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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF DESFERRIOXAMINE

PHARMACOKINETIC AND METABOLIC STUDIES

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SUMMARY

A high-performance liquid chromatographic method has been developed that permits determination and quantitation of desferrioxamine and metabolites as their iron (III) complexes in small samples of mammalian plasma at levels encountered with ion-specific chelation treatments. The technique permits measurement of desferrioxamine and metabolite concentrations which can be used in pharmacokinetic studies. A human study is presented as an example.

INTRODUCTION

Desferrioxamine (Desferal®, Ciba Geigy; DFO) is the drug of choice for ion-specific removal of aluminium [1–3] and iron [4–10] from human subjects. The drug was tested and considered safe for human application [4]. However, serious side-effects were encountered upon long-term treatment [9,11] and with high-dose infusion (single dose 40 mg/kg) in patients undergoing hemodialysis [12] and in children with thalassemia [9]. Since no pharmacokinetic data were available, meaningful investigations of metabolites possibly involved in toxic side-effects could not be undertaken.

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In order to interpret observations of side-effects and the treatment protocols it is essential to know the concentrations of the parent compound (DFO), the metabolites of DFO and their metal complexes in the circulation. Idiosyncratic differences in the metabolism of drugs are a common problem [13].

DFO metabolism was first noted by Peters et al. [14] (by destruction of the chromophore) when examining DFO distribution in nephrectomized dogs. Later Meyer-Burnot and Kerberle [15] investigated in more detail the metabolic fate of DFO in several mammalian species and in man. DFO metabolism was defined as the time-dependent disappearance of the chromophore generated by Fe^{3+} complexation with the hydroxamic acid moieties of the DFO molecule. The spectrophotometric method permitted only detection of the total destruction of the chelation center. Enzyme-generated DFO fragments, or covalently modified DFO molecules retaining hydroxamic acid moieties, can form Fe^{3+} complexes with similar absorption characteristics, and thus are spectrophotometrically indistinguishable from the ferrioxamine (FO) complex.

Fig. 1 gives a schematic presentation of possible metabolic degradation steps for DFO. From this scheme it can be deduced that previous workers were measuring the activity of a hydrolytic enzyme that is capable of cleaving the hydroxamide bonds of DFO at intramolecular sites 1, 2 and 3. Enzymic cleavage at sites 4 and 5 could not be detected by the chromophore-based spectrophotometric method, since the hydroxamic acid chelation sites are still intact. Also, a mono-

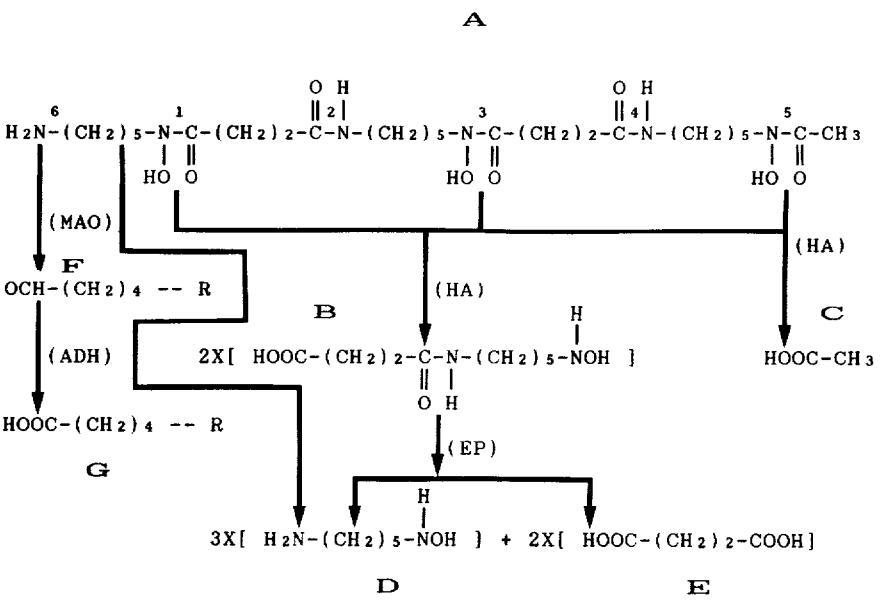

Fig. 1. Scheme of suggested enzyme-catalysed bioconversion of DFO (A) DFO; (B and C) hydrolysis products of hydroxamidase (HA) activity; (D and E) hydrolysis products of endopeptidase (EP) activity; (F) oxidation product of monoaminoxidase (MAO) and (G) aldehyde dehydrogenase (ADH) activity. End-products C, D and E are acetic acid, 1-amino-5-hydroxylaminopentane and succinic acid, respectively. The numerals 2× and 3× preceding the bracketed compounds are stoichiometric quantities based upon metabolism of one molecule of DFO.

amine oxidase (MAO)-catalysed bioconversion of the NH_2 terminus of the DFO molecule into a carboxyl group [16] would leave the chromophore intact, and thus the Fe^{3+} complex of this DFO metabolite (MFO) [1] would be included in the spectrophotometric determinations interfering with the quantification of DFO in metabolically active systems (in vivo and in vitro).

Given the increasing number of reports dealing with side-effects resulting from DFO therapy and the variation in inter-individual sensitivity to the drug, it is imperative that the methodology for determining the pharmacokinetic and metabolic products of DFO be established. Furthermore, animal models must be evaluated for pharmacokinetics and metabolism of DFO prior to embarking on toxicity studies. It cannot be assumed that both human and other mammalian species will show similar metabolic and pharmacokinetic properties. The difference in DFO metabolism between man and pig is exemplified by data given below.

Recently, we reported a high-performance liquid chromatographic (HPLC) method for measurement of DFO and its major metabolite as their Fe^{3+} complexes [1]. We now report modifications to the HPLC conditions resulting in improved resolution of chromatographic peaks and sensitivity under isocratic elution conditions.

EXPERIMENTAL

HPLC apparatus

A partially inert HPLC system was assembled using in tandem a variable-speed high-pressure pump (P3500, Pharmacia, Montreal, Canada), equipped with titanium pump heads, a general-purpose titanium HPLC valve (Pharmacia PV-7), an all-titanium column prefilter (2 μm), a stainless-steel column followed by a dual-channel Model 440 detector (Waters, Mississauga, Canada) fitted with a single-wavelength (435 nm) filter and a Waters extended-wavelength (229 nm) module. All high-pressure lines (pump to column) were 1.5 mm O.D. titanium tubing. All low-pressure lines were made of PTFE. The HPLC column body and the detector flow cells were made of stainless steel that can be a source of Fe^{3+} contamination under acidic flow conditions. Data acquisition and evaluation were acccomplished with a Waters 840 HPLC data reduction system consisting of a Waters "SYN" unit (A/D–D/A converter), a Digital professional 380 computer and appropriate software.

Column and mobile phase

The column of choice was a Waters 150 mm $\times$ 3.9 mm stainless-steel "Resolve" column packed with 5-μm spherical porous silica (part No. PN86010). The mobile phase was a mixture of 500 ml acetonitrile, 300 ml methanol, 100 ml *n*-butanol and 100 ml ion-exchanged water (all HPLC grade) with 1.5 ml of 5 *M* sodium perchlorate and 50 μl of perchloric acid added. The mixture was filtered (Millipore 0.45-μm filter) prior to usage. (Note: silica packaged by different manufacturers may have different ion-exchange properties and thus may require modification of the mobile phase composition.)

DFO and FO standards

Standard solutions of DFO and ferric chloride (Fisher, analytical grade) were prepared by dissolution of exact amounts in 10 ml deionized water to yield solutions of known concentration (0.05 *M*). Desferal was checked for purity by conversion to its Fe^{3+} complexes followed by HPLC analysis.

Calibration of HPLC system for quantitative determination of FO complexes

Test solutions of FO were prepared by mixing equal volumes of equimolar solutions of DFO and ferric chloride (0.05 *M* both) followed by appropriate dilution in deionized water. Solutions containing known amounts were injected into the 20-μl sample loop with a microsyringe equipped with a platinum needle. HPLC conditions were 1.00 ml/min continued for a total analysis time of 12 to 16 min.

Quantitation of DFO metabolite complexes

The major metabolite–iron complex (peak b, Fig. 2) showed similar spectral characteristics to FO, i.e. similar λ_{max} and ϵ_{max}. Therefore, all metabolite–iron complex concentrations were expressed in terms of FO, the latter being used as reference standard.

Calibration of HPLC system for quantitative FO analysis in plasma samples

Known amounts of 0.05 *M* aqueous FO standard solution were diluted into control serum to yield FO-containing sera of known composition ranging from 10^{-5} to $5 \cdot 10^{-2} M$. These serum calibration standards were processed according to the procedure described below.

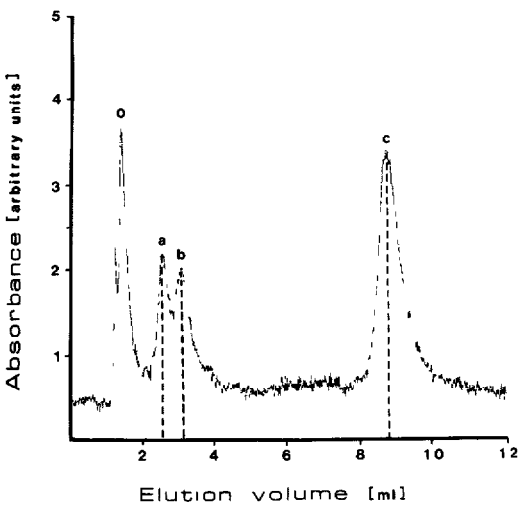

Fig. 2. Separation of a complex mixture of FO and metabolite-Fe^{3+} complexes. A human plasma sample of 20 μl was extracted as described in Experimental. The absorbance (arbitrary units) of the column eluate at 435 nm is plotted as a function of elution volume. The elution volumes of the peak maxima are 2.55, 3.08 and 7.82 ml for peak a (M_1FO), peak b (M_2FO) and peak c (FO), respectively. The height of peak a corresponds to 2.2 mV (i.e. 0.0022 absorbance units). Peak 0 represents unidentified material.

Patient's plasma and urine

DFO was administered via intramuscular injection (500 mg dissolved in sterile distilled water). Blood samples (5 ml) were obtained by venepuncture using disodium EDTA-coated test tubes (disposable/sterile) Vacutainer® EDTA; Becton Dickinson, Mississauga, Canada) and stored on ice until assay. Plasma was separated by centrifugation at 1000 *g* for 20 min at room temperature. The plasma was kept at 4 °C for up to one day. Following DFO injection a 24-h urine was collected.

Biological sample preparation for HPLC analysis

Method 1: reversed-phase adsorption method. Sep-Pak C_{18} columns (Waters) were conditioned by passing 5 ml of a solution consisting of methanol–acetic acid (80:20, v/v), followed by 5 ml methanol and 10 ml of ion-exchanged water. The plasma DFO and metabolite extraction was accomplished as shown in Table I.

Method 2: benzyl alcohol extraction. To 3 ml plasma, 50 µl of 0.05 *M* ferric chloride was added, followed by saturation with sodium chloride and vortexing for 30 s. Then 0.6 ml of benzyl alcohol was added and the sample was again vortexed for 2×30 s followed by centrifugation at 2000 *g* for 15 min yielding three layers: an aqueous layer with salt deposit, covered with large amounts of protein precipitate and a small amount of supernatant benzyl alcohol. The protein layer was broken up with a glass rod and the sample was again centrifuged at 2000 *g* for 15 min yielding sufficient benzyl alcohol extract for HPLC analysis.

Patient's urine was analyzed as described in ref. 1. Having added 100 µl of 0.05 *M* ferric chloride, urine samples (5 ml) were saturated with sodium chloride and extracted with benzyl alcohol (1 ml). The benzyl extract was used for HPLC

TABLE I

ISOLATION OF DFO AND METABOLITES FROM PLASMA

Step	Procedure
1	600-µl plasma sample in a 1.5-ml plastic tube (Eppendorf)
2	add 12 µl of 0.05 *M* ferric chloride in ion-exchanged water
3	add 600 µl chloroform
4	vortex-mix for 1 min
5	centrifuge for 10 min at 8000 *g*
6	take 500 µl (upper, aqueous layer), discard chloroform layer and apply onto conditioned Sep-Pak C_{18} columns
7	wash, 3×5 ml ion-exchanged water (by gravity)
8	wash, 400 µl methanol (by gravity)
9	elute with 1.5 ml of methanol–acetic acid (80:20, v/v) (by gravity) into 1.9-ml conical plastic centrifuge tubes (Eppendorf)
10	vacuum-dry in centrifugal vacuum dryer (Roto vap)
11	reconstitute pellet in 100 µl of a solution containing 80% (v/v) running solvent and 20% (v/v) acetic acid, by vortexing for 30 s followed by sonication for 30 s and by centrifugation for 2 min at 8000 *g*
12	use supernatant for HPLC analysis

analysis. Calibration curves were obtained by addition of known amounts of FO to control urine followed by benzyl alcohol extraction and HPLC analysis.

Pig plasma and urine samples

A three-month-old pig (22 kg) had femoral cannulae inserted bilaterally under anesthetic procedures. DFO (10.5 g in physiological saline) was infused at a rate of 240 mg/kg per h over a 2-h period through one cannula and blood samples were drained from the other into plastic test tubes. The blood samples were processed as described above (method 1).

Urine was obtained by continuous drainage into receiving containers from a urethra catheter and treated as for human subjects except that the urine was diluted 100-fold with distilled water prior to benzyl extraction (because of the presence of high concentrations of DFO and metabolites).

DFO pharmacokinetics in a human

DFO (500 mg) was administered intramuscularly to a patient taking part in a DFO treatment trial (approved by the ethics committee of the University of Toronto) who was suffering from senile dementia of the Alzheimer type but who was otherwise in good health. Blood was collected via venepuncture pre-injection and at intervals of 0.5, 1.0, 1.5, 2.0, 4.0 and 6.0 h, and processed as described by HPLC analysis.

RESULTS

Determination of elution times of the Fe^{3+} complexes of DFO and its metabolites from human plasma

The mobile phase composition reported previously [1] was modified to improve resolution under isocratic conditions. Plasma from a patient under DFO treatment supplemented with ferric chloride was subjected to HPLC analysis; the resulting 435-nm absorbance chromatogram is presented in Fig. 2. The peaks labeled a, b and c represent metabolite 1–iron, metabolite 2–iron and DFO–iron complexes with respective elution volumes of 2.55, 3.08 and 8.70 ml. The group of absorption peaks labeled 0 are (quantitatively) variable between samples and are generally present in control plasma without DFO treatment but with ferric chloride added. Peak c (Fig. 2) was previously identified as FO [1] and coincides with FO standards analyzed under identical conditions. Peaks a and b are the iron complexes of the products of in vivo metabolism of DFO and are designated metabolites M_1FO and M_2FO, respectively.

Inertness of HPLC system

The HPLC system was stripped of Fe^{3+} contamination by injecting a 0.05 *M* stock solution of DFO (20 μl) and completing the analysis cycle, followed by two baseline (deionized water injection) cycles. A test run with 20 μl of $10^{-4}M$ DFO showed no FO peak, thus the system was judged Fe^{3+}-free. This Fe^{3+} decontamination cycle was performed prior to analysis of samples (DFO and metabolites) that had no extra ferric chloride added.

FO calibration of HPLC system

The HPLC equipment and the peak integration software of the Waters 840 system were calibrated using freshly prepared FO (in deionized water). Individual sample solutions were prepared, containing 0.65, 3.25, 6.5, 16.25, 32.5, 65.0, 162.5, 325 and 650 μg/ml FO, and were analyzed. The data were subjected to linear least-squares regression analysis using the straight-line model $y=mx+b$. The resulting slope m was 1910 area units per μg/ml FO and the intercept was -2991 area units. The correlation coefficient and the standard error were 0.999 and 7.6, respectively.

FO calibration of plasma samples

Standard FO solution, 0.05 M in deionized water, was added to plasma to give final concentrations of 0.65, 3.25, 6.5, 32.5 and 65 μg/ml, and subjected to the solid-phase extraction method and analyzed by HPLC as described. The data were subjected to linear least-squares regression analysis using the straight-line model $y=mx+b$. The resulting slope was 1873 area units per μg/ml FO and the

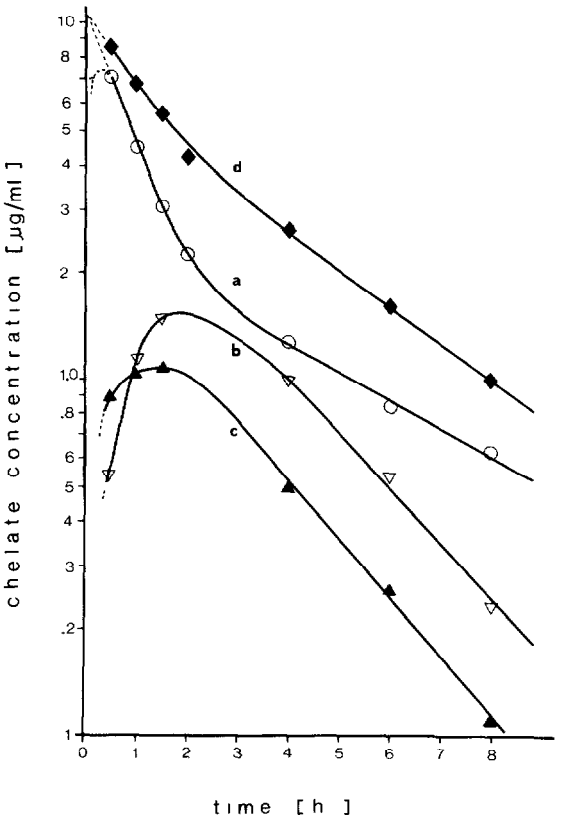

Fig. 3. DFO pharmacokinetics and metabolism of DFO in patient J.B. Curves a–c represent the amount of plasma DFO, the major and the minor metabolites complexed to Fe^{3+} as a function of time. Curve a, FO; curve b, major metabolite (M_1FO); curve c, minor metabolite (M_2FO) complexed with Fe^{3+}. Curve d represents the total amount of DFO-derived species in plasma complexed to Fe^{3+}.

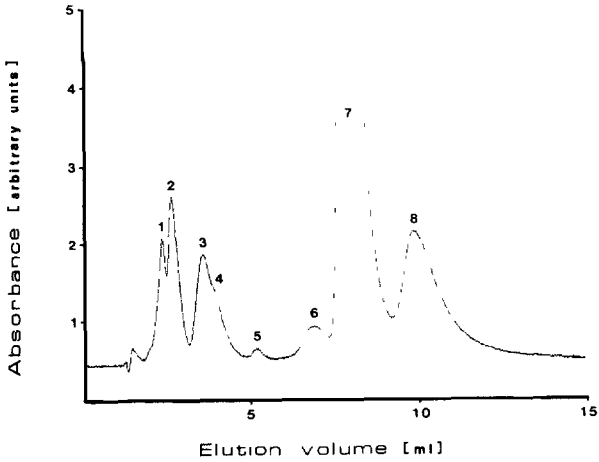

Fig. 4. Separation of a complex mixture of FO and metabolite-Fe^{3+} complexes. The 20-μl sample was extracted as described in Experimental from pig treated with DFO. The absorbance (arbitrary units) of the column eluate at 435 nm is plotted as a function of elution volume. Peak 7 is FO, the remaining peaks were not characterised.

intercept was −6472 area units. The correlation coefficient and the standard error were 0.999 and 23.5, respectively. Comparison of the latter slope with that obtained from the aqueous standard curve yields an extraction efficiency of $92 \pm 3\%$.

FO extraction efficiency into benzyl alcohol

Standard FO solution, 0.05 *M* in ion-exchanged water, was added to human urine saturated with sodium chloride, and extracted into benzyl alcohol and analyzed by the HPLC method described. The data were subjected to linear least-squares regression analysis using the straight-line model $y = mx + b$. The resulting slope was 1548 area units per μg/ml DFO and the intercept was 3311 area units. The correlation coefficient and the standard error were 0.998 and 35.9, respectively. Comparison of the slopes of the aqueous standard curve and the benzyl alcohol-extracted standard curve yielded an extraction efficiency of $84 \pm 3\%$.

DFO pharmacokinetics

Blood was drawn from a patient 15 min before injection and 0.5, 1.0, 1.5, 2.0, 4.0, 6.0 and 8.0 h after injection, and was analyzed for DFO and metabolites by HPLC (method 2). Fig. 3 shows the kinetics of removal of DFO and metabolites b and c from the circulation. All DFO and metabolites b and c were converted to their F^{3+} complexes and quantified by their absorbance at 435 nm. Curve a represents the amount of DFO plus FO in plasma. The graph is biphasic indicating an initial high rate of metabolism of DFO. The half-life is 4.5 h, 4 h after injection. During the initial 2-h period DFO decreases and DFO metabolites b and c increase (curve a). Both these metabolites chelate Fe^{3+} to form complexes which absorb at 435 nm similar to the FO complex. Metabolites b and c reach a maxi-

mum concentration at 2 h and then decrease with half-lives of 100 min. Curve d represents the sum of the Fe^{3+}-bound chelators (FO, metabolite b and c).

Pig blood metabolite analysis

During the 2-h DFO infusion period (10.4 g DFO in 10 ml physiological saline per 22-kg pig; 240 mg/kg per h), blood samples were drawn at 15-min intervals and subjected to HPLC analysis. In Fig. 4 the chromatogram of the 1 h 45 min post-injection Fe^{3+}-saturated sample is shown. This chromatogram shows both the diversity of metabolic products (DFO metabolism) and the resolving power of the analysis method described.

DISCUSSION

Since our initial publication [1] of a silica-based HPLC analysis capable of separating the parent drug and a metabolite, no other work describing DFO metabolite analysis and metabolism has appeared in the literature. We have increased the resolution of the silica HPLC method. The chromatograms in Figs. 2 and 4 show the capability of this method in achieving separation of several products arising from in vivo metabolism of DFO. Efforts have been made to reduce the blood sample size as much as possible. The improved method has sufficient sensitivity for DFO and metabolite detection and quantitation in samples where blood volume is limited. At doses of 7 mg/kg, plasma samples of 500 μl are sufficient to measure circulating levels over the range 0.3–65.0 μg/ml if a 5:1 concentration of plasma components is chosen in the extraction (method 1) prior to HPLC analysis. Alternatively, where larger volumes of plasma are available (as in adults), the benzyl alcohol extraction method (method 2) can be employed. This method is more economical and it involves fewer steps than method 1.

Pharmacokinetic data shown in Fig. 3 indicate that DFO is rapidly converted into two metabolites, both of which are capable of binding iron and/or aluminium. As the parent molecule DFO decreases, the metabolites increase and reach a maximum concentration at 2 h. As the amount of metal-free DFO decreases the rate of metabolite generation decreases and elimination of the metal–DFO complexes by the kidney becomes the controlling factor [16].

The initial half-life for DFO elimination (50 min) corresponds to that reported by Keberle [16] and Allain et al. [10]. However, the initial combined half-life for DFO, metabolites a and b is 100 min in this patient changing to 120 min for the period 4–8 h post-injection. Our data (Fig. 3) indicate further that FO is retained preferentially by the kidney (half-life 4.5 h), while metabolites a and b are eliminated with a half-life of 100 min.

It should be noted that methods for DFO analysis used by both Keberle [16] and Allain et al. [10] did neither permit separation of parent drug from the metabolites nor resolution of the latter.

Pharmacokinetic and metabolic studies of DFO on other patients (data not shown) indicate marked differences in metabolite formation (Fig. 1, A to G) and elimination kinetics. There are indications that metabolite pharmacokinetics can be related to the occurrence of DFO treatment side-effects. These studies will be published in detail elsewhere.

Metabolites a and b in Fig. 2 give rise to curves c and b in Fig. 3, respectively. We suggest metabolite b corresponds to the major metabolite isolated by Keberle [16] (compound G in Fig. 1), i.e. a product of oxidative deamination of DFO by sequential activity of MAO and aldehyde dehydrogenase activity. Compound a in Fig. 2 appears to be an aldehyde (suggested by Feulgen reaction) and would be the intermediate aldehyde F (MAO reaction product) in the bioconversion of DFO to compound G (Fig. 1). Similar spectral characteristics of the chromophores of the iron complexes a, b and c (Fig. 2) provide further support in the identification of metabolites a and b (Fig. 2) as N-terminal modified DFO reaction products.

In contrast to humans, the pig shows (Fig. 4) very diverse metabolic enzyme activity producing a total of eight metabolic products, all of which carry sufficient functional groups to form Fe^{3+} chelates. Several of these metabolites (2–5, Fig. 4) are also found in humans at low concentration in relation to the parent drug and metabolites a and b.

The chromatographic conditions described provide for the separation of metabolic products of DFO. Further, the silica C_{18} absorption method permits pharmacokinetic and metabolic studies on small sample volumes (0.5 ml) at DFO concentrations of 7 mg/kg.

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