Journal of Chromatography, 573 (1992) 178–182
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6101

Short Communication

High-performance liquid chromatographic determination of 1,2-diethyl-3-hydroxypyridin-4-one and its 2-(1-hydroxyethyl) metabolite in rat blood

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(First received March 19th, 1991; revised manuscript received July 17th, 1991)

ABSTRACT

A sensitive and selective high-performance liquid chromatographic (HPLC) method for the analysis of 1,2-diethyl-3-hydroxypyridin-4-one (CP94, I) and its 2-(1-hydroxyethyl) metabolite (II) in rat blood is described. I, II and the internal standard, 1-propyl-2-ethyl-3-hydroxypyridin-4-one (CP95, III) were extracted into dichloromethane (3 × 5 ml, with the addition of 1 g of sodium chloride) from blood (0.25 ml plus 0.75 ml of pH 7.0 morpholinopropanesulphonic acid buffer). Extractability approached 100% for I and III, and approximately 65% for II under these conditions. Chromatographic analysis was carried out using a Hypercarb porous graphitised carbon HPLC column (10 cm × 0.46 cm). The mobile phase was 14:86 (v/v) acetonitrile–NaH_2PO_4 buffer (10 m*M*, containing 2 m*M* EDTA, pH adjusted to 3 with phosphoric acid) and detection was by ultraviolet at 280 nm. Calibration curves were linear (correlation coefficient > 0.99) and reproducible over the concentration range 0–80 μg/ml and the coefficient of variation was less than 16% even at low (1 μg/ml) concentrations. The minimum quantifiable level was 0.5 μg/ml for both I and II.

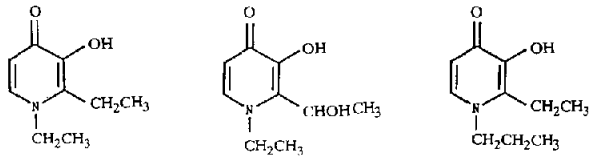
INTRODUCTION

Patients who suffer from haemoglobinopathic disorders such as β-thalassaemia major are heavily iron-overloaded due to their dependence on frequent blood transfusions. The only widely used drug to treat this disorder is desferrioxamine. Desferrioxamine, although a relatively safe and effective drug when used with care, suffers from a lack of oral activity which inevitably leads to poor patient compliance.

Over the last fifteen to twenty years a whole range of compounds which include catecholates, aminocarboxylates, or the substituted phenolates and hydroxypyridinones have been investigated as orally active iron-chelating alternatives to desferrioxamine. Many of these compounds have been rejected due to lack of sufficient oral activity, poor iron mobilisation ability or unacceptable toxicity.

The 3-hydroxypyridin-4-ones (Fig. 1) are currently the main candidates for development as orally active alternatives to desferrioxamine [1]. Despite their relatively simple structure, the hydroxypyridinones show a remarkable degree of selectivity for mobilisation of ferric iron. These compounds can generally be divided into the 2-methyl and 2-ethyl series. Introduction of the ethyl function at the 2-position markedly reduces the inhibitory properties of these compounds towards metalloenzymes catalyzing phenol- and catechol-based reactions.

0378-4347/92/$05.00



I II III

Fig. 1. Structures of I, II [the 2-(1-hydroxyethyl) metabolite of I], and III, the internal standard, CP95.

As part of the general drug safety evaluation programme, we have initiated a study of the absorption, distribution, metabolism and excretion of these compounds in laboratory animals. This necessitated the development of a sensitive and selective high-performance liquid chromatographic (HPLC) assay for the quantification of these compounds and their potential metabolites in biological matrices. The lead compound in this series, I, was chosen on the basis of its relative low toxicity and high efficiency in mobilisation of both the intra- and extracellular iron [2].

Investigations into the metabolism of the 3-hydroxypyridin-4-ones in rodents have revealed major differences between the 2-methyl (*e.g.* CP20) and the 2-ethyl compounds (*e.g.* CP94, I). The 2-methyl compounds are extensively glucuronidated (~42% of dose) at the 3-hydroxy position; in contrast, I from the 2-ethyl series is only poorly conjugated (14% of dose). The major circulating blood and urinary metabolite of I in the rat is II (Fig. 1) [3].

Analysis of iron chelators in general is difficult because of the inherent ability of the chelators to bind iron impurities present in the chromatographic system. The 3-hydroxypyridin-4-ones in addition interact extensively with free silanol groups on silica-based reversed-phase columns. We have recently described methods for overcoming the above problems associated with the chromatography of these compounds [4]. The method developed utilises the unique properties of a recently developed material, porous graphitised carbon (PGC, Hypercarb). We now report on a method, together with a validation of the assay procedure, for the recovery of I and its hydroxylated metabolite from rat blood and their quantitation by HPLC.

EXPERIMENTAL

Materials

Compound II was isolated from rat urine using a preparative HPLC procedure [3]. All the 3-hydroxypryridin-4-ones were synthesised as previously described [5]. Morpholinopropanesulphonic acid (MOPS) buffer (sodium salt) was purchased from Sigma (Poole, UK).

EDTA (trisodium salt, Convol) was obtained from BDH (Poole, UK). All HPLC solvents and reagents were either HPLC or analytical grade and were purchased from Fisons Scientific Apparatus (FSA, Loughborough, UK).

The pellicular guard column packing material (Co:Pell ODS) was purchased from Whatman International (Maidstone, UK). The Hypercarb PGC column was purchased from Shandon Scientific (Runcorn, U.K.). The MOPS buffer (60 m*M*) was prepared by dissolving the appropriate amount in 100 ml af distilled water; the pH was adjusted to 7.0 with 1 *M* NaOH. The $NaH_2PO_4 \cdot 2H_2O$ solution (10 m*M*) was prepared by dissolving the appropriate amount in 1 l of distilled water; 2 ml of 1 *M* EDTA trisodium were added before the solution was made up to volume and the pH adjusted to 3 with phosphoric acid. Glass double distilled water was used for preparation of all buffers.

Preparation of standards

Standard solutions of I, II and the internal standard III were prepared in HPLC-grade methanol at a concentration of 1.0 mg/ml. These solutions were stored in the fridge at 4°C for use in subsequent procedures, *e.g.* extractability studies, preparation of calibration curves; all samples were stable for several months under these storage conditions.

Recovery of compounds from blood

The recoveries of I, II and III were assessed by spiking 0.25, 10 and 20 μg of the respective compounds into tubes containing 0.25 ml of the control rat blood sample and 0.75 ml of 60 m*M* MOPS buffer (pH 7). This was followed by the addition of 1 g of NaCl into each tube, this "salting out" procedure being essential for improving the extractability of II. These aqueous samples

were extracted using dichloromethane (3 × 5 ml).

The pooled organic extracts were evaporated to dryness in a water bath at 40°C and the residues then reconstituted in 1 ml of MOPS buffer. Aliquots (20 μl) of these samples were injected onto the HPLC column.

The recoveries were calculated by comparing peak areas obtained for these samples, with those obtained from injection of 20 μl of the aqueous samples prior to dichloromethane extraction.

Preparation of calibration standards

To facilitate the measurement of both I and II accurately during the latter phase post drug administration, when drug levels are expected to be low, it was necessary to construct both high and low calibration curves. The low calibration for the late time points ranged from 0 to 4 μg/ml and the high calibration curve relevant to the early time points ranged from 5 to 80 μg/ml. Calibration curves were constructed by spiking known amounts of I and II into extraction tubes containing 0.25 ml of rat blood and 0.75 ml of MOPS buffer pH 7. The internal standard, III (10 μl of a 1 mg/ml methanolic solution for the high calibration range and 20 μl of a 25 μg/ml solution for the low calibration range) was added to each tube before extraction as described above.

The residues from the pooled organic extracts were reconstituted in 100 μl of mobile phase and 20-μl aliquots injected onto the HPLC column. Peak-area ratios were then plotted against concentrations of I and II.

Assay validation

The accuracy and precision of the assay were determined by spiking both I and II into control rat blood at two concentrations: 1 and 40 μg/ml. The study was carried out on four separate occasions. Assay precision and accuracy were indicated by the coefficient of variation (C.V.) and mean percentage difference (M%D), respectively. The minimum quantifiable level (MQL) was taken as the concentration affording a signal three times that of the background signal [6].

Chromatography

The HPLC system was from LDC (Stones, UK) and comprised of the following: a Consta-Metric 3000 pump, a SpectroMonitor 3100 variable-wavelength UV detector and a Model CI-4000 integrator. Samples were introduced onto the column via a Rheodyne injector fitted with a 20-μl sample loop.

HPLC was carried out on a Hypercarb PGC column (10 cm × 0.46 cm) with a precolumn (5 cm × 0.25 cm) packed with pellicular ODS. The mobile phase consisted of 14:86 (v/v) acetonitrile–$NaH_2PO_4 \cdot 2H_2O$ buffer (10 m*M*, containing 2 m*M* EDTA, pH 3 adjusted with phosphoric acid). The eluent was monitored at 280 nm and the flow-rate was 1.0 ml/min.

Storage and stability

The stability of I and II in rat blood was investigated by spiking these compounds (40 μg/ml) into several tubes containing 0.25 ml of blood and 0.75 ml of MOPS buffer. Some tubes were stored at room temperature for up to 8 h and the rest were kept at −20°C and analysed subsequently after one- and two-month periods to determine long-term stability. Immediately prior to analysis the tubes were spiked with III (internal standard) and subjected to the extraction and analysis procedure as outlined above.

Preliminary pharmacokinetic studies

Preliminary pharmacokinetic studies were carried out in conscious rats. The carotid artery and jugular vein were cannulated under anaesthesia using a mixture of fluanisone, fentanyl and hypnovel at a dose of 10, 0.315 and 5 mg/kg, respectively.

The animals were then left to recover overnight. Compound I was administered intravenously via the jugular cannulae (100 mg/kg) with saline as the vehicle and blood samples (0.3 ml) collected at pre-determined time intervals up to 8 h via the intra-arterial cannulae. Aliquots (0.25 ml) of collected blood were dispensed into extraction tubes and the pH was adjusted to 7.0 by adding 0.75 ml of the MOPS buffer. These samples were subjected to the extraction and assay procedure described above.

RESULTS AND DISCUSSION

The chromatograms of I, II and III (Fig. 2) show single symmetrical peaks with complete baseline resolution between components. The retention times of II, I and III were 3, 8 and 15 min, respectively. HPLC analysis of dichloromethane extracts of control rat blood revealed the absence of any coeluting peaks at the retention times of interest, indicating that dichloromethane was a suitable solvent for extraction. The recoveries (Table I) of I and II in dichloromethane were satisfactory under the assay conditions described in this paper, I and III being almost 100% extractable, and extractability of II approaching 65%. There was no difference in recoveries across the three levels studied. The "salting-out" procedure was found to increase the extractability of II from 35 to 65%, hence sodium chloride (1 g) was included as a standard procedure for all extractions. The calibration curves show good linearity over the concentration ranges 0–4 and 5–80 μg/ml, and correlation coefficients for both the low and high calibration ranges were greater than 0.99. The assay precision, as indicated by the C.V., and the accuracy, as shown by M%D, are given in Table II; the C.V. for the validation at the higher concentrations (40 μg/ml) was less than 2% for I and less than 5% for II. The C.V. for the validation at the lower concentration (1.0 μg/ml) was less than 16% for I and less than 7% for II. The M%D for validation at both concentrations for I and II was less than 12%. The storage stability data (not shown) indicated that the concentrations of I and II showed no significant change during the storage period (two months) in the freezer at −20°C.

The preliminary pharmacokinetic results (Fig. 3) show that the disposition of I in rat is bi-exponential, with an initial distribution phase which

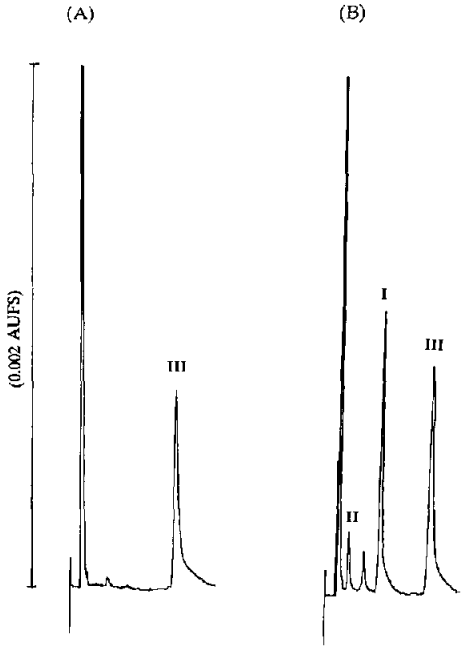


Fig. 2. HPLC analysis of dichloromethane extracts of control rat blood (A) and blood spiked with I (retention time = 8 min) and II (retention time = 3 min) at a concentration of 1 μg/ml and III, the internal standard (retention time = 15 min) (B).

TABLE I

EXTRACTION RECOVERY OF I, ITS METABOLITE II AND THE INTERNAL STANDARD FROM RAT BLOOD

Compound	Recovery[a] (mean ± S.D.) (%)	C.V.[b] (%)	*n*
I	94 ± 7.4	7.9	4
II	62 ± 4.7	7.5	6
Internal standard	106 ± 4.3	4.0	4

[a] Extraction recoveries at 1, 40 and 80 μg/ml levels were identical.

[b] C.V. = S.D./mean × 100%.

TABLE II

PRECISION AND ACCURACY OF THE HPLC ASSAY FOR I AND ITS METABOLITE II IN RAT BLOOD

Concentration spiked (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)		C.V.[a] (%)	M%D[b]	*n*
I					
1.0	1.04	0.17	15.8	4	4
40.0	43.0	0.75	1.7	7.5	4
II					
1.0	1.12	0.08	6.8	12	4
40.0	36.8	1.50	4.1	8	4

[a] C.V. = S.D./mean × 100%.

[b] M%D = 100 × (mean − spiked concentration)/spiked concentration.

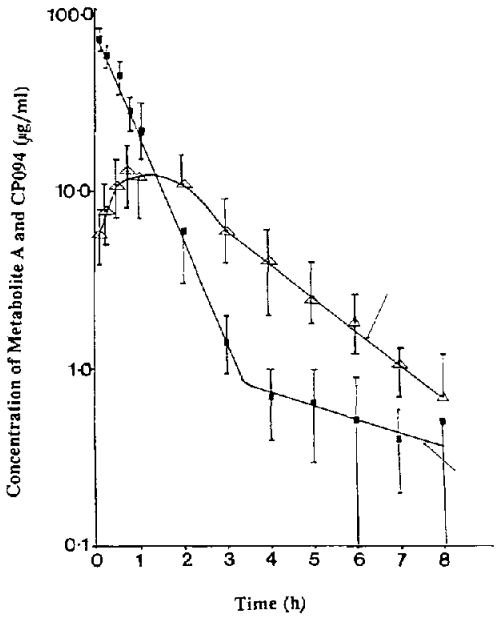


Fig. 3. Pharmacokinetic profile of I (■) and its 2-(1-hydroxyethyl) metabolite (II) (△) in rats dosed with I (100 mg/kg, intravenously, $n = 8$).

has a rate of 1.62 ± 0.21 h^{-1} and a terminal elimination phase with a rate of 0.25 ± 0.17 h^{-1}. The $t_{1/2\alpha}$ and $t_{1/2\beta}$ were 0.43 ± 0.05 and 3.62 ± 1.9 h, respectively. Compound II peaked very rapidly at about 45 min, which suggests rapid formation of metabolite.

Compound I, in contrast to compounds from the 2-methyl series of the 3-hydroxypyridin-4-ones, is only poorly conjugated at the 3-hydroxy position in rats, resulting in a much lower loss of iron-binding capacity. This is largely because the hydroxylated metabolite of I, in contrast to compounds in the 2-methyl series, do not form non-chelating glucuronide conjugates. The formation of a hydrophilic metabolite with similar pharmacological potential as that of the parent drug possibly accounts for the superior efficacy of I in iron mobilisation, compared to compounds from the 2-methyl series (*e.g.* CP20).

Compound I, which is efficiently absorbed and distributed, is likely to gain access to the main iron stores such as the liver where the drug has the opportunity to chelate intracellular iron; alternatively I could be metabolised to the hydrophilic II in this organ. II in turn can either chelate intracellular iron or is effluxed from cells where it is able to intercept iron released from the reticulo-endothelial system (RES) or bound for intracellular stores. II is therefore likely to have the ideal properties for maximal urinary iron excretion with minimal redistribution to other tissues.

In conclusion a sensitive and selective assay has been developed for the quantitation of I and II in rat blood. The method developed is highly reproducible with minimal inter- and intra-assay variation. We are currently using this assay on a routine basis to fully elucidate the pharmacokinetics of I both in rats and thalassaemic patients.

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