Journal of Chromatography, 301 (1984) 247–252
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,897

SEPARATION OF FERRICHROMES AND OTHER HYDROXAMATE SIDEROPHORES OF FUNGAL ORIGIN BY REVERSED-PHASE CHROMATOGRAPHY

M. A. F. JALAL, RAMAN MOCHARLA and DICK VAN DER HELM*
Department of Chemistry, University of Oklahoma, Norman, OK 73019 (U.S.A.)
(Received May 10th, 1984)

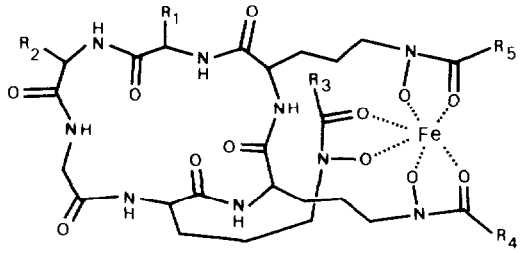
SUMMARY

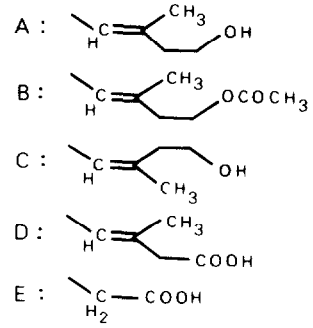
Iron(III) chelates of nineteen trihydroxamate siderophores of fungal origin, including ferrichromes, coprogen and triacetylfusarinine C, were separated on a preparative scale with a reversed-phase column using the octadecyl silica gels LRP-1 or LRP-2 as the stationary phase and a water–methanol gradient as the mobile phase. Using this system in combination with silica gel column chromatography, most siderophores can be obtained in pure form. Factors affecting the mobility of these compounds in the reversed-phase system are discussed.

INTRODUCTION

Microbial siderophores are a unique class of natural compounds that exhibit both hydrophilic and lipophilic properties. These dual properties are needed to carry out their function of (a) chelating iron in the extracellular aqueous environment and (b) transporting iron(III) ions through the outer lipid membrane of the cell with the help of membrane-bound transport proteins[1]. However, the combination of the contrasting physical properties of these compounds together with the small differences in their large molecular structures rendered many of them unamenable to conventional separation methods. We describe here a preparative separation procedure for the siderophores, based on reversed-phase chromatography in combination with silica gel column chromatography. A large number of the compounds used in this study are members of the ferrichrome family (I–XV), which are ubiquitous in fungi[1–3]. Two members of the coprogen family, coprogen (XVI)[4] and neocoprogen I (XVII; X-ray crystal structure to be published soon) are also included. N,N′,N″-Triacetylfusarinine C, which is a member of the fusarinine family, is a cyclic triester of N^{α}-acetyl-N^{δ}-hydroxy-N^{δ}-(*cis*-5-hydroxy-3-methylpent-2-enoyl)-L-ornithine[5,6]. Des(diserylglycyl) ferrirhodin (DDF) is a linear tripeptide made of three units of N^{δ}-hydroxy-N^{δ}-(*cis*-5-hydroxy-3-methylpent-2-enoyl)-L-ornithine (compound I in ref. 3; its structure, based on ^{1}H and ^{13}C NMR spectroscopy, will be published elsewhere).

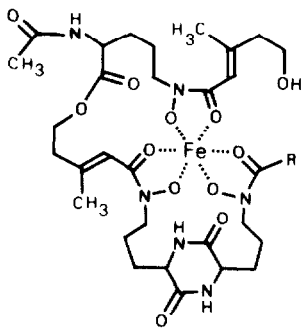
0021-9673/84/$03.00





		R_1	R_2	R_3	R_4	R_5
I	Ferrichrome	H	H	CH_3	CH_3	CH_3
II	Ferrichrome A	CH_2OH	CH_2OH	D	D	D
III	Ferricrocin	H	CH_2OH	CH_3	CH_3	CH_3
IV	Ferrichrysin	CH_2OH	CH_2OH	CH_3	CH_3	CH_3
V	Ferrirubin	CH_2OH	CH_2OH	A	A	A
VI	Asperchrome A	CH_2OH	CH_3	A	A	A
VII	Asperchrome B1	CH_2OH	CH_2OH	CH_3	A	A
VIII	Asperchrome B2	CH_2OH	CH_2OH	A	CH_3*	A*
IX	Asperchrome B3	CH_2OH	CH_2OH	A	A*	CH_3*
X	Asperchrome C	CH_2OH	CH_2OH	B	A	A
XI	Asperchrome D1	CH_2OH	CH_2OH	A	CH_3	CH_3
XII	Asperchrome D2	CH_2OH	CH_2OH	CH_3	A*	CH_3*
XIII	Asperchrome D3	CH_2OH	CH_2OH	CH_3	CH_3*	A*
XIV	Asperchrome E	CH_2OH	CH_2OH	C*	A*	A*
XV	Malonichrome	H*	CH_3*	E	E	E

* Positions not confirmed.



XVI coprogen R = $CH=C(CH_3)-CH_2-CH_2OH$ (*trans*)

XVII neocoprogen I R = CH_3

EXPERIMENTAL

Materials

The iron(III) chelates of the siderophores were isolated and purified by the methods described earlier[3,6,7] from the iron-starved cultures of various fungi grown

in this laboratory: ferrichrome and ferrichrome A from *Ustilago sphaerogena*, ferricrocin and neocoprogen I from *Curvularia subulata*, malonichrome from *Fusarium roseum*, coprogen from *Neurospora crassa*, ferrirubin, ferrichrysin, des(diserylglycyl) ferrirhodin (DDF) and all asperchromes from *Aspergillus ochraceous* and iron(III) N,N′,N″-triacetylfusarinine C from a *Penicillium* sp. The purities of these standard siderophores were established by co-chromatography, X-ray crystallography and ^{1}H and ^{13}C NMR spectroscopy.

Two types of reversed-phase support material, LRP1 and LRP2, were purchased from Whatman. Both are octadecyl (C_{18} bonded) silica gel, but they differ in the particle size and the percentage of C-bonding (LRP-1, 13–24 μm, 12% C; LRP-2, 37–53 μm, 16% C).

Preparation of the column

A Michel–Miller HPLPLC (high-performance low-pressure liquid chromatography) glass column (30 × 2.2 cm I.D., maximum pressure capacity 300 p.s.i.; Ace Glass) was connected via a coupling to an upper reservoir column (35 × 3.7 cm I.D.). The adsorbent was suspended in methanol (500 ml per 100 g), soaked overnight and the supernatant was decanted. The material was resuspended in sufficient solvent (300–400 ml) and the slurry was poured into the column, which was pre-filled with a small volume of the solvent. The outlet of the column was kept open and the packing material was allowed to settle inside the complete length of the lower column. Then the reservoir column was disconnected and a pre-column (13 × 2.2 cm I.D.) was fitted in its place via the coupling. The pre-column was filled with the slurry and when the adsorbant had settled in approximately two thirds of its length, the top of the pre-column was filled with the solvent. A pump was connected to the pre-column and the pressure was slowly increased to 50 p.s.i. so that the solvent attained a flow-rate of 2 ml/min. After passing 2 l of methanol, the solvent was gradually changed to distilled water.

Separation procedure

The sample solution containing the iron(III) siderophores (5–10 mg each) in a small volume of water was layered carefully over the adsorbent, underneath water, in the top of the pre-column (the pump was disconnected prior to loading). When the colored siderophore layer entered the stationary phase, more water was added to fill the pre-column completely and the pump was reconnected. A pressure of 40–60 p.s.i. was maintained inside the column throughout the run. Separation was carried out by changing the water–methanol gradient from 0 to 40% of methanol. The initial flow-rate (in water) was 1.5 ml/min and varied considerably with the composition of the mobile phase owing to the change in its viscosity[8]. The eluates were collected in 5-ml fractions and an elution profile was obtained by determining the absorbance of the fractions at 440 nm.

Other chromatographic methods

Thin-layer chromatography (TLC) on silica gel 60 (0.25 mm) (E. Merck) was used to follow the separation of the iron(III), siderophores from the reversed-phase column. The solvent systems routinely used for the TLC were (I) chloroform–methanol–water (35:12:2) and (II) butanol–acetic acid–water (4:1:5) (upper phase).

Separation of the iron(III) siderophores was also carried out by conventional column chromatography using silica gel 60H (TLC grade, E. Merck) and solvent system I.

RESULTS

Separation in thin layers and a silica gel column

Nineteen iron(III) siderophores were subjected to silica gel TLC using solvent systems I and II. Their R_F values are given in Table I; these values vary considerably, depending on the age of the solvent system used. In solvent II, the R_F values are close to one another, but the system offered a very sharp resolution of most of the compounds and the R_F values are reproducible.

The compounds elute from the silica gel column (with solvent I) in the same order as they move in TLC. However, asperchrome C elutes earlier than ferrichrysin and ferricrocin, although they move together in TLC.

Separation in a reversed-phase column

Fig. 1 shows the elution profile of the iron(III) siderophores from the C_{18} bonded silica gel (LRP-1) column run in a water–methanol gradient. Malonichrome is the first and iron(III) N,N′,N″-triacetylfusarinine C the last compound to be eluted from the column. Similar results were obtaining using LRP2 as the stationary phase, except that DDF eluted earlier (with asperchrome B1). The stationary phase did not

TABLE I

R_F VALUES OF THE IRON(III) SIDEROPHORES ON SILICA GEL 60 THIN LAYERS

Iron(III) siderophore	R_F *value*	
	*Chloroform–methanol–water (35:12:2)**	*Butanol–acetic acid–water (4:1:5) (upper)*
Ferrichrome	0.50	0.10
Ferrichrome A	0.01	0.17
Ferricrocin	0.45	0.11
Ferrichrysin	0.44	0.11
Ferrirubin	0.33	0.22
Asperchrome A	0.42	0.23
Asperchrome B1	0.36	0.20
Asperchrome B2	0.36	0.17
Asperchrome B3	0.36	0.18
Asperchrome C	0.44	0.25
Asperchrome D1	0.39	0.13
Asperchrome D2	0.39	0.16
Asperchrome D3	0.39	0.15
Asperchrome E	0.39	0.21
Malonichrome	0.00	0.03
Triacetylfusarinine C	0.67	0.25
Coprogen	0.37	0.17
Neocoprogen I	0.37	0.15
Des(diserylglycyl)ferrirhodin	0.14	0.16

* Age of the solvent, 4 days. In freshly prepared solvent, the R_F values of all compounds are much lower.

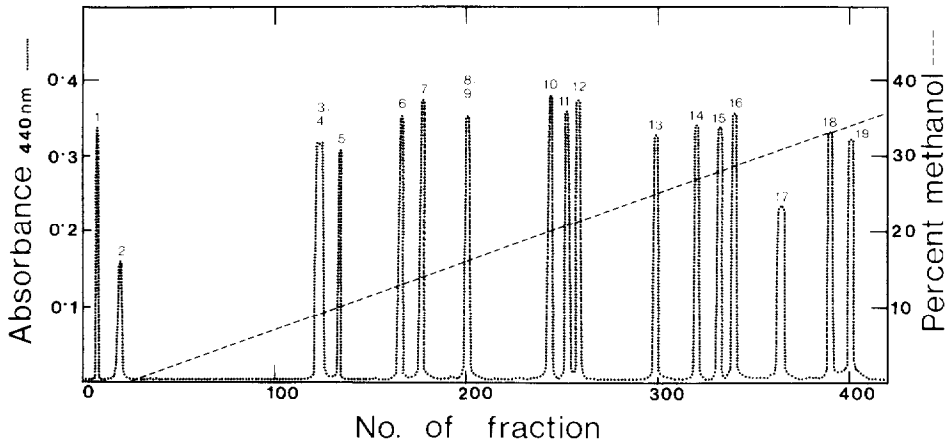


Fig. 1. Elution diagram of the iron(III) siderophores using the LRP-1 reversed-phase column run with a water–methanol gradient (5-ml fractions, initial flow-rate 1.5 ml/min). 1, Malonichrome; 2, ferrichrome A; 3, ferricrocin; 4, ferrichrysin; 5, ferrichrome; 6, neocoprogen I; 7, asperchrome D1; 8, asperchrome D2; 9, asperchrome D3; 10, coprogen; 11, asperchrome B3; 12, asperchrome B2; 13, asperchrome B1; 14, ferrirubin; 15, asperchrome E; 16, asperchrome A; 17, DDF; 18, asperchrome C; 19, triacetylfusarinine C.

show permanent binding with any compound and all siderophores were quantitatively eluted from the column.

DISCUSSION

It is apparent from the elution diagram (Fig. 1) that the iron(III) siderophores, in general, obey the usual retention rules in reversed-phase systems[8]. The replacement of a seryl $-CH_2OH$ by an alanyl $-CH_3$ increases the lipophillic character of the compound and as a result ferrirubin (serine substituent) elutes earlier than asperchrome A (alaninine substituent). Similarly, the presence of a hydrophilic group, $-CH_2OH$ in ferricrocin causes it to elute earlier than ferrichrome, which does not contain that group. Replacement of the terminal $-CH_2OH$ groups in the ornithyl N-acyl chains of ferrirubin by the more hydrophillic $-COOH$ groups, as in ferrichrome A, causes the latter to move much faster. In a similar way, acetylation of a terminal $-CH_2OH$ group of ferrirubin (to produce asperchrome C) increases the lipophilicity and, as a result, reduces the mobility.

Based on the observation that neocoprogen I moves faster than coprogen and that some ferrichromes show a regular elution order (ferrichrysin–asperchrome Ds–asperchrome Bs–ferrirubin), it may be concluded that the mobility of these compounds in this system is inversely correlated with the number of N^{δ}-(*trans*-5-hydroxy-3-methylpent-2-enoyl) residues (A) present in the molecules. When an increasing number of these long acyl groups (A) are present instead of the shorter N-acetyl group, the compound moves more slowly, probably owing to an increase in the lipophilicity or in the molecular size or both.

Apart from the hydrophilic–lipophilic interaction (or the interaction due to the molecular size) between the compound and the system, the molecular shape of the

compound also plays an important role in the separation process of the ferrichromes. Asperchrome B1, B2 and B3 separate from one another although they have the same molecular formula and contain the same number of various functional groups. Each of these three compounds has two long ornithyl N-acyl groups (A) and a shorter N-acetyl group. The isomers differ from one another on the basis of the position of the odd N-acyl group, because the three ornithines occupy different positions in the hexapeptide ring. The compounds B1, B2 and B3 are expected to have different molecular shapes and the reversed-phase system seems to recognize this difference and allows them to travel at different rates. A comparison of the elution characteristics of asperchrome B1–B3 and D1–D3 shows that the position R_3 (in ornithine 1) is much different from the other two positions, R_4 (ornithine 2) and R_5 (ornithine 3), and that a short N-acyl (acetyl) group in this position markedly increases the retention of the compound. X-ray crystallographic analysis of the structures of various ferrichromes (ref. 7 and unpublished results on ferrirubin, ferricrocin and asperchromes) revealed that the side-chains of ornithine 2 and 3 are indeed different from that of ornithine 1, because the former side-chains are continuous with the plane of the hexapeptide ring (roughly equatorial in position), whereas the ornithine 1 side-chain is perpendicular to that plane (axial in position).

Almost all of the compounds described here can be separated from one another using reversed-phase chromatography, in combination with silica gel column chromatography (in solvent I). However, ferricrocin and ferrichrysin are difficult to separate in these systems, as they both tend to travel at the same rate. Excellent separation of these compounds was achieved with the cellulose thin layers (E. Merck) [impregnated with acetone–water–saturated sodium chloride (6:3:1)] using *tert.*-butanol–0.004 *M* HCl–saturated sodium chloride (2:1:1, upper phase)[9].

The separation method described here will find use in the purification of various fungal siderophores from crude mixtures. Its application may also be extended to separate hydroxamate siderophores of non-fungal origin, such as the ferrioxamines and the schizokinens.

ACKNOWLEDGEMENT

This work was supported by a grant from the National Institute of General Medical Sciences (GM-21822).

REFERENCES

1 J. B. Neilands, *Inorg. Biochem.*, 1 (1973) 167.
2 T. Emery, *Biochim. Biophus. Acta*, 629 (1980) 382.
3 M. A. F. Jalal, R. Mocharla, C. L. Barnes, M. B. Hossain, D. R. Powell, D. L. Eng-Wilmot, S. L. Grayson, B. A. Benson and D. van der Helm, *J. Bacteriol.*, 158 (1984) 683.
4 W. Keller-Schierlein and H. Diekman, *Helv. Chim. Acta*, 53 (1970) 2035.
5 R. E. Moore and T. F. Emery, *Biochemistry*, 15 (1976) 2719.
6 M. B. Hossain, D. L. Eng-Wilmot, R. A. Loghry and D. van der Helm, *J. Amer. Chem. Soc.*, 102 (1980) 5766.
7 D. van der Helm, J. R. Baker, D. L. Eng-Wilmot, M. B. Hossain and R. A. Loghry, *J. Amer. Chem. Soc.*, 102 (1980) 4224.
8 Cs. Horváth, in C. F. Simpson (Editor), *Techniques in Liquid Chromatography*, Wiley, New York, 1982, p. 229.
9 W. Keller-Schierlein, *Helv. Chim. Acta*, 46 (1963) 1920.