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Ninhydrin reaction on thiol-reactive solid and its potential for the quantitation of D-penicillamine

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ABSTRACT

While aminothiols produce weak purple-colored reactions with ninhydrin, we demonstrate for the first time that this color could be intensely developed. Using a D-penicillamine paradigm, adsorption of this compound via a disulfide bond onto thiol-reactive solid prior to ninhydrin reaction allowed spectrophotometrical monitoring of the supernatant at 570 nm. Compared with off-solid method, this approach expanded the linear concentration range to 50–600 μg mL^{-1} and enhanced the sensitivity so that D-penicillamine with the concentrations of less than 100 μg mL^{-1} could be accurately quantitated by using a second-order polynomial calibration curve. Additionally, this assay was unaffected by disulfide adduct interference, highlighting its potential for the analysis of D-penicillamine as well as other aminothiols.

1. Introduction

Since its discovery by Siegfried Ruhemann in 1910, the reaction of ninhydrin (triketohydrindene hydrate) with a primary amino group to form the purple-colored product diketohydrindylidene–diketohydrindamine (Ruhemann's Purple; RP) has been extensively used for the qualitative and quantitative analysis of amino acids in diverse disciplines including biochemical, clinical, environmental, food, forensic, histochemical and protein sciences [1,2]. In pharmaceutical analysis, the ninhydrin reaction has been used for the assay of many drugs, e.g. tolutamide [3], famotidine [4] and cefaclor [5]. This reaction is unique in that it results in the formation of the same soluble RP products with nearly all amino acids, which can be visualized from the characteristic purple color or spectrophotometrically measured at the maximum absorption wavelength of 570 nm. The ninhydrin reaction with aminothiols, such as cysteine, cysteamine and D-penicillamine, however, generally produces much lower color yields than that with typical amino acids [2,6,7]. Instead, amine and thiol groups in juxtaposition cause spirocyclization of the ninhydrin to take place yielding colorless spirothiazolidines, whose maximum absorption is restricted to the UV range [8].

Because the colorimetric reaction offers the superior analytical advantage of forming a color that is generally distinctive and not affected by the background of samples, we investigated this approach to efficiently produce colored RP from the reaction between ninhydrin and aminothiols, using D-penicillamine or 3,3-dimethylcysteine as a model. It was found that the preceding adsorption of the compound on a thiol-reactive solid prior to ninhydrin reaction resulted in a remarkable development of intense purple color. Herein, we have clarified the mechanism of RP formation from D-penicillamine via this route and studied the factors influencing the color formation. That the approach is of analytical interest for further development as a quantitation method for this compound, as well as other aminothiols owing to its capability of expanding analytical concentration range, sensitivity and specificity, is also discussed.

2. Experimental

2.1. Instrumentation

The spectra and absorbance values were measured by an Agilent G1103A model UV–vis spectrophotometer (Agilent, USA) using a semi-micro type cuvette (1 cm path length). Shaking of sample solutions with solid were done by using an Intelli-mixer (Elmi, Latvia) and the supernatants were separated from the solid by

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centrifugation in a Spectrafuge 240 (Labnet international Inc., UK). The HPLC system consisted of an Agilent 1100 Series system and a diode array detector (Agilent, USA). The column used was a reverse phase VertiSep^{TM} GES ODS column, 5 μm, 150 mm × 4.5 mm (Verical Chromatography, Bangkok, Thailand).

2.2. Reagents and chemicals

D-Penicillamine, D-penicillamine disulfide and ninhydrin were purchased from Sigma–Aldrich (St. Louis, MO, USA). D-Penicillamine capsules (Cuprimine®, 250 mg/capsule) were from Merck & Co., Inc. (PA, USA). The thiol-reactive solid was a Thiopropyl sepharose 6B resin from GE Healthcare Bio-Sciences (Uppsala, Sweden). Deionized (DI) water was used throughout the experiments. All other chemicals and solvents were of analytical grade from Merck (Darmstadt, Germany).

2.3. Protocol for colorimetric ninhydrin reaction on thiol-reactive solid

The standard protocol was as follows: 1 mL of aqueous D-penicillamine standard, or sample solution, was placed in a 1.5-mL microcentrifuge tube and 0.1 mL of 0.5 M sodium phosphate buffer pH 7.0 was added and the resulting solution was mixed. Twenty milligrams of Thiopropyl sepharose 6B resin, in its original dry state, was added to the solution and the mixture was shaken at room temperature (24–27 °C). After 10 min, the solution was centrifuged at 5000 rpm for 2 min and the supernatant was discarded. The collected solid was then washed twice with DI water and drained. To develop the purple color, 0.2 mL of 1% ninhydrin solution prepared in 50% (v/v) ethanol/water was added to the solid. After vortex mixing for 10 s the reaction was left in a 95 °C water bath for 5 min. While heated, the mixture was occasionally shaken to prevent sedimentation of the solid. The reaction was then cooled down, centrifuged at 5000 rpm for 2 min, and the purple-colored supernatant was collected by a pipette and kept on ice. Dilutions were made with DI water to obtain a solution whose absorption could be measured by a spectrophotometer at 570 nm using a semi-micro cuvette (1 cm path length).

To study the factors influencing the color formation and stability, the experiments were conducted by varying one factor to be investigated and keeping the others constant. The factors which were examined in this study included the quantity of thiol-reactive resin (10, 20, 40, 60 mg per reaction), pH of the adsorption reaction (3, 4, 5, 6, 7, 8, 9), time of mixing between the sample and resin (2.5, 5, 10, 20, 30, 45, 60 min), amount of 1% ninhydrin reagent (10, 50, 100, 200 μL) and heating time (5, 10, 15, 20 min) at 95 °C in the color development step, as well as the temperature at which to store the colored supernatant (4 and 25 °C) until the absorbance was measured.

2.4. Preparation of standard curve and assay of commercial D-penicillamine capsules

The standard curves plotted between the concentrations of standard D-penicillamine (X) versus absorbances at 570 nm (Y) were prepared in two concentration ranges. One was in a high concentration range from 50 to 600 μg mL^{-1}. The other covered lower concentrations between 0 and 120 μg mL^{-1}. The best fit X–Y relationships in high and low concentration standard curves were found to be linear and second-order polynomials, respectively.

In the analysis of real samples, commercial capsules labeled as 250 mg per capsule were used. The contents of 20 capsules were weighed and a quantity of the capsule's contents was transferred into a volumetric flask, dissolved and adjusted to the volume with DI water. Further dilutions were made to obtain concentrations of about 300 μg mL^{-1} or 80 μg mL^{-1} which were analyzed by the colorimetric reaction on thiol-reactive solid protocol, using the linear standard curve (50–600 μg mL^{-1}) or second-order polynomial standard curve (0–120 μg mL^{-1}), respectively. The result of analysis was reported as the percent of labeled amount, calculated from the equation below.

$$\%\ \text{labeled amount} = \frac{\text{actual drug content from the assay}}{\text{drug content claimed on the label}} \times 100$$

The assay results obtained from the proposed method were compared with those from the pharmacopoeial HPLC method described in the United States Pharmacopeia, 2008 [9].

3. Results and discussion

3.1. Characteristics of the ninhydrin reaction with D-penicillamine on a thiol-reactive solid

Unlike typical amino acids, D-penicillamine, as well as other aminothiols, produce weak purple color of RP adducts with ninhydrin [2,6,7]. In our experiment, it was found that faint purple color could be formed in this reaction if solutions of D-penicillamine at sufficiently high concentrations were used (Fig. 1a, open circles). The relationship between the absorbance at 570 nm and the concentration showed a narrow linear range restricted to 100–300 μg mL^{-1}. At higher concentrations than 300 μg mL^{-1}, the color intensity appeared to be steady. In contrast, if D-penicillamine solution was pre-adsorbed on thiol-reactive resin, namely Thiopropyl sepharose 6B, and then washed to get rid of unbound species, D-penicillamine remaining on the resin could react noticeably with ninhydrin to form an intense purple color. After eightfold dilution of the colored supernatant and subsequent spectrophotometric measurement, it was revealed that this approach could broaden the linearity range to cover 50–600 μg mL^{-1} with an excellent linear relationship ($Y = 1.80 \times 10^{-3}X - 0.0322$; $r^2 = 0.9994$, Fig. 1a, closed circle). The features of the on- and off-solid ninhydrin reactions were further studied at lower D-penicillamine concentrations (0–120 μg mL^{-1}) and it was found that direct (off-solid) reaction between the D-penicillamine solution and ninhydrin hardly produced color (Fig. 1b, open circle), whereas the reaction happening through the solid support gave an obvious absorbance, demonstrating the enhanced sensitivity of this method. The absorbance of twofold diluted colored supernatant from on-solid reactions showed a good second-order polynomial correlation with concentrations, as described by $Y = 4.22 \times 10^{-5}X^2 + 7.59 \times 10^{-4}X + 0.004826$; $r^2 = 0.9995$ (Fig. 1b, closed circle).

The absorption spectra of the ninhydrin reactions with 50 μg mL^{-1} D-penicillamine are illustrated in Fig. 2. As can be clearly seen, the supernatant from on-solid reactions showed a characteristic absorption maximum at 570 nm, confirming the formation of RP adducts. The solid-free reaction did not produce these products, but instead produced colorless spirothiazolidine adducts with maximum absorption wavelengths at 231 and 246 nm. These spirothiazolidines were observed to form via spirocyclization between the proximal α-amine and the β-thiol nucleophiles of D-penicillamine toward ninhydrin (Fig. 3) as previously described by Prota and Ponsiglione [8]. The formation of this peculiar product was reported to exploit in the protection of amino-terminal cysteine in synthesized peptides [10] and in the enantiomeric HPLC resolution of DL-penicillamine [11] and DL-cysteine [12]. From our findings, this explanation can be made for the unusual formation of RP from aminothiols. Fig. 4 depicts the mechanism, starting from the adsorption of D-penicillamine on Thiopropyl sepharose 6B via the disulfide exchange reaction between the sulfhydryl groups of

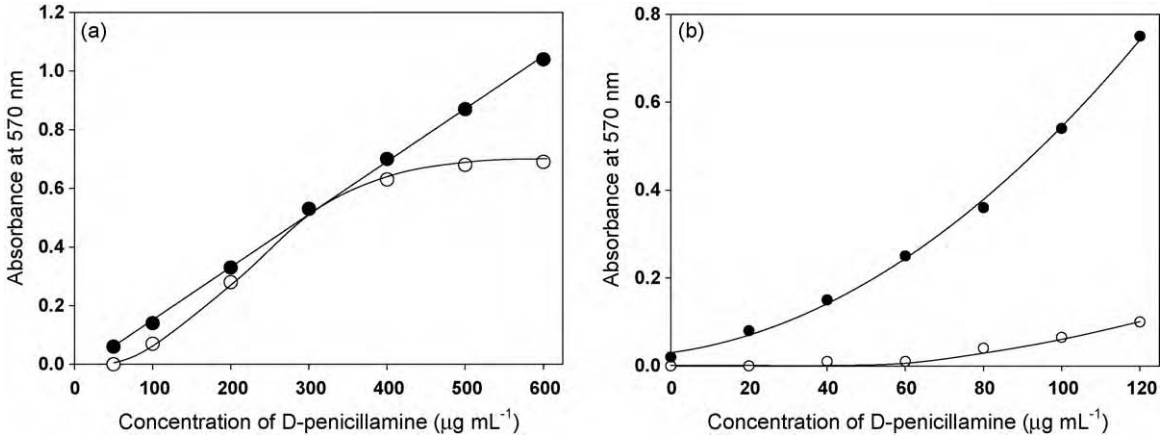

Fig. 1. The relationship of the initial concentration of D-penicillamine and absorbance at 570 nm due to Ruhemann's Purple produced from off-resin (●) and on-resin (○) ninhydrin reaction in high (a) and low (b) concentration ranges. Note that the absorbance values of on-resin reaction plotted in (a) and (b) were from the solutions after eightfold and twofold dilution, respectively, while those of off-resin reaction were from undiluted solutions.

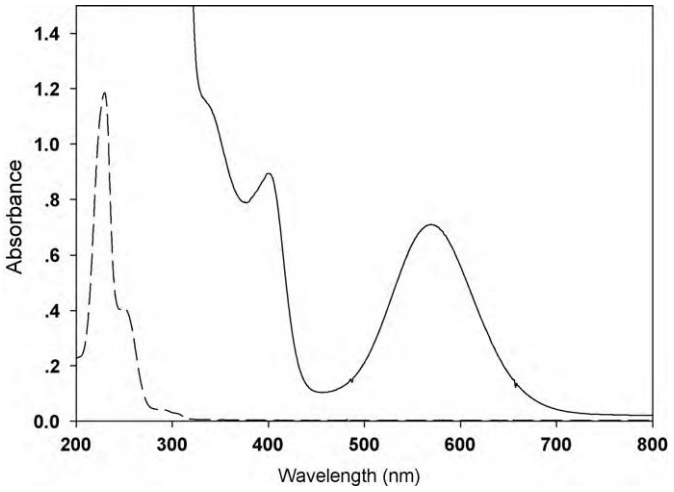

Fig. 2. Absorption spectra of the solutions obtained from the off-resin (- - - - -) and on-resin (——) ninhydrin reaction using 50 μg mL^{-1} D-penicillamine.

D-penicillamine and the 2-pyridyl disulfide on the resin. Using the reducing agent β-mercaptoethanol to cleave the linkage between D-penicillamine and the resins prior to the ninhydrin reaction and then observing that the treated resins failed to produce any purple color proved this hypothesis. Because the free sulfhydryls of D-penicillamine were unavailable for spirothiazolidine formation, ninhydrin favorably reacted with D-penicillamine in the same way as it reacted with typical amino acids to form the major purple-colored RP product. The pre-adsorption of aminothiol solution on the solid offered advantages not only in providing the possibility of subsequent colorimetric reactions but also in enhancing the analytical sensitivity of the method. This result relied on preconcentration because the targeted species were enriched in the small volume of the solid matrices. As a result, the solutions of D-penicillamine at relatively low concentrations, which could not be measured by regular ninhydrin reactions, could produce a detectable absorbance by the on-solid approach. In fact, this basis is similar to that of solid-phase spectrophotometry (SPS), in which the analyte is selectively adsorbed on solid matrices, often ion exchanger resins, and then its intrinsic absorbance is measured either directly or with the aid of a chromogenic reagent. In our case, however, the RP product was released into the supernatant because this chromophore has been known that it is not chemically bound to the protein or other insoluble material, nor is it lost when the insoluble substrate is removed after the reaction is completed [2]. The intensity of color was thus not measured on the solid, but in the supernatant. From our experience, the measurement of absorbance in solution was much easier than that with solid because the dilution could be made as needed. In addition, the errors associated with uneven packing of solid sample in a special type cuvette with a 1-mm path length which is often used in SPS are eliminated.

3.2. Factors influencing the color formation and stability

The different experimental factors influencing the intensity and stability of the developed color were extensively investigated in order to determine the optimal conditions. All optimization studies were conducted using 1 mL of solution containing 600 μg D-penicillamine. It was revealed that 20 mg of resin in its commercially supplied form was sufficient to adsorb the analyte and produce the most intense color (Fig. 5). The use of higher amounts of resin did not further enhance the color intensity, but it increased operational costs.

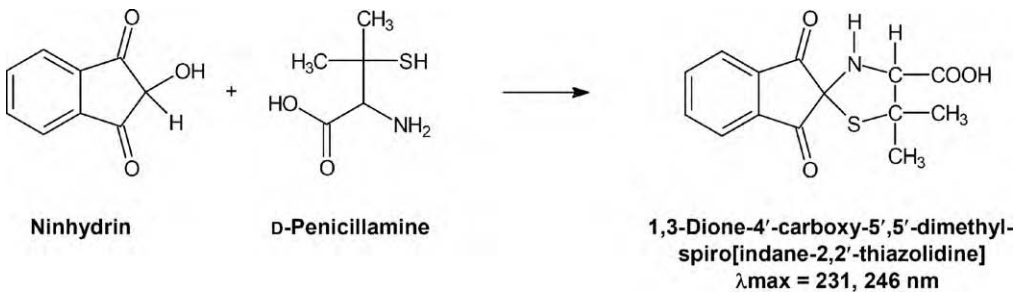

Fig. 3. Spirocyclization of D-penicillamine with ninhydrin to form the spirothiazolidine compound 1,3-dione-4′-carboxy-5′,5′-dimethyl-spiro[indane-2,2′-thiazolidine].

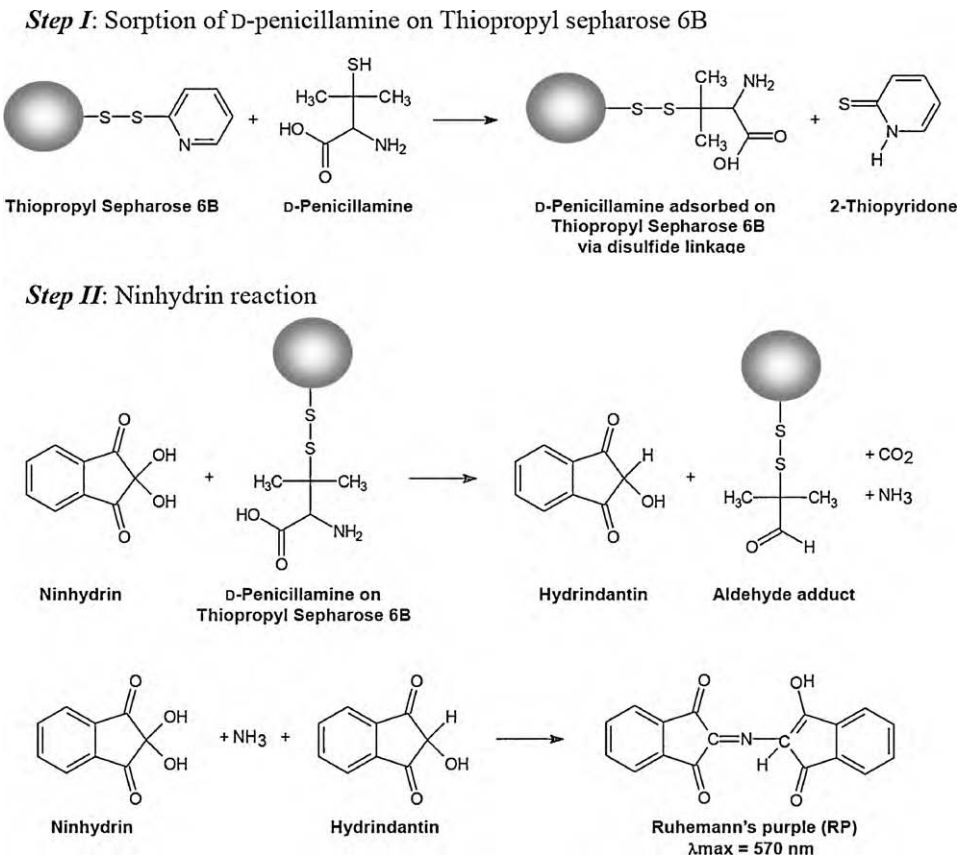

Fig. 4. Colorimetric reaction of D-penicillamine with ninhydrin to form Ruhemann's Purple via the sorption on Thiopropyl sepharose 6B.

The effect of pH was investigated over the range 3.0–9.0. It was found that the optimum pH for the adsorption of D-penicillamine on the resin fell in the pH range above 6.0 (Fig. 6). At acidic pH, D-penicillamine may not have been well fixed on the resin and thus gave lower color intensity in the colorimetric reaction. This observation can be explained by using the thiol-disulfide exchange chemistry in which the protonated thiol form (–SH) is unreactive and cannot attack disulfide bonds. Hence, thiol-disulfide exchange is inhibited at low pH where the protonated thiol form is predominantly present relative to deprotonated thiolate form ($-S^-$) [13]. From this result, the optimal pH chosen was 7.0 to maximize both the color formation and the pH-stability of the resin.

In the adsorption step, mixing time is another factor required for the complete binding between aminothiol and resin. The absorbance was found to be practically independent of the mixing time from 10 min, at which the maximum absorbance was reached, up to 45 min, the highest mixing time tested (Fig. 7). Consequently, it can be said that the adsorption of the analyte on the resin was fast and a mixing time of 10 min was chosen for subsequent experiments.

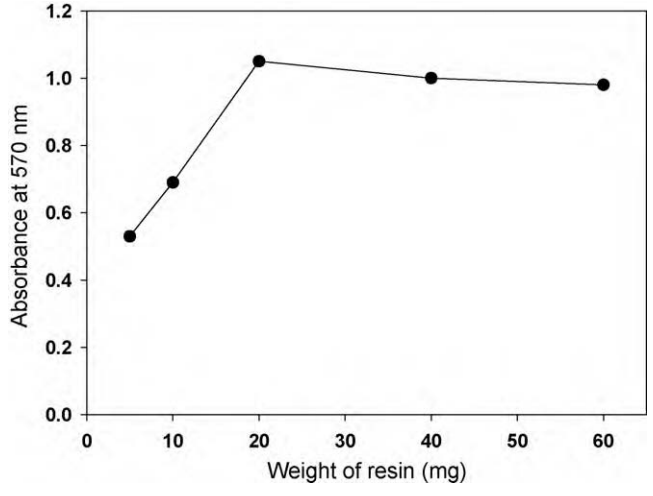

Fig. 5. Influence of the resin amount on the color formation as measured by the absorbance at 570 nm.

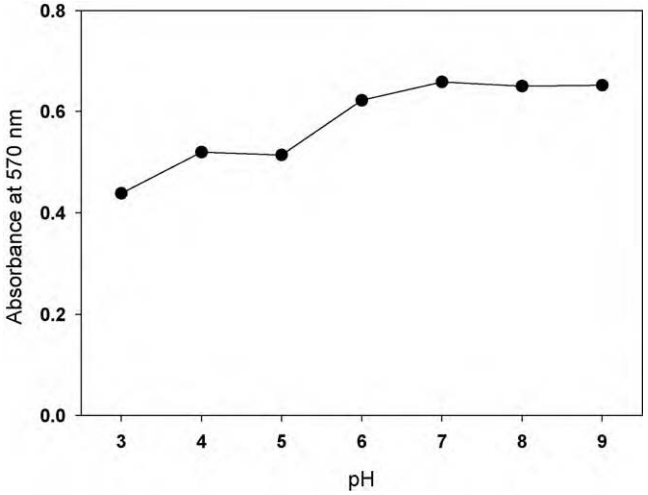

Fig. 6. Influence of the pH of sorption condition on the color formation.

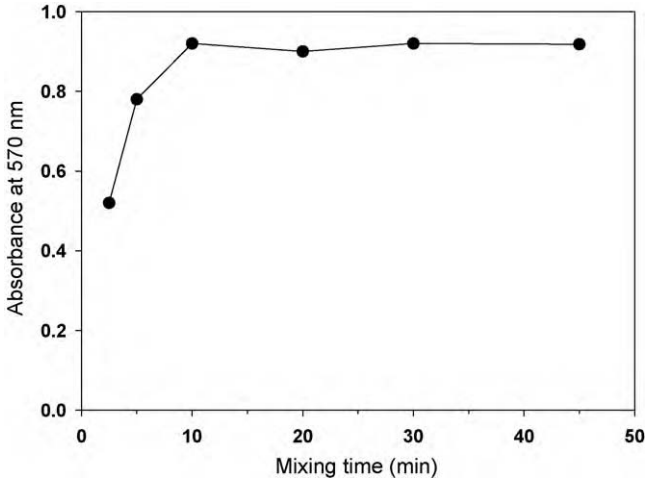

Fig. 7. Influence of the mixing time in the sorption step on the color formation.

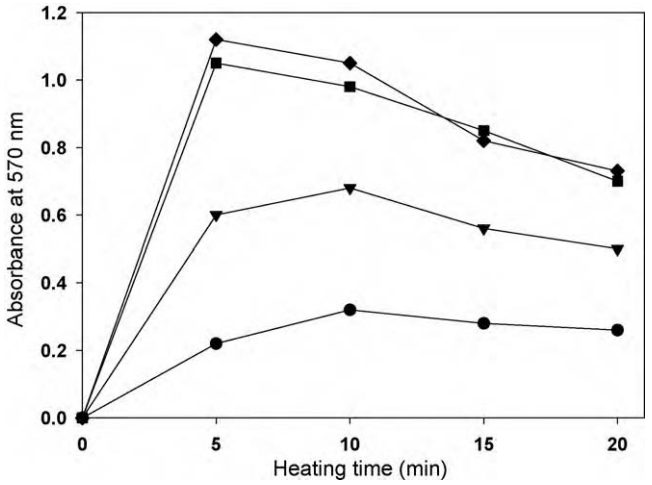

Fig. 8. Influence of the heating time on the color formation when 50 μL (●), 100 μL (▼), 200 μL (■) and 300 μL (♦) of 1% ninhydrin were used.

The concentration of ninhydrin as well as the heating time at 95 °C had a significant effect on the color formation. Different absorbance values were obtained when varied volumes of 1% ninhydrin and heating times were used. It is apparent from Fig. 8 that complete color development was attained after the resin was heated with 200 μL of 1% ninhydrin for 5 min and then remained constant up to approximately 10 min. However, the color of solution then faded if the reaction was heated for a longer time. Therefore, 5 min of heating at 95 °C using 200 μL of 1% ninhydrin was used as the optimal condition for the color development. After the colorimetric reactions, the stability of the formed color was also evaluated. For this purpose, two conditions (at room temperature of 28 °C versus on ice) were used in keeping the colored supernatant prior to the absorbance measurement. It was found that the maintenance of colored solution on ice could effectively stabilize the color so that its absorbance was apparently unchanged over a 1-h period. On the other hand, 30% of the initial absorbance was lost if the solution was left at room temperature over the same time period.

3.3. Analytical performance

From an analytical point of view, the proposed method exhibited not only a satisfactory relationship between color intensity (absorbance at 570 nm) versus concentration over a broader range as well as an enhanced sensitivity as previously described, but also an adequate specificity against disulfide interference. The method was proven to be unaffected by D-penicillamine disulfide, a major degradative impurity commonly found with D-penicillamine. While 300 μg mL^{-1} D-penicillamine disulfide produced a purple color in the ninhydrin reaction and could interfere with the assay of D-penicillamine that was performed by the off-solid method, it did not produce any color when the adsorption step was done beforehand. In addition, 100 μg mL^{-1} D-penicillamine disulfide did not show any interference on the assay when it co-existed with 80 μg mL^{-1} D-penicillamine (data not shown). This improved specificity was undoubtedly due to the adsorption step because only species with free sulfhydryl groups were able to form disulfide bonds with the resin. The interfering disulfides were unbound and washed out before reacting with ninhydrin. Therefore, the selectivity of this approach may be useful for stability-indicating assays.

The proposed approach has been applied for the assay of D-penicillamine in commercially available capsules. The sample solutions were prepared from the capsule contents to have high (300 μg mL^{-1}) and low (80 μg mL^{-1}) concentrations of D-penicillamine. By using a linear standard curve (50–600 μg mL^{-1}) or second-order polynomial standard curve (0-120 μg mL^{-1}), the percent of labeled amounts were 99.92 ± 2.42 and 99.72 ± 1.30%, respectively (n = 6). These were in good agreement with 99.76 ± 0.59% (n = 6), which was assayed by an HPLC method of the United States Pharmacopeia, 2008. It should be noted that while the prepared concentrations of samples analyzed by the proposed method were 0.3 and 0.08 mg mL^{-1}, those prepared for the USP method were of 1.2 mg mL^{-1}.

The applicability of the proposed method to other aminothiols was further demonstrated. By testing with the reduced glutathione and D-cysteine at the concentrations of 100 μg mL^{-1}, the results were similar to those tested with D-penicillamine. While the solutions of these aminothiols could not produce color with ninhydrin, the pre-sorption of these compounds onto the resin could develop the obvious purple color in the subsequent ninhydrin reaction.

Concerns about the cost of using a solid support resin should not be raised in the long run because the used resins can be cleaned by elution to remove the absorbed analyte using a common thiol reducing agent such as β-mercaptoethanol or dithiothreitol. The resin can then be reactivated through the use of a 2,2′-dipyridyl disulfide solution in ethanol or isopropanol. These recycle processes can therefore help lower costs.

4. Conclusion

This is the first report that introduces a novel route to efficiently produce an intense Ruhemann's Purple in the ninhydrin reaction from aminothiols using a D-penicillamine paradigm. The adsorption of the compound via a disulfide bond on the resin prior to the ninhydrin reaction accounted for the color-forming capability of this approach. In addition, it resulted in a broader analytical range as well as enhanced sensitivity and specificity against the disulfide interference after the experimental factors were optimized. The satisfactory analytical performance of this approach proved its potential for the development of colorimetric assays for D-penicillamine and other aminothiol compounds.

References

[1] S. Moore, W.H. Stein, J. Biol. Chem. 176 (1948) 367–388.
[2] M. Friedman, J. Agric. Food Chem. 52 (2004) 385–406.
[3] K.K. Kaistha, W.N. French, J. Pharm. Sci. 57 (1968) 459–464.
[4] N. Rahman, M. Kashif, Il Farmaco. 58 (2003) 1045–1050.
[5] A. Raza, A.S. Ijaz, S. Ahmad, Quím. Nova 32 (2009) 1180–1183.

[6] J.R. Whitaker, Nature 189 (1961) 662–663.
[7] M. Friedman, A.T. Noma, J.R. Wagner, Anal. Biochem. 98 (1979) 293–304.
[8] G. Prota, E. Ponsiglione, Tetrahedron 29 (1973) 4271–4274.
[9] The United States Pharmacopeia, US Pharmacopeial Convention, Rockville, MD, 2008, pp. 2927–2928.
[10] C.T. Pool, J.G. Boyd, J.P. Tam, J. Pept. Res. 63 (2004) 223–234.
[11] S. Sotgia, A. Zinellu, E. Pisanu, G.A. Pinna, L. Deiana, C. Carru, J. Chromatogr. 1205 (2008) 90–93.
[12] R. Bhushan, R. Kumar, J. Chromatogr. 1216 (2009) 3413–3417.
[13] H.F. Gilbert, Methods Enzymol. 251 (1995) 8–30.