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Biotoxicity Based on Microtox Fast Testing System and Correlative Material Basis of Shenmai Injection

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Abstract

Microtox assay based on luminous bacterium *Vibrio fischeri* CS234 was introduced for the first time for quality control of Shenmai injection. Contents of ginsenoside Rb_1, Re and Rg_1 in each Shenmai injection purchased from different manufacturers were assayed by HPLC-DAD and the corresponding IC_{50} values of these Shenmai injections were determined by microtox assay. We found a significant positive correlation between ginsenoside Re content and IC_{50} of Shenmai injections, which suggested that determination of IC_{50} by microtox assay is potentially a reliable new quality control method for ginsenoside Re in Shenmai injections. However, whether our work would yield useful information for predicting warnings, such as allergic reactions during clinical application of Shenmai injections requires more research.

Keywords: Shenmai injection; Microtox assay; Ginsenoside Re

Introduction

Shenmai (SM) injection is a redeveloped preparation based on the well-known Traditional Chinese Medicine formula Shengmai San. It is widely used for treating coronary heart disease, viral myocarditis, chronic pulmonary heart disease, granulocytopenia and tumors by tonifying *Qi* and nourishing *Yin*. The prescription of SM injection is composed of 100 g Red Ginseng and 100 g Radix Ophiopogonis. Red Ginseng is a processed product of *Panax ginseng* C A Meyer, which mainly contains saponins, volatile oil, polysaccharides, and other ingredients. Ginsenoside Rb_1, ginsenoside Re and ginsenoside Rg_1 are defined as three index components in National Drug Standards (WS3-B-3428-98-2010Z) of SM injection issued by State Food and Drug Administration (SFDA) [1]. Those ginsenosides inhibited inflammatory responses in atherosclerotic diseases like stroke and coronary heart disease et al. Recently, *Panax notoginseng* saponins contained ginsenoside Rg_1, and ginsenoside Rb_1 and so on inhibited an apparent phenotype switching induced by down regulated Notch 3 with a siRNA [2]. Ginsenoside Rg_1 inhibited the phosphorylation of IKK to reduces NF-κ B translocation [3].

Materials and Methods

Test samples

SM injections were purchased from Anhui Hyey Pharmaceutical Co., LTD, which were produced by 1) Sichuan Sunnny hope Pharmaceutical Co., LTD (Sichuan, China) with the batch numbers of 1501402, 1503417, and 1412406; 2) Ya'an Sanjiu Pharmaceutical Co., LTD (Sichuan, China) with the batch numbers of 141203020, 150203020, and 140107020; 3) Hebei Shineway Pharmaceutical Co., LTD (Hebei, China) with the batch numbers of 150808F1, 141121F2, and 1508253; 4) Chiatai Qingchunbao Pharmaceutical Co., LTD (Zhejiang, China) with the batch numbers of 1501171, 1501251, and 1505091; 5) Yunnan Phytopharmaceutical Co., LTD (Yunnan, China) with the batch numbers of 20150308 and 20150319; 6) Dali Pharmaceutical Co., LTD (Yunnan, China) with the batch number of 1508034.

Reagents

Rehydration solution (3% NaCl) and osmotic pressure regulator (20% NaCl solution) were provided by Beijing Hamamatsu Photon Techniques INC. (Langfang, China) with the batch numbers of 20150717 and 20150717, respectively. HPLC-grade acetonitrile was purchased from Tedia (Fairfield, OH, USA).

Ultrapure water for HPLC analysis was prepared by Milli-Q system (Milford, MA, USA). Reference substances of ginsenoside Rg_1 ($C_{42}H_{72}O_{14}$) with the batch number of 110703-201530,

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Table 1: Contents of ginsenoside Rg_1, Re and Rb_1 in SM injection from different manufacturers (n = 1).

Manufacturer	Batch numbers	Rg_1 (mg/ml)	Re (mg/ml)	Rb_1 (mg/ml)
Sunnnyhope	1501402	0.13	0.08	0.16
	1503417	0.14	0.08	0.16
	1412406	0.13	0.10	0.28
Sanjiu	141203020	0.17	0.10	0.23
	150203020	0.17	0.10	0.24
	140107020	0.16	0.11	0.28
Shineway	150808F1	0.13	0.14	0.33
	15082531	0.12	0.13	0.31
	141121F2	0.12	0.14	0.33
Chiatai	1501251	0.16	0.08	0.23
	1505091	0.18	0.09	0.22
	1501171	0.18	0.09	0.26
Yunnan	20150319	0.11	0.07	0.16
	20150308	0.12	0.08	0.14
Dali	1508034	0.15	0.14	0.27

Inhibitory activity on luminescent bacteria of SM injections (Microtox assay)

The concentrations of test sample (X%) - inhibitory effects on luminescent bacteria (Y%) curves were fitted and correlation coefficient (R^2) and IC_{50} values are shown in Table 2.

Table 2: Inhibition activity of SM injection from different manufacturers (n = 2).

Manufacturer	Batch number	Regression equation	R^2	IC_{50}(%)
Sunnny hope	1501402	y = 62.05lnx-222.8	0.988	--[a]
	1503417	y = 56.90lnx-212.8	0.992	--[a]
	1412406	y = 65.4lnx-238.3	0.972	--[a]
Sanjiu	141203020	y = 52.61lnx-156.9	0.973	51.0
	150203020	y = 51.76lnx-161.3	0.983	58.9
	140107020	y = 41.33lnx-114.1	0.997	53.0
Shineway	150808F1	y = 68.91lnx-245.0	0.972	72.3
	15082531	y = 55.18lnx-180.8	0.992	65.5
	141121F2	y = 50.86lnx-160.5	0.993	62.7
Chiatai	1501251	y = 48.31lnx-143.1	0.995	54.4
	1505091	y = 37.48lnx-97.89	0.996	51.7
	1501171	y = 48.50lnx-136.5	0.961	46.8
Yunnan	20150319	y = 63.88lnx-211.2	0.990	59.7
	20150308	y = 47.50lnx-136.7	0.991	50.9
Dali	1508034	y = 47.33lnx-151.0	0.992	69.9

[a]Percent inhibition of less than 50% at the highest concentration

Re ($C_{48}H_{82}O_{18}$) with the batch number of 110754-201525 and Rb_1 ($C_{54}H_{92}O_{23}$) with the batch number of 10704-201424 were purchased from the National Institute for Food and Drug Control (Beijing, China).

Luminous bacteria

Vibrio fischeri CS234 kits with the batch numbers of D15G053, D15G054, D15I019, D15I027, D15I028, and D15I004 were provided by the Beijing Hamamatsu Photon Techniques INC. (Langfang, China).

Instruments

The various instruments used in this study were: LUMIStox 300 luminometer with LUMIS-therm thermostatically controlled thermo-block (Dr. Bruno Lange GmbH, German); KCL-2000 incubator (Eyela, Japan); Integral 5 ultrapure water system (Milford, USA); Agilent series 1200 infinity HPLC instrument coupled with G1311A solvent delivery pumps, G1315D diode array detector, G1329A automatic injector and ChemStation (Agilent, USA).

Chromatographic and ginsenoside Rg_1, Re and Rb_1 spectrometric conditions [1].

Chromatographic separation was carried out at 30°C on a Gemini-C18 column (4.60 mm × 250 mm, 5 µm, Phenomenex, USA). The mobile phase consisted of acetonitrile (A) and ultrapure water (B). The gradient elution conditions were: 0-30 min, 0-10 % A, 100-90 % B; 30-40 min, 10-23 % A, 90-77% B; 40-50 min, 23 % A, 77% B; 50-85 min, 23-60 % A, 77-40% B; 85-95 min, 60-100 % A, 40-0% B and flow rate was 1.0 ml/min. The DAD detector scanned at 203 nm

Table 3: Normality tests of Rg_1, Re, Rb_1 and IC_{50} in SM injections (n = 12)[b].

Indexes	Kolmogorov-Smirnov[a]		Shapiro-Wilk	
	df	Sig.	df	Sig.
Rg_1	12	0.200	12	0.099
Re	12	0.200	12	0.117
Rb_1	12	0.200	12	0.526
IC_{50}	12	0.200	12	0.482

[b]SM injection manufactured by Sunnny hope were excluded because of unknown IC_{50}

Table 4: Correlation analysis of Rg_1 contents and IC_{50} (n = 12)[b].

		IC_{50}	Rg_1
IC_{50}	Pearson's correlation	1	-0.372
	Sig. (two-tailed)		0.233
	df	12	12
Rg_1	Pearson's correlation	-0.372	1
	Sig. (two-tailed)	0.233	
	df	12	12

[b]SM injection manufactured by Sunnny hope were excluded because of unknown IC_{50}

Table 5: Correlation analysis of Re contents and IC_{50} (n = 12)[b].

		IC_{50}	Re
IC_{50}	Pearson's correlation	1	0.823
	Sig. (two-tailed)		0.001[c]
	df	12	12
Re	Pearson's correlation	0.823	1
	Sig. (two-tailed)	0.001[c]	
	df	12	12

[b]SM injection manufactured by Sunnny hope were excluded because of unknown IC_{50}. [c]P< 0.01

Table 6: Correlation analysis of Rb_1 contents and IC_{50} (n = 12)[b].

		IC_{50}	Rb_1
IC_{50}	Pearson's correlation	1	0.531
	Sig. (two-tailed)		0.076
	df	12	12
Rb_1	Pearson's correlation	0.531	1
	Sig. (two-tailed)	0.076	
	df	12	12

[b]SM injection manufactured by Sunnnyhope were excluded because of unknown IC_{50}

Table 7: Nonparametric statistics of Rg_1, Re and Rb_1 contents in SM injections from different manufacturers (n = 12).

Null hypothesis (H_0)	Alternative hypothesis (H_1)	Statistical analysis	Sig. (two-tailed)	Selected
Contents of Rg_1 had no difference	Contents of Rg_1 had differences	Kruskal-Wallis	0.058	H_0
Contents of Re had no difference	Contents of Re had differences	Kruskal-Wallis	0.035[d]	H_1
Contents of Rb_1 had no difference	Contents of Rb_1 had no difference	Kruskal-Wallis	0.060	H_0

[d]P< 0.05

Table 8: One-way analysis of variance (ANOVA) for IC_{50} in different manufacturers (n = 12).

	Sum of squares	df	mean square	F	Sig.(two-tailed)
Inter classes	434.693	4	108.673	5.041	0.031[d]
Intra classes	150.893	7	21.556		
Sum	585.587	11			

[d]P< 0.05

and sample size was 10 μL. Peaks of ginsenoside Rg_1, R_e and R_{b1} in each sample were analyzed according to their corresponding reference substances.

Preparation of reference substances

Reference substances of ginsenoside Rg_1, R_e and Rb_1 were dissolved in 20 % acetonitrile concentrations of 0.10 mg/ml Rg_1, 0.08 mg/ml Re and 0.20 mg/ml Rb_1.

Microtox assay [4]

Vibrio fischeri CS234 vials were placed in a thermostat at 15±1°C for 15 min and then 1 ml of rehydration solution (3% NaCl solution) was poured quickly into each vial. The contents of the vials were blended into suspensions. After a waiting time of 10 min, added 100 μl suspensions into the test tube to measure the luminescence intensity, immediately before the addition of rehydration solution or test samples. The same time intervals (10s) were used for later intensity measurement. After a contact time of 10 min, the luminescence intensity of each vial was measured again. The inhibitory effect of test sample was calculated using the equation (1) – (4) as follows:

$$fkt=Ikt/I_0 \quad (1)$$

$$D=(fkt\pm fkt)/\ fkt\times 100\% \quad (2)$$

$$Ict=Ic\times fkt \quad (3)$$

$$Ht=(Ict-It)/Ict\times 100\% \quad (4)$$

Where *fkt* is the correction factor for the contact time of 10 min; *Ikt* is the luminescence intensity in the control group after the contact time of 10 min; I_0 is the initial luminescence intensity of the control group suspension; fkt is the mean value of *fkt*; D is the deviation of the individual from the means; *Ict* is the corrected value of I_0 for test sample suspension; *Ic* is the initial luminescence intensity of the test sample group suspension; *It* is the luminescence intensity of the test sample after the contact time of 10 min; *Ht* is the inhibitory effect of a test sample after the contact time of 10 min.

Statistical analysis

Excel 2007 was used for fitting sample curves and calculating half maximal inhibitory concentration (IC_{50}). IC_{50} value represents the concentration of inhibitor required to decrease luminescence intensity by 50%. Statistical analysis was carried out using SPSS 17.0 for normality test, one-way variance analysis (ANOVA), Pearson's correlation analysis and nonparametric test. P< 0.05 was defined as significant.

Results

Quantification of ginsenosides

According to the National Drug Standards (WS3-B-3428-98-2010Z) it is required that content of ginsenoside Rb_1 and the total contents of ginsenoside Rg_1, Re should not be less than 0.10 mg/ml, and the sum of the three should not be less than 0.20 mg/ml-0.90 mg/ml. Contents of ginsenoside Rg_1, Re, and Rb_1 in SM injection from different manufacturers all met the requirements of WS3-B-3428-98-2010Z (Table 1).

Correlation analysis between content assay and microtox assay

First, normality tests were conducted to check normality distribution of ginsenoside contents and IC_{50} values. Second, Pearson's correlation analysis revealed the relationship between them.

Normality test

Contents of ginsenoside Rg_1, Re, Rb_1 in SM from 5 manufacturers (12 batches) and their IC_{50} values all met normality distribution (P >0.05, Table 3).

Correlation analysis

The Pearson's correlation coefficient (r) for Rg_1 contents and IC_{50} in 12 batches was -0.372, which is not statistically significant (P >0.05, Table 4).

The Pearson's correlation coefficient (r) for Re contents and IC_{50} in 12 batches was 0.823 which was statistically significant (P< 0.01, Table 5). Moreover, there was a positive correlation between the concentration of Re and IC_{50} of microtox assay (Table 5).

The Pearson's correlation coefficient (r) for Rb_1 contents and IC_{50} in 12 batches was 0.531, which is not statistically significant (P >0.05, Table 6).

Discussion

The microtox assay is a technology which considers reduction of bioluminescence in luminous bacteria as a measure of toxicity. It is widely used in water quality determination and the screening of antibiotics and narcotic drugs [5-7]. Our research group introduced microtox assay for quality control of Traditional Chinese Medicine (TCM) injections in China and abroad for the first time in 2014 [8]. Firstly, we compared reaction conditions of *Photosbacterium phosphoreum* 502, *Vibrio qinghaiensisi* Q67, and *Vibrio fischeri* CS234 to optimize microtox assay for TCM. We found that *Vibrio fischeri* CS234 was more suitable than others for TCM injections detection [9]. Secondly, we selected $ZnSO_4$. $7H_2O$ as a reference substance and investigated the methodology to make sure microtox assay adequately meets the needs for measuring toxicity of TCM injections. The results showed that *Vibrio fischeri* CS234 micotox assay system was specific, stable, sensitive, accurate and adaptable for quality control of TCM injections [10].

From 2014-2015, adverse drug reactions (ADR) caused by SM injection ranked second in National Adverse Drug Reaction

Monitoring and Reporting System, behind Qingkaining injection and ahead of Xuesaitong injection. In this study, all SM injection samples we used were qualified products and contents of ginsenoside Rg_1, Re and Rb_1 all met the requirements of National Drug Standards (WS3-B-3428-98-2010Z) of SM injection (Table 1). But for all this, not only the content of Re and IC_{50} of test samples were significantly different ($P< 0.05$, Table 7 and 8), but there was also a positive correlation between them (Table 5). That is, existing quality control methods such as content quantification, asepsis inspection, microbial limit test, undue toxicity test and so forth were not enough to expose potential safety issues.

Taken together, our findings suggested that IC_{50} of microtox assay could be a new quality control method for Re in SM injection. As a biological assessment method, microtox assay was more rapid, sensitive and effective than content determination method. Whether our work would yield useful information for predicting warnings, such as allergic reactions during clinical application of SM injections requires more research.

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References

1. WS3-B-3428-98-2010Z, National Drug Standards of Shenmai injection of State Food and Drug Administration (SFDA).
2. Liu N, Shan D, Li Y, Chen H, Gao Y, Huang Y. Panax notoginseng Saponins Attenuate Phenotype Switching of Vascular Smooth Muscle Cells Induced by Notch3 Silencing. Evid Based Complement Alternat Med. 2015; 2015: 162145.
3. Gao J, Tao J, Zhang N, Liu Y, Jiang M, Hou Y, et al. Formula optimization of the Jiashitang scar removal ointment and anti-inflammatory compounds screening by NF-κB bioactivity-guided dual-luciferase reporter assay system. Phytother Res. 2015; 29: 241-250.
4. ISO11347-3:2007, water quality-determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) –Part 3: Method using freeze-dried bacteria.
5. Ali BI, Linda J, Tonya G, Dwayne B, Joseph O, Paul BT. Assessment of Individual and Combined Toxicities of Four Non-Essential Metals (As, Cd, Hg and Pb) in the Microtox Assay. Int J Environ Res Public Health. 2006; 3: 118-120.
6. Becker D, Varela Della GS, Rodriguez-Mozaz S, Schoevaart R, Barceló D, de Cazes M, et al. Removal of antibiotics in wastewater by enzymatic treatment with fungal laccase - Degradation of compounds does not always eliminate toxicity. Bioresour Technol. 2016; 219: 500-509.
7. Vighi M, Migliorati S, Monti GS. Toxicity on the luminescent bacterium Vibrio fischeri (Beijerinck). I: QSAR equation for narcotics and polar narcotics. Ecotoxicol Environ Saf. 2009; 72: 154-161.
8. Zhao JN, Yan LC. Microtox-based fast Testing Method of Comprehensive toxicity of Traditional Chinese Medicine. World Chinese Medicine. 2014; 9: 137-144.
9. Xiong JY, Li XR. Reaction conditions comparison of Photosbacterium phosphoreum 502 and Vibrio qinghaiensis Q67 in Microtox-based fast testing system for traditional Chinese medicine injection. Pharmacology and Clinics of Chinese Material Medica. 2016; 32: 227-230.
10. Xiong JY, Li XR, Yan LC, Zhao JN. Optimization of reaction conditions and methodological studies on Microtox-based fast testing system in traditional Chinese medicine injection. China J Chinese Material Medica. 2016; 41: 1623-1626.