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Determination of indomethacin in urine using molecule imprinting-chemiluminescence method

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Abstract

A soluble Mn (IV)-formaldehyde-indomethacin chemiluminescence system was found. Using a synthesized indomethacin MIP as recognition material and soluble Mn (IV)-formaldehyde-indomethacin as detection system, a new molecule imprinted-chemiluminesence method of determination of indomethacin was established. The response range of this method was between 1.0×10^{-7} and 1.0×10^{-5} g/mL with a linear correlation coefficient of 0.994. The detection limit was 4×10^{-8} g/mL. The relative standard deviation for 5.0×10^{-7} g/mL of indomethacin solution was 3.1% ($n = 7$).

Keywords: Indomethacin; Molecule imprinting-chemiluminescence method; Soluble Mn (IV)

1. Introducton

Indomethacin is a nonsteroid antiphlogistic in common use. It is usually determined by the methods such as photometry [1,2], chromatography [3–6], electrochemistry [7,8], fluorescence method [9] and acid–base determination [10]. Chemiluminescence method of determination of indomethacin has not been reported up to now. It is found that the reaction between indomethacin and soluble Mn (IV) can produce chemiluminescence (CL) and formaldehyde can enhance this CL reaction. However, there is a great deal of other substances that can react with soluble Mn (IV) to produce CL under the same conditions. The selectivity of determination of indomethacin with soluble Mn (IV)-formaldehyde-indomethacin CL system should be extremely poor because many coexistent substances may interfere with the determination and this CL method can not be used to determine indomethacin in those complicated samples directly.

Molecular imprinted polymer (MIP) has been known as a new synthetic material capable of molecular recognition [11,12]. Usually MIP is prepared by copolymerization of functional monomer with cross-linker in the presence of template molecules to produce three-dimensional network polymers. Removal of the templates molecule results in a functional polymeric matrix with recognition sites complementary in size, shape and functionality to the template molecule. The attractive features of MIP, such as good mechanical strength, ease of preparation and stability in harsh environments allow it to be used as chromatographic stationary phase [13], solid phase extraction matrices [14,15] artificial receptors for use in drug assays [16] and recognition elements in sensors [17,18]. More recently, the molecule recognition function of MIP was used by Lin and Yamada to develop CL-based sensing systems [19,20].

In CL reaction, utilizing highly selectivity and capture ability of MIP to target molecule, the target molecule would be adsorbed in MIP and separated from the interference substances. Then performing CL analysis, the selectivity of CL method can be improved greatly. Some components in the complicated samples can be determined directly by this way now [21].

In this work, the indomethacin MIP was prepared using methacrylic acid (MAA) as functional monomer and

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ethylene glycol dimethacrylate (EGDMA) as cross-linker in the presence of template molecule of indomethacin. The polymer was packed into a glass tube and made into an indomethacin MIP column. Connecting the indomethacin MIP column into the newly established soluble Mn (IV)-formaldehyde-indomethacin CL flow system, a molecule imprinting-chemiluminescence (MI-CL) method with high selectivity to determine indomethacin was established. The CL intensity was linear related to the concentration of indomethacin over 1.0×10^{-7} to 1.0×10^{-5} g/mL range with a linear correlation coefficient of 0.994. The detection limit was 4×10^{-8} g/mL. The relative standard deviation for 5.0×10^{-7} g/mL indomethacin solution was 3.1% ($n = 7$). This method has been used to determine indomethacin in human urine with a satisfying result.

2. Experiment

2.1. Apparatus

The schematic diagram of MI-CL analytical method is shown in Fig. 1. Peristaltic pumps were used to deliver all solutions and PTFE tubing (0.8 mm i.d.) was used to connect all parts in the flow system. CL measurements were performed using an IFFM-D CL analyzer (Xi'an Remax Electronic High-Tech, Ltd.). The acquisition and treatment of the data was performed with the IFFM-D CL data processing software (Xi'an Remax Electronic High-Tech, Ltd.). Absorbance was taken by a TU-1901 UV–vis spectrophotometer (Beijing Currency Instrumental, Ltd.). Ultrasonator (Kunshan Ultrasonic Instrumental, Ltd.) was used to help to mix.

2.2. Reagents

Indomethacin was purchased from Taicang Pharmacy Factory (Jiangsu, China). EGDMA were purchased from Sigma (St. Louis, MO). MAA and 2,2′-azobis(2-methylpropinitrile) (AIBN) were purchased from Shanghai Chemical Reagent Company (Shanghai, China). Other reagents were purchased from Xi'an Chemical Reagent Factory (Xi'an, China). All reagents used were of analytical reagent grade except for AIBN, which was chemical purity grade. EGDMA and MAA were redistilled and AIBN was recrystallized prior to use.

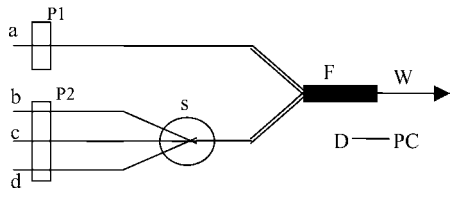

Fig. 1. Schematic diagram for MI-CL method flow system. P: peristaltic pump; D: detector; F: MIP column; PC: computer; W: waste; s: switch valve; a: Mn (IV) solution; b: water; c: formaldehyde solution; d: sample solution.

Stock standard solution of indomethacin (1.00×10^{-4} g/mL) was prepared by dissolving 50.0 mg of indomethacin with 0.1 mol/L hydrochloric acid and then diluting to 50 mL with water. Working standard solutions of indomethacin were prepared of diluting this stock solution with water.

Soluble Mn (IV) solution (4.0×10^{-3} mol/L) was prepared according to the method that had been reported previously [22]. A 2 g of newly made MnO_2, which was produced from the reaction between sodium formate and potassium permanganate, was added into 500 mL phosphoric acid. This mixture of MnO_2 and phosphoric acid were oscillated with ultrasonic for 30 min. Then it was placed at room temperature for 24 h.The concentration of this solution was determined by iodimetry and it shows no significant difference in a month.

Doubly distilled water was used throughout the experiments.

2.3. Synthesis of the polymer

A 1 mmol of indomethacin, 5 mL chloroform and 4 mmol of MAA was added into a 50 mL round-bottomed flask, the mixture was oscillated with ultrasonic for 4 h to let MAA sufficiently mixed with indomethacin molecule. Then 20 mg of AIBN and 20 mmol of EGDMA were added. The mixture was purged with nitrogen for 15 min and sealed under vacuum. The polymerization reaction was carried out at 60 °C in a water bath for 24 h. The obtained polymers were crushed, ground and sieved to collect the particles of the size between 74 and 105 μm.

2.4. Binding experiments

Before binding experiments, the indomethacin molecules in the MIP were removed by washing with the mixture of carbinol-acetic acid (4:1, v/v) until the absorbance of indomethacin had been no longer detected in the elution solution. The polymer was dried to a constant weight at 60 °C under vacuum. Then 20.0 mg MIP was mixed with 5.0 mL of various concentration of indomethacin solution in a 10 mL conical flask and oscillated for 12 h at room temperature. After centrifuging at 3000 rpm for 10 min, the concentration of free indomethacin in the supernatant was detected by UV spectrophotometry. The amount of indomethacin bound to the polymer was calculated by subtracting the concentration of free indomethacin from the initial indomethacin concentration. The data obtained were used for the Scatchard assay.

2.5. Preparation of the indomethacin MIP column

Indomethacin MIP column was a 4 mm i.d. × 15 mm length colorless glass tube whose shape just likes a capital letter Y. A 20.0 mg of above collected polymer was packed in the straight part of the Y-tube and plugged with a small amount of glass wool at both ends. The column was connected into CL system during determination. For a

new MIP column, indomethacin template molecules in the MIP have not been removed. Before the MIP column was used, let the combining stream of soluble Mn (IV) solution and formaldehyde flowed through the column, reacted with indomethacin to produce CL. The CL signal declined with the waste of indomethacin in the MIP. It can be not considered that indomethacin in the MIP had all been reacted until the signal declined to the baseline. Then, the column was washed by water to clean the reaction products for using.

2.6. Procedures for MI-CL method

The schematic diagram for MI-CL method was shown in Fig. 1 and the procedure could be summarized as four steps:

- Step 1: adsorption of indomethacin. In this step, pump 1 was stopped; switch valve was in connection of sample solution. Pump 2 pumped indomethacin solution through the MIP column for 50 s and indomethacin was selectively adsorbed in the cavities of the polymer.
- Step 2: washing to remove other substances except indomethacin. In this step, pump 1 was stopped; switch valve was in connection of formaldehyde solution. Pump 2 pumped formaldehyde solution through the MIP column continuously for 80 s to remove other substances except indomethacin.
- Step 3: chemiluminescence detection. In this step, switch valve was in connection of formaldehyde solution. Pumps 1 and 2 were both started to pump the combined stream of soluble Mn (IV) solution and formaldehyde solution flowed through the MIP column for 30 s and reacted with indomethacin adsorbed in the MIP to produce CL until the signal declined to the baseline.
- Step 4: cleaning the MIP column. In this step, pump 1 was stopped; switch valve was in connection of water. Pump 2 pumped water through the MIP column continuously for 40 s to clean the polymer for the next determination.

3. Results and discussions

3.1. Binding characteristic of the MIP

The binding characteristic of the indomethacin MIP was investigated by equilibrium binding experiments.

In molecule imprinting technique, the Scatchard equation is usually used to estimate the binding parameters of the MIP. The Scatchard equation can be expressed as $Q/[\mathrm{MET}] = (Q_{\max} - Q)/K_{\mathrm{d}}$, in which K_{d} was the dissociation constant of binding sites, $Q_{\max}$ was the apparent maximum combining amount and [MET] was the equilibrium concentration of indomethacin. The combining amount of the MIP while indomethacin concentration was in the range of 1×10^{-5} to 10×10^{-5} g/mL was determined by the equilibrium binding experiments. The obtained data were plotted according to the Scatchard equation as it was shown in Fig. 2.

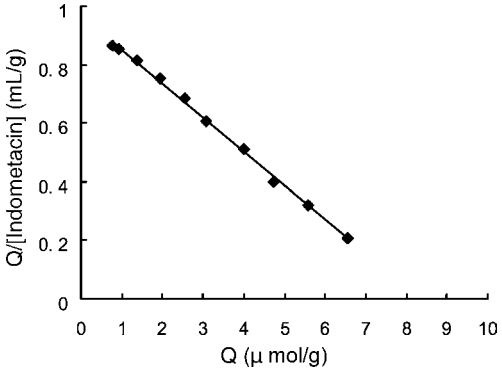

Fig. 2. Scatchard plot to estimate the binding nature of indomethacin-imprinted polymer Q: apparent combining amount (μmol/g); [Indomethacin]: equilibrium concentration of indomethacin (μmmol/L).

In Fig. 2, $Q/[\mathrm{MET}]$ was linear to Q within whole indomethacin concentration range, which indicated that there was one kind of binding sites in the MIP. The dissociation constant K_{d} and the apparent maximum combining amount $Q_{\max}$ of this kind of binding sites can be calculated to be 8.59 mmol/L and 8.32 μmol/g for dry polymer from the slope and intercept of the Scatchard plot.

3.2. Shape and size of the MIP column

In MI-CL analysis, soluble Mn (IV) solution and formaldehyde solution had to be combined first before they flowed through the MIP column and reacted with indomethacin adsorbed in the MIP producing CL. The CL signal would have been very low if soluble Mn (IV) solution and formaldehyde solution had been combined together for a relative long time. Perhaps it is because of the waste of the two each other. In order to improve the selectivity of determination, time of the combination before soluble Mn (IV) solution and formaldehyde solution flowed into the MIP column must be shortened. A Y-tube has been developed aiming at this. A 20.0 mg of indomethacin polymer were packed in the straight part of the Y-tube and plugged with a small amount of glass wool at the both ends, and then it was connected into CL system for detection. Soluble Mn (IV) solution and formaldehyde solution were mixed just in front of the column. The mixture entered into the column immediately and reacted with indomethacin adsorbed in the MIP producing CL.

3.3. Conditions of CL reaction

3.3.1. Concentration of soluble Mn (IV) solution

Soluble Mn (IV) solution was used as the oxidant in this CL reaction. Its concentration can effect on the intensity of CL signal. The effect of soluble Mn (IV) solution was examined in the range of 8.0×10^{-5} to 8.0×10^{-4} mol/L. The suitable concentration of soluble Mn (IV) solution was 4.0×10^{-4} mol/L.

3.3.2. Concentration of enhancer

The enhanced effects were compared of some substances such as sodium dodecanesulfonic, beta-cyclodextrin, sodium sulfite and formaldehyde. It was observed that formaldehyde was the best suitable enhancer of this system. In view of the blank signal and enhanced effect, the suitable concentration of formaldehyde should be 4%.

3.4. Procedures of determination

A series of experiments were conducted to optimize the experimental conditions of indomethacin analysis.

3.4.1. Adsorption time (T_a)

The adsorption time is the time standard solution or sample solution flowing through the MIP column. It determines the amount of indomethacin adsorbed in the MIP column, and then determines the sensitivity of the detection and the linear range of the method. The adsorption time is relevant to the concentration of indomethacin, the binding capacity of the polymer and the flow rate. When the amount of polymer was 20 mg, the flow rate was fixed at 1.5 mL/min and the concentration of indomethacin was 5.0×10^{-7} g/mL, the relation between the CL intensity and the adsorption time within the range 10–100 s was examined (Fig. 3). The CL intensity was increased with the increase of adsorption time. Above 60 s, the CL intensity almost remained constant. Considering analytical efficiency and the linear range of this method, 50 s was finally selected as adsorption time.

3.4.2. Washing time (T_w)

Following the adsorption step, it is necessary to wash the MIP column to remove the other substances absorbed by nonspecific interaction. A suitable washing time should remove other substances completely and did not cause the loss of indomethacin adsorbed in MIP column. To select the washing time, trimethoprim (TMP), which usually coexists with indomethacin and can also react with soluble Mn (IV) and formaldehyde producing CL, was selected as interference indicator and added into the indomethacin standard solution (indomethacin 5.0×10^{-7} g/mL, TMP 5.0×10^{-7} g/mL). Formaldehyde was used as washing reagent. The effect of washing time was examined in the range 30–140 s with the flow rate at 1.5 mL/min. The experimental results showed that when the washing time was beyond 80 s, the interference indicator can be effectively removed. The CL intensity showed no obvious change with that of the same concentration (5.0×10^{-7} g/mL) of indomethacin standard solution in the absence of TMP. So, 80 s was selected as the washing time.

3.4.3. Reaction time (T_r)

When the stream of CL reagent flowed through the column, the indomethacin adsorbed on the polymer reacted with soluble Mn (IV) and formaldehyde producing CL. The indomethacin was completely exhausted when the CL signal declined to the stable baseline. The experimental results showed that the whole process needs 40 s.

3.4.4. Cleaning time (T_c)

After CL reaction between soluble Mn (IV) and indomethacin adsorbed in the polymer, the molecule structures of indomethacin had been destroyed and indomethacin was desorbed from the MIP. Therefore, the reaction products can be easily removed from MIP column with water flowed through the MIP column and the cavities can be emptied out for the next determination. The effect of the cleaning time in the range 20–60 s was examined by comparing the blank signals produced before adsorption step and the blank signals produced after adsorption step. It was observed that when the cleaning time was over 40 s, the blank signals had no obviously differences with each other. So 40 s was selected as the cleaning time.

3.5. Analytical parameter

At the optimized conditions, using the flow system depicting in Fig. 1, the relation between the CL intensity and the concentration of indomethacin was examined. The CL intensity was linearly related to the concentration of indomethacin over 1.0×10^{-7} to 1.0×10^{-5} g/mL with a linearly regression equation of $I = 6.15C + 58.93$ ($r = 0.994$), where I was the CL intensity (relative unit) and C was the concentration of indomethacin ($\times 10^{-7}$ g/mL). The relative standard deviation for 5.0×10^{-7} g/mL of indomethacin solution was 3.1%

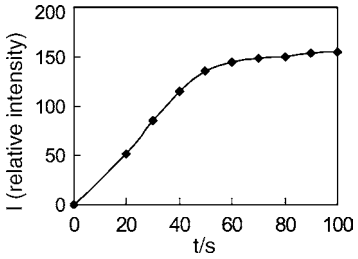

Fig. 3. Effect of adsorption time on CL reaction. Flow rate: 1.5 mL/min; concentration of indomethacin: 5.0×10^{-7} g/mL.

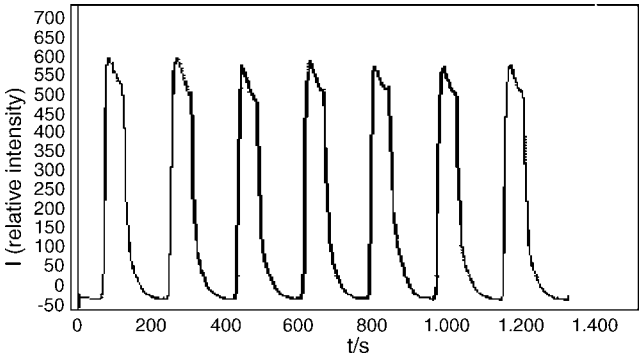

Fig. 4. CL intensity of seven times to detect indomethacin (5.0×10^{-7} g/mL).

Table 1
Tolerable ratio of interfering species

Species	Tolerable ratio with MIP	Tolerable ratio without MIP
Trimethoprim	1	0.05
Sulfanilamide	1	0.05
Epinephrine	5	0.01
Fe^{2+}	50	0.1
Tryptophan	10	0.1
Uric acid	5	0.05
Norfloxacin	1	0.01
Ascorbic acid	10	0.02
Carbamide	–	1000
Starch	–	1000
Glucose	–	1000
Pyruvic acid	1000	50
Na^+, K^+, Mg^{2+}, Cu^{2+}, Fe^{3+}	–	1000
Ca^{2+}	1000	100

($n = 7$) (Fig. 4). According to suggestion of IUPAC, the detection limit can be determined as 4.0×10^{-8} g/mL.

3.6. Selective experiment

In this study, the interferences for the determination were investigated with 5.0×10^{-7} g/mL indomethacin using the MI-CL method and the FI-CL method, respectively. The interferences for the determination of indomethacin were selected from those foreign species normally existed in urine and the substances having CL behaviors in soluble Mn (IV)-formaldehyde CL system. The results were shown in Table 1, with the tolerable limit of an interfering species taken as a relative error less than 5%. From Table 1, it is obvious that this method exhibited high selectivity compared with the FI-CL method. In CL assay, the selectivity of the determination can be improved very much by the use of MIP.

3.7. Sample determination

3.7.1. Determination of blank sample

A 5 mL blank urine was collected from three healthy volunteers respectively and centrifuged at 3000 rpm/min for 30 min. The supernatant was transferred into a 50 mL volumetric flask and diluted to the graduation with doubly distilled water. It was regarded as the sample solution and was determined by MI-CL and FI-CL method, respectively. The results were shown in Table 2. As can be seen from Table 2, the different urine samples have no significant difference with the blank signal by MI-CL determination. It means that the other species coexisted in the urine do not interfere determination of indomethacin. That is to say the recovery test can be used to estimate the accuracy of the MI-CL method. But the results are complete the reverse to the FI-CL method.

Table 2
Comparison of the CL signal for different methods

Method	Sample		
	Double distilled water	Sample 1	Sample 2
FI-CL	87	480	460
MI-CL	509	514	512

The values in the table denote the CL intensity.

3.7.2. Determination of real sample

The MI-CL method was applied to the determination of indomethacin in the urine of volunteers who had had indomethacin tablets 8 h before. The urine was treated in the same way and determined with MI-CL method. The results of the recovery test are showed in Table 3. As can be seen from Table 3, the recoveries of added indomethacin can be quantitative and *t*-test assumed that there is no significant difference between recovery efficiency and 100% at confidence level of 95%.

3.8. Possible mechanism of the CL reaction

The mechanism of soluble Mn (IV)-formaldehyde-reducing agent CL reaction had been discussed previously [23]. According to the conclusion in that paper, the possible mechanism of the CL reaction can be shown as following:

$$\mathrm{Mn(IV) + HCHO + H^+ + indomethacin} \rightarrow {}^1\mathrm{O_2}({}^1\Delta_g) + \mathrm{H_2O} + \mathrm{other\ resultants}$$

$$2\,{}^1\mathrm{O_2}({}^1\Delta_g) \rightarrow {}^1\mathrm{O_2}\,{}^1\mathrm{O_2}({}^1\Delta_g\,{}^1\Delta_g)$$

$${}^1\mathrm{O_2}\,{}^1\mathrm{O_2}({}^1\Delta_g\,{}^1\Delta_g) \rightarrow 2\,{}^3\mathrm{O_2}({}^3\Sigma_g) + h\nu$$

Table 3
Results of determination of urine samples

Sample	Initially present[a] ($\times 10^{-7}$ g/mL)	Added ($\times 10^{-7}$ g/mL)	Found[a] ($\times 10^{-7}$ g/mL)	Recovery (%)
1	5.86	2.00	7.81	97.5
		4.00	9.93	102
		6.00	10.9	101
2	6.35	2.00	8.3	97.5
		4.00	10.4	102
		6.00	12.5	103
3	6.50	2.00	8.46	98.0
		4.00	10.4	98.0
		6.00	12.7	103

[a] Average of three measurements.

4. Conclusion

In this work, a new soluble Mn (IV)-formaldehyde-indomethacin CL system had been found and an indomethacin MIP had been synthesized. Using the indomethacin MIP column as the recognition element of the CL system, a MI-CL method had been established. This MI-CL method shown an excellent selectivity to the determination of indomethacin and it had been used to determine indomethacin in urine successfully. As it can be seen, the selectivity of CL method would be improved greatly when MIT was introduced to CL method.

Acknowledgements

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