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Improved spectrofluorimetric methods for determination of penicillamine in capsules

Research Article

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Abstract: Two simple, sensitive and specific fluorimetric methods have been developed for the determination of Penicillamine (PNC), a sulphur containing compound. Method (I) involves the reaction of PNC with 2',7'-bis(acetoxymercuri)-fluorescein (AMF) in the presence of Kolthoff's buffer, pH 8.2, with subsequent measurement of fluorescence spectra at 520 nm (λ_{Ex} 497 nm). Method (II) is based on PNC being oxidized into penicillaminic acid using Cerium (IV) in an acidic medium. Method sensitivity has been improved using sodium triphosphate which enhances the luminescence intensity of Ce(III). Fluorescence spectra were then measured at 348 nm (λ_{Ex} 293 nm). The reaction conditions and the fluorescence spectral properties have been investigated for both methods. Under the described conditions, the proposed methods were applicable over the concentration ranges 0.0048 – 0.0288 μg mL^{-1} and 0.096 – 0.288 μg mL^{-1} with mean percentage recoveries 99.95 ± 1.29 and 100.04 ± 1.10 for methods I and II, respectively. The proposed methods were validated in terms of accuracy, precision, LOD and LOQ and robustness and then were successfully applied to the determination of PNC in bulk powder and in capsules as well as in the presence of the related disulphide. The results obtained were determined to be in good agreement with those obtained using a previously reported method.

Keywords: *Fluorimetric determination • Penicillamine disulphide • Acetoxymercurifluorescein • Sodium triphosphate • Capsules dosage form*

1. Introduction

Penicillamine[(2S)-2-amino-3-methyl-3-sulphanylbutanoic acid] [1] is a disease modifying antirheumatic drug used mainly in the treatment of severe active rheumatoid arthritis. It is also a chelating agent used in the treatment of Wilson's disease, heavy metal poisoning and cystinuria [2].

The BP [1] describes a non aqueous titrimetric method for the determination of penicillamine in bulk powder and a mercurimetric titration for its determination in tablets, while the USP [3] describes an HPLC method for determination of PNC in bulk powder, tablets and capsules. A review on titrimetric, electrochemical, spectroscopic and chromatographic methods for the analysis of PNC and its determination in body fluids and tissues is described in the analytical profile as well as a review on physical properties of PNC including its stability is presented in the same report [4]. Numerous analytical procedures have been reported for the determination of PNC in pure form, in pharmaceuticals and in biological fluids. These methods are focused on several spectrophotometric methods including reaction with 2,6-dichlorophenol indophenol [5], FTIR spectrophotometry [6], and kinetic spectrophotometric determination where the absorbance of Fe(II)- phenanthroline complex formed was measured at 510 nm [7]. Cerium(VI) reagent was used for the spectrophotometric determination of PNC based on measuring the decrease in cerium(VI) absorbance at 317 nm [8]. PNC has also been determined in its dosage forms through kinetic potentiometric determination using iodide ion- sensitive electrode [9], and other methods which include voltammetry [10,11], capillary electrophoresis [12,13], normal phase TLC [14], flow injection spectrophotometry [15,16] and atomic absorption spectrophotometry [17]. HPLC has found a wide application in the determination of PNC using either fluorimetric [18,19], UV/Vis [18,20], or DAD [21] detection after precolumn derivatization using various reagents.

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Spectrofluorimetry and chemiluminescence were utilized in the analysis of PNC. Fluorescence assays were carried out based on the reaction with 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole [22], 1,2-naphthoquinone-4-sulphonic acid [23] or 9- fluorenylmethyl pentafluorophenyl carbonate [24]. Several flow injection chemiluminescence methods were adopted using thalium in hydrochloric acid medium [25], fluorescamine [26], sodium periodate and hydrogen peroxide in dilute phosphoric acid medium [27], tris(bipyridyl) ruthenium(II) and peroxydisulphate system [28] or Quinine - Ce(IV) system [29] with a concentration range 2 – 200 µmol L^{-1}.

The suggested methods in this work are still more sensitive than the above mentioned fluorescence related methods. These suggested methods are also simple, less expensive and can be used in the routine analysis while the former methods required sophisticated instruments.

In method (I), PNC reacts with acetomercurifluorescein (AMF) instantaneously at room temperature leading to fluorescence quenching in the emission spectra of AMF producing a decrease in fluorescence intensity proportional to the concentration of PNC. Moreover, method (II) involves the oxidation of PNC with cerric (IV) in sulphuric acid medium and has been improved by the use of sodium triphosphate which greatly enhanced the fluorescence of the resulting cerium(III).

2. Experimental Procedure

2.1. Instrumentation

1. All fluorimetric measurements were made on a model RF-1501 Shimadzu spectrofluorophotometer version 3.0 (Kyoto, Japan) using 150 W Xenon lamp and 1-cm quartz cell.
2. Digital pH meter 3310 Jenway.
3. Thermostated water bath (Köttermann Hänigsen, Germany).

2.2. Materials and reagents

All chemicals, solvents and reagents were of analytical grade.

- Penicillamine, Sigma Chem. Co., Milwaukee-Wi-USA.
- Artamin capsules, Sandoz (Batch number 46013) were purchased from the local market, labeled to contain 250 mg penicillamine USP.
- Acetomercurifluorescein (AMF) solution (Laboratory made) 1×10^{-4} M solution was used as follows [30]: 82.3 mg of AMF was dissolved in the least amount of 0.1 M NaOH and then diluted with 100 mL 0.1 M boric acid, finally the volume was made up to 1 L using distilled water.
- Kolthoff's borax-phosphate buffer pH 8.2 [31].
- Sodium hydroxide (El-Nasr), 1M solution.
- Cerium(IV) solution 1×10^{-3} M was prepared from $Ce(SO_4)_2$. 4 H_2O (BDH, Pool, UK) in 1 M sulphuric acid.
- Sodium triphosphate pentabasic (Fluka, Sigma-Aldrich, Germany) solution (2×10^{-4} M) was prepared by dissolving $Na_5P_3O_{10}$ in distilled water.

2.3. Preparation of standard solution

PNC 120 µg mL^{-1} in distilled water was prepared as stock solution (For method I). The stock solution was further diluted using the same solvent to obtain a working solution 12 µg mL^{-1} (For method II).

2.4. General procedures

2.4.1. Method I

Different aliquots of standard stock solution were accurately transferred into a set of 25-mL volumetric flasks and; 5 mL Kolthoff's borax-phosphate buffer pH 8.2, and 3 mL AMF were added to each flask. The solutions were mixed, left for 10 min at room temperature then diluted to volume with distilled water. From these solutions, 2-mL aliquots were transferred into a set of 100-mL volumetric flasks, 10 mL 1 M NaOH were added and finally the volume was made up with distilled water. The relative fluorescence intensities of the resulting solutions were measured at 520 nm using 497 nm as excitation wavelength.

2.4.2. Method II

To a set of 25-mL test tubes, solutions were added in the following order: 0.6 mL Ce(VI) solution (1×10^{-3} M), 2 mL sulphuric acid (1M) and PNC standard working solution (0.2 – 0.6 mL aliquots). The mixtures were heated in a thermostated water bath at 80°C for 30 min, cooled to room temperature and transferred quantitatively into a set of 25-mL measuring flasks. A volume of 3 mL sodium triphosphate (2×10^{-4} M) was then added into the mixture and the volume was completed with distilled water. The fluorescence intensity was measured in a 1-cm quartz cell with excitation and emission wavelengths of 293 and 348 nm, respectively.

2.5. Procedure for capsules

The contents of ten capsules were carefully opened, mixed thoroughly, and an accurate weight equivalent to 12 mg PNC was quantitatively transferred into a

100-mL volumetric flask using distilled water. The flask was sonicated for 5 min and the volume was completed to the mark with the same solvent. Aliquots of this solution were then assayed as described under general procedures.

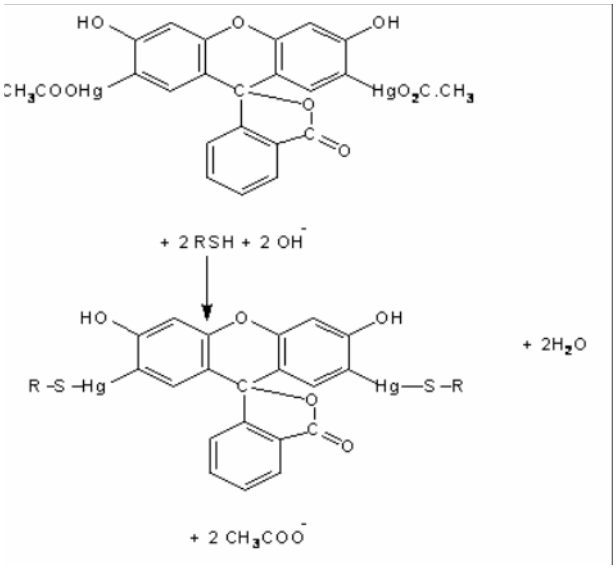
3. Results and Discussion

3.1. Method (I) using acetoxymercurifluorescein (AMF)

It has been found that the fluorescence of di and tetra acetoxymercuri fluorescein is quenched by compounds containing sulphhydril group and other organic sulphur compounds (*e.g.* diphenylthiourea) (Wronski reaction) [32]. This reaction is classified as a complex formation reaction [32] in which ligand exchange of the acetoxy group by sulphhydril in AMF molecule may be assumed to occur (Scheme 1). This reaction has been also used to determine the presence of sulphide [33,34], cyanide and iodide ions [30].

AMF in alkaline solution is yellow colored with green fluorescence; on adding PNC solution fluorescence, quenching was observed. The relative emission spectra are shown in Fig. 1. The decrease in fluorescence intensity was proportional to the concentration of the added PNC. The complex formed between PNC and AMF was found to exhibit fluorescence quenching at λ_{Ex} / λ_{Em}: 497 /520 nm.

Preliminary studies were carried out in order to optimize the assay parameters regarding buffer species, pH and volume, AMF concentration, reaction time and the diluting solvent used. Several buffers have been tried: acetate, borate, phosphate and borax-phosphate. The best results were obtained by addition of 5 mL Kolthoff's borax-phosphate buffer pH 8.2 and 3 mL AMF solution (Fig. 2). As the pH of the buffer increases, more quenching was observed reaching a maximum at pH 8.2. PNC reacts with AMF rapidly at room temperature; so it was found that 10 min reaction time is enough to obtain stable and reproducible results. The effect of diluting solvents on the fluorescence intensity such as double distilled water, buffer pH 8.2, 0.1 M boric acid, 0.005 M sulphuric acid and 0.1 M NaOH, was investigated and it was found that the latter extensively enhanced the fluorescence of AMF. Distilled water and buffer pH 8.2 can be used as diluents with reasonable sensitivity. But it was determined that NaOH has a better effect on the sensitivity of the fluorescence with a reasonable although slightly higher than that of water background readings but much better than that obtained when using buffer as a diluent. The use of different diluents had no effect on the position of λ_{Ex} and λ_{Em} of the reaction or the reproducibility of the results. The reaction product was stable for at least 30 min.



Scheme 1. Reaction pathway of AMF and PNC.

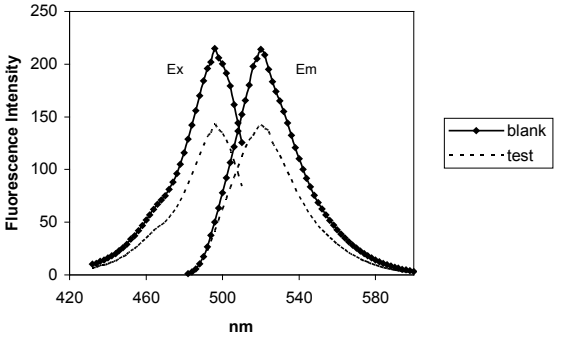


Figure 1. Excitation and emission spectra of AMF in absence and in presence of 0.0288 μg mL^{-1} PNC.

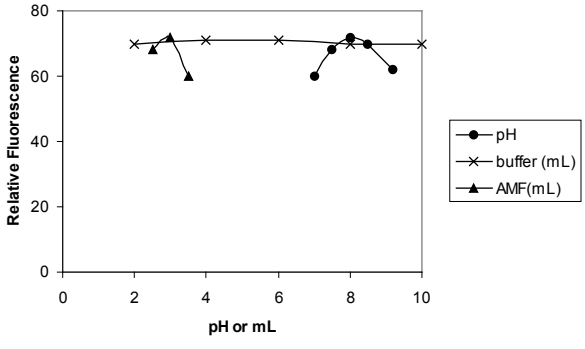


Figure 2. Effect of Kolthoff's borax- phosphate buffer pH, volume of buffer and AMF (1×10^{-4} M) on the reaction between AMF and 0.0288 μg mL^{-1} PNC.

The stoichiometry of the reaction was studied by the Job's method of continuous variation and it was found to be 2 PNC: 1 AMF. This ratio may be explained by ligand exchange of the acetoxy group in AMF by the sulphhydril group of the drug.

3.2. Method (II) using cerium(VI)

The method is based on the oxidation of PNC using Ce (IV). In the presence of excess of Ce(IV), PNC was completely oxidized to penicillaminic acid [8]. The development of the reaction has been monitored by the fluorescence of Ce(III) which can be greatly enhanced using sodium triphosphate solution, the latter acts as a specific reagent for enhancing the fluorescence of Ce(III) ion [35]. Fig. 3 shows the excitation and emission spectra of the formed Ce(III) ion in sodium triphosphate solution. Maximum excitation and emission wavelengths were observed at 293 and 348 nm, respectively, where the high background of the blank was the limiting factor for the measurements.

Table 1. Analytical parameters for the determination of penicillamine using the proposed methods.

Parameter	Method I	Method II
Wavelength (nm)	λ_{Ex} 497 λ_{Em} 520	λ_{Ex} 293 λ_{Em} 348
Concentration range (μg mL^{-1})	0.0048 – 0.0288	0.096 – 0.288
Intercept (a)	10.69	- 44.30
S_a[a]	0.552	5.60
Slope (b)	2145.12	2055.46
S_b[b]	29.54	28.45
RSD% of the slope	1.38	1.38
Correlation coefficient (r)	0.9996	0.9995
$S_{y/x}$[c]	0.593	4.84
LOD[d] (μg mL^{-1})	0.0008	0.0080
LOQ[e] (μg mL^{-1})	0.0027	0.0267

[a] *Standard deviation of the intercept*
[b] *Standard deviation of the slope*
[c] *Standard deviation of residuals*
[d] *Limit of detection*
[e] *Limit of quantification*

The conditions for the production of analytically useful fluorescence measurement were optimized to achieve maximum and reproducible measurements. The effect of acidic solution of Ce (IV) concentration on 0.24 µg mL^{-1} of PNC is shown in Fig. 4. The results showed that, as the concentration of Ce(IV) and sulphuric acid were increased, the relative fluorescence readings were increased to reach a maximum fluorescence at 0.6 mL of 1×10^{-3} M Ce (IV) and 2 mL 1 M sulphuric acid, after which the signals started to decrease. The effects of different temperatures and heating times on the redox reaction between Ce (IV) and PNC were shown in Fig. 5; the reaction is very slow at room temperature, at higher temperatures the fluorescence develops more rapidly. A thermostated water bath adjusted at 80°C was selected for heating and the optimum heating time was 30 min. The effect of sodium triphosphate concentration on the fluorescence intensity was studied. Ce(III) and sodium triphosphate can form a complex at 10°C–50°C [36], so room temperature was recommended. The fluorescence sensitivity increased in the presence of sodium triphosphate by 2 fold. Fig. 6 shows that the maximum fluorescence intensity and a reasonable blank reading were obtained upon addition of 3 mL of

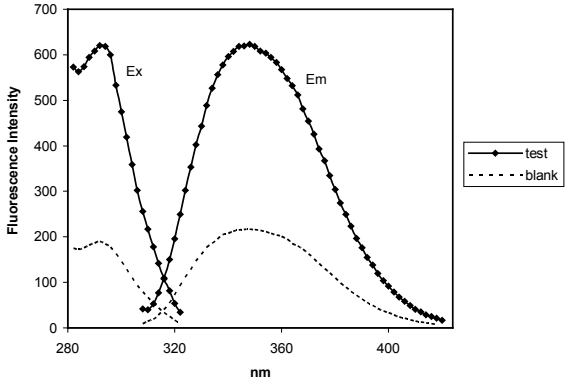


Figure 3. Excitation and emission spectra of Ce(III) formed in absence and in presence of 0.12 μg mL^{-1} PNC.

Table 2. Precision and accuracy for the determination of penicillamine in bulk form using the proposed methods.

	Nominal value (μg mL^{-1})	Within-day % Recovery ± SD[a] (μg mL^{-1})	RSD(%)[b]	E_r(%)[c]	Between-day % Recovery ± SD[a] (μg mL^{-1})	RSD(%)[b]	E_r(%)[c]
Method I	0.0048	99.46 ± 1.12	1.12	-0.54	100.73 ± 2.06	2.05	0.73
	0.0192	101.10 ± 1.4	1.39	1.10	100.78 ± 1.75	1.74	0.78
	0.0288	98.70 ± 0.93	0.95	-1.30	100.26 ± 1.65	1.65	0.26
	0.12	99.45 ± 1.46	1.47	- 0.55	99.45 ± 1.62	1.63	-0.55
Method II	0.216	100.15 ± 0.47	0.47	0.15	99.92 ± 0.91	0.91	- 0.08
	0.24	100.67 ± 0.51	0.51	0.67	99.19 ± 1.46	1.47	- 0.81

[a] *Mean ± standard deviation for three determinations.*
[b] *% Relative standard deviation.*
[c] *% Relative error.*

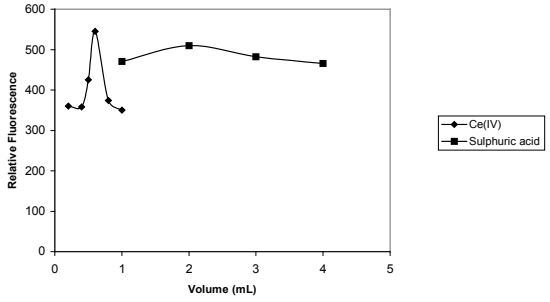


Figure 4. Effect of volume of Ce(IV) (1×10^{-3} M) and sulphuric acid (1 M) on the fluorescence of Ce(III) with 0.24 μg mL^{-1} PNC.

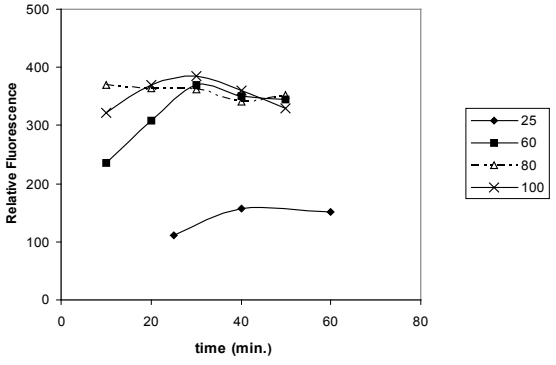


Figure 5. Effect of heating temperature and time on the redox reaction between Ce(IV) (1×10^{-3} M) and 0.216 μg mL^{-1} PNC.

2×10^{-4} M sodium triphosphate solution. The solutions were allowed to stand under normal laboratory conditions and found to be stable for at least 40 min.

3.3. Analytical performance of the proposed methods

3.3.1 Linearity range

The calibration graphs for the quantitation of PNC (using a series of different concentrations) analyzed with the two suggested methods were constructed under the optimum experimental conditions. The linearity was checked by a linear least squares treatment of calibration data. The slopes and intercepts of the calibration graphs, with the correlation coefficients (>0.999), concentration ranges, standard deviations of the intercept (S_a), slope (S_b) and standard deviations of residuals ($S_{y/x}$) are assembled in Table 1. In addition, linearity can be evaluated by calculation of the RSD% of the slope values which did not exceed 1.4%.

3.3.2 Precision and accuracy

The precision (within-day) and accuracy for the described methods were examined at three concentration levels using three replicate determinations for each concentration within one day. Similarly, the between-day precision and accuracy were tested by analyzing the same three concentrations using three replicate determinations repeated on three days. Recoveries were calculated using the corresponding regression equations. Recovery values, RSD% and E_r% were found satisfactory (Table 2).

Table 3. Application of the proposed methods to the analysis of Penicillamine in capsules.

Preparation	Method I	Method II	Reference Method[d]
Artamin® capsules			
%Recovery ± SD[a]	98.76 ± 1.759	99.55 ± 1.089	99.54 ± 1.989
RSD%[b]	1.78	1.09	1.99
E_r(%)[c]	- 1.24	- 0.45	- 0.46
	t= 0.65, F=1.28	t= 0.01, F= 3.33	

ANOVA (single factor)

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2.045326	2	1.022663	0.372328	0.696817	3.88529383
Within Groups	32.9601	12	2.746675			
Total	35.00543	14				

Theoretical values for t and F at P=0.05 are 2.31 and 6.39, respectively
[a] *Mean ± standard deviation for five determinations.*
[b] *% Relative standard deviation.*
[c] *% Relative error.*
[d] *[8].*

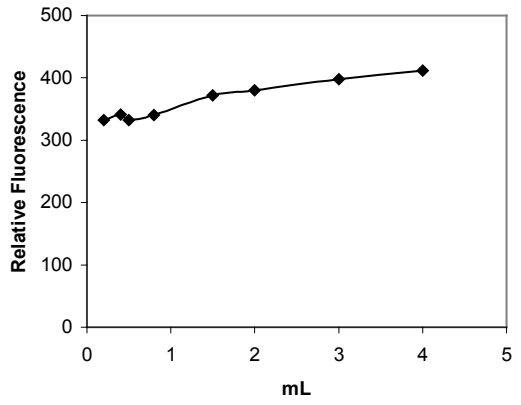


Figure 6. Effect of the volume of sodium triphosphate (2×10^{-4} M) on the fluorescence intensity of Ce(III) with 0.216 μg mL^{-1} PNC.

3.3.3 Specificity

The AMF complexation reaction is specific to unoxidized PNC. It was found that PNC can be determined using the AMF suggested method in presence of its disulphide, the latter being devoid of a sulphhydril group. On the other hand, penicillamine disulphide is easily oxidized by cerium(IV), so it will interfere with the accuracy of the proposed oxidation procedure, (Method II). The latter can determine penicillamine in presence of penicillaminic acid without any interference [8]. So, the two suggested methods are considered to be stability indicating methods for the determination of PNC.

3.3.4 Detection and quantification limits

The limits of detection (LOD) and quantification (LOQ) were calculated as 3 σ and 10 σ divided by the slope, respectively (σ = standard deviation of blank) and are presented in Table 1.

3.3.5 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations of experimental parameters and provides an indication of its reliability during normal usage. Robustness was examined by evaluating the influence of small variations in different conditions such as heating temperature (±3°C), heating time (±5 min) and volume of sulphuric acid (±0.5 mL). No significant difference was obtained in the results in this study using the small variances.

3.3.6 Stability

The stability of final measured sample solutions was examined for one hour at room temperature resulting in stable fluorescence readings. Also, the stock solutions and reagents were stable for at least a week when refrigerated at 4°C on condition that AMF solution should be kept protected from light.

3.4. Analysis of Capsules

The proposed methods were applied to the analysis of PNC in capsules. The results were in good agreement with those of the reference spectrophotometric method [8], and recoveries, SD and RSD% were satisfactory. The results are presented in Table 3.

Results of the proposed methods were statistically compared with those obtained by the reference method [8] using single factor analysis of variance (ANOVA) test which is considered a powerful tool used to compare recoveries obtained from more than two methods [37]. The obtained F value did not exceed the critical F value indicating good harmony between the proposed methods together with the previously reported method.

4. Conclusion

The two suggested spectrofluorimetric methods are simple, rapid and accurate. Comparing the proposed methods with the other fluorimetric reported methods, revealed that the former are more sensitive; moreover, they are more selective than the official titrimetric methods. The AMF complexing method could be successfully considered as a stability indicating assay for penicillamine in the presence of its corresponding disulphide.

The oxidation procedure has some distinct advantage over the flow injection chemiluminescence method [29]; the proposed method is a simpler, faster and much more sensitive with a concentration range 0.6 – 1.8 µmol L^{-1} compared to 2 – 200 µmol L^{-1} for the chemiluminescence reported method [29]; in addition, it does not need any elaborate instruments. Nevertheless, it is a universal method that can be applied for the spectrofluorimetric determination of substances which have a clear reducing character having a standard oxidation potential E less than 1.44. Both procedures can be applied to the determination of penicillamine either in pure form or in capsules.

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