

# Development of Sequential Injection Analysis for Determination of 1-(3-Dimethylaminopropyl)-3-Ethylcarbodiimide Hydrochloride (EDC·HCl) Based on Condensation Reaction between Malonic Acid and Ethylenediamine

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## Abstract

A sequential injection analysis (SIA) using a specific condensation reaction between malonic acid and ethylenediamine with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) in aqueous media was developed for EDC·HCl determination. EDC·HCl can act as a dehydration or condensation reagent for the formation of amide (peptide). The proposed system, whose operation was fully automated by a lab-made computer-controlled program, consisted of a syringe pump, a selection valve, a spectrophotometer and a computer. The product in the condensation reaction accelerated at 60°C could be detected at 262 nm. The calibration graph of EDC·HCl showed a good linearity: the regression equation and correlation coefficient (*r*) were  $y = 1.5 \times 10^{-3}x - 3 \times 10^{-4}$  (*y*, absorbance; *x*, M concentration of EDC·HCl) and  $r = 0.998$ , respectively. The limit of detection (LOD) was  $2.0 \times 10^{-5}$  M and the sample throughput was 17 samples per hour. The automated SIA for EDC·HCl determination has improved the reaction efficiency and the sensitivity by coupling with forward and backward mixing in the reaction coil.

**Keywords** EDC·HCl determination, sequential injection analysis, condensation reaction, malonic acid, ethylenediamine

## 1. Introduction

Condensing and dehydrating agents are indispensable for synthesis in chemistry fields. Carbodiimide reagents, such as 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC·HCl), *N,N'*-dicyclohexylcarbodiimide (DCC) and *N,N'*-diisopropylcarbodiimide (DIC) are often used in synthesis of esters, amides and peptides [1–5]. They have often been used for polynucleotide synthesis [6, 7], anhydroxydation [8, 9] and lactonization [10]. Of these, EDC·HCl has been widely used for polyaniline-carbon nanotube preparation for cholesterol biosensor [11], pre-column derivatization of aliphatic amines for HPLC [12], molecular beacons formation for DNA research [13], sensor preparation for calcium detection [14], fluorescent determination of carboxylic acids [15, 16] and solid-phase microsequencing of peptides [17]. As EDC·HCl is soluble in water and less toxic than DIC and DCC, it is one of the most useful reagents for dehydration and condensation reactions especially in the field of biochemistry and medicinal chemistry. In comparison with other carbodiimide reagents, EDC·HCl which is a smooth powdery crystal, is easy to handling and the by-product in dehydration and condensation reactions with EDC·HCl is easy to soluble in various kinds of solvent, especially in water.

In order to estimate the EDC·HCl concentration in the purity test, quality control and process control, some methods have been developed [5, 18, 19]. A spectrophotometric method coupled with a titration technique [19] is popular, but causes lower reproducibility. To improve the reproducibility, a spectrophotometric/flow injection analysis (FIA) has been developed for the determination of EDC·HCl in aqueous medium [20].

Though EDC·HCl was measured in a short time, its analytical procedure was not automated. In this work, an FIA system based on the reaction with pyridine and ethylenediamine in acidic solution (0.1 M HCl) in the presence of EDC·HCl was proposed, and applied to the monitoring of the residual EDC·HCl, which remained after the dehydration of phthalic acid itself and the esterification of acetic acid. The absorbance of EDC·HCl at 400 nm, decreased along with an increase in the concentration of the main product in the dehydration and esterification. Its sensitivity, however, was not so good (LOD, 0.1%). Recently, we proposed the novel condensation reaction between malonic acid and ethylenediamine in the presence of EDC·HCl, which was applied to the EDC·HCl measurement by a manually operated FIA [21]. Though this FIA was more sensitive and simple (two flow lines system), the reaction efficiency was not so good even accelerating at 60°C.

In this study, a sequential injection analysis (SIA) detecting the condensation reaction product between malonic acid and ethylenediamine in the presence of EDC·HCl was developed for the determination of EDC·HCl. The proposed system was fully automated by a lab-made program and composed of a syringe pump, a selection valve, a detector and a computer. The reaction efficiency was improved by forward and backward mixing in the reaction coil and the rapid measurement, simple procedure and sensitive and reproducible detection could be accomplished.

## 2. Experimental

### 2.1 Reagents and chemicals

A condensation and dehydration agent, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) was obtained from Osaka Synthetic Chemical Laboratories, Inc.

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(Hyogo, Japan). Malonic acid and ethylenediamine were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ultrapure water ( $18.3 \text{ M}\Omega \text{ cm}^{-1}$ ) prepared by Milli-Q System (Nihon Millipore, Tokyo, Japan) was used throughout. Stock solutions of malonic acid (0.1 M) and ethylenediamine (0.1 M) were prepared by dissolving them in water. The reagent solution (RS) was prepared by mixing malonic acid and ethylenediamine: each concentration is  $2.0 \times 10^{-3} \text{ M}$ . Standard solutions of EDC·HCl were prepared just before the experiment.

## 2.2 Apparatus

A SIA system was shown in Fig. 1, which composed of a syringe-type pump (SP: Hamilton, Reno, NV, USA), a syringe valve (SV: Hamilton, Reno, NV, USA), a six-port selection valve (SL: Hamilton, Reno, NV, USA), a holding coil (HC: 0.8 mm i.d.  $\times$  5 m), a reaction coil (RC: 0.8 mm i.d.  $\times$  4 m) and UV-VIS detector (S-3702, Soma Optics, LTD. Japan). PTFE tubings ( $\phi$ , 0.8 mm) were used for all flow lines. The developed SIA system was fully controlled by the laboratory-made program, which was written with Visual Basic. The computer was connected with USB cable. A spectrophotometer (UV-2400PC, Shimadzu, Japan) was used for the measurement of absorption spectra.

## 2.3 Procedures

The operating procedures of the developed SIA system (Fig. 1) were shown in Table 1. At the first step (cleaning step), HC was washed with each sample solution: sample solution (200  $\mu\text{L}$ ) was aspirated at the flow rate of  $100 \text{ }\mu\text{L s}^{-1}$  into HC through the port 3 of SL. After the position of SL was turned to the port 1, the sample in HC was dispensed and wasted. At the second step (mixing step), the sample and reagent solution (RS) were aspirated into the HC: RS (300  $\mu\text{L}$ ), sample (200  $\mu\text{L}$ ) and again RS (300  $\mu\text{L}$ ) were aspirated in this order into HC through the port 3 or 4 of SL, respectively, at the flow rate of  $100 \text{ }\mu\text{L s}^{-1}$ . The sample was put between two RS zones in HC, and next water (1700  $\mu\text{L}$ ) was then aspirated at the flow rate of  $200 \text{ }\mu\text{L s}^{-1}$  into SP through the port IN of syringe valve (SV) for cleaning to the next measurement.

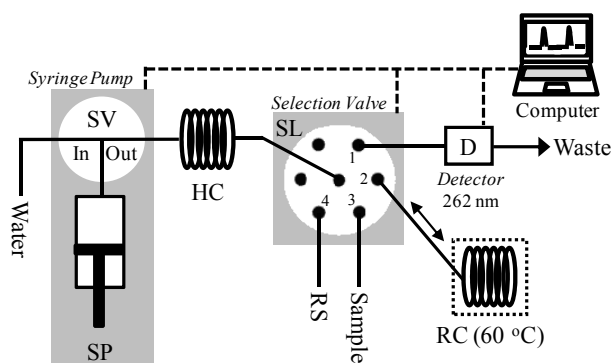


Fig. 1 Schematic SIA diagram for the determination of EDC·HCl. SP, syringe pump; SV, syringe valve; HC, holding coil (0.8 mm i.d.  $\times$  5 m); SL, 6 ports selection valve; RC, reaction coil (0.8 mm i.d.  $\times$  4 m) in air bath (60 °C); D, Detector (262 nm); RS, reagent solution (mixed solution of  $2.0 \times 10^{-3} \text{ M}$  malonic acid and  $2.0 \times 10^{-3} \text{ M}$  ethylenediamine).

Table 1 Operating procedure of the proposed SIA system for EDC·HCl determination.

Sequence	SV Port	SL Port	SP Action	Flow Rate/ $\mu\text{L s}^{-1}$	Volume / $\mu\text{L}$
Cleaning					
1	Out	3	Aspirate	100	200
2	Out	1	Dispense	100	200
Mixing					
3	Out	4	Aspirate	100	300
4	Out	3	Aspirate	100	200
5	Out	4	Aspirate	100	300
6	In	4	Aspirate	200	1700
Reacting					
7	Out	2	Dispense	50	1000
8	Out	2	Aspirate	100	1000
9	Out	2	Dispense	50	1000
10	Out	2	Aspirate	100	1000
11	Out	2	Dispense	50	1000
14	Out	2	Standing (60 s)	—	—
15	Out	2	Aspirate	100	1000
Measuring					
16	Out	1	Dispense	100	2500

At the third step (reacting step), EDC·HCl in sample proceeded the dehydration between malonic acid and ethylenediamines in RS. The sample zone and two RS zones in the HC, was injected at the flow rate of  $50 \text{ }\mu\text{L s}^{-1}$  into RC through the port 2 of SL. Next, in order to mix the sample and RS zones effectively in RC, the solutions were moving forth and back in RC and this sequences were repeated twice: a portion of sample and RSs zones was aspirated at the flow rate of  $100 \text{ }\mu\text{L s}^{-1}$  and dispensed at the flow rate of  $50 \text{ }\mu\text{L s}^{-1}$  in the sequence 8 – 11 (Table 1). Then, the mixture was kept for 60 s in RC and aspirated at the flow rate of  $100 \text{ }\mu\text{L s}^{-1}$  into the HC through the port 2 of SL for cooling. At the final step (measuring step), it was introduced at the flow rate of  $100 \text{ }\mu\text{L s}^{-1}$  into the detector (D) through the port 1 of SL, and the absorbances were measured at 262 nm. The absorbances of the peak height were used for calculating the concentration.

## 3. Results and Discussion

### 3.1 Properties of EDC·HCl in aqueous solution and condensation agent

EDC·HCl shows the absorbance around 214 nm in aqueous solution, but the absorbance decreases along with the standing time at room temperature. The time course of the absorbance in aqueous media was tested as shown in Fig. 2. It was decreased by about 3% per an hour and almost EDC·HCl (initial concentration,  $2 \times 10^{-4} \text{ M}$ ) was decomposed after 24 h, which was caused by the hydrolysis of EDC·HCl. Therefore, the absorbance of EDC·HCl in aqueous solutions should be measured rapidly after preparation and/or reaction. In our previous work [21], EDC·HCl took part in a condensation reaction agent between malonic acid and ethylenediamine, and the predicted reaction scheme was shown in Fig. 3. The condensation reaction product might have a ring structure, because the absorption maximum was longer than EDC·HCl, and malonic acid and ethylenediamine seemed to form a stable ion pair by the electrostatic interaction and hydrogen bonding before the reaction which was easy to form a ring structure. The product in the condensation reaction showed the maximum absorbance at 262 nm as is shown in Fig. 4.

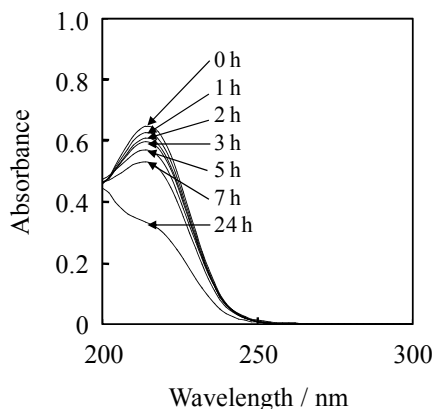


Fig. 2 Time course of EDC·HCl itself in aqueous solution at room temperature. Initial concentration of EDC·HCl is  $2 \times 10^{-4}$  M.

Its molar absorptivity ( $\epsilon$ ) at 262 nm, was  $2.8 \times 10^3$  L mol $^{-1}$  cm $^{-1}$ , and larger than that of EDC·HCl itself (almost 0 at 262 nm and  $1.7 \times 10^3$  L mol $^{-1}$  cm $^{-1}$  at 214 nm). The absorbance at 262 nm was increased with an increase in the standing time of condensation reaction between malonic acid and ethylenediamine in the presence of EDC·HCl, whereas malonic acid and ethylenediamine did not have the absorption band around 262 nm. Therefore, the absorption around 262 nm might be derived from the product in Fig. 3 and the effective adsorption might be correlated with the resonance structure of the product. In this study, the proposed condensation reaction for the determination of EDC·HCl was applied to the automated SIA method with effective mixing.

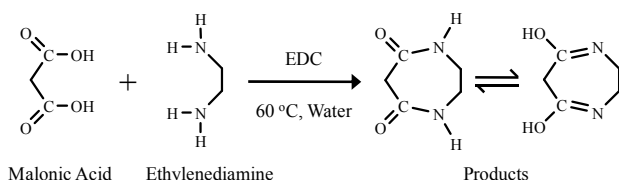


Fig. 3 Scheme of the condensation reaction between malonic acid and ethylenediamine with EDC·HCl at 60 °C in water. Cited from Ref. [21].

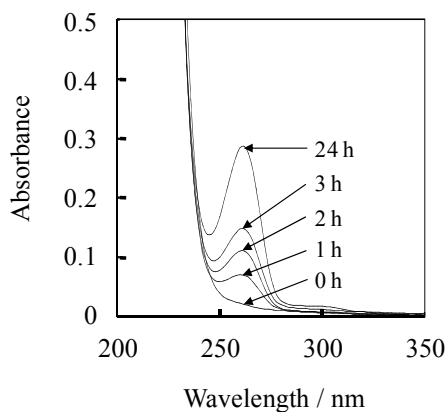


Fig. 4 Effect of the reaction time on the adsorption of the product of the condensation reaction between malonic acid and ethylenediamine with EDC·HCl at room temperature. Sample contains malonic acid (0.05 M), ethylenediamine (0.05 M) and EDC·HCl ( $1.0 \times 10^{-4}$  M).

### 3.2 Optimization of flow conditions

The proposed SIA flow conditions were optimized. The temperature of the condensation reaction between malonic acid and ethylenediamine with EDC·HCl was examined in our previous study [21] by varying it from 25 to 80 °C. The reaction was accelerated more effectively at 60 °C than at room temperature. Therefore, the reaction coil (RC) in Fig. 1 was kept in the water bath at 60 °C. To increase the reaction efficiency, the sample solution zone was sandwiched with RSs in RC (malonic acid and ethylenediamine). In order to mixing the zones effectively, they were shaken to move forward and backward in RC, as SIA can change the flow direction go and back in the same tubing by operating aspiration and dispense of the syringe pump (SP). The reaction efficiency was examined by repeating the procedure of syringe moving backward and forward as shown in Fig. 5.

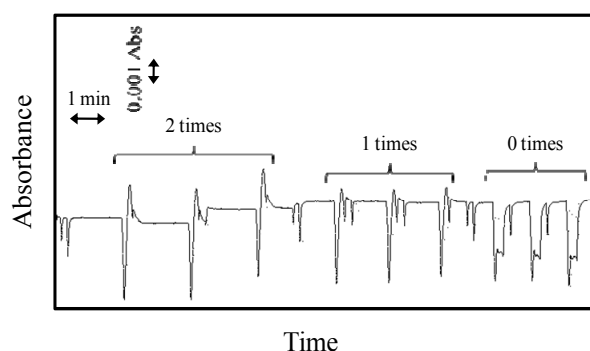


Fig. 5 Effect of the time of moving backward and forward on the reaction efficiency. Samples contain malonic acid ( $2 \times 10^{-3}$  M), ethylenediamine ( $2 \times 10^{-3}$  M) and EDC·HCl ( $2.0 \times 10^{-4}$  M).

When backward and forward moving of the sample and RSs zones were carried out two times, the peak height of the product became almost identical. Considering the effective reaction and sensitivity, two times repeating of the zones moving was selected: this procedure was added to the operating procedures in Table 1 at the sequence 8 – 11. Figure 6 shows the effect of the standing

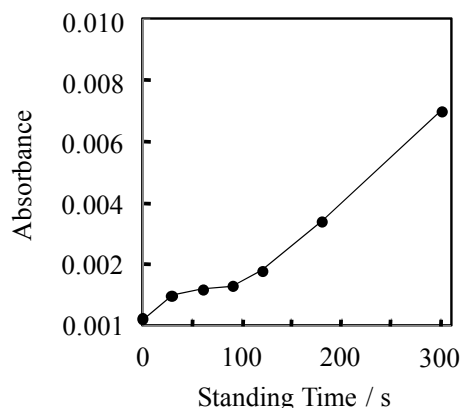


Fig. 6 Effect of standing time on the peak height of the product. Sample was the same as in Fig. 5.

time in RC at 60 °C on the absorbance of the product. The absorbances gradually increased to 120 s, and after that abruptly increased over next 120 s though arising the large deviation of measurement values. The absorbance was almost identical from

30 to 90 s. Considering the sensitivity, reproducibility and sample throughput, the standing time was set to 60 s.

### 3.3 Calibration graph and analytical performance

Figure 7 show the typical flow signals for EDC·HCl in the range from 0 to  $10.0 \times 10^{-4}$  M by the proposed SIA system. The calibration graph with the absorbance of the peak height of the product showed good linearity. The regression equation and correlation coefficient ( $r$ ) were as follows:  $y = 1.5 \times 10^{-3} x - 3 \times 10^{-4}$  ( $y$ , absorbance;  $x$ , EDC·HCl concentration) and  $r = 0.998$ , respectively. Figure 8 shows the peak profiles of the product (0 to  $10.0 \times 10^{-4}$  M EDC·HCl). Each peak top appeared at the same time, and the blank signal was not seen. The negative peaks appeared at both of the front and rear of each peak, which were caused by the shock of the syringe movement: such negative peaks did not affect the peak height of the product. The proposed SIA was much more improved than the previous FIA [21]. As the base line signal was almost 0, the limit of detection (LOD) was calculated with  $3 \sigma$  of  $2.0 \times 10^{-4}$  M EDC·HCl signals of 10 measurements was  $2.0 \times 10^{-5}$  M. The sample throughput was  $17 \text{ h}^{-1}$ .

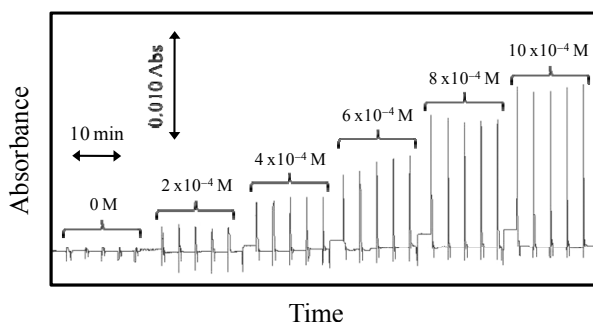


Fig. 7 Typical flow signals for the calibration graph of EDC·HCl. Samples, EDC·HCl ( $0 \text{ M} - 10.0 \times 10^{-4} \text{ M}$ ); RS, malonic acid ( $2 \times 10^{-3} \text{ M}$ ) and ethylenediamine ( $2 \times 10^{-3} \text{ M}$ ).

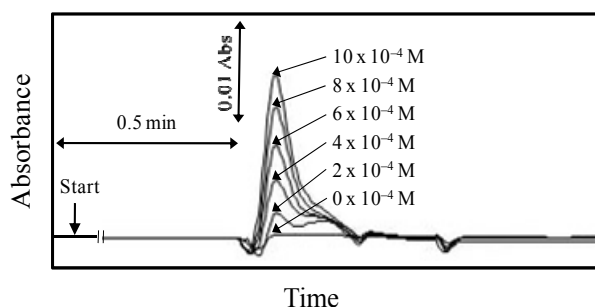


Fig. 8 Peak profiles of each flow signal for calibration graph. Samples were the same as in Fig. 7.

## 4. Conclusions

The SIA method based on the condensation reaction between malonic acid and ethylenediamine in the presence of EDC·HCl was developed for EDC·HCl determination. Backward and forward mixing procedure was effective. The analytical merits are: (1) rapid measurement (sample throughput:  $17 \text{ h}^{-1}$ ), (2) automated procedure by computer controlling and (3) good

reproducibility and relatively high sensitivity (LOD:  $2.0 \times 10^{-5} \text{ M}$ ).

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