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ORIGINAL ARTICLE

Pre-concentration of Penicillamine using Fe_3O_4NPS and analysis of it in drug formulations through spectroscopic method

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Abstract

First, an effective pre-concentration of penicillamine was done via Fe_3O_4NPS. Then, a simple, rapid and sensitive spectrophotometric method is described for the determination of penicillamine by 2, 6-dichlorophenolindophenol (2, 6-DCPIP), as the chromogenic agent in bulk drug and formulations. It produces a bluish green coloured compound with maximum absorbance 610 nm. Beer's law was obeyed in the concentration range 0.005-0.100 ppm with molar absorptivity 1.97 × 103 L/mol/cm and RSD 0.3-0.82%. Statistical comparison of the results with those from the reference method reveals excellent agreement and accuracy and precision was ±0.481 to ±0.33%. This method is also applied on the whole blood and serum samples collected from the confirmed patients. The reaction of penicillamine with 2, 6-DCPIP was confirmed by FTIR and ^{1}H NMR. The main advantage of 2, 6-DCPIP was its stability as a reagent solution, resulting in reliable and reproducible results.

Keywords: *Fe_3O_4NPS; Magnetic Nanoparticle; Penicillamine; Spectrophotometry; Serum; 2, 6-Dichlorophenolindophenol.*

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INTRODUCTION

Penicillamine is the characteristic acid degradation product of β-lactam antibiotics. It is a chelating agent, which is used to aid the elimination of copper in the treatment of hepatolenticular degeneration (Wilson's disease) [1]. It has been also used in cystiuria, in heavy metal poisoning and for the treatment of rheumatoid arthritis [2]. The presence of the amino, the carboxyl and the thiol groups of the compound offer several possibilities for interaction with organic and inorganic species. Different direct and indirect methods have been proposed for the analysis of penicillamine in both pharmaceutical preparations and biological samples. These methods include colorimetry [3-11], titrimetry [12], spectrophotometric [13], spectrofluorimetry [14], high performance liquid chromatography [15], flow injection analysis [16], sequential injection spectrophotometric method [17] and NMR spectroscopy [18].

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2, 6-dichloro-N-(-p-hydroxy phenyl)-p-benzo-quinone amine (2, 6-DCPIP) is a well-known titrant of vitamin C in biochemical studies [19, 20], in redox titrations [21], in variety of reactions of pharmaceutical and analytical importance [22]. The oxidized form of the indicator changes its colour according to the pH of the solution. In acidic medium the colour is red, while in alkaline medium it changes to blue. The colour change is very sharp and easy to observe.

Due to the lack of chromophore and auxochrome in-penicillamine molecule, a reagent to convert it to such a product which is spectrophotometrically determined is required. Variety of colorimetric methods has been reported but most of these methods are time consuming [11] or lack sensitivity due to the problem of interference with degradation product of colouring agents [10]. The proposed method circumvents, the shortcomings of earlier reported methods for

the determination of penicillamine. The proposed method involves spectrophotometric assay of penicillamine by oxidation of its sulphydryl group to the corresponding disulphide with 2, 6-dichlorophenolindophenol, having stoichiometry of 2 : 1 following the reaction given in Fig. 1. Magnetic nanoparticles offer many advantages over the traditional sorbents. They have very large surface area, highly active surface sites, and a short diffusion route. These particles tagged to the target can be removed from a matrix quickly by applying a magnetic field and do not agglomerate after removal of the field and can be reused or recycled easily; however, these nanometer sized metal oxides are not target-selective; therefore, overcoming this limitation modification of these magnetic nanoparticles is necessary [23-25]. Hemimicelles and admicelles are formed by the adsorption of ionic surfactants on surface of mineral oxides such as alumina, silica, titanium dioxide, and iron oxides [26-27] and have recently been employed as useful sorbent for the SPE of some organic compounds [28]. Few SPE methods based on surfactant-coated Fe_3O_4NPS have been reported [29].

At this paper, we have used magnetic nanoparticles for pre-concentration of penicillamine and then the concentration of penicillamine was determined using spectroscopic method.

MATERIALS AND METHODS

All the chemicals were of analytical grade and double distilled water was used throughout the study. A Helios ð model digital spectrophotometer provided with 1 cm glass cells was used for absorbance measurements. The FTIR spectra were recorded in Nicolet FTIR spectrophotometer in the range 4000-400 cm-1 using KBr pellets.1H NMR spectra were recorded in Varian 300 MHz using $CDCl_3$ as solvent.

Preparation of penicillamine standard solution and its formulations

Pharmaceutical grade penicillamine was purchased from some Iranian companies. Formulations containing penicillamine were purchased from local commercial sources. Stock standard solution containing 0.005 M was prepared by dissolving the weighed amount of penicillamine in deionized water and stored in refrigerator.

Analysis of pharmaceutical formulations

A stock solution was prepared by crushing 10 tablets and dissolving an amount equivalent to 200 mg of penicillamine in 150 mL of deionized water with sonication at room temperature for 5 min. The solution was filtered through Whatmann no. 42 filter paper into a 250 mL volumetric flask and residue was washed well with deionized water several times and the filtrate plus washings were diluted to the mark in a 250 mL calibrated flask.

Pre-concentration Procedure

By the addition of appropriate volume of the drug's stock solution in 20mL of distilled water, the aqueous solution of each drug (100 ng/mL) was prepared and then 0.75mL of the MNP suspension (containing 10 mg of Fe_3O_4NPs) was added to the drug's solution and the pH was adjusted to 8.5. Then, 0.5mL of the 10 mg/mL−1 CTAB was added and the mixture was shaken for 5 min to enhance the drug's adsorption efficiency and then by use of a strong magnet Fe_3O_4NPs placed at the bottom of the beaker was separated quickly from sample solution.

The magnet was removed and the supernatant water was decanted. Finally the drugs were desorbed with 500 μL methanol from MNPs. Calculation of ER% showed that desorption of drugs was completed during 30 s in ultrasonic bath and 30 s in vortex. The magnet was used again to settle the MNPs and the eluent was decanted into a microtube. Then, 20 μL of the solution was injected into the HPLC instrument for analysis. All the experiments were carried out at the room temperature.

Preparation of leuco 2, 6-dichlorophenolindophenol (2, 6-DCPIP) solution

To 20 mL of a 0.005 M solution of the sodium salt of oxidizing salt, 5 mL of phosphate buffer was added and the blue color of the solution was bleached by adding 0.005 M of ascorbic acid solution dropwise. The reduced indicator was oxidized when exposed to air and it is essential to decolourize the solution with ascorbic acid before use. Phosphate buffer (pH = 7) was prepared by dissolving 117.7 g of K_2HPO_4 and 44.1 g of KH_2PO_4 in 1 L of doubly distilled water.

Preparation of clinical samples

The subjects were two male patients suffering with rheumatoid arthritis aged 40 and 52 years. They weighed 70 to 80 kg. The patients received no medication for at least two weeks before the

study. Each subject was orally given a single tablet of penicillamine 125 mg with water. The clinical studies were carried out on patients kept on fast for 3 h.

Two blood samples were collected from patients suffering from rheumatoid arthritis. First blood sample, 10 mL (without drug), was collected before giving the drug. Second blood sample, 10 mL (with drug), was collected after 0.5 h of administration of penicillin, a 125 mg tablet. All samples were frozen in dry ice-bath until analyzed. Serum samples can be separated by treating pure blood and drug containing blood separately with 1.0 mL of trichloroacetic acid. The sample was then centrifuged for 0.75 h at 360 rpm. The supernatant clean liquid is the serum and it was taken in varying aliquots. The content left after separating serum is whole blood, which is diluted and used for further study by the recommended procedure.

Analysis of penicillamine

Different aliquots in the concentration range of penicillamine (0.005 M), from 0.05-3.00 ppm were pipetted into 5 mL measuring vials. 2, 6-dichlorophenolindophenol solution (0.005 M) 1.0 mL was added to each flask and the solutions were diluted to volume with doubly distilled water. The contents were mixed well and kept at room temperature for 15 min. Absorbance was measured at 610 nm against the reagent blank prepared simultaneously by omitting the drug. The amount of drug in each sample was calculated from the calibration graph or the regression equation.

Analysis of penicillamine in whole blood and serum samples

For clinical studies different sets of aliquots containing 0.2-8.0 mL of whole blood sample containing 0.6-20.7 ppm of drug were taken in 150 mL Erlenmeyer flasks. To each aliquot 10 mL of 2.46×10^{-5} 2, 6-DCPIP, $\varepsilon = 8 \times 103$ L mol^{-1} cm^{-1} was added. It was diluted to 18 mL by adding deionized water. The absorbance values of all such aliquots were recorded at 610 nm and unknown concentrations as mentioned in the above procedure. The same procedure is followed for the serum with drug-injected samples.

RESULTS AND DISCUSSION

2, 6-Dichlorophenolindophenol is a well-known oxidant. Its oxidative behaviour resembles to that of coenzyme-Q and acts through its quinoid moiety. Its use in Co^{60} moiety has also been reported [23].

Basford and Huennekens [24] first reported its reaction with sulphydryl group bearing compounds, thiols, which were later, reported by Hadler *et al.* [25]. These findings were exploited in biochemical researches to determine the membrane sulphydryl group. The oxidation of sulphydryl results in the formation of disulphide, which is fundamentally important. This is largely due to the involvement of -SH group in biochemical processes such as enzyme catalysis, cell division, radiation injury as well as in cross linking of proteins. This concept was applied in present paper resulting, in the oxidation of sulphydryl group of penicillamine into disulphide and dyes itself under goes reduction of quinone group according to the following reaction given in Fig. 1.

Spectrophotometric determination of penicillamine can be successfully performed as direct method involving two-electron change. Penicillamine undergoes oxidation and the thiol functional group is converted into corresponding disulphide confirming the 2 : 1 stoichiometry. The optimum conditions for the assay of penicillamine were established via a number of preliminary experiments.

Effect of time

To investigate the effect of time on colour development, 2 mL of 0.005 M penicillamine was pipetted into a 5.0 mL measuring vials, 1 mL of 0.005 M 2, 6-DCPIP were added and the mixture was diluted to volume with doubly distilled water. The absorbance was recorded as a function of time. The results showed that absorbance became constant after 15 min and remained unchanged up to 1 h. Thus absorbance was measured within the stability period.

Effect of 2, 6-dichlorophenolindophenol concentration

To 1 mL of 0.005 M penicillamine, different volumes (0.05-3.0 mL) of 0.005 M 2, 6-DCPIP were added. The coloured product was diluted to 10 mL with doubly distilled water and absorbance was measured against the corresponding reagent blank after 10 min. The results showed that the highest absorbance was obtained with 1 mL and remained constant for larger amounts of 2, 6-DCPIP. Thus, 0.5 mL of 0.005 M 2, 6-DCPIP was added for colour development.

Analytical data

Under the optimum experimental conditions the main merits of the procedure for the determination of penicillamine were established by least squares treatment of the results. The absorbance at 610 nm was found to be linearly dependent on the concentration of penicillamine up to 0.05 to 3.0 ppm with a molar absorptivity 1.97×10^{-3} L mol^{-1} cm^{-1}.

Regression analysis of Beer's law plot was performed to evaluate intercept, slope and correlation coefficient (r) and the values were found to be 0.27×10^{-3}, 4.0×10^{-3} and 0.999, respectively which yielded the regression equation, $A = 0.27 \times 10^{-3} + 4.0 \times 10^{-3}$ C (where A is the absorbance and C is the concentration of penicillamine in µg mL^{-1}). The detection limit for the proposed method was 0.94 µg mL^{-1}. The high value of correlation coefficient and small value of the intercept on the ordinate, which was close to zero, validated the linearity of calibration curve whereas detection limit and slope indicated the good sensitivity of the method. The small amount of scattering of the experimental data points around the line of regression was also confirmed by the small value of variance, i.e. 5.33×10^{-6}.

Effect of Magnetic Nanoparticles of Fe_3O_4

Due to high volume of different species in blood and serum, a preconcentration must be done before analysis. Alone, bulk magnetite sorbent cannot be used for quantitative and selective extraction of penicillamine in solution. By using Fe_3O_4NPS, because of its tendency to form a complex with penicillamine at suitable pH (pH = 8.5), it is possible to have quantitative and selective extraction of penicillamine. Moreover, Fe_3O_4NPS in complex with penicillamine prevents the agglomeration of analyte and proteins in this pH and induces the stabilization of the ligand on the sorbent.

Application on serum and whole blood samples

Human serum albumin (HSA) is an abundant plasma protein that binds a remarkably wide range of drugs, there by restricting their free, active concentrations. The problem of overcoming the binding affinity of lead compounds for HSA represents a major challenge in drug development.

Many commonly used drugs with acidic or electronegative features (e.g., warfarin, diazepam, ibuprofen) also bind to HSA, usually at one of two primary sites (1 and 2), located in sub domains IIA

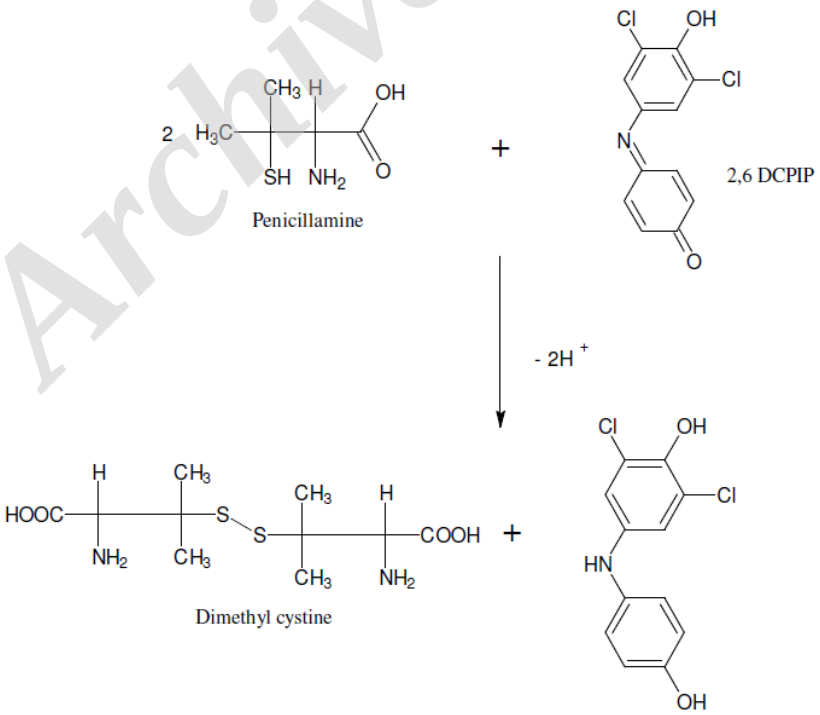

Fig. 1. Formation of dimethyl cystine and reduction of 2,6-DCPIP.

and IIIA, respectively [26]. A degree of albumin-binding may be desirable in helping to solubilize compounds that would otherwise aggregate and be poorly distributed. Drugs with an excessively high affinity for the protein (0.95 % bound) require correspondingly higher doses to achieve the effective concentration in vivo, can be slow to distribute to sites of action and may not be efficiently eliminated [27].

The object of analyzing whole blood and serum samples is to investigate the percentage extent of assimilation and distribution of penicillamine in the serum and whole blood. It also establishes, beyond doubt that all the peaks corresponding to various structural features of the penicillamine in its FTIR spectrum when subtracted from the spectrum of the penicillamine injected whole blood, serum sample leave behind no peak corresponding to sulphydryl group.

In case of clinical samples of whole blood, linear plots are observed in the range 0.025-0.37 ppm and in the serum samples from 0.005-0.10 ppm. The proposed method showed the error of 0.79% for whole blood and 0.99% for serum samples (Tables 1 and 2).

The metabolite of penicillamine formed in the body needs to be removed. This prompted us to develop a method for finding out the blood and serum level of drug assimilation and also the level of unassimilated drug. For this purpose, solid products of the clinical samples were analyzed by FTIR and ^{1}H NMR studies.

FTIR spectrum of pure penicillamine as KBr pellets indicates characteristic frequencies at 3236 cm^{-1} due to NH_3^+ stretching; sharp bands at 1354, 893 and 546 cm^{-1}, are stretching vibrations of aliphatic -C-CH_3 deformation, -C-N and -C-S stretch, respectively. The band at 1597 cm^{-1} show carboxylate ion -COO- (of COOH group). Characteristic peak at 2519 cm^{-1} indicate the presence of -SH group of penicillamine.

FTIR spectrum of 2, 6-DCPIP shows a broad band at 3407 cm^{-1} indicating -OH stretch of phenol group, a band at 2940 cm^{-1} showing -CH_2 vibration. The band at 1505 cm^{-1} showing C=N vibration and at 841 cm^{-1} showing -C-Cl group stretching. In the drug injected whole blood patient and on oxidation of the sample with 2, 6-DCPIP, a characteristic peak

Table 1: Spectrophotometric determination of penicillamine injected whole blood with 2, 6 dichlorophenolindophenol.

Range of sample (ppm)	Weight taken (ppm)	Weight found	Error (%)	Standard deviation	Coefficient of variance (%)
0.025-0.375	0.025	0.0252	0.79	6.32×10^{-4}	2.500
0.050-0.375	0.050	0.0500	0.00	6.32×10^{-4}	1.264
0.075-0.375	0.075	0.0751	0.13	6.32×10^{-4}	0.841
0.100-0.375	0.100	0.1002	1.20	6.32×10^{-4}	0.631
0.125-0.375	0.125	0.1252	0.16	6.32×10^{-4}	0.504
0.150-0.375	0.150	0.1506	0.40	6.32×10^{-4}	0.419
0.175-0.375	0.175	0.1750	0.00	6.32×10^{-4}	0.361
0.200-0.375	0.200	0.2006	0.30	6.32×10^{-4}	0.315
0.225-0.375	0.225	0.2250	0.00	6.32×10^{-4}	0.281
0.250-0.375	0.225	0.2500	0.00	6.32×10^{-4}	0.253

Table 2: Spectrophotometric determination of penicillamine injected serum with 2, 6-dichlorophenolindophenol.

Range of sample (ppm)	Weight taken (ppm)	Weight found (ppm)	Error (%)	Standard deviation	Coefficient of variance (%)
0.005-0.100	0.005	0.0050	0.00	5.47×10^{-4}	10.94
0.010-0.100	0.010	0.0100	0.00	5.47×10^{-4}	5.47
0.015-0.375	0.015	0.0150	0.00	5.47×10^{-4}	3.64
0.020-0.100	0.020	0.0201	0.49	5.47×10^{-4}	2.72
0.025-0.100	0.025	0.0252	0.79	5.47×10^{-4}	2.17
0.030-0.100	0.030	0.0303	0.99	5.47×10^{-4}	1.80
0.035-0.100	0.035	0.0352	0.56	5.47×10^{-4}	1.55
0.040-0.100	0.040	0.0402	0.49	5.47×10^{-4}	1.36
0.045-0.100	0.045	0.0450	0.00	5.47×10^{-4}	1.22
0.050-0.100	0.050	0.0500	0.00	5.47×10^{-4}	1.09

Table 3: Characteristic 1h NMR chemical shift (300 MHz) of pure serum: 2, 6-dcpip and drug serum: 2, 6-dcpip.

NMR of pure serum: 2,6-DCPIP (ppm)	NMR of drug serum: 2,6- DCPIP (ppm)	Assignment of signals
0.8-1.0	0.9-1.1	R-CH_3 protons of serum overlapping with CH_3 of drug
1.15-1.25	–	Serum protons
1.3	–	-CH_2-Cl protons of 2,6-DCPIP and CH-Cl of serum
–	1.5, 1.9-2.2	Serum protons
1.45	–	R-CH_2 Serum protons
2.40	–	C_6H_5-CH protons of 2,6-DCPIP
2.7-3.0	–	Weak serum proton signal
–	2.60, 2.65, 2.70, 2.75	Overlapping serum proton signal with non-equivalent protons of indole group of 2,6-DCPIP
2.8	–	O=C (R)-CH_3 of serum protein
–	3.2	-(S-CH_2)2 Disulphide proton signals
–	7.9	Aromatic proton of 2,6-DCPIP

Table 4: Determination of penicillamine in dosage forms by the standard addition method.

Pharmaceutical preparation	Amount taken ($\mu g\ mL^{-1}$)	Amount added ($\mu g\ mL^{-1}$)	Total amount found ($\mu g\ mL^{-1}$)	Recovery (%)	RSD (%)
Pencip	025	025	049.85	099.71	0.73
	150	150	300.20	100.10	0.30
Kaypen	025	025	050.15	100.30	0.82
	150	150	300.94	100.30	0.45
Procanine	025	025	049.10	099.40	0.75
	150	150	300.20	100.10	0.67

Table 5: Comparison of results from the proposed method with those from other spectrophotometric methods for the determination of penicillamine in pharmaceutical formulations.

Reagent	λ_{max} (nm)	Beer's law limit (µg/mL)	RSD (%)	Reference
$NiSO_4$	270	1-20	0.07	13
$CoCl_2$	291	2-20	0.14	13
$Pb(CH_3COO)_2$	267	2-25	0.11	13
2,6-Dichloroquinone-4- chlorimide (DCQ)	431	4-20	1.57	14
2,6-DCPIP	610	0.005-0.1 ppm	0.30-0.82	Present method

at 3414 cm^{-1}, indicating -OH group gets shifted by 7.4 cm^{-1}. Peak at 2920 cm^{-1} shows -CH_2 stretch of benzene, at 2861 cm^{-1} show CH_2-COO^- stretch of haemoglobin, at 2130 cm^{-1} indicate C=C stretch of haemoglobin, band at 1130 cm^{-1} shows -C_6H_5OH stretching frequency. Band at 939 cm^{-1} indicates -C-Cl bond.

In FTIR spectrum of drug injected whole blood sample with 2, 6-DCPIP a new peak appears at 654 cm^{-1} confirming the oxidation of -SH group to S-S (disulphide). Peak at 1525, 1249 and 1097 cm^{-1}, is due to ν-C=C nonconjugated alkenes, ν(CN), ν(C-CH_3) of drug. A band at 428 cm^{-1} indicates ν(S-S) bond, confirming of formation of disulphide.

In FTIR spectrum of drug-injected serum with 2, 6-DCPIP, bands at 3315, 2920 and 2532 cm^{-1}, indicate free -NH and -OH group of 2, 6-DCPIP and of drug moiety. Sharp peaks around 1657, 1538, 1262 and 1071 cm^{-1}, corresponds to ν(C=N), ν(C=C) of serum while 1406 is due to C-N of serum and 2, 6-DCPIP. The band at 1262 cm^{-1} is due to the formation of sulphonic acid hydrate, which form hydronium sulphonate salts in serum. The peak at 1071 cm^{-1}, is due to -C-CH_3 of 2, 6-DCPIP and band at 880 cm^{-1}, is due to -C-N of serum. A sharp peak around 465 cm^{-1} show formation of -S-S bond of penicillamine disulphide.

H NMR spectroscopic technique was adopted for further quantitative measurements of pharmaceutical and in clinical samples. Results of spectral studies are summarized in (Table 3). A persual of seven NMR spectra of pure drug, reagent, serum sample, drug injected serum samples, whole blood, drug injected whole

blood, confirm the derivatization of -SH group by disappearance of its signal at 1.9 ppm and also appearance of signals at 3.2 ppm and a singlet at 7.9 ppm confirming C-S bond of oxidized drug and aromatic protons of 2, 6-DCPIP. Various oxidizing agents like N-bromocaprolactam (NBCL), iodine monochloride (ICl), N-chlorosuccinamide (NCS) and 2,6-DCPIP have been considered for determining the drug by titrimetry also. Stoichiometry of the products by various halogenating reagents like NBCL, NCS showed 1 : 1 combination while ICl and 2, 6-DCPIP indicated 1 : 2 stoichiometry with penicillmaine.

CONCLUSION

The method was successfully applied to the determination of penicillamine in tablets available locally. Satisfactory results (Table 4) were obtained for the recovery of drug and were in a good agreement with the label claim. The results of the proposed method were statistically compared with those obtained by the reference method [21,22]. Statistical evaluation indicated there was no significant difference between the methods compared (Table 5).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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