

## Spectrophotometric Determination of Penicillamine with 2,6-Dichlorophenolindophenol in Drug Formulations

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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  $1.97 \times 10^3$  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  $\pm 0.481$  to  $\pm 0.33$  %. 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  $^1\text{H}$ NMR. The main advantage of 2,6-DCPIP was its stability as a reagent solution, resulting in reliable and reproducible results.

**Key Words: Spectrophotometry, 2,6-Dichlorophenolindophenol, Penicillamine, Serum.**

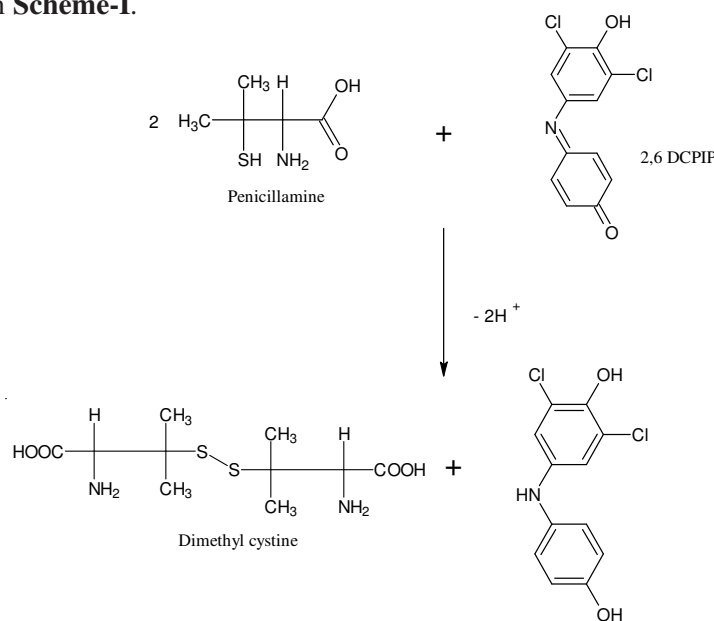
### INTRODUCTION

Penicillamine is the characteristic acid degradation product of  $\beta$ -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)<sup>1</sup>. It has been also used in cystiuria, in heavy metal poisoning and for the treatment of rheumatoid arthritis<sup>2</sup>. 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<sup>3-11</sup>, titrimetry<sup>12</sup>, spectrophotometric<sup>13</sup>, spectrofluorimetry<sup>14</sup>, high performance liquid chromatography<sup>15</sup>, flow injection analysis<sup>16</sup>, sequential injection spectrophotometric method<sup>17</sup> and NMR spectroscopy<sup>18</sup>.

2,6-Dichloro-N-(-*p*-hydroxy phenyl)-*p*-benzoquinone amine (2,6-DCPIP), a well-known titrant of vitamin C in biochemical studies<sup>19,20</sup> in redox titrations<sup>21</sup> and in variety of reactions of pharmaceutical and analytical

importance<sup>22</sup>. 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<sup>11</sup> or lack sensitivity due to the problem of interference with degradation product of colouring agents<sup>10</sup>. 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 sulphhydryl group to the corresponding disulphide with 2,6-dichlorophenolindophenol, having stoichiometry of 2:1 following the reaction given in **Scheme-I**.



**Scheme-I:** Formation of dimethyl cystine and reduction of 2,6-DCPIP

## EXPERIMENTAL

All the chemicals were of analytical grade and double distilled water was used throughout the study. A Helios  $\delta$  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  $\text{cm}^{-1}$  using KBr pellets. <sup>1</sup>H NMR spectra were recorded in Varian 300 MHz using  $\text{CDCl}_3$  as solvent.

**Preparation of penicillamine standard solution and its formulations:**

Pharmaceutical grade penicillamine was gifted by Cipla Pharmaceutical Co., Mumbai, India and was used as received. 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.

**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 colour 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 clear 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  $2.46 \times 10^{-5}$  2,6-DCPIP,  $\epsilon = 8 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$  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<sup>60</sup> moiety has also been reported<sup>23</sup>.

Basford and Huennekens<sup>24</sup> first reported its reaction with sulphhydryl group bearing compounds, thiols, which were later, reported by Hadler *et al.*<sup>25</sup>. These findings were exploited in biochemical researches to determine the membrane sulphhydryl group. The oxidation of sulphhydryl 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 sulphhydryl group of penicillamine into disulphide and dye itself under goes reduction of quinone group according to the following reaction given in **Scheme-I**.

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  $1.97 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$ . Regression analysis of Beer's law plot was performed to evaluate intercept, slope and correlation coefficient ( $r$ ) and the values were found to be  $0.27 \times 10^{-3}$ ,  $4.0 \times 10^{-3}$  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  $\mu\text{g mL}^{-1}$ ). The detection limit for the proposed method was  $0.94 \mu\text{g mL}^{-1}$ .

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 where as 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.*  $5.33 \times 10^{-6}$ .

**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 and IIIA, respectively<sup>26</sup>. While 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<sup>27</sup>.

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 sulphhydryl 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).

TABLE-1  
SPECTROPHOTOMETRIC DETERMINATION OF PENICILLAMINE  
INJECTED WHOLE BLOOD WITH 2,6-DICHLOROPHENOLINDOPHENOL

Range of sample (ppm)	Weight taken (ppm)	Weight found (ppm)	Error (%)	Standard deviation	Coefficient of variance (%)
0.025-0.375	0.025	0.0252	0.79	$6.32 \times 10^{-4}$	2.500
0.050-0.375	0.050	0.0500	0.00	$6.32 \times 10^{-4}$	1.264
0.075-0.375	0.075	0.0751	0.13	$6.32 \times 10^{-4}$	0.841
0.100-0.375	0.100	0.1002	1.20	$6.32 \times 10^{-4}$	0.631
0.125-0.375	0.125	0.1252	0.16	$6.32 \times 10^{-4}$	0.504
0.150-0.375	0.150	0.1506	0.40	$6.32 \times 10^{-4}$	0.419
0.175-0.375	0.175	0.1750	0.00	$6.32 \times 10^{-4}$	0.361
0.200-0.375	0.200	0.2006	0.30	$6.32 \times 10^{-4}$	0.315
0.225-0.375	0.225	0.2250	0.00	$6.32 \times 10^{-4}$	0.281
0.250-0.375	0.225	0.2500	0.00	$6.32 \times 10^{-4}$	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	$5.47 \times 10^{-4}$	10.94
0.010-0.100	0.010	0.0100	0.00	$5.47 \times 10^{-4}$	5.47
0.015-0.375	0.015	0.0150	0.00	$5.47 \times 10^{-4}$	3.64
0.020-0.100	0.020	0.0201	0.49	$5.47 \times 10^{-4}$	2.72
0.025-0.100	0.025	0.0252	0.79	$5.47 \times 10^{-4}$	2.17
0.030-0.100	0.030	0.0303	0.99	$5.47 \times 10^{-4}$	1.80
0.035-0.100	0.035	0.0352	0.56	$5.47 \times 10^{-4}$	1.55
0.040-0.100	0.040	0.0402	0.49	$5.47 \times 10^{-4}$	1.36
0.045-0.100	0.045	0.0450	0.00	$5.47 \times 10^{-4}$	1.22
0.050-0.100	0.050	0.0500	0.00	$5.47 \times 10^{-4}$	1.09

Basically, 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 unassi-

milated drug. For this purpose, solid products of the clinical samples were analyzed by FTIR and  $^1\text{H}$  NMR studies.

FTIR spectrum of pure penicillamine as KBr pellets indicates characteristic frequencies at  $3236\text{ cm}^{-1}$  due to  $\text{NH}_3^+$  stretching; sharp bands at  $1354$ ,  $893$  and  $546\text{ cm}^{-1}$ , are stretching vibrations of aliphatic  $-\text{C}-\text{CH}_3$  deformation,  $-\text{C}-\text{N}$  and  $-\text{C}-\text{S}$  stretch, respectively. The band at  $1597\text{ cm}^{-1}$  indicating carboxylate ion  $-\text{COO}^-$  (of  $\text{COOH}$  group). Characteristic peak at  $2519\text{ cm}^{-1}$  indicate the presence of  $-\text{SH}$  group of penicillamine.

FTIR spectrum of 2,6-DCPIP shows a broad band at  $3407\text{ cm}^{-1}$  indicating  $-\text{OH}$  stretch of phenol group, a band at  $2940\text{ cm}^{-1}$  showing  $-\text{CH}_2$  vibration. The band at  $1505\text{ cm}^{-1}$  showing  $\text{C}=\text{N}$  vibration and at  $841\text{ cm}^{-1}$  showing  $-\text{C}-\text{Cl}$  group stretching. In the drug injected whole blood patient and on oxidation of the sample with 2,6-DCPIP, a characteristic peak at  $3414\text{ cm}^{-1}$ , indicating  $-\text{OH}$  group gets shifted by  $7.4\text{ cm}^{-1}$ . Peak at  $2920\text{ cm}^{-1}$  shows  $-\text{CH}_2$  stretch of benzene, at  $2861\text{ cm}^{-1}$  show  $\text{CH}_2-\text{COO}^-$  stretch of haemoglobin, at  $2130\text{ cm}^{-1}$  indicate  $\text{C}=\text{C}$  stretch of haemoglobin, band at  $1130\text{ cm}^{-1}$  shows  $-\text{C}_6\text{H}_5\text{OH}$  stretching frequency. Band at  $939\text{ cm}^{-1}$  indicates  $-\text{C}-\text{Cl}$  bond.

In FTIR spectrum of drug injected whole blood sample with 2,6-DCPIP a new peak appears at  $654\text{ cm}^{-1}$  confirming the oxidation of  $-\text{SH}$  group to  $\text{S}-\text{S}$  (disulphide). Peak at  $1525$ ,  $1249$  and  $1097\text{ cm}^{-1}$ , is due to  $\nu-\text{C}=\text{C}$  non-conjugated alkenes,  $\nu(\text{CN})$ ,  $\nu(\text{C}-\text{CH}_3)$  of drug. A band at  $428\text{ cm}^{-1}$  indicates  $\nu(\text{S}-\text{S})$  bond, confirming of formation of disulphide.

In FTIR spectrum of drug-injected serum with 2,6-DCPIP, bands at  $3315$ ,  $2920$  and  $2532\text{ cm}^{-1}$ , indicate free  $-\text{NH}$  and  $-\text{OH}$  group of 2,6-DCPIP and of drug moiety. Sharp peaks around  $1657$ ,  $1538$ ,  $1262$  and  $1071\text{ cm}^{-1}$ , corresponds to  $\nu(\text{C}=\text{N})$ ,  $\nu(\text{C}=\text{C})$  of serum while  $1406$  is due to  $\text{C}-\text{N}$  of serum and 2,6-DCPIP. The band at  $1262\text{ cm}^{-1}$  is due to the formation of sulphonic acid hydrate, which form hydronium sulphonate salts in serum. The peak at  $1071\text{ cm}^{-1}$ , is due to  $-\text{C}-\text{CH}_3$  of 2,6-DCPIP and band at  $880\text{ cm}^{-1}$ , is due to  $-\text{C}-\text{N}$  of serum. A sharp peak around  $465\text{ cm}^{-1}$  show formation of  $-\text{S}-\text{S}$  bond of penicillmaine disulphide.

$^1\text{H}$  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  $-\text{SH}$  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  $\text{C}-\text{S}$  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

TABLE-3  
CHARACTERISTIC  $^1\text{H}$  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 <sub>3</sub> protons of serum overlapping with CH <sub>3</sub> of drug
1.15-1.25	–	Serum protons
1.3	–	-CH <sub>2</sub> -Cl protons of 2,6-DCPIP and CH-Cl of serum
–	1.5, 1.9-2.2	Serum protons
1.45	–	R-CH <sub>2</sub> Serum protons
2.40	–	C <sub>6</sub> H <sub>5</sub> -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 <sub>3</sub> of serum protein
–	3.2	-(S-CH <sub>2</sub> ) <sub>2</sub> Disulphide proton signals
–	7.9	Aromatic proton of 2,6-DCPIP

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 penicillamine.

### 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<sup>13,14</sup>. Statistical evaluation indicated there was no significant difference between the methods compared (Table-5).

TABLE-4  
DETERMINATION OF PENICILLAMINE IN DOSAGE FORMS BY  
THE STANDARD ADDITION METHOD

Pharmaceutical preparation	Amount taken ( $\mu\text{g mL}^{-1}$ )	Amount added ( $\mu\text{g mL}^{-1}$ )	Total amount found ( $\mu\text{g mL}^{-1}$ )	Recovery (%)	RSD (%)
Pencip	25	25	49.85	99.71	0.73
	150	150	300.20	100.10	0.30
Kaypen	25	25	50.15	100.30	0.82
	150	150	300.94	100.30	0.45
Procaine	25	25	49.10	99.40	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	$\lambda_{\max}$ (nm)	Beer's law limit ( $\mu\text{g/mL}$ )	RSD (%)	Reference
$\text{NiSO}_4$	270	1-20	0.07	13
$\text{CoCl}_2$	291	2-20	0.14	13
$\text{Pb}(\text{CH}_3\text{COO})_2$	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

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