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Application of the desulfurization of phenothiazines for a sensitive detection method by high-performance liquid chromatography

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Abstract

A simple and sensitive high-performance liquid chromatographic (HPLC) method for the determination of phenothiazine (PHE) is described. PHE is converted to diphenylamine (DIP) by desulfurization with Raney nickel catalyst. DIP is highly sensitive to electrochemical detection. The calibration graph for PHE quantification after desulfurization was linear between 0.1 and 2.0 ng per injection. The detection limit (signal-to-noise ratio = 3) of PHE after desulfurization was 10 pg, which is twenty times higher than that of the parent compound PHE. The proposed desulfurization technique was applied to other PHE-related compounds. The structural confirmation of the desulfurized product of PHE was carried out by LC–MS using atmospheric pressure chemical ionization.

1. Introduction

Many compounds of pharmaceutical interest, such as aliphatic amines, carboxylic acids, alcohols, or thioethers, are difficult to detect with high sensitivity by measurement of their optical absorbance, fluorescence, or electrochemical responses. Precolumn or postcolumn derivatization procedures often present an inexpensive and effective approach to overcome this problem. Derivatization reaction are extensively used for HPLC analysis of many pharmaceuticals.

Raney nickel catalyst is available for the hydrogenation of organic compounds. The activity of the catalyst depends on the preparation of the catalyst from Raney nickel–aluminium alloy. Additional hydrogen is not required, since a large quantity is adsorbed on, or dissolved in, the W-2 catalyst [1]. The authors have prepared tritium-labeled dethiobiotin by desulfurization of biotin with Raney nickel W-2 [2].

Diphenylamine, a redox indicator, is oxidized to the colorless diphenylbenzidine. The first step of the oxidation is non-reversible; the second step, however, giving a highly colored (violet) oxidized form, diphenylbenzidine violet, constitutes the actual indicator reaction.

Phenothiazine (PHE), also known as thiodiphenylamine, could be converted to diphenylamine (DIP) by treatment with Raney nickel as a reductive desulfurization catalyst.

By introducing substituents into the original

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PHE ring, several medicinal drugs or pigments can be prepared.

Antipsychotic drugs, promethazine, chlorpromazine, and others, can be obtained by introduction of functional groups at the 10 position, or the 2 and 10 positions in the PHE ring. The introduction of a substituent dimethylamino group at C-3 and C-7 positions in the PHE ring forms pigments, such as methyleneblue. Thus, PHE is important in the formation of major chemical medicines or products.

The authors have previously reported a sensitive method for the determination of DIP compounds, mefenamic and flufenamic acids, in serum by HPLC with electrochemical detection (ED) [3].

The HPLC methods used for PHE analogs include ultraviolet absorption [4,5], fluorometric [4,6] or electrochemical [4,7,8] detection systems. This paper describes the reductive desulfurization of PHE and the applicability of DIP for HPLC-ED, and is also concerned with the structural confirmation of the desulfurized products of PHE analogs using LC–atmospheric pressure chemical ionization (APCI) mass spectrometry (MS).

2. Experimental

2.1. Chemicals and stock solutions

Phenothiazine (PHE) and 1-naphthol (NAP, analytical reagent grade) were obtained from Wako Pure Chemicals (Osaka, Japan); both were recrystallized from toluene and water, respectively. Raney nickel was also obtained from Wako Pure Chemicals, and activated by the procedure of Mozingo et al. [9]. DIP and 2-methoxyphenothiazine were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). HPLC-grade methanol was purchased from Kanto Chemical (Tokyo, Japan). All other chemicals were of analytical reagent grade.

Water was purified with a Millipore Q system (Waters, Eshborn, Germany) after deionizing and distillation. Original stock solutions of PHE, DIP and NAP were prepared by dissolving each compound in methanol. Working solutions were obtained by diluting stock solutions to appropriate concentrations with methanol prior to use.

2.2. HPLC analysis

The HPLC system used in this work consisted of a Hitachi L-6000 pump equipped with a Shodex EC-1 amperometric detector with glassy carbon working electrode and Ag/AgCl reference electrode (Showa Denko, Tokyo, Japan), and a Shimadzu C-R6A integrator (Shimadzu, Kyoto, Japan). The applied potential was set at 0.8–1.05 V. A Shim-pack CLC-ODS column (15 cm × 4.6 mm I.D., Shimadzu) with 5-μm particle size was used. The column temperature was maintained at 35°C in a column oven. The detector was set at 2.5–40 nA full scale.

The optimum applied potential for DIP and NAP was examined over the range of 0.7–1.3 and 0.65–1.2 V, respectively.

The mobile phase consisted of a mixture of 1000 ml of 75% methanol containing 0.05 *M* sodium perchlorate and 5.0 ml of glacial acetic acid. The mobile phase was filtered through a 0.4-μm membrane filter prior to use. The flow-rate of the mobile phase was 0.6 ml/min. The PHE concentrations in the sample solutions were measured by the peak-height method for chromatograms obtained after desulfurization of PHE.

2.3. Desulfurization procedure

To 100 μl (5–100 ng) of methanolic solutions of PHE or its analogs, 20 μl of 5% sodium carbonate and ca. 35 mg (80 μl) of ethanolic Raney nickel (W-2) suspensions were added, and the resulting solution was mixed well. The mixtures were heated at 70°C for 15 min with stirring. Then, the reaction mixtures were cooled with ice-water. To the reaction mixture, 50 μl of NAP (20 ng) was added, as an internal standard, and mixed well. The mixtures were passed through a membrane filter (0.45 μm), and 5 μl of the filtrate were injected onto the HPLC system.

2.4. LC–APCI-MS

The desulfurized samples were injected onto the HPLC system, which was interfaced with a Hitachi M-1000 quadrupole mass spectrometer via an APCI system. The chromatographic separation was performed using a Shim-pack CLC-ODS, 15 cm × 4.6 mm I.D. column (5-μm particle size). The mobile phase was a mixture of 1000 ml of 75% methanol and 5 ml of glacial acetic acid. The flow-rate was 1.0 ml/min. The injection volume was 10 μl. In the negative-ion mode, the drift voltage of the mass spectrometer was set at 20 V, and the temperatures of the vaporizer and desolvator were 200 and 399°C, respectively. The multiplier voltage was 1800 V. The mass chromatograms were obtained in the cyclic scan mode.

3. Results and discussion

3.1. Optimal applied potential and its measurement

Fig. 1 shows the electrochemical characteristics (oxidation potentials) of DIP and NAP (internal standard) for HPLC measurement of PHE. The optimal applied potentials for electrochemical detection of the analytes were examined over the range of 0.7–1.3 and 0.65–1.2 V, respectively, by injecting 3.0 ng of DIP (50 nA full scale) and 0.5 ng of NAP (10 nA full scale), respectively. The maximum response for the measurement of DIP and NAP was obtained at a potential higher than 1.05 V. The voltamperograms verify that the phenolic group (NAP) is more readily oxidized than the aromatic amines (DIP) at the applied potentials.

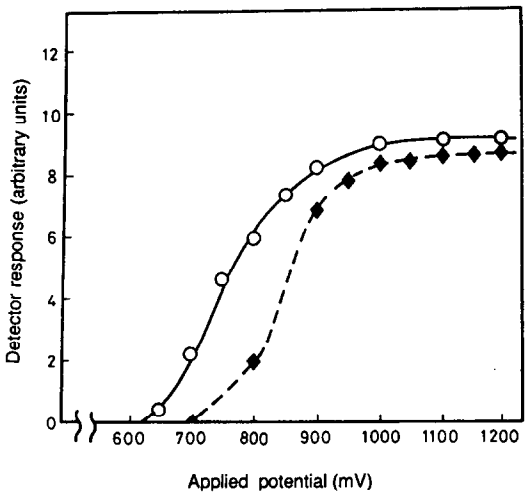

Fig. 1. Voltamperograms of 1-naphthol and diphenylamine on a glassy carbon electrode (injected amount: 0.5 ng per 5 μl for diphenylamine (DIP) and 0.3 ng per 3 μl for 1-naphthol (NAP), respectively). (◆) Diphenylamine (DIP), (○) 1-naphthol (NAP).

3.2. HPLC and internal standard

Several chromatographic conditions were evaluated to optimize the separation of DIP, NAP and PHE. Optimum separations of these analytes were achieved using the previously described mobile phase and a Shim-pack CLC-ODS column with a flow-rate of 0.6 ml/min, as shown in Fig. 2.

3.3. Desulfurization conditions for PHE

Fig. 3 shows the effect of the reaction time on the desulfurization of PHE. The peak height of PHE was significantly decreased at a reaction time of 5 min. In contrast, the peak height of DIP was increased. Desulfurization conditions of PHE were examined with regards to reaction temperatures (25–90°C, at 5°C intervals), reaction periods (0–25 min, at 2.5–5 min intervals), and volumes of Raney nickel (W-2) suspensions (10–100 μl, in 10–20 μl increments). Consequently, the optimal reaction conditions were chosen as follows; to 100 μl of PHE solution (5–100 ng), 20 μl of 5% sodium carbonate, 80 μl of Raney nickel (W-2, ca. 35 mg) suspension were added, and the mixture was shaken well. A heating period of 15 min proved to be sufficient for complete desulfurization of PHE.

As the desulfurization reaction proceeded, peak heights of DIP increased, while that of PHE decreased or disappeared (Fig. 4). When PHE was desulfurized for a period of 2.5 min, the PHE peak still appeared clearly on the chromatograms at t_R of 14.5 min, while after a

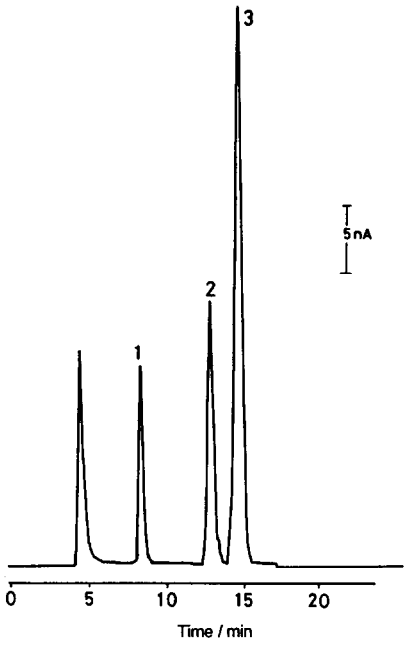

Fig. 2. Chromatograms of diphenylamine, 1-naphthol and phenothiazine. Peaks: 1 = 1-naphthol (NAP); 2 = diphenylamine (DIP); 3 = phenothiazine (PHE). The chromatographic conditions were as follows: mobile phase: 75% methanol containing 0.05 *M* sodium perchlorate–acetic acid (1000:5, v/v); column: Shim-pack CLC-ODS (15 cm × 6.0 mm I.D.); flow-rate: 0.6 ml/min; applied potential: 800 mV, glassy carbon electrode (Ag/AgCl).

reaction period of 15 min, the PHE peak had disappeared from the chromatogram, while the DIP peak height had increased. The completion

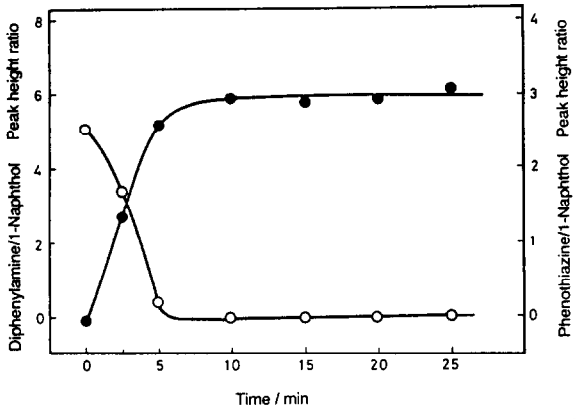

Fig. 3. Effect of reaction time on the desulfurization of phenothiazine (PHE). Reaction temperature: 70°C. (●) Diphenylamine (DIP), (○) phenothiazaine (PHE).

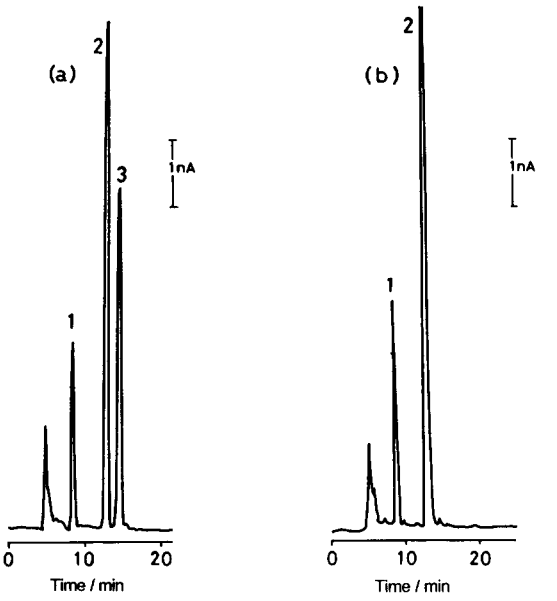

Fig. 4. Chromatographic monitoring of the disappearance of phenothiazine (PHE) with the desulfurization reaction. (a) Reaction period of 2.5 min, (b) reaction period of 15 min. Peaks: 1 = 1-naphthol (NAP), 2 = diphenylamine (DIP), 3 = phenothiazine (PHE).

of PHE desulfurization can be recognized by disappearance of the PHE peak.

3.4. Calibration curve of PHE

PHE concentrations were obtained from the calibration curve of DIP derived from the desulfurization of PHE. The calibration curve for PHE was constructed by plotting the peak-height ratio of PHE against the internal standard. It is clear that the dependence of the peak-height ratio on DIP concentration was linear in the range of 10–5000 pg per injection ($y = 2.8031 \cdot 10^{-3}x + 0.0692$, $r = 1.0000$). The calibration curve for PHE combined with the desulfurization of PHE was also linear over the range of 0.1–2.0 ng per injection ($y = 1.0722x + 0.0247$, $r = 0.9987$).

The detection limits of PHE and DIP at a signal-to-noise ratio of 3 are 10 and 0.5 pg per injection, respectively, and were twenty times higher than that of intact PHE. Jane et al. reported that the approximate detection limit of

PHE sulfur, under electrochemical oxidation conditions, was 0.1 ng [4].

3.5. LC–MS

Mass spectrometry provided powerful evidence in the structural confirmation of the product which was derived from PHE by Raney nickel desulfurization. Fig. 5 shows the mass chromatogram of a mixture of DIP (M.W. 169) and PHE (M.W. 199). As the desulfurization reaction proceeds, the peak at m/z 200 clearly diminishes and the peak at m/z 170 increases.

3.6. Application of the desulfurization to PHE-related compounds

The application of the method for PHE-related compounds was investigated (2-methoxyphenothiazine, prochloroperazine, trifluoroperazine, pericyazine, promazine, levomeprazine, prochlorperazine and trifluoroperazine). The reaction product, treated by the same pro-

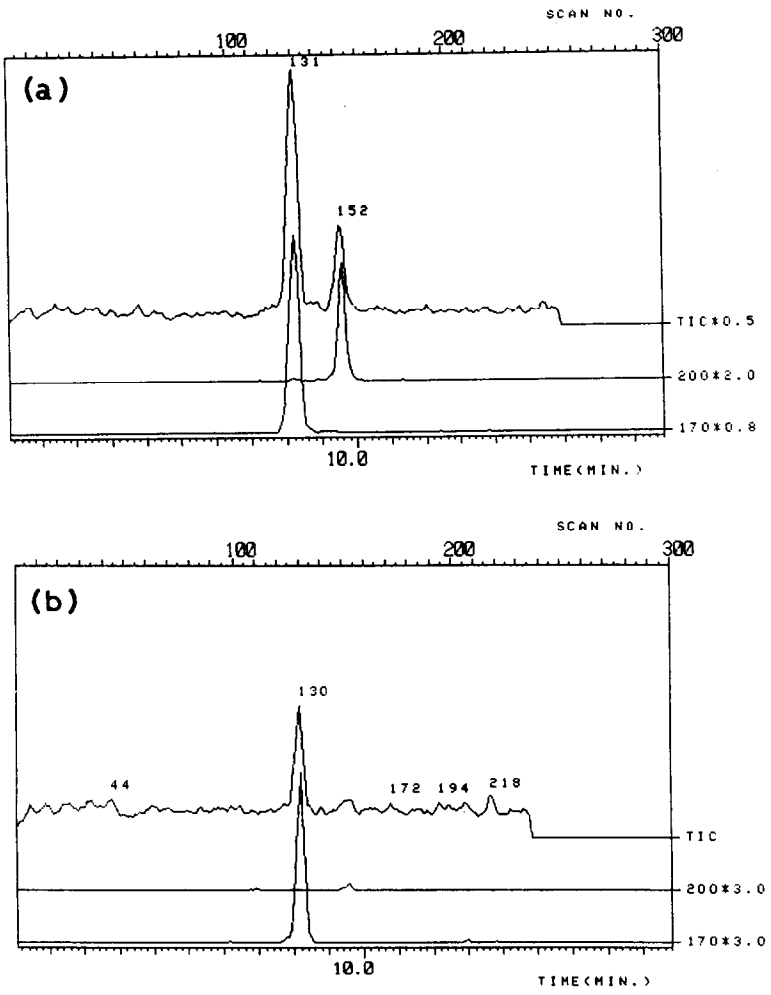

Fig. 5. Mass chromatograms of a mixture of authentic phenothiazine (PHE, M.W. 199) and diphenylamine (DIP, M.W. 169). The chromatographic conditions were as follows: mobile phase: 75% methanol–acetic acid (1000:5, v/v); column: Shim-pack CLC-ODS (15 cm × 6.0 mm I.D.); flow-rate: 1.0 ml/min; MS conditions are in Experimental. (a) Mixtures of phenothiazine (PHE) and diphenylamine (DIP), (b) desulfurized product of phenothiazine (PHE).

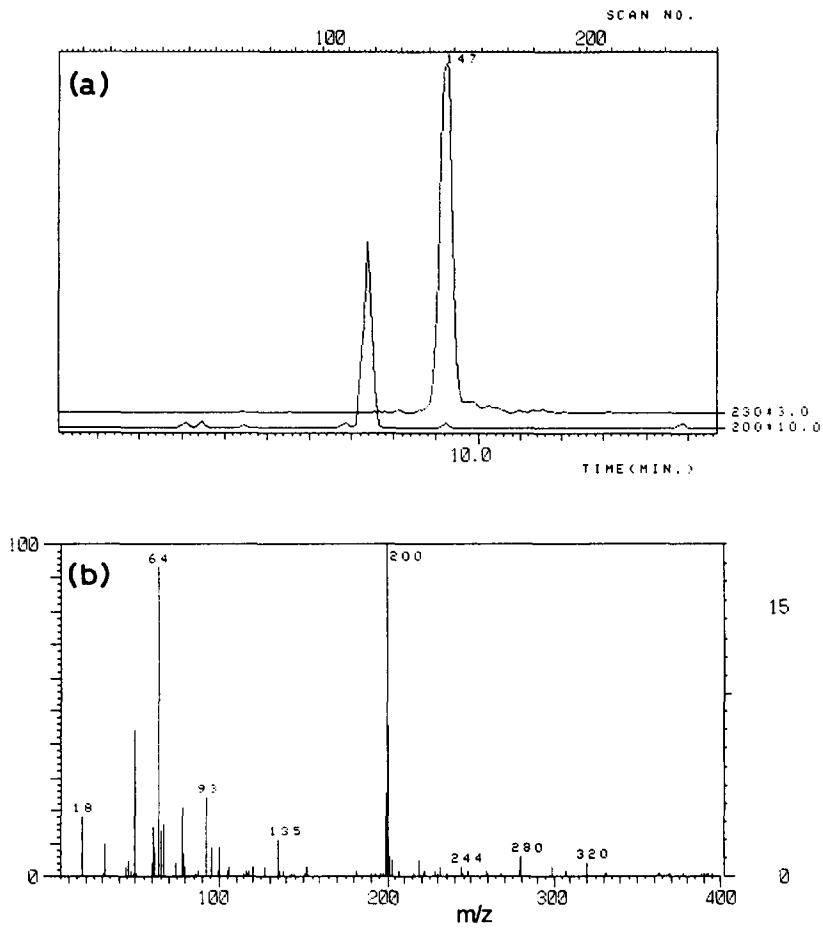

Fig. 6. Mass chromatogram of 2-methoxyphenothiazine (M.W. 229) and its desulfurized product (2-methoxydiphenylamine, M.W. 199), and mass spectrum of 2-methoxydiphenylamine. The chromatographic conditions were as follows: mobile phase: 75% methanol–acetic acid (1000:5, v/v); column: Shim-pack CLC-ODS (15 cm × 6.0 mm I.D.); flow-rate: 1.0 ml/min; MS conditions are in Experimental. (a) Mass chromatogram of 2-methoxyphenothiazine and its desulfurized product (2-methoxydiphenylamine), (b) mass spectrum of 2-methoxydiphenylamine.

cedure as used for PHE, revealed new peaks on the chromatograms at the different t_Rs. Fig. 6 shows the mass spectrum and mass chromatogram obtained from the desulfurization of 2-methoxyphenothiazine.

4. Conclusions

The proposed method is a new application of the use of Raney nickel for the determination of phenothiazine using voltammetric techniques.

With the growing importance of HPLC-ED techniques for microdeterminations in analytical chemistry, the desulfurization reaction becomes essential to overcome the analytical limitations encountered in the determination of low concentrations of drugs in biological fluids.

The sensitivity of the proposed method compares favorably with ordinary labeling techniques. In addition, without the use of labeling reagents, the introduction of simple desulfurization reactions for sulfur-containing compounds results in highly sensitive products, diphenylamine (DIP) or diphenylbenzidine, for electrochemical detection. The chemical structures of

the compounds thus produced could be confirmed on-line by LC–MS techniques.

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