ORIGINAL ARTICLE

Selection of Acid Salts: A Critical Step in Creating an Acidic Condition for Plasma Iron Release and Measurement

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ABSTRACT

Introduction: Iron deficiency anaemia (IDA) is the most common cause of anaemia worldwide. Determination of body iron status is necessary to diagnose IDA. This can be measured using a biochemistry assessment of the serum/plasma. Plasma/serum iron quantitation is also important in diagnosing iron overload disorders. However, iron studies are limited due to high cost and lack of access to biochemical analysers. Therefore, a cost- and technical-effective method is needed to measure human plasma iron concentration. Plasma iron is mainly transferrin-bound and an acidic plasmic condition is necessary to release the iron. This study investigated various candidate acid salts to achieve the acidic condition needed for plasma iron release. **Method:** Ten powdered or crystallised acid salts were studied for their water solubility as well as their pH reduction capability in revised simulated body fluid (r-SBF) and commercially available human plasma without any change in colour or form. **Results:** Six acid salts studied were discontinued from further investigation because they were insoluble in water. Another two candidates were unsuitable as they precipitated in r-SBF and human plasma. Maleic acid formed a jelly-like texture after a certain amount of time in human plasma. Only citric acid met all the criteria of a suitable acid salt to be investigated further as part of the reagent for a spontaneous plasma iron measurement. **Conclusion:** Citric acid, which is a colourless and odourless acid salt, was selected to lower the human plasma pH to an acidic condition for transferrin-bound iron release.

Keywords: Acid salts, Plasma iron, Citric acid, Iron deficiency anaemia, Human plasma

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INTRODUCTION

Anaemia is a public health problem that affects almost two billion people globally, in both non-industrialised and industrialised countries, with approximately 50% attributed to iron deficiency anaemia (IDA) (1,2). The lack of oxygen supplied by the red blood cells (RBCs), for the body's normal physiological needs, causes anaemia (2,3). Causes of IDA include lack of dietary iron, iron malabsorption, chronic gastrointestinal bleeding, and the maternal and perinatal period of iron deficiency anaemia, hookworm infestations and certain infectious diseases like malaria (4).

High-risk groups that are most vulnerable to iron deficiency are infants, young children, teenagers, women of reproductive age and seniors in terms of physiological demand (age and gender-related factors), vegetarians due to dietary habit, individuals in resource-poor area in terms of socioeconomic influences and others (3,5). In Malaysia, almost two million women of reproductive age are anaemic (6). A major concern in IDA includes impairment of oxygen delivery that may lead to weakness, lethargy, dyspnoea, unusual headaches, taste disturbances, difficulty in concentration, poor work capacity and productivity as well as decreased cognitive performance and physical development (5,7).

IDA is characterised by smaller RBCs (microcytic), reduced haemoglobin (Hb) content (hypochromic) with pencil-shaped and target cells seen in blood films and both reduced mean cell volume (MCV) and mean

cell haemoglobin (MCH) in red cell indices (3,5,8). However, other diseases such as thalassaemia, chronic anaemia and sideroblastic anaemia have similar laboratory findings; microcytic hypochromic anaemia in terms of blood film and red cell indices (3). Therefore, misdiagnosis is easy unless the iron status is assessed.

Serum/plasma ferritin is commonly used to assess the body's iron status (9). However, the serum/plasma ferritin test might only be available in some areas and has to be quantified using an automated biochemistry or enzyme-linked immunosorbent assay (ELISA) analyser and trained staff are required for blood collection and assay runs. Not only that, the sample quality may be compromised during transportation and the results take a while to be released. Thus far, there is no low-cost and efficient iron tool for body iron screening in the market, especially in field studies. Furthermore, to target rural areas, a simple iron tool might be useful, accompanied by simple blood collection (10). Approaches involving multiple complex steps and sophisticated machines are not only time-consuming, but also not as cost-effective as an absorbance approach, which can be applied in serum iron quantitation.

Serum iron measurement is also important in iron overload disorders for example, hereditary haemochromatosis albeit affecting a much smaller population. Patients with iron overload have increased iron stores which exceed the body's ability to eliminate it, leading to soft tissue damage e.g. liver cirrhosis and cardiomyopathy. The diagnosis of iron overload disorders can be made using serum iron studies, liver biopsy and assessment of response to phlebotomy if clinically indicated (11).

Serum/plasma iron is abundant in blood plasma, which includes all iron, especially from transferrin-bound iron apart from ferritin-bound iron. Transferrin-bound iron is readily released in acidic conditions compared to ferritin-bound iron which requires ferritin cage degradation (12). This study aims to reduce the pH level in commercially available human plasma to pH 2, which is suitable for transferrin-bound iron release for spontaneous plasma iron detection (unpublished data).

Generally, there are three steps involved in serum or plasma iron determination: (i) liberation of Fe^{3+} from Transferrin-Fe-complex, (ii) reduction of Fe^{3+} from Fe^{2+} and (iii) formation of Fe^{2+}•coloured complexes **[Equation 1]**.

Transferrin•Fe-complex+pH effect apotransferrin+ Fe^{3+} (i)
Fe^{3+}+ reducing agents/iron chelator Fe^{2+} (ii)
Chromophore + Fe^{2+} → Fe^{2+} •coloured complexes (iii)
[Equation 1]

This study investigated various acid salts to achieve pH 2 in revised-simulated body fluid (r-SBF) and commercially available human plasma for transferrin-bound iron release as the first step in producing a low-cost single-step plasma iron detection method.

MATERIALS AND METHODS

Ethical approvals were obtained from the Medical Research Ethics Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia [UPM/TNCPI/RMC/1.4.18.1(JKEUPM)/F2] and Medical Research and Ethics Committee, Ministry of Health Malaysia (KMM/NIHSEC/P15-1181).

Revised-simulated body fluid (r-SBF) preparation

r-SBF buffer was prepared in a total volume of 1000 mL containing reagents: 5.376 g NaCl, 0.736 g $NaHCO_3$, 2.056 g Na_2CO_3, 0.224 g KCl, 0.230 g K_2HPO_4•$3H_2O$, 0.305 g $MgCl_2$•$6H_2O$, 11.976 g HEPES, 0.293 g $CaCl_2$ and 0.072 g Na_2SO_4. All reagents were purchased from either Merck & Co., Kenilworth, NJ, USA or Nacalai Tesque Inc., Kyoto, Japan. Subsequent reagents were added accordingly after each prior reagent had dissolved completely. After that, pH was adjusted to pH 7.40 with 1.0 M NaOH and topped up to 1000 mL with deionised water. After a defined storage period, ion concentration and pH of r-SBF were assessed using elemental analysis and a pH meter, respectively, to examine the quality of prepared r-SBF in term of each ion concentration and pH of the r-SBF.

Quality assessment of revised-simulated body fluid

Inductively coupled plasma-optical emission spectroscopy (ICP-OES) and atomic absorption spectroscopy (AAS) were used to quantitate element of interest in a solution by referring to a calibration curve of the desired element. An iron standard curve and quality control standard were constructed briefly. In order to overcome matrix effects due to sample viscosity and analytes, the sample was run with a spike of yttrium as an internal control. Analysis was run in triplicates and results were analysed by taking the dilution factor into consideration. Sodium (Na), potassium (K), and magnesium (Mg) cations were assessed using ICP-OES while calcium (Ca) and phosphate (P) cations were assessed using AAS.

Screening of acids salts

For analytical sample preparation, human blood plasma can be adjusted by mixing with a strong acid or base to the desired pH (15). Since iron release can be induced effectively in acidic conditions, various acid salts, which allow the pH of r-SBF and human plasma to reach pH 2, were screened. Powdered as well as crystallised acids were chosen as the single-step plasma iron detection method, as the detection method, which will be subsequently developed requires lyophilised reagents.

Ten candidate acids were chosen; citric acid, benzoic acid, maleic acid, nicotinic acid, molybdic acid, oxalic acid, 2,4-dichlorophenoxyacetic acid, salicylic

acid, succinic acid and tartaric acid. These acids were dissolved in water to achieve 2 M, 1 M or 0.5 M. Acids that were unable to dissolve spontaneously in water were excluded from further experiments. The dissolved acid pH was measured using a pH meter. After that, selected acids were used to adjust r-SBF (1 mL) and plasma (1 mL) to pH 2, respectively.

RESULTS

Revised-simulated body fluid preparation

SBF was prepared in order to mimic the physiological condition, in which the ion concentrations are almost similar to human plasma, as human plasma would be used as buffer for the single-step reaction. r-SBF was prepared according to Oyane et al. (2003) and stored at 4˚C for quality assessment (14). The prepared r-SBF was clear without any visible precipitation after storage up to 1 month in 4˚C and the pH ranged from 7.4-7.45 at room temperature.

Quality assessment of revised-simulated body fluid

The content of Na^+, K^+, Mg^{2+}, Ca^{2+} and PO_4^{3-} elements were 5.1 mM (101.7% recovery), 1.4 mM (95.3% recovery), 1.4 mM (57.0% recovery), 0.8 mM (75.1% recovery) and 99.8 mM (70.3% recovery) in the prepared r-SBF; respectively in comparison to commercially available plasma (Fig. 1). The prepared r-SBF was similar to human plasma especially batches 6 and 7, which were used for subsequent experiments (Fig. 1).

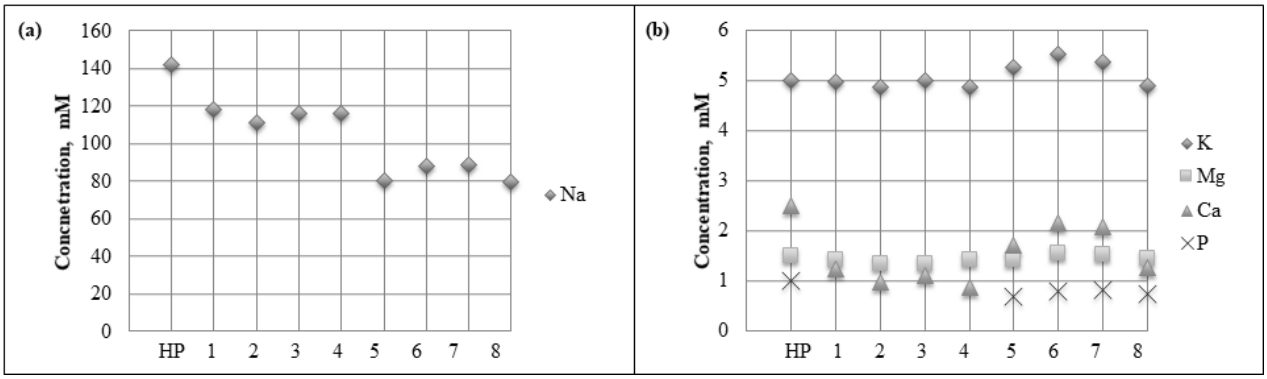

Fig. 1 : Concentration of (a) Na element and (b) K, Mg, Ca, P elements in prepared r-SBF, assessed by ICP- OES or AAS. HP: Human plasma (positive control); 1 – 8: different batches of r-SBF. Abbreviation: r-SBF- revised simulated body fluid, ICP-OES-inductively coupled plasma-optical emission spectroscopy, AAS- atomic absorption spectroscopy.

Screening of acids salts to adjust the r-SBF and human plasma pH

Ten different acid salts were screened to reduce the pH level of r-SBF and human plasma to pH 2. Benzoic acid, nicotinic acid, molybdic acid, 2,4-dichlorophenoxyacetic acid, salicylic acid, and succinic acid were not completely soluble in water even in concentrations as low as 0.5 M. They were discontinued from subsequent experiments. Oxalic acid and tartaric acid were turbid when dissolved in r-SBF as well as human plasma and were excluded from further experiments. Maleic acid was able to lower the r-SBF and human plasma pH level to pH 2 but formed a jelly-like appearance as time elapsed. It was also excluded from further studies. Only citric acid was able to fulfil all criteria of dissolving in r-SBF as well as human plasma without any precipitations or turbidity and able to lower the pH of 1 mL of r-SBF and human plasma to pH 2 by adding 700 µL and 625 µL of 1 M solution, respectively (Table I).

DISCUSSION

Iron deficiency anaemia (IDA) is the most common cause of anaemia affecting almost 2 billion individuals worldwide (6). Hb level has been widely used to study the global anaemic population, notably when portable HemoCue® was introduced in 1982 (17). Since then, the prevalence of iron deficiency is highly dependent on Hb levels. However, the clinical manifestations of IDA and thalassaemia are very similar leading to frequent misdiagnosis in the absence of further investigations. In thalassaemia, there is a decrease or absence in Hb production but iron absorption is normal or even in some cases, increased. Therefore, to accurately diagnose IDA or thalassaemia, the body's iron status is measured using various parameters for example serum ferritin, total iron binding capacity, soluble transferrin receptor and others. However, each parameter has its own limitations (3). Serum/plasma iron quantitation is also important in diagnosing iron overload disorders. Complications of these disorders include liver cirrhosis, hepatocellular carcinoma, diabetes and cardiomyopathy (11).

The gold standard for iron status determination is to measure the bone marrow iron level, however, it is too invasive and costly to be a practical first-line diagnostic evaluation. Currently, amongst available iron parameters, not one parameter alone can be used to confirm iron deficiency. Instead a combination of several indicators are needed for a definite conclusion (10). If serum ferritin level is normal or at the borderline, serum iron and transferrin are the next assays to be ordered for iron deficiency anaemia diagnosis (11). Serum/plasma iron is abundant in blood plasma, which includes all iron, especially from transferrin-bound iron apart from ferritin-bound iron. Transferrin-bound iron is

Table I : Analysis of candidate acid salts for lowering r-SBF and human plasma pH to pH 2

Form	List of acid	Dissolution in water	pH	Dissolution in r-SBF	Achieved pH 2 in 1 mL of r-SBF? (1M of acid)	Appearance in r-SBF	Achieved pH 2 in 1 mL of human plasma? (1M of acid)	Appearance in human plasma
Crystal	Citric acid	Dissolved at 2M	1.22	Yes	Yes, 700 µL	Clear	Yes, 625 µL	Clear
Powder	Benzoic acid	Not dissolved at 0.5 M	2.82			Discontinued		
Crystal	Maleic acid	Dissolved at 2M	0.89	Yes	Yes, 165 µL	Clear	Yes, 175 µL (pH1.95)	Initially clear, jelly-like as time elapsed
Powder	Nicotinic acid	White precipitates at 0.5M	3.36			Discontinued		
Powder	Molybdic acid	White precipitates at 0.5M	5.05			Discontinued		
Crystal	Oxalic acid	Dissolved at 1M	0.53	Turbid	Yes, 110 µL, turbid appearance	Undissolved appearance	Yes, 135 µL (pH1.95)	Undissolved appearance
Powder	2,4-dichloro-phenoxyacetic acid	White precipitates at 0.5M	2.74			Discontinued		
Crystal	Salicyclic acid	White precipitates at 0.5M	2.34			Discontinued		
Crystal	Succinic acid	Incomplete even at 0.5M	2.01			Discontinued		
Powder	Tartaric acid	Dissolved at 1M	0.99	Turbid	Yes, 475 µL, turbid appearance	Undissolved appearance	Yes, 575 µL	Undissolved appearance

readily released in acidic conditions while iron release from ferritin is not clear (12). However, plasma iron is affected by diurnal variation. Diurnal variation leads to a higher iron level in the morning as compared to afternoon, evening and midnight (18-21). Nevertheless, diurnal variation is not seen at values below 45 µg/dL (11).

To create a single-step plasma iron detection reagent, production of an acidic environment is necessary to release transferrin-bound iron. In bioanalytical sample preparation for a desired pH, either 1M HCl or 1M NaOH is titrated to obtain the desired pH for optimum reaction, whereby ionisable compounds are taken into consideration (15). This prototype would not involve any buffer addition, thus, it is crucial to find chemicals that would result in the desired pH as well as be stable in lyophilised form. Initially, as a cost-effective measure, r-SBF was used to mimic the human plasma as the experimental buffer.

Oyane et al. (2003) has modified and assessed three different SBFs compared to conventional SBF (c-SBF) for bioactivity of artificial materials and the formation of bone-like apatite on various substrate evaluation (14). c-SBF has ion concentrations equal to those found in blood plasma except with slightly higher chloride ion (Cl-) and lower bicarbonate ion (HCO_3^-) concentrations. r-SBF has ion concentrations that are equal in all aspects to those of blood plasma while ionised-SBF (i-SBF) has dissociated ions concentrations equal to those of blood plasma. Modified-SBF (m-SBF) on the other hand have a decreased HCO_3^- while the rest of the ion concentrations are equal to those of blood plasma (14). As with Oyane et al. (2003), the formation of calcium phosphate and calcium carbonate clusters was found in the fluid. Calcium phosphate clusters have a hydrodynamic diameter of ~0.5 nm (Oyane et al. reported ~1 nm) and calcium carbonate clusters have a hydrodynamic diameter of ~500-1000 nm (Oyane et al. reported 10-30 nm after 1-day storage and 100-200 nm after 7-day of storage). It was speculated that calcium carbonate formation in the r-SBF was due to its supersaturation of calcite (16). The remaining ion concentrations were similar to human plasma. It is stipulated that r-SBF is unsuitable for prolonged duration stability usage (16). As calcium carbonate does not affect the acidity of acid salts and the r-SBF was not intended for long-term bioactivity assessment or long-term use, r-SBF was chosen for subsequent experiments.

Ten available acid salts that are found in powdered or crystallised forms were assessed. Benzoic acid, molybdic acid, 2,4-dichlorophenoxyacetic acid, nicotinic acid, succinic acid and salicylic acid were not able to dissolve completely in r-SBF. Benzoic acid is a monobasic aromatic acid that is a highly alcohol soluble, however, does not solubilise well in water and has a high melting point of 122°C while molybdic acid is a yellow solid monohydrate which is moderately soluble in hot water (22-23). These acids were excluded from further experiments. Nicotinic acid, also known as niacin or vitamin B3, is slightly water soluble and dissolves easily in hot water (24). The saturated aqueous solution was at a pH of 3.36 which was not acidic enough for spontaneous reaction. Salicylic acid is a natural compound found in plants and a type of beta hydroxy acid. It has a role as an anti-infective agent as well as antifungal, however, it sinks and dissolves slowly in water (25). 2,4-dichlorophenoxyacetic acid on the other hand, does not occur naturally in the environment but it is widely used in herbicides. It has low solubility in acid form thus herbicides use the more soluble forms (26). Succinic acid is a dicarboxylic acid and is able

to achieve pH 2.01 in aqueous solution. It is by nature soluble in water, however, it did not dissolve completely even at 0.5 M in this study. Perhaps the acid salt used was not stored properly or has passed its expiration date.

Oxalic acid on the other hand is able to dissolve in water and is an organic compound with the formula $C_2H_2O_4$. It is known as a reducing agent as it has an acid strength much greater than that of acetic acid. With only 110 µL, it was able to reduce the pH of 1 mL r-SBF to 2 and needed 135 µL to achieve pH 1.95 in 1 mL of human plasma. However, it did not dissolve completely in r-SBF nor human plasma as it forms precipitates with calcium ions leading to the formation of calcium oxalate, which has been noted in oxalate poisoning in man and animals (27). Tartaric acid is found in many plants as a dicarboxylic acid (28). It is water soluble and was able to reduce the pH of r-SBF and human plasma to 2 using 475 µL and 575 µL of 1M tartaric acid, respectively. However, similar to oxalic acid, it forms crystalline precipitates with calcium ions (29). Thus, these 2 acid salts were not suitable for plasma iron quantitation.

Maleic acid was water soluble and was able to reduce r-SBF and human plasma pH levels to 2. However, after a few hours, the human plasma with maleic acid formed a jelly-like substance. Maleic acid is a multifunction chemical intermediate that is applied in nearly every field of industrial chemistry including producing synthetic glue. The experiment suggested that maleic acid in human plasma polymerised to form oligomeric poly(maleic acid) which is an effective cross-linking agent. Poly(maleic acid) has a high molecular weight and is liable to gelatinise through chelation with cation (such as Ca2+) (30). This could have caused the coagulation factors found in human plasma to coagulate and form a gelatinous solution in the human plasma after prolonged exposure.

The final remaining acid was citric acid. Citric acid is highly water soluble and is odourless as well as colourless. This is important as it does not produce a background noise during plasma iron quantitation using a colorimetric technique on a spectrophotometer. A weak acid, citric acid was able to lower the pH level of 1 mL r-SBF and human plasma to pH 2 by adding a higher amount of 1 M citric acid (700 µL and 625 µL in r-SBF and human plasma, respectively). From the environment viewpoint, citric acid is a good candidate as it breaks down quickly in water and does not present any hazards to the environment or human health (31) compared to some previous acids which are toxic to human in high amounts i.e. maleic acid can cause skin and severe eye irritation (30) while tartaric acid acts as a muscle toxin (28).

CONCLUSION

Among the ten tested acids, citric acid was the only candidate in this study that was suitable to be used for further development of the low-cost, spontaneous plasma iron quantitation development due to its high water solubility, colourless characteristics as well as the ability to reduce the pH of r-SBF and human plasma to an acidic condition which is appropriate for the release of transferrin-bound iron. The optimum level of citric acid in plasma would be 62.5% v/v to reduce the plasma pH to 2.

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