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Reversed-phase high-performance liquid chromatography of non-transferrin-bound iron and some hydroxypyridone and hydroxypyrone chelators

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Abstract

The pursuit of orally available Fe(III) chelating agents has resulted in several clinical trials of 1,2-dimethyl-3-hydroxypyrid-4-one (CP20). Chromatography of this and related Fe chelators on silica-based columns has proven difficult due to unwanted interactions with the stationary phase, including with contaminating Fe bound to silanol groups. By addition of Fe^{3+} (50 μM ferric ammonium citrate) to an acidified aqueous mobile phase, we have successfully separated a series of hydroxypyridones–including CP20–and the related pyrones maltol and ethylmaltol by HPLC on μBondapak C_{18}. Complexation occurs with these agents even at low pH, and they elute in an order consistent with the partition coefficients of their Fe(III) complexes. By the reverse strategy of adding ethylmaltol to the mobile phase, chelatable Fe was chromatographed and the peak response at 500 nm was linear down to a detection limit below 0.5 μM. This method was applied to pooled serum and to serum spiked with Fe after filtration at 10 kDa cut-off. The direct determination of non-transferrin-bound Fe at micromolar concentrations in serum is possible with this approach.

1. Introduction

Excessive accumulation of metal ions may require chelation therapy. In patients with an Fe overload caused by repetitive blood transfusions for the treatment of thalassemia, chelation with desferrioxamine is a prominent feature of the medical treatment. Interest in 3-hydroxypyrid-4-ones as orally available and affordable alternatives to desferrioxamine has prompted several clinical trials of the 1,2-dimethyl derivative (1,2-dimethyl-3-hydroxypyrid-4-one; also referred to as deferiprone, L1, and CP20) [1–5]. Iron is normally transported in the circulation as a stable complex with transferrin. One concern in Fe overload is that saturation of transferrin results in a low molecular mass, exchangeable pool of non-transferrin-bound Fe (NTBI) that is capable of generating harmful oxygen-centered radicals through the Fenton reaction [6,7]. Removal of NTBI with chelators such as CP20 is one of the goals of chelation therapy. The occurrence of NTBI is no longer in doubt [6,8,9] although its chemical nature remains to be elucidated [8,10,11]. Moreover, development of

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methods for NTBI measurement depends upon its definition.

Chromatographic methods for Fe measurement may be useful in biological samples where removal of matrix interferences is a prerequisite to measurement at low detection limits [12]. Ion chromatographic methods such as ion-exchange [12–15] and–less frequently–reversed-phase chromatography with complexing agents in the mobile phase [13,16–20] have been used. The bidentate 3-hydroxypyrid-4-ones, owing to their high affinity for Fe(III), have proven useful for Fe measurement by reversed-phase HPLC. Thus, Singh et al. [21] added nitrilotriacetate (NTA) to serum and separated NTBI from transferrin by ultrafiltration of its NTA chelate. HPLC on Chrom-Spher-ODS with 1-propyl-2-methyl-3-hydroxypyrid-4-one (CP22) then converted Fe to the stronger CP22–Fe chelate, visible at 450 nm. However, Al-Refaie et al. [9] have noted the difficulty of removing contaminating traces of Fe from NTA preparations, a problem we have encountered also. This leads to apparently negative values of NTBI in normal serum. Furthermore, the method depends on the inability of NTA to remove Fe from transferrin under conditions where it fully complexes NTBI. This was achieved in serum from healthy individuals [21]. However, Al-Refaie et al. [9] found significant values of NTBI in serum from thalassemia patients with only partially saturated transferrin, raising the possibility that in the serum of Fe-overloaded individuals NTA accesses Fe from sources other than low-molecular-mass forms capable of binding to apo-transferrin. These possibly include transferrin itself.

Because there is clinical interest in measuring NTBI and the hydroxypyridone chelators, we explored complementary methods of measuring both based on their mutual complexation that avoid the use of other chelating agents such as NTA. To this end, we describe here the chromatographic properties of some 3-hydroxypyrid-4-one and 3-hydroxy-4-pyrone derivatives and their Fe(III) chelates by reversed-phase chromatography with spectrophotometric detection. We have developed a method for quantitating NTBI in human serum based on these compounds, and demonstrate resolution of the compounds themselves on an octadecyl-derivatized silica column.

2. Experimental

2.1. Chemicals

All solvents and reagents used were either of HPLC or analytical grade. Water was obtained from a Milli-Q Organex-Q purification system (Millipore, Bedford, MA, USA). Acetonitrile was purchased from Mallinckrodt (Mississauga, Ont., Canada), ferric nitrate was from BDH (Toronto, Ont., Canada), trifluoroacetic acid and ferric ammonium citrate were from Sigma (St. Louis, MO, USA), nitric acid (GR, max 0.00002% Fe) was from Merck (Darmstadt, Germany), and sodium hydroxide pellets (puriss. p.a.) from Fluka (Ronkonkoma, NY, USA). Maltol (3-hydroxy-2-methyl-4-pyrone) was from Aldrich (Milwaukee, WI, USA) and ethylmaltol (3-hydroxy-2-ethyl-4-pyrone) was from Pfizer (New York, NY, USA). All 3-hydroxypyrid-4-ones were synthesized from the appropriate alkylamine and the corresponding maltol or ethylmaltol with benzoyl ether protection, recrystallized twice, and characterized by melting point and 500 MHz proton NMR as previously described [22]. The 1,2-dimethyl-, 1-ethyl-2-methyl-, 1-methyl-2-ethyl-, and 1,2-diethyl-derivatives are designated CP20, CP21, CP93 and CP94, respectively [23].

2.2. Apparatus

The HPLC apparatus consisted of a Waters 600 multisolvent delivery system with 600 E pumps, a Waters U6K universal liquid chromatograph injector with 2-ml sample loop, and a Waters Lambda-Max Model 481 LC spectrophotometer with output to a Goerz SE120 chart recorder. The stainless steel analytical column (300×3.9 mm I.D.) was packed with 10-μm particle size octadecyl-bonded silica (μBondapak C_{18}) and was in series with a 30×3.9 mm

I.D. guard column (μBondapak C_{18}/Corasil), also from Waters.

2.3. Sample preparation and chromatography

A freshly prepared 1 m*M* Fe stock solution was calibrated by graphite furnace atomic absorption spectrometry and appropriate dilutions were made with the mobile phase to provide standard solutions ranging from 0.1 to 10 μM. Pooled human serum from the Canadian Red Cross blood testing program was stored at −20°C until used. Serum ultrafiltration was achieved using 10 kDa or 30 kDa M_r cut-off filters (Centricon-10 or Centricon-30; Amicon, Beverly, MA, USA), prerinsed and spun at 200 *g* twice with 2 ml of water, then with 2 ml of 1 m*M* ethylmaltol in order to remove possible Fe contamination, and finally with an additional 2 ml of water. Two ml of serum were then loaded into the Centricon and spun for 1 h (Centricon-10) or less (Centricon-30) at 4000 *g* at 5°C in a fixed-angle rotor. The filtrate was combined with two volumes of the mobile phase and 250 μl was injected onto the HPLC system. Serum Fe values were corrected for a blank prepared by treating 2 ml water in a similar manner, which usually gave values below 1 μM. Measurement of iron was performed isocratically at a flow-rate of 1.0 ml min^{-1} and ambient temperature. Unless otherwise stated the mobile phase was prepared by dissolving 1 m*M* ethylmaltol in a mixture of trifluoroacetic acid (TFA)–acetonitrile–water (0.04:10:89.96, v/v), pH 2.3. The mobile phase was filtered under negative pressure and sparged with helium during chromatography.

3. Results and discussion

3.1. Chromatographic behaviour

Epemolu et al. [24] noted difficulties in the chromatography of CP20 on ODS-bonded silica, with peak broadening and asymmetry. Similar problems were encountered with mimosine [25] and 3-hydroxypyrid-4-one [26]. Contributing factors probably include interaction of the chelator with free silanol groups in the stationary phase, as well as with Fe sequestered by the silica and originating from the buffers or stainless steel parts of the chromatographic system. Improvements in peak shape with addition of EDTA or CP20 itself to the mobile phase [24] support these ideas. Furthermore, the use of silica columns with high carbon loading and non-silica based columns also improved the chromatographic behaviour [24]. Ion-pairing agents allowed separation of CP20 on a silica-based reversed-phase HPLC column [27], but resolution was poor and several 3-hydroxypyrid-4-one derivatives could not be separated.

We considered whether addition of Fe^{3+} to the mobile phase would overcome the above problems of reversed-phase chromatography of these agents by masking the effects of column-bound Fe and minimizing non-specific adsorption/desorption effects. Addition of 50 μM ferric ammonium citrate to a mobile phase of 0.04% TFA in water proved satisfactory for separation of the N- and C2-substituted hydroxypyridinone chelators (Fig. 1). Under these conditions of excess Fe and pH < 3 the predominant species with CP20 is the mono complex (ligand:Fe 1:1) [28]. Because the pK_a values and Fe complex stabilities are similar for CP20, CP21, CP93, and CP94 (Table 1) the speciation of each system is expected to be similar. Increasing the concentration of Fe in the mobile phase will not change this speciation, and retention is independent of the concentration of ferric ammonium citrate (Table 2). The retention times measured here parallel the octanol:water partition coefficients of the neutral $(ligand)_3Fe$ complexes (Table 1). Again, the similarities in the proton and metal stability constants of the CP compounds predict the same order of partition coefficients in the less fully coordinated species, consistent with hydrophobic interactions dominating retention on the column. Elution of CP93 before CP21 implies that N-substitution is more important than C2-substitution in determining phase partitioning. This may reflect the orientation of the N-substituents away from the β-hydroxy ketone functionality. Increasing the pH increases the re-

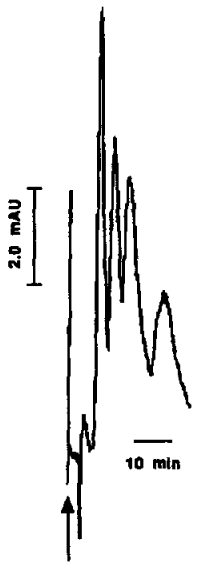

Fig. 1. Separation of hydroxypyridones on μBondapak C_{18} in the presence of Fe^{3+}. Chromatographic conditions are as in Table 1, note [d], with monitoring at 280 nm. One hundred microliters of a solution with 2 μM of each chelator was injected at the arrow. Peaks eluting after 8 min are, from left to right, CP20, CP93, CP21, and CP94.

tention time of CP20 (Table 3) as more of the neutral 3:1 complex is formed [28]. Above pH 4, addition of acetonitrile or methanol is required to effect elution.

Table 2
Dependence of retention times of Fe and CP20 on concentration of complexing agent in the mobile phase

CP20 chromatography		Iron chromatography	
[Fe] (m*M*)	Retention time (min)	[Ethylmaltol] (m*M*)	Retention time (min)
0	13	0.2	11
0.05	11	1.0	9
0.1	11	2.0	10
0.2	11		
0.5	11		

CP20 (250 μl of a 1 μM solution) was chromatographed in 0.04% TFA containing various amounts of ferric ammonium citrate. Iron (25 μl of a 17 μM solution) was chromatographed in 0.04% TFA–10% acetonitrile containing different concentrations of ethylmaltol. In both cases, the flow-rate was 0.8 ml min^{-1} and the pH was 2.4. In this experiment, CP20 was monitored at 280 nm and Fe-ethylmaltol was monitored at 310 nm.

The chromatographic characteristics of the Fe chelates of the above compounds suggested that the complementary approach of adding chelator to the mobile phase would be useful for measuring Fe by silica-based reversed-phase chromatography. The same dependence of retention time on complex partition coefficient is seen when an Fe-containing sample is chromatographed with

Table 1
Properties and retention characteristics of some Fe chelates

Compound	K_{part}(Fe)[a]	pK_{a1}[b]	logβ_3[c]	Retention time (min)	
				Chelator[d]	Iron[e]
CP20	0.0009	3.84	36	8	17
CP21	0.03	3.79	36	16	–
CP93	0.03	3.74	36	12	43
CP94	0.07	3.93	36	23	>100
Maltol	–	5.39	28.5	–	30
Ethylmaltol	–	–	–	–	45

[a] Partition coefficient between *n*-octanol and Tris-HCl at pH 7.4. From Ref. [23].
[b] Dihydroxypyridinium pK_a values from Ref. [22]. Maltol pK_a from Ref. [31].
[c] Stability constants of iron complexes with hydroxypyridones from Ref. [23] and of maltol from Ref. [31].
[d] Retention time following injection of 100 μl of 2 μM chelator: mobile phase 0.04% TFA–99.96% H_2O–50 μM ferric ammonium citrate, pH 2.4. Flow-rate 1.0 ml/min, λ = 280 nm.
[e] Retention time following injection of 100 μl of 10 μM Fe: mobile phase 0.04% TFA–99.96% H_2O–400 μM chelator, pH 2.4. Flow-rate 1.0 ml/min, λ = 500 nm.

Table 3
pH Dependence of CP20 and Fe retention times

pH	CP20 retention (min)	Fe retention (min)
1.95	–	8
2.28	–	9
2.40	11	–
2.65	–	12
2.75	16	–
4.85	20	–
3.00	29	–
≥4	>120	>120

CP20 (250 μl of a 5 μ*M* solution) was chromatographed in 0.2 m*M* Fe as ferric ammonium citrate, while Fe (25 μl of a 17 μ*M* solution) was chromatographed in 10% acetonitrile containing 0.5 m*M* ethylmaltol. In both buffers, the indicated pH was achieved by varying the TFA concentration from 0.02% to 0.1% and adjusting with NaOH as necessary. Other conditions are as in Table 2.

different chelators in the mobile phase (Table 1). Because of the increasing importance of the neutral 3:1 complex in the presence of excess chelator, retention times were increased and addition of 10% acetonitrile was beneficial in achieving shorter times of analysis. Ethylmaltol was chosen because of the better interaction of its Fe complex with the column and its commercial availability. Under these conditions, the earlier elution of CP20 compared to the Fe complex of ethylmaltol (Table 1) means that Fe and CP20 will not give interfering peaks in the presence of excess ethylmaltol; this system could in principle be used to monitor both metal and free chelator.

Both pH and chelator concentration are expected to affect the sensitivity of the method for Fe. At low pH mono- and bis-complexes are favoured, but increasing either the pH or the concentration of the chelator relative to Fe shifts the equilibrium in the direction of the more fully coordinated species. This should also improve the sensitivity as the 3:1 complex has a higher extinction coefficient than the less fully coordinated species. However, as above, the retention time increases with pH. The retention time of Fe is independent of the concentration of the chelator, implying that the speciation does not change with an increasing excess (Table 2). Standard conditions of 10% acetonitrile–0.04% TFA–1 m*M* ethylmaltol, pH 2.3, were adopted for the mobile phase for measuring Fe. Typical chromatograms obtained under these conditions (Fig. 2) show a linear response of peak height to concentration (see below).

Under all conditions, the intensity of the Fe peak recorded at 280 nm was five times that recorded at 500 nm, consistent with the extinction coefficients of the complexes and indicating

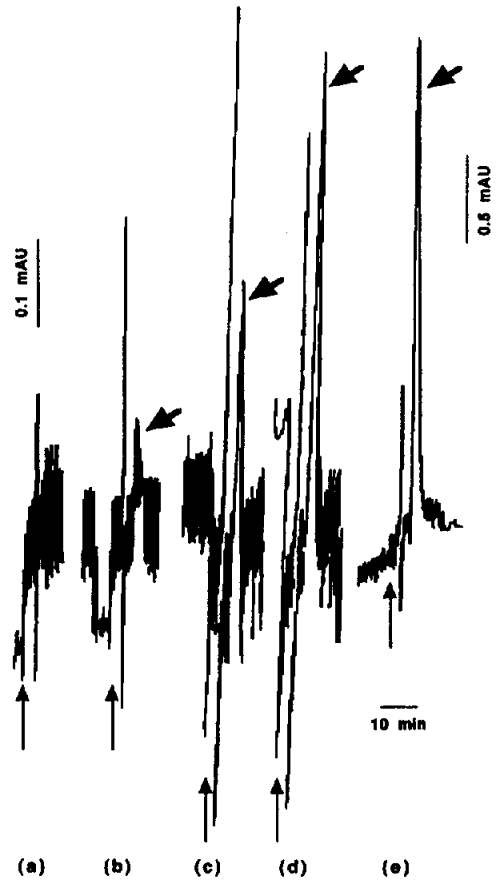

Fig. 2. Chromatograms of ferric ammonium citrate eluted with ethylmaltol. Injection volume was 250 μl in all cases. Isocratic elution at 1.0 ml/min with 0.04% TFA–10% acetonitrile–1 m*M* ethylmaltol (pH 2.3) was monitored at 500 nm. Vertical arrows indicate the point of injection, fat arrows mark the Fe chelate peak. Full scale deflection was 1 mAU for (a) to (d) and 5 mAU for (e). Iron concentrations were (a) 0.0 μ*M*, (b) 0.1 μ*M*, (c) 0.5 μ*M*, (d) 1 μ*M*, (e) 5 μ*M*.

that greater sensitivity could be achieved monitoring in the UV. However, the lower wavelength is more prone to interference in biological samples, and when chelator is included in the mobile phase monitoring at the lower wavelength is precluded by excessive background absorption.

3.2. Analytical performance for Fe

A signal-to-noise ratio of 4 was obtained at 0.5 μM Fe. From 32 samples (1–5 μM Fe) measured three to five times each over two months, a mean long-term imprecision of 7.7% (coefficient of variation) was found. Day-to-day reproducibility of the standard curve was good and the response was linear between 0.05 and 5 μM Fe; calibration curves on consecutive days had slopes of $(5.24 \pm 0.09) \cdot 10^5$ and $(5.39 \pm 0.15) \cdot 10^5$ mAU M^{-1}, with intercepts of (0.109 ± 0.018) and (0.053 ± 0.032) mAU, respectively (S.E. of estimate = 0.062, $r = 0.997$, $n = 21$ on day 1; S.E. of estimate = 0.093, $r = 0.996$, $n = 14$ on day 2). CP20 elutes well before the ethylmaltol peak and does not interfere with the measurement when present at five times the concentration of Fe. Copper and zinc at five times the concentration of Fe also did not interfere. Although equimolar Cu displaces Fe from CP20 at neutral pH, facilitated by formation of Fe hydrolysis products [29], the low pH and excess of chelator in the present study prevent this.

The 70 kDa serum protein transferrin effectively sequesters exchangeable Fe, preventing its toxicity to the organism. Except in cases of pathological Fe overload, transferrin is present in excess in human plasma and NTBI should be minimal or absent. When normal serum was filtered at 10 kDa cut-off and then spiked with Fe, recovery was excellent between 0.1 and 10 μM. Calibration curves constructed with Fe dissolved in filtered serum or mobile phase containing 2 mM ethylmaltol, pH 2.1, had slopes of $(7.48 \pm 0.21) \cdot 10^5$ and $(7.26 \pm 0.10) \cdot 10^5$ mAU M^{-1}, with intercepts of (0.33 ± 0.10) and (0.21 ± 0.05) mAU, respectively (S.E. of estimate = 0.175, $r = 0.998$, $n = 6$ in serum; S.E. of estimate = 0.092, $r = 1.00$, $n = 6$ in mobile phase). A value of 0.5 μM Fe was measured in the pooled serum, near the detection limit of the method with the noise of the present detector. This noise level seems typical of optical detection and therefore limits the usefulness of this approach for measuring NTBI in serum from healthy individuals. It should be noted that electrochemical detection should significantly improve this detection limit [30] and facilitate application of the method to a reference population. Nevertheless, in situations of clinical Fe overload where measurement of NTBI is generally undertaken, values several-fold higher and even up to 10 μM are observed [9].

When the pooled serum was spiked with ferric ammonium citrate prior to filtration, no excess Fe was recovered from spikes of less than 15 μM, representing therefore the transferrin saturation point in this sample. Recovery of excess Fe above this point was linear. Identical amounts of Fe were measured when a 30 kDa cut-off filter was used for spiked or unspiked samples. Therefore, 0.5 μM NTBI recovered in the 10 kDa filtrate represents the total pool of Fe that is not sequestered in higher molecular mass proteins. Addition of chelating agents like NTA to the sample seems unnecessary to recover this pool, and the risks of direct sample contamination and removal of Fe from high-molecular-mass sources are avoided.

4. Conclusions

A reversed-phase HPLC method has been described for chromatography of the Fe complexes of some 3-hydroxypyr(id)-4-ones on μBondapak C_{18}. Addition of Fe to the mobile phase allows separation of the chelators as their Fe complexes, and use of chelator in the mobile phase has provided the basis for an analytical method for micromolar concentrations of non-transferrin-bound Fe in serum. This fraction can be measured following direct ultrafiltration and dilution in the mobile phase.

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