Curcumin Contributes to *In Vitro* Removal of Non-Transferrin Bound Iron by Deferiprone and Desferrioxamine in Thalassemic Plasma

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Abstract: Non-transferrin-bound iron (NTBI) is detectable in plasma of β-thalassemia patients with transfusional iron overload. This form of iron may cause oxidative tissue damage and increased iron uptake, into several vital organs. Removal of NTBI species is incomplete and transient using standard intermittent desferrioxamine (DFO) or deferiprone (DFP) monotherapy. Combinations of these or other chelators may improve the protection time from NTBI and increase removal of harmful NTBI species. Curcuminoids from *Curcuma longa* L. is a naturally occurring phytochemical which shows a wide range of pharmacological properties including anti-oxidative, anti-inflammatory, anti-cancer and iron-chelating activities.

In this study, the curcuminoids was investigated for NTBI chelation in thalassemic plasma *in vitro* and for the potential to improve NTBI removal when used with other chelators.

Curcumin bound Fe^{3+} to form a Fe^{3+}-curcumin complex with a predominant absorption at 500 nm. The chemical binding of curcumin was dose- and time-dependent and more specific for Fe^{3+} than Fe^{2+}. Using a HPLC-based NTBI assay without an aluminium blocking step, curcumin shuttled the iron from Fe^{3+}-NTA complex, giving underestimated NTBI values. At equivalent concentrations DFO, DFP and curcumin decreased plasma NTBI with the order of DFP>DFO>curcumin. None of these chelators removed NTBI completely, but curcumin appeared to increase the rate of NTBI removal when added to DFP. It is proposed that the β-diketo moiety of curcumin participates in the NTBI chelation.

Key Words: Curcumin, cucuminoids, deferiprone, desferrioxamine, iron chelator, labile plasma iron, non-transferrin bound iron, thalassemia.

INTRODUCTION

Iron is a trace transition element essential for the physiological functions of living organisms. Disorders of iron balance such as iron deficiency and iron overload can lead to human diseases [1]. Secondary iron overload associated with anemia in β-thalassemia patients is commonly caused by increased iron absorption and multiple blood transfusions. The existence of an excessive toxic form of iron is able to catalyze the generation of free radicals leading to damage of cellular biomolecules, and consequent impairment in cellular functions and integrity [2]. phlebotomy is effective in removing excessive iron in hereditary hemochromatosis while iron chelation therapy is required for β-thalassaemia to prolong life expectancy.

The term non-transferrin-bound iron (NTBI) refers to all forms of iron in the plasma bound to ligands other than transferrin. It was first demonstrated as a non-specific chelatable iron in serum of β-thalassemia patients, appearing when large amounts of iron released into plasma exceed the transferrin iron binding capacity [3]. However, NTBI can be detected in unsaturated plasma from patients with sickle cell anemia, hereditary hemochromatosis, African dietary iron overload, receiving bone marrow transplants, myelodysplastic syndromes undergoing cytotoxic chemotherapy, megaloblastic anemia and congenital dyserythropoietic anemia type I [4-10]. This highly toxic, labile form of iron is present in concentrations of 1-10 μM in β-thalassemia patients and potentially causes oxidative damage to cell membranes and biomolecules [11]. The exact chemical nature of NTBI has not been identified, but is proposed as a large proportion of iron loosely bound to plasma proteins (such as albumin) and a small proportion of iron complexed with citrate, phosphate and some amino acids [12]. A clinical study showed that a decrease of plasma NTBI in β-thalassemia patients occurs when they are infused with desferrioxamine (DFO) [13]; however, removal of NTBI is incomplete [14]. Furthermore, a labile redox active subfraction of NTBI (so called labile plasma iron, LPI) [15] is incompletely removed with monotherapy using either DFO or deferiprone (DFP), partly due to the short plasma residency with either monotherapy regimen. However, the sequential combination of chelators can increase the period of protection from NTBI [16] and in principle this could also be achieved by adding curcumin to the above chelation regimens.

Plant flavonoids like catechin, quercetin and diosmetin possess both iron-chelating and iron-reducing properties that can selectively inhibit 5-lipoxygenase [17, 18]. Curcuminoids from *Curcuma longa* L (tumeric) contains curcumin (diferuloylmethane), demethoxycurcumin (*p,p'*-dihydroxy-dicinna-

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moyl-methane) and *bis*-demethoxycurcumin (*p*-hydroxy-cinnamoyl-feruloyl-methan), with curcumin being the dominant compound [19]. Curcumin is a major active component that can interact with iron and inhibit iron-catalysed lipid peroxidation [20]. This iron-chelating activity is due to the interaction of its β-diketo moiety with ferric ion [21, 22]. Additionally, synthetic curcuminoids can chelate copper using a hydroxy group on the ring [23]. So far a direct interaction or contribution of curcumin with plasma NTBI has not been studied. Therefore, the *in vitro* kinetic removal of NTBI by DFO and DFP concomitant with curcumin was investigated in β-thalassemic plasma.

MATERIALS AND METHODS

Patient Plasma Samples

The study complied with the Declaration of Helsinki and the Faculty of Medicine Ethics Committee for Human Research in Chiang Mai University approved the protocol for blood collection (Reference number 0605(8)/217/2003). Written informed consent was obtained from all patients. Fifteen β-thalassemia patients (seven females and eight males) attending the Thalassemia Clinic at Maharaj Nakorn Chiang Mai Hospital, Department of Medicine, Chiang Mai University, Chiang Mai, Thailand were recruited into the study. All patients were regularly transfused at 4-week intervals with the purpose of maintaining a pretransfusion hemoglobin (Hb) concentration above 9.0 g/dl. Five patients had never been administered with any iron chelators, and ten patients used to receive DFO chelation and stopped their chelation at least 72 hours before blood collection. Venous blood was collected in heparinized tubes and centrifuged at 3,000 rpm (660*g*, *Hettich* Centrifugation, Germany) 4°C for 15 min. Plasma was removed and kept frozen at –80°C until analysis. The NTBI concentration and transferrin saturation were determined in the thalassemic plasma samples before being used in the experiments.

Measurement of Chemical Iron Binding: Spectral Analysis

A 100 μM curcumin solution was prepared by dissolving curcumin powder (purity 65-70%; Sigma-Aldrich Co., St. Louis, MO, USA) in 50 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (Sigma-Aldrich Co., St. Louis, MO, USA) solution, pH 7.0 and gentle heating at 80°C for 10 minutes. The solution (1 ml) subsequently mixed with solutions of ammonium ferrous sulfate (E. Merck, Darmstadt Germany) and stock ferric nitrate solution (AAS iron reagent, 1000 ppm. in 0.5% HNO_3; APS Finechem, Seven Hills, Australia) (10 μl) in polypropylene tubes to obtain the indicated concentrations and incubated at room temperature (25°C). After 10 min, the absorbance of curcumin alone, Fe^{3+}-curcumin complex and Fe^{2+}-curcumin complex was monitored between 400-800 nm using the Shimadzu double-beam UV-VIS scanning spectrophotometer (Shimadzu Corporation, Analytical & Measuring Instruments Division, Kyoto, Japan).

Kinetic Formation of Iron-Curcumin Complex

Various concentrations of ferric nitrate solution (final concentrations of 20-100 μM) were incubated with the curcumin solution (a final concentration of 100 μM) at room temperature for 0-30 min. The absorbance of Fe^{3+}-curcumin complex was measured at 500 nm.

Chemical binding of Fe^{2+} and Fe^{3+} was investigated by incubating the ammonium ferrous sulfate and ferric nitrate solutions (final concentrations of 0-200 μM) with the curcumin solution (a final concentration of 100 μM at room temperature. After 10 min, the absorbance of iron-curcumin complex was measured at 500 nm.

HPLC-Based Assay of Plasma NTBI

Plasma NTBI measurement was undertaken essentially as described by Singh and colleagues [24] with minor varieties as following. Briefly 0.45 ml of plasma was mixed and incubated with 800 mM nitrilotriacetic acid (NTA), pH 7.0 solution (50 μl) at room temperature for 30 min. to transform NTBI into Fe-NTA. Afterwards plasma proteins were removed by centrifugation of the treated plasma using an ultracentrifugation filtration device (NanoSep®, 30-kDa cut off, polysulfone type; Pall Life Sciences, Ann Arbor, MI USA) at 12,000 rpm (10620*g*, Hettich Centrifugation, Germany), 15°C for 45 min. The ultrafiltrate was analyzed using a non-metallic HPLC system. The HPLC conditions were as follows: a dual-piston high pressure pump (ConstaMetric 3500 LDC Analytical, Inc., Florida, USA), a glass analytical column (ChromSep ODS1, 100x10 mm, 5 μm; Chrompack International, Middleburg, the Netherlands), mobile-phase solvent containing 3 mM CP22 (1-methyl-2-propyl-3-hydroxy-pyridin-4-one) in 19% acetonitrile (HPLC grade, E. Merck, Darmstadt, Germany) buffered with 5 mM MOPS, pH 7.0 at a flow rate of 1.0 ml/min. Column effluents were monitored at 450 nm using a flow-cell detector (SpecMonitor2300; LDC Milton-Roy Inc., Florida, USA) and conducted with the BDS software (BarSpec Ltd., Rehovot, Israel). The NTBI peak was calculated from a calibration curve of standard iron solutions (0-16 μM Fe-NTA in 80 mM NTA, pH 7.0). The NTA solution was prepared by mixing one volume of N,N-bis[carboxymethyl]glycine disodium (purity minimum 99%, Sigma-Aldrich Co., St. Louis, MO, USA) solution (800 mM) with one volume of N,N-bis[carboxymethyl]glycine trisodium (purity 98%, Sigma-Aldrich Chemie GmbH, Steinheim, Switzerland) solution (800 mM) to reach a final pH of 7.0.

However, a recent study has showed that excess unbound DFO interfere the NTBI assay when they were present in the plasma. These molecules can shuttle the iron from preformed Fe-NTA complex, leading to an underestimated NTBI concentration [14]. The NTBI measurement was therefore slightly modified by including an aluminum blocking step to saturate free iron-binding sites on iron chelator molecule and prevent an iron shuttling by NTA when excess unbound iron chelator remained in chelator-treated plasma. The plasma was then quantified for NTBI as described above.

RESULTS

Chemical Iron Binding of Curcumin

Spectral analysis demonstrated that a Fe^{3+}-curcumin complex exhibited a predominant peak at 500 nm and a Fe^{2+}-curcumin complex gave very low absorption at this wave-

length. In comparison, curcumin alone gave a typical absorption peak at around 420 nm consistent with a previous report [25]. The absorption intensity increased dose-dependently at their specific wavelengths (Fig. (**1**)).

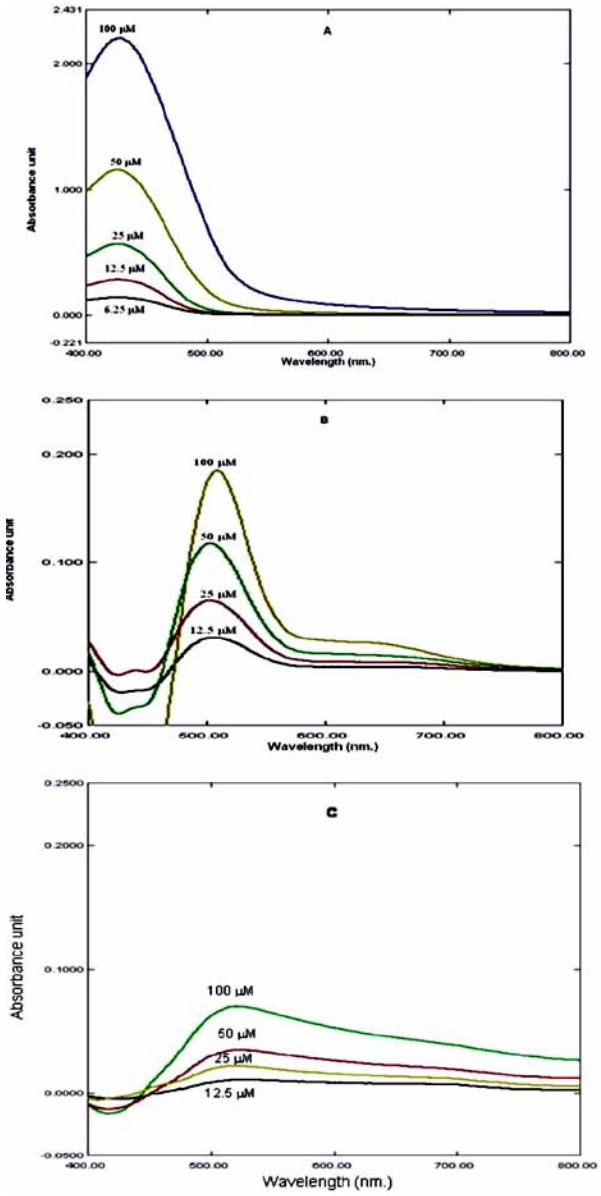

Fig. (1). Spectra of curcumin alone (A), Fe^{3+}-curcumin complex (B) and Fe^{2+}-curcumin complex (C).

The binding of iron to curcumin was time dependent for the first 10 min and complete following incubation for 15 min, and also concentration-dependent (Fig. (**2**)). Interestingly curcumin had higher binding affinity for Fe^{3+} than Fe^{2+}, and formed a red Fe^{3+}-curcumin complex. The binding was dependent upon the concentrations of ferric ion (0-100 µM) and seemed to be saturated at higher concentrations (100-200 µM) (Fig. (**3**)). The results were consistent with previous studies that reported that curcumin formed a 1:1 complex with iron and behaved as an iron-chelating agent through 1,3-diketo part in the molecule [21, 26].

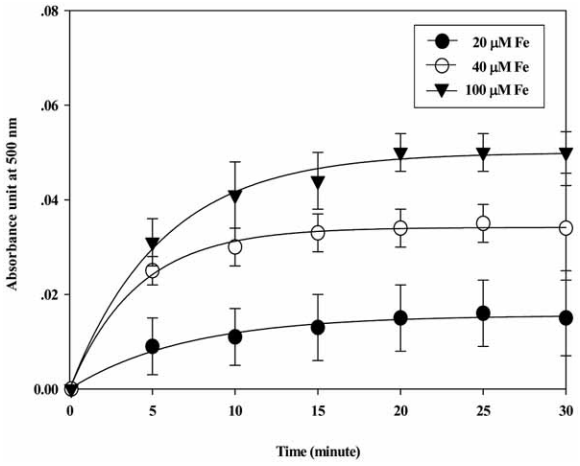

Fig. (2). Kinetics of Fe^{3+} binding to crucumin. Data are obtained from a triplicate experiment and shown as mean ± standard error of the mean (SEM).

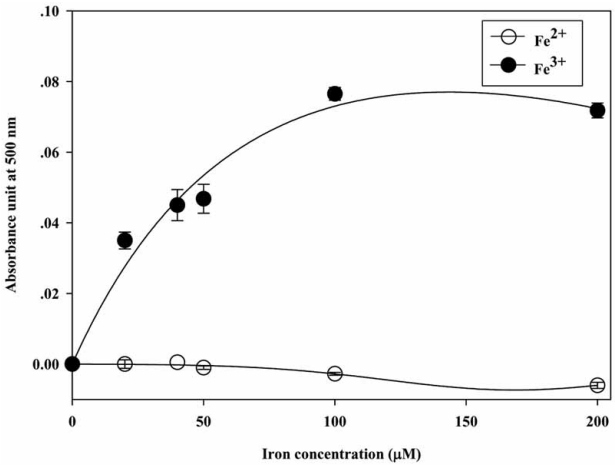

Fig. (3). Fe^{2+} and Fe^{3+} binding affinity of curcumin (100 µM). Data are obtained from a triplicate experiment and shown as mean ± SEM.

HPLC-Based Assay of NTBI in Chelator-Treated Plasma

In a modified HPLC-based NTBI assay, plasma (0.45 ml) from β-thalassemia patients (TS) was first incubated with DFP (0-80 µM) at 37°C for 1 hour. Subsequently, a 200 µM aluminium chloride solution (purity 99%; APS Finechem, Seven Hills, Australia) was added to and further incubated at room temperature for 1 hour. Finally, the plasma was measured for NTBI concentration as described above. DFP (1,2-dimethyl-3-hydroxypyrid-4-one) (kindly donated from Dr. Chada Phisalaphong, Government Pharmaceutical Organization, Bangkok, Thailand) solutions were freshly prepared in 50 mM MOPS solution, pH 7.0.

NTBI concentrations assayed using the modified HPLC with an aluminium blocking step were higher than those obtained from the method without blocking step (Fig. (**4**)). We

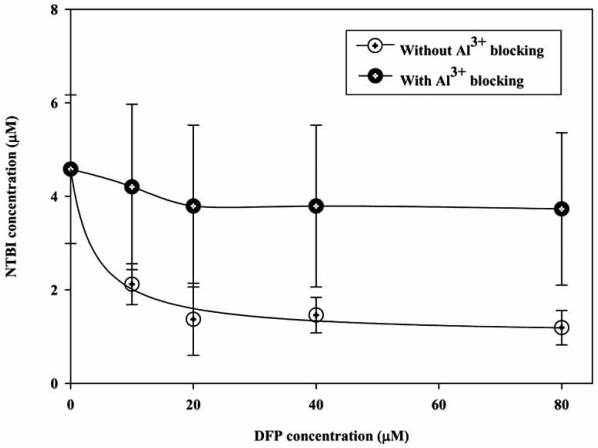

Fig. (4). Plasma NTBI concentrations assayed using the HPLC with and without the aluminium blocking step. Data were obtained from three individual thalassemic plasmas incubated with DFP (0-80 μM) for 1 hour and shown as mean ± SEM.

found that the aluminium concentration at 200 μM did not displace iron from the Fe^{3+}-curcumin complex. This phenomenon is similar to the one observed when NTBI was measured in DFO-treated thalassemic serum [14]. When the Al^{3+} was added immediately prior to the NTA addition and not allowed enough time for incubation, it did not block properly. The NTA was therefore able to shuttle NTBI onto the free chelator and this led to underestimated NTBI values. It was found that ferrioxamine (FO) and Fe^{3+}-DFP complex did not interfere this HPLC-based NTBI assay. The modified HPLC method was therefore necessary for quantification of NTBI in the chelated plasma samples.

Kinetics of Plasma NTBI Removal by DFP, DFO and Curcumin

Plasma (0.45 ml) from β-thalassemia patients (TS) was first incubated with 100 μM DFP, 100 μM DFO and 100 μM curcumin at 37°C for the indicated time. The remaining NTBI concentration was measured using the modified HPLC-based assay with the aluminium blocking step as described above. DFO (Novartis Pharmaceutical, Basel, Switzerland) solutions were freshly prepared in 50 mM MOPS solution, pH 7.0. Dose-response removal of NTBI was also examined by incubating the TS with DFP, DFO, and curcumin solutions (0-100 μM) at 37°C for 1 hour. NTBI concentration was assayed using the modified HPLC-based assay.

NTBI was rapidly removed at the first hour and progressively decreased until 8 hour. The initial rate of NTBI removal was 4.78 μM/hour, 3.38 μM/hour and 2.01 μM/hour by DFO, DFP and curcumin respectively. With equivalent concentration at 100 μM, NTBI was decreased by DFP, DFO and curcumin in a time-dependent manner (Fig. (**5**)). Concentration dependencies of NTBI removal in aluminium-blocked thalassemic plasma by DFP, DFO and curcumin are shown in (Fig. (**6**)). DFP was the most efficient and DFO was the second efficient in chelation of NTBI. The NTBI was approximately 50% reduced by all concentrations of DFP. DFO removed NTBI in dose-dependent manner. Only

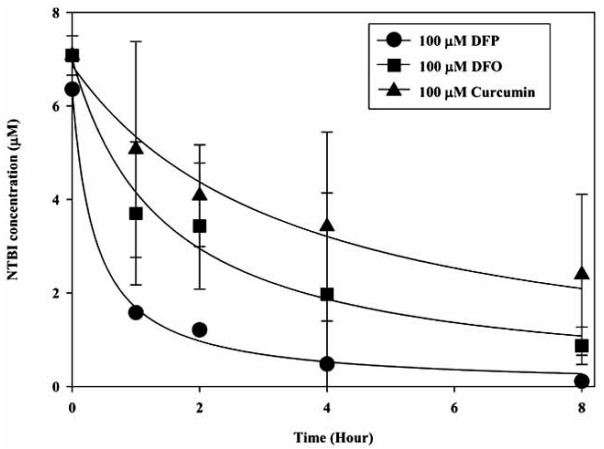

Fig. (5). Time-course removal of NTBI by DFP, DFO and curcumin. Data were obtained from three individual thalassemic plasmas and shown as mean ± SEM.

22.4% NTBI was removed by curcumin (final concentrations of 12.5-100 μM). This rapidly chelated NTBI probably represents mono-meric and dimeric iron-citrate species, and the remaining NTBI likely represents oligomeric and polymeric forms of NTBI that may be bound to plasma proteins. On a molar basis of iron binding, bidentate DFP was less effective than hexadentate DFO.

Effects of Curcumin and Ascorbic Acid on Plasma NTBI Removal by DFP

Ascorbic acid (Sigma-Aldrich Co., St. Louis, MO, USA) (100 μM) and curcumin (100 μM) solutions were first added to the TS, followed by the addition of DFP (100 μM), and samples were then incubated at 37°C for 0-8 hours. The NTBI concentration was measured using the modified HPLC-based assay. As shown in (Fig. (**7**)) the rate of NTBI removal by 100 μM DFP alone (ΔNTBI = 4.78 μM/hour) was slightly slower than that included 100 μM curcumin (ΔNTBI = 5.33 μM/hour), but faster than that included 100 μM ascorbic acid

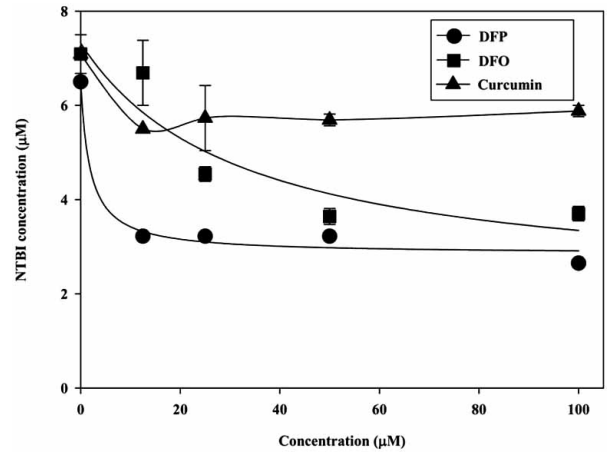

Fig. (6). Dose-response removal of NTBI by DFP, DFO and curcumin for 1 hour. Data were obtained from three individual thalassemic plasmas and shown as mean ± SEM.

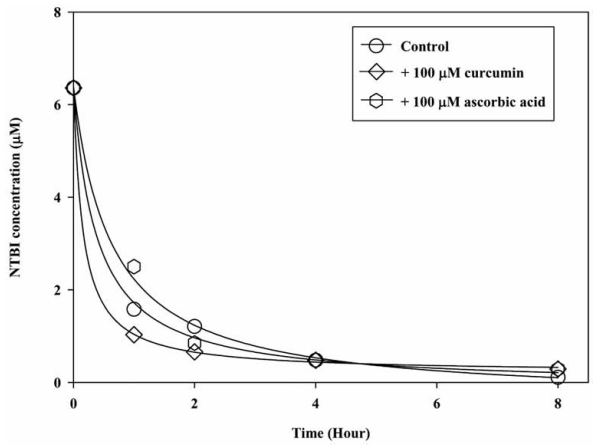

Fig. (7). Time-course effect of curcumin and ascorbic acid on NTBI removal by 100 μM DFP (control) for 1 hour. Data were obtained from three individual thalassemic plasmas and shown as an average NTBI value.

(ΔNTBI = 3.86 μM/hour). However, both curcumin and ascorbate did not influence the NTBI chelation by 100 μM DFP when incubated for 4-8 hours.

For a dose response, the ascorbic acid (100 μM) and curcumin (100 μM) solutions were first added to the TS, followed by an addition of DFP (12.5-100 μM), followed by incubation at 37°C for 1 hour. The NTBI concentration was measured using the modified HPLC-based assay. Within 1 hour, about 40-50% of NTBI content was removed by 12.5 μM DFP. NTBI was not decreased further although DFP concentrations were increased up to 100 μM. Using 100 μM DFP, curcumin seemed to enhance the reduction of NTBI slightly at 60 minute while ascorbic acid did not (Fig. (**8**)). Curcumin (100 μM) and ascorbic acid (100 μM) existing in the system did not accelerate the decrease of NTBI by DFP at these concentrations.

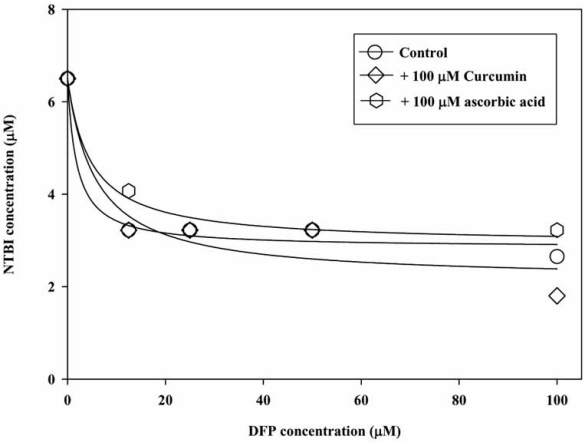

Fig. (8). Dose-response effect of curcumin and ascorbic acid on NTBI removal by DFP (control) for 1 hour. Data were obtained from three individual thalassemic plasmas and shown as an average NTBI value.

DISCUSSION

It is known that DFO and DFP can bind ferric ion to form a Fe^{3+}-chelator complex exhibiting distinct absorption peaks at 430 nm and 450 nm respectively. Spectral analysis indicates that curcumin bound ferric ion to form a Fe^{3+}-curcumin complex with a predominant absorption peak at 500 nm, and curcumin itself in 50 mM MOPS, pH 7.0 solution give an absorption peak at 420 nm. Curcumin could be more specific for Fe^{3+} than Fe^{2+}. A recent study showed that curcumin has a red color (maximum absorption at 261 and 463 nm) in basic aqueous solution (0.5 M NaOH) while it has a yellow color (maximum absorption at 422 nm) in glacial acetic acid [27]. The chemical binding of iron onto the chelator molecule was both time- and concentration-dependent. Curcumin also had a higher affinity for ferric ions than ferrous ions to form an iron-curcumin complex in MOPS buffer pH 7.

Using an NTA capture HPLC-based NTBI assay technique, DFP chelated plasma NTBI slowly over 1 hour incubation and the NTBI concentration was not changed at higher DFP concentrations. In principle DFP can act as a recipient for iron shuttled from Fe^{3+}-NTA complexes during the assay, leading to underestimated NTBI concentrations unless any free binding sites of DFP are blocked by iron or a similar metal. Previous studies have shown that free iron-binding sites on transferrin or DFO molecules can be saturated with some trivalent metal ions such as Co^{3+} [5] or Al^{3+} [28] respectively. As Al^{3+}-DFO and Al^{3+}-DFP complexes exhibited distinct UV absorption peak at 230 nm and 297 nm respectively, which did not interfere with the HPLC-based NTBI measurement detecting at 450 nm. An addition of 200 μM Al^{3+} to saturate the free iron-binding site on unbound DFP molecules can prevent thus iron shuttling in the chelated plasma.

Like DFO, DFP and curcumin were able to decrease plasma NTBI concentrations in a time-dependent manner, and DFP was the most efficient at an equivalent concentration. At 12.5 μM and 25 μM, DFP decreased plasma NTBI more rapidly than DFO, probably due to the smaller molecule of DFP being more accessible for interaction with the NTBI target than DFO. NTBI removal by curcumin (12.5-100 μM) was only 22%, which was less effective than both of the established chelators. An independent study showed that administrations (500 mg/day) of curcuminoids by mouth are for 6 months significantly decreased NTBI concentrations from 5.48±1.84 μM to 0.06±1.52 μM in splenectomized β-thalassaemia/HbE patients (n =13) and from 2.34±2.22 μM to -2.05±1.73 μM in non-splenectomized β-thalassaemia/HbE patients (Kalpravidh *et al.*, in preparation). Phenolic, methylene and β-diketone groups contribute to both iron-chelating and radical-scavenging properties [29]. The β-diketo moiety within curcuminoids molecule could be a potential Fe^{3+}-complexing site with an iron-binding affinity ($K_a = 10^{30}$) close to that of DFO ($K_a = 10^{31}$) [21]. Recently, a hexadentate structure of curcumin chelated with Fe^{3+} has been proposed in a at 1:1 molar ratio with the chelating activity of Fe^{3+} belonging to the β-diketo groups of the curcumin molecule [22].

Not all NTBI was removed by these chelating agents at all concentrations. However it is clear that curcumin either alone or in combination with DFP can decrease plasms NTBI. The lack of complete removal of iron with any chelator is consistent with previous observations by ourselves and others that only a proportion of plasma NTBI is directly

chelatable by chelators at clinically relevant concentrations [14, 16]. This is because plasma NTBI is heterogenous and exists in different pools, only a fraction of which is redox active and sufficiently labile for rapid chelation, the so called the labile plasma ion (LPI) component of NTBI [15]. With current monotherapy using DFP or DFO in standard regimes, either during the day or at night respectively, this form of iron is incompletely removed, mainly because either chelator is not present in the plasma compartment 24 hour per day [9]. By using these chelators sequentially [16] or by providing with an orally active chaltor with a longer plasma half life such as deferasiorox (ICL670, Exjade) [30] further protection against labile NTBI species can be achieved [31]. The data presented here provide evidence that curcumin is a chelator that can remove NTBI in thalassaemic plasma *in vitro* and this is consistent with preliminary observations that NTBI can be decreased *in vivo* (Kalpravidh *et al.*, in preparation**).** Further work is needed to determine the conditions under which curcumin competes with DFO and DFP for NTBI species when combined with these chelators. It will also be important to understand the plasma kinetics of curcumin so as to determine whether the period of protection from NTBI can be enhanced when curcumin is added to DFP or DFO. Further work will also be required to establish the fate of iron bound to curcumin. In particular it will be important to understand whether iron complexed to curcumin is eliminated in urine and/or feces and whether iron-curcumin complexes are redox active.

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