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Short communication

Evaluation of the antimicrobial mode of berberine by LC/ESI-MS combined with principal component analysis

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Abstract

In this investigation, metabolic profiles of *Staphylococcus aureus* treated by berberine and nine antibacterial substances with known modes of action were acquired by HPLC/ESI-MS. After data pretreatment, those profiles acquired were reduced into several MS vectors. Then, principal component analysis was carried out upon those vectors to classify those drugs according to their mechanisms. From the result obtained by principal component analysis, the possible antibacterial mode of berberine was evaluated.

Keywords: Berberine; *Staphylococcus aureus*; LC/ESI-MS; Metabolic profile; Principal component analysis

1. Introduction

Metabolic profile is a very sensitive indicator of environmental influences and might be used to detect and analyze changes of the total metabolic state of microbe due to patho-physiological stimuli [1], which makes it an attractive candidate for mode-of-action studies [2]. Nowadays, metabolic profile is being used to evaluate the pharmacological mechanism of the drugs or drug candidates. The method is particularly useful when a moderate number of different outcomes (e.g., modes-of-action, disease states) can be pre-defined [3].

In this work, we investigated the antibacterial mode of berberine on *Staphylococcus aureus*. With the help of HPLC/ESI-MS, metabolic profiles of *S. aureus* treated by berberine and nine antibacterial substances with known modes of action (Table 1) were acquired. After data pretreatment, those profiles acquired were reduced into several MS vectors containing 900 *m/z* values. Then, principal component analysis (PCA) was carried out upon those metabolic profiles in order to classify those drugs according to their mechanisms. From the result obtained by PCA, the possible antibacterial mode of berberine was explored.

2. Materials and methods

2.1. Chemicals

All authentic reference compounds (berberine, chloromycetin, streptomycin, acheomycin, rifampicin, cefataxime, vancomycin, norfloxacin, erythromycin, and lincolmensin) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (China).

2.2. Validation of the antibacterial mode of berberine

2.2.1. Bacterial strains and growth conditions

S. aureus CCTCC AB9105 was used in this study. All tests were performed in Mueller–Hinton broth (MHB). The growth of microbe cultures was monitored by measuring optical density at 600 nm in a UV–visible spectrophotometer. Microbe strain was cultured into 50 mL of MHB, and incubated in a shaker incubator (300 rpm) overnight at 37 °C. The overnight cultures were diluted to a concentration of approximately 10^8 cfu/mL and used as the source of inoculums. Solutions of each drug were prepared with concentration ranging from 1.25 to 400 μg/mL. After preparation, 1 mL solution was diluted with 9 mL MHB and the final concentrations of each drug were shown in Table 2. At each passage, a volume of 100 μL of the inoculum was

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Table 1
Modes of actions of selected known antibiotics

Drug/class	Function inhibited	Molecular target
Chloromycetin	Protein synthesis	50S ribosomal subunit
Erythromycin	Protein synthesis	50S ribosomal subunit
Lincolmensin	Protein synthesis	50S ribosomal subunit
Streptomycin	Protein synthesis	30S ribosomal subunit
Acheomycin	Protein synthesis	30S ribosomal subunit
Norfloxacin	DNA replication/transcription	Gyrase and topoisomerase IV
Rifampicin	Transcription	RNA polymerase
Cefataxime	Peptidoglycan synthesis	Transpeptidases and carboxpeptidases
Vancomycin	Peptidoglycan synthesis	Cell wall peptidoglycan

re-inoculated into solutions containing different concentrations of each drug and the cultures were again incubated overnight with shaking at 37 °C. The inhibition of each drug to cells growth was between the approximate ranges of 40–80% to ensure that any change in the metabolic profiles could be ascribed to the mode of action of the inhibitor and not changes in growth rate [4].

2.2.2. Extracting metabolites from S. aureus

After 24 h growth when the majority of cultures appeared to have entered stationary phase, 30 mL cultures treated with each drug were centrifuged (Eppendorf centrifuge, 4000 rpm for 10 min at 4 °C). The supernatant was discarded and the pellet was suspended in 5 mL buffer (Tris–HCl pH 7.4). Then, the suspension was centrifuged at 4000 rpm for 10 min again and the cultures were separated. This step was repeated three times to remove residual broth. Then the pellet was resuspended in 10 mL cold (−20 °C) absolute methanol in a tube. After rapid mixing, the tube was transferred into dry ice for 30 min. The sample was then thawed in an ice bath for 10 min and centrifuged (Eppendorf centrifuge, 12,000 rpm for 10 min at 4 °C). After this step, the supernatant was transferred to a new tube. To the extracted pellet, 5 mL cold (−20 °C) methanol–water (50:50, v:v) was added to extract any metabolites left after the first extraction. The first and the second extracts were combined and concentrated for 6 h in vacuum at 4 °C to approximately 0.5 mL. In order to ensure the reproducibility of the method, every sample was repeated for three times. The sample was stored at −40 °C until LC/ESI-MS analysis.

2.2.3. HPLC/ESI-MS conditions

A Shimadzu LC system, consisting of a Shimadzu (Kyoto, Japan) LC-10ADvp solvent delivery pump, an FCV-10ALvp low pressure gradient unit, a DGU-14A degasser, a CTO-10Avp column oven, a 8125 Rheodyne manual injector (CA, USA), coupled to a LCMS-2010 single quadrupole equipped with electrospray ionization (ESI) probe were used in this study. The temperatures were maintained at 250, 250 and 200 °C for the probe, CDL and block, respectively. The voltages were set at 4.5 kV, −30 V, 25 V, 150 V and 1.5 kV for the probe, CDL, Q-array 1, 2, 3 bias, Q-array RF and detector, respectively. The flow rate of nebulizer gas was 4.5 L/min. The ions of selection monitoring were decided by positive scanning from *m/z* 50 to 1000 [5]. The separation was performed on a C18 (Thermo Hypersil-Keystone Hypurity C18, 150 mm × 2.1 mm, 5 μm) analytical column, and the oven temperature was set at 40 °C. The mobile phase consisted of (A) water and (B) methanol (containing 0.2% formic acid), was first filtered through a membrane of 0.22 μm and degassed ultrasonically. The mobile phase underwent a gradient elution procedure of 5–28% B in 10 min, 28–55% B in 10–20 min, 55–72% B in 20–30 min, 72–85% B in 30–40 min, 85–90%B in 40–50 min, 90–85% B in 50–60 min, 85–5% B in 60–130 min. The flow-rate was set at 0.2 mL/min. The data were collected and processed using LC/MS Solution software 2.02.

Table 2
Concentrations of antibiotics, and berberine used in the inoculation of individual samples/cultures of *S. aureus* (the sample symbols refer to the individual metabolite profiles represented as points in PCA projections)

Drugs used	Concentration (μg/mL)	Solvent/dilution	Sample symbols
Controls			+
Norfloxacin	4, 2	0.1 mol/L NaOH/H_2O	◯
Acheomycin	0.25, 0.125	H_2O/H_2O	◊
Lincolmensin	16, 8	H_2O/H_2O	▲
Cefataxime	0.25, 0.125	H_2O/H_2O	*
Vancomycin	0.5, 0.25	H_2O/H_2O	×
Rifampicin	4, 2	Methanol/H_2O	☆
Erythromycin	2, 1	95% ethanol/H_2O	△
Chloromycetin	8, 4	95% ethanol/H_2O	▽
Streptomycin	16, 8	H_2O/H_2O	▼
Berberine	40, 20	H_2O/H_2O	□

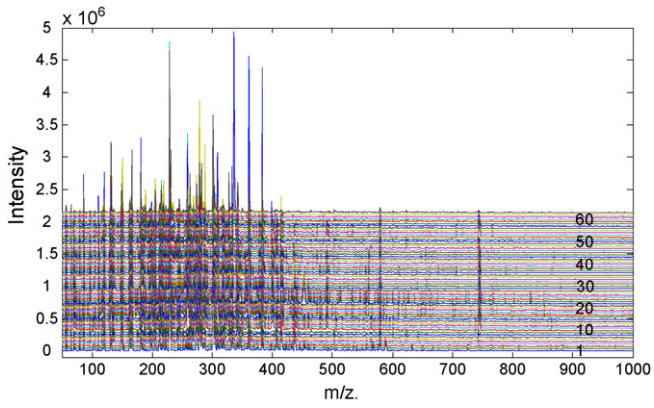

Fig. 1. After data preprocessing, *m/z* of controls (numbers 1–3) and cultures treated with berberine, nine antibiotics (rifampicin (4 μg/mL, 4–6; 2 μg/mL, 7–9), cefataxime (0.25 μg/mL, 10–12; 0.125 μg/mL, 13–15), vancomycin (0.5 μg/mL, 16–18; 0.25 μg/mL, 19–21), erythromycin (2 μg/mL, 22–24; 1 μg/mL, 25–27), chloromycetin (8 μg/mL, 28–30; 4 μg/mL, 31–33), streptomycin (16 μg/mL, 34–36; 8 μg/mL 37–39), acheomycin (0.25 μg/mL, 40–42; 0.125 μg/mL, 43–45), berberine (40 μg/mL, 46–48; 20 μg/mL, 49–51), lincolmensin (16 μg/mL, 52–54; 8 μg/mL, 55–57), norfloxacin (4 μg/mL, 58–60; 2 μg/mL, 61–63)).

2.3. Pretreatment of data and principal component analysis

PCA, which has been widely used for exploratory data analysis [6,7], may be a useful and convenient means for possible evaluation of modes of action. The objective of PCA is not only to describe the data in a space of lower dimensionality, but also to determine the minimum dimensionality needed to reproduce the information within experimental measurement error [8]. Thus, PCA could enable us to represent objects or variables on a graph, with different objectives to study the proximity of objects in order to classify them and to detect atypical objects, and also to analyze the position of objects in varied representations and to assign new objects in a representation characterizing the population. Here PCA was performed on the metabolic profiles to classify those selected drugs and try to find out the possible antimicrobial mode of berberine.

Before PCA, data pretreatment is necessary. Each ESI-MS array was reduced into a single 'aggregate' MS vector by summing the ion counts of a given *m/z* ratio over the total scan cycle. Thus, after this initial data reduction an ESI–MS spectrochromatogram with MS range 50–1000 *m/z* will be reduced to a single vector having 900 values [9]. Then PCA was performed on the MS matrixes obtained. All programs were coded in MATLAB 6.5 for windows.

3. Results and discussion

In order to investigate the possible antibacterial mode of berberine, the culture treated with berberine was harvested. Metabolites were then extracted from cultures and analyzed by HPLC/ESI-MS to acquire metabolic profiles (see supplementary information). The cultures treated with nine antibiotics (chloromycetin, streptomycin, acheomycin, rifampicin, cefataxime, vancomycin, norfloxacin, erythromycin, and lincolmensin) were also performed with the same steps.

Fig. 1 depicts the *m/z* profiles of all samples obtained after preprocessing. Obvious differences could be found between these metabolic profiles, hence allowing classification of the drugs according to their metabolic profiles.

PCA was performed on all the *m/z* vectors obtained from each metabolic profile treated with the different drugs (berberine and nine antibiotics) and controls. The results are shown in Fig. 2. The result of PCA shows six site-related clusters. Although no two drugs produce exactly the same pattern of loadings, the map gives a clear indication of the classification for all drugs and controls. From those plots, one could see

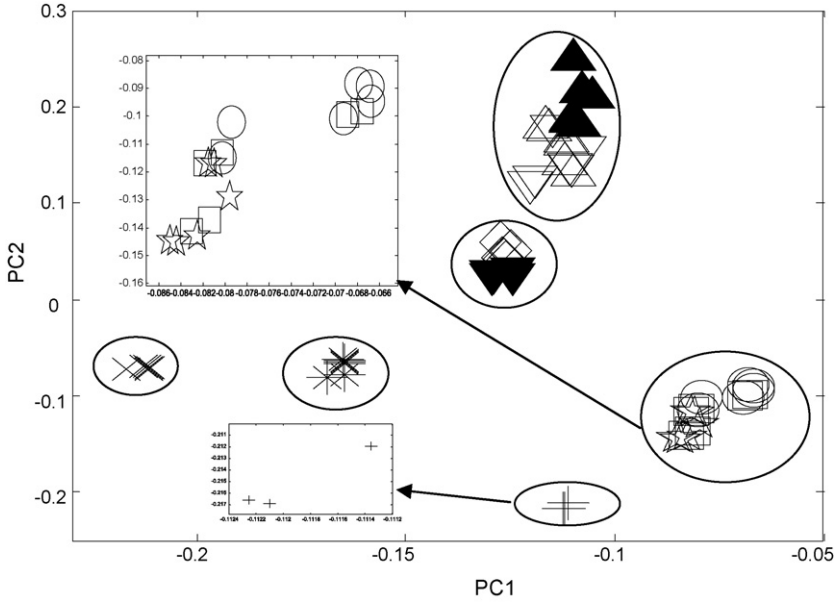

Fig. 2. PCA projection of metabolic profile of controls and cultures treated with berberine, nine antibiotics (controls (+), acheomycin (◊), lincolmensin (▲), erythromycin (△), chloromycetin (▽), streptomycin (▼), cefataxime (*), vancomycin (×), rifampicin (☆), norfloxacin (◯), berberine (□)).

clearly that the cultures treated with each drug are well separated from the control samples. Similarly, the points representing the cultures treated with drugs of similar targets will cluster together in PCA projections. For instance, acheomycin (◊) and streptomycin (▼) are clustered together. Lincolmensin (▲), erythromycin (△) and chloromycetin (▽), are clustered together as well. As we know from Table 1, lincolmensin, erythromycin and chloromycetin have effects on 50S ribosomal subunit; streptomycin and acheomycin act on 30S ribosomal subunit. That is, the mode of acheomycin and streptomycin and the mode of lincolmensin, erythromycin and chloromycetin are possibly similar, which are quite close with each other as shown in Fig. 2. In the same way, cefataxime (*), whose target is on transpeptidases and carboxpeptidases, forms a single group, which means its mode is different with others. Similarly, vancomycin (×), whose target is on cell wall peptidoglycan, is clustered as a single group.

The PCA results support the hypothesis that modes of actions of a drug could be identified by the metabolic profiles acquired. This may provide the basis to classify the metabolic profiles by PCA. Furthermore, one might find out the antimicrobial mode of berberine from the classification.

Based on the discussion above, it is interesting to see that the points representing berberine at both concentrations (□) are close to that of rifampicin (☆) and norfloxacin (◯), whose targets are on RNA polymerase and gyrase and topoisomerase IV, both on nucleic acid. According to the principle of PCA, the mode of berberine should be similar with that of Rifampicin and Norfloxacin. That is, its target is possibly on nucleic acid, which also has been documented by other methods [10–16].

4. Conclusion

This work has shown that it is possible for LC/MS technique to discriminate the modes-of-action of several different antibiotics with the help of PCA. Furthermore, the possible antibacterial mode of berberine could be identified by this technique. The conclusion of antibacterial mode is identical with the reports acquired by other means, which prove the combined technique is feasible to investigate the antimicrobial mechanism of new drugs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2007.02.018.

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