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Application of an unusual ninhydrin-based reaction for the indirect chiral resolution of D,L-penicillamine

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ABSTRACT

An unusual reaction involving ninhydrin and aminothiols was exploited to set an indirect method for the chiral recognition of stereoisomers of penicillamine. Separation of diastereoisomers was achieved on a C18 column in isocratic mode by using a mixture of propionic acid (pH 3.0)/acetonitrile/water (10:10:80, v/v/v) as a mobile phase. Diastereoisomers were detected by a fluorescence detector in fairly short times (about 7 min) and with a good resolution. The lowest detectable amount of toxic isomer of penicillamine (L-enantiomer) in samples of the D-enantiomer, was around 0.01%. The method was also suitable for the indirect chiral recognition of other aminothiols such as cysteine and cysteinylglycine.

1. Introduction

Due to the absence of functional groups with fluorescence or strong absorption properties in the UV/vis spectrum, functionalization using a pre- or post-column derivatizing agent is frequently needed for detection [1–4]. Moreover, derivatization may also be useful to enhance the chances of resolution between the isomers. In fact, when pre-column derivatizing reagent is chiral, isomers can be separated indirectly as diastereomers which show quite different physical and chemical properties from one another, allowing an easier separation also on achiral HPLC columns [5]. Indirect methods can then resolve some drawbacks of direct chiral separation methods, such as expenses of the chiral stationary phases (CSPs) or decreasing of the detection limit and restriction of the choice of the detection system produced by chiral mobile phases (CMPs). Thus, derivatization is more than a simple analytical option.

Ninhydrin (2,2-dihydroxyindane-1,3-dione), a well-known post-derivatizing reagent, condenses with primary amino groups to form a deep blue or purple colour soluble chromophore known as Ruhemann's purple (RP) [6,7]. RP dye is the same with all amino acids, therefore, ninhydrin can be commonly used only as a post-column derivatizing reagent. As an exception, amino acids with another nucleophilic group, such as SH-group, besides the α-amino group may react with ninhydrin in a "non classical" way to form exotic products that nevertheless may be of analytical interest [7]. In this light, we have recently developed an original method based on the atypical reaction between thiol-containing amino acids and ninhydrin [1]. Because of the proximity of α-amine and β-thiol nucleophiles, aminothiols such as cysteine, penicillamine, and cysteamine, react with ninhydrin to form spirothiazolidine derivatives which are unique for each amino acid [7,8] and show fluorescent and strong absorption properties in the UV/vis spectrum. Based on this peculiarity, we have previously developed an original approach that for the first time employs ninhydrin as an achiral pre-column derivatizing agent for direct resolution of D,L-penicillamine [1]. Once derivatized, enantiomers were separated as spirothiazolidine adducts on an achiral C18 column by means of chiral ligand-exchange chromatography [9,10]. In the course of our attempts to improve the performance of this method, it was discovered that under certain conditions, ninhydrin can act also as a chiral derivatizing reagent (CDR). To our best knowledge, this feature of ninhydrin is not described yet. In this work, for the first time, this discovery is exploited to set up an original method for the diastereoisomeric reversed high-performance liquid chromatography recognition of stereoisomers penicillamine (Pen, 3,3-dimethylcysteine), a non-coded sulfur-containing amino acid structurally related to the cysteine and principally used as a drug to treat an autosomal recessive disorder of copper transport known as Wilson's disease [11].

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2. Experimental

2.1. Chemicals

Acetonitrile (CH_3CN) HPLC grade and ethanol (EtOH) HPLC grade were obtained from Carlo Erba Reagenti (Milan, Italy). Propionic acid, pure enantiomers (DPen, LPen) and racemate (DLPen) of penicillamine, D-penicillamine disulfide (DPenDPen), L-cysteine (LCys), N-acetyl-L-cysteine, S-methyl-L-cysteine as well as ninhydrin were purchased from Sigma Aldrich Italia (Milan, Italy). High-purity water was obtained by a Milli-Q water purification system (Millipore, Milford, MA, USA).

2.2. Solutions

Racemic and pure enantiomers standard solutions of penicillamine were prepared as 15 mmol/l stock solutions in ultrapure water and stored at −80 °C until use. Fresh working penicillamine standard solutions were prepared by diluting the stock solutions in ultrapure water. L-Cysteine was prepared as 20 mg/ml solution in ultrapure water while ninhydrin was prepared by dissolving a weighed amount in EtOH to form a 2% (w/v) solution.

2.3. Derivatization procedure

Procedure 1. A 200 μl volume of aqueous penicillamine standard solution was mixed with 20 μl of an ethanolic 3% (w/v) ninhydrin solution. After vortex-mixing, 20 μl of an aqueous H_2SO_4 5 mol/l solution were added, then the reaction mixture was left for 5 min at 100 °C in a thermoblock heater [1].

Procedure 2. A 200 μl volume of an aqueous 20 mg/ml L-cysteine solution was mixed with 100 μl of a penicillamine standard solution. After vortex-mixing, 20 μl of the ethanolic 2% (w/v) ninhydrin solution were added, then the reaction mixture was left for 5 min at 100 °C in a thermoblock heater.

2.4. Apparatus and chromatographic conditions

Chromatographic experiments were performed on a Waters (Milford, MA, USA) HPLC system model Alliance 2695 equipped with a Waters 474 fluorescence detector and a Waters 2487 UV/Visible dual-wavelength absorbance detector. The separation was achieved by using a 150 mm × 4.6 mm Waters XBridge C18 5 μm column with a 4.6 mm × 20 mm guard column cartridge. The mobile phase consisted of a mixture of propionic acid (pH 3.0)/CH_3CN/water (10:10:80, v/v/v) filtered through a disposable 0.22-μm filter (Millipore, Milford, MA, USA) to remove any particulate matter prior to its use. The eluent was delivered to the column isocratically at a flow-rate of 1.8 ml min^{-1} and separation was carried out at room temperature (about 23–24 °C). Samples were held at room temperature in the autosampler and the amount injected was 5 μl. Column eluates were detected by fluorescence detector with the gain set at ×1000 scale expansion and excitation and emission wavelengths set at 380 and 460 nm, respectively.

3. Results and discussion

The new feature of ninhydrin was discovered by comparing the chromatograms obtained after derivatization by the procedure described in our previous paper [1] of racemate and pure enantiomers solution of penicillamine. As can be seen in Fig. 1, pure enantiomers of DPen and LPen gave only one peak while, unexpectedly, an additional peak was, instead, obtained when racemate solution was running. Taking into account the achiral chromatographic environment as well as the achirality of ninhydrin, immediately, we discarded the idea that it could be due to the

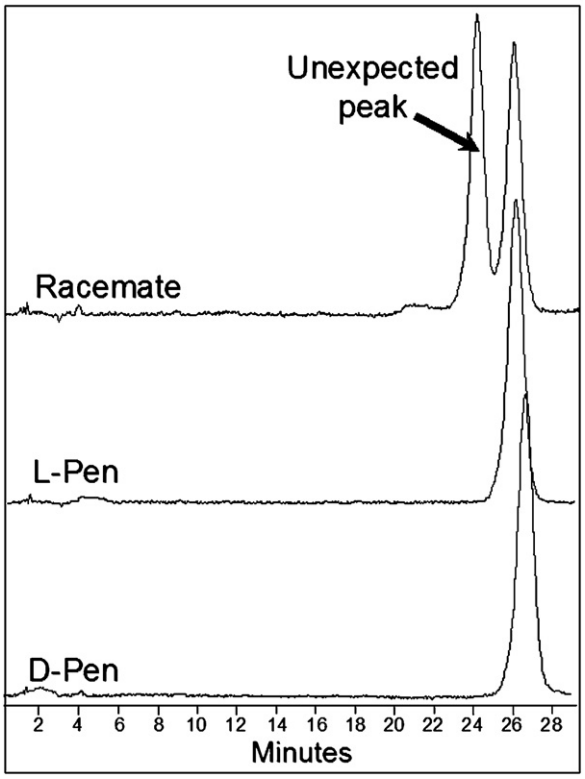

Fig. 1. Chromatographic elution profiles of racemate and pure isomers of penicillamine after derivatization with ninhydrin without addition of cysteine, as described in Section 2 (Procedure 1) and previously reported by Sotgia et al. [1].

separation of the two enantiomers of penicillamine as diastereoisomers. On the other hand, as shown in Fig. 1b and c, when pure enantiomers were derivatized individually, their retention time was the same. To explain this behaviour, we then considered the possibility that unexpected peak could be due to the formation of the mixed disulfides between DPen and LPen (DPenLPen) due to the spontaneous oxidation of the enantiomers in the racemate solution or as a result of the reaction with ninhydrin. This could be plausible in the view of the oxidizing properties of ninhydrin. However, we had some indications against this hypothesis. First of all, any attempt to increase the formation rate of disulfide, e.g., by copper and H_2O_2 addition, did not produce any kind of effect. Moreover, by proposed derivatization procedure, a solution of disulfide DPenDPen, which is commercially available, does not undergo derivatization. However, these elements were not decisive to exclude that unexpected peak could be due to the derivatization of the mixed disulfide DPenLPen and we are currently trying to characterize the adduct. Regardless from the mechanism of reaction and from the real nature of the unexpected peak, it seemed obvious that it was due to a complex that contained both isomers of penicillamine (DPen∼LPen). Starting from this evidence, we conjectured that it was possible to exploit this unknown reaction for the chiral recognition of stereoisomers of penicillamine. For this purpose, we hypothesized that the addition of another chiral aminothiol, such as D- or L-cysteine, could serve as a chiral selector by the formation of an adduct containing, depending on the cysteine isomer employed, D- or L-cysteine and one or the other of the isomer of the penicillamine. As shown in Fig. 2, the hypothesis was correct, in fact, by adding L- or D-cysteine, stereoisomers of penicillamine were easily separated in less than 7 min on an achiral C18 column by using a mixture of propionic acid (pH 3.0)/CH_3CN/water (10:10:80, v/v/v) as a mobile phase. Compared to our earlier paper, derivatization conditions were slightly

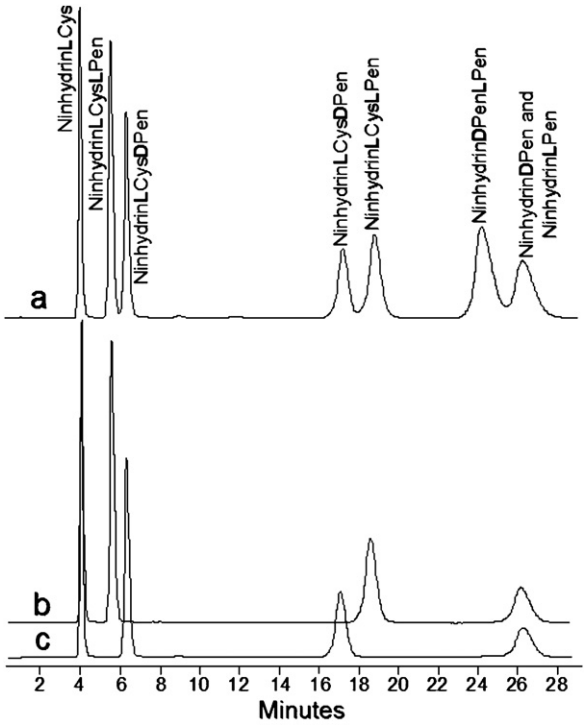

Fig. 2. Chromatographic elution profiles of (a) racemate and pure (b) LPen and (c) DPen solutions of penicillamine after derivatization with ninhydrin and L-cysteine as described in Section 2 (Procedure 2).

modified and, in order to maximize sensitivity, the most suitable combination ratio between reagents were found to use the 10:5:1 LCys:DLPen:ninhydrin. Because of the two chiral centres of the adduct, on the bases of the general rule whereby if a structure has n stereogenic centres it will have potentially 2^n stereoisomers, four peaks might be expected from an enantiomeric mix of DPen or LPen with LCys (or DCys). However, although another series of diastereoisomers were eluted around 16–20 min, it is more likely that this additional pair of peaks are due to structural isomerism. Curiously, in this series of diastereoisomers, elution order of DPen and LPen isomers were reversed with respect to the first series of peaks. Elution order was also reversed when DCys was employed instead of LCys. As suspected, only the addition of aminothiols able to form spirothiazolidine adducts with ninhydrin, such as D or L isomer of cysteine or cysteinylglycine, were useful for the chiral recognition of DLPen, thus reinforcing the idea that the adduct is not due to the derivatization of a disulfide. Moreover, the failure of N-acetyl-L-cysteine and of S-methyl-L-cysteine, respectively, with amino function and thiol group protected, highlights that both these groups are necessary for the reaction. Column eluates were monitored by fluorescence detector at an excitation wavelength of 380 nm and emission wavelength of 460 nm. At the same concentration, a higher derivatization yield was observed for the L-isomer compared to D. This would suggest that the reaction between ninhydrin and LCys with DPen is more difficult and thus slower. However, under the selected conditions both linear responses for each isomer was obtained and no significant differences in the signals were observed when the D-isomer was contained in the sample as a raceme or as a single isomer. Under the described experimental conditions, the calibration curve of a duplicate set of five non-zero calibration standards, ranging from 0.1 to 0.9 mM, constructed by plotting peak correct area (area/migration time) against the corresponding LPen or DPen concentration, showed a good coefficient of determination ($R \geq 0.99$) ensuring a linear response over the concentrations tested. The diastereoisomer adducts were quite stable at room temperature and the intra- and inter-assay reproducibility expressed as relative standard deviation (RSD%) of triplicates of a racemate solution of penicillamine were under 5%. The lowest detectable amount of toxic isomer of penicillamine (L-enantiomer) [12] in samples of the D-enantiomer, evaluated by a signal-to-noise ratio of 3:1, was around 0.01%.

4. Conclusion

To our best knowledge, for the first time, a new reaction of the aminothiols with ninhydrin was described. Although the mechanism of reaction must be clarified, this discovery was successfully applied to set up an original method for the indirect chiral recognition of the stereoisomers of penicillamine. After pre-column chiral derivatization, stereoisomers were easily detected at high sensitivity by a fluorescence detector within fairly short times on an achiral C18 column. Moreover from some tests, resulted that by suitably modifying the method, it is also possible to separate the isomers of other aminothiols such as cysteine and cysteinylglycine. On the whole, developed method was original, easy, fast, and provides a sensitivity comparable to other assays.

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