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A microcalorimetric method to determine antimicrobial effects of two bile acid derivatives on *Staphylococcus aureus*

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Abstract Using microcalorimetry, the characteristic metabolic heat flow power-time curves of *S. aureus* growth affected by Ursodesoxycholic acid and Hyodeoxycholic acid were measured at 37 °C. The thermal-kinetic parameters such as, growth rate constant k, the maximum power output (P_m), the time corresponding to the maximum power output (t_p), total heat-production Q_t, half inhibitory concentration of the drugs (IC_{50}) were calculated from the growth curves. For both HDCA and UDCA, with the increasing of concentration, k, P_m, and Q_t decreased, meanwhile, k–c fit a linear equation, t_p was prolonged correspondingly. Principle component analysis, the results indicated t_p might be the main parameter in evaluating the antibacterial activity of HDCA and UDCA in microcalorimetric method. Combining with t_p and IC_{50}, the results revealed that the differences and trends of antibacterial activity of these bile acid derivatives were: HDCA > UDCA. Structure-activity relationship (SAR) analysis showed that the α-OH at C-3 and C-6 position at equal pace on the steroid nucleus enhanced the hydrophilicity of HDCA, which led to a stronger antibacterial effect than UDCA. In this study, a useful tool was provided to accurately evaluate the antibacterial effects of bile acid derivatives. The thermolysis curve recorded by microcalorimetry could provide a lot of kinetic and thermodynamic information for the study of growth process of living microbial, which could be helpful in the screening of high efficacy antibacterial agents.

Keywords Antibacterial activity · Microcalorimetry · *Staphylococcus aureus* · Hyodeoxycholic acid · Ursodesoxycholic acid

Introduction

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium. It is a virulent pathogen that is currently the most common cause of clinical infections [1]. *S. aureus* can be found in water, dust and air, food has placed a heavy burden on the health care system. *S. aureus* can enter many organ systems and cause many types of serious diseases, even life-threatening infections, including endocarditis, pyohemia, septic arthritis, osteomyelitis, systemic infection, skin, and soft-tissue infections [2–5]. In the past decades, the tremendous increase in the frequency of bacterial and fungal infections [6–8] and the emergence of multi-drug-resistant microorganisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA) [9] and penicillin-resistant *Streptococcus pneumoniae* (PRSP) [10] have become a serious problem in medical community. The resistance to antibacterial agents is a pressing concern for human health [11], the emergence of multidrug-resistant

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bacteria has created a situation in which there are few or no treatment options for infections with certain microorganisms [12, 13].

The antibacterial effect of steroidal compounds has long been recognized and recently attracts more and more attention [14–16], whereas bile acid derivatives are investigated less frequently [17, 18]. The derivatives of two bile acid, Hyodeoxycholic acid (HDCA), and Ursodeoxycholic acid (UDCA), were reported with strong antimicrobial effects [19, 20]. As the resistant pathogens are becoming increasingly difficult to treat, it is necessary to screen new antibacterial agents with high efficacy and low toxicity by using appropriate approaches. Several techniques are available to determine the antimicrobial activity, such as serial dilution, micro-dilution, antimicrobial circle, and agar cup method [21, 22], especially antibiotic drugs. These methods can be used to assay the antibiotic activities; however, all those methods are intrusion detection in that they require sampling of the microbiological sample at specific time points. So, it is of great significance to find a more suitable approach to evaluate the antibacterial activity. In this study, a new method was applied to assay drug bioactivity by microcalorimetry.

It has been recently demonstrated that calorimetric methods could be used for monitoring microbial activities [23–25]. Microcalorimetry is a non-destructive, quantitative, inexpensive, and versatile method for measuring the heat production in many fields that can be applied to various reactions of applied biology, chemistry, life science, and environmental science [26–30]. Actually, microcalorimetry is especially suitable for the survey of heat-flow of slight 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 process and has been extensively used to investigate the interaction between drug and cultured cell [21–33]. Microcalorimetry provides a continuous real-time electronic signal proportional to the amount of heat being produced by an ampoule containing microorganisms. It is an online, kinetic, and precise method to measure the bioactivity of drugs, by analyzing the heat-flow power-time curves, the kinetic parameters, such as rate constant for bacterial growth, peak power for microbial activity, could be obtained to evaluate the effect of compounds on microbes [34, 35]. Therefore, Microcalorimetry potentially provides a high accuracy for dynamic measurements of bacterial metabolic energy that cannot be achieved with some conventional methods.

In this article, microcalorimetry was used to investigate the inhibitory effect of HDCA and UDCA on *S. aureus* growth. The relationships of thermokinetic parameters of *S. aureus* growth were discussed so as to compare the inhibitory effects of the presence of the two compounds. Also the structure–function relationship of antibacterial activities of HDCA and UDCA had been preliminarily discussed in this article. In addition, a useful tool was provided to accurately evaluate the antibacterial effects of bile acid derivatives. The thermolysis curve recorded by microcalorimetry could provide a lot of kinetic and thermodynamic information for the study of growth process of living microbial, which could be helpful in the screening of high efficacy antibacterial agents.

Experimental

Reagents

UDCA (3α,7β-dihydroxy-5β-cholanoic acid, UDCA) and HDCA (3α,6α-dihydroxy-5β-cholanoic acid, HDCA) (purity ≥ 98%) were purchased from National Institute for the Control of Pharmaceutical and Biological Products, Beijing 100051, P. R. China. The structures are given in Fig. 1. Methanol (MeOH) was of analytical purity. UDCA and HDCA were dissolved in methanol before experiments.

Materials

Bacterial strain culture

Staphylococcus aureus (*S. aureus* CCTCCAB910393) was provided by China Center for Type Culture Collection (Wuhan University, Wuhan, P. R. China). The bacterial strain was propagated in broth culture medium, which was

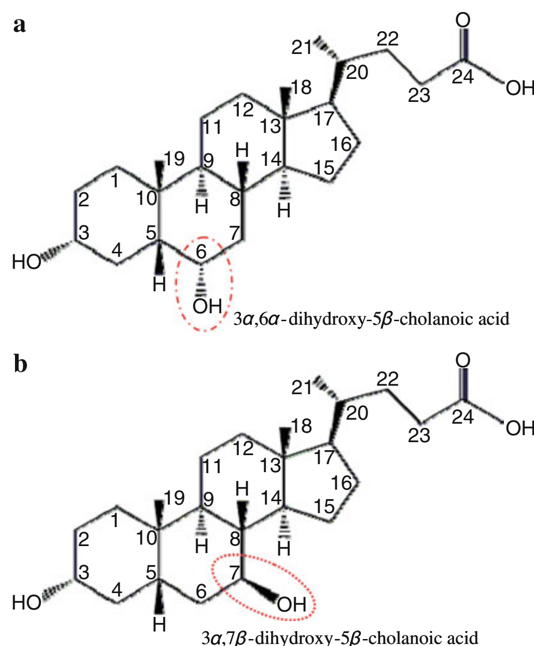

Fig. 1 Chemical structures of HDCA and UDCA. **a** HDCA **b** UDCA

a solution per 1,000 mL (pH 7.2–7.4) containing NaCl (5 g), peptone (10 g), and beef extract (6 g). The medium was sterilized in high-pressure steam at 121 °C for 30 min. The bacteria were prepared for microcalorimetric measurements.

The Luria-Bertani broth (LB) culture medium containing per 1,000 mL (pH 7.2–7.4): peptone (10 g), yeast extract (5 g), and NaCl (5 g) was sterilized in high-pressure steam at 121 °C for 30 min. The strain was stored at 4 °C before use. The sterilized culture medium oxidation processes might be not involved to microcalorimetry with the term degradation. This culture medium was prepared for microcalorimetric measurements.

Instrument

A 3114/3236 thermal activity monitor (TAM) air isothermal calorimeter (Thermometric AB, Sweden) was used to measure the heat production of *S. aureus* growth. It was equipped with eight twin calorimetric channels with one side for the sample and the other for an inert reference. The measurements were carried out in sealed 20 mL glass ampoules and the thermostat was maintained at 37 °C with an absolute accuracy of 0.02 °C. The heat flow signal generated was recorded and processed by the software of PicoLog TC-08, which was supplied to TAM air. For details of the performance and structure of the instrument, see the instruction and report of Kong et al. [36].

Methods

Experimental procedure

The microcalorimetric measurement was made with ampoule method. In all of the microcalorimetric experiments, the equipment was thermostated at 37 °C and the glass ampoules were completely cleaned and sterilized. The procedure was as follows: all the ampoules containing the bacterial suspension which was inoculate in 10 mL LB culture medium, which containing *S. aureus* at an initial density of 2×10^6 colony forming units (CFU) mL^{-1}, different concentrations of HDCA and UDCA were added into designated ampoules at equal volume, respectively. The ampoules were sealed up to insure the isothermal and isochoric conditions, then were put into the calorimeter's eight channel calorimeter block, signals of heat flow during growth were recorded after about 30 min (the temperature of ampoules reached 37 °C and the baseline stabilized). All data were collected continuously until the recorder returned to the baseline using the dedicated software package. All samples were repeated three times, the data were expressed as means ± SD of triplicate measurements.

Quantitative thermokinetic parameters for *S. aureus* growth

Exponential model was used to describe the *S. aureus* growth, obeyed the following equation [37]:

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

Here P_0 represented the heat-flow power at time 0, and P_t represented the power at time t. Thus, data $\ln P_t$ and t were taken from the curves to fit a linear equation. According to Eq. 1, the rate constant k of microbial growth, with or without impact, could be calculated using the data P_t and t obtained from the heat flow power-time curves.

$$Q_t = \text{Q}_0 \exp(kt) \text{ or } \ln Q_t = \text{Q}_0 + kt \quad (2)$$

As could be seen from Fig. 2, the heat flow power-time curves were integrated by the software of OriginPro 7.5 (OriginLab, USA), the exponential part of the heat over time curve fitted with the Eq. 2, the quantitative thermokinetic parameters such as the maximum power output P_m, the appearance times of the maximum power output t_p and total heat-flow Q_t calculated by analyzing the heat flow power-time curves.

Half inhibitory concentration (IC_{50}) determination

The inhibitory ratio I and the half inhibitory concentration (IC_{50}) determination were performed by a microcalorimetric method, inhibitory ratio I was defined as [38]:

$$I = [(k_0 - k_c)/k_0] \times 100\% \quad (3)$$

where k_0 was growth rate constant of the control, k_c was rate constant in the exponential phase of bacterial growth inhibited by inhibitor concentration is c. When the inhibitory ratio I is 50%, the corresponding concentration of the inhibitor is called the half inhibitory concentration (IC_{50}).

Principle component analysis

Principle component analysis (PCA) is the simplest of the true eigenvector-based multivariate analysis; it could reveal the internal structure of the multivariate dataset by "shadow" the variables in a lower-dimension picture. As a standard data reduction technique, it extracts data, removes redundant information, highlights hidden features, and visualizes the main relationships that exist between observations among a large number of variables in terms of a smaller number of underlying factors (principal components or PCs) without losing much information [39]. Here, PCA was performed on the mean-centered data with no scaling of many quantitative parameters taken from the heat power-time curves to find out the main parameters using software of Windows SAS 8.0 (SAS for Windows

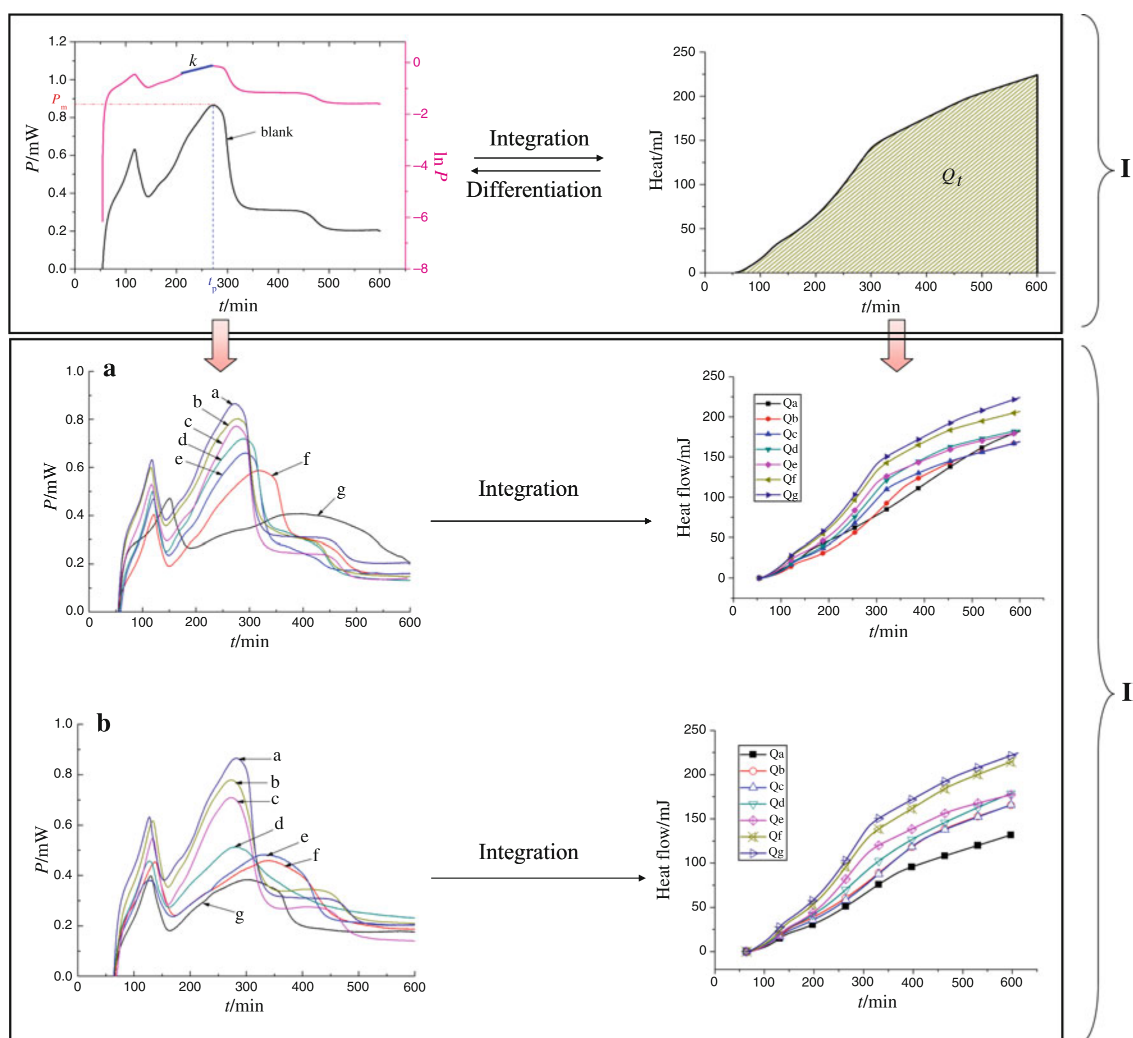

Fig. 2 Sketch showing the relationships between actual calorimetric measurements [i.e., heat flow (≈thermal power) and heat], their biological equivalents (microbial activity and products resulting from microbial activity). Heat flow is an excellent proxy for microbial activity, Heat flow and activity reflect metabolic rates and, on the other hand, heat is an indication of the quantity of substrate consumed or metabolic product released. Moreover, in certain cases, heat is also directly linked to the electron acceptors consumed, and their reduced counterpart produced. (I) The curve *of S. aureus* growth: the heat flow power-time curve was a typical profile of *S. aureus* cultured in LB medium supplemented without any substance, monitored by the microcalorimeter at 37 °C. (II) The power-time curves of *S. aureus* growth under different concentrations of **a** HDCA: *a* control *b* 24.9 *c* 31.0 *d* 38.8 *e* 48.6 *f* 60.7 75.6 μg mL^{-1} **b** UDCA: *a* control *b* 55.8 *c* 70.9 *d* 83.7 *e* 111.6 *f* 125.6 140.0 μg mL^{-1}, respectively. The figures were performed using the software of OriginPro 7.5 (OriginLab, USA)

8.0, SAS Inc., USA). These main parameter(s) is/are this/these furthest away from the main cluster of variables.

Results

Antibacterial activity determination

The heat flow power-time curves of *S. aureus* growth under different concentrations of HDCA and UDCA are shown in Fig. 2. As could be seen from the profiles of the curves, the growth of *S. aureus* was influenced by the presence of the two bile acid derivatives. Compared to the control group, the growth of *S. aureus* was influenced by the two compounds in a dose-dependent manner, illustrating the inhibition effect of HDCA and UDCA on *S. aureus* growth. Then the quantitative thermokinetic parameters such as, growth rate constant k, the maximum power output (P_m), the time corresponding to the maximum power output (t_p), total heat-production Q_t were obtained from the heat flow power-time curves were shown in Table 1.

Half inhibitory concentration (IC_{50}) determination

In order to demonstrate the inhibitory effect of various cholalic acids on *S. aureus*, the values of I were also shown

in Table 1. The IC_{50} had been calculated 61.9 μg mL^{-1} for HDCA and 130.6 μg mL^{-1} for UDCA, respectively. Considering the values of growth rate constant and half-inhibitory ratio, it could be easily concluded that HDCA showed stronger inhibitory effect on *S. aureus* than UDCA.

Relationship between quantitative thermokinetic parameters and concentration of HDCA and UDCA

In order to further show the results in a more quantitative way, the authors calculated the growth rate constants k, the maximum power output P_m, the appearance times of the maximum power output t_p, the heat-flow Q_t from the power-time curves of *S. aureus* growth under different concentrations of HDCA and UDCA using software of OriginPro 7.5 (OriginLab, USA). These results were also listed in Table 1, and the relationships between these quantitative thermokinetic parameters and concentrations (c) of the two bile acid derivatives are shown in Fig. 3.

Relationship between k and c

The values of rate constant k in Table 1 and Fig. 3a illustrated that the inhibition of HDCA and UDCA on *S. aureus* growth was clearly dose-dependent. The rate constants decreased gradually with increasing concentration of inhibitors. The results in this study revealed that HDCA and UDCA had possibly altered the growth process of *S. aureus* and the descending of k values suggested the accrescence of antibacterial activity of the two compounds.

Linear regression of k versus c was used to describe the inhibition of HDCA, which could be expressed as following equation:

$$k = \left(-7 \times 10^{-5}\right)c + 0.0082(0 \sim 75.6\,\mu\text{g mL}^{-1})$$
$$R = 0.9597$$

In the case of *S. aureus* growth under UDCA, the descending tendency of rate constant was resemble to that of HDCA. The correlations between k and c could be expressed as following equation:

$$k = \left(-5 \times 10^{-5}\right)c + 0.0104(55.8 \sim 140.0\,\mu\text{g mL}^{-1})$$
$$R = 0.9906$$

Relationship between P_m and c

As could be seen from Table 1 and Fig. 3b, the value of P_m decreased with the increasing concentration of HDCA. The relationship between P_m and c was linear and could be described as:

$$P_m = -0.0054\,c + 0.9111 \quad R = 0.9768$$

The relationship between P_m and c of UDCA could be expressed as the following equation:

$$P_m = -0.0037c + 0.9062 \quad R = 0.9670$$

Relationship between t_p and c

With the concentration of HDCA and UDCA increasing, the growth of *S. aureus* was inhibited, resulting in t_m postponed (Table 1). However, when the concentration of UDCA was below 125.6 μg mL^{-1}, the t_p value was gradually retarded, but with a concentration above 125.6 μg mL^{-1}, the t_p value decreased sharply and the growth phase was decurtated obviously. The result suggested that UDCA might inhibit growth of *S. aureus* in relatively higher dosage.

Table 1 Thermokinetic data for *S. aureus* growth at 37 °C affected by different concentrations of HDCA and UDCA (Mean ± SD, $N = 3$)

Compounds	c/μg mL^{-1}	k/min^{-1}	R^*	P_m/mW	t_p/min	Q_t/mJ	I%
HDCA	0	0.00784 ± 0.00007	0.9958 ± 0.0032	0.8360 ± 0.0423	273.7 ± 6.5	227.2 ± 8.4	–
	24.9	0.00653 ± 0.00006	0.9919 ± 0.0043	0.8031 ± 0.0354	277.3 ± 6.0	206.6 ± 7.8	15.63 ± 0.015
	31.0	0.00662 ± 0.00008	0.9938 ± 0.0035	0.7721 ± 0.0218	274.0 ± 6.7	174.4 ± 9.8	14.47 ± 0.018
	38.8	0.00541 ± 0.00005	0.9941 ± 0.0033	0.7215 ± 0.0350	289.0 ± 6.9	189.9 ± 10.2	30.10 ± 0.021
	48.6	0.00529 ± 0.00007	0.9960 ± 0.0027	0.6610 ± 0.0405	292.3 ± 6.7	168.9 ± 7.4	31.65 ± 0.032
	60.7	0.00456 ± 0.00006	0.9972 ± 0.0019	0.5869 ± 0.0377	318.7 ± 6.5	169.1 ± 7.3	41.08 ± 0.017
	75.6	0.00232 ± 0.00009	0.9923 ± 0.0045	0.4713 ± 0.0335	394.7 ± 6.4	162.1 ± 6.9	70.02 ± 0.018
UDCA	55.8	0.00754 ± 0.00005	0.9954 ± 0.0036	0.7790 ± 0.0267	273.7 ± 6.9	215.0 ± 9.6	2.58 ± 0.023
	70.9	0.00718 ± 0.00008	0.9920 ± 0.0053	0.7090 ± 0.0311	273.0 ± 8.1	177.6 ± 9.5	7.24 ± 0.032
	83.7	0.00687 ± 0.00009	0.9928 ± 0.0047	0.5132 ± 0.0287	279.7 ± 7.4	173.4 ± 10.5	11.24 ± 0.019
	111.6	0.00525 ± 0.00007	0.9935 ± 0.0038	0.4821 ± 0.0334	326.3 ± 7.2	166.7 ± 11.3	32.17 ± 0.023
	125.6	0.00464 ± 0.00007	0.9960 ± 0.0029	0.4610 ± 0.0314	337.3 ± 8.5	165.9 ± 9.5	40.05 ± 0.032
	140.0	0.00378 ± 0.00006	0.9948 ± 0.0033	0.3841 ± 0.0256	304.3 ± 8.3	132.3 ± 8.7	51.29 ± 0.022

* R correlation coefficient

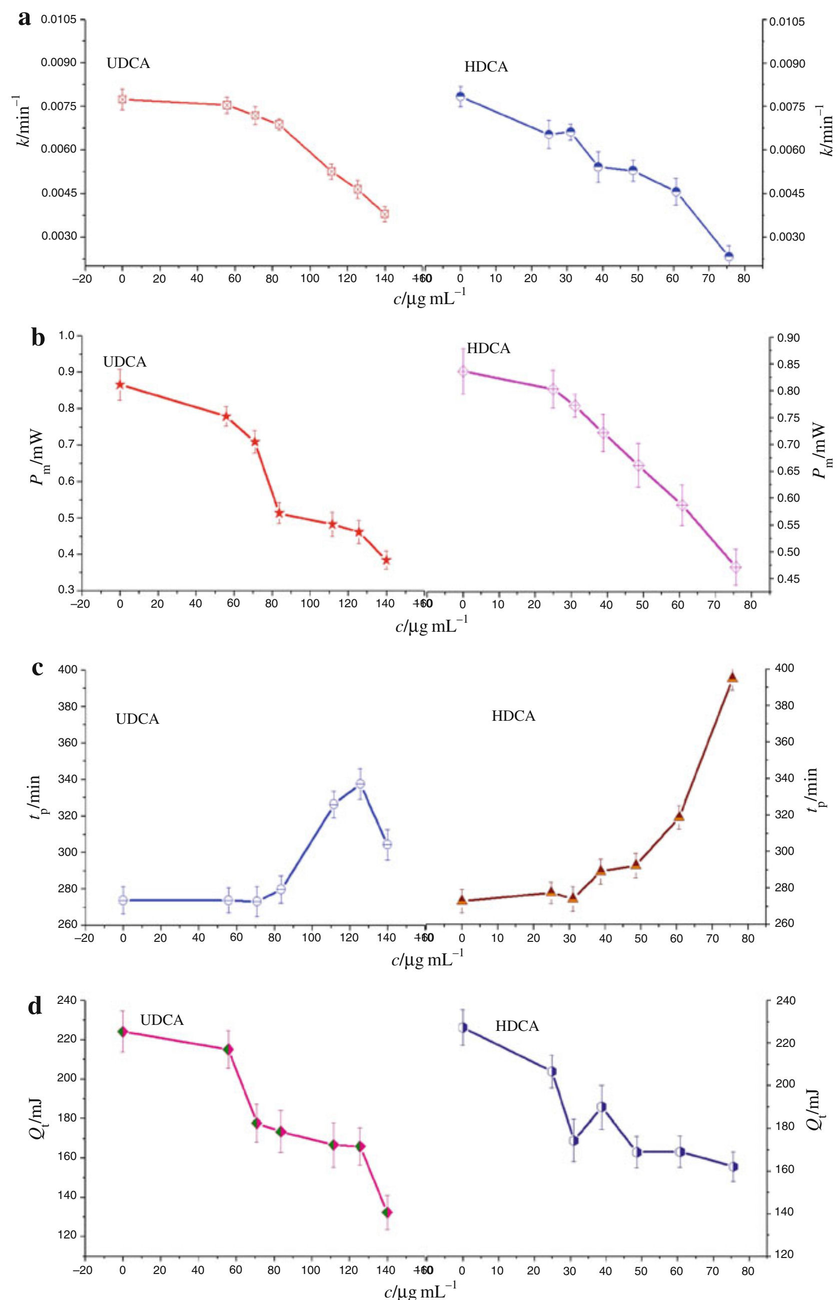

Fig. 3 Relationship between thermokinetic parameters and concentration of HDCA and UDCA. The plots of the four quantitative thermokinetic parameters **a** Growth rate constant k. **b** Peak values of the highest peak P_m. **c** appearance time of the highest peak t_p. **d** Total heat-flow Q_t were performed using the software of OriginPro 7.5 (OriginLab, USA)

Linear regression equation for HDCA:

$$t_p = 0.0358c^2 - 1.2861c + 278.2 \quad R = 0.9789$$

Linear regression equation for UDCA:

$$t_p = -0.0002c^3 + 0.0471c^2 - 2.2409c + 274.97 \quad R = 0.9155$$

Relationship between Q_t and c

The heat-flow Q_t of microbial growth process could reflect the inhibitory potency of drugs. The smaller the value of Q was, the stronger the antibacterial activity the drug possessed. As could be seen from Table 1 and Fig. 3d, with the concentration of HDCA and UDCA increasing, the growth of *S. aureus* was inhibited and led to the decreasing of the Q_t value. For HDCA, the correlation between Q_t and c was as following equation:

$$Q_t = 0.0076c^2 - 1.4153c + 225.51 \quad R = 0.9270$$

With the concentration of UDCA increasing, the value of Q_t decreased. The relationship between Q_t and c obeyed the following equation:

$$Q_t = -0.0017c^2 - 0.3711c + 225.31 \quad R = 0.9327$$

Analyzing the multivariate variables in Table 1, the authors could find that the four parameters have different change trends (increasing or decreasing) with the increase of concentrations of the two compounds, making it difficult to accurately compare the antibacterial effects of HDCA and UDCA. So, it was necessary to find out the main parameter(s), which played the most important role in evaluating the antibacterial effects of HDCA and UDCA. By analyzing the change of the main parameter(s), whether the two the bile acid derivatives have intrinsically different antibacterial effects or not and the tendency of these differences could be explained better and faster. Therefore, principal component analysis was introduced in the next part.

PCA

The four quantitative parameters (k, P_m, t_p, and Q_t in Table 1) were analyzed by PCA. With the first two components explaining 94.841% of the variability in the original four parameters, the loading plot in Fig. 4 showed the distribution of these four parameters. This plot indicated that parameters t might be the main parameter, which played more important role in evaluating and comparing the antibacterial effects of the two the bile acid derivatives. Returning to the main parameter t_p in Table 1, it could be found that there were no significant or major differences between them. Then, the box plot in Fig. 5 of t_p for HDCA and UDCA provided some help. This plot markedly showed the distribution of the data of t_p for HDCA and UDCA. It could be perspicuously seen that the minimum and maximum values, the mean, 25% quartile and 75% quartile values of t_p for HDCA were bigger than those of UDCA, showing that the antibacterial effect of HDCA was stronger than UDCA. The IC_{50} of 61.9 μg mL^{-1} for HDCA and 130.6 μg mL^{-1} for UDCA further validated the above results.

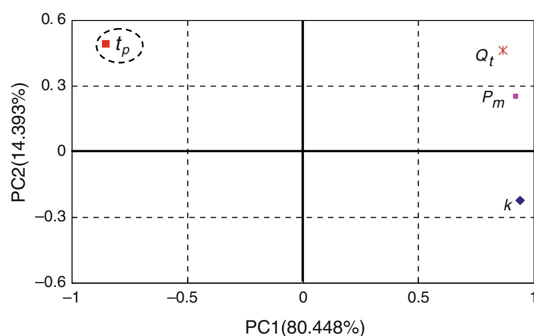

Fig. 4 Loading plot generated from PCA on the four quantitative parameters. This plot was obtained using software of Windows SAS 8.0 (SAS, USA). The main parameter was marked with a *circle*

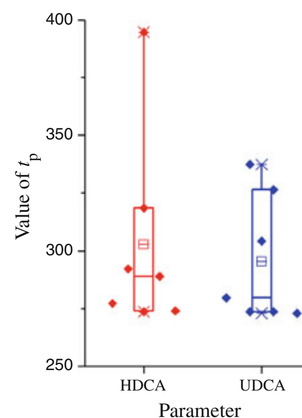

Fig. 5 *Box* plot of the main parameter t_p for HDCA and UDCA. This plot was made using software of Origin 7.5 and showed the minimum and maximum value, the mean, 25% quartile, 75% quartile and the range

Discussion

In this study, the antibacterial effects of HDCA and UDCA on the growth of *S. aureus* were investigated and compared by microcalorimetry. The heat flow power-time curves and thermokinetic parameters calculated from these curves of *S.aureus* growth in the presence of the two compounds were selected as quantitative indicator for this comparison. Relationships between quantitative thermokinetic parameters and concentration showed that $k-c$ and P_m-c were both fit linear equations. By the values of k and P_m in Table 1 and the relationship of k—c, P_m—c in Fig. 3a and b, it is clearly that the antibacterial activity of these two bile acid derivatives on *S. aureus* growth was increased with the increasing concentration. PCA results indicated t_p might be the main parameter in evaluating the antibacterial activity of HDCA and UDCA in microcalorimetric method. Combining with the values of the IC_{50} of HDCA (61.9 μg mL^{-1}) and UDCA (130.6 μg mL^{-1}) and t_p in Fig. 5, the differences and trends of antibacterial effects of HDCA and UDCA were successfully compared: the HDCA has much stronger inhibition effects than UDCA on the growth process of *S. aureus*.

For the compounds' structure (seen from Fig. 1), HDCA and UDCA are steroids analogs with a 24-carbon atoms possessing an acidic side chain and two hydroxyl groups, which are α or β oriented in the 3, 6 or 7 positions. The α-OH at C-3 and C-6 position on the steroid nucleus of HDCA is substituted by a β-OH at C-7 for UDCA. However, the number and orientation of hydroxyl groups on the steroid backbone influence the compound's susceptibility [40]. The common steroid nucleus of the bile acid derivatives leads to a facial amphiphilic nature of a hydrophobic and a hydrophilic face [41, 42]. The change of side chains on the steroid nucleus alters the facial hydrophobicity or

hydrophilicity, and further alters the facial amphiphilic nature of these compounds. In addition, Roda et al. [43] reported the lipophilicity of bile acids (BA) was in relation to their structure, the order of lipophilicity is related mainly to the position of hydroxy groups 3α, $7\beta > 3\alpha$, 6α on the steroid nucleus. Then, the α-OH at C-3 and C-6 position at equal pace enhances the hydrophilicity of compound HDCA, the facial activity is accordingly enhanced. So, HDCA could penetrate and disrupt the integrity of the bacterial membrane more easily, resulting in cell death much faster than UDCA.

In summary, microcalorimetry was successfully used to investigate and compare the antibacterial effect of HDCA and UDCA on *S. aureus* growth. The power-time curves and some thermokinetic parameters k, $P_{\max}$, t_{p}, and Q_t from these curves of *S.aureus* growth could be determined simultaneously. The consistent results in this study of the biologic effects of two bile acid derivatives on *S. aureus* growth showed that HDCA has much stronger antibacterial effect than UDCA. However, whether such differences would lead to the variances of their therapeutic efficacies remains to be further evaluated. This result inspired that some new antibacterial agents with high efficacy could be synthesized and screened out by changing the functional groups of steroid. This study also provided a useful method to evaluate the antibacterial effects of steroidal compounds accurately (e.g., bile acid derivatives): the recorded curve could be used to describe the bioactivity of drugs, which might be helpful in the screening of high efficacy antibacterial agents.

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