Iranian Journal of Pathology (2007)2 (3), 104-108

Original Article

A Novel High-Performance Liquid Chromatography Method for Detection of Alginate in *Pseudomonas aeruginosa*

Parviz Owlia[1], Effat Souri[2], Qurban Behzadian-Nejad[3]

1. Dept. of Microbiology, Faculty of Medicine, Shahed University, Tehran, Iran
2. Dept. of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
3. Dept. of Microbiology, Faculty of Medical Sciences, Tarbiat Modarres University,Tehran, Iran

ABSTRACT

Background and Objective: **The opportunistic pathogen *Pseudomonas aeruginosa* secrets a capsule-like polysaccharide called alginate which is important for evasion of host defenses, especially in patients with suppressed immunity. Method of alginate determination has an important role in the study of microbial alginate. In this study, a novel method for alginate determination by high-performance liquid chromatography (HPLC) was introduced.**

Materials and Methods: **Standard alginate was used for construction of standard curve and standard mucoid and non-mucoid strains of *Pseudomonas aeruginosa* were used as positive and negative samples respectively. The method of Toyoda was modified for determination of microbial alginate. HPLC determination was performed using a Resolve C18 column (3.9 × 150 mm, Waters, Milford, MA) and acetonitrile-water-butyl acetate (55: 42: 3) as the mobile phase at a flow rate of 0.6 ml/min and detection at 565 nm.**

Results: **The obtained data indicated that minimal detectable concentration of alginate by this method is 20 µg/ml. The method was linear over the range of 1-1000 µg/ml of alginate. The retention time was about 10 min.**

Conclusion: **The proposed method was used for determination of alginate in standard mucoid and non-mucoid strains of *Pseudomonas aeruginosa*. The results of this study showed that the proposed method is a simple and valid method for bacterial alginate assay.**

Key words: Alginate, HPLC, *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen par excellence that causes severe and life-threatening infections in immuno-compromised hosts such as patients with respiratory diseases, burns, cancers undergoing chemotherapy, and cystic fibrosis (CF). Virulence factors produced by *P. aeruginosa* include numerous extracellular toxins, proteases, hemolysins, and exopolysaccharides. The most striking feature of *Pseudomonas aeruginosa* strains infecting the CF pulmonary tract is their highly mucoid phenotype, which is due to alginate overproduction (1,

Received: 12 Augest 2007
Accepted: 15 september 2007
Address communications to: Dr. Parviz Owlia Department of Microbiology, Faculty of Medicine, Shahed University, Tehran, P.O. Box: 14155-7435, Iran.

2). Alginate may confer several selective advantages on the bacterial invader, which have been reviewed (3) and include increased resistance to phagocytosis (a property typically associated with bacterial capsules) and reduced susceptibility to antibody-dependent bactericidal mechanisms (4-7). Alginate also provides a polyanionic barrier that may exclude cationic peptide antibiotics (8, 9). Structurally, alginate is a simple, un-branched polysaccharide with a very high molecular weight which is composed of two uronic acids: ß-D-mannuronic acid and its C5 epimer α-L-guluronic acid. In *Pseudomonas aeruginosa*, alginate is produced as a capsule-like exopolysaccharide that loosely adheres to *Pseudomonas aeruginosa* cells, and so most of it is found in the culture supernatant (10). In most research studies, it had been shown that a few agents (for example antibiotics) can reduce the alginate production (11). Determination of alginate is very important in these studies and developing and modification of methods of alginate assay is strongly recommended. Usual methods of alginate assay are microscopic observation, colorimetric method, and HPLC method. In this study, we introduced a simple method for extraction and assay of alginate in *Pseudomonas aeruginosa* by HPLC.

Materials and Methods

Bacteria

Mucoid strain of *Pseudomonas aeruginosa* 8821M was kindly donated by Dr. Isabel Sa- Corria, Instituto Superior Tecnico, Lisboa, Portugal. The standard strain of *Pseudomonas aeruginosa* ATCC 27853 was used as non-mucoid strain. These strains were maintained in skimmed milk at -80 °C.

Chemicals and reagents

Alginate and naphtoresorcinol was purchased from Sigma (Sigma chemical Co., St Louis, MO). Butyl acetate and acetonitrile (analytical grade) were purchased from Merck (Merck, Germany). The culture media were Difco products (Difco laboratories, Detroit, MI).

Apparatus

The HPLC system consisted of a model 510 HPLC Pump, a model 880 UV detector and a model 476 integrator (Waters, Milford, MA). Determination was performed on a Resolve C18 column (3.9 × 150 mm, Waters, Milford, MA) using acetonitrile-water- butyl acetate (55: 42: 3) as the mobile phase at a flow rate of 0.6 ml/min and detection at 565 nm.

Standard curve

A standard solution of alginate was prepared by dissolving of alginate in distilled water (0.5 mg/ml). Standard solutions of alginate for calibration curve (1-1000 µg/ml) were prepared by subsequent dilution with distilled water. Distilled water without alginate was used as negative control. Calibration curve was constructed by plotting the measured peak area of alginate versus concentrations of standard samples. This experiment was repeated 3 times in different days.

Experimental procedure

The modified methods of Toyoda (12) and Ichimiya (13) were used for preparing the samples. After 8 h incubation at 37 °C in Mueller-Hinton broth, the mucoid *Pseudomonas aeruginosa* 8821M strain was adjusted to 103 CFU/ml with 0.06M phosphate-buffered saline (PBS; pH 7.2) and 0.1 ml of the resultant suspension was inoculated onto Mueller-Hinton agar plates. Inoculated plates were incubated for 24 h at 37 °C. The formed colonies were collected on a cotton swab and suspended in 10 ml of PBS. Each suspension was centrifuged at 500 g for 5 min to remove bacteria. The supernatant was used for the determination of alginate by HPLC as follow: Two milliliters of a copper-hydrochloride solution (1 ml of 2.5% copper sulphate solution, 9 ml distilled water, and 40 ml of 36% hydrochloric acid) and 1 ml of 0.4% (W/V) naphthoresorcinol were added to 1 ml of sample. The mixture was vortexed and heated in a hot water bath at 100 °C for 40 min, then cooled to room temperature in an ice bath. The solution was shaken with 4 ml of butyl acetate. After centrifugation, the aqueous layer was discarded and the organic layer was washed with 3 ml of 20% (W/V) aqueous NaCl. Organic layer (100 µl) was diluted with 200 µl of acetonitrile and 25 µl was injected into the HPLC system. These experiments were repeated 6 times in different days.

Results

The results of plotting of standard curve showed that the method is linear over the range of 1-1000 µg/ml of alginate. Minimal detectable concentration of alginate by this method was 20 µg/ml (Fig. 1). Application of the method for standard mucoid and non-mucoid strains of *Pseudomonas aeruginosa* showed that the mucoid strain has a relevant alginate peak, but in non-mucoid strain, the peak was not observed (Fig. 2). The retention time of alginate was about 10 min.

Repeating the experiments in different days showed the same results and no significant difference was observed.

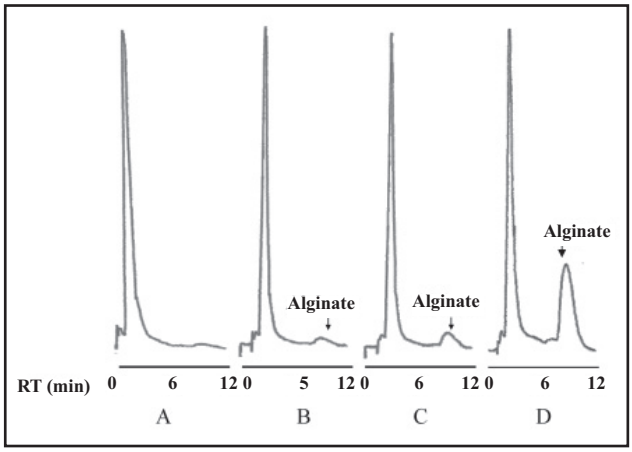

Figure 1. The HPLC chromatograms of standard alginate solutions at concentrations of 5 (A), 10 (B), 20 (C), and 80 (D) µg/ml

RT = Retention time

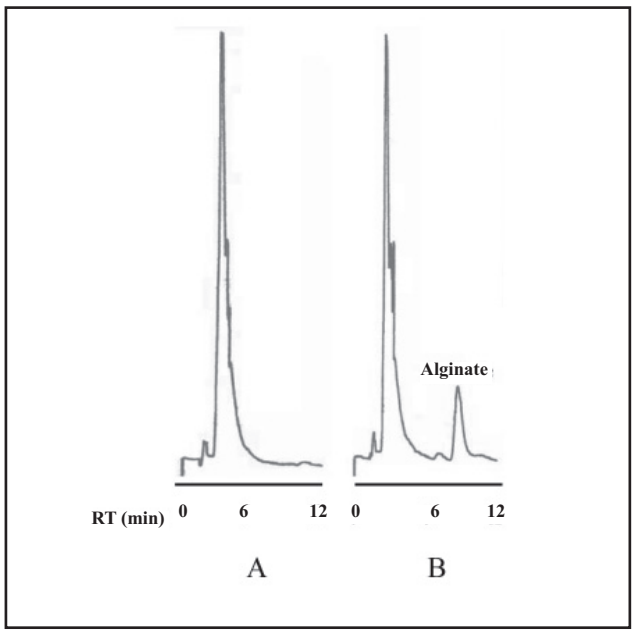

Figure 2. Representive HPLC chromatogams of non-mucoid *P. aeruginosa* 27853 ATCC (A) and mucoid *P. aeruginosa* 8821M (B)

RT = Retention time

Discussion

The standard curve indicated that minimal detectable concentration was 20 µg/ ml and this level is acceptable for detection of microbial alginate. Other methods of microbial alginate assay are not exact, for example, microscopic studies are qualitative methods, because these methods are based on the observation of thickness of alginate around the microbial cell (14). Carbazol method is based on determination of the rate of color creation by interaction between indicator and alginate using a colorimeter (15). Usually, a few other substances can react with indicator and develop false positive results. But, in this study, naphtoresocinol just reacted with alginate and we had not false positive reaction. In the method of Toyoda (12), the alginate assay by HPLC was based on a 25 cm, 4.6 mm internal diameter Finepak SILC18 column, and acetonitrile-water-butyl acetate (75: 20: 5) as the mobile phase. The flow rate was 1.2 ml/min, the wavelength 565 nm, and 20 µl of sample was used for injection. In the Toyoda method, the retention time is 5-7 min. The difference between our method and Ichimiya (13) method for isolation of bacterial alginate is only in one step. In the Ichimiya method, after centrifugation of bacterial suspension, the supernatant will be filtered, but in our method, we eliminate this step because alginate may precipitate on filter and decrease alginate in sample. The limitation of this method was related to low concentration of alginate. Concentrations lower than 20 µg/ml are not detectable by this method.

Conclusion

The results of this study showed that this method is a simple method for bacterial alginate assay. Extraction and identification of bacterial alginate in this method is easy and preference of the method is related to use of usual and available column (resolve C18). We can use this method for evaluation of the effects of different chemical and physical agents (for example antibiotics) on alginate production in *Pseudomonas aeruginosa*. Identification of alginate by HPLC is recommended, because this method is quantitative and the effects of troublesome factors are less.

Acknowledgment

We wish to thank Prof. H. Farsam (Department of Medicinal Chemistry, School of Pharmacy, Tehran University of Medical Sciences) for advising on the HPLC method.

References

1. Evans LR, Linker A. Production and characterization of the slime Polysaccharide of *Pseudomonas aeruginosa*. J Bacteriol 1973; 116: 915– 24.

2. Martin DW, Schurr MJ, Mudd MH, Govan JRW, Holloway BW Deretic V. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proc Natl Acad Sci USAb1993; 90: 8377– 81.

3. Govan, JRW, Deretic V. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and Burkholderia cepacia. Microbiol Rev 1996; 60: 539– 74.

4. Baltimore RS, Mitchell M. Immunologic investigations of mucoid strains of *Pseudomonas aeruginosa*: comparison of susceptibility to opsonic antibody in mucoid and nonmucoid strains. J Infect Dis 1982; 141: 238– 47.

5. Pier GB, Coleman F, Grout M, Franklin M. Ohman DE. Role of alginate O acetylation in resistance of mucoid *Pseudomonas aeruginosa* to opsonic phagocytosis. Infect Immun 2001; 69: 1895– 901.

6. Pier GB, Small GJ. Warren HB. Protection against mucoid *Pseudomonas aeruginosa* in rodent models of endobronchial infections. Science 1990; 249: 537– 40.

7. Schwarzmann S, Boring JR. Antiphagocytic effect of slime from a mucoid strain of *Pseudomonas aeruginosa*. Infect. Immun. 1971; 3: 762– 7.

8. Pedersen SS. Lung infection with alginate-producing, mucoid *Pseudomonas aeruginosa* in cystic fibrosis. APMIS Suppl 1992; 28: 1– 79.

9. Fux CA, Costerton JW, Stewart PS. Stoodley P. Survival strategies of infectious biofilms. Trends Microbiol 2005; 13: 34– 40.

10. Sumita J, Ohman ED. Role of an alginate lyase for alginate transport in mucoid *Pseudomonas aeruginosa*. Infect Immun 2005; 73: 6429– 36.

11. Molinari G, Guzman CA, Pesce A. Schito GC. Inhibition of *Pseudomonas aeruginosa* virulence factors by subinhibitory concentration of azitromycin and other macrolide antibiotics. J Antimicrob Chemother 1993; 31: 681- 8.

12. Toyoda M, Yomota C, Ito Y. Harada M. High performance liquid chromatographic method for the determination of sodium alginate in food. J Food Hyg Soc Jpn 1985; 26: 189- 94.

13. Ichimiya TR, Yamasaky T, Nasu M. In-vitro effects of antimicrobial agents on *Pseudomonas aeruginosa* biofilm formation. J Antimicrob Chemother 1994; 34: 331- 41.

14. Owlia P, Behzadiyan-Nejad Q, Souri E, Saderi H. Microscopic study of the effects of sub-inhibitory concentrations of gentamicin on capsule production of *Pseudomonas aeruginosa*. Arch Iranian Med 2001; 4: 18- 20.

15. Knutson CA, Jeanes A. A new modification of the carbazole analysis: application to heteropolysaccharides. Annals Biochem 1968; 24: 470– 81.