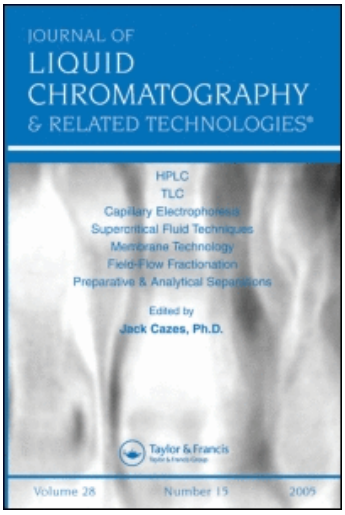

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Sensitive and Fast Method for the Determination of Polyamines in Biological Samples. Benzoyl Chloride Pre-Column Derivatization High-Performance Liquid Chromatography

Ying-Hua Mei[a]
[a] Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts

To cite this Article Mei, Ying-Hua(1994) 'A Sensitive and Fast Method for the Determination of Polyamines in Biological Samples. Benzoyl Chloride Pre-Column Derivatization High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 17: 11, 2413 — 2418
To link to this Article: DOI: 10.1080/10826079408013489
URL: http://dx.doi.org/10.1080/10826079408013489

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(11), 2413-2418 (1994)

A SENSITIVE AND FAST METHOD FOR THE DETERMINATION OF POLYAMINES IN BIOLOGICAL SAMPLES. BENZOYL CHLORIDE PRE-COLUMN DERIVATIZATION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

YING-HUA MEI
Department of Cellular and Developmental Biology
Harvard University
Cambridge, Massachusetts 02138

ABSTRACT

A sensitive, rapid pre-column derivatization HPLC procedure for determination of biologically important polyamines is described. Benzoylated putrescine, cadaverine, norspermidine, spermidine, norspermine and spermine were separated on reverse-phase column using 42:58 acetonitrile:water as mobile phase and detected by UV absorption at 198 nm. The detection limits were: 0.8 pmol for putrescine, 1 pmol for spermidine and 1.3 pmol for spermine. The method was successfully applied to the analysis of polyamines in small samples of unicellular organisms.

INTRODUCTION

Our recent studies [1] on the biological function of polyamines led us to investigate whether the concentration of polyamines in cultures of algae and other unicellular organisms depends on the time in their cell cycle, hence to seek a fast and sensitive polyamine assay.

Many polyamine analysis methods are based on those for amino acids, using both pre- and post-column derivatization high-performance liquid chromatography [2,3]. Post-column derivatization uses ion-exchange resins as stationary phases; this method suffers from long retention time and interference from amino acids [3,4]. Pre-column derivatization using dansyl chloride or tosyl assays [2,5,6] are highly sensitive, but again interference from amino acids and poor separation of derivatives on reverse phase column prove to be problems. Redmond and Tseng

[7] reported a benzoyl chloride pre-column derivatization method based on the Schotten-Baumann reaction combined with ether extraction to eliminate interference from amino acids; it uses 60: 40 methanol: H_2O as mobile phase and 254 nm absorption detection. This method has found applications in body fluid [8], mammal tissue [9] and plant [10] analysis. Although this method has the advantage of fast separation, its low sensitivity has limited its application in our studies. In the present paper, we report a sensitive and fast quantitative method for polyamines based on the benzoylated polyamine separation on reversed phase column, using 42:58 acetonitrile: H_2O as mobile phase and 198 nm absorption detection, which allows faster separation and more sensitive detection.

EXPERIMENTS

Materials and Equipments

Materials 1,3-diaminopropane, putrescine and norspermidine (3,3'-iminobispropylamine) were from Sigma. Cadaverine, 1,6-hexanediamine, spermidine and spermine were from Aldrich. Norspermine (N,N'-bis[3-aminopropyl]-1,3-propanediamine) was a gift from Dr. S. Marvin Friedman. Benzoyl chloride (99%, A.C.S.reagent, Aldrich) was used without further purification. Anhydrous ethyl ether (A.R.) was from Mallinckrodt. Methanol, acetonitrile and water used for separation were all of HPLC grade.

Equipment A Beckman HPLC equipped with Model 110B high-pressure pump and Model 163 variable wavelength absorbance detector was used throughout the study, with a Kipp & Zonen recorder (Model BD41). A Beckman 5μm C_8 reverse-phase column (25cm x 4.6mm) was used to separate the benzoylated polyamines. Sample injection was carried out using either 20 μl sample loop (Beckman) or 25 μl micro-syringe (Hamilton).

Sample Treatment, Polyamine Derivatization and HPLC Separation

Chlamydomonas reinhardtii, Gonyaulax polyedra, Pyrocystics lununa and *Plasmodium falciparum* were grown and treated as described previously [1a]. Briefly, after addition of a polyamine internal standard to 10~500 mg wet cell pellets (cadaverine as internal standard for *Chlamydomonas reinhardtii* and *Plasmodium falciparum* ; 1,6-hexanediamine for *Gonyaulax polyedra* and *Pyrocystics lununa*), the cells were broken by sonicator or beadbeater in 1~3 ml water (check under microscope to make sure all the cells were broken), extracted with 2 ml 5% trichloroacetic acid and centrifuged at 8,000 *g* at ca. 4°C for 20 min. The pellets were resuspended in 1 ml water and 2 drops of 2 N NaOH and recentrifuged in 2 ml 5% trichloroacetic acid.

To *ca.* 5 ml pooled supernatant of sample, 1.5 ml 2N NaOH was added to adjust the pH >13, followed by 5 μl benzoyl chloride. The mixture was magnetically stirred for 20 minutes for complete reaction. 2 ml NaCl (sat.) was added and the resulting solution was extracted twice with 1ml ether. Pooled ether fractions were evaporated by a stream of nitrogen and the residue (benzoylated polyamines) was dissolved in 0.2 ml 42% acetonitrile/water (for acetonitrile/water mobile phase) or 60% methanol/water [8] (for methanol/water mobile phase). The resulting solution was subjected to HPLC separation. Polyamines standards, dissolved in distilled water, were derivatized with NaOH and benzoyl chloride and separated by the same procedure.

RESULT AND DISCUSSION

It has been shown [7] that the benzoylated derivatives of putrescine, cadaverine, spermidine and spermine are well separated on reversed-phase column using 60:40 methanol: H_2O as the mobile phase. We confirmed this. Fig. 1 shows a typical chromatogram of these benzoylated polyamines, together with derivatives of norspermidine and norspermine. However, when we used this method to study the time dependence of the polyamine level in *Chlamydomonas reinhardtii* and *Gonyaulax polyedra* as a function of the cell cycle, requiring frequent collection of samples of limited amount, it became important to increase both detection sensitivity and separation speed. It was even more imperative when assaying for possible traces of norspermine in very small samples of the parasitic *Plasmodium falciparum*, which is phylogenetically related to dinoflagellates.

We found that the absorbance of benzoylated polyamines increases *ca.* 50 times from 254 to 198 nm in acetonitrile as solvent, which means that the detection sensitivity of the method could be greatly increased by a change of detection wavelength. However, the use of methanol/water as mobile phase precluded this possibility because of the UV cut-off point of methanol at 205nm.

Our result showed that benzoylated polyamines are well separated on a RP-C_8 column when using 42% acetonitrile/water as mobile phase (Fig. 2). This allows detection at 198 nm without significant background noise, and only an overlap of the peaks of hexanediamine and spermidine

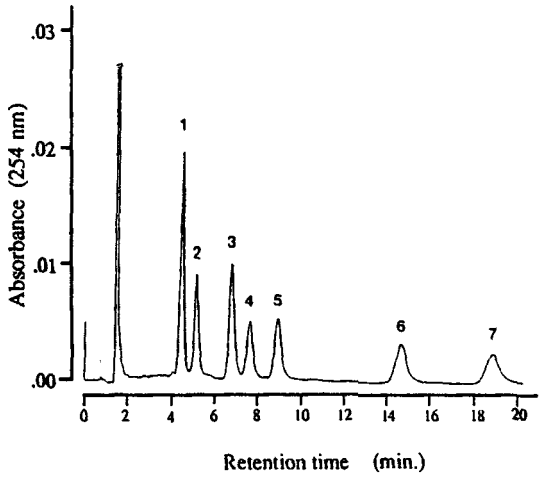

Fig. 1 A chromatogram of a standard mixture of benzoylated polyamines (containing *ca.* 2 nmol each), with methanol/water 60:40 as a mobile phase and detection at 254 nm.. 1. putrescine, 2. cadaverine, 3. hexanediamine, 4. norspermidine, 5. spermidine, 6. norspermine and 7. spermine.

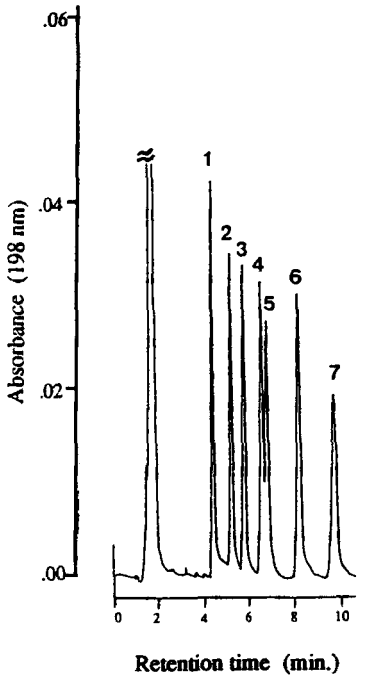

Fig.2 Separation of a standard mixture of benzoylated polyamines with 42% acetonitrile/water as mobile phase and detection at 198 nm. Each peak contains *ca.* 0.1 nmol polyamines. 1. putrescine, 2. cadaverine, 3. norspermidine, 4. spermidine, 5. hexanediamine, 6. norspermine and 7. spermine.

as drawback. Since hexanediamine is not a natural polyamine, but serves only as potential internal standard, this overlap is of no consequence. By using this mobile phase, the retention time of spermine is about a half of its value in methanol/water. The detection limits are: 0.8 pmol for putrescine, 1 pmol for spermidine and 1.3 pmol for spermine, therefore 60 times less than with the methanol/water system [8]. In both mobile phase systems, however, diaminopropane overlaps with putrescine and they are difficult to separate by simply changing the ratio of mobile phases.

Improper use of dissolving solvent may cause poor resolution [8]. Our experiment showed that 42% acetonitrile/water is suitable for benzoylated polyamines. The solution can be kept for up to six months at room temperature without significant change.

Per gram of wet weight, we found that *Chlamydomonas reinhardtii* cells contained 5.5 μmol putrescine, 0.9 μmol norspermidine and 0.2 μmol spermidine in average; *Gonyaulax polyedra* and *Pyrocystics lununa* contained only norspermine (0.06 and 0.016 μmol, respectively). Polyamine concentrations in *Plasmodium falciparum* were 0.01 for putrescine, 0.03 for spermidine and 0.005

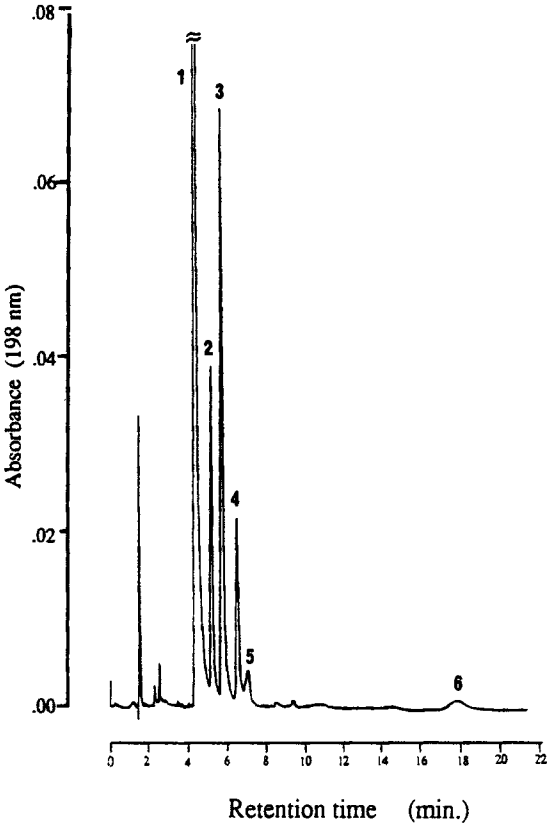

Fig. 3 Chromatogram of a sample of *Chlamydomonas reinhardtii* (treatment of sample and derivatization, see text). Mobile phase: 42% acetonitrile in water. Absorption detection wavelength: 198 nm. 1.putrescine, 2. cadaverine (internal standard), 3. norspermidine, 4. spermidine; 5 and 6. unknown.

for spermine (all in nmol per 10^6 cells); no norspermine was found above our detection limit. The recovery rates of polyamines were satisfactory for the samples (>90%). A typical chromatogram of *Chlamydomonas reinhardtii* polyamine separation is shown in Fig. 3.

CONCLUSION

By using 42% acetonitrile/water as mobile phase and UV detection at 198 nm, benzoylated putrescine, cadaverine, norspermidine, spermidine, norspermine and spermine can be satisfactorily

separated on C_8 reverse-phase column in 10 minutes, with detection limit of less than 1.3 pmol. The mobile phase also serves as dissolving solvent. This method can been used in the polyamine analysis in some unicellular prokayotes (*Chlamydomonas reinhardtii, Gonyaulax polyedra, Pyrocystics lununa* and *Plasmodium falciparum*) and would work well, we expect, in other biological samples.

ACKNOWLEDGEMENTS

I thank Dr. Thérèse Wilson for her suggestions and advice throughout this study. This work was supported by National Institute of Mental Health (46660 to J.W. Hastings).

REFERENCES

1. a). Y.-H. Mei, A. U.Khan, T. Wilson, *Quimica Nova*, 16, 000(1993) b). A. U. Khan, Y.-H. Mei, T. Wilson, *Proc. Natl. Ca. Sci. USA*, 89, 11426-7(1992).
2. For reviews: a) N. Seiler, *Methods in Enzymology*, Vol. 94: *Polyamines*. H. Tabor and C. W. Tabor Ed., Academic Press, New York, 1983, pp 3-54. b) R. Durie, *Progress in Cancer Research and Therapy*, Vol. 8: *Polyamines as Biochemical Markers of Normal and Malignant Growth*, Raven Press, New York, 1978, pp 121-127.
3. V. R. Villanueva, R. C. Adlakha, *Anal. Biochem.*, 91, 264-75(1978).
4. K. Hamana, S. Matsuzaki, *J. Biochem.*, 91, 1321-8(1982).
5. N. Seiler, B. Knodgen, F. Eisenbeiss,, *J. Chromatogr.*, 145, 29-32(1978).
6. T. Hayashi, T. Sugiura, S. Kawai and T. Ohno, *J. Chromatogr.*, 145, 141-6(1978).
7. J. W. Redmond, A. Tseng, *J. Chromatogr.* 170, 479-81(1979).
8. S. Wongyai, P. J. Oefner, G. K. Bonn, *J. Liq. Chromatogr.*, 12, 2249-61(1989).
9. R. Porta, R. L. Doyle, O. Sellinger, S.B. Tatler, T.E. Wilens, R.A. Schatz and O.Z. Sellinger, *J. Neurochem.*, 37, 723-9(1981).
10. D. R. Roberts, M. A. Walker, E. B. Dumbroff, *Phytochem.*, 24, 1089-90(1985).

Received: September 3, 1993
Accepted: December 21, 1993