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Quantification of desferrioxamine, ferrioxamine and aluminoxamine by post-column derivatization high-performance liquid chromatography
Non-linear calibration resulting from second-order reaction kinetics

Hans-Joachim Kraemer[1], Henning Breithaupt*

Department of Internal Medicine, Justus-Liebig-University, Klinikstrasse 36, 35385 Giessen, Germany

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Abstract

Desferrioxamine B is widely used as therapeutic agent for removal of excess body iron and, more recently, for removal of aluminium. A HPLC-based method for direct sensitive and reliable determination of ferrioxamine, desferrioxamine, aluminoxamine and related metabolites has been developed for use in pharmacokinetic studies. The method consists of complete separation of the analytes by an optimized mobile phase avoiding conversion of desferrioxamine to ferrioxamine by the analytical system and overcoming problems due to peak tailing properties of desferrioxamine. A post-column derivatization reaction with colourless fluoro-complexed iron converts all iron free species to ferrioxamine and allows quantification at 430 nm avoiding interference of UV-absorbing coelutes. This reaction might be of interest for other analytical procedures concerning iron chelators. The influence of the post-column reaction system on the column plate number is characterized. As the reaction rate of desferrioxamine and aluminoxamine with iron(III) is of second-order kinetics, a quadratic calibration function is observed, resulting from a compromise between residence time and peak broadening. A solid-phase extraction procedure is presented for extraction of the analytes from plasma. Limits of detection (S/N ratio of 3) were determined as 1.95 ng for ferrioxamine, 3.9 ng for aluminoxamine and 15.7 ng for desferrioxamine, expressed as on-column load. A new iron-free metabolite was identified with fast atom bombardment-mass spectrometry as *N*-hydroxylated desferrioxamine.

Keywords: Desferrioxamine; Ferrioxamine; Aluminoxamine

1. Introduction

Desferrioxamine B (DO) is isolated from cultures of *Streptomyces pilosus* as iron-chelate ferrioxamine B (FO, [1,2]), an octahedral hydroxamate-complex [3]. Its strong and selective iron(III) and aluminium(III) chelating properties (log k for iron 31, for aluminium 26, [4–6]) are clinically used for removal of iron and aluminium overload [7–25]. The chemical structures of desferrioxamine B and of the metal-chelate are presented in Fig. 1.

Since the therapeutic introduction of DO there

*Corresponding author.

[1]This manuscript includes portions of a doctoral thesis by H.-J. Kraemer.

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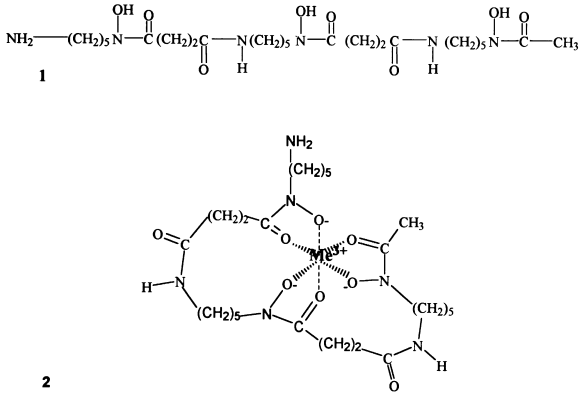

Fig. 1. Structure of the analytes (1 = desferrioxamine, 2 = chelate with Me^{3+}).

exist only a few methods selective and sensitive enough for its determination and that of the related chelates in biological fluids [26–42]. On the other hand, pharmacokinetic and metabolic studies are still of interest, especially concerning removal of iron and aluminium and optimizing dosage to avoid adverse side effects [43–49].

Indirect methods for determination of DO and related compounds are based on inductively coupled plasma atomic emission spectrometry (ICP-AES) [28] or atomic absorption spectrometry (AAS) [27]. Both make use of estimation of extractable, organic bound iron and are thus prone to erroneous co-determination of other chelated iron-species.

Direct methods for determination of DO and its derivatives are based on high-performance liquid chromatography (HPLC) [29–42], and suffer from similar difficulties mainly due to the extraordinary chelating property of the DO molecule. A general approach to avoid conversion of DO to FO due to contact with the stainless steel apparatus is the use of iron-free HPLC devices [34]. A more practicable approach seems to be the addition of a chelating agent such as nitrilotriacetic acid (NTA) to the mobile phase [40,41], thus reducing the availability of free iron in the chromatographic system. Although the binding constant for NTA as well as ethyl-enediamin-tetraacetate (EDTA) for iron is largely lower compared to DO, it has to be taken special care to avoid a major influence of the additional iron chelator on the complex equilibrium of DO and its derivatives. Another DO related problem is the large peak tailing properties of DO in most of the described HPLC methods thus leading to low sensitivity and reproducibility in quantification. It was our aim in this study to develop a routine HPLC method for separation and quantification of DO, FO, AO and its major metabolites in biological fluids with reliable reproducibility and accuracy thus permitting pharmacokinetic studies. Furthermore, the described derivatization reaction might be of interest for other analytical methods concerning iron chelators.

A combination of solid-phase extraction (SPE) and HPLC with a mobile phase containing the modifier *N*,*N*-dimethylformamide minimizing peak tailing was used. Detection of DO, AO and major iron-free metabolites was achieved by a post-column derivatization related method, which consists of an addition of fluoro-complexed iron after separation of the components. The iron-hexafluoro-anion reacts readily with DO, AO and iron-free metabolites to form FO or the respective iron-containing metabolite. A second-order reaction rate for the reaction of the chelator with iron(III) results in a slightly non-linear calibration function, resulting from the used compromise between residence time for completion of the reaction and peak broadening. Nevertheless, all components are easily detectable at 430 nm thus avoiding interference with coelutes which absorb in the UV region. The iron-hexafluoro-anion has a negligible absorption at 430 nm, thus does not influence the detection of the compounds of interest. A sensitive and reliable method for determination of DO and metabolites was thus achieved allowing pharmacokinetic studies.

An iron-free metabolite of DO was identified as *N*-hydroxylated DO after preparative isolation and purification by means of fast atom bombardment-mass spectrometry (FAB-MS).

2. Experimental

2.1. Chemicals

DO methanesulfonate was obtained from Ciba-Geigy (Basel, Switzerland). Ferric-nitrate-nonahydrate, sodium fluoride, sodium azide, nitric acid, sulfuric acid, ammonia (25%), ammonium sulfate, aluminium and iron standard solution (1

mg/ml) were purchased from Merck (Darmstadt, Germany) in analytical quality. *N,N*-Dimethylformamide, methanol and acetonitrile were of HPLC-grade and obtained from Baker Chemicals (Deventer, Netherlands). Sodium heptanesulfonate was from Aldrich (Steinheim, Germany). Octadecylsilica (ODS)-silica (3 μm and 5 μm) was from Shandon (Frankfurt/Main, Germany), C_{18} SPE columns (3 ml) were from Varian (Frankfurt/Main, Germany). Aqua ad injectabilia in glass containers was from Pfrimmer (Erlangen, Germany).

2.2. Sample preparation and storage

Blood was collected in heparinized tubes and centrifuged (3000 *g*, 10 min) immediately after drawing. Dialysate was obtained by clinical routine plasma dialysis with Cuprophan capillary membranes (type RC55 8/200, Akzo Nobel Faser, Wuppertal, Germany). Dialysis fluid was the electrolyte solution SK-F 219 (Fresenius Medical Care, Bad Homburg, Germany). Plasma, urine and dialysate samples were stored at −20°C until analysis, for at least six months. Stability of the analytes was not affected during this period of storage.

2.3. HPLC system

Separation was achieved on a coupled column consisting of two stainless steel columns (15 cm×4.6 mm, Bischoff Analysentechnik, Leonberg, Germany) filled with ODS-silica (3 μm, Shandon) and attached to each other by a low-dead volume coupling (Bischoff). Another column (25 cm×4.6 mm) was filled with ODS-silica (5 μm, Shandon). The columns were packed according to the ''balanced density slurry'' method. Mobile phase was delivered by a HPLC pump Gynkothek 600 (Gynkothek, Munich, Germany) at a flow-rate of 1 ml/min. Samples were applied to the column by means of an automatic sampler (Kontron HPLC autosampler 465, Kontron, Munich, Germany) with a sample volume of 50 μl. Back-pressure of this column was about 260 bar with a slight rise of 10 bar when in use with biological specimen. Post-column derivatization reagent was delivered by a second Gynkothek HPLC pump at a flow-rate of 1 ml/min and was admixed to the mobile phase shortly after the separation column by means of a hydraulic micro-mixer (Lee Visco-jet 10-μl, Lee Hydraulische Miniaturkomponenten, Frankfurt/Main, Germany). In order to obtain a reasonable back-pressure for the pump a 15×4.6 cm HPLC-column filled with 3 μm ODS-silica was mounted in the reagent delivery path. The capillary length after the micro-mixer t-piece was 20 cm towards the detector. Detection at 430 nm was achieved by Kratos Spectroflow 773 with 8 μl cell (Kratos, Ramsey, NJ, USA), equipped either with a tungsten or a deuterium lamp. Chromatograms were recorded by a Shimadzu CR6a integrator (Shimadzu, Kyoto, Japan).

The mobile phase used was a water–acetonitrile–*N,N*-dimethylformamide (1000:185:40, v/v/v) mixture, to which 0.6 g of sodium heptanesulfonate, 0.4 g ammonium sulfate and 0.1 g di-sodium EDTA were added. The pH was adjusted to 1.5 by use of 25% sulfuric acid. Dissolved gases were removed by treatment for 10 min in an ultrasonic bath (Bandelin, Berlin, Germany). The acidic mobile phase was tolerated by the columns in daily use and under reflux conditions when not in use, leading to a mean life-time of four months without loss of resolution.

Post-column derivatization was achieved by a mixture of mobile phase–iron-fluoro solution (1000:120, v/v). The iron-fluoro solution was prepared from 40 g ferric-nitrate-nonahydrate (0.1 *M*) and 12.6 g sodium fluoride (0.3 *M*) dissolved in 1 l water containing 2 ml of concentrated nitric acid thus avoiding hydrolysis of the ferric-nitrate.

2.4. Characterization of the column

The above described apparatus was used with a mobile phase consisting of acetonitrile–water (60:40, v/v), which was also delivered by the second pump connected after the column by means of the t-pieced hydraulic micro-mixer. Both the 3 μm coupled column and the 5 μm column were employed for testing the column's properties, flow-rates were as above. Test solution was a mixture of acetone, phenol, 2,3-dimethyl-phenol and phenetol dissolved in acetonitrile at a concentration of 1 mg/100 ml of each compound. Samples of 10 μl were injected to the column and were integrated at an attenuation of 10. The integrator data were used for calculating

column plate numbers, for the 3 μm column with and without supply of post-column flux.

2.5. Standard solutions

A standard solution of 200 μg each of DO, FO and AO was prepared by dissolving 57.6 mg DO-methansulfonate in water, adding 789 μg of aluminium(III) and 1.571 mg of iron(III), 2 ml Tris-buffer (0.1 *M*) and 1 ml sodium azide (5% in water), finally adjusting the volume to 100 ml. The standard solution was stored at 4°C for two weeks before renewal. After that period a considerable decay of the analytes took place.

2.6. Solid-phase extraction

C_{18} SPE columns were conditioned with 3 ml of methanol in a Baker vacuum manifold (10 columns, Baker), followed by 3 ml of Tris buffer (0.05 *M*, pH 9). One ml of Tris buffer (0.1 *M*, pH 9) was added to 1 ml plasma and the mixture was applied to the preconditioned extraction columns. Following absorption the columns were washed with 1 ml of Tris buffer (0.05 *M*, pH 9).

Application of vacuum (100 mbar) for 10 min removed traces of humidity prior to elution with subsequent 2×500 μl of a mixture of methanol–acetic acid–water (90:5:5, v/v/v). The eluates were collected in 1.5-ml Eppendorf tubes and dried under nitrogen at ambient temperature. The residue was dissolved in 1 ml of Tris buffer (0.05 *M*, pH 9), vortexed and centrifuged for 10 min at 3000 *g* prior to HPLC analysis.

2.7. Sample preparation for urine and dialysate

Urine was diluted 1:9 (v/v) with Tris buffer (0.05 *M*, pH 9) and directly used for HPLC analysis.

Dialysate (100 ml) was adjusted with Tris buffer (1 *M*, pH 9) to pH 9 and applied to the preconditioned SPE columns. Further processing was as described above.

2.8. Validation

In order to show the reproducibility of the chromatographic system a repetitive analysis of a standard solution of 30 μg/ml of each analyte in Tris buffer (0.05 *M*, pH 9) was performed.

The limit of detection (LOD) was determined by analysis of standard solutions containing 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078 and 0.039 μg/ml of the analytes. Signal-to-noise (S/N) ratio was determined by setting the analysis parameter of the integrator to the lowest possible value (1 for width, minimal peak area, slope and attenuation).

Recoveries of the analytes from plasma, urine and dialysate were obtained by spiking drug-free standard plasma samples with the appropriate concentrations and submitting to the extraction procedure followed by HPLC analysis.

Intra- and inter-day variability of the method was tested by analysis of five samples of spiked plasma and 10 samples of spiked urine (30 μg/ml of each analyte) on five days.

2.9. Atomic absorption spectrometry

Iron and aluminium in plasma samples were determined with a Beckman AAS 1272 (Beckman Instruments, Munich, Germany) system with Massmann-cuvette. Iron was determined at 248.3 nm, aluminium at 309.2 nm. A standard solution in the range of 0.5 to 6 μg/ml for iron and 10 to 500 μg/ml for aluminium was used to calibrate the analysis. A sample of 10 μl plasma was introduced in the graphite cuvette, dried for 30 s at 200°C, heat-decayed for 20 s at 800°C and atomized for 28 s at 2400°C, in case of aluminium for 30 s at 2800°C. The cuvette was purged at 3000°C for 2 s.

Inert gas was nitrogen, the appropriate spectral lamps were set at 25 mA, slit width was set at 10 nm. The height of the resulting peaks was used for quantification.

2.10. Preparative isolation of an iron-free metabolite

A 2-l volume of urine from a DO-treated patient was adjusted to pH 9 with NaOH and filtered over 50 g C_{18} silica gel in batch mode (40 μm, Merck). The reddish mass was washed with 200 ml water and dried in a slight vacuum (100 mbar). Elution was achieved with 200 ml methanol, thus solubilizing the

red coloured substances. The extract was diluted with 800 ml water and freeze–dried.

The residue was dissolved in 5 ml water and submitted to a semi-preparative HPLC column (25 cm×1.4 cm) filled with C_{18} silica gel (5 μm, Shandon). The mobile phase of the analytical procedure was used for elution but without EDTA and sodium heptanesulfonate. Flow-rate was 3.6 ml/min. A 2-ml sample was applied to the column, and the eluates were collected in 0.5 ml fractions (elution time 20 min). Absorption at 430 nm was determined by manual photometry before and after off-line addition of post-column derivatization reagent to an aliquot of each fraction. Iron binding eluates were detected by strong differences in absorption following the addition of the iron-reagent.

The corresponding fractions were submitted to the analytical HPLC process, and fractions containing the metabolite were combined, adjusted to pH 7 with ammonia and freeze–dried.

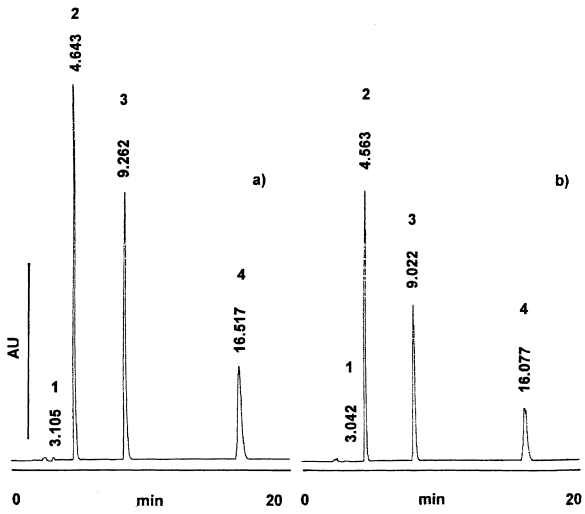

Fig. 2. Chromatogram of the test mixture (1=acetone, 2=phenol, 3=2,3-dimethylphenol, 4=phenethole, 10 μg of each compound) (a) without (b) with post-column flux.

2.11. FAB-MS

Fractions containing the iron-binding metabolite were freeze–dried and the residue was submitted to FAB-MS with a Finnigan Mat 900 instrument (Finnigan, San Jose, CA, USA). The sample was loaded in an *m*-nitrobenzyl alcohol–trifluoroacetic acid mixture (1:1, v/v) and bombarded with Cs atoms having an energy of 20 keV. The mass range was set at m/z 20 Da to 1000 Da, with a sensitivity of 1 Da to 1000 Da (a mass difference of 1 Da detectable at a molecular mass of 1000 Da), calibration was done with a perfluorocarbon mixture with known mass range.

3. Results

3.1. HPLC

3.1.1. Characterization of the columns and of the influence of the post-column reaction system on the column's plate number

Fig. 2 shows a chromatogram obtained with the test solution before and after admixture of the post-column flux, showing only marginal peak broadening due to the used post-column flux. The obtained integrator data and the resulting plate numbers of this column as well as those of a 5-μm column are documented in Table 1. Plate numbers were calculated according to $N=2\pi[(h_P t_R)/A_P]^2$, were h_P is the peak height, t_R is the retention time and A_P is the peak area of the corresponding peak.

3.2. Analytical HPLC

The described mobile phase containing the ionic pair reagent heptanesulfonate for interaction with the terminal amino-group of DO and related compounds, and the modifier *N*,*N*-dimethylformamide [50] allowed baseline separation of the analytes within 16 min (Fig. 3), with minor peaks resulting from trace contaminants in the drug. Conversion of free DO to FO through contact with the steel components of the HPLC apparatus was shown to be negligible by injection of a DO solution (50 μg/ml), resulting in a peak of FO less than 2% of the peak area of the DO peak.

3.3. Post-column derivatization

The iron-free analytes AO and DO are detected after separation by means of conversion to FO.

Table 1
Number of plates of the 3-μm ODS silica column determined with the test mixture without and with influx (data in parentheses) of the post-column mobile phase and data for a corresponding 5-μm ODS silica column with comparable length

Substance	Retention time (min)	Peak height (μV)	Peak area (μV s)	Plate No.
3-μm ODS silica column				
Acetone	3.103 (3.042)	12 471 (4057)	193 170 (64 806)	907 (819)
Phenol	4.643 (4.563)	1 114 586 (737 148)	8 744 505 (5 753 834)	7918 (7726)
2,3-Dimethylphenol	8.212 (8.092)	731 094 (420 995)	7 704 176 (4 582 973)	13 897 (12 338)
Phenetol	16.517 (16.077)	255 097 (144 963)	4 939 826 (2 854 292)	16 447 (15 072)
5-μm ODS silica column				
Acetone	3.197	6804	154 918	445
Phenol	4.727	1 077 887	8 808 832	7563
2,3-Dimethylphenol	8.24	669 436	7 619 835	11 847
Phenetol	16.182	238 992	4 856 395	14 337

Flow-rates both 1 ml/min, mobile phase acetonitrile–water (6:4, v/v).

Therefore linearity and detection limit of the process has to be demonstrated. Solutions with known amounts of the analytes were prepared by dilution of the standard solution and injected onto the HPLC column. The resulting standard curves of both, peak area and height, against varying concentrations of FO resulted in linear characteristics with a correlation coefficient of 0.9996 in the concentration range of 1 to 50 μg/ml, with a linear regression of the peak heights (Y) against the concentration (X) according to $Y=-54.88(\pm49.61)+860.12(\pm10.84)X$ (mean±S.D.). The corresponding standard curves of both, peak area and height, against varying concentrations of AO and DO resulted in slightly non-linear characteristics well approximated by a second-order polynomial [data given for the peak heights (Y), concentration (X), mean±S.D.]:

$$\text{AO: } Y = 203.50(\pm140.84) + 251.63(\pm27.11)X + 1.78(\pm0.77)X^2, \quad r = 0.999$$

$$\text{DO: } Y = -1563.08(\pm240.81) + 923.45(\pm55.32)X + 6.13(\pm1.84)X^2, \quad r = 0.999$$

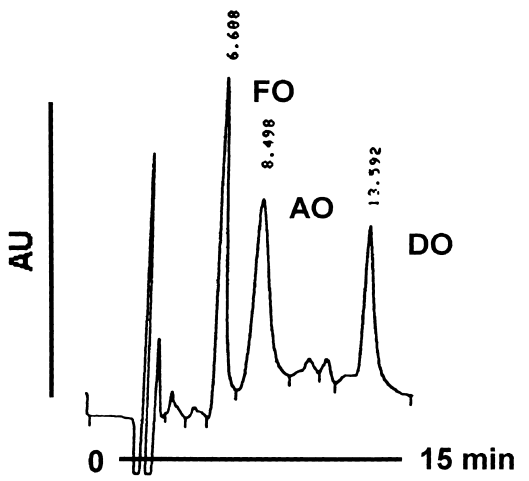

Fig. 3. Chromatogram of the analytes in an aqueous solution (15 μg/ml of each compound).

The calibration curves for all analytes are presented in Fig. 4.

Data obtained by 10 consecutive injection of a standard solution in Tris buffer are given in Table 2 demonstrating the stability of the post-column derivatization reaction.

Detection limits (tungsten lamp) were 0.039 μg/ml for FO, 0.078 μg/ml for AO and 0.313 μg/ml for DO ($S/N=3$), according to an on-column-load of 1.9 ng for FO, 3.9 ng for AO and 15.7 ng for DO. For the deuterium lamp these values were twice as high corresponding to the approximately doubled noise level. Limits of quantification according to the

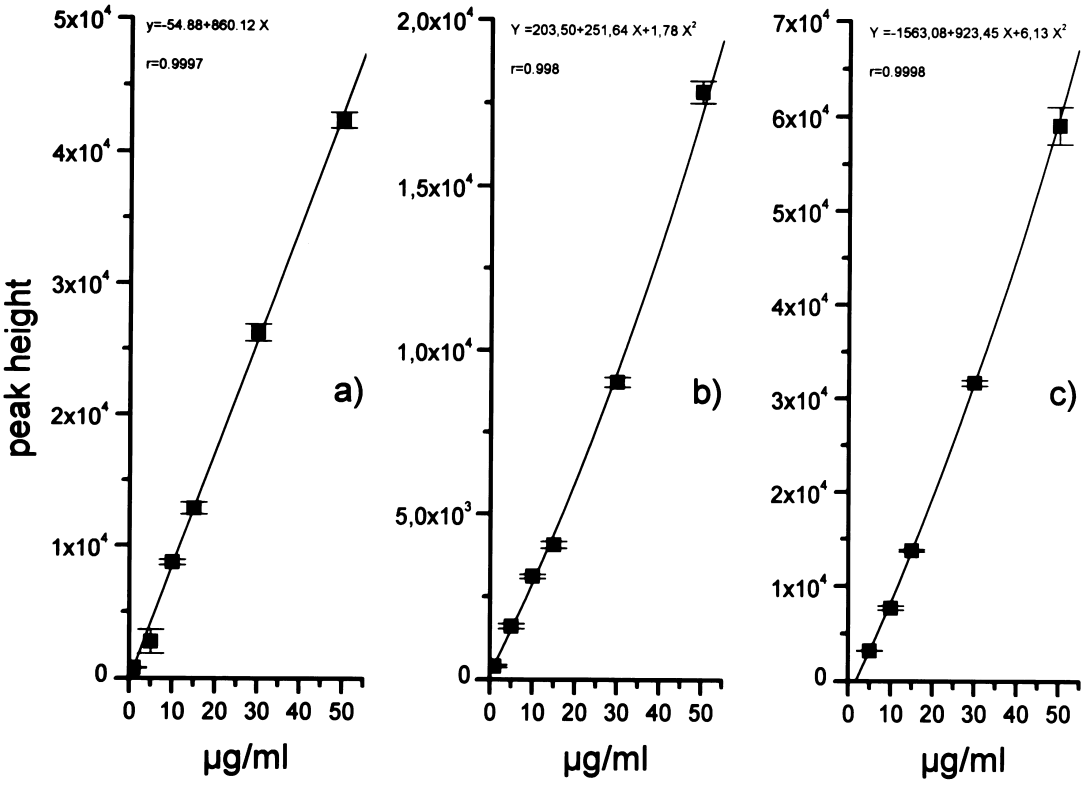

Fig. 4. Calibration functions of the analytes (a) ferrioxamine, (b) aluminoxamine, (c) desferrioxamine.

definition were the 10-fold amounts at a signal-to-noise-ratio of 30.

3.4. Solid-phase extraction

The SPE of plasma and dialysate was strongly dependent on the pH of the buffer used for adjusting the sample pH prior to extraction. In order to optimize the pH for extraction a mixture of equal amounts of the analytes in plasma were submitted to the process with varying pH of the sample, elution was carried out under the described conditions. In the pH range from 3 to 6 there was a very low recovery of FO and AO of less than 30% but a high recovery of DO in the range of 300% (Fig. 5), thus indicating nearly stoichiometric decay of the metal complexes to free DO. At pH values >7 there was

Table 2
Reproducibility of the chromatographic system

Substance	Retention time±S.D. (min)	Peak height±S.D. (μV)
FO	6.58±0.01	26 388±38
AO	8.50±0.01	14 677±19
DO	13.49±0.03	30 620±38

A standard solution containing 30 μg/ml of each analyte in Tris buffer (0.05 *M*, pH 9) was repetitively ($n=10$) analyzed.

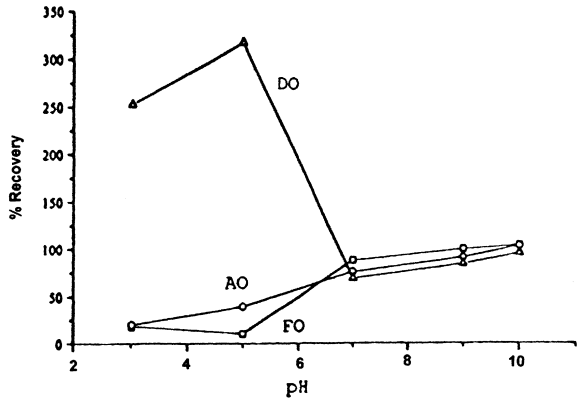

Fig. 5. Recovery of the analytes from solid-phase extraction (plasma) at different pH values.

an approximation of recovery to more than 95% for all analytes, indicating stability under these conditions. The optimal pH of 9 was used in the further investigations.

Recoveries were calculated by comparison of the signal of the extracted or diluted sample to the signal of the corresponding solution in water. In case of the non-linear calibration curves the quadratic polynomials were resolved for the concentration X after transformation of $Y=A+BX+CX^2$ to $0=(A-Y)+BX+CX^2$, taking the positive square root as solution.

Recovery data for the different matrices are given in Table 3.

Intra- and inter-day variability is within acceptable limits as shown in Table 4.

Limits of quantification after extraction from

Table 3
Recoveries of the analytes from different matrices

Theoretical concentration (μg/ml)	Concentration found (plasma) (μg/ml±S.D.)	Recovery (plasma) (%±S.D.)	Concentration found (urine) (μg/ml±S.D.)	Recovery (urine) (%±S.D.)	Concentration found (dialysate) (μg/100 ml±S.D.)	Recovery (dialysate) (%±S.D.)
Ferrioxamine						
1	1.15±0.065	115.0±6.5				
5	4.91±0.53	98.2±10.6				
10	9.79±0.68	97.9±6.8	8.76±0.02	87.60±0.2		
15	14.54±1.44	96.9±9.6	13.51±0.06	90.10±0.4		
30	28.33±1.46	94.4±4.9	28.21±0.65	94.10±2.2	27.21±0.41	90.7±1.4
50	46.33±3.48	92.7±7.0	48.16±0.23	96.32±0.5		
100			98.54±1.29	98.54±1.3		
Aluminooxamine						
1						
5	5.89±1.00	117.8±20.0				
10	10.83±1.75	108.3±17.5	8.59±0.48	85.90±4.8		
15	15.53±0.89	103.5±5.7	13.93±0.68	92.86±4.5		
30	29.90±1.83	99.7±6.1	28.27±0.41	94.23±1.4	20.81±0.15	69.4±0.5
50	49.21±3.56	98.4±7.1	48.37±0.71	96.74±1.4		
100			100.68±0.99	100.68±1.0		
Desferrioxamine						
1						
5	5.31±0.62	106.2±12.4				
10	9.13±0.99	91.3±9.9	10.62±0.73	106.20±7.3		
15	16.22±0.52	108.1±3.5	15.6±0.95	104.60±6.3		
30	25.91±2.11	86.4±7.1	29.01±1.69	96.70±5.6	20.18±0.31	67.2±1.0
50	48.37±1.80	96.7±3.6	51.29±0.31	102.58±0.6	37.93±0.61	75.9±1.2
100			107.70±0.57	107.70±0.6		

All data are given as mean±S.D. for $n=5$, for the dialysate $n=3$.

Table 4
Intra- and inter-day variability of the analytical procedure

Day	*n*	FO (μg/ml)	AO (μg/ml)	DO (μg/ml)
Plasma				
1	5	29.76±2.67	29.38±2.22	29.85±3.07
2	5	30.14±2.99	27.43±1.20	30.86±1.95
3	5	29.87±0.91	29.15±1.64	28.97±3.42
4	5	27.89±0.64	30.78±0.96	29.08±2.84
5	5	30.44±0.41	30.22±0.44	31.24±1.13
Mean±S.D.		29.62±1.52	29.39±1.92	29.95±2.48
Urine				
1	10	32.48±0.26	30.54±0.30	28.08±0.20
2	10	29.56±0.47	31.17±0.51	31.08±0.57
3	10	30.83±1.87	29.09±1.11	27.26±0.57
4	10	30.19±0.60	30.04±0.97	30.97±0.59
5	10	31.28±0.47	30.27±1.54	30.27±1.54
Mean±S.D.		30.87±0.73	30.22±0.89	29.43±0.46

Samples were spiked with a concentration of 30 μg/ml for each analyte. Plasma was analyzed after solid-phase extraction, urine was diluted 10-fold prior to analysis.

plasma with acceptable reproducibility were 1 μg/ml for FO and 5 μg/ml for both AO and DO.

3.5. Validation of SPE/HPLC analysis by atomic absorption spectrometry

Plasma samples of patients undergoing DO therapy were analyzed by both methods (HPLC and AAS). The iron content of the resulting FO concentration in plasma was calculated and compared to the iron content detected by AAS.

The resulting correlation was linear for 12 samples with a correlation coefficient of 0.936 and a slope of 1.19, thus indicating fairly well, coincidence of the two methods (data depicted in Fig. 6). Aluminium could not be detected in these samples either by HPLC or by AAS.

3.6. Application to patients samples

Two patients undergoing DO chelation therapy due to hemosiderosis were investigated by the proposed method of analysis. The pharmacokinetics following subcutaneous and intravenous DO application are shown in Figs. 7 and 8.

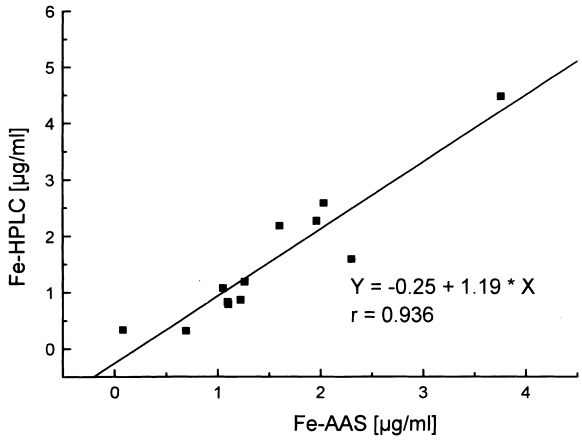

Fig. 6. Comparison of iron content in plasma of patients undergoing desferrioxamine-therapy detected by HPLC and AAS.

3.7. FAB-MS of an iron-binding metabolite

The predominant iron binding metabolite detected in various plasma and urine samples was isolated in a nearly pure state (Fig. 9). FAB analysis of this metabolite showed the presence of a species with a molecular mass of 577 (*m*/*z*) (Fig. 10).

4. Discussion

The new HPLC method allows separation and quantification of DO, FO and AO and further metabolites in one run. Baseline separation without major peak tailing was achieved. The conversion of DO to FO by interference with labile iron from the stainless steel apparatus was quantitatively suppressed. The HPLC method is sensitive and reproducible with no disadvantages in resolution due to the post-column derivatization. The relative broad peaks resulted from the compounds' properties and not from the admixture of the post-column reagent. The methods' applicability to clinical samples is demonstrated. Following rapid intravenous application free DO was detectable in plasma, whereas by means of slow subcutaneous infusion only FO appeared in the plasma, thus indicating a quantitative conversion of DO to it's iron complex in vivo under this condition. Some methodological aspects are discussed in detail:

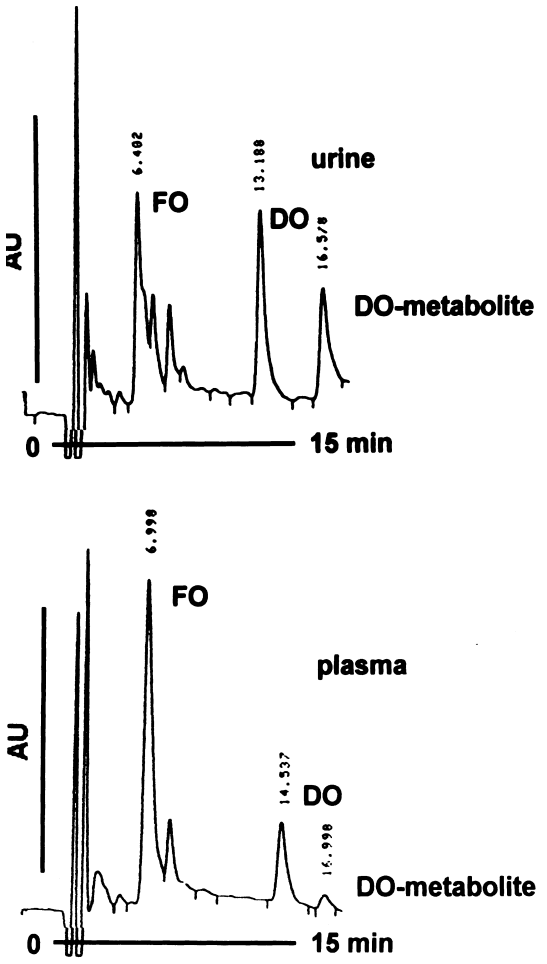

Fig. 7. Time course of DO and FO in plasma following intravenous application of 1.85 g DO in 1 h (a), subcutaneous infusion of 1.65 g DO in 12 h (b), respectively.

4.1. HPLC and post-column reaction system

EDTA was used to bind labile iron from the stainless steel equipment. In contrast to Singh et al. [40] or Glennon and Senior [41], in our system EDTA did not or only slightly remove iron from FO, especially at lower pH. Furthermore, the pH of the mobile phase is low enough to effect some decay of the analytes (cf. Fig. 5). As separation of FO and AO starts at a pH of 2 with baseline separation at a pH of 1.5, a pH dependent decay of AO is a putative mechanism of chromatographic separation by this mobile phase. Omission of the ionic pair reagent also abandoned separation of the analytes, indicating the essential role of the heptane sulfonate. FO remains mainly unchanged, as experiments with stopped addition of the derivatization reagent demonstrated, leading to a detectable peak of FO. Quantitative statements concerning the decay of FO during the chromatographic process are not possible, because of alteration of the detector signal after the addition of the post-column reagent, thus not allowing comparison of the peak signal with and without iron(III)–admixture, revealing a putative decay of the chelate. Anyway, overall any decay of the analytes was surpassed by the addition of excess iron to the elution volume of the analytes, thus saturating all chelators with iron and avoiding underestimation of the analytes. In contrast to other proposed methods, our system allows the detection of the analytes without use of dual detectors or use of radioactivity thus rendering it more suitable for routine analysis. A slightly inconvenience is the quadratic characteristics of the calibration for AO and DO. This is strongly due to the reaction kinetics of the reaction between iron and the chelators and a short connection to the detector. As for the reaction of DO with iron a second-order kinetic is described [51], and, secondly, the release of iron(III) out of the fluoride-complex is likely to be very fast and thus not rate-determining, therefore our data are readily explainable. Increase of residence time of the reaction mixture would have eliminated the influence of the reaction kinetics, but would have also increased peak broadening due to diffusion. From principal aspects, our system is a contradictory example to the general statement, that in post-column derivatization conditions are not suitable to finish the reaction quantitatively [52–54]. This strongly depends upon the underlying reaction kinetics and has to be evaluated for the used system. As the peaks of the analytes were rather broad in spite of the use of a small particle stationary phase and addition of modifiers to the mobile phase, we have used the system as described.

Comparing the chromatograms of the urine sample with the plasma sample of the patient one can realize some shift in retention times of DO. This point is to our opinion due to the different specimen matrices used (direct injected diluted urine vs. extracted plasma). The composition of the samples differ thus

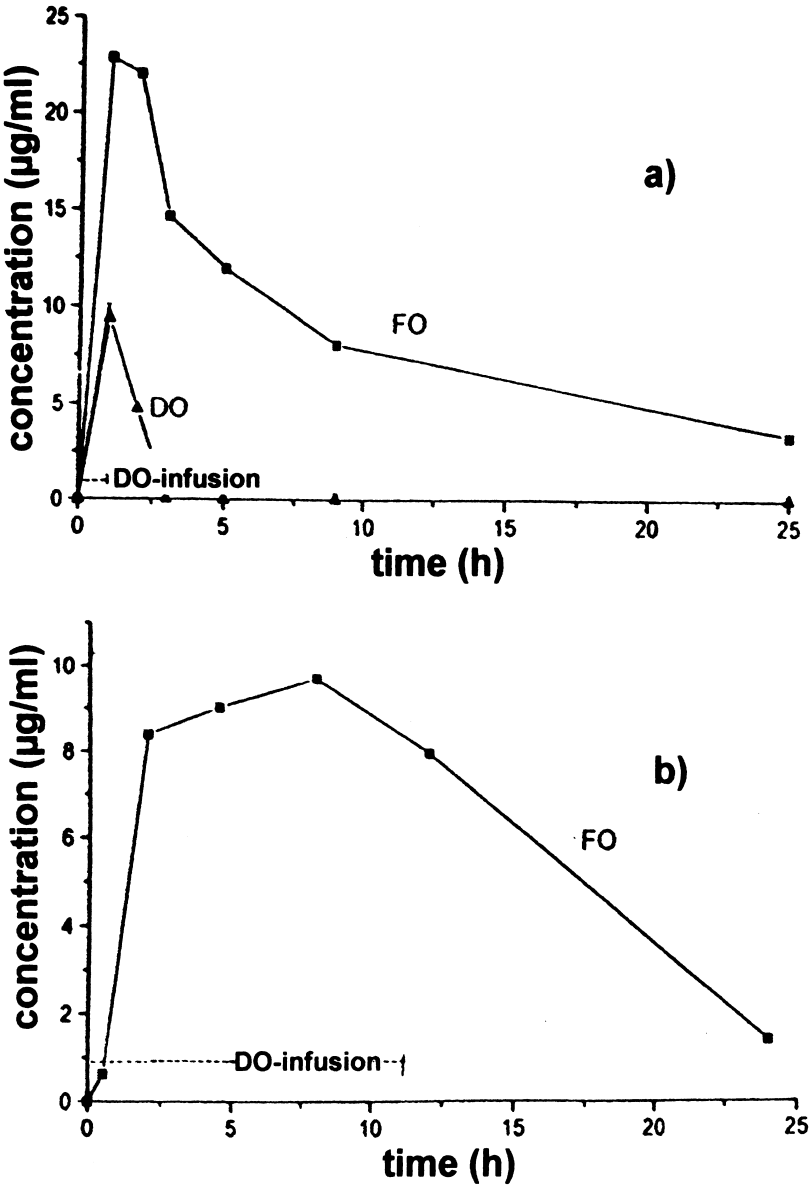

Fig. 8. Chromatograms of plasma and urine of a patient following intravenous DO-therapy (1.85 g DO/1 h).

influencing the retention behaviour of the analytes. But this point is, in our opinion, not very crucial, as the detection is rather specific and quantitative analysis is done with external standardization from the corresponding matrices, so any shift in retention time also affects these calibration samples excluding an influence on the quantitative analysis. The same argument is valid concerning the shift of retention times of the isolated metabolite and the original urine sample.

4.2. Solid-phase extraction

SPE efficacy was shown to be poor at low pH values, thus resulting in bad recoveries of the metal-chelates. Therefore stabilization of the sample pH

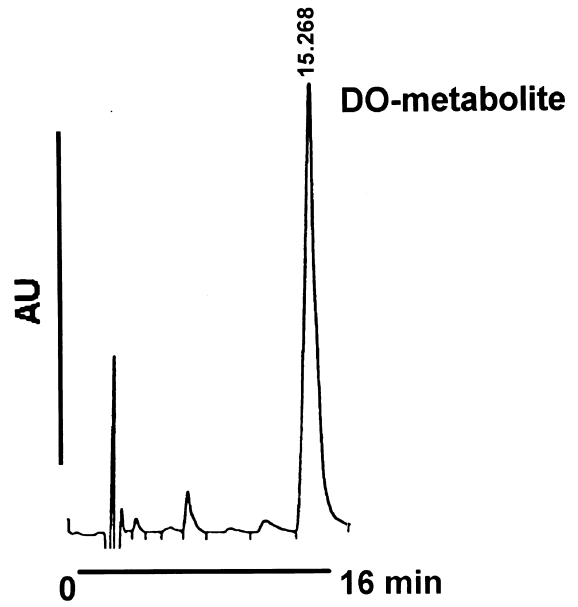

Fig. 9. HPLC chromatogram of the purified iron-binding metabolite.

prior to extraction by addition of sufficient buffer is noteworthy for efficient recoveries.

Concerning the elution of the solid-phase columns with an acidic medium (containing 5% acetic acid) we found no decay under the described conditions. The acetic acid and the water were added after trials with pure methanol resulted in only poor recoveries of the analytes. As the red-coloured FO leads to a visible absorption zone on the loaded columns, the process of desorption could be easily followed up by

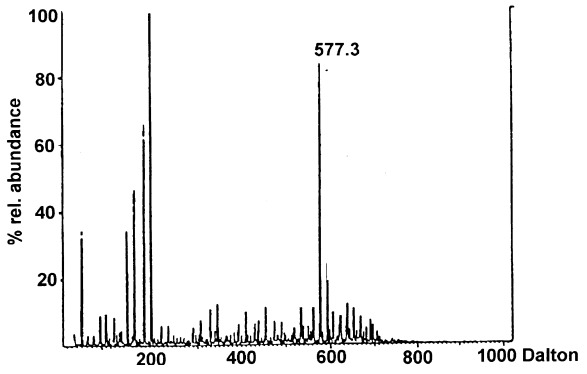

Fig. 10. FAB mass spectrum of the purified iron-binding metabolite.

vision. The amount of acetic acid was stepwise increased to the minimum amount necessary for immediate desorption of the coloured zone. We conclude from this behaviour of the analytes, that they are absorbed on the column in the free base form due to the samples' pH of 9, thus having the most lipophilic properties. Elution with the slightly acidic methanol–water mixture results in a protonisation of the free bases and also in a protonisation of the columns' free cation-exchange moieties, which were also in the proton-free state after absorption of the analytes.

Overall, the acidic component of the elution buffer is partly neutralized and thereafter no decay of the analytes was detectable.

Recoveries from dialysate are included in Table 3. Only one or two concentrations were investigated because preliminary studies with dialysate from patients undergoing desferrioxamine-therapy prior to dialysis (bolus of 500 mg i.v.) had shown no measurable occurrence of the drug in the dialysate under these condition. However, the data were reproducible enough to be included in the manuscript, for documentation of extraction efficiency from larger specimen volumes and for application under other conditions (higher dosage, other application).

Internal standardization would enhance the stability of the analytical method, but we failed to find an appropriate standard. A number of acylated and alkylated derivatives were tested for use as internal standard, but their retention times were too long as compared to the analytes (over 25 min) or they coelute with the analytes (e.g., acetylated DO). The use of iron complexes of different derivatives (modified Schotten–Baumann reaction for acylation and Eschweiler–Clarke- or Michael-reaction for alkylation) was not practicable, since their affinity to iron was too low as compared to unsubstituted DO thus leading to conversion of original iron free DO to FO. Therefore, external standardization was used.

In the plasma and urine samples of one patient an iron-free metabolite was detected which not been described before. Isolation and purification of this metabolite allowed us to perform FAB-MS for further characterisation of this compound. The molecular mass (M_r) of the compound was found to be 577 Da, thus indicating an additional functional

group of molecular mass 16 compared to DO (M_r 561). This change is in accordance to the previously described metabolic pathways of DO, one of them resulting in *N*-hydroxylation of the amino group [40]. Characteristically, this metabolite was only detectable in that patient undergoing intravenous application of DO, which was followed by an interim appearance of free DO in plasma.

Our proposed analytical method seems to be a useful tool in further investigations of the pharmacokinetics and metabolic fate of DO. The method is applicable for drug monitoring in patients undergoing regular therapy with DO.

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References

[1] H. Bickel, E. Gäumann, W. Keller-Schierlein, V. Prelog, E. Vischer, A. Wettstein, H. Zähner, Experientia 16 (1960) 129.
[2] H. Bickel, R. Bosshard, E. Gäumann, P. Preusser, E. Vischer, W. Voser, A. Wettstein, H. Zähner, Helv. Chim. Acta 43 (1960) 2118.
[3] H. Bickel, G.E. Hall, W. Keller-Schierlein, V. Prelog, E. Vischer, A. Wettstein, Helv. Chim. Acta 43 (1960) 2129.
[4] G. Schwarzenbach, K. Schwarzenbach, Helv. Chim. Acta 46 (1963) 1390.
[5] G. Anderegg, F. L'Eplattenier, G. Schwarzenbach, Helv. Chim. Acta 46 (1963) 1400.
[6] G. Anderegg, F. L'Eplattenier, G. Schwarzenbach, Helv. Chim. Acta 46 (1963) 1409.
[7] M.J. Pippard, Birth Defects 23 (1988) 35.
[8] R.N. Matthews, Birth Defects 23 (1988) 117.
[9] N. Lerner, F. Blei, F. Bierman, L. Johnson, S. Piomelli, Am. J. Ped. Hematol./Oncol. 12 (1990) 56.
[10] H.S. Maurer, J.D. Lloyd-Still, C. Ingrisano, F. Gonzales-Crussi, G.R. Honig, Am. J. Dis. Child. 142 (1988) 287.
[11] C.H. Hershko, G. Link, A. Pinson, Blood Rev. 4 (1990) 1.
[12] M.J. Pippard, Bailliere's Clin. Hematol. 2 (1989) 323.
[13] A.V. Hoffbrand, B. Wonke, Bailliere's Clin. Hematol. 2 (1989) 345.
[14] E.A. Llados, I. Vallory, R. Girot, M. Payet, P. Houze, R. Bourdon, Eur. J. Pediat. 148 (1989) 503.
[15] J. Kovarik, H. Hirschik, H. Graf, W. Woloszczuk, V. Meisinger, W. Linkesch, H.K. Stummvoll, Contrib. Nephrol. 49 (1985) 45.
[16] Ch. Schneider, H. Richter, J.L. Fischer, W. Larcher, Internist 30 (1989) 335.
[17] E. Rocchi, M. Cassanelli, E. Ventura, Br. J. Dermatol. 117 (1987) 393.
[18] A.C. Alfrey, G.R. Legendre, W. Kaehny, New Engl. J. Med. 294 (1976) 184.
[19] H.L. Elliot, A.I. Macdougall, G.S. Fell, Lancet, June 3 (1978) 1203.
[20] P. Ackrill, A.J. Ralston, J.P. Day, K.C. Hodge, Lancet, Sept. 27 (1980) 692.
[21] P. Ackrill, J.P. Day, Clin. Nephrol. 24 (1985) 94.
[22] D.S. Milliner, G. Hercz, J.H. Miller, J.H. Shinaberger, A.R. Nissenson, J.W. Coburn, Kidney Int. 29 (1986) 100.
[23] B. Winterberg, A.E. Lison, H.P. Bertram, C. Spieker, H. Kellinghaus, H. Zumkley, Trace Elem. Med. 1 (1984) 111.
[24] L.G. Weiss, B.G. Danielson, B. Fellström, B. Wikström, Nephron 51 (1989) 325.
[25] P. Altmann, F. Marsh, D. Plowman, J. Cunningham, Lancet, May 7 (1988) 1012.
[26] H. Keberle, Pharma-Doku-Zentrale Ciba Geigy, 1963.
[27] P. Allain, Y. Mauras, G. Beaudeau, P. Hingouet, Analyst 111 (1986) 531.
[28] P.C. D'Haese, L.V. Lamberts, M.E. De Broe, Clin. Chem. 35 (1985) 884.
[29] S.M. Cramer, B. Nathanael, C. Horvath, J. Chromatogr. 295 (1984) 405.
[30] G. Matiello, F. Rizzo, R. Zoccolan, M. Andriani, Trace Elem. Med. 2 (1985) 1490.
[31] T.P.A. Kruck, W. Kalow, J. Chromatogr. 341 (1985) 123.
[32] A. Van Der Horst, P.N.F.C. De Goede, H.J.J. Willems, A.C. Van Loenen, J. Chromatogr. 381 (1986) 186.
[33] H. Hughes, L.E. Hagen, E.C. Cameron, R.A.L. Sutton, Clin. Chim. Acta 157 (1986) 115.
[34] S. Venkataram, Y.E. Rahman, J. Chromatogr. 411 (1987) 494.
[35] T.P.A. Kruck, K. Teichert-Kuliszewska, J. Chromatogr. 433 (1988) 297.
[36] H.B. Jenny, H.H. Peter, J. Chromatogr. 438 (1988) 433.
[37] J. Gower, G. Healing, C. Green, Free Rad. Res. Commun. 5 (1989) 291.
[38] J.D. Gower, G. Healing, C.J. Green, Anal. Biochem. 180 (1989) 126.
[39] A. Tesoro, J.S. Leeder, Y. Bentur, J. Klein, M. Freedman, G. Koren, Ther. Drug Monit. 11 (1989) 463.
[40] S. Singh, R.C. Hider, J.B. Porter, Anal. Biochem. 187 (1990) 212.
[41] J.D. Glennon, A.T. Senior, J. Chromatogr. 527 (1990) 481.
[42] S. Sing, N. Mohammed, R. Ackerman, J.B. Porter, R.C. Hider, Anal. Biochem. 203 (1992) 116.
[43] J.B. Porter, E.R. Huehns, Bailliere's Clin. Haematol. 2 (1989) 459.
[44] A. Cases, J. Kelly, F. Sabater, A. Torras, M.C. Grino, J.L. Lopez-Pedret, L. Revert, Nephron 56 (1990) 19.
[45] S. De Virgilis, M. Congia, M.P. Turco, F. Frau, C. Dessi, F. Argiolu, R. Sorcinelli, Arch. Dis. Child. 63 (1988) 250.

[46] R. Albera, F. Pia, B. Morra, M. Lacilla, L. Bianco, V. Gabutti, A. Piga, Audiology 27 (1988) 207.
[47] H. Pall, D.R. Blake, P. Winyard, J. Lunec, A. Williams, P.A. Good, E.E. Kritzinger, A. Cornish, R.C. Hider, Br. J. Ophthalmol. 73 (1989) 42.
[48] M.H. Freedman, M. Boyden, M. Taylor, B. Skarf, Toxicology 49 (1988) 283.
[49] S. De Virgilis, M. Congia, F. Frau, F. Argiolu, G. Diana, F. Cucca, A. Varsi, G. Sanna, G. Podda, M. Fodde, G. Pirastu, A. Cao, J. Pediatr. 113 (1988) 661.
[50] M. Ryba, Chromatographia 15 (1982) 227.
[51] D.J. Lentz, G.H. Henderson, E.M. Eyring, Mol. Pharm. 6 (1973) 514.
[52] R.W. Frei, H. Jansen, U.A.Th. Brinkman, Anal. Chem. 57 (1985) 1529A.
[53] R.W. Frei, A.H.M.T. Scholten, J. Chromatogr. Sci. 17 (1979) 152.
[54] G. Schwedt, Angew. Chem. 91 (1979) 192.