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Comparison of high-performance liquid chromatography and capillary electrophoresis for the determination of some bee venom components

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Abstract

HPLC and capillary electrophoretic (CE) methods were compared for the determination of phospholipase A_2 and melittin in bee venom. Size-exclusion chromatography on a Tessek Separon HEMA-BIO 40 column requires the use of a denaturing eluent (0.2% trifluoroacetic acid in 20% acetonitrile) to overcome non-specific interactions of some components, e.g., melittin. Reversed-phase HPLC on a HEMA-BIO 1000 C_{18} column with gradient elution using water–acetonitrile mobile phases containing trifluoroacetic acid and UV spectrophotometric detection at 215 nm permits the identification and determination of the main bee venom components and their preparative chromatography. CE analysis for bee venom components is optimum with electrolyte system of 150 m*M* phosphoric acid (pH 1.8) with UV spectrophotometric detection at 190 nm. In comparison with HPLC, the CE method is cheaper and faster (6 min vs. 45 min) and the separation is more efficient.

1. Introduction

The effects of bee venom have been known since prehistoric times, but its composition was established only 10–20 years ago [1,2]. It contains low-molecular-mass components, e.g., histamine (M_r 111, 0.1–1.5%), oligopeptides (M_r 200–1000), phospholipids and saccharides (about 25%), polypeptides, e.g., melittin (M_r 2840, 50% of dry venom), neurotoxic apamine (M_r 2038, 2%) and a mast cell degranulating (MCD) peptide (M_r 2593, 2%), and proteins e.g., phospholipase A_2 (M_r 19 000, 12–15%) and hyaluronidase (M_r 45 000–50 000, 1–3%). Whereas phospholipase and hyaluronidase (and also melittin, slightly) are allergens, the polypeptides are highly toxic.

Many methods have been described for characterization of bee venom, (e.g., [3–12]), including biological tests, chemical approaches based on typical protein reactions and separation techniques. Common preparative and analytical methods, such as gel filtration, ion-exchange chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), are relatively time consuming and, therefore, are often replaced by high-performance liquid chromatography (HPLC) [4–11], whose principal advantages are speed of analysis (about 30 min) and high separation efficiency.

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Capillary electrophoresis (CE), although widely used for the separation and analysis of complex biological products, has not yet been used for this purpose. This paper deals with the identification and determination of predominant bee venom components using HPLC on new polymeric, biocompatible stationary phases and CE and critically compares these two approaches.

2. Experimental

2.1. Chemicals

Dry bee venom samples from various sources (Czech Republic, Russian Federation, Bulgaria) and fractions isolated by size-exclusion chromatography were obtained from Sevac (Prague, Czech Republic). Standard samples of phospholipase A_2 (isolated from bee venom), melittin (purity 86% by HPLC) and hyaluronidase (isolated from bovine testes) and molecular mass standards human serum albumin (HSA) (68 459), bovine serum albumin (BSA), ovalbumin (45 000), myoglobin (17 400), cytochrome *c* (13 500), ribonuclease A (13 700), aprotinin (6500), vitamin B_{12} (1355.4) and cytidine-5-monophosphate (340) were obtained from Sigma (St. Louis, MO, USA).

All other chemicals were of analytical-reagent grade from Lachema (Brno, Czech Republic) and were used as received.

2.2. Instrumentation and experimental conditions

HPLC measurements were performed using a PU 4100 LC liquid chromatograph, with a PU 4110 variable-wavelength UV–Vis detector (Philips Pye Unicam, Cambridge, UK) and a Model 7125 injector (Rheodyne, Cotati, CA, USA). The data were collected and processed using a CSW data station (DataApex, Prague, Czech Republic).

For size-exclusion chromatography (SEC), a steel column (250 mm × 8 mm I.D.) containing Separon HEMA-BIO 40, 10 μm (Tessek, Prague, Czech Republic) was used at a flow-rate of 0.5 ml/min, with UV absorbance detection at 215 nm. The mobile phases consisted of 0.1 *M* phosphate buffer (pH 7.0) or 0.2% trifluoroacetic acid (TFA) in 20% acetonitrile (ACN).

For reversed-phase chromatography (RP-HPLC) with gradient elution, a Compact Glass Cartridge (CGC) column (150 mm × 3.3 mm I.D.) packed with Separon HEMA-BIO 1000 C_{18}, 10 μm (Tessek) was used at a flow-rate of 0.5 ml/min, with UV absorbance detection at 215 nm. Eluent A was 0.22% TFA in water, eluent B was 0.2% TFA in acetonitrile. The gradient was linear from 0 to 50% B in 20 min, followed by a rise to 100% B in 5 min. After 5 min at 100% B, resetting followed to 0% B in 1 min and re-equilibration for 14 min. The column was thermostated at 37°C. Preparative separation was performed on a steel column (80 mm × 8 mm I.D.) packed with the same sorbent. The conditions were the same except for a flow-rate of 1 ml/min and a gradient time of 40 min.

Capillary electrophoresis (CE) was carried out on a Crystal CE Model 310 instrument, with a variable-wavelength UV spectrophotometric detector (ATI Unicam, Cambridge, UK). The total length of the fused-silica capillary (L_c) was 75 cm, the length to the detector (L_D) was 60 cm and the I.D. was 75 μm. A constant potential of 20 kV was applied. UV spectrophotometric detection was used at 205 or 190 nm depending on the composition of the electrolyte system. The electrolyte systems consisted of (A) 20 m*M* phosphate buffer (pH 5.0), (B) 20 m*M* Tris buffer–50 m*M* SDS (pH 9.0) and (C) 150 m*M* phosphoric acid (pH 1.8)

The calibration plots for HPLC were prepared by injecting standard mixtures of phospholipase and melittin in the running mobile phase at concentrations of 0.125, 0.20, 0.25 and 0.5 mg/ml. Solutions of Czech, Russian and Bulgarian bee venom were prepared by dissolving 5 mg of the dried material in 1 ml of mobile phase and diluting tenfold before injection. Volumes of 10 μl were injected manually with a 25-μl syringe (Hamilton, Reno, NV, USA). The standards for

the calibration plots and samples of bee venom for CE were dissolved in deionized water at a concentration of 1 mg/ml and diluted before use. Samples were injected pneumatically for 6 s at an overpressure of 30 mbar.

3. Results and discussion

3.1. Size-exclusion chromatography

One of the common methods for the separation of bee venom components, used also for preparative purposes, is size-exclusion chromatography on soft, hydrophilic gels such as Sephadex G-50 [9]. These separations are slow and have poor efficiency. Separations on rigid, macroporous sorbents suitable for high-performance size-exclusion chromatography (HP-SEC) can significantly speed up the analysis. We tried to use the HEMA-BIO 40 sorbent, an additionally hydrophilized hydroxyethyl methacrylate–ethylene dimethacrylate-based macroporous copolymer with an exclusion limit of M_r 40 000–70 000. The separation on this sorbent was found to be strongly affected by its interactions with the analytes. Under common conditions (0.1 M phosphate buffer, pH 7), when the standard globular proteins yielded acceptably linear calibration plots, phospholipase was eluted later than expected on the basis of its molecular mass and melittin and some other components were strongly retained (Fig. 1). Polypeptides and proteins usually have a hydrophobic interior covered by a largely hydrophilic shell. In bee venom, the strongly basic melittin has hydrophilic residues unevenly distributed and therefore such a shell cannot be formed [10]. This characteristic was probably the main cause of the strong retention of melittin. We attempted to eliminate the interactions by adding an organic solvent to the mobile phase and decreasing the pH. Finally, a mobile phase containing 20% acetonitrile and 0.2% TFA yielded a separation according to molecular mass (Fig. 2). Previous results [6,13] indicate similar problems on other polymeric HP-SEC sorbents.

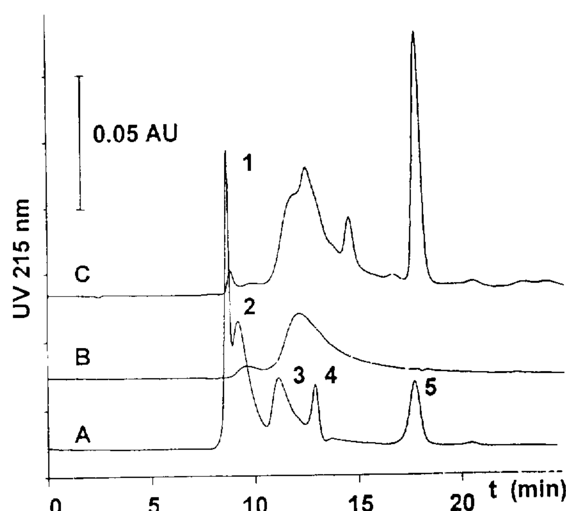

Fig. 1. HPLC separation of bee venom components by SEC. Column, 250 mm × 8 mm I.D. Separon HEMA-BIO 40 (10 μm); mobile phase, 0.1 M phosphate buffer (pH 7.0); detection, UV at 215 nm; injection volume, 20 μl. (A) Protein standard mixture: 1 = thyroglobulin; 2 = bovine serum albumin; 3 = cytochrome c; 4 = cytidine-5-monophosphate; 5 = low-molecular-mass impurity. (B) Phospholipase A, fraction V, by RP-HPLC. (C) Czech bee venom, 1.0 mg/ml.

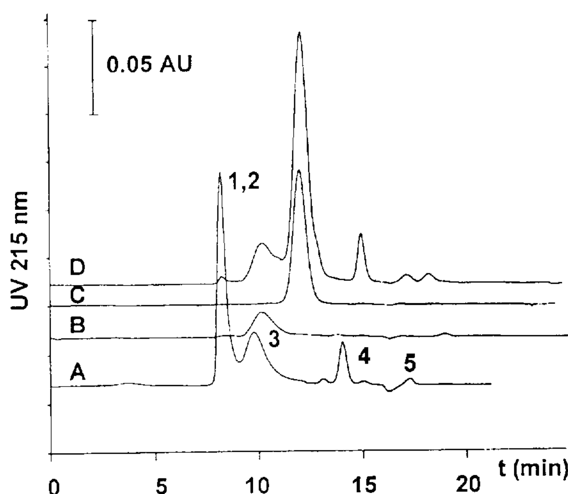

Fig. 2. HPLC separation of bee venom components by SEC. Column, 250 mm × 8 mm I.D. Separon HEMA-BIO 40 (10 μm); mobile phase, 0.2% TFA in 20% ACN; detection, UV at 215 nm; injection volume, 20 μl. (A) Protein standard mixture: 1 = thyroglobulin; 2 = bovine serum albumin; 3 = cytochrome c; 4 = cytidine monophosphate; 5 = low-molecular-mass impurity, 1.0 mg/ml. (B) Phospholipase A. (C) Melittin. (D) Czech bee venom.

3.2. Reversed-phase chromatography

Reversed-phase chromatography with gradient elution is usually the method of choice for separating mixtures of proteins and peptides. Melittin was not eluted from a common silica-based reversed-phase sorbent (Separon SGX C_{18}), probably owing to its interactions with residual silanol groups. A very good separation of phospholipase and melittin was achieved on a polymer-based reversed-phase sorbent, Separon HEMA-BIO 1000 C_{18} (150 mm × 3.3 mm I.D. column). Gradient elution (see Fig. 3) allowed the resolution of venom components with retention times of 14.24 min for phospholipase and 18.12 min for melittin. Owing to the lack of a suitable standard we were not able to identify hyaluronidase. The hyaluronidase standard from Sigma gave a very complex array of peaks, the reason for this apparently being its completely different origin (bovine testes).

Preparative isolation of venom components was performed on an 80 mm × 8 mm I.D. steel column packed with Separon HEMA-S 1000 C_{18}

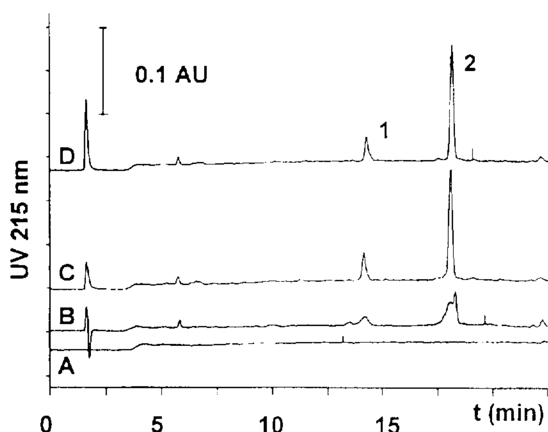

Fig. 3. HPLC separation of the components in Czech, Russian and Bulgarian bee venoms by RP-HPLC. CGC column, 150 mm × 3.3 mm I.D. HEMA-BIO 1000 C_{18} (10 μm); Eluent A, 0.22% TFA in water; eluent B, 0.2% TFA in ACN; gradient from 0 to 50% B in 20 min, from 50% to 100% B in 5 min. Detection, UV at 215 nm, 0.5 AUFS; injection volume, 10 μl of 0.5 mg/ml bee venom solution. (A) Blank run; (B) venom from Bulgaria; (C) venom from Russian Federation; (D) venom from Czech Republic. Peaks: 1 = phospholipase A_2; 2 = melittin.

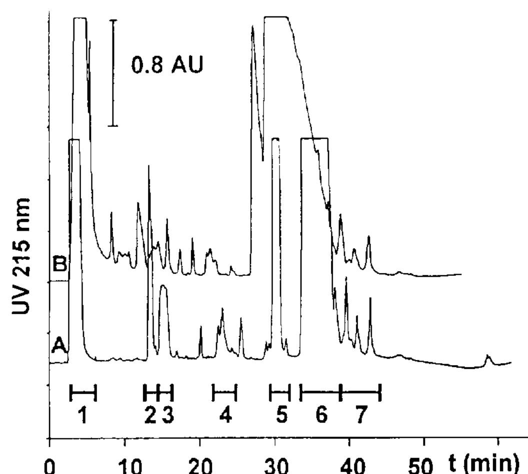

Fig. 4. Preparative HPLC separation of components in Czech bee venom by RP-HPLC. Steel column, 80 mm × 8 mm I.D. HEMA-BIO 1000 C_{18} (10 μm). Eluent A, 0.22% TFA in water; eluent B, 0.2% TFA in ACN; gradient from 0 to 50% B in 40 min, from 50% to 100% B in 5 min. Detection, UV at 215 nm, 2.0 AUFS. Injection: (A) 5 mg in 1 ml; (B) 20 mg in 2 ml.

(10 μm) sorbent with 5 and 20 mg of bee venom. Seven fractions were collected and evaluated for enzyme activity (Fig. 4). Fraction V was identified as phospholipase with a high activity and fraction VI was identified as melittin. An amount of 4 mg of phospholipase was obtained after lyophilization of pooled fractions from both runs. Hyaluronidase activity was not found in any collected fraction. Probably hyaluronidase was not stable under the separation conditions used.

3.3. Capillary electrophoresis

CE has been established as a very efficient and convenient method for the separation of peptides and proteins. Therefore, we tested it for the quantitative analysis of bee venom. Different electrolyte systems were evaluated (Fig. 5). First, 20 m*M* phosphate buffer (pH 5.0) was used (system A). The separation of bee venom components was satisfactory and venom samples of different origin could be distinguished, but the sensitivity of measurement gradually decreased during the analyses owing to adsorption of pro-

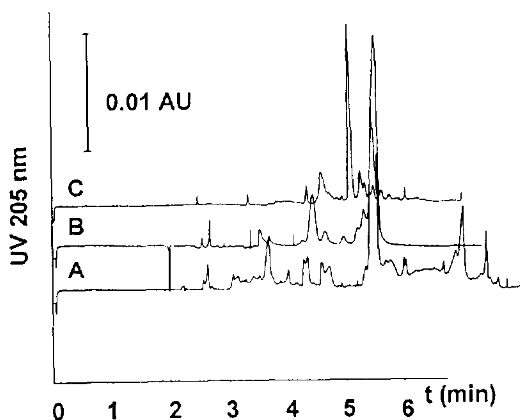

Fig. 5. Comparison of different electrolyte systems for CE separation of components in Czech bee venom. (A) 20 m*M* phosphate (pH 5.0); (B) 20 mM Tris–50 m*M* SDS (pH 9.0); (C) 150 m*M* phosphoric acid (pH 1.8). For other conditions, see Experimental.

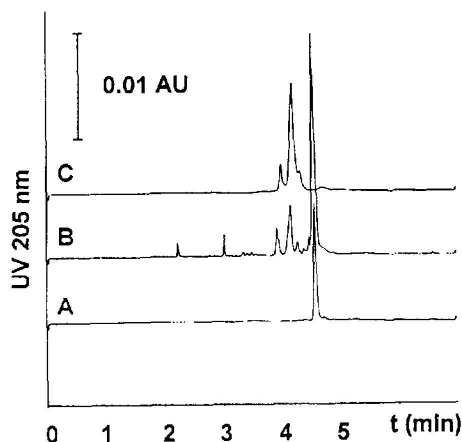

Fig. 6. CE identification of bee venom components. Electrolyte, 150 m*M* phosphoric acid (pH 1.8). (A) Melittin; (B) extract of Czech bee venom; (C) phospholipase. For other conditions, see Experimental.

teins on the column wall. Washing the capillary with NaOH after each injection did not improve the results.

With 20 m*M* Tris buffer–50 m*M* SDS (pH 9.0), as the electrolyte (system B), problems similar to those with phosphate buffer were encountered. The components could be distinguished but the peaks were broader.

Finally, a 150 m*M* phosphoric acid electrolyte system of pH 1.8 (system C) gave the best results. Undesirable effects of solute adsorption on the capillary wall were not observed. The precision of the measurement was very good (see Table 2). The low absorbance of this eluent even permitted the more sensitive UV detection at 190 nm. Two main components, phospholipase A_2 and melittin, were identified on the basis of peak matching with the standard solutes (Fig. 6). The resolving power of CE for phospholipase is demonstrated in Fig. 7. The standard from Sigma, apparently homogeneous with a minor impurity according to HPLC measurements, shows the presence of at least three different components, the main component amounting to only ca. 85%. Hyaluronidase could not be identified as no standard isolated from bee venom was available (cf., the analogous situation in HPLC measurements).

3.4. Quantitative analysis

Quantitative analysis was performed using absolute calibration with standard solutions of phospholipase and melittin. The results obtained by RP-HPLC are given in Table 1. All the three bee venoms have similar contents of phospholip-

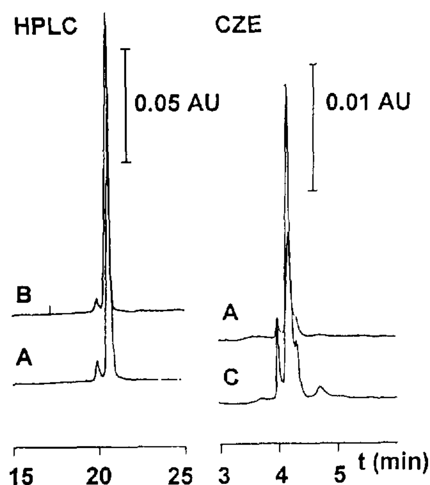

Fig. 7. Determination of phospholipase purity by RP-HPLC and CE. Conditions for RP-HPLC as in Fig. 3 and for CE as in Fig. 6. (A) Phospholipase A_2 standard (Sigma); (B) isolated by RP-HPLC; (C) isolated by SEC on Sephadex G-10.

Table 1
Comparison of determination of phospholipase A_2 and melittin in different bee venoms by RP-HPLC and CE

Bee venom	Content (%) ($n = 5$)			
	Phospholipase A_2		Melittin	
	RP-HPLC	CE[a]	RP-HPLC	CE[a]
Czech	15.4	14.5	41.6	46.5
Russian	17.1	15.8	42.9	47.3
Bulgarian	–	15.2	–	47.9

[a] CE with electrolyte system (C).

ase and melittin. The HPLC and CE analyses correlate for the Czech and Russian samples. However, the HPLC analysis of the Bulgarian sample yielded unresolved peaks and for this reason the HPLC results for Bulgarian bee venom could not be compared with those obtained by the CE method. The differences among venoms from individual honeybees were studied recently [12].

The parameters of RP-HPLC and CE analysis are given in Table 2. The lower precision of the HPLC method was probably caused by using manual injection; the use of an overfilled loop or an autosampler would probably lead to a precision comparable to that of CE. The detection limits were calculated as the ratio of three times the standard deviation of the peak-to-peak noise, s_{xy}, and the slope of the calibration plot, $3s_{xy}/S$. The concentration detection limit for the HPLC method was calculated for a 10-μl injection, however, it can be decreased to a certain extent by using larger injection volumes. The system used (gradient reversed phase of proteins) will tolerate large injection volumes (up to several ml) without an adverse effect on the resolution provided that the sample is dissolved in a low-strength eluent (aqueous TFA in this instance). The solutes are retained at the top of the column until the strength of the eluent increases sufficiently to elute them. This permits a very efficient preconcentration.

4. Conclusions

The results obtained indicate that both HPLC and CE can be readily used to differentiate bee venoms obtained from different sources. However, identification of individual components is often prevented by the lack of suitable standard compounds (standards obtained from materials other than bee venom may have completely different compositions). Size-exclusion chromatography can be used for the rough characterization of bee venom, but its separation efficiency is insufficient for quantitative purposes. Reversed-phase HPLC gives satisfactory results, using gradient elution on a polymer-based C_{18} station-

Table 2
Comparison of the parameters for Czech bee venom by RP-HPLC and CE

Analytical Parameter	RP-HPLC		CE[a]	
	Phospholipase A_2	Melittin	Phospholipase A_2	Melittin
R.S.D. (%)				
Peak area	5.2	2.8	2.1	1.5
Retention time	1.4	1.0	6.5	5.6
Detection limit:				
ng	56	30	0.4	0.15
μg/ml	5.6	3	4.5	1.6
Linear dynamic range (orders of analyte concentration)	3		2	
Analysis time (min)	45		6	

[a] CE with electrolyte system (C).

ary phase, which is suitable for the separation of strongly basic polypeptides and stable over a wide pH range.

Compared with the HPLC method, the CE analysis described is faster, the separation efficiency is better and the running costs are much lower. The precision and detection limits of the CE measurements are better than or comparable to those obtained by gradient elution in HPLC (see Table 2). The main advantage of CE over HPLC is the better resolution, permitting quantification even in cases when HPLC fails. Therefore, CE seems to be generally preferable to HPLC for bee venom analyses.

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