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Chromatography of 3-hydroxypyridin-4-ones: novel orally active iron chelators

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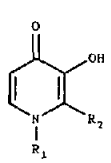
ABSTRACT

Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) of 3-hydroxypyridin-4-ones has been investigated. These novel orally active iron chelators display extremely poor chromatographic behaviour on conventional bonded-silica phases. This poor chromatography in HPLC is characterised by non-symmetrical, often multiple peaks, and in TLC by streaking. This appears to be due to interaction of the compounds with saturable free silanol groups and residual metal impurities on the bonded silica phases. This problem in HPLC can partially be solved by mobile phase manipulations (*e.g.* addition of the analyte to the mobile phase, 5 mg/l). TLC also improves dramatically when analyte is added to the solvent system (5 mg/50 ml). However in HPLC, due to poor selectivity, a better solution is the use of non-silica-based columns of columns with high carbon loading. The recently developed material, porous graphitised carbon (PGC Hypercarb®), is in particular an excellent HPLC phase for the separation of a series of these hydroxypyridinone iron chelators.

INTRODUCTION

The requirement for an orally active, non-toxic alternative to desferrioxamine has stimulated considerable research effort into the design of specific iron chelators [1]. The 3-hydroxypyridin-4-ones (Fig. 1) have emerged as a promising series of compounds, being non-toxic, orally available and very effective in drecreasing iron overload in experimental animal models and cultured hepatocytes [2]. As a continuation of our interest in the metabolism of nitrogen heterocycles [3–5], and as part of the general drug safety evaluation programme, we initiated a study of the absorption, distribution, metabolism and excretion (ADME) of some candidate compounds in



General structure of the 3-hydroxypyridin-4-ones

Compound	Code	R_1	R_2
1	CP028	-H	$-CH_3$
2	CP020	$-CH_3$	$-CH_3$
3	CP051	$-(CH_2)_2OCH_3$	$-CH_3$
4	CP094	$-C_2H_5$	$-C_2H_5$
5	CP040	$-(CH_2)_2OH$	$-CH_3$
6	CP052	$-(CH_2)_3OC_2H_5$	$-CH_3$
7	CP099	-H	$-C_2H_5$

Fig. 1. Structures of some 3-hydroxypyridin-4-one iron chelators.

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this series. This necessitated the development of methods for the isolation and quantitation of the candidate compounds and its potential metabolites using chromatographic techniques.

The main problem with chromatographing iron chelators is primarily due to their ability to bind iron impurities present in the chromatographic system. All buffers, unless pretreated with a chelating resin, contain variable amounts of iron. Another important source of iron which can adversely affect chromatographic behaviour is iron bound to free silanol groups of the silica stationary phase. Small amounts of iron can also be removed from the mechanical parts of the high-performance liquid chromatographic (HPLC) system which is made up of stainless steel.

Roberts *et al.* [6] who recently examined the HPLC separation of some potential iron chelators such as β-diketones and heterocyclic acids reported difficulties which arose due to interaction with iron. These workers also reported difficulty in chromatographing these chelators on bonded-silica phases. The free silanol groups present on the stationary phase lead to extensive peak broadening and tailing for iron chelators which have amine-like properties. Likewise Tangendjaja and Wills [7] and Acamovic *et al.* [8] had earlier reported poor chromatographic behaviour of mimosine and 3-hydroxypyridin-4-one respectively, on such conventional phases.

More recently Goddard and Kontoghioghes [9] have developed an HPLC method for the separation of these compounds using silica-based reversed-phase columns, with the incorporation of ion pairing agents. Their method, however, suffers from poor resolution and is unable to separate hydroxypyridinones from closely related structures. A major limitation of all methods described to date is their inability to chromatograph the corresponding complexes. This requirement is absolutely critical in view of the potential clinical use of these compounds as iron chelators. Levels of the drug both in the free and iron-bound forms need to be determined to study pharmacokinetics in iron-overloaded patients.

Some of the above problems can be eliminated by using an "iron-free" HPLC system as used by Venkataram and Rahman [19]. However, in order to be a truly "iron-free" system, stainless-steel parts of the HPLC system, such as the pump heads, injection system, connecting tubing, columns and frits have to be made from inert material such as glass, PTFE or titanium. In addition, the mobile phase should also be pre-treated with a chelating resin to remove iron contamination which would otherwise bind to the silanol groups of the stationary phase and build up to relatively high concentrations. However, all the above problems can be overcome by the simple incorporation of a complexing agent such as EDTA in the mobile phase [11]. We report here our systematic studies on the chromatography of the 3-hydroxypyridin-4-ones on conventional phases which demonstrate that a judicious choice of mobile phase and column is required to achieve acceptable chromatography. We also report on the use of the recently developed porous graphitised carbon (PGC) material for the HPLC of the free 3-hydroxypyridin-4-ones and their corresponding iron complexes.

MATERIALS AND METHODS

Materials

All HPLC solvents and all chemical reagents were either of HPLC or analytical

grade and were purchased from Fisons Scientific Apparatus (FSA, Loughborough, U.K.). All the 3-hydroxypyridin-4-ones were synthesised as previously described [12]. A Hewlett-Packard (Berkshire, U.K.) HPLC system Model 1090M complete with an auto-injector, and diode-array detector attached to a HP900-300 data station was used throughout the study. Samples were introduced onto the column via the auto-injector. Solutions of complexes (1 m*M*) of the 3-hydroxypyridin-4-ones were prepared by adding 0.6 ml of 50 m*M* free ligand to 5.0 ml of MOPS buffer (60 m*M*, pH 7.0), and subsequent addition of 1.0 ml of ferric nitrate (10 m*M* in 0.1 *M* HCl), and the volume made up to 10 ml with water.

High-performance liquid chromatography

Various columns were investigated for the analysis of these novel compounds, *viz.* Hypersil 5 ODS (10 × 0.46 cm I.D.) from Thames Chromatography (Berkshire, U.K.), Ultracarb® 30 ODS (15 × 0.46 cm I.D.) from Phenomenex (Rancho Palo Verdes, CA, U.S.A.), polymer reverse phase PLRP® (15 × 0.46 cm I.D.) from Polymer Laboratories (Church Stretton, U.K.) and finally, Hypercarb® (10 × 0.46 cm I.D.) PGC from Shandon, (Runcorn, U.K.). A 30-μl of sample of a 1-mg/ml solution of CP020 in methanol was routinely injected and the chromatographic behaviour of this test substance was monitored by UV absorption at 280 nm. If the peak shape was asymmetrical, attempts were made to improve the symmetry mostly by various types of mobile phase manipulations, *e.g.*, altering the composition, the pH, the ionic strength, the flow-rate or adding mobile phase additives such as triethylamine, analyte at 5 mg/l or picolinic acid [6], only when these approaches failed, did we investigate another column.

HPLC of the iron complexes required the addition of the free ligand (2.5 m*M*) to the mobile phase so as to prevent on-column dissociation of the 3:1 iron complex. The mobile phase consisted of phosphate buffer (pH 7.00, 60 m*M*)–acetonitrile (70:30, v/v) to which was added the free ligand. A 30-μl sample of the appropriate iron-complex solution was injected onto the column, and column eluent monitored at 450 nm, the λ_{max} of the complex.

Thin-layer chromatography

Thin-layer chromatographic (TLC) plates (20 × 10 cm) were obtained from Macherey-Nagel (Düren, F.R.G.). The plates were pre-coated to a thickness of 0.25 mm with silica gel C_{18}, containing a fluorescent indicator for UV 254 nm. A 10-μl sample of the stock solutions (1 mg/ml in methanol was applied onto the TLC plates 2 cm from the bottom and 1 cm apart using disposable glass microcapillary tubes and dried in warm air. The plate was then allowed to run up to the 15-cm solvent front in a saturated TLC tank before being air-dried and examined under UV light of 254 nm.

The choice of best solvent system was arrived at by using the prima method of Nyiredy *et al.* [13]. The prima method classifies possible developing solvents into groups based on similarities between their chemical structures and their dipole moments. Each solvent is assigned a solvent-strength value with respect to hexane which is given a value of zero. By optimising the prisma method, the best solvent systems for the resolution of the 3-hydroxypyridin-4-ones was found to be a mixture of glacial acetic acid–ethyl acetate–hexane–tetrahydrofuran (10.2:36.2:20.2:33.4, v/v), as well as 5 mg of CP094 in 50 ml of the solvent system.

RESULTS AND DISCUSSION

The chromatography of the 3-hydroxypyridin-4-ones is difficult on ODS bonded-silica columns. On such silica columns, chromatography is often characterised by broad asymmetrical and sometimes multiple peaks (Fig. 2a), possibly due to adsorption–desorption phenomena [14]. Several approaches were investigated to improve the symmetry of the peaks. These included (a) change of pH and ionic strength of buffers, (b) the addition of competing organic compounds such as triethylamine and (c) the addition of picolinic acid [6], or the addition of analyte itself (5 mg/l, Fig. 2b). These approaches, with the exception of the addition of analyte were unsuccessful. Even the addition of analyte was only partially successful, as the complications associated with negative elution peaks were frequently experienced, negating the use of this approach for routine analysis.

The use of silica columns with high carbon loading or of non-silica columns greatly improved the peak symmetry. Columns such as Ultracarb, Polymer PLRP and Hypercarb PGC drastically improved both peak shape and symmetry (Fig. 3a, b and c, respectively). These observations suggested that silanol binding sites were

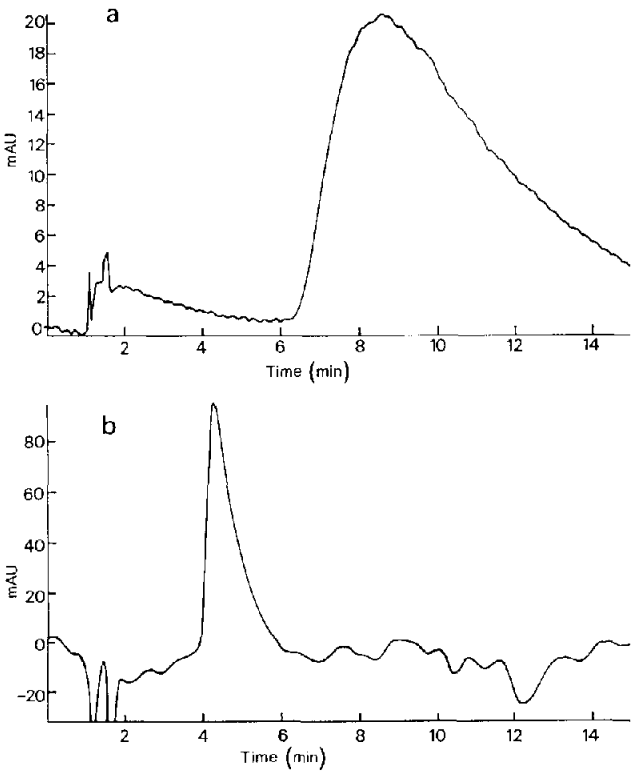

Fig. 2. (a) HPLC chromatogram of CP020 on Hypersil 5 ODS. Mobile phase, NaH_2PO_4 (10 m*M*)–acetonitrile (95:5, v/v), pH 3 adjusted with H_3PO_4, UV detection at 280 nm, flow-rate 1.0 ml/min. (b) HPLC chromatogram of CP020 on Hypersil 5 ODS. Conditions as in (a), except the addition of 5 mg/l of CP020 to the mobile phase.

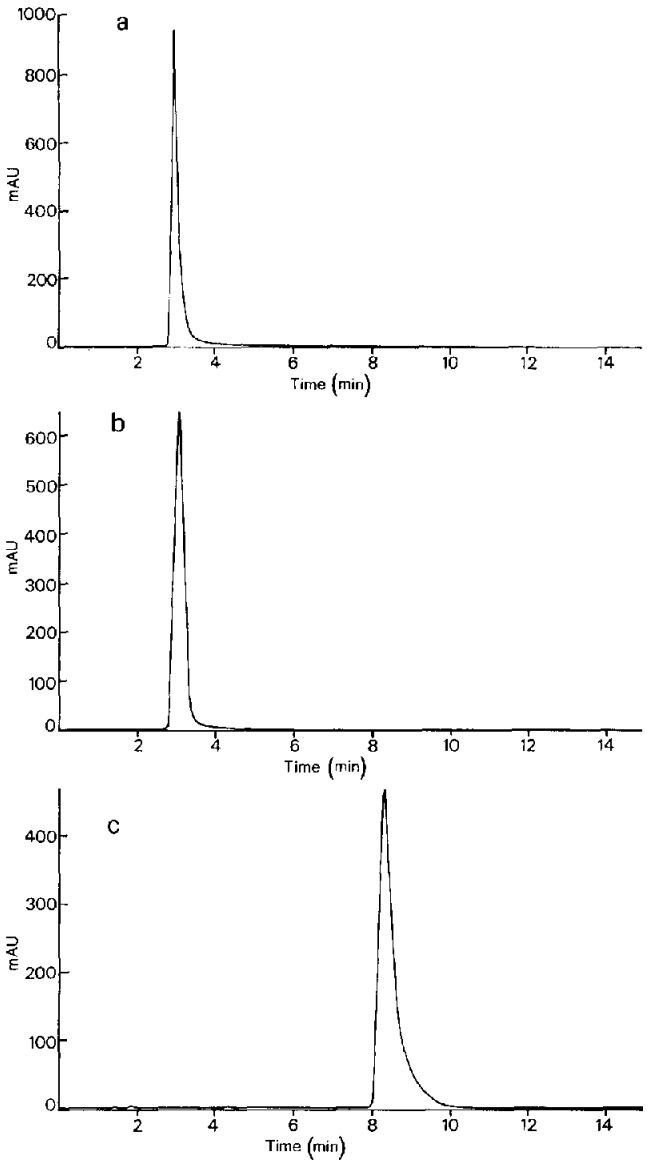

Fig. 3. (a) HPLC chromatogram of CP020 on Ultracarb 30 ODS. Mobile phase, NaH_2PO_4 (10 m*M*)–acetonitrile, (95:5, v/v), pH 3 adjusted with H_3PO_4 UV detection at 280 nm, flow-rate 1.0 ml/min. (b) HPLC chromatogram of CP020 on polymer reverse phase PLRP. Conditions as in (a). (c) HPLC chromatogram of CP020 on Hypercarb PGC. Mobile phase, NaH_2PO_4 (10 m*M*)–acetonitrile (90:10, v/v), pH 3 adjusted with H_3PO_4.

probably the major cause of non-symmetrical peaks seen with ODS bonded-silica phases (Fig. 2a).

The above problem is not confined to HPLC alone, and is also observed in the

TLC analysis of the hydroxypyridinones. These compounds do not chromatograph as compact spots on TLC but streak, even when C_{18} plates are employed. The streaking was eliminated in a similar manner to that of the broadening encountered in the HPLC, *i.e.*, TLC plates were pre-conditioned in a solvent system containing 5 mg of hydroxypyridinone 50 ml of solvent. Under these conditions, the 3-hydroxypyridin-4-ones migrated as compact spots, and it was possible to use this TLC system for the separation of various hydroxypyridinones (Table I) for our chemical and biological studies.

The non-symmetrical peaks and streaked spots observed in HPLC and TLC respectively are probably due to saturable interaction with component(s) of the chromatographic system, *e.g.* stationary phase, solvent or, given the iron-chelating property of these hydroxypyridinones, interaction with the stainless-steel tubing and frits present in the system or even with iron impurities in the bonded-silica phase. Addition of analyte to the mobile phase or solvent system improves peak symmetry and spot compactness by saturating these "active sites" and consequently prevents interaction with the injected or spotted compounds.

It seems unlikely that the residual silanol groups alone are responsible for the poor chromatographic pattern observed with this new series of compounds on bonded-silica phases, since triethylamine addition to the mobile phase failed to improve the peak symmetry. However, because the absence of silica also improved the peak shape, it is not easy to elucidate the role played by free silanol groups in the analysis of the 3-hydroxypyridin-4-ones. Whilst the involvement of silica might not be very clear, the part played by iron impurities is very evident because addition of analyte to the HPLC mobile phase or the TLC solvent system dramatically improved chromatography.

The effect of the addition of 2 m*M* EDTA to the mobile phase can clearly be seen in Fig. 4a and b. In Fig. 4a where there was no addition of EDTA to the mobile phase, the effect of iron contamination can be seen, by the formation of hydroxypyridinone–iron complex which absorbs at 450 nm. In the presence of EDTA however (Fig. 4b), there was no observable formation of the hydroxypyridinone–iron complex. EDTA thus prevents on-column formation of the iron complex. Fig. 4c on the other hand, shows the on-column dissociation of the iron complex, under the conditions employed for the free ligand. The dissociation of the 3:1 hydroxypyridinone–

TABLE I

REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY OF SOME 3-HYDROXYPYRIDIN-4-ONES[a]

Compound code	R_F
CP020[b]	0.33
CP028	0.43
CP051	0.55
CP052	0.61
CP094	0.55

[a] Solvent system composition: Glacial acetic acid–ethyl acetate–hexane–tetrahydrofuran (10.2:36.2:20.2:33.4, v/v).

[b] See Fig. 1 for compound codes.

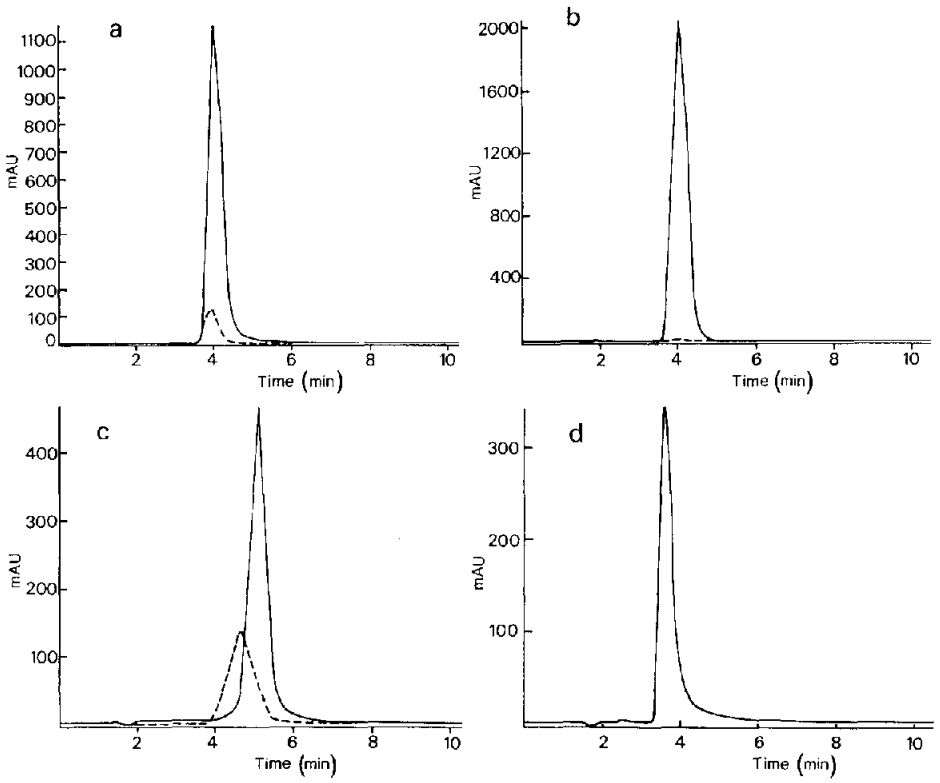

Fig. 4 (a) HPLC chromatogram of CP094 on Hypercarb PGC. Mobile phase, NaH_2PO_4 (10 m*M*)–acetonitrile (82:18, v/v), pH 3 adjusted with H_3PO_4, flow-rate 1.0 ml/min. UV detection 280 and 450 nm. (dotted lines indicate the peak of 450 nm). (b) HPLC chromatogram of CP094 in Hypercarb PGC. Other conditions as in (a) except the addition of 2 m*M* EDTA. (c) HPLC chromatogram of CP094–Fe complex on Hypercarb PGC. Other conditions as in (a) except the addition of 2 m*M* EDTA. *Note CP094–Fe complex breaks down on column.* (d) HPLC chromatogram of CP094–Fe complex on Hypercarb PGC. Mobile phase, phosphate buffer (pH 7, 60 m*M*)–acetonitrile (70:30, v/v) and 2.5 m*M* CP094. UV detection at 450 nm. Other conditions as in (a).

iron complex can be prevented by the addition of the free ligand (2.5 m*M*) to the mobile phase.

This allows rapid ligand exchange to take place [15]; in addition it serves to remove the iron contamination from both the mobile phase and column. This mobile phase manipulation therefore afford the direct analysis of the iron complex (Fig. 4d), with no detectable on-column dissociation. Since both the free ligands and the complexes are readily extractable into organic solvents (*e.g.* dichloromethane), there is the possibility of designing assays for the determination of both these species in biological fluids.

In conclusion, it appears that the absence of silica and interaction with iron impurities are the pre-requisites for the analyis of the 3-hydroxypyridin-4-ones and such conditions are fully met by the Hypercarb column (being carbon based), and incorporation of EDTA in the mobile phase. This column is also suitable for the

chromatography of 3-hydroxypyridin-4-one iron complexes (Fig. 4d). Work is now in progress to apply these TLC and HPLC methods for the routine analysis of the 3-hydroxypyridin-4-ones in biological matrices.

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