J Therm Anal Calorim (2012) 109:1069–1075
DOI 10.1007/s10973-011-1804-2

Detection of microorganisms in different growth states based on microcalorimetry

A challenging test for establishing a rapid and reliable sterility method

Manrong Tan · Yongsheng Ren · Dan Yan · Xianghong Meng · Longhu Chen · Lingling Qiu · Yan Yan · Jianyu Li · Xiaohe Xiao

Received: 7 May 2011 / Accepted: 14 July 2011 / Published online: 30 July 2011

Abstract A challenging method for sterility test which was rapid and reliable had been established to assess the adaptability and robustness of the microbial under different conditions. There were material and energy metabolism or exchange with microbial on microcalorimetry, as a result this method can be served as one of the optimization of thermodynamics sterility test. Thermal power-time curves under various environmental conditions (including processing temperature, storage time, and drugs inhibition) were determined. Typical microbial growth thermal power-time curves were obtained. The curves were analyzed qualitatively and quantitatively by similarity values of bio-profiles and thermodynamics parameters, such as the exponential growth rate constant (k), detection time (T_d). The similarity showed that microbial growth curves of low processing temperature, short storage time (1 month), and Traditional Chinese Medicine injection (Shuanghuanglian, contained native compounds) inhibiting were match better with the normal than other circumstance. Thermodynamic parameters indicated that the microcalorimetric method could detect the positive bacteria within 18 h (less than 10 cfu), and more quickly identify the different states of the bacterium growth and metabolism than routine sterility. In conclusion, characterized by of the specific and strong two-dimensional information, microcalorimetry could supply thermograms as biological profiles to describe the microbial activity under different conditions, which were not only used as a rapid and reliable identification of microbial, but also as a method for sterility test of microcalorimetry optimization.

Keywords Microcalorimetry · Sterility test · *Staphylococcus aureus* · *Escherichia coli* · *Aspergillus niger* · *Common environmental strains* · Growth states

Manrong Tan and Yongsheng Ren contributed equally to this study.

M. Tan · Y. Ren · D. Yan (✉) · L. Chen · L. Qiu · Y. Yan · J. Li · X. Xiao (✉)
China Military Institute of Chinese Materia Medica, 302 Military Hospital of China, 100 West 4th Ring Middle Road, Beijing 100 039, People's Republic of China
e-mail: yd277@126.com

X. Xiao
e-mail: pharmcy302@126.com

M. Tan
Pharmacy College, Jiangxi University of TCM, Nanchang 330 004, People's Republic of China

Y. Ren
Pharmacy College, Chengdu University of TCM, Chengdu 610 075, People's Republic of China

X. Meng
The 309th Military Hospital of China, Beijing 100 094, People's Republic of China

Introduction

Sterility test is the routine inspection item claimed for security guarantee of aseptic preparation and ultimately aseptic preparations, which plays an important part in determining the production cycle. Currently, there are strict requirements of the national pharmacopoeia sterility test for injection, and basically forms a consistent inspection standards and operating procedures, so that we can effectively improve the evaluation of the level of sterile preparations. Provisions of the current pharmacopoeia sterility test methods include membrane filtration and direct inoculation [1], which also can effectively sterility test of

samples. However, the methods are limited by certain disadvantages: low sensitivity of observing culture turbidity, subjective misjudgment rate, and error-prone negative/positive results; low degree of automation, required to repeatedly involved in observation; heavy workload, increased the risk of secondary contamination; long inspection period and so on. Hereby it is high time to establish a fast method for microbial contamination of sterile preparations.

As reported, there were materials and energy metabolism or exchange with all living beings [2, 3]. Although infinitely low thermogenesis of a single microbe, the exponential replication in culture made them easy to be detected by microcalorimetry [4, 5]. Microcalorimetry was a nonspecific analytical method with high sensitivity, accuracy, and simplicity [6], which had been extensively applied in physics, chemistry, life sciences, and other fields [7–12]. Former study showed that microcalorimetry could be adopted to portray the metabolism character of different microbes, and the thermal activity fingerprint could sensitively reflect the change of culture condition or drugs affect on them [13–17], which indicted the potential application of microcalorimetry in sterility test by measuring the thermogenesis of bacterial contamination.

In this study, TAM air isothermal microcalorimeter was applied to record thermal metabolism of bacterial growth. Selecting *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, and *environmental strains* (laboratory cultured) as illustrations, we had investigated the growth of diverse microbial under different storage time, processing temperature, and drug inhibition. That the conditions were set based on the characteristics of the bacteria. The article objectively and numerically evaluated the growth status of microorganisms by thermodynamic parameters such as the exponential growth rate constant (k), detection time (T_d). Furthermore, the adaptability and robustness of microorganisms under different conditions were also probed into. As well the methods were served as one optimization of microcalorimetric sterility test.

Instruments and materials

Instruments

A TAM air 3114 isothermal microcalorimeter with TAM assistant workstation (TA Instrument, US) [18] was applied to measure the power–time curve of microorganism metabolism. This instrument included thermostat, 8 calorimetric channels with twin configuration and date logger system, could work at 5–90 °C, with measure ranges set at 600 mW, the detection limit was 2 μW, baseline drift over 24 h was less than ±10 μW [7]. Other instrument included SW-CT-2FD double-sided clean bench (Purification Equipment Factory of Suzhou).

Materials

Three strains were purchased from National Center for Medical Culture Collections (CMCC) as typical microorganisms: *S. aureus* (CMCC B26003, G^+, aerobic bacteria), *E. coli* (CMCC B44103, G^-, facultative aerobic bacteria), *A. niger* (CMCC F98003, fungi). *Environmental strains* in this study, which were a yellow mycophenolate, were separated from agar medium. Strains were stored at −70 °C with cryovial bead preservation system.

Medium: thioglycollate medium contained 15 g casein peptone, 5 g yeast extract, 5 g glucose, 0.5 g L-Cystine, and 0.75 g agar. Medium pH was adjusted to 7.2–7.4 before autoclaving. Then it was sterilized at 121 °C for 30 min, cooled rear, and stored at 4 °C for use. Modified Martin medium [Modified martin medium (MMM), lot number 090915].

Drugs: Ceftizoxime Sodium Injection (CZX) (Lot: B200908009 Specification: 0.75 g, General Pharmaceutical Factory of Harbin Pharmaceutical Group). Shuanghuanglian freeze-dried injection (SHL) (Lot: 1012241, specification: 600 mg, Harbin Pharmaceutical Group Plant Chinese medicine, It consists of *Honeysuckle*, *Baikal Skullcap*, and *Forsythia.* There are sereral principal ingredients, chlorogenic acid, baicalin, forsythin, and wogonoside).

0.9% sterile sodium chloride solution (Shijiazhuang Pharmaceutical Group IV, China).

Experimental methods

Microorganism culture

S. aureus, *E. coli*, and *environmental strains* were cultured in nutrient broth medium at 30–37 °C for 18–24 h. The bacteria were then suspended in 0.9% sterile sodium chloride solution to the approximate concentration of 10 cfu. *A. niger* were inoculated in modified martin medium at 23–28 °C for 5–7 days. The spores were eluted using 0.9% sterile sodium chloride solution. Then, the elution was diluted to the approximate concentration of 10 cfu. Take couple samples of 1.5 mL of the above-mentioned bacterium to 5 mL plastic tube individually, sealed up completely. One pot was placed at 50 °C for 2 h; the other was placed at −20 °C for 2 h. To assess the long-term and short-term status of the bacteria, the growth was calculated with different storage times such as 1 and 2 months. Then all experiments were conducted at room temperature.

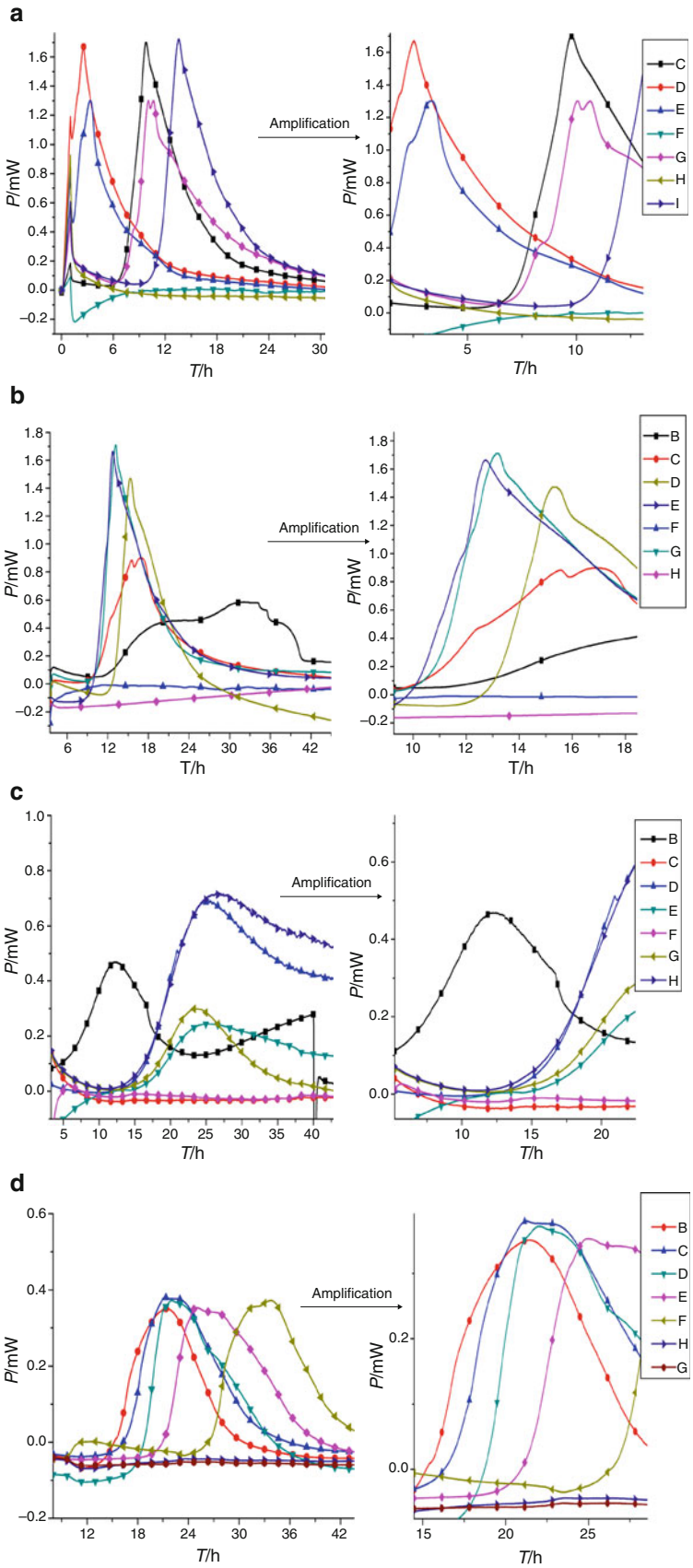

Fig. 1 The power–time curve of *bacteria growth* with different states. **a** (*C*) *E. coli* in the normal states, (*D*) the growth of *E. coli* with 1 month, (*E*) the growth of *E. coli* with 2 months, (*F*) the growth of *E. coli* with ceftizoxime sodium injection, (*G*) the growth of *E. coli* with Shuanghuanglian freeze-dried injection, (*H*) the growth of *E. coli* at 50 °C with 2 h, (*I*) the growth of *E. coli* at −20 °C with 2 h. **b** (*B*) the growth of *S. aureus* with 2 months, (*C*) the growth of *S. aureus* with 1 month, (*D*) the growth of *S. aureus* at −20 °C with 2 h, (*E*) the growth of *S. aureus* in the normal states, (*F*) *S. aureus* with ceftizoxime sodium injection, (*G*) *S. aureus* with Shuanghuanglian freeze-dried injection, (*H*) *S. aureus* at 50 °C with 2 h. **c** (*B*) the growth of *A. niger* with 1 month, (*C*) the growth of *A. niger* with ceftizoxime sodium injection, (*D*) the growth of *A. niger* at −20 °C with 2 h, (*E*) the growth of *A. niger* with 2 months, (*F*) the growth of *A. niger* at 50 °C with 2 h, (*G*) the growth of *A. niger* with Shuanghuanglian freeze-dried injection, (*H*) the growth of *A. niger* in the normal states. **d** (*B*) the growth of *environment strain* with 1 month, (*C*) the growth of *environment strain* in the normal states, (*D*) the growth of *environment strain* with 2 months, (*E*) the growth of *environment strain* at −20 °C with 2 h, (*F*) the growth of *environment strain* with Shuanghuanglian freeze-dried injection, (*H*) the growth of *environment strain* at 50 °C with 2 h, (*G*) the growth of *environment strain* with ceftizoxime sodium injection

Microcalorimeter determination

Microcalorimeter ampoule system was applied at 37 °C under sterile conditions. Aliquots of 10 mL of the cultures were added into 20 mL sterilized ampoules containing positive bacteria (to yield 10^6 beads mL^{-1} concentration). As well, antibiosis injection Shuanghuanglian solution and injection ceftizoxime sodium (lyophilized) were differently introduced to two of the bacteria solutions (to yield 10^6 beads mL^{-1} concentration). Then sealed tightly. Similarly, 10 mL of sterile de-ionized water was joined into as reference. All the ampoules were put into microcalorimeter to record the heat power–time curves. The record stopped until the curve re-back to baseline and extracted as microcalorimetric parameters, mapping by Origin 8.0 software.

Routine observation for sterility test

Direct inoculation was adopted as routine sterility test. The routine sterility test and microcalorimeter were parallel operated. Aliquots of 10 mL of the cultures were added into 20 mL sterilized ampoules containing positive bacteria (to yield 10^6 beads mL^{-1} concentration), two of the bacteria solution was differently added with antibiosis injection Shuanghuanglian solution and injection ceftizoxime sodium (lyophilized), then sealed tightly. Take one ampoule directly inlet sterilized medium as negative control for each test. The medium for *S. aureus*, *E. coli*, *A. niger*, and *environmental strains* were thioglycollate medium and cultured at 37 °C, All the ampoules were put into a thermostat, and the turbidity was observed by unaided eyes every half hour to determine the detection time [1].

Data processing

The experiments were run at 37 °C for bacteria, and the thermodynamic curves were recorded until the recorder returned to baseline. All data were collected continually using the dedicated software package. The results of *E. coli*, *S. aureus*, *A. niger*, and *environmental strains* were shown in Fig. 1a, d.

The growth and reproduction process of microbial was divided into several phases: lag phase, exponential phase, and decline phase. All were reflected by the changes of the peak time t, the heating power P, and growth rate constant k. And in the process of microbial growth, heat production of "Power-time" exponential growth curve between the culture time consistent with the *formula* 1 [19]:

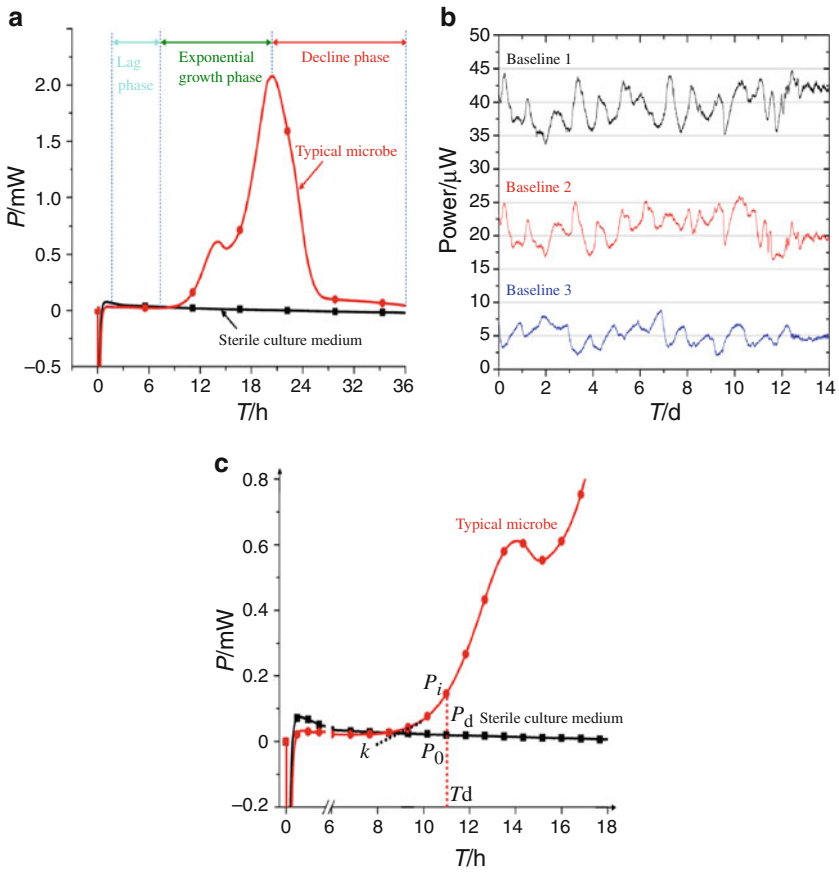

Fig. 2 Pictorial diagram for sterility test result determination; **a** the stage of a typical bacterial thermogenesis curve contains lag phase, exponential growth phase, and decline phase; **b** the baseline of sterile medium for 14 days showed that the fluctuation of baselines were less than ±10 μW; **c** the calculation method T_d

$$P_t = P_0\exp(kt) \text{ or } \ln P_t = \ln P_0 + kt \quad (1)$$

Where P_0 is the heat-output power at time 0, and P_t at time t. The thermogenic curve formula of the exponential phase of growth was Eq. 1. The calorimetric power (P), which reflects the multiplication of the cells, can be used as a parameter to characterize the growth of cells. Since $P = dQ/dt$, the area under the curve records the heat output Q released during the experimental period. The growth rate constant k was obtained by fitting ($\ln P_t - \ln P_0$) and t to a linear equation. The linear fitting with the computer can get the exponential growth rate constant k of *E. coli*, *S. aureus*, *environmental strains*, and *A. niger*.

According to Fig. 2a, b, the essential requirements of positive discrimination standard for bacterial contamination could be defined as the start of exponential growth phase by calculating the slope (k) of per 15 min curve. The exact detection time (T_d) was defined as $P_d/|P_0| \geq 3$, according to the requirement of signal–noise ratio ≥ 3 ($S/N \geq 3$) for positive signals, where $P_d = P_i - P_0$, P_i was the power of sample, and P_0 was the power of parallel baseline, shown in *Formula* 2 [20, 21]. For the fluctuation of baselines were about 10 μW, so P_d should be higher than 30 μW at least which is shown in Fig. 2c

$$T_{\mathbf{d}} = \text{Time } (P_d/|P_0| \geq 3). \quad (2)$$

Table 1 The growth rate constant of *E. coli*, *S. aureus*, *A. niger*, and *E. strain* in different states (μW s^{-1})

Different states	*E. coli*	*S. aureus*	*A. niger*	*E. strain*
Normal (37 °C, fresh)	0.15	0.16	0.02	0.03
Temperature				
50 °C	–	–	–	–
−20 °C	0.13	0.16	0.02	0.03
Time				
1 month	0.14	0.05	0.02	0.02
2 month	0.13	0.02	0.01	0.02
Drugs				
Ceftizoxime	–	–	–	–
Shuanghuanglian	0.10	0.18	0.01	0.02

"–" indicated almost nil

Discussion

The full spectrum of reaction heat of the same species of bacteria under the given conditions reflects the characteristics of bacteria. By dealing with varied states of different bacteria, the growth rate was different, which indicated that the bacteria's ability to adapt each different state was different. Similarly, in the different menstruum and processing temperatures, the growth of bacteria was alterable. In the former investigation, the cultural conditions for microcalorimetric sterility test were investigated, and it was showed that at optimal condition as 10 mL thioglycollate medium with 10 mL sterile air at the temperature of 37 °C, all positive bacteria and fungi contamination (respectively, represented gram-positive/gram-negative bacteria, aerobic/facultative aerobic/anaerobic bacteria and fungi) could detect early (less than 19 h) and could avoid the miscarriage by routine observation method.

In the paper, that treated 50 and −20 °C as critical temperature to duplicate temperature-damaged microbe model was based on that four bacteria were mid-temperature bacteria, and their tolerated temperature ranged from 20 to 42 °C. In general, bacteria preserved at 4 °C should be inoculated every month to keep theirs' vitality. Therefore, different storage time bacteria such as 1 and 2 months without inoculation were investigated to. In order to study the detection capability of microcalorimetry for drug-damaged microbe, a typical bacteriostatic ceftizoxime

Table 2 The detection time of *E. coli*, *S. aureus*, *A. niger*, and *E. strain* in different states by microcalorimetric and routine observation (h)

Different states	Microcalorimetric				Routine observation			
	E. coli	*S. aureus*	*A. niger*	*E. strain*	*E. coli*	*S. aureus*	*A. niger*	*E. strain*
Normal (37 °C, fresh)	7.48	9.33	16.17	18.36	9.50	11.00	36.50	32.00
Temperature								
50 °C	–	–	–	–	–	–	–	–
−20 °C	7.80	12.72	16.38	21.59	10.50	13.00	37.00	34.50
Time								
1 month	1.35	3.56	5.29	17.32	3.00	4.50	18.50	16.00
2 month	1.91	3.71	19.24	19.87	4.00	5.50	25.50	27.50
Drugs								
Ceftizoxime	–	–	–	–	–	–	–	–
Shuanghuanglian	10.67	9.77	18.31	28.06	15.00	17.50	38.00	35.50

"–" indicated negative

sodium (CZX) and Traditional Chinese Medicine injection Shuanghuanglian (SHL) were chosen.

The similarity showed that thermogenic curve of low temperature, short storage time (1 month), and SHL matches more with the normal (shown in Fig. 1). What have brought this about? The reason for this was may that the properties had been changed in the conditions: under the effect of low temperature, enzyme activity would be inhibited and cell metabolic activity declined; extension storage time had certainly changed the nature of the cells and slowed the operation of matter and energy; SHL inhibited the growth and metabolism of microbial [22–28]. With the impact of high temperature, lengthened storage time and CZX inhibition, bacterial growth rate was significantly different from the normal (shown in Fig. 1). Processing temperature could influence microbial growth, reproduction, and metabolism, not only by changing the characteristics of the protein and nucleic acid, but also by changing the properties of the cell structures such as fluidity and integrity of membrane. Take high temperature (50 °C), for example, microbial enzyme activity was damaged in vivo [29]. As is known to all, a long time could accelerate the aging of the bacteria; Ceftizoxime sodium made bacteria growth weak by converting bacteria-shaped, such as filaments or spherical body [28] Table 1.

Conclusions

Using microcalorimetry, the impact on growth and metabolism of *S. aureus*, *E. coli*, *A. niger*, and *environmental strains* were investigated under different conditions. All samples were analyzed qualitatively and quantitatively by similarity values of biological profiles and thermodynamics parameters, such as the exponential growth rate constant (k), detection time (T_d), which was good enough to reflect the adaptability of *E. coli*, *S. aureus*, *A. niger*, and *environmental strains* in different conditions. It showed that: first, all the normal positive bacteria could be detected within 18 h, except for *environmental strain*, the detect time was 18.36 h (shown in Table 2), while the blank media could not be detected. It was much more sensitive than routine sterility test (shown in Table 2). Second, along a curve differentiation with the extension of storage time, the growth rate constant k reduced gradually, take *E. coli* for example, as the treatment temperature ascended, the k value decreases, sometimes cannot be even detected, whereas the influence was so minor but the detected time was delayed at low temperatures. Finally, all of them were more vulnerable to the CZX than SHL. The results of yellow mycophenolate isolated from the environment were consistent with conclusions above.

The challenges of research of microcalorimetric bacteria in different states, on one hand, could examine the robustness of microorganisms in different states; On the other hand, the investigation in adaptability and robustness of bacteria can establish a fast and reliable challenge method of microorganisms, as well as can get a lot of thermokinetic information. Not only does the information provide a scientific basis for the cultivation of bacteria, but also supplies a scientific reference for the microcalorimetric method based on the sterility test. The sterility test based on microcalorimetry is a simple, high sensitive, and accurate method. Furthermore it is of great significance to security assessment of aseptic preparation.

References

1. Republic of China Pharmacopoeia (Part one). Beijing: Chemical Industry Press; 2005. p. 213–14.
2. Ames AM, editor. Thermal and energetic studies of cellular biological systems. Bristol: Wright; 1987.
3. Wadsö I, Goldberg RN. Standards in isothermal microcalorimetry. Pure Appl Chem. 2001;73(10):1625–39.
4. Trampuz A, Salzmann S, Antheaume J, et al. Microcalorimetry: a novel method for detection of microbial contamination in platelet products. Transfusion. 2007;47:1643–50.
5. Charlebois SJ, Daniels AU, Lewis G. Isothermal microcalorimetry: an analytical technique for assessing the dynamic chemical stability of UHMWPE. Biomaterials. 2003;24:291–6.
6. Von Stockar U, Maskow T, Liu J, Marison IW, Patino R. Thermodynamics of microbial growth and metabolism: an analysis of the current situation. J Biotechnol. 2006;121:517–33.
7. Ingemar W. Isothermal microcalorimetry in applied biology. Therm Acta. 2002;394:305–11.
8. Shoghi E, Romero L, Reta M, et al. Enthalpies and constants of dissociation of several neutral and cationic acids in aqueous and methanol/water solutions at various temperatures. J Pharm Biomed Anal. 2009;49:923–30.
9. Dai J, Zhang YZ, Liu Y. Microcalorimetric investigation on metabolic activity and effects of La (III) in mitochondria isolated from Indica rice 9311. Biol Trace Elem Res. 2008;121:60–8.
10. Ingemar W. Isothermal microcalorimetry for the characterization of interactions between drugs and biological materials. Therm Acta. 1995;267:45–59.
11. Graham B. Applications of isothermal microcalorimetry in the pharmaceutical sciences. Therm Acta. 1995;248:117–29.
12. Kong WJ, Li ZL, Xiao XH, Zhao YL, Zhang P. Activity of berberine on *Shigella dysenteriae* investigated by microcalorimetry and multivariate analysis. J Therm Anal Calorim. 2010;102:331–6.
13. Yan D, Jin C, Xiao XH. Antimicrobial properties of berberines alkaloids in *Coptis chinensis* Franch by microcalorimetry. J Biochem Biophys Methods. 2008;70:845–9.
14. Kong WJ, Zhao YL, Xiao XH. Investigation of the anti-fungal activity of coptisine on *Candida albicans* growth by microcalorimetry combined with principal component analysis. J Appl Microbiol. 2009;107:1072–80.
15. Kong WJ, Zhao YL, Shan LM, Xiao XH, Guo WY. Thermochemical studies on the quantity-antibacterial effect relationship of four organic acids from Radix Isatidis on *Escherichia coli* growth. Biol Pharm Bull. 2008;31:1301–5.

16. Yan D, Jin C, Xiao XH, Dong XP. Investigation of the effect of berberins alkalokds in *Coptis chinensis* Franch on *Bacillus shigae* growth by microcalorimetry. Sci China Ser B Chem. 2007;50(5): 638–42.
17. Luo JY, Yan D, Ren YS, et al. Investigation on the acrasintitration method by biothermoactivity. Acta Pharmcol Sin. 2009;44: 1136–9.
18. 3114/3236 TAM Air Isothermal Calorimeter, Thermometric, Instruction Manunal. p. 2–8.
19. Liu Y, Tan AM, Xie Cl. Cell kinetics—III. Bacterial growth arithmetic progression type thermal power. J Phys Chem. 1996; 12(5):451–6.
20. Andrej T, Andrea S, Matthias W, Stephen LL. Rapid diagnosis of experimental meningitis by bacterial heat production in cerebrospinal fluid. BMC Infect Dis. 2007;7:116.
21. Ruiz L, Reves N, Duany L, Franco A, Aroche K, Hardy Rando E. Long-term stabilization of recombinant human interferon alpha 2b in aqueous solution without serum albumin. Int J Pharm. 2003; 264(1–2):57–72.
22. Nan ZD, Xiang Y, Zeng XC, et al. Studies on thermokinetics of bacterial growth under limited conditions. J Sichuan Univ (Nat Sci). 1999;36(4):795–9.
23. Yang LN, Sun LX, Xu F, Zhang J, Zhao JN, et al. Inhibitory study of two cephalosporins on *E. coli* by microcalorimetry. J Therm Anal Calorim. 2010;100(2):589–92.
24. Li XY, Shao WH, Zhang HL, et al. Inhibition study on E.coli by temperature, pH and natural drug with microcalorimetric method. Food Sci Bio-tech. 2007;28(6):252–5.
25. Zhao YL, Yan D, Wang JB, Zhang P, Xiao XH. Anti-fungal effect of berberine on *Candida albicans* by microcalorimetry with correspondence analysis. J Therm Anal Calorim. 2010;102: 49–55.
26. Wang K. Bacterial biofilm formation and the effect of shuanghuanglian injection on bacterial biofilms, a master's degree thesis. 2009.
27. Li X, Liu Y, Qu SS, et al. Microcalorimetric study on the effects of selenium on the growth of *E. coli*. J Phys Chem. 2000; 16(6):568–71.
28. Shen XS, Liu Y, Zhou CP, Zhao RM, Qu SS. The dose–effect relationship of fluoroquinolones inhibition of *E. coli* by thermochemistry. J Chem. 2000;58:1463–6.
29. Watanabe K, et al. The effect of the Ceftizoxime sodium on antibacterial activity of human serum. Chemotherapy. 1986;32(2): 113–7.