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Analysis of ^{55}Fe-labeled hydroxamate siderophores by high-performance liquid chromatography

ROBERT J. SPEIRS and GREGORY L. BOYER*

Faculty of Chemistry, State University of New York, College of Environmental Science and Forestry, Syracuse, NY 13210-2786 (U.S.A.)

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ABSTRACT

The radiolabeled iron(III) chelates of four different hydroxamate siderophores (desferrioxamine B, rhodotorulic acid, desferriferrichrome and desferriferrichrome A) were analyzed by high-performance liquid chromatography using an absorbance or radioactivity detector. Optimal conditions consisted of a polymeric PRP-1 column and a water to acidified acetonitrile gradient as mobile phase. Silica based columns gave similar resolution, but adsorption of unchelated iron-55 to the silica support limited the desirability of this approach. Detection limits based on radioactivity of the labeled compounds were consistently two to three orders of magnitude greater than that observed using visible absorbance at 436 nm. This method provides a generalized approach for the detection of high affinity iron(III) chelators (*e.g.* siderophores), regardless of their structural type.

INTRODUCTION

Iron, an essential trace metal for the growth of microorganisms, is an element of low biological availability. To overcome this problem, blue-green algae, bacteria, and fungi secrete high affinity iron chelators termed siderophores [1–3]. These compounds play a dual role in microbial ecology. First, they solubilize needed ferric iron which is then absorbed by the cell through specific uptake receptors [4]. Second, they may tie up iron in a form that is unavailable to competing species [5,6]. An understanding of the biochemical ecology of these compounds is essential for our understanding the biochemical ecology of natural systems.

Siderophores exist in a wide variety of structural types [3]. They are usually divided into two groups depending on the nature of their iron-binding ligand: the catechol siderophores (*e.g.* enterobactin), and the hydroxamate siderophores (*e.g.* desferriferrichrome, rhodotorulic acid and desferrioxamine B). These ligands also serve as the basis for the common chemical tests for the detection of siderophores; the Arnow test for catechols and phenolics [7] and the Csaky test for hydroxamates [8]. Recently, several important siderophores have been elucidated that do not fall into either of these structural categories. These include phytosiderophores (*e.g.* mugineic acid [9]) produced by graminaceous plants and rhizobactin produced by the nitrogen

fixing bacterium, *Rhizobium meliloti* [10,11]. Lacking either a hydroxamate or phenolic ligand, these compounds are not detected using the classical chemical assays. Thus there is considerable interest in developing a general procedure for the detection of strong iron-binding compounds.

High-performance liquid chromatography (HPLC) has been used for the separation and analysis of hydroxamate [12,13] siderophores. These compounds are best detected by monitoring the rose-colored ferric complex at approximately 425–440 nm [14]. Detection of the iron complexes at lower wavelengths, such as 220 nm, works well with purified compounds, but may be too non-specific for use in natural samples [15]. Fewer reports have appeared on the use of HPLC for the detection of phenolic-based or mugineic acid-like siderophores. Lacking a distinctly colored iron complex, the phytosiderophores are more difficult to distinguish from interfering compounds.

We report here an HPLC-based assay for the general analysis of siderophores. A common feature of these compounds is their ability to strongly bind iron, hence detection in this procedure is by liquid scintillation counting of the ^{55}Fe-labeled iron complex. While this work focuses on its use for the analysis of hydroxamate siderophores, the technique provides a general approach for the analysis of all siderophores and strong iron-binding compounds, regardless of their structural type.

EXPERIMENTAL

Materials

Rhodotorulic acid (RA) was isolated from iron-limited cultures of *Rhodotorula pilimanae* [16]. Ferrichrome (FC) and ferrichrome A (FCA) were isolated from iron-limited cultures of *Ustilago sphaerogena* [17] using published procedures and identified on the basis of their spectroscopic and thin-layer chromatographic behavior. Desferrioxamine B mesylate salt (Desferal, DES) was a gift from Ciba-Geigy (Suffern, NY, U.S.A.). [^{55}Fe]iron(III) chloride in 0.1 *M* hydrochloric acid (80 MBq/mg Fe) was purchased from Amersham (Arlington Heights, IL, U.S.A.). Methanol was glass distilled prior to use. Acetonitrile, Scintiverse LC, and other chemicals were HPLC grade or better and purchased from Fisher Scientific (Rochester, NY, U.S.A.).

Equipment

Our HPLC system consisted of two Waters Assoc. (Milford, MA, U.S.A.) M6000A pumps and a Model 660 gradient controller. The system was equipped with a Rheodyne 7125 injector and a Waters Assoc. Model 440 fixed-wavelength detector using a 436-nm filter. No effort was made to use a metal-free system or components. For the determination of radioactivity, 1-min samples were collected using a Gilson F-80 fraction collector (Gilson, Madison, WI, U.S.A.) connected to the outlet of the UV–VIS detector. These fractions were subsampled, mixed with Scintiverse LC counting cocktail, and then counted in a Packard Tricarb Model 4400 liquid scintillation counter using the preset ^{3}H channel. In some applications, continuous detection of the ^{55}Fe complexes was done using a Radiomatic A-120 HPLC–liquid scintillation counter (Radiomatic Instrument and Chemical Co., Tampa, FL, U.S.A.) equipped with a 0.5-ml flow cell. The column eluent was mixed 1:3 with scintillation fluid prior to the flow cell and total counts recorded at 6 s intervals.

HPLC conditions

The following reversed-phase columns were used; a silica-based μBondapak C_{18} column (Waters Assoc.; 10 μm; 300 × 3.9 mm), a polymeric polystyrene divinyl benzene-based PRP-1 column (Hamilton, Reno, NV, U.S.A.; 10 μm; 150 × 4.1 mm), and a polymeric fluorocarbon-based Poly F column (DuPont, Boston, MA, U.S.A.; 20 μm; 80 × 6.2 mm]. The mobile phase consisted of a 20-min linear gradient from distilled water to either acetonitrile or methanol containing 1% (v/v) glacial acetic acid. Injection volumes were routinely 20 μl. Flow-rates were systematically investigated at 0.5, 1.0 and 2.0 ml/min and 1.0 ml/min was selected as optimal for most applications.

Methods

^{55}Fe complexes of desferal and rhodotorulic acid were formed by adding the iron to the deferrated siderophore. Ferrichrome and ferrichrome A were isolated as their ^{56}Fe complexes and radiolabeled by ^{55}Fe exchange. A 50-μl volume of 1 *M* sodium hydroxide solution was added to 200 μl of a ferrichrome or ferrichrome A solution to dissociate the iron complex. This was followed by 2 μl of ^{55}Fe standard (containing *ca.* 3 kBq ^{55}Fe) and the pH was adjusted back to neutral with 50 μl 1.0 *M* hydrochloric acid. Samples were allowed to stand for 1 h prior to analysis by HPLC.

To determine total recoveries of radioactive iron, 86 pmol total iron (containing 385 Bq ^{55}Fe) with or without excess chelator were injected on the HPLC system and the entire 20-min gradient collected in a round-bottom flask. The eluent was then concentrated by rotary evaporation to 6 ml and counted via liquid scintillation counting. To correct for possible effects on counting efficiency, the control for this experiment consisted of collecting a blank gradient (no injection) and adding ^{55}Fe directly to the flask prior to concentration.

For the determination of standard curves, the ^{55}Fe standard was diluted 1:1000 with 0.1 *M* hydrochloric acid. This solution was mixed with an equal volume of deferrated siderophore solution (final concentration of Fe = 4.3 μ*M*) and 20 μl injected on the HPLC system. Peaks corresponding to the radiolabeled siderophore were collected and counted by liquid scintillation counting. All points on the standard curves were run in triplicate.

RESULTS

The separation of four different hydroxamate siderophores was investigated using three different columns. Only minor differences were observed between their chromatography on the silica-based μBondapak C_{18} column and the polymeric Hamilton PRP-1 column (Table I). Either a 0–50% acidified methanol or 0–30% acidified acetonitrile gradient was suitable to cleanly separate the four iron chelates. Detection could be accomplished by monitoring the absorbance of the colored ferric complex at 436 nm (Fig. 1 top). Use of the smaller Poly F column resulted in earlier elution of the larger siderophore complexes (DES, FC, FCA), however, the rhodotorulic acid iron complex was not retained by the column using these mobile phases.

Siderophores are characterized by having a very high affinity for ferric iron (log of the formation constants for their iron complexes are between 29 and 32, depending

TABLE I

RETENTION TIMES OF FOUR SIDEROPHORES ON THREE DIFFERENT COLUMNS

HPLC conditions: 20-min linear gradient from water to methanol or acetonitrile containing 1% (v/v) glacial acetic acid, 2 ml/min flow-rate. Detection was at 436 nm.

Gradient	Retention time (min)			
	RA	DES	FC	FCA
μBondapak C_{18} (Waters Assoc.)				
0–50% acidified methanol	7.2	16.4	13.6	19.4
0–30% acidified acetonitrile	7.4	15.2	13.4	17.6
PRP-1 (Hamilton)				
0–50% acidified methanol	3.2	12.0	17.0	22.8
0–30% acidified acetonitrile	3.8	8.8	10.4	15.8
Poly-F (DuPont)				
0–50% acidified methanol	1.0	5.2	4.4	9.2
0–15% acidified acetonitrile	1.0	6.4	4.6	13.0

on the individual siderophores [18]). ^{55}Fe is a weak electron-capture radionuclide and can be counted by liquid scintillation counting using techniques similar to those used for tritium. Unlike the γ emitter ^{59}Fe, ^{55}Fe requires only minimal shielding and has a long (2.6 year) half life. While the addition of chelators is sometimes used to improve its counting efficiency [19], this was not necessary in our experiments. Addition of the chelators DES or RA at concentrations between 0 and 100 μg per vial (1000 × molar excess) did not affect the counting efficiency nor was quenching of the ^{55}Fe observed in these experiments (data not shown). Observed counting efficiencies (*ca.* 50%) were close to what we commonly observe for tritium under similar conditions. Thus ^{55}Fe could be used to provide a second method for the detection of the ferric siderophores. The radiochromatogram for these compounds is shown in Fig. 1 (bottom panel).

Recoveries of both chelated and unchelated iron from the three different columns are shown in Table II. The polymeric columns (PRP-1 or Poly F) showed an excellent recovery for both chelated and unchelated iron. In contrast, the silica-based column gave high though inconsistent recoveries for chelated iron but a very low recovery (44%) for the unchelated iron. This missing iron was apparently adsorbed to the silica since it was possible to strip varying amounts of ^{55}Fe off the silica by injecting iron-free EDTA through the column (data not shown).

Standard curves were prepared for each of the four siderophores (RA, DES, FC and FCA) between concentrations of 0 and 100 nmol per 20-μl injection. In each case, the total iron concentration was held constant at 86 pmol per 20-μl injection. A representative plot of the observed radioactivity in the chelator peak versus total chelator concentration for FCA is shown in Fig. 2. At chelator concentration much higher than the fixed iron concentration (Fe_T = 4.3 μM), the curve resembled a saturable curve. At chelator concentrations equal to or less than the iron concentration, observed counts in the chelator peak were linearly dependent on the chelator concentration. For a chelator-to-iron ratio of 1 or less, the correlation

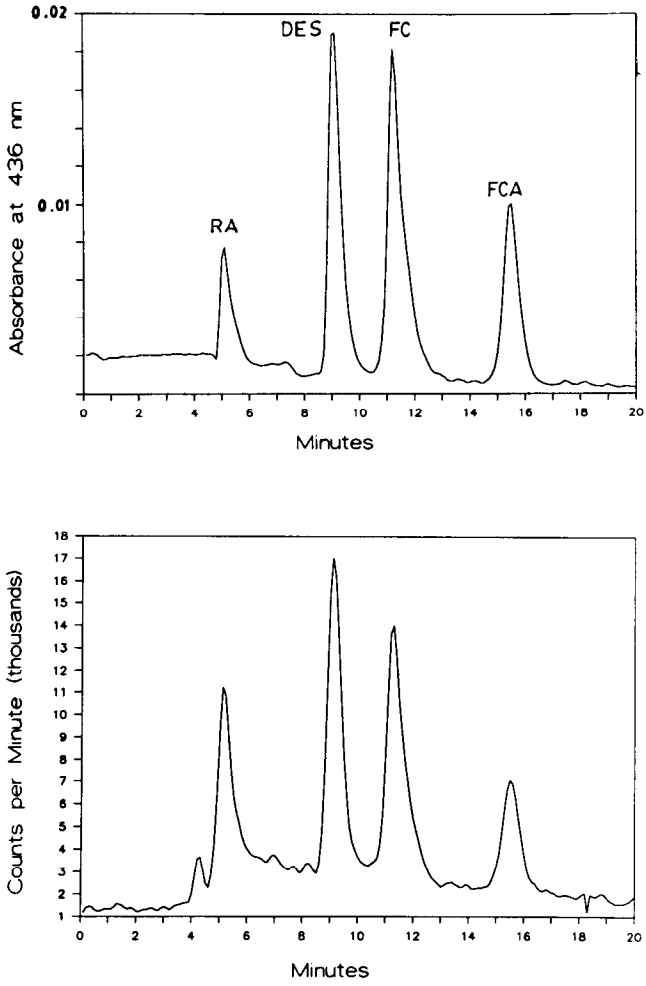

Fig. 1. Chromatograms obtained from a concentrated mixture of ^{55}Fe-labeled siderophores as determined by monitoring absorbance at 436 nm (top) or counts per min (bottom). HPLC conditions consisted of a PRP-1 column and a 20 min 0–50% acidified acetonitrile in water gradient. Flow-rate was 2 ml/min. To simultaneously observe absorbance and radioactivity on the same run, a mixed siderophore standard containing 0.25 m*M* FCA, 0.4 m*M* FC, 0.4 m*M* DES and 0.7 m*M* RA was labeled with ^{55}Fe (385 kBq) and then saturated with excess ^{56}Fe. The ordinate in the top panel is in arbitrary units but represents approximately 0.02 a.u. full scale.

coefficient for FCA was 0.995. This general pattern was true for both DES and FC as long as the total iron concentration was greater than the total chelator concentration (correlation coefficients for DES and FC were 0.985 and 0.994, respectively). At this iron concentration, the detection limit for the three chelators was 10 pmol per 20-μl injection. This limit was well below that observable by absorbance detection. Only at the higher siderophore concentrations (>10 nmol per 20-μl injection) was there a measurable peak at 436 nm. Determination of RA concentrations at these low

TABLE II

RECOVERY OF CHELATED AND UNCHELATED ^{55}Fe FROM THE THREE DIFFERENT COLUMNS AT 1 AND 2 mg/ml CHELATOR CONCENTRATION (± S.D)

Recoveries were determined by counting the entire gradient as described in the *Methods* section. HPLC conditions consisted of a 20-min water-to-acidified acetonitrile gradient at 1 ml/min flow-rate. Molar concentrations: EDTA: 1 mg/ml = 2.7 m*M*; 2 mg/ml = 5.4 m*M*; DES: 1 mg/ml = 1.6 m*M*; 2 mg/ml = 3.3 m*M*; RA: 1 mg/ml = 2.9 m*M*; 2 mg/ml = 5.8 m*M*. Total iron concentration: 4.3 μM.

Column	Unchelated ^{55}Fe	Recovery (%)					
		RA (mg)		EDTA (mg)		DES (mg)	
		1	2	1	2	1	2
PRP-1	85% ±12%	104% ±4%	105% ±5%	91% ±8%	100% ±5%	102% ±11%	101% ±1%
μBondapak C_{18}	44% ±5%	n.d.	n.d.	87% ±7%	116% ±36%	97% ±2%	115% ±7%
Poly F	103% ±21%	101% ±1%	96% ±5%	98% ±3%	92% ±8%	97% ±2%	96% ±7%

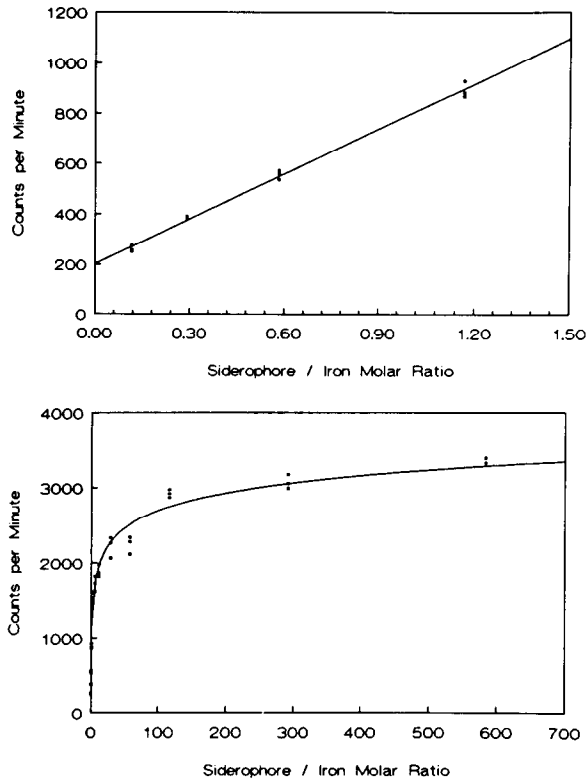

Fig. 2. Counts per min in the ferrichrome A peak as plotted against the siderophore/iron ratio. The total iron concentration was held constant at 4.3 μM. HPLC conditions as in Fig. 1.

concentrations was more difficult due to overlap of the free iron peak with the ferric-RA peak and the fact that RA forms a 2:3 iron–siderophore complex. Its detection limit was generally 10-fold higher than that observed for ferrichrome A. The detection limit of the HPLC assay could be increased by labeling the siderophores with ^{55}Fe of a higher specific activity. In contrast, the linear range of the standard curve could be increased by the addition of cold (^{56}Fe) ferric iron (*e.g.* Fig. 1). This did, however, result in a corresponding loss in sensitivity at low siderophore concentrations.

DISCUSSION

Siderophores may play an important role in microbial ecology. They exist in a wide variety of structural types, thus new methods are needed to detect these compounds in natural systems. Recently HPLC has been applied to the study of these compounds. Jalal *et al.* [12] studied nineteen different hydroxamate siderophores of the ferrichrome type and successfully separated them using a reversed-phase column. This approach was used to study siderophore formation in culture filtrates from four different fungi known to produce hydroxamate siderophores. These complexes all have very strong absorption maxima in the 420–450 nm range and can readily be detected using photodiode array detection. This approach would be less successful for the detection of siderophores such as rhizobactin, mugineic acid and possible unknown siderophores which do not form highly colored complexes. For this reason, the ability to detect the siderophore complex based on their affinity for iron rather than on the spectroscopic properties of the iron complex is desirable. The use of radiolabeled siderophores described here is several orders of magnitude more sensitive than the spectroscopic approach and provides for the quantitative determination of siderophore concentrations provided sufficient iron is present to saturate all chelators. As such, it provides a very general method for the quantitative and qualitative analysis of siderophores.

In a similar approach, Glennon *et al.* [20] used a metal-free system and amperometric detection for the direct analysis of siderophoric iron. This technique has both advantages and disadvantages compared to radiolabeling. For the detection of siderophoric iron, an applied potential of 1.0 V was optimal. This high potential greatly shortens electrode life and required that the electrode be polished after every 70 plus injections. This high potential also limits the chromatographic system to isocratic mobile phases, decreasing the diversity of compounds that can be analyzed in a single run. When applied to their *Pseudomonas fluorescens* cultures, problems of background oxidation of EDTA in the culture media forced Glennon *et al.* to use 0.5 V to work with culture supernatants. In contrast, radiolabeling the siderophores using ^{55}Fe suffers few of these problems. Gradient mobile phases can easily be used to separate complex mixtures of chemically distinct iron complexes. Since detction is of the iron–siderophore complexes, and these complexes are stable on the HPLC time frame, exchange of ^{55}Fe with cold iron that may be present in the chromatographic system was not a problem. This minimizes need for metal-free systems that have been used by other workers [21,22]. Silica-based C_{18} columns and polymeric-based columns (PRP-1) gave comparable resolution. However, there was a marked difference in the recovery of radioactive iron from the two column types. Adsorption of iron to the silica-based columns did occur and could be a potential source of interference, even in the presence

of 1000-fold excess chelator. This was not a problem when polymeric-based columns were used. Others [23] have also reported the use of polymeric columns for the separation of iron and aluminum complexes of desferrioxamine B. While the larger PRP-1 column was generally the preferred of the two polymeric columns, the smaller Poly F based column may be advantageous under limited circumstances. This column would be especially suitable for the quick analysis of ferrichrome analogs due to its short retention times. It may also be useful for the early screening of unknown siderophores due to its high and consistent recoveries of ^{55}Fe.

In summary, HPLC and radiochemical detection after labeling with ^{55}Fe provides a rapid and general assay for the quantitative and qualitative analysis of siderophores based only on their ability to bind iron. Unlike other general assays for siderophore detection [20,24], this approach does not appear to be affected by the presence of other weak metal chelators such as citric acid or EDTA commonly present in culture supernatants [25]. While only its use with hydroxamate siderophores is reported here, it provides a rapid and general method for the detection of a wide variety of different strong iron-binding compounds.

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