Thermochemical Studies on the Quantity–Antibacterial Effect Relationship of Four Organic Acids from *Radix Isatidis* on *Escherichia coli* Growth

Weijun KONG,[a,b] Yanling ZHAO,*[,a] Limei SHAN,[a] Xiaohe XIAO,[a] and Weiying GUO[b]

[a] *Institute of Chinese Materia Medica, 302 Hospital of People's Liberation Army; Beijing 100039, China: and* [b] *Pharmacy College, Liaoning Medical University; Jinzhou Liaoning 121001, China.*

Received January 25, 2008: accepted April 4, 2008; published online April 30, 2008

In this report, we have investigated the inhibitory action of four organic acids from *Radix Isatidis* on *Escherichia coli* growth was investigated at 37 °C by using a microcalorimeter. The four organic acids were: syringic acid, 2-amino-benzoic acid, salicylic acid, benzoic acid. In accordance with thermokinetic model, the pertaining relationships of the drugs, such as growth inhibitory ratio *vs.* concentration, maximal power-output *vs.* growth rate constant, growth rate constant *vs.* concentration, were obtained. Half-inhibitory concentration of the drugs, IC_{50}, was obtained by quantitative analysis. From the view of thermodynamics and molecular structure, the relationship between quantity and effect of the four organic acids has been discussed. The functional groups on phenyl ring had important influence on the antibacterial activities. Our work suggests that microcalorimetry is a fast, simple and more sensitive method that can be easily performed and applied to study the anti-bacterial activities of organic acids from *Radix Isatidis* on microorganism compared to other biological methods.

Key words *Radix Isatidis*; organic acid; *Escherichia coli*; antibacterial activity; microcalorimetry

Radix Isatidis (Banlangen in Chinese) is a Traditional Chinese Medicinal (TCM) herb, and is officially recorded in the Chinese Pharmacopoeia.[1] *Radix Isatidis*, the root of *Isatidis indigotica* FORT, is mainly distributed in Hebei, Jiangsu, Zhejiang, Fujian, Henan and Guangxi provinces of China. As a medicinal herb, it has been used for more than 2000 years.[2] It has strong activities in clearing away heat, cooling blood, removing toxin and eliminating inflammation.[3,4] The main chemical components of *Radix Isatidis* were sophisticated and their contents in *Radix Isatidis* were low. Moreover, the structures of most ingredients have not been determined, which limits the thorough study of *Radix Isatidis*. Nevertheless, the four organic acids (OAs) among the main chemical components, syringic acid, 2-amino-benzoic acid, salicylic acid and benzoic acid have been segregated and purified to crystal from *Radix Isatidis*.[5,6] The contents of these four OAs in *Radix Isatidis* have been determined.[7,8] It has been showed that the four OAs have strong antiendotoxic activity.[9,10] However, that whether these four OAs possess antibacterial activities remains unexplored.

Escherichia coli (*E. coli*) is one of the most common pathogenic bacteria in clinic.[11] The culturing technique of this organism in laboratory is relatively easy and readily available, and it has been used in searching the bioactive part of folium of *Isatis indigotica*.[12]

Microcalorimetry is a quantitative, inexpensive and versatile method for measuring the heat production in many systems that can be applied to various fields of physics, chemistry and life sciences.[13] As an important method of thermodynamics, microcalorimetry has been used to study on interaction between T4 phage and *E. coli* B.[14] Meanwhile, chloramphenicol is a well-defined, broad-spectrum antibiotics, it capable of inhibiting strongly the growth of *Escherichia coli*, *Salmonella* and *Staphylococcus aureus*, *etc*.[15,16] Hence, in this study, chloramphenicol was selected as a standard positive control agent to study the antibacterial activity of the four OAs from *Radix Isatidis*. In this report, we investigated the relationship between quantity and antibacterial effect of these four OAs on *E. coli* growth based on thermo-kinetic model by quantitative analysis of the thermodynamics data. This work presents a novel, yet powerful method for studying the pharmacodynamic action of *Radix Isatidis*. The principle of this method may be applied to the study of other herb medicine and natural products.

MATERIALS AND METHODS

Apparatus A new type of heat-flow microcalorimeter, the Thermal Activity Monitor (TAM) Air Isothermal Calorimeter (Thermometric AB, Sweden) was used to measure the heat output of the metabolism of *E. coli*. This isothermal microcalorimeter is an eight-channel twin instrument. It is designed to monitor continuously a wide variety of processes and complex systems over a temperature range of 5—60 °C. A schematic representation of the calorimetric system is shown in Fig. 1.

Each measuring cylinder normally contains a sample and a reference in separate measuring cups (twin system). The heat output from the sample flows *via* the thermoelectric detector to a large heat sink (in close contact with the water bath). In order to minimize the systematic errors and disturbance effects, a differential or twin detector system is used. The system is very sensitive, with the detection limitation of 2 μW and baseline stability (over a period of 24 h) is 6 μW. The performance of this instrument and the details of its construction have been described previously.[17] Picolog software (Pico Technology Ltd.) was used to proceed the data.

Material *Radix Isatidis* (No. 070306), which was accredited by Professor Xiaohe Xiao, one of the authors, Institute of Chinese Materia Medica, 302 Hospital of PLA (People's Liberation Army), Beijing, 100039, PR China, was the dried root of *Isatis indigotica* FORT, collected from Anguo city, Hebei province, China. Syringic acid, 2-amino-benzoic acid, salicylic acid and benzoic acid were extracted and puri-

* To whom correspondence should be addressed. e-mail: zhao2855@263.net

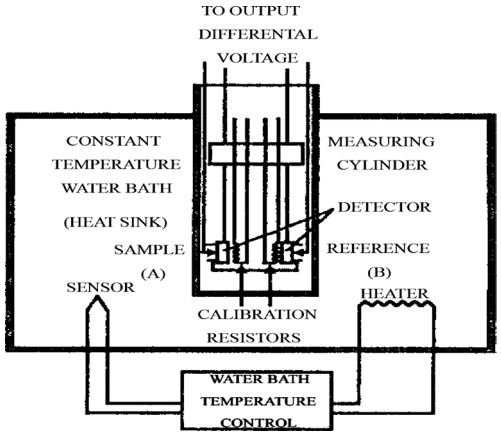
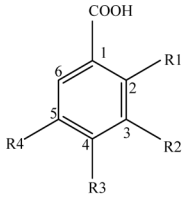


Fig. 1. Thermal Activity Monitor (TAM) Air Isothermal Calorimeter (Thermometric AB, Sweden)

It is an eight-channel twin instrument with the detection limit of 2 μW and baseline stability (over a period of 24 h) of 6 μW. It is designed to monitor continuously a wide variety of processes of complex systems in a temperature range of 5—60 °C.



	R1	R2	R3	R4
benzoic acid	H	H	H	H
salicylic acid	OH	H	H	H
2-amino-benzoic acid	NH_2	H	H	H
syringic acid	H	OCH_3	OH	OCH_3

Fig. 2. The Chemical Structures of Investigated Organic Acids (OAs) in *Radix Isatidis*

The four OAs have different substituted groups R1, R2, R3, R4 at C1, C2, C3, C4 and C5 of phenyl ring, and the substituted groups were carboxyl, hydrogen, hydroxyl, amino and methoxyl, respectively.

fied from *Radix Isatidis* by the Institute of Chinese Materia Medica, Beijing, 100039, PR China. Their structures (Fig. 2) and purities (all exceeded 98%) were analyzed and accredited with the standard by Professor Peigen Xiao, the National Institute for the Control of Pharmaceutical and Biological Products, Beijing 100051, PR China.

A batch of *E. coli* strain (CMCC B44103) and the standard chloramphenicol (No. 071126) were provided by the Chinese Center for Type Culture Collections, National Institute for the Control of Pharmaceutical and Biological Products, Beijing 100051, PR China. *E. coli* was inoculated into the peptone medium with 2×10^6 cells per ml and preserved at −4 °C. Peptone medium containing 10 g peptone, 6 g beef extract and 5 g NaCl dissolved with deionized water to 500 ml. Medium pH was adjusted to 7.0—7.2 with 1 mM NaOH before autoclaving. Lactose Broth (LB) culture medium containing 10 g peptone, 5 g yeast extract and 5 g NaCl dissolved with deionized water to 500 ml and medium pH was adjusted to 7.0—7.2 with 1 mM NaOH before autoclaving. The peptone and LB culture media were both sterilized in high pressure steam at 121 °C for 30 min.

Experimental Procedure The metabolic thermogenic curves of *E. coli* were determined by using ampoule method. Briefly, 100 μl *E. coli* suspension culture was added into 50 ml LB culture medium, and 5 ml bacterial suspension was put into a 20 ml glass ampoule that was pre-cleaned and -sterilized,. The fresh prepared OAs water solutions with different concentrations were added to the cell suspension sequentially. The metabolic thermogenic curves of *E. coli* growth in the presence of tested OAs were determined by using a computer program, monitoring the process continuously until the recorder returned to the baseline. The temperature of the calorimeter system and the isothermal box were set at 37 °C.

RESULTS AND DISCUSSIONS

Growth Rate Constant (*k*) of *E. coli* Figure 3 showed the power–time curve for growth of *E. coli* at 37 °C, a typical growth curve for *E. coli* and could be divided into four phases, *i.e.*, the first exponential phase (A–B), lag phase (B–C), the second exponential phase (C–D) and decline phase (D–E). The exponential model of *E. coli* metabolism could be used in the two growth processes[18]:

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

where P_0 was the heat output power at time 0, and so was P_t at time t. The thermogenic curve formula of the exponential phase of growth was set as Eq. 1. The growth rate constants (k) were obtained by fitting $\ln P_t$ and t to a linear equation (Table 1).

Table 1 showed $k=(0.010\pm0.006)\,\text{min}^{-1}$ and R all exceeded 0.9960, indicating good reproducibility and dependability of the results.

Positive Control Figure 4 showed the power–time curves of *E. coli* growth in the presence of 50 μg/ml of chloramphenicol and the four OAs. It was evident that the addition of these agents delayed the maximum peak-time (t_m), suggesting that chloramphenicol and four OAs all have inhibitory effect on *E. coli* compared to the control. At the same time, the maximum heat power-output (P_m) decreased correspondingly (as could be seen from the heights of the highest peaks in Fig. 4). The sequence of P_m was chloramphenicol<syringic acid<2-amino-benzoic acid<salicylic acid<benzoic acid<negative control, which meaned that the strength of anti-*E. coli* growth was chloramphenicol>syringic acid>2-amino-benzoic acid>salicylic acid>benzoic acid. 50 μg/ml of chloramphenicol was used as positive control throughout the experiments.

The Power–Time Curves and Growth Rate Constant *k* of *E. coli* with the Four OAs Figure 5 showed the power–time curves of *E. coli* growth in the presence of the four OAs at different concentrations. The generation time (t_G) of *E. coli* could be obtained from the formula: $t_G=(\ln 2)/k$. Table 2 showed the values of k, t_G and P_m of *E. coli* growth in the presence of the four OAs.

Growth Inhibition Ratio *I* and the Half-Inbibitory Concentration IC_{50} Growth inhibitory ratio (I) was defined as:

$$I\%=[(k_0-k_c)/k_0]\times100\% \tag{2}$$

where k_0 was the growth rate constant at concentration 0, so was k_c at concentration c. Table 2 showed the I% of *E. coli* by different drugs. When the inhibitory ratio I is 50%, the corresponding concentration of inhibitor is termed IC_{50}. IC_{50} can be regarded as the inhibiting concentration of causing a 50% decrease of the *E. coli* growth rate constant. It could be obtained by fitting I% and c to a linear equation.

Table 1. Growth Rate Constant (k) of *E. coli* Growth at 37 °C

Experiment	1	2	3	4	5	6	7	8
k/min^{-1}	0.009	0.008	0.010	0.011	0.008	0.012	0.014	0.011
$R^{a)}$	0.9964	0.9978	0.9986	0.9991	0.9987	0.9988	0.9979	0.9980

a) Coefficient correlation.

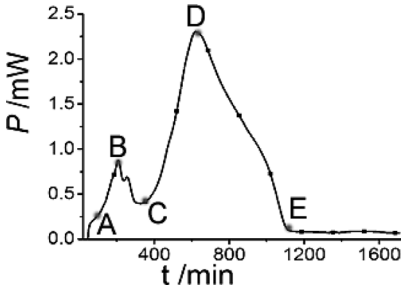


Fig. 3. The Power–Time (P–t) Curves for Growth of *Escherichia coli* (*E. coli*) at 37 °C

E. coli was cultured in Lactose Broth (LB) medium and monitored using a TAM Air Isothermal Calorimeter by ampoule method at 37 °C.

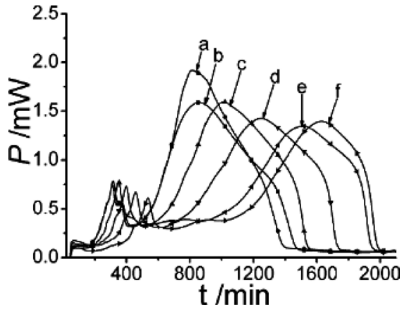


Fig. 4. The Power–Time (P–t) Curves for *E. coli* Growth at 37 °C without Drug (a) and with 50 μg/ml of Benzoic Acid (b), Salicylic Acid (c), 2-Amino-benzoic Acid (d), Syringic Acid (e) and Chloramphenicol (f)

E. coli was cultured in Lactose Broth (LB) medium and monitored using a TAM Air Isothermal Calorimeter by ampoule method at 37 °C.

For syringic acid: $I\%=0.120c+43.989$, $R=0.945$
$IC_{50}=50.1$ μg/ml (25—300 μg/ml)

For 2-amino-benzoic acid: $I\%=0.269c+29.842$, $R=0.926$
$IC_{50}=74.9$ μg/ml (25—300 μg/ml)

For salicylic acid: $I\%=0.207c+32.095$, $R=0.935$
$IC_{50}=86.5$ μg/ml (25—300 μg/ml)

For benzoic acid: $I\%=0.219c+0.988$, $R=0.974$
$IC_{50}=223.8$ μg/ml (25—300 μg/ml)

***P_m–k* Relationship** P_m–k relationship could be obtained by fitting P_m and k to a linear equation.

For syringic acid: $P_m=133.97k+1.048$, $R=0.949$ (25—300 μg/ml)

For 2-amino-benzoic acid: $P_m=76.149k+1.305$, $R=0.953$ (25—300 μg/ml)

For salicylic acid: $P_m=113.8k+1.206$, $R=0.974$ (25—300 μg/ml)

For benzoic acid: $P_m=117.66k+0.962$, $R=0.951$ (25—300 μg/ml)

***k–c* Relationship** k–c relationship could be obtained by fitting k and c to a linear equation.

Table 2. The Thermokinetic Parameters of *E. coli* Growth in the Presence of Tested OAs from *Radix Isatidis* at Various Concentrations (37 °C)

OAs	c (mg·ml^{-1})	k (min^{-1})	t_G (min)	P_m (mW)	I (%)
Syingic acid	0	0.022	31.5	2.226	0
	0.50	0.014	49.5	1.842	36.4
	1.00	0.012	57.8	1.803	45.5
	1.50	0.010	69.3	1.654	54.5
	2.00	0.008	86.6	1.541	63.6
	2.50	0.007	99.0	1.369	68.2
	3.00	0.006	115.5	1.372	72.7
	3.50	0.004	173.2	1.368	81.8
2-Amino-benzoic acid	0.50	0.016	43.3	2.101	27.3
	1.00	0.014	49.5	1.872	36.4
	1.50	0.013	53.3	1.674	45.5
	2.00	0.012	57.7	1.625	45.8
	2.50	0.010	69.3	1.523	54.5
	3.00	0.009	77.0	1.512	59.1
	3.50	0.007	99.0	1.519	68.2
Salicylic acid	0.50	0.018	38.5	2.140	18.2
	1.00	0.016	43.3	2.014	27.3
	1.50	0.014	49.5	1.827	36.4
	2.00	0.012	57.7	1.696	45.8
	2.50	0.011	63.3	1.627	50.0
	3.00	0.009	77.0	1.573	59.1
	3.50	0.007	99.0	1.582	68.2
Benzoic acid	0.50	0.021	33.0	2.219	4.50
	1.00	0.019	36.5	2.143	13.6
	1.50	0.018	38.5	1.943	18.2
	2.00	0.016	43.3	1.792	27.3
	2.50	0.015	46.2	1.653	33.8
	3.00	0.013	53.3	1.578	41.9
	3.50	0.010	69.3	1.582	53.5

For syringic acid: $k=0.006—2\times10^{-5}c$, $R=-0.968$ (25—300 μg/ml)

For 2-amino-benzoic acid: $k=0.007—3\times10^{-5}c$, $R=-0.975$ (25—300 μg/ml)

For salicylic acid: $k=0.007—4\times10^{-5}c$, $R=-0.978$ (25—300 μg/ml)

For benzoic acid: $k=0.010—5\times10^{-5}c$, $R=-0.977$ (25—300 μg/ml)

"Power–Time" Curves The power–time curves of *E. coli* growth in Fig. 5 for the four OAs were similar and the curves could still be divided into four phases. They had same profiles but different peak-heights.[19] Some similarities and differences could be observed from a qualitative point of view. The curves demonstrated that the lag phase was prolonged and the highest peak degraded with the increasing concentrations of OAs compared to the control. The curves of *E. coli* growth in the presence of syringic acid in Fig. 5A had the lowest peak-height and shortest peak time among the four OAs. The results in Table 2 showed that the values of k and P_m decreased and t_G increased with the increasing concentration of OAs compared to the control. The general results from Fig. 5 and Table 2 indicated that four OAs bona fide inhibited the growth of *E. coli*. But, with the differences

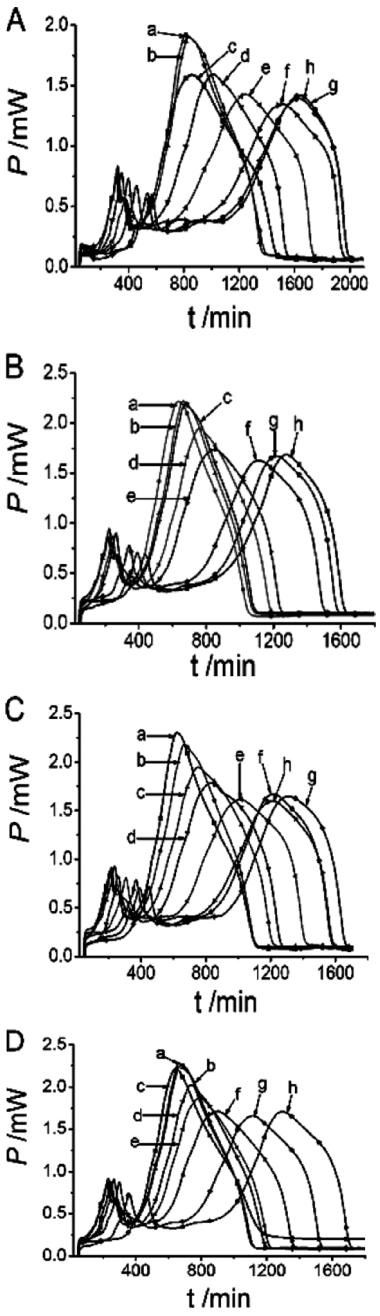


Fig. 5. The Power–Time (*P–t*) Curves of *E. coli* Growth in the Presence of Various Concentrations of Syringic Acid (A), 2-Amino-benzoic Acid (B), Salicylic Acid (C), Benzoic Acid (D)

E. coli was cultured in LB medium supplemented with different concentrations of tested OAs respectively, and monitored using a TAM Air Isothermal Calorimeter by ampoule method at 37 °C. (A) The concentrations of syringic acid: control (a), 25 μg/ml (b), 50 μg/ml (c), 75 μg/ml (d), 100 μg/ml (e), 125 μg/ml (f), 150 μg/ml (g), 300 μg/ml (h). (B) The concentrations of 2-amino-benzoic acid: control (a), 25 μg/ml (b), 50 μg/ml (c), 75 μg/ml (d), 100 μg/ml (e), 125 μg/ml (f), 150 μg/ml (g), 300 μg/ml (h). (C) The concentrations of salicylic acid: control (a), 25 μg/ml (b), 50 μg/ml (c), 75 μg/ml (d), 100 μg/ml (e), 125 μg/ml (f), 150 μg/ml (g), 300 μg/ml (h). (D) The concentrations of benzoic acid: control (a), 25 μg/ml (b), 50 μg/ml (c), 75 μg/ml (d), 100 μg/ml (e), 125 μg/ml (f), 150 μg/ml (g), 300 μg/ml (h).

of k, P_m and t_G values, the four OAs had different antibacterial activities. Syringic acid with the least k, IC_{50} values and biggest I value among the four OAs had the strongest antibacterial activity, and benzoic acid had the poorest antibacterial activity.

Structure–Effect Relationship The power–time curves of *E. coli* growth affected by various OAs from *Radix Isatidis* indicated that the four OAs all possessed inhibitory effects on the tested strain of *E. coli*. The lag phase of bacterial growth prolonged with the increasing concentrations of tested OAs, indicating that the four OAs all have the capacity of inhibiting the growth of *E. coli* and the inhibitory potency varied with different OAs. This result was likely due to excess inhibiting or killing the bacteria by these agents. Syringic acid and 2-amino-benzoic acid showed stronger inhibitory effects on *E. coli* than the other two OAs and benzoic acid showed the weakest inhibitory effects on *E. coli*, illustrating that the functional groups on phenyl ring improve the antibacterial activity. There were different substituted groups at C2, C3, C4 and C5 of phenyl ring (see Fig. 2). The only carboxy group on phenyl ring (benzoic acid) had negligible influence on the antibacterial activity. Meanwhile, the functional groups methoxyl at C3 and C5 enhanced antibacterial activity more effectively and efficiently than other functional groups, and the functional amino group at C2 improved antibacterial activity more effectively than hydroxyl at C2 on phenyl ring. These results showed that the number, position and type of functional groups on phenyl ring played significant role in the antibacterial activity of the four OAs. The different antibacterial effect on *E. coli* growth of these four OAs might result from the different efficiency of a detoxification mechanism, with the bacterial DNA-helicase being inhibited. The organic acids can connect to cellular vestibule in cytoplasm which is shaped by DNA and ToPopase to form a DNA-drugs-ToPopase ternary complex. The complex affects the duplication of DNA and thus microbial growth is inhibited.[20] Possibly, there are alternative detoxification mechanisms, such as ATPase inversion in cells under acid toxicity. It is due to energy depletion resulted from the diversion of ATPase function as a proton pump that creates the sub-toxicity effect and non-lethal acid concentrations. All these may contribute to the strong antibacterial activity of the OAs.

CONCLUSIONS

The antibacterial effect of *Radix Isatidis* has been studied extensively in a number of laboratories,[4,21,22] but the exact antibacterial components and action remain ambiguous. In this study, we investigated the antibacterial effect of the four OAs in *Radix Isatidis* on *E. coli* growth by microcalorimetry based on biothermokinetics. The co-linear relationship between the quantity-antibacterial effect of the OAs provides more references and insights for studying the action mechanism of these four natural products, the relationship between drug and bacterium metabolism. IC_{50} and k are used to represent the sensitivity of bacteria to drugs. The smaller the values of IC_{50} and k are, the stronger antibacterial activity the drugs possess. From the linear relationships I–c, P_m–k and the data in Table 2, the potency sequence of the tested OAs on anti-growth of *E. coli* was obtained that was: syringic acid>2-amino-benzoic acid>salicylic acid>benzoic acid. From the power–time curves in Fig. 5, we can find that the values of k, P_m, IC_{50} of syringic acid were the smallest among the tested OAs in the same scope of concentration of 25—300 μg/ml, showing that syringic acid possessed the strongest antibacterial effect. These results were helpful for

search for the suitable antimicrobial concentration quantitatively and antibacterial effect qualitatively. The molecular structures of the tested OAs demonstrated that these four compounds share basic structure, but have different functional groups on phenyl ring, respectively. Our results depicted that the number, position and type of functional groups on phenyl ring have great impacts on the antibacterial activities of the four OAs. These facts may provide helpful hints in searching for more bioactive components and discovering the action mechanism of *Radix Isatidis* and other natural products.

Our experiments have further proven that microcalorimetry is a powerful tool for investigating the anti-microbial activity and the relationship between drug quantity and drug effects of medicinal herbs. In any living system, such as cell, the various metabolic events are all biochemical reactions producing heat. By monitoring the heat power-output P_m in cell growth with powerful microsensor, microcalorimetry can directly detect the biological activity of a living system and provide a continuous measurement of heat production, thereby giving much information about the metabolism of organism in both qualitative and quantitative ways. Analyzing the power–time curves obtained from microcalorimetric measurement can reveal much temporal details about the microbial metabolism not observable by other methods. Compared with cup-plate method and nephelometry, microcalorimetry not only supplies a new point of view for the evaluation of bioactivity of drugs but also provides more information about the microbial growth.[23] The values of P_m and k for the power–time curves are determined simultaneously, which could describe the heat growing production and metabolic process of microbes dynamically and precisely. The essential feature of microcalorimetry method bases on the universal heat exchange involved in any biochemical reaction. It implies the possibility of using this method in research a wide range of research drugs to determine their antibiotic activity and supplies these thermograms as a "fingerprint" to investigate the bioactivity of herb medicines. By applying microcalorimetry in drug research, several different and important goals may be reached in studying the energy flow in natural environments.[24]

Acknowledgments The authors are grateful to the support of Foundation of State Administration of Traditional Chinese Medicine (No. 04-05ZP70).

REFERENCES

1) China Pharmacopoeia Committee, "Chinese Pharmacopoeia," 1st Div., China Chemical Industry Press, Beijing, 2005, p. 142.
2) Peng J. Y., Fan G. R., Wu Y. T., *J. Chromatogr. A*, **1091**, 89—93 (2005).
3) Xiao S. S., Jin Y., Sun Y. Q., *J. Shenyang Pharm. Univ.*, **20**, 455—458 (2003).
4) Tang J., Shi C. Y., Xu H., *Chin. Hosp. Pharm. J.*, **23**, 327—328 (2003).
5) Liu Y. H., Wu X. Y., Fang J. G., Xie W., *Central South Pharm.*, **1**, 302—305 (2003).
6) Fang J. G., Wang S. B., Xu H., Liu Y. H., Liu Y. W., *Chin. Tradit. Herb Drugs*, **8**, 845—846 (2004).
7) Fan G. R., Hu J. H., Li B. H., Zhang H. M., Lin M., An D. K., *J. Chin. Pharm. Univ.*, **31**, 111—114 (2000).
8) Li X., Lu H. B., Liu A. F., Zhu Z. M., Zheng X. H., *WCJ PS*, **19**, 114—117 (2004).
9) Wang Y., Yin C., Qiao C. Z., Liu S., *Acad. J. Sec. Mil. Med. Univ.*, **6**, 374—376 (1999).
10) He L. W., Li X., Chen J. W., *ITCM*, **22**, 37—40 (2005).
11) Liu Z. T., Masaki Y., Shoyama H., *Planta Med.*, **72**, 151—157 (2006).
12) Wu Y. W., Xiao X. H., Gao W. Y., Zhao Y. L., Jin C., Liu Y., *China J. Chin. Mater. Med.*, **31**, 676—679 (2006).
13) Ingemar W., *Thermochim. Acta*, **6**, 394—398 (2002).
14) Li G. S., Liu Y., Chen X. D., Liu P., Shen P., Qu S. S., *J. Virol. Methods*, **112**, 137—143 (2003).
15) Wang X. Y., Liu Z. Z., Deng X. B., Han X. P., Fu X. W., *Jiangxi J. Anim. Husb. Vet. Med.*, **1**, 8 (2000).
16) Yang Y., Zhu J. C., Liu Y., Shen P., Qu S. S., *J. Biochem. Biophys Methods*, **62**, 183—189 (2005).
17) Wadso I., *Thermochim. Acta*, **269—270**, 337—350 (1995).
18) Li X., Liu Y., Wu J., Liang H. G., *Thermochim. Acta*, **387**, 57—61 (2002).
19) Yang Y., Zhu J. C., Liu Y., Shen P., Qu S. S., *J. Biochem. Biophys. Methods*, **62**, 183—189 (2005).
20) Mitsuhashi S., *Rev. Infect. Dis.*, **10**, 27—30 (1988).
21) Hao Y. Y., Zhao Z. J., Zhao M., *Modern Trad. Chin. Med.*, **26**, 57—58 (2006).
22) Bao C. P., Chen B. Q., Xu Q. T., *J. Henan Univ.*, **22**, 25—27 (2003).
23) Xie C. L., Tang H. K., Song Z. H., Qu S. S., *Thermochim. Acta*, **123**, 33—41 (1988).
24) Gustafsson L., *Thermochim. Acta*, **251**, 69—70 (1995).