Science in China Series B: Chemistry

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Microcalorimetric investigation of the effect of berberine alkaloids from *Coptis chinensis* Franch on *Staphylococcus aureus* growth

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The inhibitory effects of three berberine alkaloids (BAs) from rhizome of *Coptis chinensis* Franch, a traditional Chinese medicinal (TCM) herb, on *Staphylococcus aureus* growth were investigated by microcalorimetry. The power–time curves of *S. aureus* with and without BAs were acquired; meanwhile the extent and duration of inhibitory effects on the metabolism were evaluated by studying the growth rate constant (k), half inhibitory ratio (IC_{50}), maximum heat-output power (P_{max}), peak time of maximum heat-output power (t_p) and total heat production (Q_t). The value of k of *S. aureus* in the presence of the three BAs decreased with the increasing concentrations of BAs. Moreover, P_{max} was reduced and the value of t_p increased with increasing concentrations of the three drugs. The inhibitory activity varied with different drugs. The values of IC_{50} of the three BAs are respectively, 101.4 μg/mL for berberine, 241.0 μg/mL for palmatine and 792.3 μg/mL for jateorrhizine. The sequence of antimicrobial activity of the three BAs is: berberine > palmatine > jateorrhizine. It is suggested that the functional group methylenedioxy or methoxyl at C2 on the phenyl ring could possibly improve antimicrobial activity more strongly than hydroxyl at C2 on the phenyl ring.

berberine alkaloid, microcalorimetry, *Staphylococcus aureus*, inhibitory effect

1 Introduction

Coptis chinensis Franch (Huanglian in Chinese) is a traditional Chinese medicinal (TCM) herb, and is officially listed in the Chinese Pharmacopoeia[1]. The *C. chinensis* Franch extract has strong antibacterial activity and is used for treating dysentery, cholera, leukemia, diabetes and lung cancer[2]. The major active components of the herb are berberine alkaloids (BAs), which are often used as criteria in the quality control of Huanglian products. BAs are also active components in large numbers of plant-derived drugs such as antimicrobials from *Berberidaceae* and *Rutaceae* family. In this study, the inhibitory effects of three BAs, i.e. berberine, palmatine and jateorrhizine, on *Staphylococcus aureus* (*S. aureus*) were investigated by microcalorimetry.

S. aureus is one of the most common pathogen bacteria. Its clinical course is very extensive, ranging from transient uncomplicated bacterial infection to a complicated course of septic embolization[3].

Microcalorimetry is a quantitative, inexpensive and versatile method for measuring the heat production in many fields that can be applied to various reactions of physics, chemistry and life science[4,5]. Actually, microcalorimetry is very suitable for the survey of heat-output of slightly exothermic or endothermic processes, such as the heat production of microbial cells, organelles, tissues and organs. It can provide a general analytical tool for characterization of cell growth processes and has been extensively used to investigate the interaction between

Received January 25, 2008; accepted February 4, 2008
doi: 10.1007/s11426-008-0072-x
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Supported by the National Natural Science Foundation of China (Grant No. 30625042)

drug and cultured cells. Any substances that could modify cellular metabolism would change the power-time curve (*P-t*) obtained from the microcalorimeter and from the heat output curves, not only thermodynamics data but also kinetic data can be obtained[6−9]. Although calorimetry is completely nonspecific, it often affords a valuable property for the monitoring of complex and poorly characterized biological systems. Calorimetry is useful and has been used to monitor cellular metabolism and the whole organism for a long time[10−15].

As we know, the diameter of the inhibition zone in the cup-plate method and the turbidity value of nephelometry are usually used to evaluate the bioactivity of drugs in vitro, especially antibiotic drugs. Both methods can reveal antibiotic activity, but they can hardly show how the antibiotic process happens kinetically.

In this work, we propose a new method to assay drug bioactivity by using microcalorimetry. It focuses on the energy change of microbial growth. It is an online, kinetic and precise method to measure the bioactivity of drugs. We report here, for the first time, the development of microcalorimetry method to TCM for the investigation of effect of BAs in *Coptis chinensis* on the growth of *S. aureus* and discuss the structure-function relationship preliminarily.

The heat-production plots of three tested BAs on *S. aureus* were determined by the thermopile of a heat conduction microcalorimeter. The inhibition of biochemical reactions in the cells by drugs was studied. The growth rate constant k, maximum heat-output power P_{max}, peak time of maximum heat-output power t_p and the total heat production Q_t of different drugs were calculated. According to these values, the antibacterial activities of three tested BAs were evaluated quantitatively.

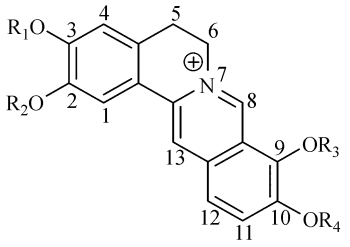
2 Materials and methods

2.1 Instrumentation

An 8-channel heat conduction calorimeter (TAM air, Thermometric AB, Sweden) was held together in a single removable block for heat flow measurements in the milliwatt range under isothermal conditions. This block was placed in an air thermostat, which kept the temperature within 0.02℃ indicating temperature gradient to be very low. All calorimetric channels were of twin type, consisting of a sample and a reference vessel. Each vessel was connected to the surrounding heat sink by a Peltier module, and when heat was produced or consumed due to any process, the temperature of the sample vessel changed. The temperature of surrounding was constant and thus a temperature gradient across the Peltier module was developed. This would generate a measurable voltage and the voltage was proportional to the heat flow across the Peltier module and to the rate of the processes taking place in the sample vessel. Such voltage signals were recorded continuously and in real-time through an 8-channel data logger. The software supplied to the calorimeter was used to monitor and record the heat flow over the Peltier module when the baseline drift was less than 20 μW over 24 h.

2.2 Materials

S. aureus (CCTCC AB910393) was provided by the Chinese Center for Type Culture Collections, Wuhan University. *S. aureus* was grown in a peptone culture medium, which contained 10 g peptone, 5 g beef extract and 5 g NaCl per liter. Medium pH was adjusted to 7.0 −7.2 with 1 mol/L NaOH and 1 mol/L HCl before autoclaving. Berberine, palmatine and jateorrhizine were supplied by National Institute for the Control of Pharmaceutical and Biological Products in Beijing (purity > 95%). The three BAs were extracted from *C. chinensis* Franch and their structures are given in Figure 1.



	R_1	R_2	R_3	R_4
Berberine	—CH_2—		CH_3	CH_3
Palmatine	CH_3	CH_3	CH_3	CH_3
Jateorrhizine	H	CH_3	CH_3	CH_3

Figure 1 Chemical structures of three tested BAs.

2.3 Preparation of the samples

At the beginning of the experiments, *S. aureus* was inoculated into the peptone medium, with 2×10^6 cells per mL. Cells were suspended in the peptone culture me-

dium, and the fresh prepared BAs solutions by using water with different concentrations were added to the cell suspension.

2.4 Experimental procedure

The microcalorimeter was thermostated at 37℃, and the measurement was made with the ampoule method. All the ampoules containing the cell suspension of *S. aureus* and one of the BAs were sealed up and put into the 8-channel calorimeter block. After about 30 min (the temperature of ampoules reached 37℃), the thermogenic curves were recorded until the recorder returned to the baseline. Since the bacterial metabolic process was monitored under the isothermal and isochoric conditions, the nutrient and oxygen consumed by cells were surely limited. All data were collected continuously by using the dedicated software package.

3 Results

3.1 The growth rate constants of *S. aureus* with and without drugs

Figure 2 shows the thermogenic curves of *S. aureus* without drugs at 37℃. Since the *S. aureus* metabolic process was monitored in isothermal and isochoric conditions, the nutrient and oxygen consumed by cells were supplied limitedly. The growth curve of *S. aureus* could be divided into four phases: exponential phase (A-B), lag phase (B-C), stationary phase (C-D) and decline phase (D-E)[8]. The exponential metabolism model of *S. aureus* could be used in the growth processes[16]:

$$\ln(P_t/P_0) = kt \quad (1)$$

where P_0 represents the heat output power at the beginning of baseline and P_t represents that at time t. The thermogenic curves of the exponential phase of growth correspond to Eq. (1), while the growth rate constant k can be obtained by fitting ln P_t and t to a linear equation. The growth rate constant k of *S. aureus* is shown in Table 1, and $k = (1.22682 \pm 0.00218)\ h^{-1}$ indicates a good reproducibility.

The thermogenic curves of *S. aureus* with drugs were monitored by the microclarometer. The *P-t* curves of *S. aureus* at different concentrations of drugs show that the peak time of maximum heat-output power t_p was prolonged and the growth rate constant k decreased with the increase of concentrations of BAs. All correlation coefficients were larger than 0.9680, indicating a good linear correlationship between k and the corresponding concentration.

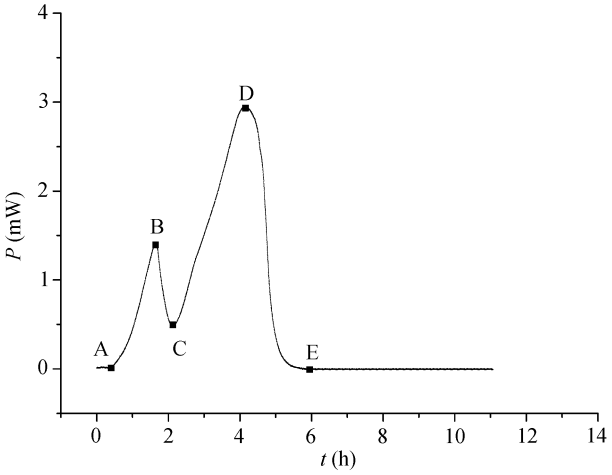


Figure 2 *P-t* growth curves of *S. aureus* cultured in the peptone culture medium and monitored by the microcalorimeter at 37℃.

These results demonstrate that all the three BAs inhibited the growth of *S. aureus* and additionally, the inhibitory activities increased with the increase of concentration of the tested drugs. Thus, the growth rate constant decreased gradually, and the reduction degree of k indicates the antimicrobial activities of the drugs. However, the duration of the stationary phase observed was suppressed more remarkably than the exponential growth phase in the presence of BAs compounds. The different inhibitory activities of three drugs are demonstrated in Figure 3.

3.2 Peak time t_p and total heat production Q_t

It is possible that the exponential phase is aerobic metabolism and the stationary phase is anaerobic metabolism. It might be because that BAs killed some of the bacteria so that it took longer time to generate detectable signals. With the increase of concentrations of three BAs, the values of t_p became longer. Accordingly, the total heat production Q_t decreased gradually. Therefore, the values of t_p and Q_t are also listed and used to show antibacterial activities of three BAs (Table 2).

Table 1 Growth rate constant k of *S. aureus* cultured in the peptone culture medium and monitored by the microcalorimeter at 37℃

Experiment No.	1	2	3	4	5	6	RSD[a] (%)
k/h^{-1}	1.22786	1.22581	1.22613	1.22893	1.22329	1.22890	0.18

a) RSD is relative standard deviation.

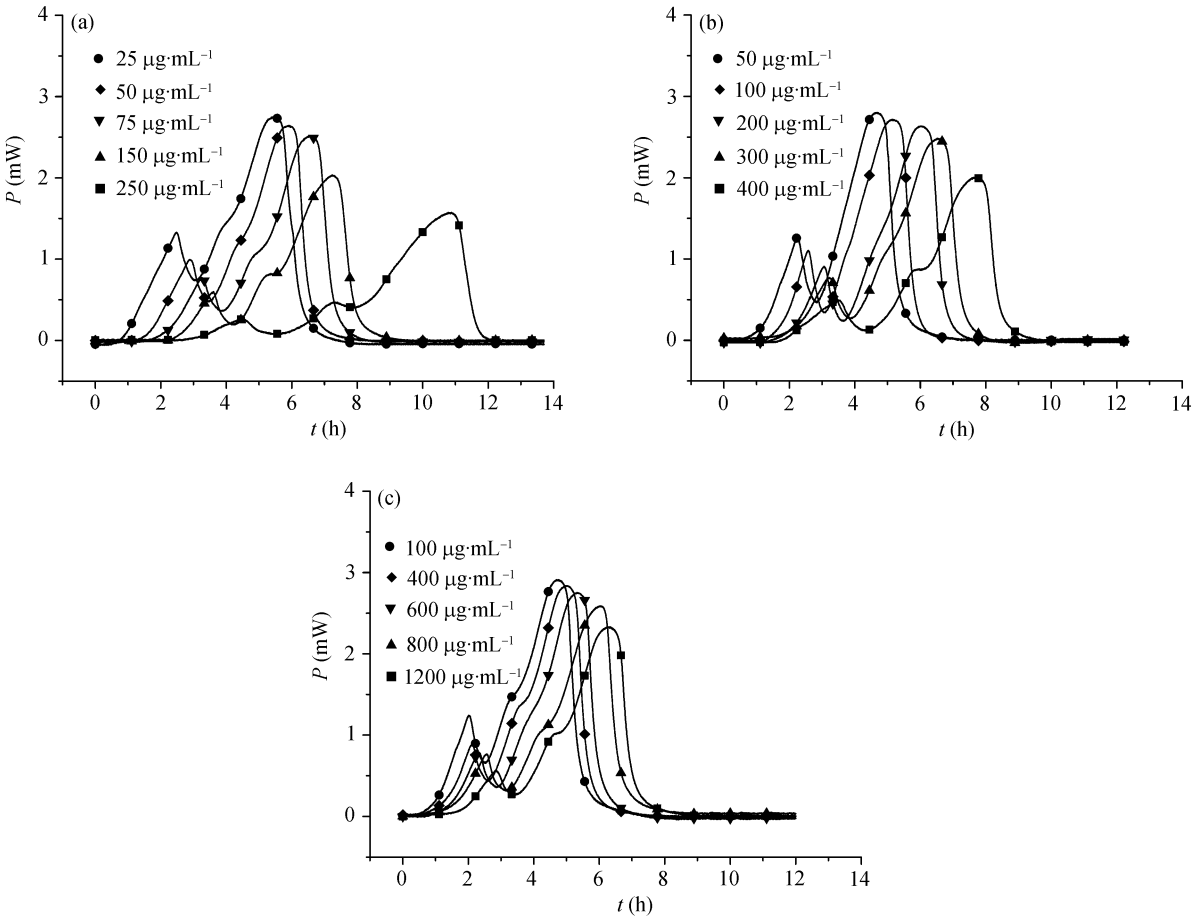


Figure 3 *P-t* curves of *S. aureus* growth with different concentrations of berberine (a), palmatine (b) and jateorrhizine (c). *S. aureus* was cultured in peptone culture medium supplemented with different concentrations of three BAs respectively, and monitored by the microcalorimeter at 37℃.

Table 2 The values of k, peak time t_p, maximum heat-output power P_{max}, inhibitory ratio I and total heat production Q_t of *S. aureus* with different concentrations of three BAs ($\overline{X} \pm SD$, $n = 6$)

Compd.	c (μg·mL^{-1})	k (h^{-1})	t_p (min)	P_{max} (mW)	Q_t (J)	I (%)
Control	–	1.22682±0.00218	249±1.78	2.95±0.13	25.6±0.15	–
Berberine	25	0.82512±0.00167	326±2.15	2.75±0.17	24.7±0.21	32.7±0.92
	50	0.76318±0.00211	354±1.76	2.64±0.12	22.2±0.11	37.8±1.12
	75	0.64489±0.00098	396±1.75	2.52±0.08	20.8±0.19	47.4±0.77
	150	0.53078±0.00143	435±1.81	2.03±0.10	17.5±0.14	56.7±1.58
	250	0.18530±0.00286	647±2.42	1.57±0.05	17.0±0.09	84.9±1.21
Palmatine	50	0.97273±0.00271	281±1.58	2.80±0.20	21.5±0.07	20.7±0.66
	100	0.94172±0.00219	309±1.67	2.71±0.14	20.2±0.18	23.2±0.79
	200	0.64276±0.00189	362±1.75	2.63±0.07	19.5±0.12	47.6±1.02
	300	0.50282±0.00152	391±1.85	2.48±0.13	18.9±0.15	59.0±0.64
	400	0.31117±0.00249	462±1.82	2.01±0.18	16.1±0.08	74.6±1.82
Jateorrhizine	100	0.99649±0.00282	285±1.92	2.91±0.11	24.6±0.04	18.8±0.95
	400	0.77422±0.00301	301±1.76	2.84±0.08	22.2±0.12	36.9±1.62
	600	0.69371±0.00223	319±1.70	2.75±0.15	21.1±0.17	43.5±1.42
	800	0.54614±0.00334	361±1.64	2.59±0.23	20.5±0.06	55.5±0.89
	1200	0.47125±0.00260	377±1.73	2.33±0.09	19.8±0.19	61.6±1.76

3.3 Inhibitory ratio *I* and the half inhibitory concentration IC_{50}

The inhibitory ratio I is defined as in eq. (2), where k_0 is the rate constant of the control in the exponential phase; k is the rate constant in the exponential phase of bacterial growth inhibited by inhibitors with a concentration of c[8].

$$I = [(k_0 - k)/k_0] \times 100\% \quad (2)$$

IC_{50} means that ratio I is 50%, which can be regarded as the inhibiting concentration to cause a 50% decrease of the *S. aureus* growth rate constant. To demonstrate the inhibitory effects of various BAs on *S. aureus*, the values of I are also shown in Table 2. Then IC_{50} can be calculated to be 101.4 μg/mL for berberine, 241.0 μg/mL for palmatine and 792.3 μg/mL for jateorrhizine, respectively. Considering the values of the growth rate constant and the half inhibitory concentration, it can be easily concluded that berberine and palmatine showed stronger inhibitory effects on *S. aureus* than jateorrhizine. Thus, the sequence of antimicrobial activity of the tested BAs is berberine > palmatine > jateorrhizine. Basically, these antibacterial activity results are the same as those in ref. [17].

4 Discussion

The thermogenic curves of *S. aureus* growth affected by various BAs from *C. chinensis* Franch indicate that all the tested drugs have inhibitory effects on the tested bacteria.

When the growth rate constant k, total heat production Q_t and P_{max} decrease and t_p is prolonged, the antibacterial activity of drugs is enhanced. As to three BAs, these alterations of parameters demonstrated that they can inhibit the growth of *S. aureus*. Compared with the control (peptone culture medium without drug), P_{max} and k of the same concentration of berberine, palmatine and jateorrhizine increased while the t_p values of them were shortened gradually. This implies that not only the pharmacological antibacterial activity of the tested BAs has the following order: berberine > palmatine > jateorrhizine, but also their antibacterial activities are weakened gradually.

The t_p was prolonged with the increasing concentrations of all the tested BAs. Berberine and palmatine showed stronger inhibitory effects on *S. aureus* than jateorrhizine. All the three BAs are berberine alkaloids with only differences in the substituents at C2 and C3 of the phenyl ring (Figure 1). It is suggested that the functional group methylenedioxy or methoxyl at C2 on the phenyl ring can possibly improve antimicrobial activity more strongly than hydroxyl at C2 on the phenyl ring. Thus, the functional group methylenedioxy or methoxyl at C2 can be the major groups inducing the action of bacteriostasis of BAs.

Some traditional techniques can also be used to evaluate the antimicrobial activity of three BAs, but they all have some disadvantages, such as complexity, time consuming, insufficient information and so on. However, microcalorimetry is an established procedure to offer quantitative measurements and distinct advantages over traditional antimicrobial test methodologies; calorimetric measurements are continuous and real-time, thus the dynamic response of microorganisms to antimicrobial agents can be observed in situ. Microcalorimetry can be alternatively utilized to evaluate antibacterial activity of bioactive components extracted from TCM or other medicinal plants.

5 Conclusions and simplified description of the method

In this work, we propose a new microcalorimetric method to assay drug bioactivity. This method is appropriate to determine the heat production curve of microbes, such as *S. aureus* at a constant temperature of 37℃ and to obtain thermodynamic parameters. Additional advantages are that it generates thermodynamic information of the microbe-drug reaction when drugs are added to the culture medium. Compared with the control, the curves are usually different from each other when different drugs are added to the culture medium.

Compared with the cup-plate method and nephelometry, microcalorimetry not only offers a new point of view for the evaluation of bioactivity of drugs but also provides more information about the microbial growth. By using it, the energy changes of four growing periods of *S. aureus*, which represent the regularity of microbial population growth, such as the lag phase, logarithmic phase, stationary phase and decline phase, can be distinguished from the heat production curve. Values of P_{max} (maximum heat-output power), t_p (peak time of maximum heat-output power), k (growth rate constant), as well as AUC (area under curve) for power-time

curves are all determined simultaneously which can describe the heat growing production and the metabolic process of microbes dynamically and precisely.

The essential features of the microcalorimetry method are based on the universal heat exchange are involved in all biochemical reactions. Characterized by two-dimension, it can reflect the microbe growth and state completely and more directly. It can provide the possibility of using this method in a wide range of drugs to determine their antibiotic activity and supply these thermograms as a profile characterized with fingerprint to research the bioactivity of drugs.

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