Introduction

Penicillamine (PEN) is a sulfur amino acid with chelating properties. Since 1956 it has been introduced for the treatment of Wilson’s disease, and subsequently it found use in the management of cystinuria and rheumatoid arthritis.\(^1\) PEN’s therapeutic effectiveness has been investigated and well-documented.\(^2\)–\(^6\)

Several analytical methods have been developed for PEN determination in pharmaceutical preparations, including spectrophotometry,\(^7\)–\(^9\) voltammetry,\(^10\) potentiometry,\(^11\) amperometry,\(^12\) fluorometry,\(^13\)\(^,\)\(^14\) capillary electrophoresis,\(^15\) and chromatography.\(^16\)\(^,\)\(^17\) There are several flow systems using a spectrophotometric detector\(^18\)–\(^21\) for the determination of PEN, among them, only one is a SIA method based on complex formation between Fe(III) ion and PEN.\(^20\)

The method presented in this work is based on the reaction of a formation complex between nickel ion and PEN. One batch procedure based on the same reaction was previously reported.\(^9\) However, in this work the developed method has been presented, and the analytical applicability of the reaction of complexation was enhanced with particular emphasis on all advantages arising from the SIA system: simplicity of procedure, sensitivity, reliability and excellent sample throughput. In addition, the developed method allows the determination of PEN in the concentration range 3.0 \(\times\) \(10^{-6}\) – 2.0 \(\times\) \(10^{-4}\) mol L\(^{-1}\), while 8.9 \(\times\) \(10^{-7}\) mol L\(^{-1}\) was the limit of detection.

Experimental

Reagents and chemicals

All solutions were prepared using analytical-reagent grade substances and Milli-Q deionized water.

A stock solution of D-penicillamine (PEN), 1.0 \(\times\) \(10^{-2}\) mol L\(^{-1}\), was prepared by dissolving 37.3 mg of PEN (Sigma-Aldrich, Munich, Germany) in deionized water up to 25.0 mL volume.

A stock solution of Ni(II), 1.0 \(\times\) \(10^{-1}\) mol L\(^{-1}\), was prepared by dissolving 1.3143 g of NiSO\(_4\)·6H\(_2\)O (Fluka, Buch, Switzerland) in deionized water up to 50.0 mL volume. The stock solutions were stored in refrigerator, and were stable for seven days.

Working solutions were prepared daily by appropriate dilution of the stock solutions with borate buffer, pH = 8.0.

Borate buffer, pH = 8.0, was prepared by mixing 138.5 mL of 0.1 mol L\(^{-1}\) solutions of H\(_3\)BO\(_3\) and NaOH with 223.0 mL of 0.1 mol L\(^{-1}\) mol L\(^{-1}\) HCl and diluting to 1000.0 mL with deionized water. All chemicals for preparations of this buffer were purchased from Kemika (Zagreb, Croatia). The final pH was adjusted by adding the following solutions: 0.5 mol L\(^{-1}\) HCl or 0.5 mol L\(^{-1}\) NaOH. Also, by mixing appropriate volumes of the same solutions (H\(_3\)BO\(_3\), NaOH, HCl) we prepared borate buffers used throughout the optimization (pH \(=\) 6.0 – 8.5).

Metalcaptase (Heyl) and Artamin 250 (Sandoz) were commercial available pharmaceutical samples. The same procedure for preparation of stock solutions was carried out for all pharmaceutical samples. Twenty tablets were accurately weighed and smashed. The quantity of their contents was dissolved in an adequate volume of deionized water and filtered. Then, it was transferred to a 100.0-mL volumetric flask, and diluted to a nominal volume with deionized water. Solutions of pharmaceuticals with a final concentration of PEN suitable for analysis were prepared by additional dilution of stock solutions in a borate buffer, pH = 8.0.

Notes

A simple and sensitive spectrophotometric method, based on reaction between Ni(II) ion and D-penicillamine (PEN), was developed. The proposed SIA system enhanced the analytical applicability of the reaction of complexation, and allowed the determination of PEN in the concentration range of 3.0 \(\times\) \(10^{-6}\) – 2.0 \(\times\) \(10^{-4}\) mol L\(^{-1}\) with a sampling rate of 200 h\(^{-1}\).

With the proposed SIA system, PEN could be accurately determined up to 0.9 nmol quantity. The method was successfully applied to the determination of PEN in laboratory samples and pharmaceuticals.

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from a syringe pump M6-Pump VICI (Valco Intruments, Houston, TX), a 10-port electrically actuated selection valve C25-3180EMH Cheminert (Valco Instruments, TX), and a Shimadzu UV mini-1240 (Shimadzu, Kyoto, Japan) UV-Vis spectrophotometer equipped with a flow cell (Hellma, Müllheim, Germany) of 80 μL internal volume and 10 mm optical path. Spectrophotometric data acquisition and control of the measurement were achieved by coupling a detector with a personal computer, and using UVmini-1240 data management software and a plug-in memory card with kinetics program, both from Shimadzu (Shimadzu, Japan). M6-LHS-M6 Liquid Handling Software (Valco Instruments, Houston, TX) has been used for writing sequences of analysis and for the pump and the valve control.

**Recommended procedure**

Before beginning of the analysis, all reagents and samples/standards must be aspirated in a holding coil (HC), and washed from a HC dispensing carrier with flow reversal. This is necessary for filling all flow lines.

The analysis was performed following sequences that had been written using the software (mentioned above) M6-LHS. The steps of analysis are summarized as follows:

1. The valve position 1: the syringe pump aspirated 50 μL of 5.0 × 10⁻³ mol L⁻¹ Ni(II) solutions into the holding coil with flow rate of 7.0 mL min⁻¹.
2. The valve position 2: the syringe pump aspirated 300 μL of PEN sample/standard solution into the holding coil with flow rate of 7.0 mL min⁻¹.
3. The valve position 10 (detector line): the syringe pump dispensed 1.5 mL of carrier solution with flow reversal (5.0 mL min⁻¹) for pushing the stack of zones from the holding coil into the reaction coil and then to the detector.

The absorbance of the formed complex is then monitored at λ = 270 nm against a borate buffer (pH = 8.0). The first sequence ended with step 3. For following sequences valve position 2 was replaced by valve position 3 to 9 for different solutions of the sample or standard, as required. The time needed to complete one cycle was 18 s.

The holding coil and the reaction coil (both spirally wrapped around solid base, φ = 9.0 mm) were 100.0 and 50.0 cm in length, respectively.

**Results and Discussion**

**Optimization of the reaction condition**

Preliminary batch experiments indicated that the complexation reaction of Ni(II) with PEN is pH dependent, and the optimum reaction pH can be expected at pH = 8.0. For a preliminary study, the absorption spectra in the range from 240 to 900 nm for solutions of PEN (1.0 × 10⁻⁴ mol L⁻¹), Ni(II) (1.0 × 10⁻⁴ mol L⁻¹), and mixtures of the same concentration PEN and Ni(II) were measured. Furthermore, the spectra were measured at pH values for the range of 1.0 – 10.0. The results, obtained under these experimental conditions, confirmed that only the complex between Ni(II) and PEN had the absorption maximum at λ = 270 nm.

The influence of the concentration of Ni(II), under the flow condition, was studied concerning the determination of 8.0 × 10⁻³ mol L⁻¹ and 8.0 × 10⁻⁴ mol L⁻¹ PEN. The tested concentrations of Ni(II) were in the range from 1.0 × 10⁻² to 1.0 × 10⁻⁴ mol L⁻¹. A concentration of 5.0 × 10⁻³ mol L⁻¹ Ni(II) provided the best sensitivity, and therefore was chosen as the optimal one.

The effect of the pH on the determination of 1.0 × 10⁻⁴ mol L⁻¹ PEN was also studied under the flow condition over a pH range of 5.0 – 10.0. The absorbance increased with increasing pH up to 8.0, and the precipitation of nickel hydroxide occurred at pH = 10.0. A borate buffer, pH = 8.0, was chosen both as a reaction medium and a carrier solution.

**Optimization of the SIA system**

The optimization of variables that significantly affect the SIA system included: the injection volumes, carrier flow-rate, holding coil and reaction coil lengths.

The influence of the injection volumes on the sensitivity of the proposed method was studied for the determination of 1.0 × 10⁻⁴ and 1.0 × 10⁻⁵ mol L⁻¹ PEN. These optimization experiments were performed by using the previously optimized concentration of Ni(II), 5.0 × 10⁻⁴ mol L⁻¹. The tested injection volumes were over the ranges of 20 – 300 μL for Ni(II) and 100 – 700 μL for PEN. The volumes selected as V (PEN) = 300 μL and V (Ni(II)) = 50 μL, gave the highest absorbance. Also, a somewhat higher absorbance was obtained upon the injection of a PEN solution after a solution of Ni(II). This order was selected for all further experiments.

The effects of the flow rates of the carrier were examined in the range from 1.0 to 9.0 mL min⁻¹. The analytical signal was increased by increasing the flow rate up to 5.0 mL min⁻¹. At higher flow rates the peak absorbance started to decrease, probably due to shorter residence times.

Also, the peak width decreased by upon increasing the flow rate. Therefore, a 5.0 mL min⁻¹ flow rate was selected as the optimum.

The length of the holding coil was tested over the range of 50.0 – 200.0 cm. The holding coil with a length of 100.0 cm gave the optimal result, implying: accommodation of the stacks of the sample and the reagent zone, initial dispersion, stable base line due to an uncontaminated carrier.

The reaction coil length was optimized, ranging from 25 to 90 cm. The formation of complex is practically instantaneous, and higher peaks were obtained with a shorter coil length of 50.0 cm, due to less dispersion. A longer reaction coil increased the residence time of the complex, while increasing the dispersion and decreasing the peak heights.

The inner volume of the carrier, V = 1.5 mL, was selected for the proposed SIA system. At a flow rate of 5.0 mL min⁻¹, the system enabled the measurement of one sequence within 18.0 s. Thus, the frequency of the sample measurement was 200 per hour.

**Interference studies**

The effect of some interferences was tested by analyzing solutions containing 8.0 × 10⁻⁴ mol L⁻¹ of PEN and various concentrations of the interfering substances. The tolerable molar ratio (interference/PEN) caused an error in the signal of less than ± 3%.

The tolerable molar ratio of lactose, sucrose, glucose, fructose, Na⁺, K⁺, Cl⁻ was 1000:1, and the tolerable molar ratio of Zn²⁺, SO₄²⁻, Ca²⁺, Mg²⁺ was 500:1.

The significant interferences were cysteine (CIS) and Cu(II) at very low concentrations (< 8.0 × 10⁻⁶ mol L⁻¹). This was expected due to possible reactions of complexation of CIS with Ni(II) and PEN with Cu(II). However, as far as we know, CIS and PEN are not present simultaneously in pharmaceutical formulations.

**Analytical application**

Under the optimized conditions previously given, a linear
Table 1: Results for the determination of PEN in real samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Labeled/mg</th>
<th>Found/mg</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>METALCAPTASE® 300°</td>
<td>300.0</td>
<td>296.7 ± 0.2</td>
<td>98.9</td>
</tr>
<tr>
<td>METALCAPTASE® 150°</td>
<td>150.0</td>
<td>148.8 ± 0.2</td>
<td>99.2</td>
</tr>
<tr>
<td>ARTAMIN 250°</td>
<td>250.0</td>
<td>247.7 ± 0.4</td>
<td>99.1</td>
</tr>
</tbody>
</table>

a. Other labeled ingredients: copolyvidon, talcum, titani dioxide, polysorbat 80, calciumbehain, dimeticon, macrogol 6000, methacrylsaure-copolymer.
b. Other labeled ingredients: magnesiumstearat, titani dioxide, gelatin, ferrousoxid, schellack.
c. Average of five determinations ± SD, %.

Table 2: Results for the determination of PEN in solutions of the pharmaceutical with the known addition of the standard

<table>
<thead>
<tr>
<th>Sample</th>
<th>Taken/ mg</th>
<th>Added/ mg</th>
<th>Found/mg</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>METALCAPTASE® 300°</td>
<td>4.0</td>
<td>10.0</td>
<td>13.8 ± 0.8</td>
<td>98.3</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>53.3 ± 0.4</td>
<td>98.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>103.0 ± 0.5</td>
<td>99.1</td>
<td></td>
</tr>
<tr>
<td>METALCAPTASE® 150°</td>
<td>4.0</td>
<td>10.0</td>
<td>13.8 ± 1.0</td>
<td>98.7</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>53.4 ± 0.6</td>
<td>98.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>102.6 ± 0.8</td>
<td>98.6</td>
<td></td>
</tr>
<tr>
<td>ARTAMIN 250°</td>
<td>4.0</td>
<td>10.0</td>
<td>13.8 ± 0.9</td>
<td>98.6</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>53.4 ± 0.7</td>
<td>98.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>102.9 ± 1.2</td>
<td>98.9</td>
<td></td>
</tr>
</tbody>
</table>

a. Average of three determinations ± SD, %.

calibration graph in the concentration range over \(3.0 \times 10^{-6} - 2.0 \times 10^{-4}\) mol L\(^{-1}\) was obtained. The corresponding regression calibration equation was: \(A = 4346c + 0.013\) (\(r^2 = 0.999\)), where \(A\) is the absorbance at 270 nm and \(c\) is the penicillamine concentration (mol L\(^{-1}\)).

The calculated limit of detection, LOD, \((3\sigma/m)\) was \(8.9 \times 10^{-7}\) mol L\(^{-1}\). In addition, with this method PEN could be accurately determined up to 0.9 nmol quantity (300 μL-injection volume \(\times 3.0 \times 10^{-4}\) mol L\(^{-1}\), the lowest concentration of linear dynamic range, LDR).

The proposed sequential injection system was applied under the optimized conditions for the determination of PEN in pharmaceutical preparations. The results of this analysis presented in Table 1 indicate the successful applicability of the proposed method in the analysis of real samples. Also, the accuracy of the method was checked by carrying out recovery studies for each sample by adding the standard of the compounds to the sample (Table 2).

Conclusions

The main advantages of proposed method over the previously reported flow spectrophotometric methods of analysis, based on the reaction of complex formation, are: a wider linear dynamic range, lower LOD, higher sensitivity and sample frequency.

The developed SIA procedure enables the determination up to 0.9 nmol of the PEN with a sample throughput of 200 h\(^{-1}\). The proposed method could, with good precision and accuracy, be applied for the routine analysis of pharmaceutical in quality-control laboratories.

References