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A Chemiluminescent Method for *In-vitro* Screening of Arylesterase Inhibitors*

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Abstract In our previous work, the 9-(4-chlorophenyloxycarbonyl)-10-methylacridinium triflate ester (CPOCMA) was used as a chemiluminescent substrate for determination of serum arylesterase activity successfully. Based on CPOCMA, a chemiluminescent method was developed for assessing drugs' effect on this enzyme activity. The method was first validated with a UV method based on phenyl acetate by using trinitroglycerine as a model drug. The inhibitory effects of drugs were then exemplified by three anti-inflammatory drugs (including indometacin, aspirin and acetaminophen). It was observed that the serum-mediated CPOCMA hydrolysis slowed down due to addition of the drugs individually. It means that all the three drugs were PON inhibitors. The IC_{50} values of indometacin, aspirin and acetaminophen were 0.254, 0.564 and 0.656 mmol/L, respectively; and their average inhibitory constants were 0.154, 1.38, and 2.98 mmol/L, respectively. Competitive inhibitory type was observed for all the three drugs by plotting the Lineweaver-Burk curves. According to the kinetics of the hydrolysis, IC_{50} values, inhibitory constants, and Michaelis constants, the inhibitory abilities of the three drugs were ranked as: indometacin>aspirin>acetaminophen. This chemiluminescent method is especially valuable for evaluation of those drugs which the UV method cannot work for.

Key words PON arylesterase activity, acridinium ester, chemiluminescence, inhibitor screening
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Arylesterase is able to hydrolyze arylesters, and thus the enzyme activity can be determined with an arylester. The serum arylesterase activity is mainly attributed to serum paraoxonase, although the other serum hydrolyases showed weakly catalytic ability[1]. PON (paraoxonase) received its name from its ability to detoxify paraoxon[2]. It has been regarded as a key hydrolyase for *in-vivo* metabolism and detoxification[2-5]. Low serum PON activity was found in patients suffering from many diseases such as diabetes, cancers, polycystic ovary syndrome, depression, nephritic syndrome, haemodialysis, metabolic syndrome, obstructive sleep apnea syndrome, and liver diseases. Investigation of correlations between PON activity and diseases has been a hot topic recently. There are excellent reviews available about this topic[6-9].

Decrease of serum PON activity for a people will result in diseases, and hence investigation of the drugs' inhibitory or activating effects on this enzyme is therefore necessary and useful. Beydemir and Scott have respectively investigated the *in-vitro* drug-PON interactions by measurement of the changed PON activity after addition of drugs to the system[10-13]. In the evaluation of PON-drug interactions, Beydemir and Scott employed paraoxon as a substrate for the PON activity assay by monitoring the UV absorbance of the hydrolytic product (4-nitrophenol) at 405 nm[10-13]. Thus the evaluation is hampered for a considerable number of drugs which have overlapped UV absorption around the monitored wavelength.

Phenyl acetate is another widely used substrate

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for serum arylesterase activity assay by monitoring the UV absorbance of the hydrolytic product (phenol) at 270 nm [14]. Compared with the organophosphates, it has the advantages of simple performance, easy availability and low toxicity. However, Beydemir and Scott used paraoxon rather than this substrate for the investigation of drug-PON interaction [10–13], probably because the studied drugs showed UV absorption around 270 nm overlapped with that of the monitored hydrolytic product of phenyl acetate, and so phenyl acetate was not suitable for their researches.

The chemiluminescent CPOCMA [9- (4-chlorophenyloxylcarboxyl) - 10- methylacridinium triflate ester] was previously synthesized and successfully used as a substrate for serum PON activity assay in our lab [14]. Compared with the UV method based on phenyl acetate, the chemiluminescent method based on the CPOCMA for serum PON activity assay showed high sensitivity and selectivity; and compared with the UV method based on paraoxon, it showed advantage of low toxicity, easy performance, and high sensitivity. Therefore, the chemiluminescent method based on the CPOCMA would be an alternative tool for PON-drug interaction research. Additionally, the acridinium esters and acridinium sulfonamides give off chemiluminescence around 430 nm[15–16] under proper triggering conditions [17–19]. This wavelength is longer than those UV absorptive wavelengths for the hydrolytic products of phenyl acetate and paraoxon, and hence the CPOCMA would be suitable for the drug-PON interaction research in different scope of application. Here, we intended to establish a chemiluminescent method based on CPOCMA for *in-vitro* evaluation of drug effects on PON activity.

The indometacin, aspirin and acetaminophen widely used for treatment of fever, tooth ache, nerve neuralgia and rheumatism were employed as model drugs. Evaluation of these drug effects would reveal their potential risks.

1 Materials and methods

1.1 Materials

The chemiluminescence test was performed on an IFFM-E flow-injection chemiluminecent analyzer (Ruimai, Xi'an, China); and the UV absorbance was measured on a UV spectrophotometer (Puxitongyong, Beijing, China). The CPOCMA and aspirin were self-prepared and identified with NMR. The trinitroglycerine was extracted from the trinitroglycerine pills (Yimin pharmaceutical Co. Ltd, Beijing, China) with ethyl acetate. The phenyl acetate of analytical grade was purchased from Aladdin Chemistry Co. Ltd (Shanghai, China). Indometacin (>98.5%) and acetaminophen (>99%) were made by Wuhan Xinjialin Biological Co. Ltd and Xi'an Zaolutang Pharmaceutical Co. Ltd, respectively. The $CaCl_2$, H_2O_2, CTAB, HNO_3 and NaOH made in China were of analytical grade. The blood serum samples in a routine physical examination were collected from the clinically analytic laboratory in the staff hospital in Chongqing University. Distilled-deionized water was used through out.

1.2 Chemiluminescent method for measurement of PON activity

1.2.1 The chemiluminescent test mode. A flow-injection mode was used for the tests, and the procedure was illustrated in Figure 1 [14]. The test solution, triggering reagent 1 (0.25 mol/L HNO_3+0.1% H_2O_2), and triggering reagent 2 (1.0 mol/L NaOH + 1.0×10^{-3} mol/L CTAB) were driven by peristaltic pumps *via* ports 1, 2 and 3, respectively. The test solution and triggering reagent were blended in V_1, and then triggering reagent 2 was introduced into the same passage of the above mixture by changing the valve passage position (V_2). By self-diffusion, the chemiluminescence is triggered. The chemiluminescence is recorded automatically and the intensity was calculated according to the integral of peak area.

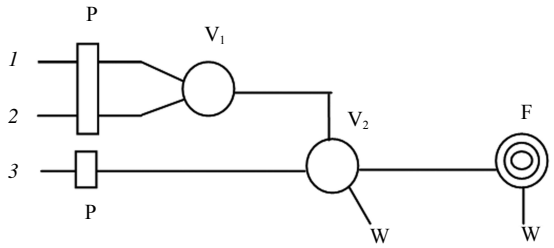

Fig. 1 The chemiluminescent test scheme for the flow-injection mode

P: Peristaltic pump; V_1: Three-way valve; V_2: Six-way valve; W: Waste solution; F: Flow cell for detection; Port *1*: Test solution; Port *2*: Triggering reagent 1 (HNO_3 +H_2O_2); Port *3*: Triggering reagent 2 (NaOH+CTAB).

1.2.2 A procedure for chemiluminescent evaluation of drug effects on PON activity. A requisite amount of CPOCMA was dissolved in acetonitrile to prepare the

stock solution (1.01×10^{-3} mol/L), followed by gradual dilution with acetonitrile to prepare the ready-to-use solution (1.01×10^{-6} mol/L). The stock solutions of drugs, Tris-HCl buffer, and serum were added to a volumetric flask, and then distilled H_2O was added to scale, followed by addition of CPOCMA. Then the mixed solution was incubated for a period at 25℃. Unless otherwise stated, the final test solution contained Tris-HCl buffer (10 mmol/L, pH 7.5), 3000-fold diluted serum, and CPOCMA (4.04×10^{-9} mol/L). Afterwards, the chemiluminescence was tested. A blank solution in absence of serum and a control solution in absence of drug were prepared, and their chemiluminescent intensities were recorded. 1U of arylesterase activity is defined as 1 μmol of CPOCMA hydrolysis per minute.

1.2.3 The UV procedure by using phenyl acetate for PON activity assay. The test solution containing 2.0 mmol/L phenyl acetate, 2.0 mmol/L $CaCl_2$, 300-fold diluted serum, and trinitroglycerine in 10 mmol/L Tris-HCl buffer pH 8.0 was incubated at 37℃. The UV absorbance of the hydrolytic product phenol at 270 nm was determined after incubation for 0, 1.5, 3.0, 4.5, 6.0, and 7.5 minutes, respectively. The reference solution was prepared with a similar procedure that used for the test solution without addition of phenyl acetate to exclude the UV absorption of serum. The molar absorptive coefficient 1.63×10^{3} L•mol^{-1}•cm^{-1} for phenol was assumed for calculation of hydrolysis[14].

2 Results and discussion

2.1 Optimization of the conditions for *in-vitro* evaluation of the drug effects

In the procedure for serum PON activity assay, stable and reliable chemiluminescent signal of the CPOCMA-serum system is crucial for evaluation of drug effect. Therefore, CPOCMA concentration and serum dilution fold were investigated.

2.1.1 The substrate concentration. The serum arylesterase assay is based on the assumption that the chemiluminescent intensity is proportional to the CPOCMA concentration. Under the instrumentally permitted conditions, the chemiluminescent signal was proportional to CPOCMA concentration in the range of 2 orders of magnitude. Good linearity between chemiluminescent intensity and CPOCMA concentration in the range $1.01\times10^{-9}\sim8.08\times10^{-8}$ mol/L was showed in Figure 2. In our research the CPOCMA concentration of 4.04×10^{-9} mol/L was used in the following section. The within-day RSD for the chemiluminescence of the CPOCMA solution (4.04×10^{-9} mol/L) was less than 1.2%(n=5). It means in this concentration range, the CPOCMA provided a stable chemiluminescent signal.

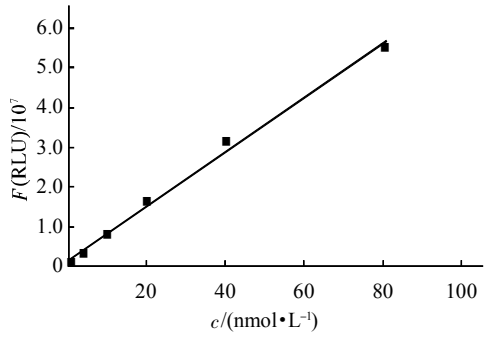

Fig. 2 The chemiluminescent intensity of the CPOCMA against its concentration

2.1.2 The serum concentration. After fixing the CPOCMA concentration, the serum concentration was optimized. Because sera from different sources (healthy subjects) possess different PON activity, it is difficult to give a specific optimized range of serum concentration. Despite of the disadvantage, we employed serum rather than pure PON, due to that serum provides a physiological environment. And the disadvantage can be overcome by fixing the reaction kinetics of CPOCMA-serum system rather than fixing the serum concentration. With a suitable serum concentration the first-order reaction kinetics for the serum-mediated and the drug-serum-mediated CPOCMA hydrolysis should be detected reproducibly within 15 min. The first-order reaction kinetics for the serum-mediated and the drug-serum-mediated CPOCMA hydrolysis were illustrated in Figure 3a.

In our research the 3000-fold diluted serum conformed to the above requirements, and the variation coefficient of chemiluminescent signal for the serum-CPOCMA system after incubation of 15 min was within 5.1%. If substrate COCMA concentration would change, the serum concentration should change consequently to ensure detectable hydrolysis.

2.2 The validation of the chemiluminescent method for PON-drug interaction with a UV method

The serum-mediated hydrolysis of CPOCMA was

strongly dependent of serum arylesterase activity (serum concentration), and so serum arylesterase activity can be detected by CPOCMA hydrolysis. However, for caution, the method would be validated with the UV method by using phenyl acetate. Trinitroglycerine is used as a model compound for validation. The reaction kinetics for CPOCMA hydrolyses and for phenyl acetate hydrolyses under various conditions is illustrated in Figure 3.

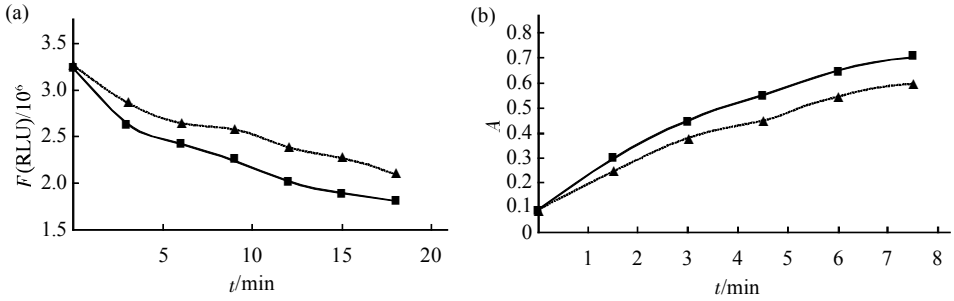

Fig. 3 The reaction kinetics for the hydrolysis of substrates

(a) The hydrolysis of CPOCMA; (b) The hydrolysis of the phenyl acetate. ■—■ The serum-mediated hydrolyses, ▲---▲ : The trinitroglycerine-serum mediated hydrolyses.

In Figure 3a, the chemiluminescence of the CPOCMA after incubation with serum decayed due to hydrolysis. The reaction velocity constants for the serum-mediate hydrolysis and for the trinitroglycerine-serum mediated hydrolysis were calculated as 0.15 and 0.13 min^{-1}, respectively (The calculation was based on the assumption that the hydrolysis followed the first-order kinetics[14]). By comparing the two curves in Figure 3a, it shows that addition of trinitroglycerine (2.20 mmol/L) slowed down the serum-mediated CPOCMA hydrolysis. It means that based on this chemiluminescent method, the trinitroglycerine is a serum arylesterase inhibitor. From the solid line in Figure 3b, the hydrolytic product (phenol) was converted from phenyl acetate under incubation with serum. By comparing the two curves in Figure 3b, it means that trinitroglycerine (2.20 mmol/L) inhibited the serum-mediated hydrolysis of phenyl acetate. It suggests that based on the UV method, trinitroglycerine is an arylesterase inhibitor. According to the conclusion drawn above, overdose intake of trinitroglycerine for long term is possibly harmful.

The inhibitory effects of the model compound at various concentrations were further investigated. The results are illustrated in Figure 4. As showed in Figure 4, the velocity constant of the serum-mediated CPOCMA hydrolysis decreased as the trinitroglycerine concentration increasing. It hints the trinitroglycerine showed enhancing inhibitory effect as its concentration increased. A similar inhibitory effect of trinitroglycerine was observed if the detection was based on the phenyl acetate as a substrate.

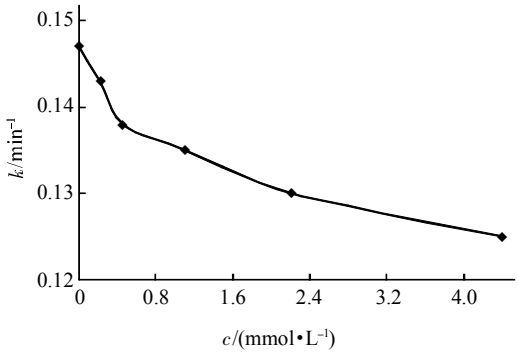

Fig. 4 The kinetic constants for the nitroglycerine-serum mediated CPOCMA hydrolysis against nitroglycerine concentration

In conclusion, the chemiluminescent method is consistent with the UV method.

2.3 *In-vitro* evaluation of the drug effects

With the chemiluminescent method, the effects of three anti-inflammatory drugs on serum PON activity were evaluated by the kinetics of the drug-serum-mediated CPOCMA hydrolysis, IC_{50} value (half inhibitory concentration) and Michaelis constant k_m.

2.3.1 The reaction kinetic curves. Figure 5 described the reaction kinetics of the drug-serum-mediated CPOCMA hydrolysis within 15 min. All the curves for the three drugs were located above the control curve. It means slowdown of the reaction kinetics caused by separate addition of each drug (0.50 g/L) and that all the drugs separately showed inhibitory effects on serum PON activity. The kinetic curves suggested that the inhibitory effects were ranked in the order: indometacin>aspirin>acetaminophen.

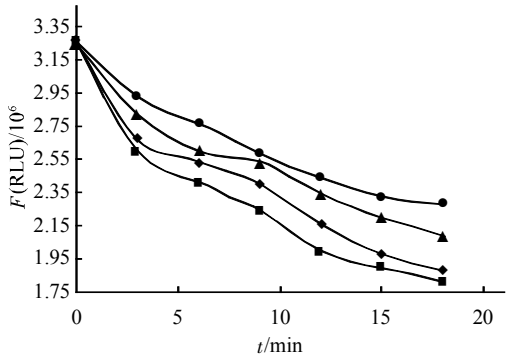

Fig. 5 The reaction kinetics of the drug-serum-mediated CPOCMA hydrolysis

The CPOCMA concentration was 4.04 nmol/L, and the serum was 3000-fold diluted. The test solution was incubated at 25℃; and the chemiluminescence of the test solutions was recorded at intervals of 3 min. ■—■: Control; ♦—♦: Acetaminophen; ▲—▲: Aspirin; ●—●: Indometacin;

2.3.2 The IC_{50} values. The inhibited serum PON activity by the drugs of different concentrations were further determined. The serum PON activity was plotted against drug concentration to give Figure 6. It showed that the serum PON activity was largely inhibited as the drug concentration increasing. The IC_{50} values for indometacin, aspirin and acetaminophen were listed in Table 1. According to the IC_{50} values the inhibitory effects were ranked as follows: indometacin>aspirin>acetaminophen. These IC_{50} values were among the dose range of effective plasma concentration.

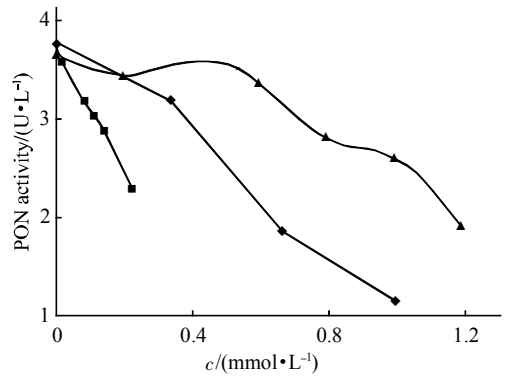

Fig. 6 The serum PON activity plotted against concentration of drugs

The CPOCMA concentration was 4.04 nmol/L, and the serum was 3000-fold diluted. The incubation period was 3 min. ■—■: Indometacin; ♦—♦: Aspirin; ▲—▲: Acetaminophen.

Table 1 The inhibitory effects of the drugs

Drug	c/ (mmol•L^{-1})	k_m'/ (nmol•L^{-1})	k_i/ (mmol•L^{-1})	Average k_i/ (mmol•L^{-1})	IC_{50}/ (mmol•L^{-1})
Acetaminophen	0.662	65.7	3.15	2.98	0.972
	0.992	73.6	2.80		
Aspirin	0.666	68.5	1.43	1.38	0.656
	0.888	77.8	1.34		
Indometacin	0.112	57.9	0.21	0.154	0.254
	0.224	123.9	0.099		

2.3.3 The Lineweaver-Burk curves and inhibitory type The inhibitory type was investigated by using the Lineweaver-Burk curves showed in Figure 7[10–13]. The three Lineweaver-Burk curves for acetaminophen at concentration of 0, 0.662, and 0.992 mmol/L intersected at the ordinate, and they showed identical V_{max} but different k_m value. It means acetaminophen displayed competitive inhibitory effect. The apparent k_m values were listed in Table 1. The increase of the apparent k_m value caused by addition of drug further demonstrated the inhibition. The Lineweaver-Burk curves for the other two drugs in Figure 7. demonstrated that the indometacin and aspirin were also competitive inhibitors of PON, and the apparent k_m values were shown in Table 1. The inhibitory effect of the discussed drugs may result from competitive binding with serum PON. For a reversible competitive inhibition, the inhibitory constant k_i was calculated according to the following formula $k_m'=k_m\times(1+[I]/k_i)$, where k_m is the original apparent Michaelis constant, and k_m' is the apparent Michaelis constant changed by an inhibitor; and the [I] is the inhibitor concentration. The k_i values for drugs of various concentrations and the average k_i values for the drugs were listed in Table 1. According to the k_i values, the inhibitory ability of the drugs was ranked, and the ranked order was same as the kinetic constants and the IC_{50} values demonstrated.

It means administration of the drugs possibly increases the risk of PON-related diseases by inducing decrease of serum PON activity.

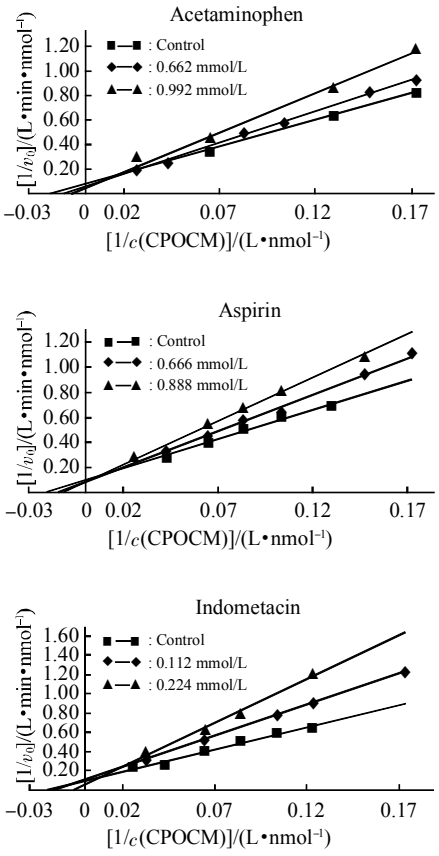

Fig. 7 The Lineweaver-Burk curves inhibited by the three drugs

The CPOCMA concentration was in the range of 5.71～40.0 nmol/L, the serum was 3000-fold diluted. The chemiluminescnce of the test solutions were measured at intervals to obtain the initial reaction velocity $1/v_0$. The drug concentration was indicated in the picture.

2.3.4 Method applicability.

Each of the three drugs demonstrated significant UV absorption around 270 nm (Figure 8). Therefore, addition of the mentioned drugs greatly affects the determination of serum PON activity based on phenyl acetate, because the monitored UV absorption of the hydrolytic product phenol was overlapped the absorption of the drugs. It means the phenyl acetate is not suitable for the evaluation of the three drug effect on PON activity.

From the UV spectra of the three drugs in Figure 8, it is clear that for indometacin there shows weak

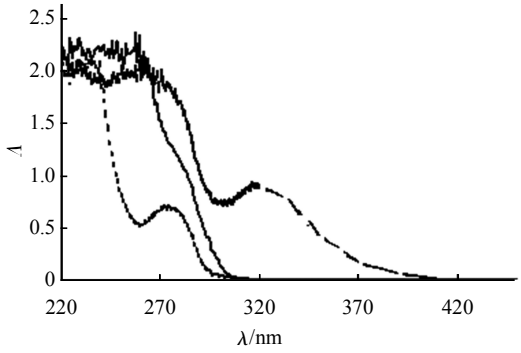

Fig. 8 The UV spectrum of the three anti-inflammatory drugs

The drug aqueous solutions of indometacin (0.126 mmol/L), aspirin (0.749 mmol/L), and acetaminophen (0.595 mmol/L) were used in the UV scanning. ----: Indometacin; ·····: Aspirin; ——: Acetaminophen.

absorption at 405 nm overlapping the absorption of the hydrolytic product of paraoxon (4-nitrophenol with λ_{max}=405 nm[9]), and so paraoxon is not a suitable substrate for the evaluation of indometacin. For aspirin and acetaminophen, they did not show any absorption around 405 nm. Hence the paraoxon possibly is a suitable substrate for evaluation of the aspirin and acetaminophen. However, purchase and use of paraoxon are generally prohibited in China due to its extremely high toxicity. In all, the UV spectrum is helpful to choose a suitable UV substrate. Fortunately, sometimes the UV absorption can be predicted by the molecular structure, because the λ_{max} of a compound can be approximately calculated according to woodward rule and Scott rules. The UV absorption at 270 nm for aspirin acetaminophen and indometacin can be foreseen by the phenyl moiety in their structures.

In the evaluation of the three drugs, the UV method based on phenyl acetate showed disability. The chemiluminescent method based on CPOCMA showed unique advantage due to the emitted wavelength of the substrate, which does not overlap with the absorption of the three drugs. Despite the advantage, CPOCMA is not suitable to evaluate the drugs which influence the CPOCMA chemiluminescence such as strong reductive or oxidative molecules, because CPOCMA chemiluminescence is triggered by oxidation of CPOCMA with H_2O_2 or reduction with Zn[15−19].

In all, a chemiluminescent method based on

CPOCMA was established for the evaluation of *in-vitro* inhibitory drug effects on PON activity.

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化学发光法用于芳香酯酶抑制剂体外筛选 *

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摘要　在前期的研究中，我们将 9-(4- 氯苯氧羰基)-10- 甲基吖啶酯三氟甲基磺酸盐(CPOCMA)用于测定血清芳香酯酶活性，取得满意结果．在此基础上，本文以 CPOCMA 为底物，建立化学发光法评估药物对芳香酯活性影响的新方法．以硝酸甘油为模型药物，比较了化学发光法与 UV 方法的一致性．并将此法应用于评价三种抗炎药吲哚美辛、阿司匹林和乙酰氨基酚对芳香酯酶活性的影响．药物的加入使血清催化 CPOCMA 水解的动力学减缓，这表明这些药物均为抑制剂．吲哚美辛、阿司匹林和乙酰氨基酚表现出的 IC_{50} 值分别为 0.254、0.564 和 0.656 mmol/L，抑制常数 k_i 分别为 0.154、1.38 和 2.98 mmol/L．加入药物后的 Lineweaver-Burk 曲线表明这三种药物对 PON 的抑制均为竞争性抑制．根据加入药物后的动力学曲线，其 IC_{50} 值、抑制常数和米氏常数的变化均表明这三种药物的抑制能力大小顺序：吲哚美辛 > 阿司匹林 > 乙酰氨基酚．CPOCMA 可以作为 PON 底物体外评价药物对 PON 的抑制能力和抑制机理．UV 法不适合评价紫外吸收峰与 UV 法的检测波长重叠的药物，而新建立的化学发光法对这类药物的筛选有独特优势．

关键词　PON 芳香酯活性，吖啶酯，化学发光，抑制剂筛选

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