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A sensitive enzyme-linked immunosorbent assay for evaluating the concentration of bee venom in rat plasma

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Abstract

A simple and reproducible enzyme-linked immunosorbent assay (ELISA) was developed to determine the concentration of bee venom in rat plasma. The intra- and inter-assay coefficients of variation for the ELISA were less then 3% between 0.1 and 1000 ng mL^{-1} venom, and the sensitivity of the detection was 0.1 ng mL^{-1}. Total recovery of the bee venom added to rat plasma was determined. Using this ELISA, serum levels of bee venom were easily determined. The rats were administered a single intravenous injection or oral dose of bee venom (1 mg kg^{-1} of body weight). The bioavailability of the bee venom under the two administrations was compared using pharmacokinetic parameters. Results showed that intravenous administration of bee venom produced high plasma concentrations with a short half-life. The area under the curve for oral administration was 10 times lower than for intravenous administration. This loss of bee venom may be due to the degradation that occurs in the enzymatic and acidic environment of the gastrointestinal tract.

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Introduction

The venom of the honey bee, *Apis mellifera*, is a complex mixture of substances with biological activity (Habermann 1972) affecting the cardiovascular system, immune system, nervous system and circulatory system. In a previous study, we reported that bee venom exhibited antitumor activity in-vitro and in-vivo (Xing et al 2002). However, lack of specific immunoreagents, low level sensitivity, lengthy incubation steps and the need for expensive equipment has hampered the widespread use of clinical routine diagnostic methods. The purpose of this study was therefore to develop a method for analysing bee venom in-vivo.

Enzyme-linked immunosorbent assay (ELISA) has been developed for venom detection and has also been used for venom antibody detection (Theakston 1983). The techniques have been extensively reviewed by various investigators up to 1986, and ELISA appears to be the ideal method for the detection of both venom and venom antibody (Ho et al 1986; Minton 1987; Ratanabanangkoon et al 1987). ELISA is the most versatile immunoassay technique so far applied to the field of venom research, having relatively high sensitivity, specificity, reproducibility and simplicity.

In order to evaluate the pharmacokinetic validity of such regimens, a simple, specific, accurate and reproducible method for the determination of bee venom concentration in serum is required.

We have developed an ELISA that can detect bee venom concentrations in-vitro and in the plasma of rats following intravenous and oral administration. The ELISA gives the basic pharmacokinetic parameters in animals for its further clinical development.

Materials and Methods

Materials

Chinese male Sprague–Dawley rats (200 ± 10 g) were purchased from the Laboratory Animal Center of Beijing. All animal experimentation was approved by the Animal

Care Committee of the Laboratory Animal Center of Beijing. Lyophilized bee venom (Sigma) was used as the standard and for the control (prepared in ELISA diluent and stored at −80 °C), and rabbit anti-bee venom antibody, ortho-phenylenediamine (OPD), horseradish peroxidase (HRP), Tween-20 and bovine serum albumin (BSA) were purchased from Sigma (St Louis, USA). Hydrogen peroxide (H_2O_2) was from Glaxo Laboratories (Bombay, India), and the polystyrene microtiter plates (96 wells) were obtained from Costar (Cambridge, USA).

Rabbit IgG anti-bee venom–HRP was conjugated by the Laboratory of Molecular Biology (Tsinghua University, China). All other chemicals and reagents used were of analytical grade.

ELISA

In all steps of this ELISA, the wells were filled to a volume of 200 μL. Microtiter wells (Costar) were coated with antisera against bee venom, freshly diluted to its optimal concentration (1:200), in carbonate/bicarbonate buffer (pH 9.6) and incubated for 18 h at 4 °C. The coated plates were washed five times with ELISA washing buffer (phosphate buffered saline (PBS) containing 0.05% Tween-20, pH 7.4) and blocked with ELISA blocking buffer (PBS containing 10% BSA, 0.05% Tween-20, pH 7.4) for 30 min at 37 °C. After the blocking, the plates were washed again five times with ELISA washing buffer then 200 μL of standards, plasma samples (diluted up to 1:10 in PBS) or controls were added to duplicate wells and incubated for 90 min at 37 °C. Following another washing cycle, rabbit IgG anti-bee venom–HRP conjugate at 1:1000 dilution was added to each microtiter well for 90 min incubation at 37 °C. After another washing cycle, the reaction was revealed by addition of 0.4 mg mL^{-1} OPD and 1.5 μg mL^{-1} H_2O_2 in 0.1 mol L^{-1} sodium citrate/phosphate buffer (pH 5.0) for 15 min at room temperature in the dark and stopped by the addition of 50 μL of 3 mol L^{-1} sulfuric acid. The absorbance was measured at 490 nm with a spectrophotometer (Bio-Rad Model 3550 Microplate Reader, USA). Free venom antigen concentrations in the plasma were determined by interpolation against a reference curve, set up as described below.

Calibration and quality control of the ELISA

For quantitation of bee venom in rat plasma the standard curve was prepared using a range of bee venom concentrations (0.1–1000 ng ml^{-1}) diluted in a pool of normal rat sera. Performance parameters of the ELISA (detection limit, linearity, precision and intra-assay, inter-assay and inter-sample coefficients of variation) were established. In order to quantify the amount of venom present in plasma samples, a titration curve was constructed by plotting a log of venom concentration against the bee venom's absorbance value. Known amounts of bee venom spiked with plasma from normal controls were included with each test to obtain a titration curve. Quantitation of bee venom was carried out by working out the difference in the absorbance values between the experimental and control wells, and by subsequent comparison with the titration curve. The venom concentration was calculated and expressed in ng mL^{-1} and pg mL^{-1} of rat plasma.

Administration and samples

Twenty Sprague–Dawley rats were used in the pharmacokinetic study. The bee venom was administered intravenously to one group (10 rats) at a dose of 1.0 mg kg^{-1}. Another group (10 rats) received bee venom in a single oral dose of 1.0 mg kg^{-1}. Blood samples of 0.2 mL were drawn before the bee venom was administered and again at 5, 10, 20, 30, 40, 60, 90, 120, 180, 240 and 360 min after administration. Blood samples were obtained by puncture of the retro-orbital blood vessels with heparinized pipettes. Plasma was separated from whole blood by centrifugation at 4000 *g* for 10 min at 4 °C. These samples were stored at −80 °C until they were analysed. Bee venom levels were then assessed by ELISA as described above.

Pharmacokinetic analysis

Serum bee venom levels were measured at appropriate intervals after treatment to determine the maximum concentration (C_{max}), the time to reach the maximum concentration (T_{max}), the elimination rate (K_e), the elimination half-life ($t_{1/2}$), the area under the curve estimated to infinity ($AUC_{(0-\infty)}$), the area under the concentration × time curve estimated to infinity ($AUMC_{(0-\infty)}$) and the mean residence time estimated to infinity (MRT). The mean plasma concentration was used to calculate the kinetic parameters. The bioavailability of the bee venom under the two types of administration was compared using the pharmacokinetic parameters C_{max}, T_{max} and $AUC_{0-\infty}$, which were estimated from the plasma concentration–time data. C_{max} and T_{max} were obtained directly from the plasma concentration values.

Compartment models of the plasma concentration–time course of the bee venom were fitted, and pharmacokinetic parameters were calculated using the Practical Pharmacokinetic Program 3P97 (Mathpharmacology Committee, Chinese Academy of Pharmacology, Beijing, China), using the F-test, r^2-value and Akaike's information criteria (Yamaoka et al 1978; Guo et al 1993) as criteria for the selection of compartment models.

Statistics

The statistical Program for Scientific Studies (SPSS 10.0) software package (for a Windows operating system) was used to perform statistical analysis of the data. A non-parametric test was used to compare multiple groups. The effects of bee venom concentration on the various parameters were analysed using a Kruskal–Wallis test. The oral and intravenous pharmacokinetic properties were compared for significance using a Mann Whitney U test. Data are presented as mean values with the standard deviation (mean ± s.d.). A *P* value of less than 0.05 was considered to be statistically significant.

Results

ELISA for estimating bee venom concentration

The curve of the bee venom concentration (in rat plasma) vs optical densities was obtained. The correlation of optical density with concentration was well linearized by log–log plot. All the results revealed high reproducibility and high accuracy in the correlation of the optical density with bee venom concentration (0.1–1000 ng mL^{-1}, $r^2 = 0.9981$), with the lower detection limit at 0.1 ng mL^{-1}. The limit of detection, that is the concentration of bee venom giving greater than two standard deviations above the optical density (490 nm) of the blank, was 0.1 ng mL^{-1} in ELISA diluent. The recovery was 100.65%. Since plasma samples were diluted 1:10 to minimize matrix interference, as little as 1 ng mL^{-1} of bee venom could be measured in the original sample (shown in Table 1). Assay precision was evaluated using ELISA diluent controls diluted to all points on the standard curve (Table 2). The coefficients of variation percentage ranged from 1.05 to 2.33% (inter-assay) and from 1.28 to 2.96% (intra-assay). Table 3 shows the ability of the ELISA to accurately recover bee venom that has been added to individual plasma samples. The recovery percentage of these samples ranged from 99 to 101.15%.

Table 1 Sensitivity test for determination of bee venom by ELISA (n = 6).

	Zero plate	Negative plate	Bee venom ($\mu g\ mL^{-1}$)			
			10^{-5}	10^{-4}	10^{-3}	10
OD value (s.d.)	0.032 (0.003)	0.321 (0.008)	0.473 (0.031)	0.665 (0.012)	1.039 (0.018)	3.01 (0.038)
Positive/negative	–	–	1.36	2.07	3.24	9.38
P	–	–	–	*	**	**

Data are mean (±s.d.) of six experiments performed in triplicate. **P* < 0.01 vs negative value, ***P* < 0.001 vs negative value (analyzed using a Nemenyi's test).

Table 2 Accuracy and precision test for determination of bee venom by ELISA.

Concentration ($\mu g\ mL^{-1}$)	Inter-assay (n = 6)		Intra-assay (n = 7)	
	Mean ± s.d.	CV (%)	Mean s.d.	CV (%)
10	3.010 ± 0.038	1.26	3.104 ± 0.072	1.96
1	2.395 ± 0.010	1.08	2.405 ± 0.039	2.13
10^{-1}	1.719 ± 0.040	2.33	1.724 ± 0.051	2.96
10^{-2}	1.489 ± 0.018	1.21	1.483 ± 0.033	2.23
10^{-3}	1.05 ± 0.011	1.05	1.047 ± 0.006	1.28
10^{-4}	0.665 ± 0.008	1.84	0.659 ± 0.010	2.70

Data are mean ± s.d. of six or seven experiments performed in triplicate (analysed using a Kruskal–Wallis test). CV, coefficient of variation.

Table 3 Recovery test for determination of bee venom by ELISA (n = 6).

Added (%) ($\mu g\ mL^{-1}$)	Recovery (%)					Mean (%) s.d.	CV
	1	2	3	4	5		
10	100.60	98.30	97.70	100.06	101.93	99.72 ± 1.72	1.73
10^{-1}	99.47	102.14	99.93	101.39	102.44	101.07 ± 1.32	1.31
10^{-3}	100.80	100.03	99.80	103.51	101.63	101.15 ± 1.50	1.48

Data are mean ± s.d. of six experiments performed in triplicate (analysed using a Kruskal–Wallis test). CV, coefficient of variation.

Pharmacokinetics of bee venom after administration

Twenty rats were used. Ten rats received a single intravenous dose of $1\,mg\,kg^{-1}$ of bee venom and another 10 rats received a single oral dose of $1\,mg\,kg^{-1}$ of bee venom. The time courses of the bee venom concentrations in the plasma were followed by ELISA. Figure 1 shows the results of assays of the plasma samples as the curve of logarithm (concentration)–time. The plasma bee venom level dropped rapidly after i.v. administration and then declined slowly. The curve fitted a two-compartment model well. The results for oral administration are also shown in Figure 1. After a rapid ascending phase, characteristic of a fast absorption, the distribution reached its plasma maximum value (C_{max}) at 60 ± 4.17 min after administration. The distribution and elimination of the plasma venom seem to follow a non-compartment model. The mean estimated pharmacokinetic parameters derived from the plasma concentration profile of bee venom after treatment with the two types of administration are shown in Table 4. The total area under the plasma concentration curve ($AUC_{0-\infty}$) decreased from 138.47 ± 0.53 to $7.23 \pm 0.26\,min\,\mu g\,mL^{-1}$, a decrease of 94.78% ($P < 0.0001$, Mann Whitney U test). On the other hand, the elimination half-life ($t_{1/2}$) decreased from 295.8 ± 0.76 to 21.16 ± 0.75 min ($P < 0.0001$, Mann Whitney U test). There was an increase both in the clearance from 0.11 ± 0.005 to $0.30 \pm 0.01\,\mu g\,kg^{-1}\,min^{-1}$ ($P < 0.01$, Mann Whitney U test) and in the MRT from 44.54 ± 0.19 to 88.74 ± 0.39 min ($P < 0.001$, Mann Whitney U test). After an intravenous dose, the MRT may be calculated as:

$$MRT = AUMC/AUC$$

Since the ratio of AUMC/AUC will change depending on the route of venom administration, some have concluded that the MRT also changes with route of administration. The mean biovailability of the oral bee venom was $5.22 \pm 3.60\%$.

As shown in Table 4, the determination of the pharmacokinetic parameters for the bee venom indicated that bee venom has rapid absorption and slow elimination.

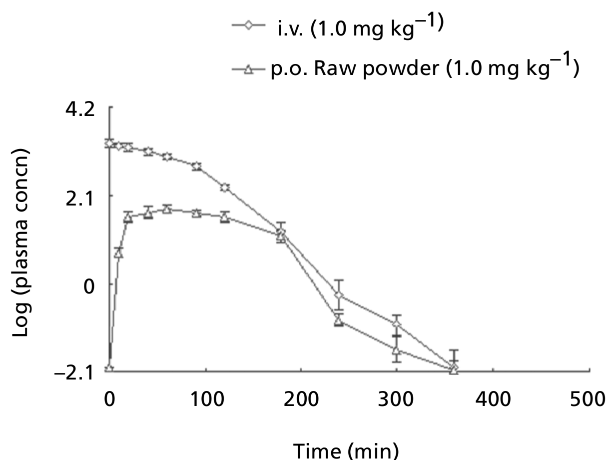

Figure 1 Pharmacokinetics of bee venom after intravenous or oral administration. Blood samples were drawn at the indicated times and centrifuged, and the plasma was collected. The concentration of bee venom in plasma samples was determined by ELISA as described in Materials and Methods. Each bar represents the mean ± s.d. of six experiments performed in triplicate.

Table 4 Pharmacokinetic parameters determined by ELISA after intravenous injections ($1\,mg\,kg^{-1}$) and oral raw powder ($1\,mg\,kg^{-1}$) of bee venom in rats (n = 10).

Parameters	Intravenous injections	Oral raw powder
α (min^{-1})	0.036 ± 0.0009	0.049 ± 0.028
β (min^{-1})	0.0023 ± 0.003	0.033 ± 0.005
Lag time (min)	–	9.10 ± 0.74
$t_{1/2\alpha}$ (min)	19.4 ± 0.02	–
$t_{1/2\beta}$ (min)	42.95 ± 7.27	–
$t_{1/2\ (Ka)}$ (min)	–	14.29 ± 0.69
$t_{1/2\ (Ke)}$ (min)	–	21.16 ± 4.57
K_{21} (min^{-1})	0.00016 ± 0.04	–
K_{10} (min^{-1})	0.036 ± 0.07	–
K_{12} (min^{-1})	0.00011 ± 0.03	–
T_{max} (min)	–	60.00 ± 4.17
C_{max} ($ng\,mL^{-1}$)	–	61.32 ± 6.30
CL ($\mu g\,kg^{-1}\,min^{1}$)	0.11 ± 0.005	0.30 ± 0.01
$AUC_{(0-\infty)}$ ($min\,\mu g\,mL^{-1}$)	138.47 ± 19.26*	7.23 ± 1.24
$AUMC_{(0-\infty)}$ ($min^2\,\mu g\,mL^{-1}$)	6167.59 ± 487*	641.58 ± 49
MRT (min)	44.54 ± 5.88*	88.74 ± 9.31

Data are mean ± s.d., n = 10. *Significant differences between two groups ($P < 0.01$, Mann Whitney U test). CL, clearance.

Discussion

Theakston et al (1977) first described the use of ELISA for the detection of snake venom and venom antibodies. During the last two decades, numerous ELISAs have been reported from various parts of the world for different purposes (Doellgast 1987; Pugh & Theakston 1987; Pe et al 1991, 1994, 1995; Moisidis et al 1996; Selvanayagam & Gopalakrishnakone 1999). Although these ELISA methods were similar to that developed by Theakston et al (1977), several important advancements have been made in the specificity, sensitivity, rapidity and simplicity of the ELISA method.

According to the ELISA method described by Theakston et al (1977), Franca et al (1994) investigated bee venom levels in five victims of multiple bee stings in Brazil. However, in his paper, the ELISA method for the detection of bee venom was not described in detail. In the ELISA investigated in the present study, the coating concentration of antibody of anti-bee venom was optimal at $0.5\,mg\,mL^{-1}$ by the criss-cross analysis, demonstrating a good result with low background and a large range of optical densities for the increasing numbers of standards (data not shown).

The pharmacokinetic results of the bee venom after intravenous injection in rats show that the plasma concentration–time course fits a two-compartment model. The short half-life of the α phase (19.4 min) indicates that the bee venom was rapidly distributed from the central to the peripheral compartment after injection. This result implies a strong affinity of the bee venom for tissue. After oral administration, the bee venom plasma level rose to a maximal concentration after about 1.0 h, and the area under the plasma concentration–time curve was 10 times lower than after intravenous injection. This result indicates that the orally administered bee venom is delivered from the gastrointestinal tract. The loss of bee venom may be due to the degradation that occurs in the enzymatic and acidic environment of the gastrointestinal tract.

In conclusion, the ELISA method for determination of the bee venom concentration in plasma in-vitro and in-vivo shows high reproducibility, high sensitivity and high efficiency, thus meeting the current requirements for a bioanalytical assay. Finally, the method was employed in a pharmacokinetic study of bee venom in rat, which demonstrates its usefulness for pharmacological research.

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