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Note

Determination of desferoxamine and ferrioxamine by high-performance liquid chromatography with direct serum injection and pre-column enrichment

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In 1976, aluminium was recognized as a toxic factor in dialysis encephalopathy, a progressive neurological disease that occurs in patients with impaired renal function undergoing chronic haemodialysis.

Since the introduction, in 1977, of desferoxamine (DFO) to remove aluminium from a patient with severe dialysis encephalopathy, this chelating agent has been used successfully in aluminium intoxication [1—3]. The ability of DFO to chelate aluminium and displace it from binding sites was demonstrated recently in a uraemic patient before and after chelating therapy with DFO [4].

In order to characterize the optimum procedure of administration before, during or after haemodialysis, it is essential to have a procedure to measure DFO in biological fluids.

Until recently, DFO was determined colorimetrically, based on the same properties as its use as a chelating drug, i.e. the formation of the ferrioxamine (FO) complex with iron(III) [5—8]. Disadvantages of this method were the need for a relatively large sample volume ($\geq$ 1 ml), a low detection limit for clinical studies ($\geq$ 5 mg/l) and interference by other substances in serum.

Recently, a high-performance liquid chromatographic (HPLC) technique was presented by Cramer et al. [9] for simultaneous determination of DFO and FO. They studied the interference by iron in the HPLC system and overcame this problem successfully by either purging the system with DFO or adding EDTA to the mobile phase. Today, no HPLC procedure for measuring DFO and FO in biological fluids is available.

We combined the HPLC system proposed by Cramer et al. [9] with a

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relatively new technique, which allows the direct injection of serum onto a reversed-phase pre-column [10—13]. DFO is determined by subtracting the FO measured in a sample, both with and without the addition of iron(III). This procedure is simple and rapid, with an absolute detection limit of 25 ng of DFO.

EXPERIMENTAL

Chemicals and reagents

Desferoxamine mesylate (Desferal) is obtained from Ciba-Geigy (Arnhem, The Netherlands). Iron(III) chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate, acetonitrile and EDTA disodium salt are purchased from Merck (Amsterdam, The Netherlands). All chemicals and solvents are of analytical grade and the water used is demineralized.

Instrumentation and chromatographic conditions

Two solvent delivery systems, a Model M-45 and a Model M-6000 A, a Model U6K liquid chromatograph injector and a Model 461 variable-wavelength detector are used (all from Millipore Waters, Etten-Leur, The Netherlands). The pumps and column are connected by a six-port switching valve, Model Valco (Chrompack, Middelburg, The Netherlands). The HPLC analytical column is a LiChrosorb RP-8 (10 µm), 25 cm × 4.6 mm I.D. (Chrompack). A Guard-Pak pre-column module with RCSS Guard-Pak C_{18} cartridges, 4 mm × 6 mm I.D. (Waters), replaces the sample loop in the Valco valve. The detector is connected to a Shimadzu Chromatopac C-R3A integrator (Packard, Delft, The Netherlands). A flow diagram of the column-switching arrangement is shown in Fig. 1.

The flow-rate of the mobile phase is 2 ml/min. The effluent from the column is monitored at a wavelength of 430 nm.

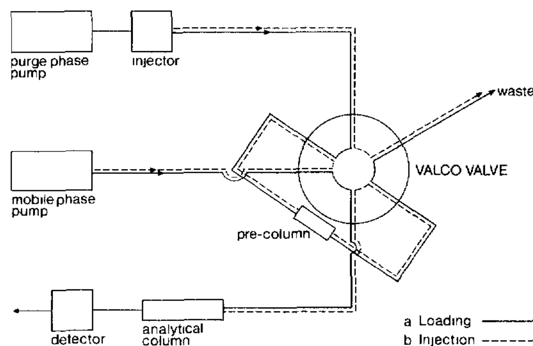

Fig. 1. Column-switching flow diagram of the direct serum injection and column enrichment

Mobile phase and purge phase

The mobile phase consists of a mixture of 0.02 *M* phosphate buffer (pH 6.6) and acetonitrile (85:15) with 2 m*M* EDTA. The purge phase is 0.1 *M* phosphate buffer (pH 6.6). The phases are filtered and degassed by vacuum before use.

Preparation of calibration standards and calculations

Stock solutions of desferoxamine mesylate are prepared in methanol and stored at 4°C. The calibration curve samples are prepared by evaporating appropriate quantities of the stock solution. These are redissolved in serum of a normal healthy volunteer. The calibration samples are stored at −20°C and thawed before use.

Sample preparation and injection

The DFO serum samples and calibration standards are centrifuged for 10 min at 5000 *g* before use. A 100-μl aliquot of serum is mixed with 10 μl of 4 m*M* iron(III) chloride solution and allowed to stand for 5 min to complete complexation. FO is determined in the same way as DFO, except that the addition of iron(III) is omitted.

Before injection, the Valco valve is positioned so that the purge phase can flow through the pre-column, which is pre-washed with at least 2 ml of the purge phase. Simultaneously, the mobile phase flows through the analytical column to the detector.

A 50-μl aliquot of sample is injected onto the pre-column and washed with 5 ml of the purge phase. The Valco valve is switched and the retained compounds are eluted onto the analytical column with the mobile phase flowing in the same direction as the purge phase. Re-equilibration of the pre-column and injection can be done after 2 min, while the analysis is in progress.

RESULTS AND DISCUSSION

The only previously published HPLC assay for DFO and FO (Cramer et al. [9]) did not have a procedure for measuring the compounds in biological fluids. We have tried to measure DFO and FO in serum using their HPLC method at a wavelength of 220 nm. However, there seemed to be too many interferences, so we complied with their suggestion of adding EDTA to the mobile phase. The next problem was the relative high UV absorbance capacity of EDTA at a wavelength of 220 nm. For this reason and because patient serum always contains a different amount of iron(III), we added an excess of iron(III) to the serum in order to measure the FO complex at a wavelength of 430 nm.

We started our experiments with sample clean-up by Sep-Pak C_{18} cartridges (Waters), but because of the lack of reproducibility, bad recovery and the time-consuming extraction step, we changed to direct serum injection onto a pre-column.

During the development of our described procedure, a second HPLC assay was presented by Kruck et al. [14], who used a straight phase system with two kinds of sample clean-up methods, which needed 1 ml of serum. Our presented method only needs 100 μl of serum and requires no prior work-up.

The addition of EDTA to the mobile phase was also useful for monitoring the effluent at 430 nm. It was proved that for suppressing any unidentified peaks due to other chelating compounds present in the serum, the concentration of EDTA should be at least 2 m*M* (Fig. 2). We also investigated the amount of

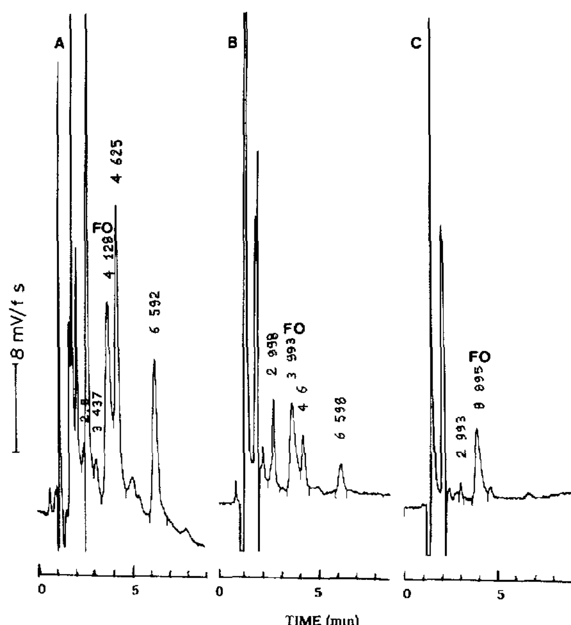

Fig. 2. Chromatograms of a serum containing 8.7 mg/l DFO (15.5 μM). (A) Mobile phase without EDTA; (B) mobile phase containing 1 m*M* EDTA; (C) mobile phase containing 2 m*M* EDTA. FO is ferrioxamine. Injection volume, 50 μl; further chromatographic conditions as described in the text.

iron(III) that must be added for complexation of DFO. Addition of a 4 m*M* iron(III) solution to serum samples containing up to 60 mg/l DFO (0.107 m*M*) was sufficient (Table I). The 5-min complexation time has already been tested [7].

It seemed to be of importance to remove solid particles from the serum by centrifugation [13]. When we started our experiments, with direct serum injection, we did not centrifuge or filter the samples. The pre-column then built-up a back-pressure of 70 bar after 20—30 injections of serum. We overcame most of this problem by centrifuging the samples before starting the work-up. Thus, we were able to do more injections, but the replacement time of the cartridges depended on the amount of solid particles that still remained in the sera, after centrifugation.

TABLE I

COMPARISON OF DFO DETERMINATIONS WITH DIFFERENT AMOUNTS OF IRON(III)

DFO (mg/l)	Counts × 1000 (n = 4)		
	2 m*M* iron (III)	4 m*M* iron(III)	8 m*M* iron(III)
20.1	52	86	83
40.2	116	188	195
60.3	113	298	313
Correlation coefficient	0.838	1.000	1.000

We chose to equilibrate the pre-column with 2 ml of purge phase, because there is a possibility that the FO may elute from the pre-column by some remaining mobile phase.

The absolute recovery of FO from the pre-column was tested by comparing the results of injection of a FO solution both with and without pre-concentration. There was no significant difference between the two procedures. The effect of proteins was tested by comparing the recovery after injection of a sample of DFO in water and serum. No effect on recovery was seen.

In most direct injection and pre-enrichment procedures, the injected compounds that remained on the pre-column are eluted by back-flushing with the mobile phase [10—12]. We found no differences in the peak area of FO when it was eluted by the mobile phase either in the normal or back-flush mode. We chose the normal mode (as seen in Fig. 1), because in this way the analytical column is protected from possible impurities remaining on the front of the pre-column. The elution time of FO from the pre-column was tested by injecting an FO solution without an analytical column. We found that the injection procedure can be started after 2 min.

Several sera of patients on dialysis, not receiving DFO, were tested for interferences, both before and after dialyses. These patients received a large variety of drugs, including magnesium hydroxide, iron fumarate and aluminium hydroxide. No interferences with FO were seen in these samples either with or without iron(III) addition (Fig. 3). In Fig. 4, two examples are given of FO determination in sera from patients receiving DFO.

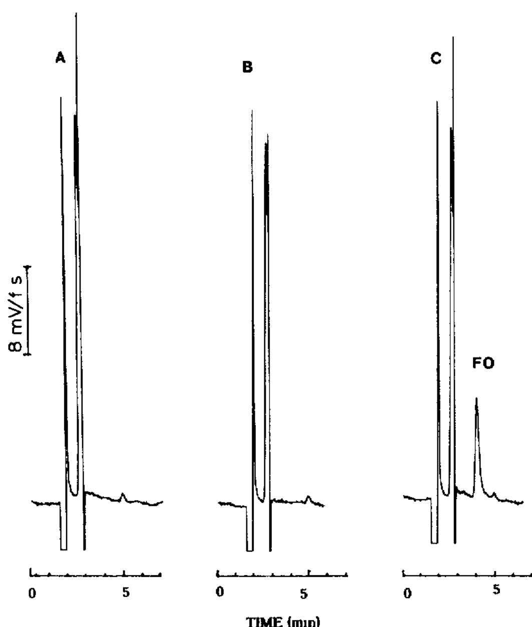

Fig. 3. Chromatograms of serum from a patient on dialysis not receiving DFO. (A) Serum with addition of iron(III) before dialysis; (B) the same patient serum with addition of iron(III) after dialysis; (C) same serum as A spiked with 8.7 mg/l DFO (15.5 μM) and addition of iron(III) chloride solution. FO is ferrioxamine. Injection volume, 50 μl; further chromatographic conditions as described in the text.

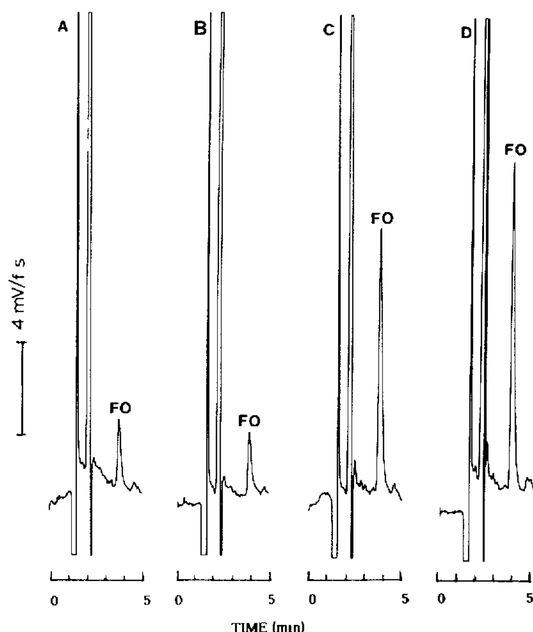

Fig. 4. Chromatograms of sera from two patients receiving Desferal. (A) Serum of a patient receiving 1000 mg per 12 h subcutaneous, without addition of iron(III) (FO concentration = 1.0 mg/l or 1.7 μM); (B) the same serum as A with addition of iron(III) (FO concentration = 1.0 mg/l or 1.7 μM); (C) serum of a patient receiving 1000 mg per 12 h intramuscular, without addition of iron(III) (FO concentration = 3.8 mg/l or 6.2 μM); (D) the same serum as C with addition of iron(III) (FO concentration = 4.8 mg/l or 7.8 μM). FO is ferrioxamine Injection volume, 100 μl; further chromatographic conditions as described in the text.

TABLE II

PRECISION DATA FOR DFO DETERMINATION

Added (mg/l)	Found (mean) (mg/l)	n	Coefficient of variation (%)
2.03	2.03	7	4.8
4.05	3.92	6	5.1
6.08	6.20	6	5.4
8.10	8.10	6	2.8
10.13	10.21	6	1.8

The precision data obtained by repeated analysis of sera, to which DFO was added, are presented in Table II.

Standard curves were prepared by plotting peak areas against the amount added initially. These curves were linear over the range of interest, 1—10 mg/l DFO. The correlation coefficient of several curves was 0.997 (n = 7).

The limit of detection of our procedure was < 0.5 mg/l DFO*. The limit of detection can be increased by injecting a larger volume of serum. The absolute limit of detection was 25 ng of DFO**.

*The relative limit of detection is equal to the concentration when the signal = 3 × relative standard deviation of the noise. The noise is the response from a blank serum measured at the retention time of FO.

**The absolute limit of detection = relative limit of detection × injection volume.

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