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A Simple and Rapid Method for the Determination of "free" Iron in Biological Fluids

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We present a convenient method for determining "free" or non-protein-bound iron in biological fluids. The new method is based on the bathophenantroline method for determination of total serum iron, and comprises binding of iron by a chromogenic chelator (bathophenantroline-disulphonate, BPS), which is specific for ferrous iron. The ferrous complex of BPS absorbs strongly at 535 nm, and the detection limit is less than 1 μM in a sample size of 50 μl. The chelator does not liberate iron from either haemoglobin or transferrin. Interference from copper or zinc in concentrations up to 50 μM does not significantly disturb measurements. The main problem when measuring in blood plasma, the high and fluctuating background in the region around 535 nm, has been overcome through filtering techniques. Data from measurements of ferrous iron in microdialysate, cerebrospinal fluid, and blood plasma in different animal models and clinical conditions are presented as illustrative examples of the usefulness of the method. The method allows the determination of ferric, as well as ferrous, iron in the same sample.

Keywords: Free radicals; Iron; Plasma; Cerebrospinal fluid; Microdialysis; Analysis

INTRODUCTION

Iron in the ferrous state is a key element in mediating transformations from less to more reactive and damaging oxygen species. In fact, all that is required for the formation of the noxious hydroxyl radical is ferrous ion in an aqueous environment also containing dissolved molecular oxygen:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot-} \quad (1)$$

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^- + HO^{\cdot} \quad (3)$$

Actually the same result can be achieved starting from ferric iron and any suitable bioreductant, including superoxide. Thus, ferric as well as ferrous iron are important determinants of the level of oxidative stress in a specific system. If the iron that catalyses formation of the hydroxyl radical is bound close to a membrane surface, the membrane destroying process of lipid peroxidation will be initiated, and will ultimately lead to cell death.

Most of the iron in living systems is, for obvious reasons, sequestered into compartments in which it does not participate in redox reactions. This strategy is reflected in the transport and storage proteins transferrin and ferritin which bind iron tightly in the ferric form, separating it from the aqueous environment. However, there are various disturbances, such as ischaemia and reperfusion, or inflammation, that may result in inappropriate liberation or translocation of iron.[1–6]

The liberated iron is generally referred to as "free" iron although no such species exists in a biological system. Instead, the iron ion will always be complexed more or less strongly by a variety of biomolecules. Examples include citrate, DNA, albumin, and phosphate groups such as those of the membrane phospholipids. The term "free iron" thus refers to iron bound in such a way that it retains its ability to catalyse the formation of reactive oxygen

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species, and it is therefore a highly relevant parameter to measure during periods of oxidative stress.

For a long time, the dominating method for analysis of free iron in biological samples has been based on the binding of iron to bleomycin, and the ability of this complex to inflict damage to DNA resulting in the formation of intensely coloured products in a reaction with thiobarbituric acid.[7,8] In recent years, however, alternative methods have emerged where the iron to be measured is bound to other chelators with characteristics that can be analysed in various ways, including Fluorescence quenching[9,10] and dequenching,[11] chromatography[12,13] and even electron spin resonance (ESR).[14] In this work, we present a spectrophotometric method for measuring free iron which is cheap, rapid and simple, and builds on standard methods[15] for determination of total iron. One particularly attractive feature of the method is that it allows specific determination of both ferrous and ferric iron in the same sample.

MATERIALS AND METHODS

Chemicals

The following chemicals were of the highest available purity and purchased from Sigma-Aldrich Chemical Company: 4,7-diphenyl-1,10-phenantroline disulphonate (bathophenantroline disulphonate (BPS) (B 1375), ferrous ammonium sulphate $[(NH_4)_2Fe(SO_4)_2]$ (F 3754), holotransferrin (T 7434), haemoglobin (H 7379). $FeCl_3{\cdot}6H_2O$ (101103Q) and ascorbic acid (103033E) were from BDH Laboratory Supplies, and the apo-transferrin (178481) was supplied by Calbiochem.

BPS is a bidentate ligand, coordinating to iron via the two nitrogen atoms in the phenantroline ring system. Since iron usually accommodates six ligands, the binding stoichiometry will be 3 BPS per Fe. The two sulphonate groups render the molecule hydrophilic, and there is practically no partition into lipophilic environments. Binding constants for Fe(II) are in the order of 10^{22} (β^3).[16] The ferrous complex exhibits strong absorption at 535 nm ($\varepsilon = 221 \times 10^3\ M^{-1}\ cm^{-1}$.[17])

Materials

Plasma samples were filtered using the Millipore MultiScreen® Filtration System (MAHV N45 10), including MultiScreen Vacuum Manifold and MultiScreen-HV Clear plates with 0.45 μm Hydrophilic low protein binding Durapore® membrane and lid. Filtered samples were transferred to polystyrene 1/2 area 96-well plates (costar® 3690). Absorbances were read with a plate reader from Molecular Devices (SPECTRA max® plus) controlled with Softmax Pro 2.4 software.

Microdialysis probes were from CMA Microdialysis AB (CMA/10, 4 mm; CMA/12, 2 mm).

Standard Curves for the Ferrous Bathophenantroline Complex

A 50 mM stock solution of the ferrous iron chelator BPS was prepared by dissolving 28.2 mg BPS in 1000 μl deionised pyrogen free water.

A standard curve was constructed by dissolving 196 mg ferrous ammonium sulphate in 500 ml deionised pyrogen free water. This 1 mM solution was further diluted with water to produce solutions of 0.1–50 μM. To 490 μl of each of these solutions was added 10 μl of the BPS stock. After mixing, the samples were left for 15 min to ensure complete formation of the complex. It has been shown previously[18] that complex formation is essentially complete within the first minute after mixing. Then 50 μl of each sample was transferred to a 96-well plate, and absorbance was read at 535 nm against a water blank.

Measurements in Microdialysate

Two neonatal pigs (<5 days old) from a local farmer were anaesthetised (i.v. chloralose) and had microdialysis probes implanted in cortex (2 mm probe), striatum and white matter (4 mm probes). Microdialysis was maintained at a flow rate of 1 μl per min (Ringer solution), before, during, and after a period of hypoxia, induced by supplying one animal with 8% oxygen for 60 min, so that the electroencephalogram was rendered isoelectric[19] while the other animal was allowed to breathe normal air throughout the experiment. Microdialysate was collected and analysed for iron content every 60 min. Of the microdialysate 48 μl was pipetted into the wells, and all samples were blanked. Then 1 μl of EGTA (250 mM) to prevent turbidity sometimes caused by precipitation of Calcium in the Ringer solution, and 1 μl of the BPS stock was added and mixed. The plate was then left for 15 min, and read at 535 nm.

Measurements in Cerebrospinal Fluid

Neonatal cerebrospinal fluid (CSF) was obtained by therapeutic ventricular taps from preterm infants with post-haemorrhagic hydrocephalus, or by lumbar puncture from healthy term infants with suspected but unconfirmed infectious disease. The latter group served as negative controls.

CSF (49 μl) was of CSF was pipetted into wells and absorbance was read at 535 nm to give the blank absorbance for each sample. Then 1 μl of the BPS

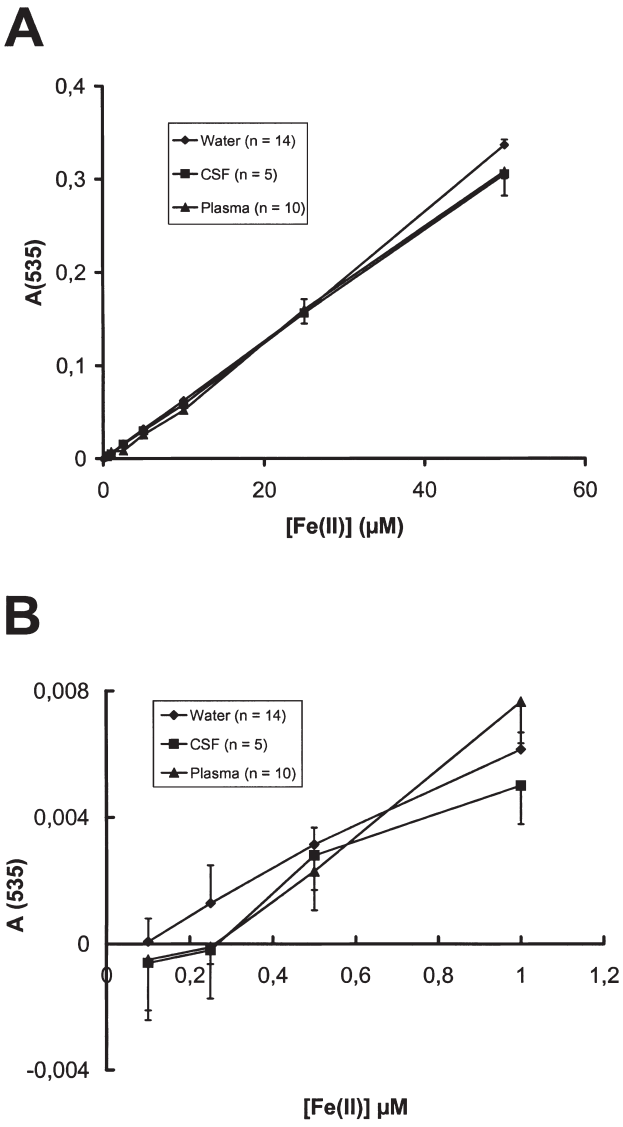

FIGURE 1 Comparison between standard curves of the Fe(II) BPS complex in water, CSF and plasma. The complex was made up in water and added in a volume of 1 μl, to 49 μl of CSF or plasma, and then filtered as described in the "Materials section". Data are given as mean ± SD of four measurements. (B): Enlargement of the area between 0 and 1 μM in (A).

stock solution was pipetted into each well. After mixing the plate was left for 15 min, and the absorbance was again read at 535 nm. After subtraction of the blank values the readings were quantified against the standard curve.

Measurements in Blood Plasma

Blood samples were obtained from four patients undergoing dialysis treatment, and from 10 healthy non-smoking volunteers. One sample was withdrawn before, and one after the dialysis session in each subject in the patient group. The blood was collected into heparinised Vacutainer® tubes and allowed to stand in room temperature about 15 min to reduce subsequent haemolysis whereafter the samples were centrifuged at 1000*g* for 10 min.

Plasma samples were analysed promptly as follows: Two portions of 73.5 μl were drawn from each plasma sample. To one of these was added 1.5 μl of the BPS stock solution. The other portion served as an individual blank and received 1.5 μl of deionised water. After allowing 15 min for the BPS–Fe^{2+} complex to form, all samples were filtered through the MultiScreen® filtration system. This procedure removed much of the background absorbance that was seen in plasma at 535 nm. More importantly, the filtration prevented the background from fluctuating with time. After filtration 50 μl of each sample and blank was transferred to the half-well plate and absorbance measured at 535 nm. The value of each respective blank was subtracted from each of the samples containing the BPS complex.

TABLE I Interference from zinc and copper*

	A_{535}	
	Zinc	Copper
0 (μM)	0.068 ± 0.001	0.068 ± 0.001
10 (μM)	0.067 ± 0.003	0.071 ± 0.006
25 (μM)	0.067 ± 0.002	0.067 ± 0.002
50 (μM)	0.074 ± 0.010	0.067 ± 0.005

*The data in the table denotes the absorbance recorded from a 10 μM solution of Fe(II) in the presence and absence of various concentrations of Zinc and Copper *upon addition of 1 mM BPS*. Data are means ± SD from five observations.

Ethical Considerations

The experiments in this study that involved animals and patients were scrutinised and approved by the local ethics committee. Before obtaining human material, informed consent were obtained from the subjects or, in the case of infants, from their parents.

Statistics

Statistical significance was tested using Student's *t*-test, or Fischer's exact probability test. A *p*-value ≤ 0.05 was considered statistically significant.

RESULTS

Standard Curves

Figure 1 shows the standard curve for the complex between BPS and ferrous iron obtained when adding 1 mM BPS to different concentrations of ferrous ammonium sulphate in water, CSF, and blood plasma. Linearity persists well below 1 μM Fe, and we consider the detection limit to be 0.5 μM for plasma and CSF, and 0.25 μM in less complex fluids,

TABLE II Interference from transferrin

Reaction mixture	*n*	$A_{535} \pm SD$
60 μM iron-saturated transferrin	5	0.046 ± 0.000
60 μM iron-saturated transferrin + 1 mM BPS	5	0.047 ± 0.001
10 μM BPS Fe	4	0.071 ± 0.004
10 μM BPS Fe + 30 μM apo-transferrin	4	0.070 ± 0.005
10 μM BPS Fe 3 h later	4	0.064 ± 0.006
10 μM BPS Fe + 30 μM apo-transferrin 3 h later	4	0.061 ± 0.006

such as microdialysate, since the respective coefficients of variation exceeded the mean values below these concentrations. It is evident from Fig. 1A and B that the BPS Fe complexes have virtually the same absorbance and show the same linearity in either one of these fluids.

Interference

Copper and Zinc

Interference from Copper and Zinc is shown in Table I. Presence of either Copper and Zinc in concentrations up to 50 μM have only small effects on the absorbance of 10 μM Fe when BPS is added. The

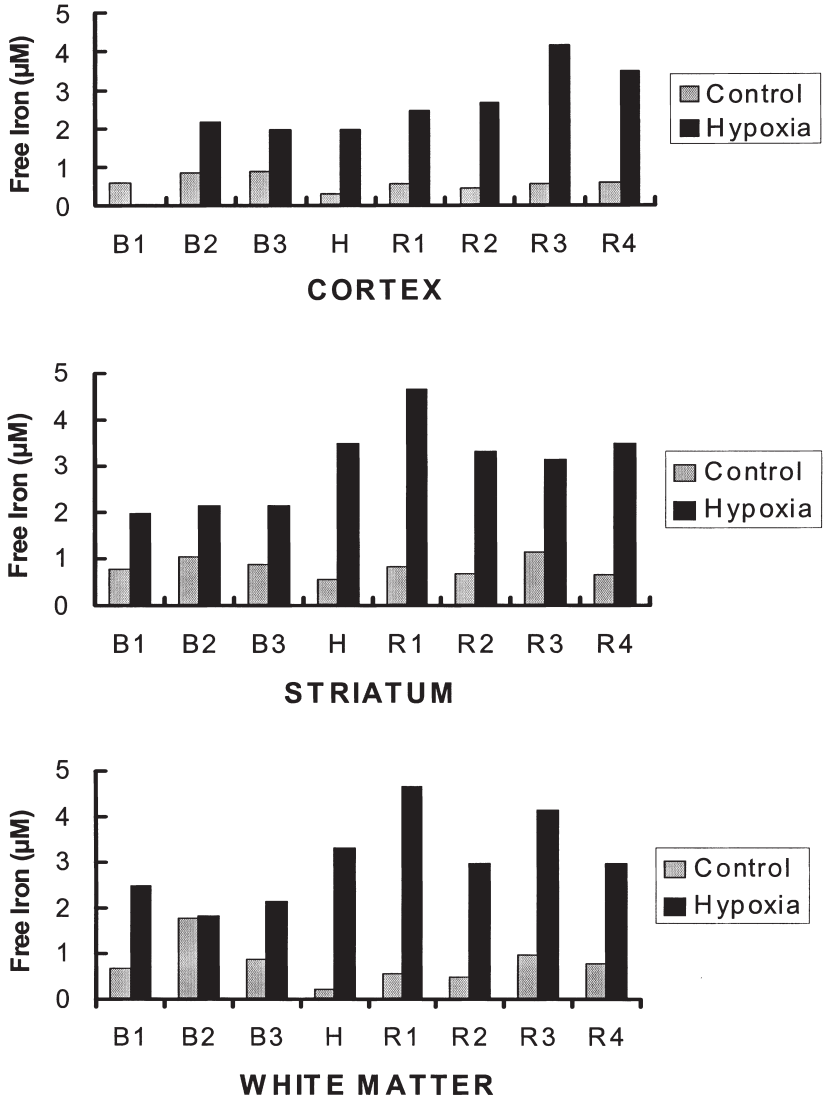

FIGURE 2 Concentrations of free ferrous iron in microdialysates obtained from probes implanted in different areas of the brain of two neonatal piglets, one of which was subjected to a 1 h period of hypoxia by ventilating it with 8% oxygen. *Legend*: B, baseline; H, hypoxia; R, restitution with air. The sampling time was 60 min at a flow of 1 μl per min. Comparison of baseline values to restitution values reveals significantly elevated iron concentrations in the hypoxic animal during restitution in striatum and white matter.

TABLE III Measurement of free ferrous iron in CSF from preterm infants suffering from post-haemorrhagic hydrocephalus (A–F), and from a group of healthy term infants (G–J) with suspected but unconfirmed infection of the cerebral system. "*n*" denotes the number of separate measurements on each sample

Patient	Concentration of free iron (μM)	*n*
A	1.7 ± 0.0	4
B	2.0 ± 0.7	5
C	2.1 ± 0.4	9
D	3.1 ± 2.2	6
E	2.3 ± 0.6	6
F	1.7 ± 0.3	10
Healthy control		
G	Not detectable	1
H	Not detectable	1
I	Not detectable	1
J	Not detectable	1

TABLE IV Determination of free ferrous iron in plasma from dialysis-dependent patients with renal disease (1–6), and from healthy volunteers (7–16)

	Iron concentration (μM)	
Patient	Before dialysis	After dialysis
1	5.8	Not detectable
2	2.0	Not detectable
3	3.3	Not detectable
4	Not detectable	Not detectable
5	Not detectable	Not detectable
6	0.5	Not detectable
Control		
7–16	Not detectable	

increase of absorbance seen with 50 μM Zinc is not statistically significant.

Transferrin

Interference from transferrin was tested in two ways: first the release of iron from saturated transferrin to BPS was measured, and then release of iron from 10 μM BPS Fe to apo-transferrin was tested. The results, summarised in Table II, show that the release of iron in both cases was minimal. Even after 3 h of incubation of the apo-transferrin and BPS Fe there were no significant uptake of iron to the protein. Interference from haemoglobin is discussed below.

Measurements in Microdialysate: Neonatal Hypoxia in the Pig

Although measurable concentrations of iron were present in microdialysate from all three localities already during baseline conditions, significantly increased concentrations of free ferrous iron in brain interstitium were found after a 1 h period of hypoxia in a neonatal piglet (Fig. 2). The increase was detected in microdialysates from striatum ($p = 0.012$) and white matter ($p = 0.032$), but not from cortex ($p = 0.126$). No increase in the level of free iron was seen in the control animal during the course of the experiment. (*p*-values were obtained by comparing the group of three baseline values to the group of four reoxygenation values for each animal).

Measurements in CSF: Preterm Post-haemorrhagic Hydrocephalus

The *upper* part of Table III shows the results from measurement, with BPS, of concentrations of free ferrous iron in CSF from six preterm infants suffering from post-haemorrhagic hydrocephalus. All subjects had detectable amounts of free iron in their CSF, while iron concentrations in the control group, consisting of five healthy term infants, were below the detection limit of the BPS method.

Blood contaminating the CSF from subjects with haemorrhagic disorders, such as those in Table III, is a possible source of artefactual iron, which could be liberated during inappropriate treatment and storage of samples. Therefore, pooled normal CSF was incubated at 37°C, with or without addition of up to 2% whole blood, for up to 7 days in a crude simulation of the *in vivo* situation in the hydrocephalic children of Table III. Freezing and thawing of the CSF induced haemolysis after 3 days, unless the samples were centrifuged before freezing, in which case haemolysis was seen only after 7 days of incubation. However, there was no sign of iron release from haemoglobin in any of the samples, although very strong haemolysis seen in a few samples may have masked small increases in free iron (data not shown).

Measurements in Blood Plasma: Elimination of Background

One problem with absorbance measurements in plasma is the high and variable background present. However, much of this background could be eliminated by filtering the samples through the system mentioned in the "Materials and methods section". This also prevented the absorbance in the samples from fluctuating with time. The recovery of the BPS Fe complex after such filtration was measured by filtering 20 aliquots of pooled plasma. To half of these 25 μM Fe BPS was added before filtering, and the other half of the samples received 25 μM Fe BPS after filtration. A filtered plasma sample without Fe BPS was used as blank. The recovery, given by the ratio between the samples filtered after and those filtered before addition of Fe BPS, was 95.5 ± 7.3% (coefficient of variation 7.6% of the mean).

Measurements in Blood Plasma: End Stage Renal Failure

We have used the BPS method to determine serum levels of free ferrous iron in a small number of patients with end stage renal disease, and undergoing haemodialysis (Table IV). A group of healthy volunteers recruited from the staff of our department served as negative controls. As seen in Table IV, the plasma from three out of the six patients contained detectable amounts of free iron when measured immediately before a dialysis session, whereas no iron could be detected shortly after the dialysis was finished approximately 4 h later. No free iron was found in plasma from any of the control subjects. The differences before and after dialysis, and between the patients and the controls were statistically significant when subjected to Fisher's exact probability test ($p < 0.05$).

Discrimination Between Ferrous and Ferric Iron

BPS forms stable complexes with ferric iron as well as with ferrous, but the former is colourless. It is, however converted to the ferrous complex by incubation with e.g. ascorbate: BPS (final concentration 1 mM) was added slowly to a solution of 100 μM ferric chloride in citrate buffer, pH 3.2. After slow neutralisation of pH 6.9, ascorbate (final concentration 1 mM) was added, and 30 min later absorbance at 535 nm was read against a blank consisting of buffer, BPS and ascorbate, and treated in the same way. Upon addition of ascorbate, ferric iron was reduced, and a pink colour developed. Absorbance at 535 nm in the samples corresponded to 100.3 ± 0.2 μM Fe^{2+} ($n = 5$), demonstrating quantitative transfer from ferric to ferrous ion.

There is a possibility that ascorbate could liberate iron bound to plasma proteins, e.g. transferrin. However, treatment of four samples of pooled plasma with 1 mM each of BPS and ascorbate, and incubated for 1 h, did not lead to any detectable release of iron.

DISCUSSION

What, exactly, is free iron? It has been branded as "non-protein-bound" "low molecular-weight", "spurious", "adventitious" or "unspecifically bound", etc. Most likely, it can be all or none of the above; actually referred to is any iron bound in a way enabling it to catalyse reactions associated with oxidative stress. These include reduction of oxygen to superoxide, formation of hydroxyl radicals from hydrogen peroxide in the Fenton reaction, and iron-dependent initiation of lipid peroxidation, all of which are catalysed by ferrous ion. All methods available for determination of free iron rely on stabilising it through chelation and subsequently determining the iron/chelate complex. Since no truly free iron, i.e. coordinated only to water, exists in biological systems, all methods involve the transfer of iron from various biological chelators to the added chelator. Because many of the biological ligands of free iron are unknown, there is no way of ascertaining that all of the free iron in the sample binds to the added chelator. Rather, different methods measure bleomycin-bound,[7,8] Desferal-bound,[9,10] nitrilotriacetic acid-bound (NTA),[13] or, indeed, BPS-bound iron. Presumably, different methods measure the same pool of iron, but this can be ascertained only by carefully designed parallel studies. Such studies lacking, we note one other report[9] of "non-transferrin bound" iron in patients with end stage renal disease. Our results in Table IV (mean 1.9, range 0–5.8 μM, $n = 6$) are compatible with data from reference[9] (mean 3.8, range 0.1–13.5 μM, $n = 68$).

The BPS-method measures directly the concentration of chelatable iron in the sample, and BPS is also used clinically e.g. for determining total serum iron. Several chromogens have been presented as an alternative to BPS,[18,20–23] all of which have somewhat higher extinction coefficients and sometimes absorption maxima at more favourable wavelengths, but BPS is easier to use: there is no need to add masking agents to reduce interference from other metals and, more importantly, the BPS Fe complex retains its extinction coefficient at physiological pH, whereas other chromogens must be assayed under acid conditions, with associated risk of liberating physiologically bound iron, e.g. from transferrin. Also, BPS does not liberate iron bound to either haemoglobin or transferrin (Table II), even during prolonged incubation. Further advantages of the method include low cost and high availability of the chemicals involved, and that the only equipment needed for analysis, a spectrophotometer, is available in virtually any lab anywhere in contrast to equipment needed for other methods, such as HPLC-instruments, ESR-spectrometers, etc.

The standard curve for BPS Fe^{2+} is linear in the interval between 0.5 and at least 50 μM (Fig. 1) in CSF and plasma, and in water linearity remains down to 0.25 μM, allowing for a lower detection limit in less complex biological fluids such as microdialysate. Also, standard curves in water may be used for measurement in other fluids. For iron concentrations below 1 μM, the quality of the spectrophotometer used becomes an important determinant of the detection limit. An instrument with a resolution of a few mAU allows detection of iron concentrations below 1 μM, under the circumstances described above, i.e. a path length of about 2.9 mm in a sample volume of 50 μl. Provided that sample volume is not

limiting, sensitivity can be improved by increasing the path length, allowing detection of iron concentrations below 100 nM. Such concentrations are likely close to the lower limit for biological significance.

A previously reported method for determining non-transferrin-bound iron[24] utilises an excess of NTA to bind free iron in a serum sample. After ultrafiltration BPS is added and chromogen is formed by competition for the iron bound to NTA in the ultrafiltrate. The detour over NTA, however, seems unnecessary in the light of the good recovery of the BPS Fe complex found in our filtration procedure. It also prolongs the time of analysis to 150 min compared with 15 min for the BPS method.

BPS forms complexes with both ferric and ferrous ion, but only the ferrous complex is coloured. The ferric complex of BPS, however, was readily and quantitatively reduced by ascorbate. Therefore, measurement of absorbance before and after adding ascorbate will give the concentration of ferrous iron and total iron, respectively, in the sample. Subtraction of the former from the latter gives the ferric concentration. Thus, the BPS-method, in contrast e.g. to the bleomycin method, is able to differentiate between "active" (i.e. ferrous) and "inactive" free iron in a biological sample, with the ferric part constituting a latent pool that can be activated through bioreduction.

When conducting measurements in biological systems, where reducing agents may occur in abundance, one must consider the redox nature of the BPS Fe(III)/BPS Fe(II) couple. The fact that the ferric complex of BPS is readily reduced by ascorbate indicates that any ferric ions in the sample might be reduced upon binding to BPS, thus leading to underestimation of the ferric/ferrous ratio in the sample before chelation. This reasoning applies to all methods for determining free iron. Also, due to its dynamic nature *in-vivo*, the ferric/ferrous ratio may not be crucial, although ferrous ion is the species directly responsible for initiating oxidative stress.

The BPS method is not particularly susceptible to interference from artefactual iron, since only the chelator, dissolved in water, is added to the sample, and since iron itself is measured, as opposed to its catalytic effect. The BPS itself typically contains 0.001–0.002% iron according to suppliers certificates of analysis. Thus, a 1 mM solution of BPS contains about 0.15 μM contaminating iron, which is below the practical detection limit of the method. This contribution is constant and may simply be subtracted from absorbance values as required.

The data from the pig model for neonatal hypoxia in Fig. 2, the preterm infants with hydrocephalus in Table III, and the haemodialysis patients in Table IV illustrate the use of the BPS-method for analysing free iron in different pathological processes. In particular, the difference compared to the respective negative control group in all three processes provide examples of the usefulness of the BPS method for determination of free iron in various *in-vivo* systems. However, no detailed conclusions concerning the importance of free iron for progression of the particular disorders mentioned above should be drawn from the scant data presented here. Rather, separate studies have been undertaken to elucidate the role played by iron and oxidative processes in the pathologies mentioned.[25,26]

Due to the simplicity and usefulness of the BPS method in a variety of biological fluids under clinical and experimental conditions, to the fact that it measures iron as such rather than its catalytic effects, and to the possibility to discriminate between ferrous and ferric iron, we conclude that the BPS method should prove a valuable alternative to other methods currently available for the assessment of "free" iron.

Acknowledgements

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