Chromatographic method for the determination of non-transferrin-bound iron suitable for use on the plasma and bronchoalveolar lavage fluid of preterm babies

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1. Non-protein-bound iron has been implicated in the aetiology of chronic lung disease of prematurity. 2. The modification of a method for the measurement of non-transferrin-bound iron in small volumes of plasma and bronchoalveolar lavage fluid from preterm babies is described. 3. The assay runs with a good degree of precision and a lower limit of detection of 0.02 µmol/l. 4. Non-transferrin-bound iron was detected in 50% of plasma samples and 11% of bronchoalveolar lavage fluid samples collected over the first week of life from babies born prematurely.

INTRODUCTION

In human plasma, iron exists mainly in the ferric (Fe³⁺) form bound to the protein transferrin. In healthy adults, the transferrin is only 20–35% saturated with iron and therefore non-protein-bound iron should be present in only trace amounts. The conversion of ferrous to ferric iron, which binds to transferrin, is facilitated by the ferroxidase activity of another plasma protein, caeruloplasmin [1].

The importance of these proteins as antioxidants relates to the potential of non-protein-bound iron to participate in reactions leading to the generation of reactive oxygen species. Of particular importance is the fact that non-protein-bound ferrous iron reacts with hydrogen peroxide to yield the highly reactive hydroxyl radical in a Fenton reaction [2]. Additionally, iron can catalyse the breakdown of lipid peroxides to form a range of products including certain radicals [3]. The pro-oxidative nature of non-protein-bound iron in vitro is well established [4–6].

Interest in the pro-oxidative nature of non-protein-bound iron led to the development of an assay for its detection. The bleomycin assay was published in 1982 by Gutteridge et al. [7] and was described as measuring 'loosely bound' iron in plasma and other biological fluids. This has formed the basis of several subsequent reports describing the presence of 'bleomycin-detectable iron' in plasma from various patients, including those with idiopathic haemochromatosis [8], adult respiratory distress syndrome [9], and β-thalassaemia [10]. Bleomycin-detectable iron is also reported to occur in some samples of synovial fluid [11] and cerebrospinal fluid [12].

More recently, interest has been shown in a possible pro-oxidative role for non-protein-bound iron in the aetiology of chronic lung disease (CLD) of prematurity. At birth, babies born prematurely have low levels of circulating transferrin [13, 14], which would tend to lead to an increase in the percentage saturation of transferrin with iron. Plasma levels of caeruloplasmin are also low, which in principle could lower the ability of the plasma to maintain iron in the ferric form, the redox state in which it binds to transferrin [15, 16]. Furthermore, it has been demonstrated that the concentration of vitamin C in the plasma of some preterm babies at birth is sufficiently high to inhibit the ferroxidase activity of caeruloplasmin [17].

Bleomycin-detectable iron was first reported in the plasma of some babies born either at term or prematurely by Evans et al. [14], and subsequently confirmed using the same method by Berger and co-workers [18–20] in the plasma of cord blood from term and preterm infants. The chemistry associated with bleomycin-detectable iron is complex and may be subject to interference, consequently a different method for the direct measurement of potentially pro-oxidative iron in very small samples of biological fluids would find considerable application, particularly in studies relating to the aetiology of chronic lung disease of prematurity. Singh et al. [21] developed an HPLC method for the direct detection of non-transferrin-bound iron (NTBI) in...
the plasma of patients with iron overload. We set out to develop this method further, for application to the small samples of plasma and bronchoalveolar lavage (BAL) fluid collected from babies born prematurely.

MATERIALS AND METHODS

Materials

Disodium nitrolitriacetic acid (NTA) was obtained from Aldrich; Mops and ferric nitrate nonahydrate were obtained from Sigma. HPLC-grade acetonitrile was obtained from Rathburns. Deionized water was produced by 'Purite Select Analyst', a Hewlett Packard reverse-osmosis deionizer on site. 3-Hydroxyl-1-propyl-2-methyl-pyridin-4-one (CP22) was synthesized in-house following the method developed by Dobbin et al. [22].

Sample collection and storage

All work was carried out with the approval of the South Sheffield Hospitals Ethics Committee. Informed consent was obtained from a parent before recruitment of an infant to the study. Blood samples were collected from 50 babies born between 24 and 36 (mean 31.5) weeks' gestation on the Neonatal Intensive Care Unit at the Jessop Hospital for women. Birthweight ranged from 555 to 2760 g with a mean of 1615 g.

Aliquots of 100 μl of plasma were taken from blood samples collected as part of the routine management of the babies over the first 7 days of life. Blood samples were collected into lithium heparin either via an established intra-arterial line or by heel prick. Blood samples were processed for storage immediately after collection. Samples were centrifuged at 1000 g at 4°C for 10 min. The plasma was removed, and stored at 4°C for a maximum of 2 h before storage at −70°C until analysis. BAL was performed on 26 ventilated infants and where possible a 50 μl aliquot of fluid collected from these washings was stored for use in this study. BAL fluid was centrifuged at 12,000 g for 10 min to remove mucus, and the supernatant stored at −70°C for later analysis.

All samples were stored in polypropylene tubes.

Equipment

Initial investigations revealed a significant iron contamination from the HPLC system, presumably a result of the action of the powerful iron chelator used in the mobile phase on stainless-steel components. The system was therefore substantially modified to minimize this; all connective tubing from the pumps to the Rheodyne injector, Rheodyne injector to column and column to detector was made from polyetherethyketone (PEEK). All in-line frits, solvent reservoir filters and the Rheodyne injector, the loop and the drain valve were made from titanium equivalent or polytetrafluoroethylene (PTFE), as were all nuts and ferrules. A glass-lined column was used.

The HPLC system consisted of two pumps, a Gilson 305 and a Gilson 306, as slave modules, the former fitted with a Microperspex peristaltic pump from LKB (S-16 16 Bromma, Sweden). Both pumps were made from stainless steel and were passivated with 7 mmol/l nitric acid. A Gilson 805 manometric module, a Gilson 811C Dynamic mixer fitted with a titanium mixing chamber and a Gilson 119UV detector with backpressure regulator in line were used. For injection a Rheodyne PEEK 9125 injector was used with a fixed 20 μl PEEK loop. The glass-lined column was an octadecylsilane (ODS) Chrompak Chromspher column (5 μm particle size), measuring 10 cm ± 3.0 mm with a stainless-steel holder and a 10 mm ± 3 mm internal diameter guard column. Column efficiency was measured at inception using the manufacturer's own test mix of fluoroanthene. For this exercise the mobile phase used was a 70:30 (v/v) mixture of acetonitrile and water, which was run at a flow rate of 0.4 ml/min. An injection volume of 50 μl was used and a detection wavelength of 254 nm.

All system operations were controlled via the Gilson model 621 Data Master Module by use of the Gilson 715 software interface version 1.21.

The HPLC system was prepurged with deionized water, acetonitrile, propan-2-ol, deionized water and finally with mobile phase to remove all contaminants before use.

Measurement of NTBI

For the measurement of NTBI the system was operated isocratically at a pressure of approximately 8.3 MPa (1200 psi) with a flow rate of 0.8 ml/min. The detection wavelength was 450 nm.

The method relies on the preferential chelation of NTBI by the use of a large excess of a low-affinity ligand, disodium NTA. This complexes all low molecular mass iron and iron non-specifically bound to serum proteins such as albumin. It does not remove iron bound to transferrin or ferritin [21]. A two-step filtration process is used: filtration with a 100-kDa molecular weight cut-off (MWCO) Whatman ultracentrifuge filter is followed by the use of a 20 kDa MWCO Whatman ultracentrifuge filter. The filtrate is analysed by direct injection onto a reverse-phase liquid chromatography system utilising precolumn derivatization with the high-affinity iron chelator CP22. The red (CP22)~iron complex formed absorbs in the visible region at 450 nm [21].

To ensure minimum contamination from salts, all glassware was prerinsed in acetone, methanol and
deionized water and dried before use. All plastic ware was prerinsed in deionized water only before use.

Reagents

The mobile phase was prepared fresh each day as follows. A 3 mmol/l solution of CP22 was prepared in 5 mmol/l Mops buffer and the solution adjusted to pH 7.0 with 1.0 mol/l sodium hydroxide before making up to volume. An 800-ml aliquot of this solution was mixed with 200 ml of acetonitrile and the resulting solution degassed by vacuum filtration followed by ultrasonication for 10 min.

NTA stock solution

A stock solution of 0.8 mol/l was prepared in deionized water and the pH adjusted to 7.0 with NaOH 1.0 mol/l before making up to volume.

Iron solutions for standard curve

A stock solution of 1.0 mmol/l ferric nitrate nonahydrate was prepared in deionized water containing NTA at a final concentration of 5 mmol/l. The pH of the solution was adjusted to 7.0 with 1.0 mmol/l sodium hydroxide before making up to volume. A series of solutions of ferric nitrate nonahydrate from 0 to 10 μmol/l was prepared fresh from the stock standard on each day of analysis.

Reagent blank

Reagent blanks were prepared from Mops buffer and NTA and run through the entire sample preparation procedure on each day of sample analysis.

Sample preparation

To 100 μl of plasma was added 10 μl of NTA stock solution, then the mixture was homogenized and allowed to stand for 20 min at room temperature to optimize chelation of NTBI. The solution was diluted in an equal volume of 5 mmol/l Mops, mixed and allowed to stand at room temperature for 10 min before filtration. A 130-μl aliquot was transferred to a 100 kDa MWCO Whatman ultracentrifuge filter and centrifuged at 12000 g for 30 min at 4°C. The filtrate was transferred to a 20 kDa MWCO ultracentrifuge filter and centrifuged for a further 30 min at 12000 g and 4°C. The final filtrate was held at 0°C over ice before injection in duplicate via Rheodyne, and resolution by HPLC.

Recovery of NTBI from ‘spiked’ samples of adult plasma

A 20-ml sample of adult human blood was taken up into lithium heparin, mixed gently, centrifuged at 400 g for 10 min at 4°C and the plasma removed. A 500 μl volume of a solution of 1.196 mmol/l ferric nitrate nonahydrate was added to 10 ml of plasma to give a final concentration of 56.94 μmol/l. The spiked plasma was stored in aliquots at −70°C. These samples were used for the determination of the recovery of NTBI from plasma and for the measurement of within-batch and between-batch assay precision. An aliquot of this spiked plasma sample was run with each batch of samples as an in-house quality control.

RESULTS

CP22-bound iron eluted at a retention time of approximately 3.4 min. The system is able to detect iron in a ferric nitrate standard at a concentration as low as 0.02 μmol/l. A signal-to-noise ratio of 8 was obtained at 0.12 μmol/l iron. Conventionally, the detection limit for an assay using HPLC is the concentration at which a signal-to-noise ratio higher than 2 is achieved [23]. For the ferric nitrate standards this gives a detection limit of less than 0.02 μmol/l. Figure 1 shows a typical standard curve obtained over the range 1.07–10.72 μmol/l ferric iron, for which regression analysis gave a correlation coefficient of 0.997.

Development work showed linearity of response between 0.55 and 480 μmol/l; the range of standards chosen (1–10 μmol/l) reflects the concentrations of NTBI found in most samples tested. Ferrous iron was found to elute at the same time; autoxidation

![Plot](image-url)
leads to quantitative conversion to the CP22-ferric iron complex.

Iron was not detected in the reagents used in the sample preparation, but we encountered low-level contamination in reagent blanks that had been processed as samples, which we traced to the filters used in the sample purification. Washing the filters with water to remove possible contamination before use with samples proved ineffective. The use of NTA and CP22 was also explored, but once either of these reagents had passed through a filter damage to the filter bed precluded further use. Contamination introduced into samples during filtration had therefore to be estimated from appropriate filtered reagent blanks. Analysis of 12 filtered reagent blanks measured on the same day gave iron concentrations in the range 1.49–2.07 μmol/l with an average of 1.88 μmol/l (SEM 0.092). The overall mean value for the contamination in 43 filtered reagent blanks run over a 9 month period was 1.66 (SEM 0.09). A correction was made for this potential interference from the filters by including three filtered reagent blanks with all runs. Over 9 months of sample analysis the average value of filtered reagent blanks run in triplicate (12 runs) ranged from 1.31 to 2.36 with a mean of 1.75 (SEM 0.12).

Analysis of the ‘spiked’ samples of adult plasma gave a recovery of between 92.0% and 99.2%. The intra-batch coefficient of variation was 5.13% and the inter-batch coefficient of variation was 9.97%.

Chromatograms showing NTBI in a processed reagent blank, a ‘spiked’ adult plasma sample and plasma from a premature baby are shown in Fig. 2.

The nature of the first peak seen in Fig. 2B was investigated by inductively coupled plasma emission spectroscopy (ICPES). The peak was identified as lithium, attributable to the exposure of the infants’ blood to lithium heparin anticoagulant. Although this was used in all blood samples collected, the relatively large volume of the adult blood samples would explain the absence of the HPLC peak in these samples.

NTBI could not be detected in any of 13 samples of adult plasma. In contrast, NTBI was detected in 55 of 111 plasma samples collected from 50 babies born at between 24 and 36 weeks’ gestation. Values for detectable NTBI fell between 0.03 μmol/l and 15.5 μmol/l, with a median of 0.45 μmol/l. Samples were obtained on the day of birth and days 2, 4, 5 and 6 of life for nine of the babies and the data from this cohort (Fig. 3) suggest that plasma NTBI may be higher on the day of birth than at any other time over the subsequent 6 days. Seven of 57 samples of BAL fluid collected over the first 6 days of life from 26 babies showed evidence of NTBI, with detectable NTBI ranging from 0.06 μmol/l to 0.99 μmol/l and a median within this range of 0.35 μmol/l.

**DISCUSSION**

This paper reports the first direct demonstration of the presence of NTBI in the plasma and BAL fluid of babies born before term. The method used has been rigorously developed and characterized.
The measurement can be made in duplicate using only 100 μl samples, making it particularly appropriate for studies in neonates.

The importance of this methodology to an understanding of the aetiology of and the pathogenesis of CLD of prematurity cannot be overestimated. Studies in babies born prematurely have identified a number of factors that appear to play a role in the development of CLD. These include aspects of neonatal care such as the use of supplemental oxygen, mechanical ventilation and transfusion of packed red blood cells [24–26]. It is now widely accepted that lung damage is mediated by the effects of increased production of reactive oxygen species. The potential involvement of 'catalytic' iron in reactions leading to the production of reactive oxygen species has been acknowledged for many years, and has been clearly demonstrated in vitro. The contribution of iron to such reactions in vivo is limited by the presence of specific iron-binding proteins both intracellularly (ferritin) and extracellularly (transferrin), and ferroxidase activity to maintain the ease of binding of iron to these proteins. This protection may be seriously compromised in babies born prematurely, in whom levels of plasma transferrin [27] and ferroxidase activity [17] are low. In a longitudinal study of antioxidant status in preterm babies, we have recently demonstrated that the antioxidant activity of the plasma at birth, measured as the ability of the plasma to oxidize and bind iron, is a powerful predictor of outcome [28]. The antioxidant activity of the plasma measured in this manner is influenced by the concentration of total ascorbic acid, which interferes with the ferroxidase activity of the plasma and thereby reduces protection from the effects of potentially 'catalytic' iron.

Other workers have reported the presence of potentially 'catalytic' iron in the plasma of babies born prematurely, using the bleomycin assay, and have implicated it in the pathogenesis of CLD. Unfortunately, the assay is time-consuming and does not lend itself to automation. The conditions of the assay, which must be conducted in unbuffered media, are critical to the interpretation of the results as outlined by the authors [29, 30]. The HPLC method described in this paper measures iron that is not bound to the plasma iron carrier transferrin, and is therefore termed NTBI. A key factor in the measurement of low concentrations of NTBI using the method described is the removal of all possible sources of iron as contaminant from the hardware itself. This is an expensive but critical aspect of the assay. The HPLC system was sufficiently sensitive to be able to detect ferric nitrate in solution at a concentration as low as 0.02 μmol/l. Solutions and glassware did not present a significant contamination problem, but the essential two-step filtration to remove plasma proteins did. As the contamination varies from one filter to another, the appropriate correction applied to samples to account for this source of iron can be determined either from a random sampling of filters at the time of each sample run (as described here) or as the average of measurements made over a longer period of time.

The technique has been applied to plasma and BAL samples collected from preterm babies over the first week of life. In contrast to the complete absence of NTBI in plasma samples from 13 adults, 50% of all plasma samples collected from 50 babies showed detectable NTBI. Serial data from nine babies suggest that high concentrations of NTBI may be more likely on the day of birth than subsequently. This would be compatible with the rapid increase in plasma transferrin and caeruloplasmin that is observed during the neonatal period in premature babies [16, 18]. It may also be relevant to the observed association between plasma antioxidant activity at birth and outcome in premature babies, which we have reported previously [28]. The detection of NTBI in BAL fluid collected from preterm babies has not been reported before. It may indicate endothelial cell damage and plasma leakage into the alveolar space. This is an application that deserves particular attention as it may provide a biochemical marker of early lung damage.

In conclusion, the automated HPLC measurement of NTBI has been developed for application to small samples of plasma and BAL fluid from preterm infants. This method could make a valuable contribution to progress in our understanding of the aetiology of CLD in preterm infants and the effectiveness of strategies to minimize lung damage in this vulnerable group.

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REFERENCES


