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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF DEFEROXAMINE AND FERRIOXAMINE: INTERFERENCE BY IRON PRESENT IN THE CHROMATOGRAPHIC SYSTEM

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SUMMARY

The assay of the complexed (ferrioxamine) and uncomplexed forms of deferoxamine, a strong iron(III) chelating agent, by reversed-phase high-performance liquid chromatography was investigated. Complex formation with trace amounts of iron in the chromatographic system hinders the assay of deferoxamine by causing the appearance of unexpected peaks, reaction zones and variations in retention time. By purging the column with deferoxamine and using EDTA in the mobile phase, these adverse effects in the determination of deferoxamine were eliminated. No interference by iron contamination of the chromatographic system was found in the assay of ferrioxamine, which is facilitated by the strong absorption of the analyte at 430 nm.

INTRODUCTION

Deferoxamine (DFA) is a naturally occurring trihydroxamic acid that reacts stoichiometrically with Fe(III) to form the octahedral iron complex ferrioxamine (FA), depicted in Fig. 1[1]. In the FA molecule the iron is surrounded by a shell of the organic moiety, which imparts great stability to the iron complex[2]. The kinetics of DFA iron complexation have been examined by Lentz *et al.*[3].

DFA is a widely used drug for the removal of iron following iron intoxication or for the reduction of excess iron in idiopathic hemochromatosis or in transfusional hemosiderosis[4]. Monitoring of the treatment requires the determination of DFA-bound iron in physiological fluids.

Standard analytical methods rely on pre-treatment of FA to release free iron, which is subsequently determined by a colorometric reaction[5–7]. Automated systems have also been developed for this assay[8,9]. Methods for the determination of FA and DFA in urine have been developed that make use of the specific adsorption of FA at 430 nm[10,11]. However, no method is currently available for the simultaneous assay of DFA and FA. Chromatographic techniques are likely candidates, yet we have found no such methods in the literature.

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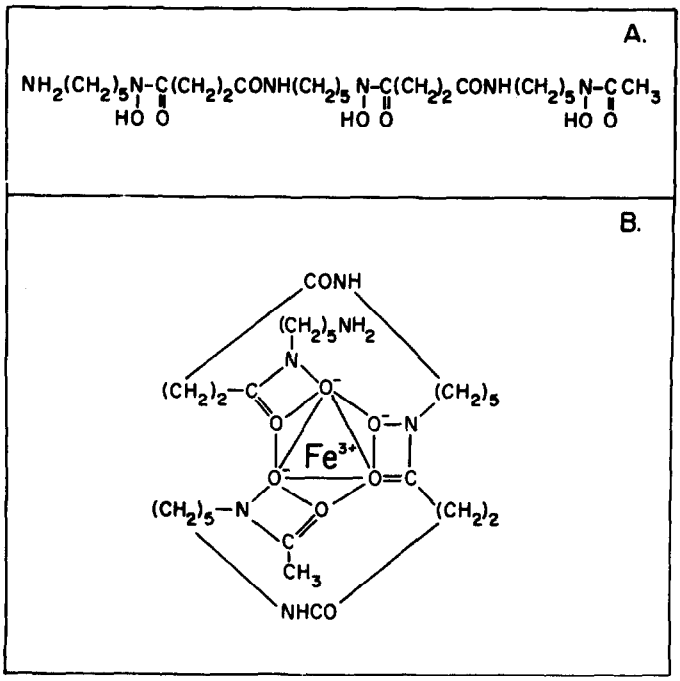


Fig. 1. Structures of (A) FA and (B) DFA.

This paper describes the use of reversed-phase high-performance liquid chromatography (HPLC) for the concommitant analysis of DFA and FA, and sheds light on the irregular chromatographic behavior of DFA observed in initial studies and shown in Fig. 2A.

EXPERIMENTAL

Materials

Deferoxamine mesylate (Desferal) was obtained from Ciba (Summit, NJ, U.S.A.), iron(III) chloride from Fisher (Fair Lawn, NJ, U.S.A.), EDTA disodium salt from Malinckrodt (Paris, KT, U.S.A.) and *o*-phthaldialdehyde from Pierce (Rockford, IL, U.S.A.). All chemicals and solvents were of analytical-reagent grade.

Equipment

A liquid chromatograph was assembled from a Model 250 pump (Kratos, Westwood, NJ, U.S.A.), a Model 7010 injection valve (Rheodyne, Berkely, CA, U.S.A.), a Model 770 wavelength detector (Kratos) and a Model C10 integrator (LDC-Milton Roy, Riviera Beach, FL, U.S.A.). A dual pump post-column reactor (Kratos) and a Model 770 fluorescence detector (Kratos) were also employed in certain experiments.

The following columns were used. Column A, octadecyl-Spherisorb having a carbon load of 14% (w%w) was prepared from 5 μm Spherisorb silica gel by the procedure of Kováts and Boksányi[12] and packed into a 150 × 4.6 mm I.D. column.

Column B was a 250 × 4.6 mm column packed with 5-μm octylsilica particles, obtained from IBM (Danbury, CT, U.S.A.).

Determination of iron in the chromatographic system

The chromatographic system was purged of Fe(III) by recirculating 100 ml of 20 m*M* DFA solution at a flow-rate of 0.3 ml/min for 12 h. The concentration of FA in the resulting solution was measured spectrophotometrically at 430 nm. In each experiment less than 1% of the total amount of DFA was converted into FA.

Reversed-phase chromatography of DFA and FA

DFA and FA were chromatographed using either of the above columns. The mobile phase was 12.5% (v/v) acetonitrile in 0.02 *M* phosphate buffer (pH 3.0) at a flow-rate of 0.8 ml/min and a column temperature of 22°C. The sample volume was 20 μl. The absorbance of the effluent was usually monitored at 220 nm because both DFA and FA show strong absorbance at this wavelength. Unlike DFA, FA has an absorption maximum at 430 nm[10]. Hence the two species could be readily distinguished by monitoring the effluent at 220 and 430 nm. In the course of the experiments samples containing 10 mg/ml of DFA were injected periodically to remove traces of iron(III) believed to be bound to the stationary phase.

In certain instances the post-column reactor was used and the combined flow-rate of the column effluent and the reagent was adjusted so as to maintain the pH at 9.0. The reagent solution contained 0.8 mg/ml of *o*-phthaldialdehyde as the fluorogenic agent and 0.2 μl/ml of mercaptoethanol in 0.2 *M* boric acid, the pH being adjusted to 10.4 with NaOH. The excitation and emission wavelengths of the fluorescence detector were 230 and 340 nm, respectively.

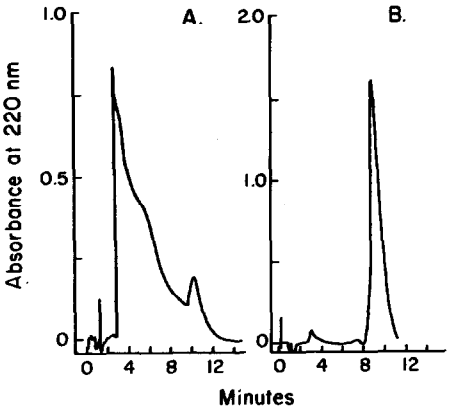
Use of EDTA in the mobile phase

Samples containing both DFA and FA were assayed quantitatively using column A with a mobile phase containing 12.5% acetonitrile and 2 m*M* EDTA in 0.02 *M* phosphate buffer (pH 6.5) under otherwise identical chromatographic conditions.

RESULTS AND DISCUSSION

Initial studies on the chromatography of DFA using column A resulted in either one early eluting peak for the injection of small samples of DFA (<2 μg), multiple peaks and a reaction zone for intermediate size samples of DFA (2–20 μg) as shown in Fig. 2A, or one later eluting peak for large samples of DFA (>100 μg) at the appropiate setting of the detector sensitivity. Such chromatographic behavior had been observed previously as a result of the interplay between chemical reactions in the column and the chromatographic retention process[13–15]. Owing to the stainless-steel equipment and columns employed in HPLC, trace amounts of iron are likely to be present in the chromatographic system[16] and could have been responsible for the observed phenomena. Indeed, purging with a DFA solution resulted in the removal of 160 μg of iron from a chromatographic system with column A, which had been used for reversed-phase chromatography for approximately 60 h.

Purging of column A improved the chromatographic results with DFA dramatically, as shown in Fig. 2B. However, after injecting 2 mg of iron in the form of



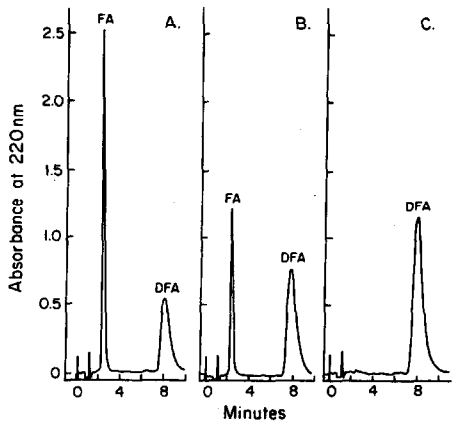


Fig. 2. Chromatograms obtained on column A by injecting 20 μg of DFA, (A) before DFA purge and (B) after DFA purge. The mobile phase was 12.5% (v/v) acetonitrile in 0.02 *M* phosphate buffer (pH 3.0) at a flow-rate of 0.8 ml/min and a column temperature of 22°C. The sample volume was 20 μl. The absorbance of the effluent was monitored at 220 nm.

Fig. 3. Chromatograms of (A) 50%, (B) 25% and (C) 0% iron-complexed DFA on column A. Chromatographic conditions as in Fig. 2.

$FeCl_3$ into column A, DFA again exhibited similiar chromatographic behavior to that observed before column purging, as shown in Fig. 2A. These findings confirmed the assumption that the reaction of DFA with trace amounts of iron in the column accounts for the anomalous chromatographic results.

The order of elution of the two species was investigated using the DFA-purged column A. DFA was complexed with iron(III) chloride to different extents and the mixtures were subsequently chromatographed, with the results shown in Fig. 3. Each peak was collected and, on the basis of their absorption spectra, it was established that FA elutes before DFA in reversed-phase chromatography.

Fig. 1 suggests that FA has a more compact molecular structure than the uncomplexed DFA and thus a smaller contact area on binding to the hydrocarbonaceous stationary phase. Consequently, in reversed-phase chromatography FA is expected to elute faster than DFA, in agreement with the experimental results.

The multiple peaks in Fig. 2A can be readily interpreted in view of the above results. The first peak in Fig. 2A is FA formed from DFA complexation with iron present in the mobile phase and/or on the stationary phase at the entrance region of the column. The final peak is unreacted DFA. Between the initial and final peaks there is a "reaction zone" containing FA that is formed from DFA as it travels down the column.

Both FA and DFA were also chromatographed using column B, which was purged of iron, and the results are depicted in Table I. Again FA eluted before DFA in this octylsilica column. Whereas the k' values of DFA in columns A and B were similar under the same mobile phase conditions, the k' values of FA were significantly different. Column B was considerably more retentive for FA than was column A, resulting in a separation factor of 1.7 compared with 3.1 for the octadecylsilica column (column A). Although this stronger retention for FA may be advantageous in

TABLE I

CAPACITY FACTORS FOR FA AND DFA

Column	*Acetonitrile concentration (%, v/v)*	k'_{FA}	k'_{DFA}*	*Separation factor*
A	12.5	3.6	11.3	3.1
B	12.5	6.1	10.2	1.7
B	16	3.4	3.9	1.2

* For injection of 2 μg of DFA.

certain physiological cases, Column A was used for this study owing to its superior selectivity for FA and DFA.

Under the described conditions, calibration graphs for FA were linear with respect to both peak area and peak height in the range from 22 ng to 22 μg when monitoring at 430 nm. Detection at this wavelength greatly facilitates the assay of FA in physiological fluids. For the simultaneous determination of FA and DFA in our experiments the detector was set at 220 nm. Post-column reactor fluorescence detection also permits the simultaneous assay of both DFA and FA with a sensitivity comparable to that of the UV detector at 220 nm. Although this detection method offers advantages with eluents that have a high absorbance at 220 nm, in our study the use of a variable-wavelength UV detector was most convenient.

Whereas the above chromatographic conditions were suitable for the analysis of FA samples, they gave rise to several anomalies in the quantitative analysis of DFA. At a sufficiently high detector sensitivity at 220 nm, injection of less than 2 μg of DFA gave rise to two small peaks and a reaction zone preceding the DFA peak, as shown in Fig. 4B. Moreover, the relative height of the peaks changed with

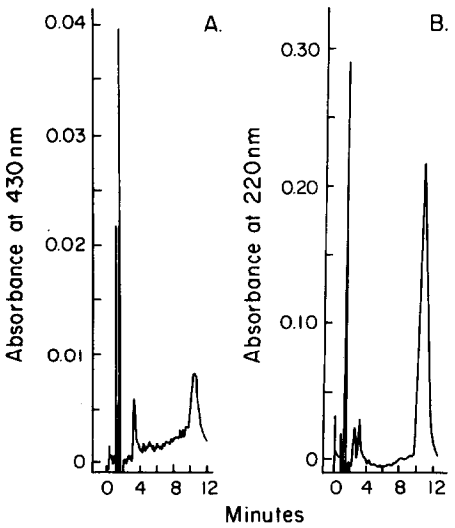


Fig. 4. Chromatogram of 2 μg of DFA on column A. (A) Detector setting at 430 nm; (B) detector setting at 220 nm. Chromatographic conditions as in Fig. 2.

time, indicating a non-linear chromatographic system. The first peak appeared only when the sample was prepared in a solution having a pH lower than 4 and was incubated for several hours prior to injection.

The second peak in Fig. 4B invariably eluted at a retention time characteristic of FA and was identified as such by monitoring the effluent at 430 nm, as shown in Fig. 4A. Detection at this wavelength also revealed the "reaction zone" and the unexpected presence of a relatively small amount of FA co-eluting with DFA. Formation of this FA must have occurred at the outlet frit or in the post-column space, as it co-eluted with DFA.

These phenomena also occurred when larger amounts of DFA, in the range 10–100 μg, were injected. However, at the relatively low detector sensitivity at 220 nm required for the analysis of such large samples the effects were too small to be observed. It is assumed, therefore, that despite the DFA purge, Fe(III) originating from the wetted stainless-steel parts of the HPLC apparatus[16] or from the mobile phase components was still present in the chromatographic system and reacted with a small fraction of DFA in the column. Evidently under these conditions no reliable results could be obtained in the chromatography of DFA.

Further, we found that the retention time of the DFA peak, as measured at the apex, increased with decreasing sample amount, ranging from $k' = 12.5$ to 7.9 for injections ranging from 0.2 to 20 μg of DFA. This observation is puzzling in that the opposite effect is expected from iron complexation of DFA. It is possible that iron is bound to the stationary phase surface such that it is not fully accessible to DFA molecules so that no complex is formed. Therefore, DFA would not be effective in the removal of such bound iron, which may still interact with DFA and give rise to an increase in its retention over that due to the solvophobic effect[17]. Thus, a dual mechanism[18] may be involved in the retention. At relatively high DFA loadings solvophobic interactions determine the magnitude of retention due to the limited number of other sites. With decreasing DFA loading, however, the more strongly binding "unremovable iron" on the stationary phase increasingly dominates the retention of DFA. Alternatively, the interaction of DFA with the stainless-steel tube, walls and frits may account for this phenomenon.

In any case, the presence of iron in the chromatographic system causes impermissible interferences in the chromatographic assay of DFA. Therefore, the use of a suitable chelating agent in the mobile phase in order to eliminate these interferences was considered. Such a chelating agent should strongly complex heavy metals potentially present in the chromatographic system and complex iron with a high but significantly lower formation constant than DFA.

The stability constants of several chelating agents were considered[2,19,20] and for our purposes EDTA appeared to be the most suitable, as it binds iron sufficiently strongly to serve as a scavanger for iron in the mobile phase but it does not compete strongly with DFA for iron when the pH of the solution is higher than 6[11]. Further, EDTA competes very strongly with DFA for other metallic ions. Therefore, in further studies EDTA was employed in the mobile phase of pH 6.5.

Complexation of DFA by iron present in the column was supressed by using 2 m*M* EDTA in the mobile phase and no FA peak or reaction zone was observed at 220 nm. Monitoring the effluent at 430 nm with the same detector sensitivity as used before confirmed the absence of the FA peak, in addition to the elimination of

the FA reaction zone and the FA peak co-eluting with DFA. EDTA in the mobile phase also had a stabilizing effect on the retention of DFA as a function of sample loadings.

With EDTA in the mobile phase and a detector setting of 220 nm we were able to quantitate DFA at levels down to 50 ng. Under such conditions both DFA and FA could be assayed in a single chromatographic run at concentration levels of physiological significance[6]. The ability to monitor FA at 430 nm offers a simple approach for the assay for FA in biological fluids by this method. Further work is needed, however, to establish a procedure for clinical applications.

The results of this study suggest that HPLC offers significant advantages over other analytical techniques for DFA and FA, and makes it possible to explore other variants of the methods described above. For instance, the determination of iron by reversed-phase chromatography with DFA or EDTA in the eluent could be carried out by incubating the sample with excess of DFA and measuring the FA peak thus obtained at a detector wavelength of 430 nm. The practicability of such an iron assay procedure is currently under investigation in our laboratory.

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