment protocol. At the time that the plasma and lung samples were collected and processed, the optimal processing and storage method had not been determined. The samples were divided into four aliquots, with each aliquot being processed and stored differently. The procedures described in Materials and Methods were subsequently validated and used to collect the data reported in Table 1. Plasma and lung samples were analyzed in duplicate. This approach allowed accurate analyses but resulted in plasma volumes too small for analysis for two rats in each dosage group. Sample volume restriction or machine error resulted in the lack of 14 individual analyses of plasma or lung samples of a potential total of 156 analyses. The *n* values given in Table 1 reflect cases in which there were no data for an animal. Since the lowest concentration for which the assay was validated is 0.33 μg ml^{-1}, this value was used for any sample measuring at that concentration or lower. Errors caused by this assumption would be small and would result in a higher than actual mean, a conservative interpretation.

DISCUSSION

The data from two independent laboratories suggest that while DFO is effective in the treatment of PCP in animal models of the disease, the doses required are higher than those tolerated by humans (4, 21). Patients with β-thalassemia and with established myocardiopathy because of iron deposition can be treated continuously with up to 100 to 125 mg of DFO kg^{-1} day^{-1}, although usual long-term prophylactic therapy is at lower dosages (from 20 to 60 mg kg^{-1} day^{-1}) (9). We examined the steady-state concentrations of DFO in the plasma and lungs of *P. carinii*-infected animals continuously infused with DFO to determine if the concentrations in plasma that are effective in the rat model also exceed those achievable in patients or are within this range.

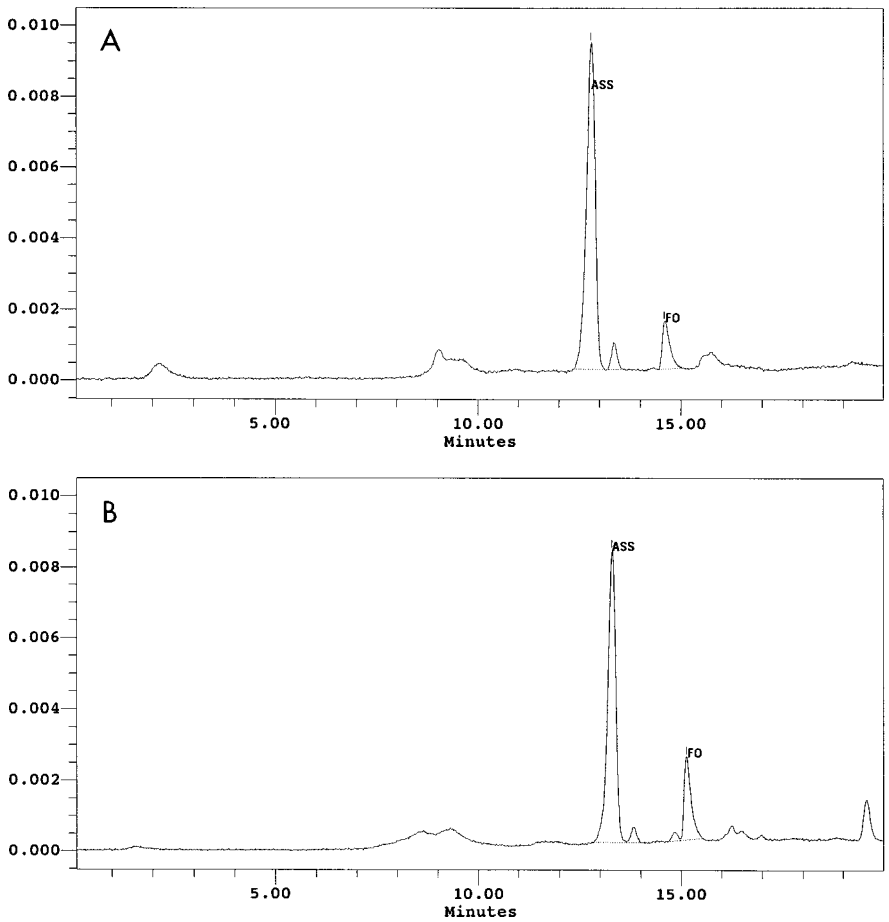

FIG. 2. HPLC profiles from plasma of *P. carinii*-infected rats treated with DFO by continuous infusion (A) and lung homogenate from *P. carinii*-infected rats treated with DFO by continuous infusion (B).

Following continuous intraperitoneal infusion of DFO mesylate for 22 days at mean doses of 335 and 195 mg kg^{-1}, we observed mean concentrations in plasma of 2.5 and 1.3 μg ml^{-1}, respectively. These concentrations are lower than the 3.5 μg ml^{-1} reported in the plasma of patients after 48 h of intravenous infusion at a dosage of 50 mg kg^{-1} day^{-1} (12). In another clinical study, infusion of 100 mg kg^{-1} over 24 h produced a steady-state concentration in plasma of about 10 μg ml^{-1} (19). Because these concentrations in human plasma are higher than those in the rat model, our data support the notion that DFO could be effective when administered to patients with PCP.

When compared with the values in the literature for plasma DFO levels in mice, the data in Table 1 also provide an explanation for the better dose response achieved in the mouse model of PCP compared with that achieved in the rat model. Continuous infusion of 170 mg kg^{-1} day^{-1} into mice for 28 days produces concentrations in plasma of about 10 μg of DFO ml^{-1} (3), or about eight times the concentration that we reported in rats administered the slightly higher dose of 195 mg kg^{-1}. Thus, to the extent that increased levels of DFO in plasma contribute to the effectiveness of DFO against *P. carinii*, the higher levels observed in mice for a given dosage suggest that it should be more efficacious in this species, as was found. This explanation for the relative difference in the responses of mice and rats supports the prediction that a therapeutic response can be achieved in patients administered tolerable doses of this drug.

Assuming that the volume of distribution in rats is similar to the 1.8-liters kg^{-1} reported in humans (12) and taking into account the drug infusion rate for the rats measured in the present study and the mean steady-state concentrations mea-

TABLE 1. DFO concentrations in plasma and lungs of *P. carinii*-infected rats after 22 days of continuous infusion of DFO mesylate

DFO dose (mg kg^{-1})[a]	Mean % suppression of total cysts relative to untreated controls[b]	Mean plasma DFO concn (μg ml^{-1})[a]	Mean lung DFO concn (μg g^{-1})[a]
335 ± 87 (205–507; n = 18)	87	2.5 ± 1.9 (0.3–7.2; n = 16)	6.0 ± 6.2 (1.6–27.2; n = 18)
195 ± 46 (142–318; n = 20)	80	1.3 ± 0.3 (0.3–2.0; n = 18)	4.9 ± 3.9 (0.3–13.4; n = 20)

[a] Values are means ± standard deviations (ranges; number of animals tested. The n values varied because data from some animals could not be obtained as described in the results.

[b] These values were taken from reference 13.

sured in the plasma of these rats, the half-lives of elimination of DFO in rats was calculated to be 0.22 and 0.20 h for daily infused doses of 335 and 195 mg kg^{-1}, respectively. Lacking a direct measurement of the volume of distribution, these values are estimates, but the half-life of elimination of DFO in rats was considerably shorter than the value reported in humans, 3.05 ± 1.30 h (12).

On the basis of a comparison of the micrograms of DFO per gram of lung with the micrograms of DFO per milliliter of plasma, the lungs of animals treated with 335 and 195 mg of DFO mesylate kg^{-1} contained approximately 2.4 and 3.8 times as much DFO, respectively, as the corresponding plasma samples (Table 1). Since the lung is the primary site of *P. carinii* infection, this is most important. While the mechanism for this apparent sequestration of DFO is not clear, it may reflect the higher rate of DFO metabolism in plasma compared with that in the lung (14). It remains to be seen if the concentration of DFO in the lungs of mice is increased as a result of the higher concentration in the plasma of this species or if the latter simply reflects the rate of metabolism or clearance from this tissue. Another possibility is that DFO is not evenly distributed in the total extracellular fluid, as indicated by its apparent volume of distribution (1.8 liter kg^{-1}) (12). No data on the DFO concentration in the lungs of patients treated with this iron chelator are available. A comparison of the DFO concentrations in various tissues may shed some light on this critical issue, although different relative rates of metabolism in different tissues could markedly change the results of such a study.

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