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DETERMINATION OF DESFEROXAMINE AND A MAJOR METABOLITE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO THE TREATMENT OF ALUMINIUM-RELATED DISORDERS

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SUMMARY

A high-performance liquid chromatography method is described that permits separation and quantification of desferoxamine, a major metabolite, the iron(III) and the aluminum(III) chelates of desferoxamine. This method now facilitates pharmacokinetic studies on desferoxamine and derivatives designed to study side-effects and metabolite patterns in patients undergoing treatment.

INTRODUCTION

Aluminium has been implicated as a toxic factor in a number of human diseases. The dialysis encephalopathy syndrome, a progressive neurological disease, occurs in patients with impaired renal function undergoing chronic haemodialysis. This condition is associated with markedly elevated brain aluminium content [1—5]. An encephalopathy following chronic peritoneal dialysis has been reported [6] and increased serum aluminium levels occur during and after peritoneal dialysis [7]. Aluminium intoxication is implicated in dialysis osteomalacia in which aluminium accumulates in bone

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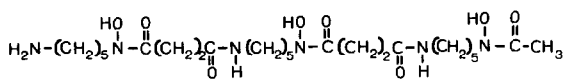
[3—5]. Increased levels of aluminium in bone, urine and plasma of patients on chronic total parenteral nutrition were reported [8]. Aluminium also appears to be a toxic factor in Alzheimer's disease, the most common cause of senile dementia. A significant increase of aluminium occurs in the cerebral cortex of brains affected by Alzheimer's disease [9—11]. Aluminium accumulation occurs in every neuron with Alzheimer's type neurofibrillary degeneration [12]. Yase [13] and Yoshimasu et al. [14] found elevated aluminium and calcium levels associated with amyotrophic lateral sclerosis in patients from the Kii penninsula of Japan and in Guam Parkinson dementia, amyotrophic lateral sclerosis syndromes. These latter neurodegenerative conditions are also associated with Alzheimer's type neurofibriliary degeneration.

One treatment strategy for patients suffering from diseases associated with elevated levels of aluminium is to remove aluminium with the chelating agent desferoxamine (DFO) (Desferal®, Ciba-Geigy). DFO is an effective trivalent metal-chelating agent capable of binding iron(III) (equilibrium constant, K 10^{31}) [15] and aluminium(III) (K ca. 10^{23}) [16] with high affinity and specificity. DFO has been widely employed in the treatment of conditions associated with iron overload [17, 18]. Ackrill et al. [19] reported successful removal of aluminium from patients with dialysis encephalopathy. More recently DFO was used to remove aluminium from patients with renal failure who were stabilized on standard haemodialysis [20]. Further DFO has been employed in an effort to remove aluminium from patients suffering from Alzheimer's disease [21]. Even though DFO has been established as a safe drug for short-term treatment [17—20], long-term exposure to the drug leads to undesirable side-effects in some elderly patients [21].

A



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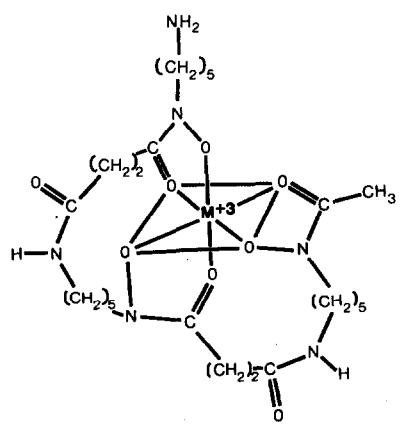

Fig. 1. (A) Molecular formula of desferoxamine, free base; (B) Suggested structure of metal^{3+} [iron(III) and aluminium(III)] chelate of desferoxamine.

Inter-individual differences in the metabolism of drugs are common occurrences [22]. In order to characterize the variability in patients' response to the drug treatment, it is essential to study the pharmacokinetics of DFO and its metabolites because long-term exposure to DFO may tend to cause deleterious side-effects.

Previously employed methods, based on the spectroscopic properties of the ferrioxamine (FO) chromophore (λ_{max} = 450 nm), are subject to interference by other iron(III)-chelating substances present in serum and urine in variable amounts. Further, the major metabolite of DFO having a chromophore similar to that of the parent compound cannot be distinguished spectroscopically, thus rendering these methods inappropriate for pharmacokinetic studies.

In this paper we describe a method that permits detailed studies on DFO and its derivatives. The molecular formula for DFO (free base) is given in Fig. 1A and a schematic representation of the metal (M^{+3}) complex is given in Fig. 1B.

EXPERIMENTAL

HPLC apparatus

An Altex variable-speed high-pressure pump fitted with a general-purpose manual valve, a 100-μl calibrated sample loop and micro-syringe injector was employed. Two chromatography columns were tested and used experimentally: (a) a commercial 20 cm × 4 mm HPLC stainless-steel column (Waters μPorasil) and (b) a 20 cm × 4 mm stainless-steel column packed with 10 μm particle size Dupont Zorbax silica gel. Sample detection was achieved at two wavelengths, 440 and 229 nm, using a Waters (Model 440) dual-fixed-wavelength detector equipped with separate flow-cells connected in tandem. The detector output was recorded on a Canlab dual-pen strip chart recorder (100 mV sensitivity).

Mobile phase

The mobile phase consisted of a mixture of 400 ml acetonitrile, 400 ml methanol, and 100 ml *n*-butanol, all HPLC grade, to which 100 ml deionized water were added and a buffering system was created by addition of 2.0 ml glacial acetic acid (BDH analytical grade) and 1.0 ml of 10 *M* sodium hydroxide (Fisher standard 10 *M* sodium hydroxide). This mixture was degassed under vacuum prior to use.

Desferoxamine, ferrioxamine and desferoxamine—aluminium complex

Standard solutons of DFO, ferric chloride (Fisher, analytical grade) and aluminium nitrate (BDH, analytical grade) were prepared by dissolving precisely weighed amounts in 10.0 ml deionized water to yield solutions of 0.0426 *M* desferoxamine mesylate, 0.0498 *M* ferric chloride, and 0.0501 *M* aluminium nitrate. Desferal was checked for purity by thin-layer chromatography (TLC) on 100-μm silica gel G-60 glass-backed plates developed in solvent of composition *n*-butanol—acetone—water—acetic acid (40:30:20:10). Detection of DFO was achieved by spraying developed TLC plates with freshly prepared 1% (w/v) solution of ferric chloride in water.

HPLC analysis of desferoxamine, ferrioxamine and desferoxamine–aluminium complex (aluminoxamine)

Solutions for HPLC analysis were prepared by dilution of appropriate amounts of stock solutions in the mobile phase. The amount of sample applied to the column was generally 100 μl.

Calibration of HPLC system for quantitative determination of ferrioxamine

Test solutions of FO were prepared by diluting DFO and ferric chloride stock solutions in mobile phase of pH 5.2. Known amounts of the calibration mixture were injected by micro-syringe into the sample loop and analysed. HPLC conditions were 135 bars at 2.0 ml/min flow-rate and chart speeds of either 10 or 20 cm/h were employed. The detection limit was arbitrarily taken to be three times the maximum noise level.

Patient sera and urine

Desferal was administered (intramuscularly 500 mg dissolved in distilled water) to a 63-year-old patient with normal renal function. Blood samples (5 ml) were withdrawn at intervals according to the schedule in Fig. 3. The blood was stored at 0°C and allowed to clot at room temperature immediately prior to analysis. Serum was separated from blood by centrifugation (1000 *g* for 10 min). Urine was collected and pooled for a 24-h period following injection of DFO.

Biological sample preparation for HPLC analysis

Method I. To 1.0 ml of serum in a 5.0-ml disposable glass test tube 1.0 ml of acetonitrile was added slowly with gentle rotation to achieve large flocculent precipitates. Then the tube was vortexed for 60 sec, twice, allowed to stand for at least 10 min and then centrifuged (3000 *g* for 5 min). The clear supernatant was suitable for direct injection into the HPLC system for determination of FO.

Method II. To 1.0 ml of serum in a 5-ml disposable glass test tube were added 300 mg sodium chloride (analytical grade) and the mixture was vortexed to achieve sodium chloride saturation. Then, 1.0 ml of benzyl alcohol was added and the mixture was vortexed for 60 sec, three times, followed by centrifugation (2000 *g* for 5 min) to achieve clear separation of layers. The benzyl alcohol layer contained the DFO and its metal ion complexes and was directly analysed by HPLC. The urine samples were treated similarly. Increased sensitivity could be obtained by increasing the sample to benzyl alcohol ratio without interference from increased extraction of coloured components absorbing in the visible region of the spectrum.

RESULTS

Determination of elution times of desferoxamine, ferrioxamine and aluminoxamine

Systematic variation of solvent composition in respect to dielectric constant, pH and ionic strength were employed to establish optimal elution volumes for separation of the three compounds, DFO, FO and aluminoxamine (AO). Then a mixture of these compounds was analysed by the HPLC system. The optimal conditions and results are given in Fig. 2. All compounds exhibited an

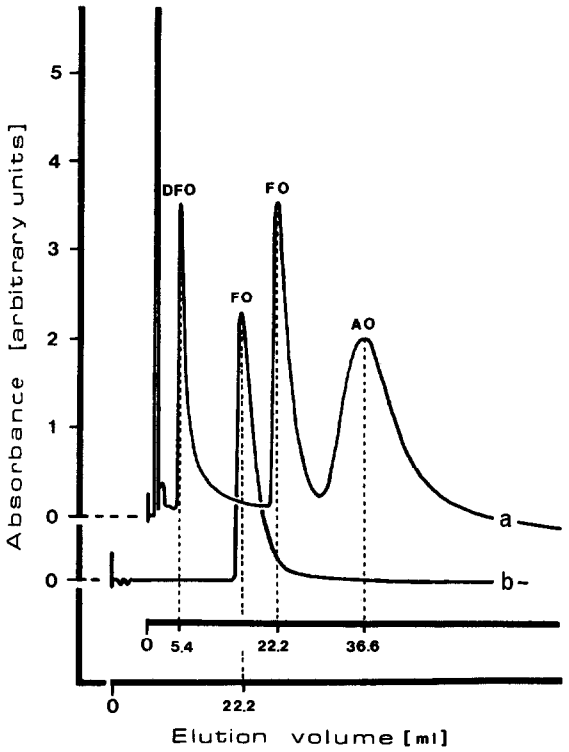

Fig. 2. Separation of a complex mixture of desferoxamine (DFO), ferrioxamine (FO) and aluminoxamine (AO) by HPLC. The input sample (20 μl) contained DFO, FO and AO in a molar ratio of 3:1:1 (150, 50 and 50 μM respectively). The absorbances of the column effluent are plotted as a function of eluting solvent volume (trace a, 229 nm, 0.2 a.u.f.s.; trace b, 440 nm, 0.05 a.u.f.s.). The elution maxima of DFO (5.4 ml), FO (22.2 ml) and AO (36.6 ml) are noted on the abscissa.

absorbance shoulder at 229 nm. FO showed an additional absorbance with a maximum at 435 nm. The UV tracing (Fig. 2) shows complete separation of DFO, FO and AO. Trace b, 440 nm, identified the FO complex. The elution volumes for DFO, FO and AO were 5.4 ml, 22.2 ml and 36.6 ml, respectively.

As concentrations of DFO in the analytical sample are decreased, increasing amounts of DFO are converted to FO presumably through a reaction with ferric ions liberated from the stainless-steel plumbing system of the HPLC apparatus by the acetic acid component of the solvent system. Generally at concentrations of less than 5 μg/ml all DFO was converted to FO.

Ferrioxamine calibration

The HPLC system was calibrated for standard normal serum by addition of varying amounts of FO. Plotting absorbance peak areas as a function of added amounts of FO resulted in a linear response ($r = 0.99$) and was reproducible for constant solvent composition (precision was S.D./x = 5.5% at the 10 μg/ml FO solvent level). No significant changes were observed on a day-to-day basis. The sensitivity of the test methods was defined in practical terms as the amount of FO giving rise to an absorbance peak of three times the average

maximum baseline noise. The detection limit taken as three times noise is 1.5 μg/ml for method I and 0.25 μg/ml for method II.

Pharmacokinetics of desferoxamine in a patient with Alzheimer's disease

Blood samples (5 ml) were analyzed according to methods I and II for disappearance of FO and DFO from blood following a single intramuscular injection of 500 mg DFO. Fig. 3 shows the amounts of FO determined in serum as a function of time in form of a semi-logarithmic plot. The half-life was 90 min. Extrapolation to zero time gave a value of 15 μg of FO per ml of serum, indicating a distribution volume of 33 l.

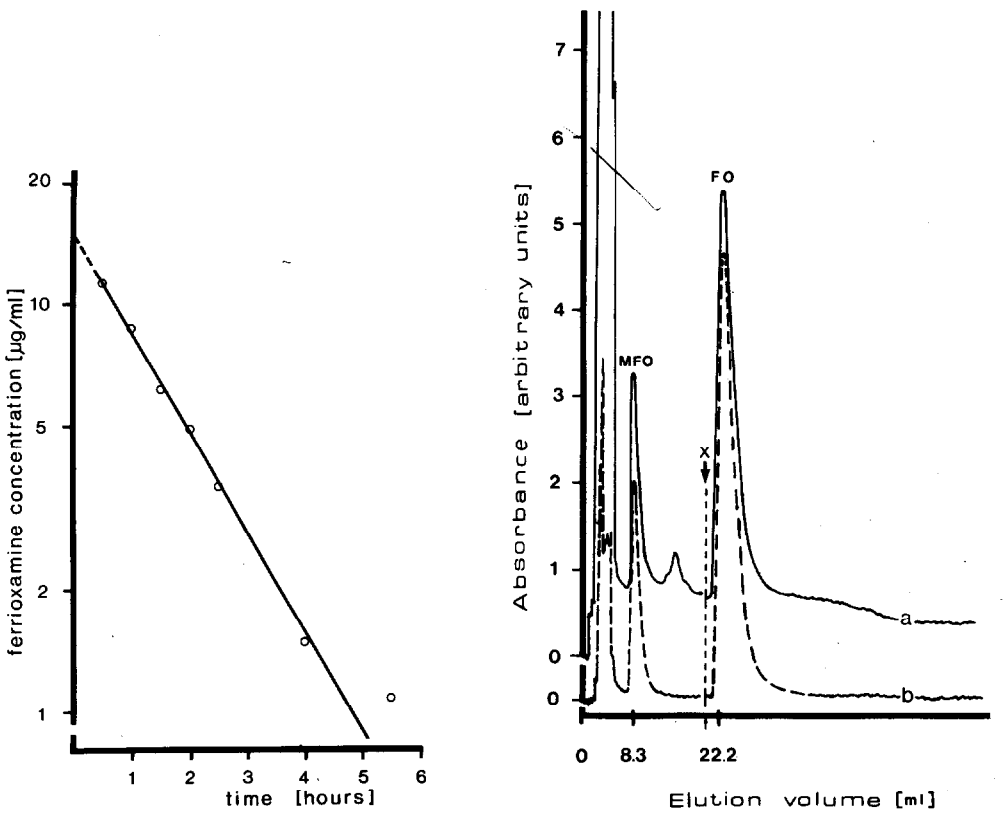

Fig. 3. Ferrioxamine elimination from the blood of an Alzheimer's disease patient (E.T.). Ferrioxamine concentrations in semilogarithmic form were plotted versus time lapsed after injection. The fitted line graph extrapolates to a desferal concentration of 15 μg/ml, indicating a volume of distribution of 33.3 l and it shows a drug half-life of 90 min.

Fig. 4. Determination of ferrioxamine and an iron(III)-binding metabolite in urine from patient with Alzheimer's disease. A urine sample was extracted with benzyl alcohol and the alcohol fraction was analyzed by HPLC. The position, marked X, on the abscissa designates where the sensitivity of the instrument was changed from 0.2 and 0.05 a.u.f.s. to 0.02 and 0.005 a.u.f.s. for 225- and 400-nm detectors, respectively. Traces a and b are the absorbance tracings at 229 and 440 nm, respectively. The peak appearing at 22.2 ml elution volume was identical to ferrioxamine (FO) and MFO (8.3 ml elution volume) was identified as an iron-binding metabolite.

Urinary excretion of desferoxamine

Initial experiments on urine employing method I showed that the UV absorbance of DFO eluting at 5.4 ml coincided with unidentified compounds absorbing also in the UV region. However, the FO peaks at 229 and 440 nm were well separated and free from interfering peaks. Method II yielded good resolution of the UV peaks. An early eluting iron(III)-binding compound, MFO, probably a DFO metabolite, was readily recognized (Fig. 4, trace a), since both compounds MFO and FO (Fig. 4) could be identified as iron(III) complexes through their absorbance at 229 and 440 nm.

DISCUSSION

Analytical methods employing spectroscopy failed to achieve DFO determination in serum of patients in the 0.5—50 μg/ml range necessary for monitoring patients undergoing long-term low-level DFO treatment. The HPLC method described here will separate both the iron(III) and the aluminium(III) complexes (Fig. 2) from other serum or urine components (Fig. 4). The method is limited in sensitivity primarily by the performance of the detection equipment. We achieved a sensitivity of 1.5 μg/ml FO using method I and 0.25 μg/ml using method II. The analytical response is linear and no zero bias is observed. Using this method to evaluate pharmacokinetic parameters of desferal in a patient with Alzheimer's disease (E.T.) we found in blood a half-life of FO of 90 min (Fig. 3), which is comparable to those reported by Keberle [15] and Wohler [17, 18]. DFO and FO are largely excreted by the kidney but lesser amounts are excreted in bile. Employing [^{14}C]DFO, Keberle [15] reported the production of three metabolites of DFO. The major component was shown to be an oxidized (COOH terminus) deamination product of DFO. Urine analysis by method II in this study revealed the presence of FO (Fig. 4) and another iron(III)-binding compound, MFO (Fig. 4) possibly the major metabolite reported by Keberle [15]. This tentative identification is suggested by the observation that both FO and MFO have 440-nm absorbance in addition to the 299-nm UV absorbance and the ratio of A_{229}/A_{440} is about equal (4—5) for both compounds.

The method described above provides a tool to study the pharmacokinetics of DFO and MFO. Further, the capability of this method to determine the aluminoxamine complex will provide a means of monitoring removal of aluminium from patients undergoing haemodialysis.

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