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Original Article

QUALITATIVE AND QUANTITATIVE EVALUATION OF MELITTIN IN HONEYBEE VENOM AND DRUG PRODUCTS CONTAINING HONEYBEE VENOM

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Abstract

A reverse-phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for the analysis of honeybee venom samples and drug products containing honeybee venom. The validation parameters were linearity, sensitivity, precision, and recovery. Melittin is the main component of honeybee venom was extracted with pure water, and then evaluated by RP-HPLC with a photodiode array (PDA) detector. Separation of the samples was achieved on a Europa Protein C_{18} column with linear gradient elution of acetonitrile and 0.4% phosphoric acid at 25°C. There was a flow rate of 1 mL/min. Detection was set at 220 nm. Limits of detection (LOD) and quantification (LOQ) for melittin were 1.1 and 3.2 µg/mL, respectively. The amount of melittin in honeybee venom samples ranged from 21.9 to 66.4 %.

Keywords: honeybee venom, melittin, method validation, RP-HPLC.

INTRODUCTION

Honeybee venom possesses diverse biological and pharmacological activities. It has been reported to have a marked effect on a variety of ailments, such as arthritis, rheumatism, cancerous tumors, pain, angiocardiopathy, multiple sclerosis, and skin diseases (Kwon et al., 2002; Kim et al., 2003; Park et al., 2004; Russel et al., 2004; Kwon et al., 2005; Putz et al., 2006; Son et al., 2007; Lee et al., 2008). Honeybee venom is collected by an electric shock instrument, sometimes coupled with stimulating the honeybee to sting the surface of the collector sheet or venom is extracted from bee glands or sticks (Rybak et al., 1995; Rybak and Skubida, 2007). It contains a complex mixture of biogenic amines and peptides. This mixture has both pharmacological and biological activities. The main components of honeybee venom are peptides (melittin, apamin, promelittin, and mast cell deregulating peptide [MCDP]), enzymes (phospholipase A_2 and hyaluronidase), histamine, sinkaline, noradrenaline and amino acids (Habermann, 1972; Gauldie et al., 1978). The major compound of the lyophilized venom is a peptide containing 26 amino acid residues. This peptide was named melittin by Ernst Habermann (Gauldie et al., 1976).

Several analytical techniques have been reported for determinating biological compounds of honeybees, such as HPLC coupled with detections of ultraviolet, diode array and mass spectrometry (Rader et al., 1987; Szokan et al., 1994; Pacáková et al., 1995; Ziyavitdinov et al., 1995; Rybak-Chmielewska and Szczęsna 2004; Kokot and Matysiak, 2009; Zhou et al., 2010), and capillary electrophoresis (CE) (Pacáková et al., 1995; Pacáková and Stulik, 2000; Matysiak et al., 2011). In pharmaceutical industry, the RP-HPLC method is one of the most useful techniques for quality control of drugs formulations. The samples of honeybee venom and drug products containing the venom

with different forms need to be analysed using an assay with the precise the RP–HPLC method. We duplicated the RP-HPLC methods mentioned above but separation was inadequate because of overlapping and intense baseline drift up, during the gradient run. Applying the materials in cream and ointment forms is rather difficult. The cream and ointment often contain excipients, osmotic regulators, and other stabilizers which can interfere with the analytes of interest. Due to the complexity of this matrix, it is difficult to obtain a satisfactory method for analyzing the active ingredients of honeybee venom in cream and ointment products by RP-HPLC. Previously, there were no methods available for assaying the active ingredients in honeybee products. Therefore, an accurate analytical method was required to meet the current analytical needs. The objective of this work was to develop and validate a RP–HPLC method for the quantitative determination of melittin in honeybee venom and honeybee venom products.

MATERIAL AND METHODS

Chemicals

HPLC-grade acetonitrile and water, and phosphoric acid (85%) were from Merck (Darmstadt, Germany). The 91.8% purity melittin was from Sigma (M2272, 119K4001).

Sample collection

During the summer of 2011, samples of honeybee venom were collected from the apiaries in: the province of Ardabil (Mount Sabalan), in Uromia (north western Iran), and in Shahrekord which is in the province of Chaharmahal va Bakhtiari (south western Iran). Venom was collected at the entrance of the hive by stimulating the honeybee with an electric shock instrument. The samples were stored in sealed amber vials at the low temperature of 4°C, until analysis. The cream for musculoskeletal pains ApiVENZ™ Relief, containing 0.1% of honeybee venom, standardized to melittin (40%), was from New Zealand, and APISARTHRON® ointment (labeled as containing 0.003% honeybee venom) was provided from Russia.

Sample preparation

One mg of powdered honeybee venom was transferred into a screw capped tube and dissolved in 10 mL of pure water by mixing with a vortex mixer for 3 min. After centrifuging at 4000 rpm for 5 min, 3 mL of supernatant was transferred to a 5 mL vial. For the HPLC analysis, this solution was filtered through a syringe filter which had a 0.45 µm membrane.

One gram of cream containing 1 mg of honeybee venom and 10 mL of 0.4% phosphoric acid in water (eluent A) were put into at a screw capped tube and heated at 70°C for 5 min and mixed. The solution was centrifuged at 4000 rpm for 10 min at ambient temperature. The supernatant was decanted, and clarified by mixing with 1 mL of hexane. The aqueous solution was filtered through 0.45 µm membrane for HPLC analysis.

Standard solution

The stock solution was prepared by dissolving the melittin in pure water to yield the concentration of 1000 µg/mL. A working standard solution of 200 µg/mL was made by diluting the stock solution with pure water and keeping this diluted solution in the dark at 4°C prior to analysis. The concentration of standard solutions was selected according to the level of the analyte expected in the samples. The calibration curve for melittin was constructed at five concentration levels.

Chromatographic conditions

The high performance liquid chromatographic analysis was performed using a Knauer UHPLC/HPLC PLATINblue instrument (Knauer, Berlin, Germany) consisting of two pumps, the column compartment, and the PDA detector. Samples were separated on a Europa Protein 300 C_{18} column (250 × 4.6 mm, 5µm) at a flow rate of 1 mL/min. The mobile phase was acetonitrile (eluent A) and 0.4% phosphoric acid in water (eluent B) at 25°C with gradient elution as follows: 5% – 52% A at 0 – 15 min, 52 – 80% A at 5 min, 80% – 100% at 5 min. The volume of all injections was 20 µL (loop 20 µL). The PDA detector scanned from 200 to 380 nm, and detection was set at 220 nm. Each sample

was injected three times on the HPLC system. The analytes were confirmed by comparing the retention times and UV spectra with standard compounds and with those reported in the literature.

Method validation

The proposed method was validated in terms of linearity, limits of detection (LOD) and quantification (LOQ), precision (intra- and inter-day), recovery, robustness and analyte stability. To establish calibration curve, linearity was tested at five concentration levels and injected three times. The limits of detection (LOD) and quantification (LOQ) were determined by injecting a series of dilute solutions with known concentrations on the basis of a signal-to-noise ratio equal to about 3 and 10, respectively. Intra and inter-day precisions were determined on day 1 and 3 consecutive days, with 3 repetitions each. The relative standard deviation (RSD) was taken as a measure of precision. A recovery test was used to evaluate the accuracy of this method. Recovery was determined by adding the standard solution at three concentration levels to the samples. Each sample was analysed in triplicate. Stability of the analyte was tested with the standard solution and sample solution stored at ambient temperature and analysed at 0 and 24 h. The robustness of the method was studied by evaluating the effect of the small but deliberate variation of the chromatographic conditions. The column temperature was changed by approximately 10% and the flow rate by 0.2 units from 1.0 to 0.8 or 1.2 mL/min.

RESULTS

The optimum condition for separation of analytes in the samples was obtained on a Europa Protein 300 C_{18} column under gradient elution using 0.4% phosphoric acid in water and acetonitrile as the mobile phase at a flow rate of 1 mL/min at 25°C. By applying a gradient of 5 - 52% acetonitrile, approximately 14 peaks could be detected within 15 min. The phospholipase A_2 and melittin as the main components and many minor peaks of honeybee venom in all samples could be easily separated with reproducible results. The wavelength of the PDA detector ranged from 200 to 380 nm and detection was set at 220 nm. The UV absorption of melittin and phospholipase A_2 were very similar. Owing to the lack of a suitable standard, we were not able to identify apamin, hyaluronidase, and MCDP. The apamin, hyaluronidase, MCDP, and phospholipase A_2 on RP-columns were eluted before melittin. The actual analysis time of all the samples was less than 15 min. The retention times of phospholipase A_2, and melittin were 10.3 and 13.3 min, respectively (Fig. 1A and 1B). The validated method was used successfully to evaluate the melittin of eight honeybee venom samples and four honeybee drug products.

The following parameters are used to validate the proposed method: selectivity, linearity, sensitivity, repeatability, recovery and stability. The parameters are shown in Table 1. The method was validated using a honeybee venom sample with melittin >40% and ApiVENZ™ cream.

A method is selective if it can be used to measure analyte in the matrices without interference from co-eluting matrix components. Selectivity of the described method was determined by analysing both the drug product and honeybee venom samples. There were no interferences from the matrix components present in samples with analyte. On the other hand, no peaks overlapped with other peaks. The UV spectra of all the components recorded by PDA detector, were similar. Peaks in UV spectra show a shoulder at 220 nm (Fig. 1D). For this reason, the peak area percentage of components (normalization method) was constant at 220 nm. The amount of melittin obtained by normalization is not the same as when using the external standard method because honeybee venom is often contaminated with inorganic salts or insoluble impurities.

The stock solution of melittin was diluted to the appropriate concentration ranges for the establishment of the calibration curve. Triplicate injections were made at five different concentrations. The linearity was ensured with values in the range of 5 – 100 µg/mL. The quantitative determination of melittin in the samples was calculated based on the calibration curve. The correlation coefficient of the detector response

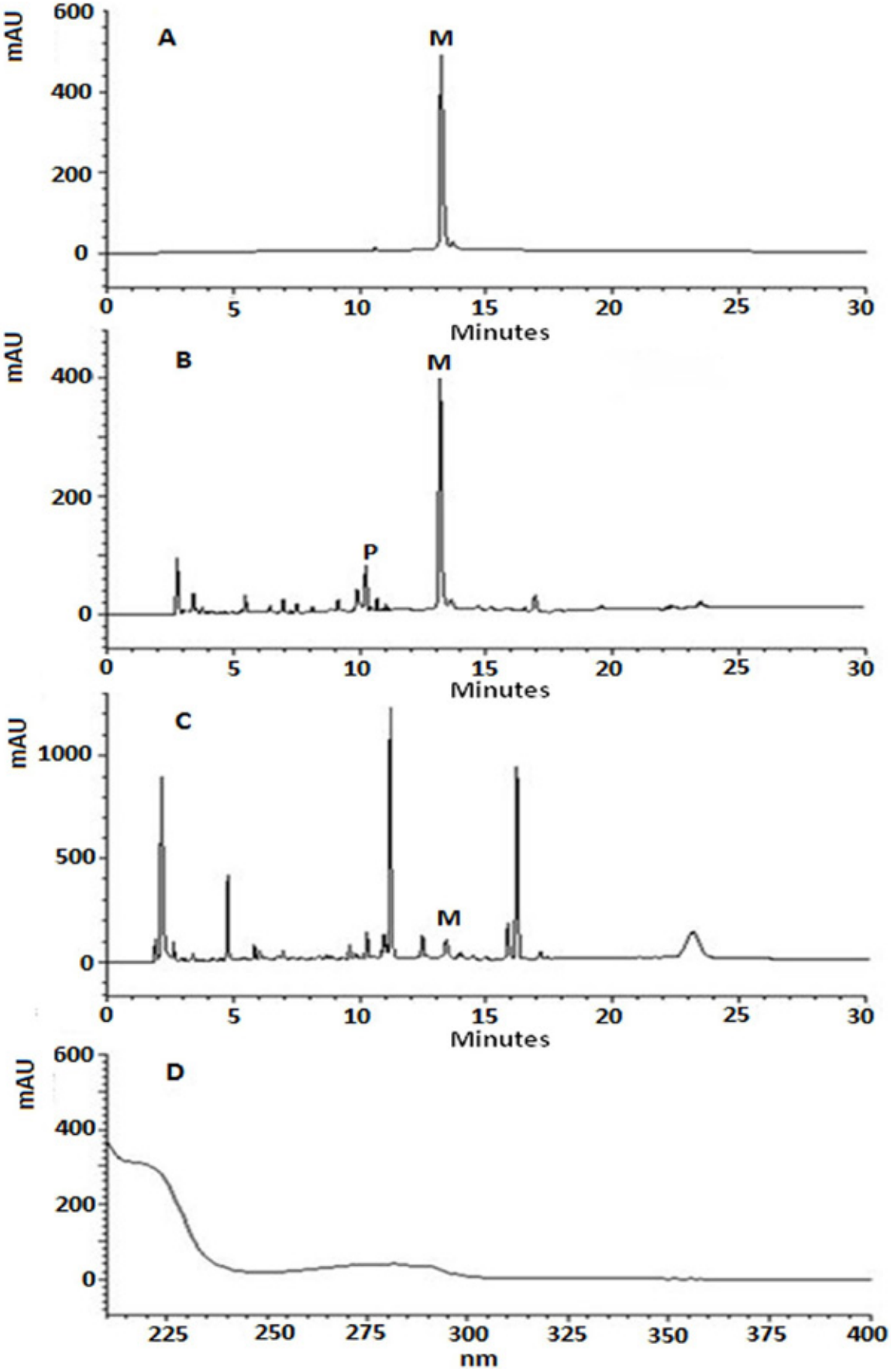

Fig. 1. HPLC chromatograms of melittin standard (A), honeybee venom (B), honeybee venom cream (C), UV spectrum of melittin standard recorded by PDA detector (D). Peaks: M (melittin), P (phospholipase A_2).

and the regression equation for melittin was 0.9997, and y = 59919x + 131690 (y is the peak area and x is the concentration [μg/mL]), respectively. The sensitivity of the method was indicated by LOD and LOQ. The lowest concentration of the analyte detected by the method is called the LOD; the minimum quantifiable concentration is the LOQ. The signal-to-noise ratio of 3:1 and 10:1 were taken as LOD and LOQ, respectively. For melittin, the LOD and LOQ were 1.1 and 3.2 μg/mL, respectively (Tab. 1).

The repeatability of the method was assessed using three individual samples within one day (three parallel samples) and on three consecutive days (two parallel samples each day). The RSD values calculated as a measurement of method repeatability were 1.68 and 1.93%. Recovery was determined by the standard addition method. The standard solutions at three different contents of melittin corresponding to 80, 100, and 120% were spiked into the samples. For comparison, an unspiked sample was concurrently prepared and analysed. Triplicate experiments were performed at each level. The results of the recovery test indicate that the established method was accurate enough for the determination of the analyte. The mean recovery was found to be 98.6%, with a mean RSD of 1.58%.

Stability of melittin in the mobile phase was determined by injecting them with venom and products 0 and 24 h post-preparation at ambient temperature while protecting the samples from direct sunlight. No peaks corresponding to the degradation products were observed and there was no significant change in the melittin peak area after 24 h of storage. Melittin was found to be stable in the mobile phase for at least 24 h (RSD less than 2.0%). Finally, because alteration of the chromatographic conditions had no significant effect on retention time or resolution, the method can be regarded as robust. All honeybee venom samples had phospholipase A_2, and melittin. The amount of melittin in the bee venom samples was 21.9, 23.7, 28.2, 31.8, 41.7, 46.4, 51.3, and 66.4%. The amount of melittin in

Table 1.

Validation data for the suggested method

Analyte	Linearity Range (μg/mL)	Regression equation	LOD* (μg/mL)	LOQ** (μg/mL)	R
Melittin	5 - 100	y = 59916x + 131690	1.1	3.2	0.9997

*-LOD – limit of detection
**- LOQ – limit of quantification

Table 2.

The sample concentration, limits of detection and quantification, values, and retention time (t_R) for melittin analyses in honeybee venom by RP-HPLC in comparison to results received by other authors

Method	Sample Concentration	LOD*	LOQ**	$t_{R\ (min)}$ ***
Present study	100 μg /mL	1.1	3.3	13.2
Szokan et al., (1994)	5000 μg/mL	-	-	31.2
Pacáková et al., (1995)	500 μg/mL	3	-	18.1
Rybak - Chmielewska and Szczęsna (2004)	5000 μg/mL	40	-	25.7
Kokot and Matysiak (2009)	300 μg /mL	4.8	14.4	16.4

* LOD – limit of detection
** LOQ – limit of quantification
*** t_R – retention time

ApiVENZ™ cream from New Zealand was found to be 4.6 mg per 100 g of cream.

DISCUSSION

Honeybee venom contains a complex mixture of peptides (melittin, apamin, promelittin, and mast cell deregulating peptide [MCDP]), enzymes (phospholipase A_2, and hyaluronidase), histamine, sinkaline, noradrenaline, and amino acids. These ingredients particularly peptides, are active compounds. The products containing these ingredients are used for reducing inflammation in joint diseases such as arthritis. The honeybee venom products contain excipients. An excipient is generally a pharmacologically inactive substance formulated with the active ingredient of a medication. Due to the complexity of the matrix, it is difficult to separate the active materials in the presence of excipients, in medicinal products containing honeybee venom. The analysis of the honeybee venom samples and drug products was done without the use of cartridges containing sorbent substances. The analysis time was shorter. More sensitivity and better separation efficiency were involved than for other methods (Tab. 2).The sample concentration, limits of detection and quantification, values, and retention times (t_R) for melittin were less than those previously reported (Szokan et al., 1994; Pacáková et al., 1995; Rybak-Chmielewska and Szczęsna, 2004; Kokot and Matysiak, 2009). There was a significant difference of melittin in samples. The amount of melittin in the honeybee venom samples collected during the summer season ranged from 21.9 to 66.4% with a mean value of 38.9%. Szokan et al. (1994) found the melittin content in bee venom samples to be 50-60%. In another study, the amount of melittin was reported to be 61 to 71% (Rybak-Chmielewska and Szczęsna, 2004). Our results may differ from those of other studies because of the geographical origin of the samples, the use of different sampling methods, the condition used for drying and keeping the samples and because of impurities. The findings showed that honeybee venoms from Ardabil and Uromia (north western Iran) was >40%. It should be noted that a validated method was used for the analysis of four marketed honeybee venom drugs. These included drug products from two different manufacturers with two different contents of honeybee venom (0.1 and 0.003%). The representative chromatogram of ApiVENZ™ cream from New Zealand, which claims to content 40 mg melittin per 100 g of cream, is shown in Fig. 1C. The melittin peak was detected as poor. The amount of melittin was estimated to be 4.6 mg per 100 g of cream. The results revealed that melittin in the formulation of this drug may be unstable and that the melittin content degrades with time. The APISARTHRON® ointment from Russia (0.003%) had a melittin level that was below the quantitative limit (BQL).

CONCLUSIONS

A reverse-phase high-performance liquid chromatographic method was developed to separate melittin in several honeybee samples and products. The analysis time was less than 15 min for all samples. The sample concentration, limits of detection and quantification, and retention time (t_R) for melittin were less than those previously reported. Baseline drift up during gradient run at a wavelength of 220 nm reduced significantly.

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