An innovative modified dispersive liquid-phase microextraction method for trace level of iron in serum samples of neurogical disorders patients prior to determine by flame atomic absorption spectrometry

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ABSTRACT

An innovative, modifieddispersive liquid-phase microextraction (MDLP-µE) to asses the iron (Fe) concentrationin blood serum samples of different neurological disorderspateints. The main objective of this work to disperse extracting solvent by using air-agitated syringe system to overcome the matrix effect and avoid the dispersion by using heat, hazardous dispersive organic solvents. The MDLP-µEconsists of twodispersive liquid-phasesteps with chloroform as an extractant solvent. In the first step,Feform complexes with a chelating reagent, 8-hydroxyquinoline (oxine)in aqueous phase and extracting into extracting solvent (chloroform). In the second step,Fe was back-extracted into the acidic aqueous phase and finally determined by flame atomic absorption spectrometry (FAAS).The variables play a key role on the extraction efficiency and reproducibility such as pH, first extractant volume, back-extractant volume, concentration of complexing agent and aspirating/dispensing cycles through a syringe were studied and optimized.Under the favorable condition enhancement factor(EF) and detection limit (LOD) were found to be 47and0.44 µg L^{-1}, respectively. Reproducibilty of the method was analysed by relative standard deviation (%RSD), which was < 5%. Accuracy of the desired procedure was analysedby blood serum of certified reference material (CRM) .The developed procedure was applied to the analysis of Fe in serum samples of patients (males) have different neurological disorders and healthy control.

INTRODUCTION

Iron (Fe) is necessary for life, and is one of the most important metal in environmental and biological systems.But both severe deficiency and excess leads tosignifcantserious health risks (1).The Fe play important role in biological activity and active centre for protein to transfer electron and oxygen in metelloenzymes such as dehydratases and oxidases (2,3).

The brain uses Fe for many essential processes as either haem iron (including the iron transport oxygen in haemoglobin,), or non-haem iron (4-7).Which is responsible for the synthesis of neurotransmitters and cellular aerobic metabolism (8,9).The accumulation of Fe in brain region substantia nigra (SN) causes degeneration of dopaminergic neuron and form complexwith neuromelanin inducing oxidative stress which leads to different nerveous system disorders such as (Alzheimers and Parkinsons) (10-14).

In central nervous system (CNS) Fe is important cofactor for different metabolic funtions including transport oxygen, nitric oxide metabolism phosphorylation oxidative (15). Due to the increased level of Fe cause imbalance of brain hemostasiscreats pathogenesis of neurodegenerative disorders (16-18).

Consequentlypreconcentration methods have been developed and employed for the analysis of Fe at lower concentration.Various analytical instruments applied for the analysisof metal and metalloid (19-21). The very common and widely used FAAS is frequently applied for metal analysis but it is not sensitive or efficient to determine low level of Fe in real samples (environmental and biological),mainly due to their complex matrixes which require, preconcentration of analyte of interest (22-24).

Recently a miniaturized solvent extraction proceduresuch as liquid–liquid extraction (LLE), co-precipitationmethod(25,26),solid-phase extraction (SPE) (27,28) hollow fiber membrane and cloud point extraction (CPE) (29,30) have been used for the separation

andenrichmentof iron inbiological matrices.The some other simple, rapid and inexpensivemethods and high extraction capability i.e. dispersive liquid-liquid microextraction (DLLME) have grabbed a great deal of space in the recent literature . The main limitation of the almost DLLME based procedure has need to be a long time to reach equilibrium. This fact create a negative effect on the extraction capability of the desired method that might be due the small contact between both medium (aqueous and the extractant) (31). The developed method MDLP-µE not only eliminate the solubility effect of organic solvent in aqueous media, but also reduce the matrix effect of the organic solvent on the target analyte.

The developed preocedurehave been applied for the extraction of trace level of Fe in blood serum samples obtained from patients having different neurological disorders for the first time. Thus eliminating the use of harmful effect of organic solvents and subjected by analysis with FAAS.In the first phase of MDLP-µE method provided extraction of the target analyte by using organic solvent, in this step interference of other elements could be present. The interference effect will be minimized in the second phase of the method. In whichthe organic phase was withdrawn from the chloroform and mixed with an acidic back axtracting solvent. For this purpose, the target analyte were ionized and extracted into the acceptor solution, while many interferences will be eliminated. The second step presented a simple and efficient back-extraction step, and in addition to the increase in the sample clean-up, the analytes were extracted into the aqueous solution. Thus, this step could cause eliminating the problem of injection of the organic solvent into the final instrument analyzer as well. On the other hand, unlike most methods coupled with MDLP-µE for improving the sample clean-up, back-extraction of the analytes was a green step in the desire MDLP-µE procedure. Inorder to make this method greener/environmental friendly, metal complex was back extracted into HNO_3.

EXPERIMENTAL

Reagents and Chemicals

For the experimental work deionized water was taken from (ELGA lab water system, Bucks, UK). Hydrogen peroxide (30%), nitric acid concentrated (65%), and HCl (37%) were acquired from Merck (Darmstadt, Germany). For the present work 1000 ppm stock solution of Fe was used, FlukaKamica (Buchs, Switzerland).The 8-hydroxyquuinoline (oxine) and other reagents were of analytical grades acquired from Merck Darmstadt(Germany).The CRM of serum was obtained from Clincheck control lyophilized ® human serum Recipe (Munich, Germany). The glassware were decontaminated to soak in 10% HNO_3 for 24 h, then washed carefully with deionized water.

Instrumentation

A Perkin-Elmer FAAS instrument, Model AAnalyst 700 (Norwalk, CT, USA) was used for the analysis.The working conditions set according to recommended by manufacturer and as stated in our former study (31).

Study Population and Sampling Protocol

In present case-control study the neuro patients (n=60), blood serum samples were collected from outdoor patients admitted in the neurological section of Liaquat National Hospital, Karachi and Hyderabad Civil Hospital. For a comparison purpose, normal referents (n=40) of similar age matched (40–70) years socioeconomic position, and residential areas (generally patients relatives) were choose. Current research criteria was accepted by review board of organization (NCEAC), and all patients were agree to participate voluntarily. For the details regarding dietary health, physical data, origin of ethnic habit, age, and a performa was also run to in order to collect sample. Almost 5 mL of venous blood samples was collected from patients suffering from any neurological disorders and normal subjects. Then blood samples 2

mL was used for biochemical tests carried out in pathological test site of clinic. Whereas for sera separation standard procedure was used (32).The all serum samples were kept at 20 °C until study.

Sample Digestion Procedure

About 0.2 mL blood serum wastaken in PTFE flasks from patient and healthy subjects to prepare triplicate samples. The combination of $HNO_3$and H_2O_2(2:1, v/v) was freshly prepared added about 2 mL in each flask then left the flaks for 10 min at room temperature. Then microwave oven (Osaka, Japan), were oxidized all serum samples .The organic matrix oxidized completely take only 2-3 min by using microwave assisted digestion procedure. The clear contents of the flasks were dissolved in 0.1 mol $L^{-1}HNO_3$ and made volume up to a mark in the volumetric flask (10 ml in capacity).The detail is reported elsewhere (32). Blank digestion protocol was carried out simultaneously.

Design of MDLP-µE Method

The MDLP-µE method is required a glass test tube with asyringe system. In the first extracting MDLP-µE step,10mL standard (10-100 µg L^{-1}) were taken into glass test tube. Then 0.2mL of desire buffer and 0.1–0.5 mL of oxine (0.113%); added and pH value was adjusted to pH 6. The extracting solvent chloroform (80 µL) was added. The syringe system (10 mL) was used to aspirated and dispersed back the portion of each standard and sample solution. Thisaspirating/dispensing cycle made the sample solution more cloudy.The mixture was centrifuged at 2500 for 4 min to extract the analyte into finely-dispersed droplets of the extractant to settle down at the bottom of the centrifuge tube.In the second step of this method, the resultedFeenriche organic phase was shifted into another glass tube. Followed by the addition of 0.5mL of the back extracting solution (1.5 mol L^{-1} of HNO_3)with the help of syring system. The centrifugation was carried outat 2500 rpm for 1 min. Finally, the aquous portion was separated

and analysed by FAAS. For each step of methodology blanks were prepared simultaneously. The proposed MDLP-µE method was applied on acid-digested serum samples of neurological disorders pateints and healthy controls subjects.

RESULTS AND DISCUSSION

Optimized Experimental Factors

The variables play a key role on the extraction efficiency and reproducibility such as pH, first extractant volume, back-extractant volume, concentration of complexing agent andaspirating/dispensing cycles through a syringe were studied and optimized.

The pH

The pH is considered to be theimportant variable in the extraction efficiency ofdesiredMDLP-µE method. The role of pH on the proposed method for Fe was carried outin the range of 3 to 8. The maximum extraction effeiciency was achieved at pH 5 as shown in Figure 1. Whereashydrolysis occur at higher pH.

Oxine Concentration

For the purposed MDLP-µE methodology, oxine was used for the complex formation of analyte (Fe). The concentration of complexing agent ranging from 0.1−0.5 mL (0.113%) was studied for the recovery of Fe as shown in Figure 2. Quantitative recovery was achieved at 0.3 mL of complexing agent and further increase in the concentration didn't show any significant effect.

Volume of Extracting Solvent

The extracting solvent has a key role on the first step of the MDLP-µEmethod.The extracting solvent should have the ability to extract thetarget metal complex due to low solubility in aqueous medium, and the cloudy solution was formed with tiny droplets. Hence, chloroform

was selected due to higher extraction efficiency. The volume of extracting solvent was studied in the range of 50 to 200 µL. Thus, 80 µL chloroform selected forthe rest of the work.

The Back Extracting Solvent

In current study, we also studied the effect of the back extracting solvent in the second step of MDLP-µE. For this purpose, HNO_3 of 0.5 to 2.0 mol $L^{-1}$was used for the back extraction of Fein aqueous media from analyte enriched organic phase. The optimum extraction of Fe was observed on 1.0 mol $L^{-1}$of HNO_3. So, HNO_3 solution (1.0 mol L^{-1}) of 0.5 mL was used for back extraction of the target analyte into the aqueous phase .

Effects of Aspirating/Dispensing Cycles

The dispersion of the extracting solvent has a major roletoachievedthe maximum extraction efficiency of the developed procedure. To disperse the organic solvent in aqueous phase, number of triggers have been used, which are mostly create a negative effect on the nature of the solvent and extractionefficiency. In the current study, weused dual-syringe based MDLP-µE coupled with FAAS as the dispersive medium for organic solvent. The effect of aspirating/dispensing cycles on the proposed method was carried out ranging fromof 2 to 10 cycles (Figure 3). It was found that maximum recovery of Fe was achieved by increasing the number of aspirating/dispensing cycles, due to higher dispersion and increased contact with theaqueous phase.Therefore, 8 aspirating/dispensing cycles were selected for furtherstudy. In the back extracting process, the Feenriched organic solvent aspirated to aqueous phase (1.0 mol L^{-1}, HNO_3)of 0.5 mL by 5 timesaspirating/dispensing cycles.

Centrifugation Time and Rate

The extraction efficiency of the proposed method was observed at different centrifugation rate (1500 to 3000 rpm) for 5 min. It was observed that 2500 rpm was adequate forFe enriched

phase. In the second phase of MDLP-µE, the centrifugation rate and time was also 2500 rpm and 5 min, respectively.

The Selectivity

The matrix ions effect were studied for the competent extraction recovery of Feby proposed, MDLP-µE. To carry out this study, 10 µg L^{-1}Fe in (10 mL) were added with co-existing ($Ca^{2+,}$ $Co^{2+,}$, K^{+}, Na^{+}, Ag^{+}, $Mg^{2+,}$ $Ni^{2+,}$ $Cu^{2+,}$ $Al^{3+,}$) at diverse analyte to interferent ratios, and used for the developed procedure. The ratios (w/w) of studied analyte and matrices ions were set according to 1 : 1000 for K^{+}, Na^{+} ; 1 : 800 for $Ca^{2+,}$ $Mg^{2+;}$ 1 : 25 , Ni^{2+}; 1 : 20 for Ag^{+}, $Cu^{2+;}$ and 1 : 500 for Al^{3+}.The acceptance limits for recovery of Fe−Oxine complex with various foreign ions was found to be <5%. The alkali and alkaline earth elements are not counted as matrix components, because they forms unstable complexes with oxine. Therefore, the proposed procedure is better selectivity for trace levels of Fe.

Analytical Capability of MDLP-µE Method

The linearity of the developed method for the preconcentration of Fe was studied in the range of 10-100µg L^{-1}as shown in Tables I. Theenhancement factor (EF) 47 was achieved from slope of calibration curves for the purposedMDLP-µE method. The LOD and LOQ which was found to be 0.44µg $L^{-1}$and 1.47 respectively.The validity of the developed procedurewas verified by the analysis of certified sample of serum have certified value for Fe (Table II). The MDLP-µE method have good sensitivity and low detection limits which recommend to analyze the concentration of Fe in blood serum.

Application

The developed procedure at optimum values of different variables was used for the analysis of Fe of trace levels in serum samples of different neurological disorders patients (Alzheimers, parkinsons, multiple sclerosis) and age matched healthy controls The resulting data

indicate that the Fe levels in serum samples of neurological disorders male patients are significantly higher than the controls of same age group (Table III). At 95% confidence intervals the ranges of Fe in the serum samples of male neurological disorders patients were observed to be (CI) for alzheimer's (CI 1403–1445 µg L^{-1}), parkinsons (CI 1535–1575µg L^{-1}), multiple sclerosis (CI 1350–1378µg L^{-1}) versus controls (CI 600–795 µg L^{-1})s .It was reported that due to excess of Fe causes mental action, learning behavior, and the susceptibility to different patients have neurologicaldisorders.Iron is vital for normal neuronal metabolism. The level of iron increased in many chronic neurological disorders including Alzheimer's disease, Parkinson's disease, and multiple sclerosisleads todeposition of iron in the brain due to the formation free radical (33-36).Excess of Fe cause cellular damage where as deficiency impair cell growth. Fe is important cofactor for enzymes involved in the neurotransmitters synthesis, neural function and development (37).

CONCLUSION

An efficient, innovative preconcentration method, MDLP-µE was purposed for the enrichment of Fe concentration of acid digested blood serum in patients having different neurological disorders such as (Alzheimer's ,Parkinson's,multiple sclerosis) before proceeding to FAAS. The resulted data indicated that the developed (MDLP-µE)procedure, having low cost and in 5 minutes extraction cleaning. Otherremarkable features of the developed method was back-extraction step very simple, achieved in less then 2 minutes.MDLP-µE method has some advantages such as good enhancement factor, low consumption of organic solvent, extraction time short, easy operation, and low generation ofwaste.The developed methodology was validated by using analysis of CRM of blood serum.The results shows that the greater concentration of Fe were obtained in serum samples compared with healthy control.

ACKNOWLEDGMENT

The authors would like to acknowledge the National Centre of Excellence in Analytical, University of Sindh, Jamshoro-PAKISTAN, for provided the required facilities to conducted this research work.

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Figure Captions

Fig. 1. Effect of pH on the recovery (%) of (MDLP-µE), 10µg L^{-1} Fe, Oxine (0.113%)0.3 mL, chloroform (80 µL), 8 aspirating/dispensing cycles of extraction first step, 5 aspirating/dispensing cycles for second step with 0.5 mL (1.5 mol L^{-1}, HNO_3) and a centrifugation time of 5 min at 2500 rpm

Fig. 2. Effect of Oxine concentration on % recovery of Fe by (MDLP-µE) using 10µgL^{-1}Fe, pH 5,chloroform (80 µL), 8 aspirating/dispensing cycles of extraction first step, 5 aspirating/dispensing cycles for second step with 0.5 mL (1.5 mol L^{-1}, HNO_3) and a centrifugation time of 5 min at 2500 rpm.

Fig. 3. Effect of aspirating/dispensing cycles on the recovery (%) of (MDLP-µE), 10µg L^{-1} Fe,pH 5Oxine (0.113%)0.3 mL, chloroform (80 µL), back extracting solution 0.5 mL (1.5 mol L^{-1}, HNO_3), a centrifugation time of 5 min at 2500 rpm.

TABLE I
Characteristics performance of the presented MDLP-μE method.

Concentration range (μg L^{-1})	10–100
LOD[a](μg L^{-1})	0.44
R^2 (correlation coefficient)	0.998
Repeatability (RSD%)[b](n=10)	3.4
Enhancement factor[c]	47

Key:

[a] Limit of detection. Calculated as three times the S.D. (3σ) of the blank signal.

[b]Fe concentration was 10μg $L^{-1}$for which the R.S.D. was obtained.

[c]Calculated as the ratio of slope of preconcentrated samples to that obtained without preconcentration.

TABLE II

Preconcentration of Fe in certified reference material (μgL^{-1})byMDLP-μE method(n=10)

CRM of Clincheck control lyophilized ® human serum	x±s[a]	% Recovery[b]	Certified value
MDLP-μE	739±0.016	99.5	742±0.014

Key:

[a] Mean±S.D

[b] $\%\text{recovery} = \frac{\text{measure values}}{\text{certified value}} \times 100$

TABLE III

The concentration of Fe in serum samples of neurological disordersmale patients and healthy control (μgL^{-1})

Element	Healthy control (n =60)	Alzheimer's (n= 20)	Parkinson's (n= 20)	Multiple sclerosis (n=15)
Fe (μg L^{-1})	660±50.5	1417±10.9	1562±11.5	1359±16.8
	P= 0.01 – 0.001			

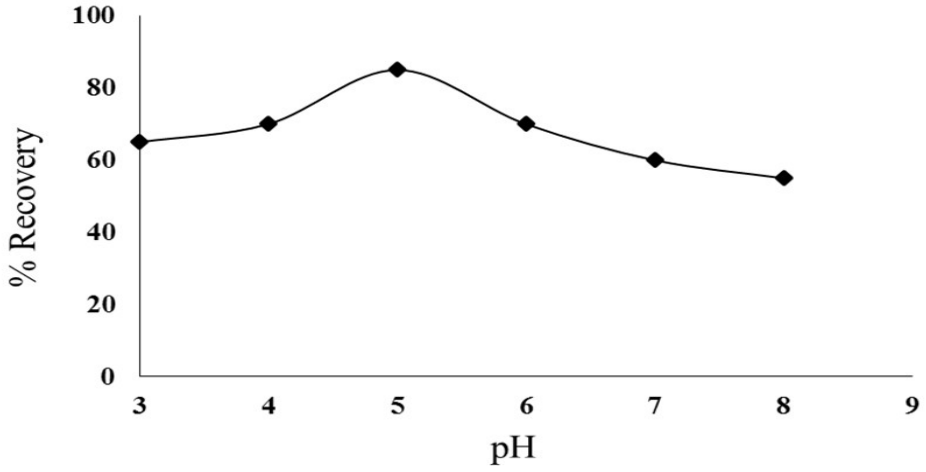

Fig.1

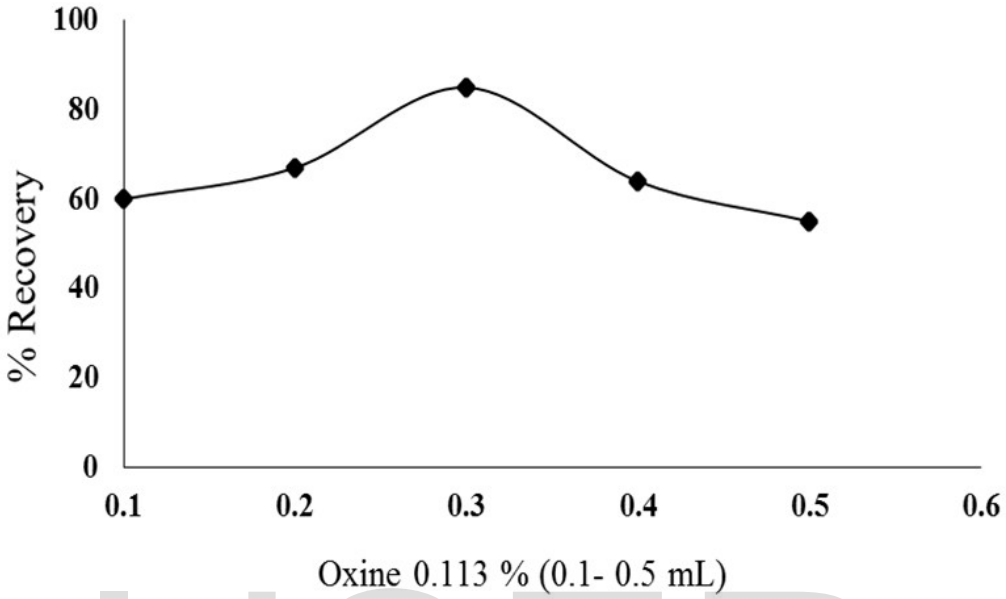

Fig.2

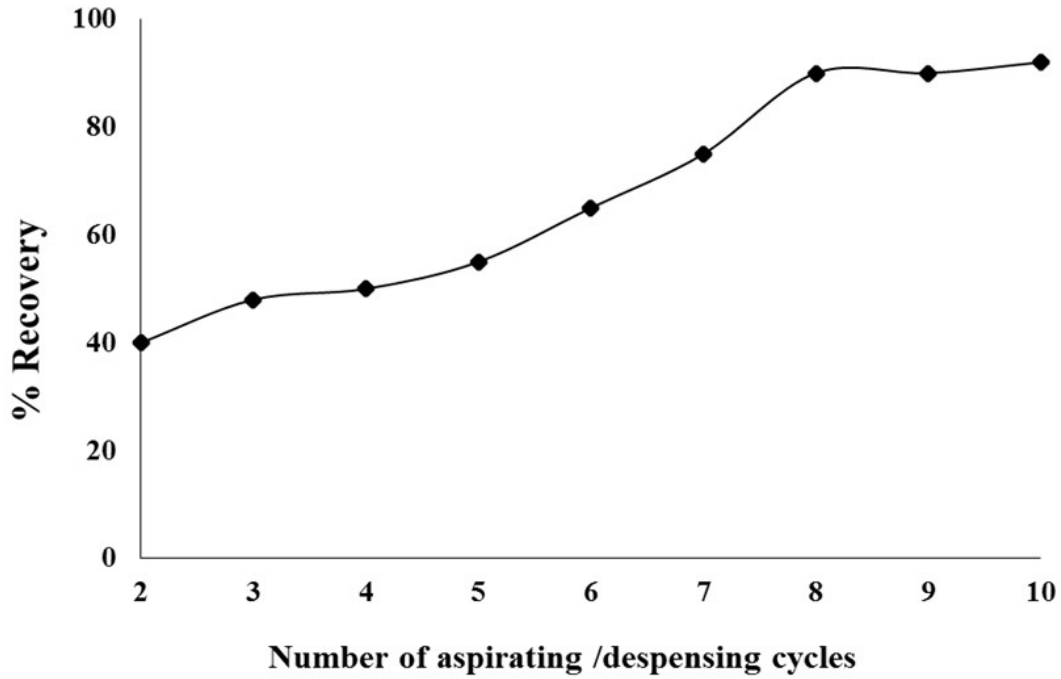
100
80
60
40
20
0
% Recovery
2
3
4
5
6
7
8
9
10
Number of aspirating /despensing cycles

Fig.3

Graphical Abstract of the (MDLP-μE for Fe)