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Simultaneous determination of *Bordetella pertussis* toxin and filamentous haemagglutinin concentrations by hydroxyapatite high-performance liquid chromatography

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ABSTRACT

A simple and rapid method for the simultaneous determination of *Bordetella pertussis* toxin (PT) and filamentous haemagglutinin (FHA) concentrations in fermentation broths has been developed. The rapid single-step analysis performed by hydroxyapatite high-performance liquid chromatography using a salt gradient with UV detection allows both the separation of PT from FHA and the measurement of their respective concentrations. The assay is highly reproducible. Over 35 lots of acellular *B. pertussis* vaccine production lots were examined and PT concentrations measured by high-performance liquid chromatography were found to be in good agreement with the values obtained by sodium dodecyl sulphate–polyacrylamide gel electrophoresis densitometry. The chromatographic conditions have been optimized to separate the intact holotoxin from its B-oligomer subunits.

INTRODUCTION

Bordetella pertussis is the microorganism causing whooping cough. *B. pertussis* toxin (PT) and flamentous haemagglutinin (FHA) have been identified as potential protective antigens against *B. pertussis* infection. Antibodies to PT have been shown to protect mice against both intracerebral and respiratory challenges with virulent organisms. On the other hand, FHA protected mice from *B. pertussis* respiratory infection only (For reviews, see refs. 1–3). Both proteins promote adherence of the bacteria to ciliated epithelial cells *in vitro*[4]. Therefore, PT and FHA are prime candidate antigens for inclusion in any new *B. pertussis* vaccine. Pertussis toxin is a hexameric protein (105 000 dalton) composed of five subunits: S1 (28 000 dalton); S2 (23 000 dalton); S3 (23 000 dalton); S4 (12 000 dalton) and S5 (11 000 dalton) assembled in a molar ratio of 1:1:1:2:1, respectively. The holotoxin is an A:B type toxin. The A subunit (S1) catalyzes the ADP-ribosylation of a family of GTP-binding regulatory proteins in eukaryotic cells[5–7]. The other subunits (S2–S5) form a non-covalent B-oligomer which mediates the binding of the toxin to its target-cell

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receptors[8,9]. FHA has an apparent molecular weight of 220 000 dalton and may be spontaneously degraded into 130 000- and 90 000-dalton proteolytic fragments[10,11].

Both antigens are secreted into *B. pertussis* culture supernatants and the relative concentrations of these proteins vary significantly depending on fermentation conditions. To achieve consistency between vaccine production lots, the relative concentrations of PT and FHA must be closely monitored during vaccine preparation. In this report, we describe a hydroxyapatite high-performance liquid chromatography (HPLC) method which allows the simultaneous determination of PT and FHA concentrations in a fermentation broth sample.

EXPERIMENTAL

Chemicals

All buffer reagents were obtained from Fisher. Chemicals for gel electrophoresis and HPLC columns (Bio-Gel HPHT column) were purchased from Bio-Rad Labs., Canada. Water was either HPLC grade (Fisher) or purified through a Milli-Q water purification system (Millipore, MA, U.S.A.).

Preparation of protein standards

FHA was isolated from the culture supernatant of *B. pertussis* strain 10536 by hydroxyapatite chromatography, as described by Sato *et al.*[10]. PT was purified by fetuin-Sepharose 4B affinity chromatography, as previously described[12]. The B-complex of PT was prepared according to Chong and Klein[12]. Both PT and FHA were stored at −20°C in glass vials.

Protein concentration determination

Protein concentrations were determined by amino acid analysis performed by the Department of Biochemistry, University of Toronto, Canada.

Chromatographic apparatus

Chromatography was performed on an analytical Bio-Gel HPHT column using a Pharmacia fast protein liquid chromatography (FPLC) system. The effluent was monitored with a UV detector (Erma Model ERC-7210) at 230 nm, and protein peaks were integrated using a Spectra-Physics SP4270 integrator.

Preparation of samples for HPLC analysis

To achieve accurate determinations of PT, the ionic strength of the sample solution has to be less than 10 m*M* potassium phosphate and 50 m*M* NaCl, since the binding of PT to hydroxyapatite is salt concentration-dependent. All samples were filtered through a 0.22-μm filter before injection. To avoid precipitation of PT at low ionic strength, samples were tested within 2 days of their preparation.

Separation and analysis of PT and its B-oligomer

PT samples and standards (100–500 μl) were subjected to chromatography on an analytical Bio-Gel HPHT column using a phosphate-salt gradient and a flow-rate of 1 ml/min (see below). Fractions (1 ml) were collected and analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Buffer A was 10 m*M*

potassium phosphate, pH 8.0. Buffer B was 200 m*M* potassium phosphate containing 1 *M* sodium chloride, pH 8.0. After equilibration of the column in buffer A at room temperature, the holotoxin and its B-oligomer were separated from other *B. pertussis* proteins under the following conditions: after sample injection, buffer A (100%) was run for 5 min, followed by a linear gradient from 0 to 12% buffer B, established in 3 min and held for 5 min to remove any contaminants. In order to elute PT, a second linear gradient from 12 to 35% buffer B was then established in 10 min. Finally, the column was re-equilibrated with buffer A for 15 min before the next injection.

HPLC analysis of PT and FHA mixtures

The chromatographic conditions were essentially as described above except for the addition of a second linear gradient of 35–85% buffer B over 15 min, in order to elute FHA.

SDS-PAGE

Protein samples (10–100 μg protein) were subjected to SDS-PAGE on a discontinuous 13.5% gel according to the method of Laemmli[13]. Two PT standards at different concentrations were included to allow for the quantification of PT in the sample preparations. Protein bands were visualized by Coomassie brilliant blue staining. After destaining, the concentration of PT was determined using a Philips PU8800 densitometer equipped with a computing analysis program.

RESULTS AND DISCUSSION

A method for the purification and separation of FHA and PT from *B. pertussis* culture supernatants using a two-step hydroxyapatite chromatography has been reported by Sato *et al.*[10]. We have modified their approach and designed a single-step hydroxyapatite HPLC method to measure simultaneously the concentrations of PT and FHA in a fermentation broth.

PT (P273) and FHA (P214) standards were prepared by fetuin-affinity and hydroxyapatite chromatography, respectively, as previously decribed[10,12]. SDS-PAGE analysis of these standards (Fig. 1) revealed that both PT (lane 1) and FHA (lane 2) standards were homogeneous. PT was shown to consist of five subunits with molecular weights similar to those reported in the literature[10,14,15]. Typical FHA protein bands at 220 000, 130 000 and 90 000 dalton were detected (Fig. 1, lanes 2 and 3). This observation is consistent with a WHO report indicating that the molecular weight of native FHA is 220 000 dalton whereas the 130 000- and 90 000-dalton bands correspond to spontaneous proteolytic fragments[11,14]. Furthermore, the amino acid analyses of PT and FHA standards (Table I) revealed that their respective amino acid compositions were in agreement with those published in the literature[10,15].

Fig. 2 depicts the typical hydroxyapatite HPLC elution profiles obtained for PT and FHA standards, individually. The HPLC analysis of the PT standard is shown in panel A. A 10-μg amount (200 μl) of PT standard (P273) was injected and the column was washed with the starting buffer (buffer A) for 5 min. A steep gradient (0–12% B in 3 min) was applied to remove any minor contaminants. PT was subsequently eluted with a shallow gradient from 12 to 35% B in 10 min. PT eluted as a sharp peak at 21.6 min which could easily be integrated for PT concentration determination. Since the

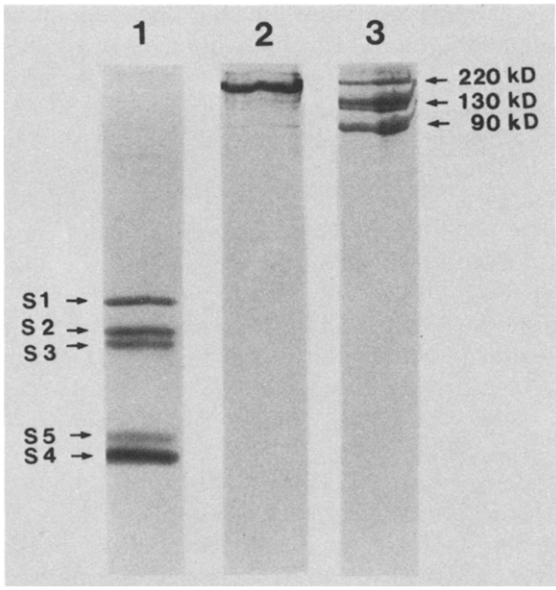

Fig. 1. SDS-PAGE analysis of native PT, native FHA and degraded FHA. Lane 1 = 6 μg of PT standard (P273); lane 2 = 8 μg of FHA standard (P214); lane 3 = 6 μg of degraded FHA sample. kD = Kilodalton.

TABLE I

AMINO ACID COMPOSITION OF PT AND FHA STANDARDS (% OF TOTAL)

Amino acid	*PT*		*FHA*	
	Expected[a]	*P273*	*Expected*[b]	*P214*
Asx	6.9	7.5	10.5	10.8
Thr	7.3	6.4	6.1	6.9
Ser	7.1	6.6	7.6	7.8
Glx	8.6	9.0	9.0	8.8
Pro	5.7	6.2	2.2	2.2
Gly	8.5	9.2	13.7	13.6
Ala	9.2	9.3	15.3	15.1
Cys	2.7	1.1[c]	0.2	0.0[c]
Val	6.9	7.5	8.3	9.1
Met	2.9	2.9	1.1	0.5
Ile	4.1	4.3	3.3	3.5
Leu	7.7	8.8	8.6	8.6
Tyr	6.5	5.3	1.3	1.0
Phe	3.4	4.0	1.4	2.0
Lys	3.4	4.0	4.6	4.9
His	1.8	2.1	1.7	0.5
Arg	6.3	5.9	5.1	4.8

[a] Published amino acid composition of PT[15].
[b] Published amino acid composition of FHA[10].
[c] Cysteine residues were directly measured without any modification.

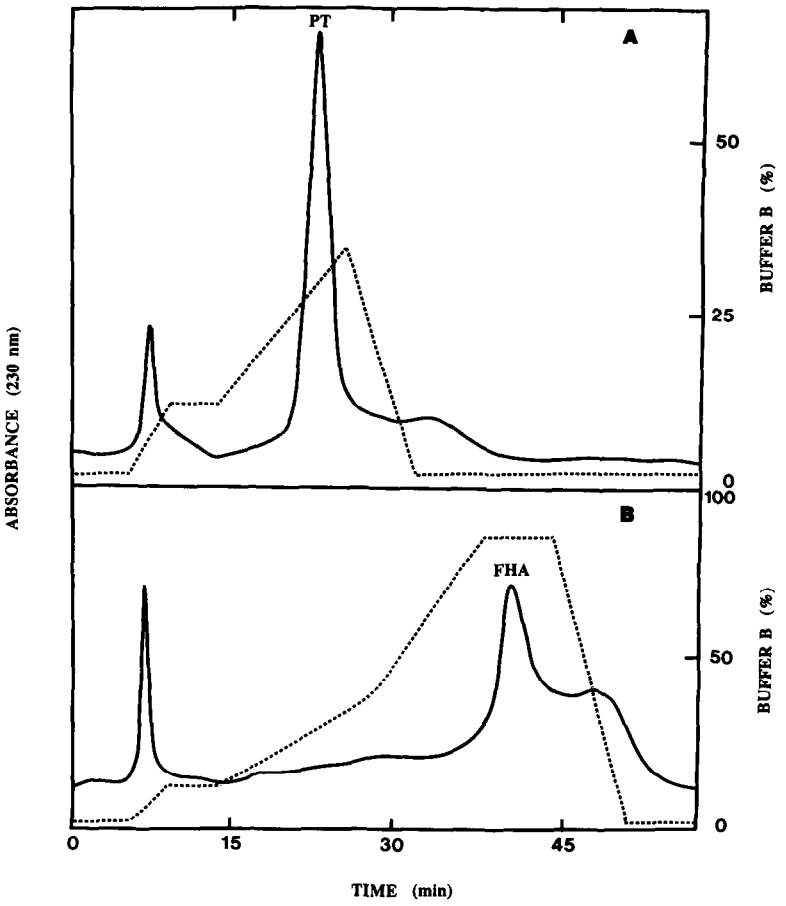

Fig. 2. Elution profile of PT and FHA standards on hydroxyapatite HPLC. Panel A, chromatogram of 15 μg of PT standard. Panel B, chromatogram of 25 μg of FHA standard. Samples were injected at time 0. The gradient program is indicated by the dotted line.

binding affinity of FHA for hydroxyapatite is very high, FHA was eluted with a steep salt gradient from 35 to 85% B within 15 min as indicated in panel B. Native FHA and its proteolytic fragments co-eluted as a single peaks at 41 min. Due to the upwards shift of the baseline, FHA peak areas could not be automatically integrated. Therefore, peak heights were used to calculate FHA concentrations. Since PT and FHA have significantly different retention times, the hydroxyapatite HPLC method allows for the identification of each protein in a sample as well as the calculation of there respective concentrations. A typical run of an FHA–PT mixture is shown in Fig. 3A. A sample (500 μl) containing 10 μg of PT and 25 μg of FHA standards was subjected to hydroxyapatite HPLC analysis. Both the PT and FHA peak were easily separated (Fig. 3A). The binding affinity of PT for hydroxyapatite was found to be salt and phosphate concentration-dependent. Essentially all of the PT binds to the column equilibrated in buffer containing less than 10 m*M* phosphate and 50 m*M* NaCl. Interestingly, we found that most *B. pertussis* proteins had very little binding affinity

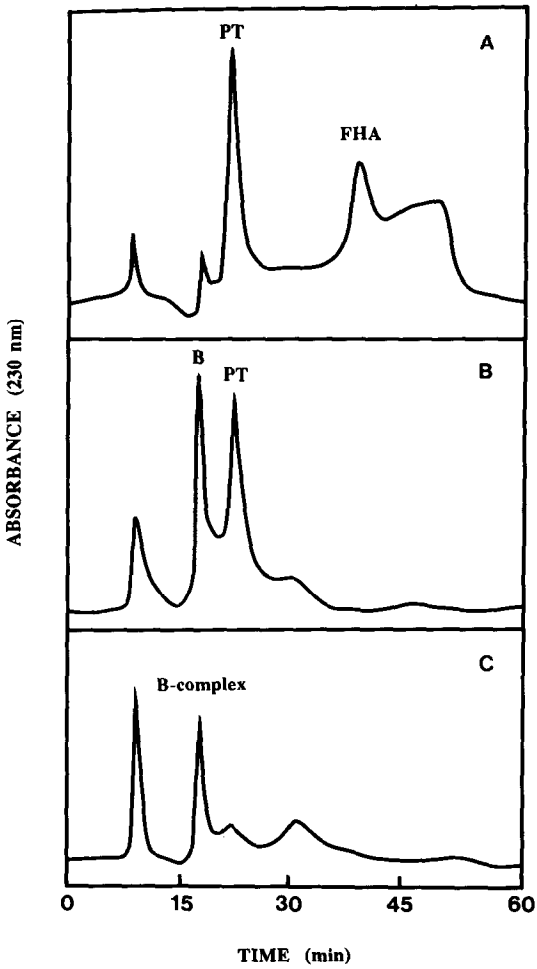

Fig. 3. Chromatographic separation of PT, FHA and B-oligomer of PT by hydroxyapatite HPLC, as described in Experimental. (A) Elution pattern of a mixture containing 10 μg of PT and FHA standards in 0.5 ml; (B) elution profile of 15 μg of a PT sample (P192) stored at 4°C for 2 weeks, peak B was collected for SDS-PAGE analysis (see Fig. 4, lane 2); (C) elution profile of 5 μg of fetuin-Sepharose-purified B-oligomer.

for hydroxyapatite and were eluted during the first 15 min. Thus, they did not interfere with the analysis of HPLC profiles.

During the course of stability studies, it was observed that the S1 subunit of PT purified by fetuin-affinity chromatography was prone to proteolytic degradation during storage at 4°C for a period of 2 weeks. Degradation of S1 and S5 subunits under similar conditions has been previously reported by Peppler *et al.*[16]. The loss of S1 cannot be detected by the conventional fetuin capture enzyme-linked immunosorbent assay (ELISA). The CHO cell clustering and the ADP-ribosyltranferase assays are necessary to demonstrate the loss of PT activity (unpublished results). When PT stored at 4°C for long periods of time was subjected to HPLC analysis, two elution peaks were

observed as shown in Fig. 3B. The first peak (B) eluted at 16.5 min was subjected to SDS-PAGE analysis and found to contain the four subunits of pertussis toxin B-oligomer (Fig. 4, lane 2). The second peak corresponded to intact PT (Fig. 4, lane 1). Fetuin-Sepharose-purified B-component subjected to HPLC analysis eluted as a single peak (Fig. 3C) with a retention time similar to that of peak B of Fig. 3B. These results suggest that the holotoxin and the B-component have different binding affinities for hydroxyapatite and can easily be separated. Thus, HPLC analysis offers a unique advantage over conventional ELISA and protein assays since it allows the simultaneous determination of the concentrations of both the holotoxin and its B-oligomer.

The sensitivity of the hydroxyapatite HPLC method was evaluated by injecting increasing amounts of PT and FHA standards (1–30 μg). The recovery of PT from the column was high (>95%) and a linear dose–response relationship was observed between 1 and 25 μg (Fig. 5). Due to the baseline shift, a linear dose-response curve could only be observed for amounts of FHA above 10 μg (Fig. 5). However, the method could be directly used for measuring PT and FHA concentrations in fermentation broths since they range from 5–20 μg/ml and 20–200 μg/ml, respectively.

To test the reproducibility of the method, PT and FHA standards were subjected to hydroxyapatite HPLC analysis at various time intervals over a 6 months period. The results in Table II show that the assay is reproducible with a variation in retention times of less than 30 s for both proteins. Moreover, less than 10% variation from the mean values was observed for both PT protein peak areas and FHA peak heights.

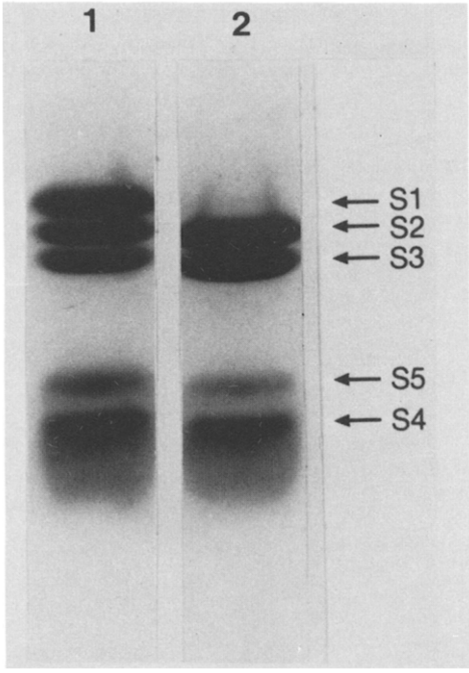

Fig. 4. SDS-PAGE analysis of the intact PT and peak B of Fig. 3B. Lane 1 = 20 μg of the intact PT collected from the HPLC run shown in Fig. 3B; lane 2 = 20 μg of peak B collected from the HPLC run shown in Fig. 3B.

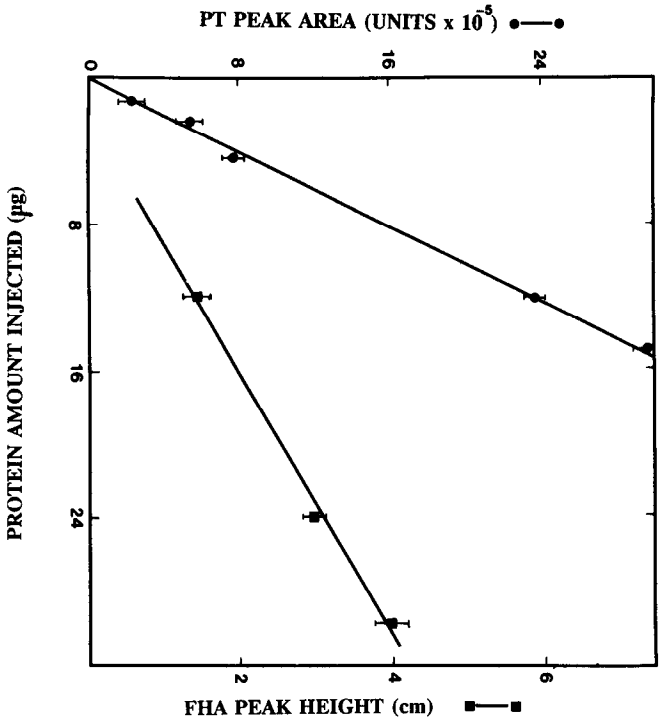

Fig. 5. Standard curves of PT and FHA obtained from hydroxyapatite HPLC analysis. Assays were performed in duplicate. PT is measured as peak area in arbitrary units (●), and FHA was reported as peak height (■).

PT and FHA concentrations were measured by both HPLC and amino acid analysis to determine the accuracy of the hydroxyapatite method. Results summarized in Table III indicate that the values obtained by both methods are in good agreement.

A comparative HPLC and SDS-PAGE analysis of 35 broth concentrates (25%

TABLE II

REPRODUCIBILITY OF THE HYDROXYAPATITE HPLC ASSAY

ND = Not determined.

Time	*Peak area of PT (P273) (mean ± S.D., n=3) (units)*[a]		*Peak height of FHA (P214) (mean ± S.D., n=3) (cm)*
	12 μg	*15 μg*	*30 μg*
0	2397 ± 25	2996 ± 36	3.8 ± 0.1
3 Days	ND	3008 ± 25	3.9 ± 0.1
3 Months	2393 ± 20	2991 ± 22	ND
6 Months	2360 ± 35	ND	4.2 ± 0.1

[a] Arbitrary units.

TABLE III

COMPARATIVE ANALYSIS OF PT AND FHA CONCENTRATIONS OBTAINED BY HYDROXYAPATITE HPLC AND AMINO ACID ANALYSIS

Samples	*Protein concentration (μg/ml)*		*Ratio of values obtained by the two assays*
	HPLC	*Amino acid analysis*	
PT			
P192	678	735	1.08
P261	128	115	0.90
P273[a]	180	180	1.00
P278	327	363	0.98
P280	350	371	1.06
FHA			
P214[b]	114	114	1.00
P258	154	173	1.12
P271	146	152	1.04
FHA-PC8	108	113	1.03

[a] Sample P273 was used as standard for all PT HPLC analyses.
[b] Sample P214 was used as standard for all FHA HPLC analyses.

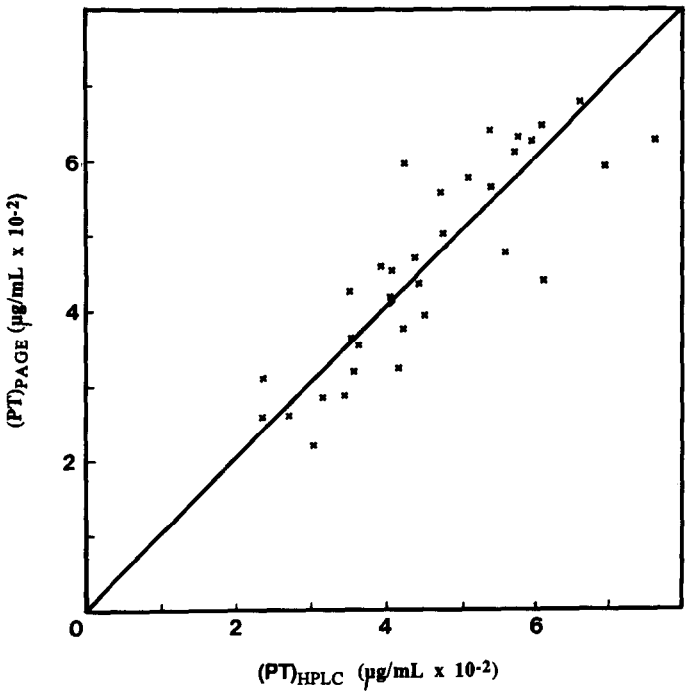

Fig. 6. Linear regression analysis of PT concentration values obtained from 35 lots of fermentation broth concentrates analysed by both hydroxyapatite HPLC and SDS-PAGE densitometry.

ammonium sulfate precipitates) from fermentors which had been optimized for PT secretion was carried out. These precipitates contained significant amounts of PT representing 70–90% of the total proteins, as judged by SDS-PAGE analysis[17]. As shown in Fig. 6, a strong positive correlation was observed between PT concentrations determined by these two methods ($r = 0.84$). Two batches of broth concentrates (lots 012 and 014) contained significant amounts of FHA protein, as judged by SDS-PAGE analysis. To determine whether the hydroxyapaptite HPLC method could be used to measure the relative concentrations of PT and FHA in these samples, a densitometric scanning analysis of SDS gels was performed on samples 012 and 014. The PT–FHA ratio was found to be 85:15 for both samples. Similar PT–FHA ratios were obtained by HPLC analysis (88:12 for lot 012 and 83:17 for lot 014), indicating good agreement between the two methods.

In conclusion, we have developed a simple, rapid and reproducible hydroxyapatite HPLC method for the simultaneous determination of PT and FHA protein concentrations in *B. pertussis* culture supernatants. In addition, the chromatographic conditions have been optimized to separate the holotoxin from its B-component. Since the analysis can be completed within 30 min, this HPLC method can potentially be used as an in-process monitoring of PT levels in fermentation broths.

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