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Short communication

Gradient ion-pair high-performance liquid chromatographic method for analysis of 3-hydroxypyridin-4-one iron chelators

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Abstract

A gradient ion-pair HPLC separation of highly hydrophilic 3-hydroxypyridin-4-one (HPO) iron chelators is described. The separation of HPOs was performed using a reversed-phase polymer HPLC column (PLRP-S 100 Å, 15×0.46 cm ID, 5 μm). The ion-pair buffer contained 1-heptanesulfonic acid (sodium salt) (5 m*M*) and the pH was adjusted to 2.0 using HCl. The gradient was 2%–35% CH_3CN in 20 min and post-run was followed for 5 min using 2% CH_3CN and 98% buffer. The flow-rate was 1 ml/min and the analytes were monitored at 280 nm. The retention times of 30 hydrophilic HPOs fell in the range of 10–18 min with sharp peak shapes, although these iron chelators possess various functional groups and distribution coefficients. The application of this HPLC method in the analysis of HPO chelators and their metabolites in rat bile and urine is described.

Keywords: 3-Hydroxypyridin-4-one; Iron chelators

1. Introduction

Frequent blood transfusion remains the basic therapeutic treatment for the survival of β-thalasaemia major patients. Such long-term blood transfusion leads to the accumulation of excess iron and the associated toxic consequences. 3-Hydroxypyridin-4-ones (HPOs) are currently the main candidates for development of orally active iron chelators [1]. Previous investigations with a range of HPO ligands, both in iron overloaded animal models and in thalassaemic patients, have demonstrated enhanced iron excretion via both urinary and biliary routes [2,3]. Since liver is the major storage organ of accumulated iron, it is essential that the iron chelators are efficiently extracted by the liver and are not appreciably distributed to other organs. The capability of drugs to penetrate biological barriers and to distribute into tissues is largely dependent on their distribution coefficients, values in the range 0.001–0.005 minimising the rate of such movement.

Previous investigations have established that one of the major reasons for the limited efficacy of HPOs is their rapid conversion to non-chelating metabolites, via the glucuronidation of the 3-hydroxyl function group. Thus the studies on the metabolism of HPOs are essential in order to reliably identify a chelator with clinical potential. Such metabolic studies rely on appropriate analytical methods which provide good separation for the highly hydrophilic

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HPO chelators, their metabolites and endogenous substances present in biological fluids. Previously the analysis of HPOs was based on the reversed-phase HPLC method developed by Epemolu et al. [4], which is unsatisfactory for extremely hydrophilic HPOs. The ion-pair HPLC method described below has been developed to cope with this difficulty.

2. Materials and methods

The 3-hydroxypyridin-4-ones (HPOs) studied in the present investigation were prepared using the methodology reported by Dobbin et al. [5]. The chemical structures of these compounds are shown in Table 1. 1-Heptanesulfonic acid (sodium salt) and EDTA were the products of Aldrich Chemical Company (Gillingham, Dorset, UK).

A Hewlett-Packard model 1090M Series HPLC system with an autoinjector, an autosampler, a diode-array detector and a reversed-phase polymer HPLC column (PLRP-S 100 Å, 15×0.46 cm ID, 5 μm, Polymer Laboratories Ltd, Church Stretton, Shropshire, UK) was used in the present study. Two buffer systems were used to produce the mobile phase: buffer A contains 1-heptanesulfonic acid (sodium salt) (5 m*M*) and the pH was adjusted to 2.0 using HCl; buffer B was a phosphate buffer (25 m*M*) containing EDTA (1 m*M*) and the pH was adjusted to 3.0 using H_3PO_4. The linear gradient was 2–35% acetonitrile (CH_3CN) in 20 min and post-run was followed for 5 min using 2% CH_3CN and 98% buffer. The flow-rate was 1 ml/min and the analytes were monitored at 280 nm.

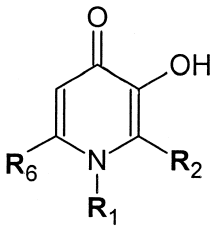
In order to observe the biliary metabolic profile of CP502 (Table 1), Wistar rats (200 g, male) were anaesthetised by the combined i.p. application of Hypnorm® (a mixture of fentanyl citrate and fluanisone) and Hypnovel® (midazolam). Cannulation of the bile duct was followed by exteriorisation of cannulae. After CP502 was administered (450 μmol/kg body weight) by gavage, bile samples were collected hourly over an 8-h period. Meanwhile, the urine samples were also collected from the same rats, while placed in the metabolism cages. The bile and urine samples were diluted 10-fold with water and the resulting solutions were filtered using syringe filters (13 mm GD/X disposable filter device, 0.2 μm pore size, Whatman) and 30 μl of the filtrates was injected directly on the HPLC column. All authentic HPOs were dissolved in water for determination.

3. Results and discussion

Due to the strong interactions between HPO compounds and the stationary phase of the HPLC columns, it was previously found that the chromatography of the 3-hydroxypyridin-4-ones was difficult on normal ODS columns as the analytes are often characterised by broad asymmetrical and sometimes multiple peaks [4]. However, the use of silica columns with high carbon loading and of non-silica columns, such as Ultracarb, Polymer PLRP and Hypercarb PGC columns, were found to dramatically improve the peak shape and symmetry [4]. HPLC procedures based on the use of these columns have been successfully applied to metabolic and pharmacokinetic studies of a series of HPOs [6,7]. In order to prevent the formation of iron complexes of the HPOs with ferric ions present in the sample and HPLC system, the mobile phase is normally adjusted to pH 3.0 and EDTA (2 m*M*) is included in the mobile phase.

Unfortunately, when this and similar HPLC methods were applied to the analysis of more hydrophilic HPOs, e.g. those with distribution coefficient at pH 7.4 ($D_{7.4}$) (see Table 1) less than 0.1, the retention times were found to be unsatisfactorily short and poor resolution from interfering substances may occur. As a result, Skinner and co-workers developed a baseline file subtraction method combined with a HPLC procedure, in order to reliably analyze the plasma samples containing 1-[ethan-1-ol]-2-methyl-3-hydroxypyridin-4-one (CP40) [8]. This baseline file subtraction method is useful when other methods cannot be adapted to adequately resolve a hydrophilic analyte from coeluting interfering substances. During the investigations of the metabolism of CP502 ($D_{7.4}$=0.02), it was also found that the separation of the analytes was poor. Thus the HPLC chromatograms of the rat bile and urine samples after oral administration of CP502 (Fig. 1A), only two major peaks were identified, when the sample was eluted using the mobile phase composed with phos-

Table 1
The structures, distribution coefficients and HPLC retention times of 3-hydroxypyridin-4-one (HPO) derivatives



HPOs	R_1	R_2	R_6	$D_{7.4}$[a]	HPLC retention time (min)
CP20	CH_3	CH_3	H	0.17	12.6
CP21	CH_2CH_3	CH_3	H	0.49	13.9
CP22	$CH_2CH_2CH_3$	CH_3	H	1.51	15.5
CP28	H	CH_3	H	ND[d]	12.0
CP38	CH_2CH_2COOH	CH_3	H	<0.001	12.7
CP40	CH_2CH_2OH	CH_3	H	0.08	12.0
CP41	$CH_2CH_2CH_2OH$	CH_3	H	0.132	12.6
CP42	$CH_2(CH_2)_3OH$	CH_3	H	0.181	13.2
CP44	$CH_2CH_2NH_2$	CH_3	H	0.028	13.6
CP60	CH_3	H	H	ND	11.4
CP61	CH_2CH_3	H	H	ND	12.5
CP90	H	H	H	ND	11.1
CP93	CH_3	CH_2CH_3	H	0.624	13.9
CP94	CH_2CH_3	CH_2CH_3	H	1.78	15.2
CP95	$CH_2CH_2CH_3$	CH_2CH_3	H	5.04	17.1
CP99	H	CH_2CH_3	H	ND	13.6
CP102	CH_2CH_2OH	CH_2CH_3	H	0.22	13.4
CP106	$CH_2CH_2CH_2OH$	CH_2CH_3	H	0.36	13.6
CP107	$CH_2(CH_2)_3OH$	CH_2CH_3	H	0.52	14.4
CP359	CH_2CH_3	CH_2OH	H	0.14	12.1
CP364	CH_3	CH_2OH	H	0.048	11.0
CP365	CH_2CH_3	$CH(CH_3)OH$	H	0.27	13.3
CP366	$CH_2CH_2CH_2OH$	CH_2OH	H	0.056	11.0
CP385	CH_3	H	CH_2OH	0.039	11.3
CP387	CH_3	CH_2OH	CH_2OH	0.042	11.1
CP501	CH_3	$CONH_2$	CH_3	ND	10.7
CP502	CH_3	$CONHCH_3$	CH_3	0.04	11.7
CP504	CH_3	$CONHCH(CH_3)_2$	CH_3	0.30	14.1
3OMeCP94[b]	CH_2CH_3	CH_2CH_3	H	ND	16.2
4OMeCP94[c]	CH_2CH_3	CH_2CH_3	H	ND	15.0

[a] Distribution coefficients at pH 7.4, which were determined in an octanol/MOPS (4-morpholinepropanesulfonic acid–NaOH buffer, pH 7.4) system using an automated continuous flow technique as described by Rai et al. [9].
[b] With –OMe substitution at 3-position.
[c] With –OMe substitution at 4-position.
[d] ND: not detected.

phate buffer and EDTA (mobile phase B). By examining the spectrometric purity of the peaks it was found that both peaks were impure (not shown). However, when the mobile phase containing the ion-pair reagent 1-heptanesulfonic acid (mobile phase A) was applied, the separation of the analytes

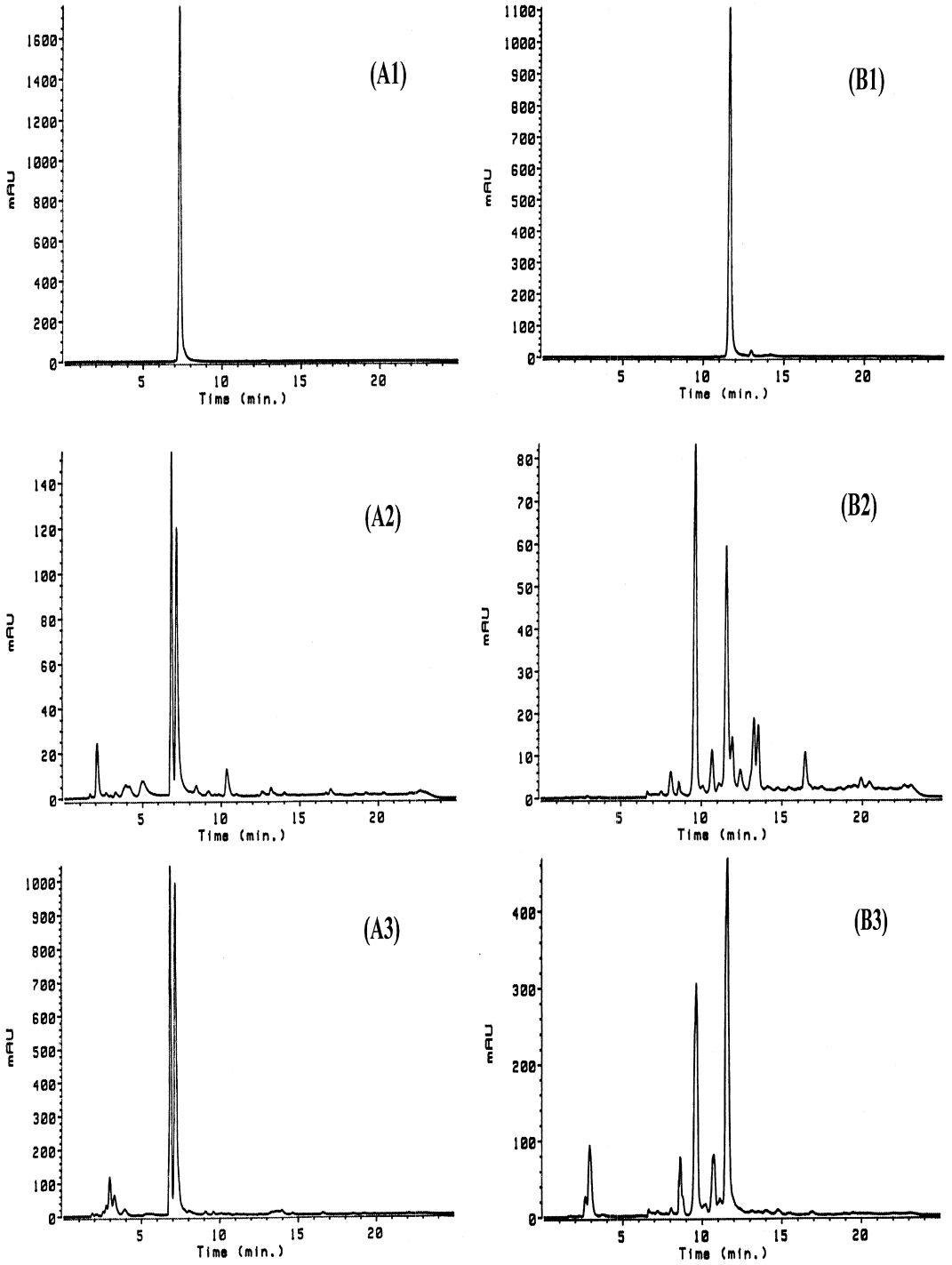


Fig. 1. The HPLC chromatograms of the rat bile and urine samples after oral administration of CP502. A1 and B1: authentic CP502; A2 and B2: bile sample; A3 and B3: urine sample. A1, A2 and A3 were chromatographed using the mobile phase (pH 3.0) containing phosphate buffer (25 m*M*) and EDTA (1 m*M*), whilst B1, B2 and B3 were chromatographed using the ion-pair buffer containing 1-heptanesulfonic acid (5m*M*)–HCl (pH 2.0). Other conditions were as described in the text.

in rat bile and urine was significantly improved thus enabling further quantitative and characteristic analysis of CP502 metabolites (Fig. 1B).

Thirty HPO derivatives, which possess considerable variation in chemical substituents and distribution coefficients (from <0.001 to 5.04), were investigated using the ion-pair HPLC method and their retention times are presented in Table 1. The advantage of the present ion-pair HPLC method is clearly demonstrated in that all the tested compounds showed retention times in the range of 10 to 18 min, in contrast to the much shorter retention times associated with the previous methods. This retention time range is generally appropriate for efficient HPLC separation and determination of analytes present in biological fluids. The peak profiles of some HPOs are presented in Fig. 2, their sharp symmetrical peaks are typical of all the tested HPOs. It is suggested that this ion-pair HPLC method may prove to be useful for the analysis of other hydrophilic HPO derivatives.

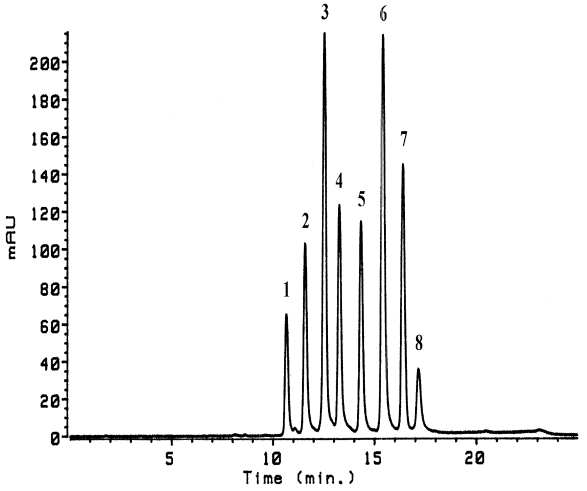


Fig. 2. The HPLC chromatogram of some 3-hydroxypyridin-4-one (HPO) iron chelators using the ion-pair HPLC conditions. 1: CP501; 2: CP502; 3: CP20; 4: CP102; 5: CP107; 6: CP94; 7: 3OMeCP94; 8: CP95.

Acknowledgements

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