ELSEVIER

Journal of Chromatography A, 946 (2002) 265–273

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Selective fluorometric detection of polyamines using micellar electrokinetic chromatography with laser-induced fluorescence detection

Richard E. Paproski, Kimberly I. Roy, Charles A. Lucy*

Department of Chemistry, University of Alberta, Gunning/Lemieux Chemistry Centre, Edmonton, Alberta, Canada T6G 2G2

Received 24 September 2001; received in revised form 12 November 2001; accepted 21 November 2001

Abstract

The polyamines putrescine, cadaverine, spermine and spermidine were separated and quantified by micellar electrokinetic chromatography (MEKC) with laser-induced fluorescence detection. The derivatization reagent, 1-pyrenebutanoic acid succinimidyl ester (PSE), allowed for the selective detection of the polyamines at 490 nm. Multiple labeling of the polyamines with PSE allows the formation of intramolecular excimers that emit at longer wavelengths (450–520 nm) than mono-labeled analytes (360–420 nm). Optimal separation of the labeled polyamines was achieved using a separation buffer consisting of 10 m*M* phosphate pH 7.2, 30 m*M* cholate, and 30% acetonitrile. Using these conditions, the four polyamines were separated in under 10 min. Limits of detection for putrescine, cadaverine, spermine and spermidine were 6, 5, 15 and 13 n*M*, respectively. These are superior or comparable to those previously reported in the literature using fluorescence detection.

Keywords: Detection, electrophoresis; Derivatization, electrophoresis; Polyamines; Amines; Pyrenebutanoic acid succinimidyl ester

1. Introduction

Putrescine, spermidine, spermine, and to a lesser extent cadaverine, are the major cellular polyamines in the human body [1]. These biogenic amines are involved in cellular growth and differentiation, regulation of nucleic acid and protein synthesis, stabilization of lipids, brain development, and nerve growth and regeneration [1]. They also play a major role in the body's response to brain injury and stress, and in the regulation of neuronal ion channels and brain neurotransmitter receptors [1]. Some studies have also shown that the high concentration of polyamines found in human milk may play a role in the apparent protective effect of human milk against allergies [2].

There has been recent evidence linking elevated polyamine levels and cancer. Nairn et al. [3] have reported high polyamine concentrations in breast cancer and colon cancer cells. McCloskey et al. [4] and Bergeron et al. [5] have demonstrated the arrest of cancer cell growth using polyamine analogues. Pentaazapentacosane pentahydrochloride, an anti-

*Corresponding author. Tel.: +1-780-492-0315; fax: +1-780-492-8231.

E-mail address: charles.lucy@ualberta.ca (C.A. Lucy).

0021-9673/02/$ – see front matter
PII: S0021-9673(01)01542-4

cancer agent, is also analogous in structure to both spermine and spermidine [6]. The antiproliferative nature of these analogues is possibly due to the disruption of polyamine metabolism in cancer cells. The potential link between polyamine levels and cancer has promoted interest in the development of sensitive analytical techniques capable of detection and quantification of biogenic amines in biological fluids.

Polyamines do not exhibit any structural features that enable their direct detection in a sensitive manner. There are some reports of native, non-derivatized polyamine detection using ion chromatography with integrated pulse amperometric detection [7], enzymatic differential assays [8], and competitive enzyme-linked immunosorbent assays (ELISA) using polyamine-specific monoclonal antibodies [9]. These techniques, however, suffer from poor sensitivity.

Therefore, most analytical techniques reported for the determination of polyamines include both a derivatization step and a separation step. Dorhout et al. [10] used capillary gas chromatography with nitrogen–phosphorous detection for the detection of spermidine, spermine, and putrescine in leukemia cells following derivatization by methylation. However, high-performance liquid chromatography (HPLC), preceded by amine derivatization, is by far the most frequently reported technique for polyamine separation and quantification [11–20]. Fu et al. [11] have used HPLC with dansyl chloride derivatization to evaluate putrescine, spermidine, and spermine levels in human prostate, and they have reported detection limits of ~10 n*M* using fluorescence detection. Other common derivatization agents used with HPLC include naphthalene-2,3-dicarboxaldehyde (NDA) [18] and *o*-phthalaldehyde (OPA) [12,14,16] with fluorescence detection, benzoyl chloride with UV detection [17], and ferrocene derivatives with electrochemical detection [15].

Since the work of Jorgenson and Lukacs in the early 1980s [21], capillary electrophoresis (CE) has become widely accepted as an analytical tool. CE offers many advantages over its counterpart, HPLC, such as higher column efficiency (10^5–10^6 theoretical plates), lower sample volume (~10 nl), and very high sensitivity with femtomole to zeptomole limits having been reported [22–25]. As for detection, laser-induced fluorescence (LIF) provides the greatest degree of sensitivity of any detector currently available for CE, with detection limits approaching the molecular level [22]. The use of CE–LIF for the separation and quantification of polyamines is becoming increasingly popular, and there are reports in the literature demonstrating the analysis of polyamines using CE–LIF with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ) [26], OPA [27–29], and fluorescein isothiocyanate (FITC) [30] derivatization. Similarly, Rodriguez et al. [31] have reported the microchannel electrophoretic separation of putrescine, cadaverine, spermidine and spermine by micellar electrokinetic chromatography (MEKC) following derivatization by FITC. The limits of detection were ~1 μ*M*.

There are some limitations to the common derivatization reagents used. For instance, FITC and AccQ are natively fluorescent and, depending on the separation, may interfere with the detection of the polyamines of interest. Further, OPA derivatives are unstable and the reaction must be carefully timed to enable quantification [32]. Another limitation is that most of the polyamine derivatization reagents also react with monoamines that are present in the samples. Without further sample pretreatment, this leads to numerous peaks that may interfere with the detection of the polyamines. Finally, polyamines labeled with NDA [33], OPA [34,35], and 5-furoylquinoline-3-carboxaldehyde (FQ) (K.I. Roy, N.J. Dovichi, unpublished results) suffer from poorer sensitivity due to intramolecular quenching. This quenching results when analytes are multiply-labeled, which places the labels in close proximity. An excited label on the analyte then has a high probability of interacting with a second non-excited label, and thereby relax from its excited state in a non-radiative manner.

Recently, Yamaguchi and coworkers [36,37] have developed sensitive and selective reversed-phase HPLC methods for the determination of polyamines using 1-pyrenebutanoic acid succinimidyl ester (PSE) as the derivatization reagent. PSE labels amines with a fluorescent pyrene group, as shown in Fig. 1. In analytes with multiple labeling sites, an excited pyrene group can form an intramolecular excimer with a second ground state pyrene group. This excimer emits at longer wavelengths (450–520

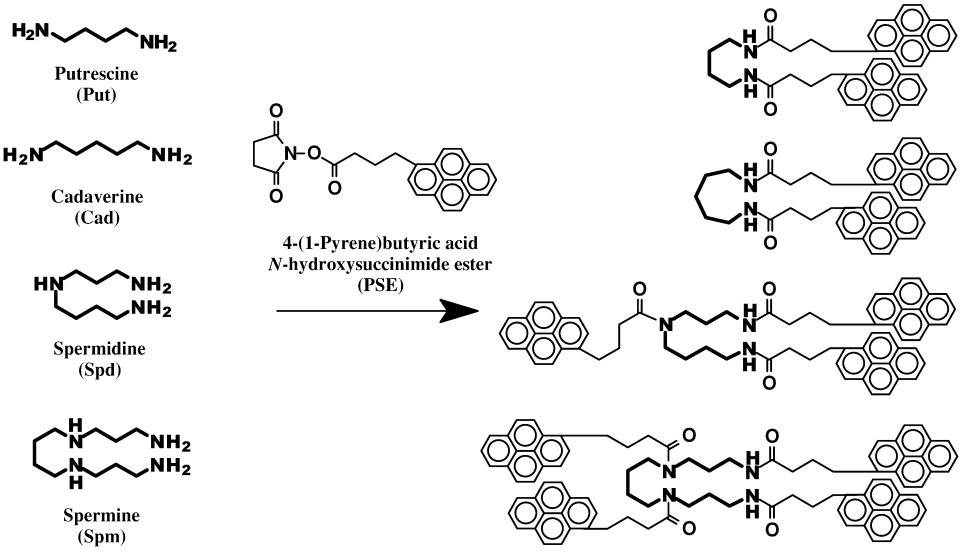

Fig. 1. Reaction scheme for the derivatization of polyamines with PSE. Adapted from Ref. [37].

nm) than mono-labeled analytes (360–420 nm) [36]. High selectivity for polyamines can thus be achieved by using PSE with detection at 490 nm. It has been shown that PSE-labeled monoamines do not give any significant response at these longer wavelengths [36], making PSE an obvious choice for use with biological samples whose matrix includes monoamines such as most amino acids.

In this paper, a method for the selective determination of polyamines is described. Polyamines of biological importance are first derivatized with PSE, followed by separation and quantification by MEKC–LIF. The multiply-labeled polyamines are selectively detected at 490 nm. To our knowledge, this is the first report of a capillary electrophoretic technique employing PSE as a derivatization reagent.

2. Experimental

2.1. Apparatus

A P/ACE 2100 (Beckman, Fullerton, CA, USA) system equipped with an LIF detector module was used for all experiments. Data acquisition and instrument control was performed with P/ACE Station software (Beckman) for Windows 95 on a 486 PC. Laser power measurements were performed with a PocketPower handheld power meter (Melles Griot, Irvine, CA, USA).

The single-wavelength He–Cd laser was operated at 325 nm with a power output of 5 mW, and was equipped with a SMA fiber optic receptacle (Model 3056-8M; Omnichrome, Irvine, CA, USA). The laser beam was coupled to the LIF detector through a 1-m multimode fiber optic patchcord with a 100/140-μm (core/cladding) diameter and SMA 906 connectors (Polymicro Technologies, Phoenix, AZ, USA). The optical power measured out of the fiber was ~2 mW. Fluorescence was collected through a 490-nm (490DF10) band pass filter (Omega Optical, Brattleboro, VT, USA), unless otherwise noted.

2.2. Reagents and solutions

All solutions were prepared in Nanopure 18-MΩ water (Barnstead, Chicago, IL, USA). Acetonitrile (ACN) was HPLC-grade and was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Dimethyl sulfoxide (DMSO) and tetrahydrofuran (THF) were reagent-grade and were obtained from ACP (Montreal, Canada) and BDH (Toronto, Canada), respectively. 1-Pyrenebutanoic acid succinimidyl ester (PSE, Fig. 1) was obtained from Molecular Probes (Eugene, OR, USA). Sodium dihydrogen orthophosphate and potassium carbonate were reagent-grade

and were obtained from BDH. Cholic acid (sodium salt) was of 99% minimum purity and was purchased from Sigma (St. Louis, MO, USA). The hydrochloride salts of putrescine (Put), cadaverine (Cad), spermidine (Spd), and spermine (Spm) were purchased from Sigma. The structures of these analytes are shown in Fig. 1.

The separation buffer was prepared by dissolving sodium dihydrogen orthophosphate in water, bringing the pH to 7.2 with concentrated aqueous NaOH (BDH), adding the required volume of acetonitrile, and finally by adding cholate. The optimized separation buffer consisted of 10 m*M* pH 7.2 phosphate, 30 m*M* cholate, and 30% acetonitrile. Fresh buffers were prepared weekly.

Polyamine stock solutions (100 m*M*) were prepared in water and stored frozen at −15 °C in plastic vials. These solutions were stable for at least 3 months. Polyamine standards were prepared from the stock solutions by diluting with a THF–DMSO–water (1:2:1, v/v) solvent mixture before use. PSE (10 m*M*) was made up in acetonitrile and stored at −15 °C under dark conditions. It was stable for at least 1 week.

An amino acid standard solution was used to evaluate the selectivity of the method. The standard solution was obtained from Sigma, and contained the following 17 amino acids: L-alanine, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-valine. Before use, the standard solution was diluted 2.5-fold with a THF–DMSO–water (1:2:1,v/v) solvent mixture. All amino acids were 25 μ*M* in the original standard solution, except L-cystine which was 12.5 μ*M*.

2.3. Derivatization procedure

This derivatization procedure is based on that by Nohta et al. [36]. Briefly, 200 μl of a polyamine standard, 10 μl of 1 *M* potassium carbonate (aqueous), and 200 μl of 10 m*M* PSE were added to a 1-ml Reacti-vial (Pierce, Rockford, IL, USA). The vial was sealed and heated in boiling water at ~95 °C for 40 min. The reaction mixture was cooled in ice water and diluted 10-fold with the separation buffer prior to injection. Sample blanks were made by replacing the polyamine standard with 200 μl of the THF–DMSO–water (1:2:1) solvent mixture. The reaction mixture and diluted samples were stable for at least 6 h when protected from light.

For the sample stacking experiments, the reaction mixtures were diluted with a solution consisting of 10 m*M* pH 7.2 phosphate, 30% acetonitrile, and 0–300 m*M* NaCl (BDH). This solution differs from the separation buffer in that the cholate is replaced by a high concentration of salt.

2.4. Capillary electrophoresis

Untreated fused-silica capillaries (Polymicro Technologies) with total lengths of 37 cm and 57 cm (30 cm and 50 cm to the detector, respectively), inner diameters of 50 μm, and outer diameters of 365 μm were used. New capillaries were flushed at 138 kPa with 1 *M* sodium hydroxide for 10 min, water for 10 min, 0.1 *M* sodium hydroxide for 5 min, and finally with water for 10 min. Before each run, the capillary was rinsed for 3 min with 0.1 *M* sodium hydroxide, 3 min with water, and 5 min with the separation buffer. Injections were performed hydrodynamically using 3.45 kPa for 3 s, and separation voltages ranged from 12 to 30 kV. Data was collected at 10 Hz with a detector response time of 0.5 s. Capillaries were thermostated at 25 °C in all experiments.

3. Results and discussion

3.1. Separation of PSE-labeled polyamines by MEKC

Separation between all four labeled polyamines was achieved using MEKC with cholate as the surfactant and acetonitrile as an organic modifier. As reported by Nohta et al. [36], the use of organic modifiers is necessary to solvate the pyrene-labeled polyamines, as was evidenced by a decrease in excimer fluorescence in media containing less than ~50% organic solvent. Cholate is a bile salt that forms helical micelles between which analyte molecules may partition based on their hydrophobicity [38–43]. Cholate was chosen over the more commonly used sodium dodecyl sulfate (SDS) because

the structure of the bile salt micelle is much more tolerant to the addition of organic solvents [39–41]. Thus cholate has a greater ability to separate highly hydrophobic analytes in the presence of high amounts of organic modifier [39–41].

Since labeled Put and Cad differ by only one methylene group (Fig. 1), they were the most difficult to separate by MEKC. Therefore, their separation was used to optimize the separation conditions. First, the optimum amount of acetonitrile for both separation and dissolution was determined. Fig. 2a and 2b show the improvement in separation between Put and Cad that was achieved upon decreasing the ACN content from 50 to 27%. Little improvement in resolution was achieved by varying the ACN content between 25 and 30%. Generally, highly hydrophobic analytes are best separated when the minimum amount of organic modifier needed for full dissolution is used [44]. This allows for complete solubility of the analytes while minimizing the organic modifier's negative impact on micelle formation. However, as the solubility of the PSE-labeled analytes decreased sharply at 25% ACN, 30% ACN was chosen for the separation of the four PSE-labeled polyamines.

Resolution between Put and Cad improved as the cholate content was increased from 15 to 30 m*M*, with little improvement observed above 30 m*M*. Therefore, to minimize Joule heating, 30 m*M* cholate was used in all further experiments. Also, since adequate resolution for quantification could not be obtained on a 37 cm capillary, the capillary length was increased to 57 cm (50 cm to the detector). The separation voltage was also increased from 20 to 30 kV in order to keep the field strength constant. The increased separation time provided by a longer capillary resulted in near baseline resolution between Put and Cad (Fig. 2c). Therefore, a 57-cm capillary was used in all further experiments involving the separation of the four PSE-labeled polyamines.

Using the optimized conditions, the four polyamines of interest were separated in under 10 min. The separation is shown in Fig. 3, where the peaks used for quantification are labeled in bold. Several peaks resulted from PSE, since this derivatization reagent is present at a sufficiently high concentration for *inter*molecular excimer complexes to be formed. Also, several distinct peaks were observed for PSE-labeled Spd and Spm. The origin of these peaks is discussed in the next section.

3.2. Characterization of labeled polyamines

It was observed that Spd and Spm gave multiple peaks corresponding to different degrees of labeling. Spm, having four available amines for labeling (Fig. 1), was used to study the effects of reaction time and PSE concentration on the extent of the reaction. Fig. 4 shows the change in Spm peak intensities while varying the PSE concentration between 5 and 20

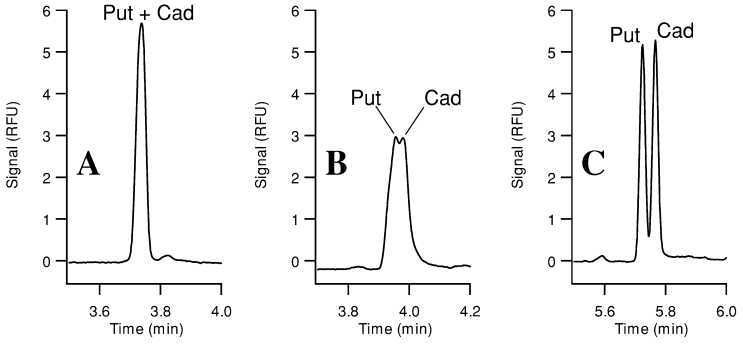

Fig. 2. Optimization of the separation of PSE-labeled Put and Cad. A mixture of 500 μ*M* Put and 500 μ*M* Cad was derivatized according to the procedure described in the Experimental section, and diluted 1000-fold with the separation buffer. (A) 37-cm capillary (30 cm to detector), 20 kV applied voltage, separation buffer: 10 m*M* phosphate pH 7.2, 30 m*M* cholate, 50% ACN. (B) 37-cm capillary (30 cm to detector), 20 kV applied voltage, separation buffer: 10 m*M* phosphate pH 7.2, 30 m*M* cholate, 27% ACN. (C) 57-cm capillary (50 cm to detector), 30 kV applied voltage, separation buffer: 10 m*M* phosphate pH 7.2, 30 m*M* cholate, 27% ACN. Detection was at 490 nm in all cases.

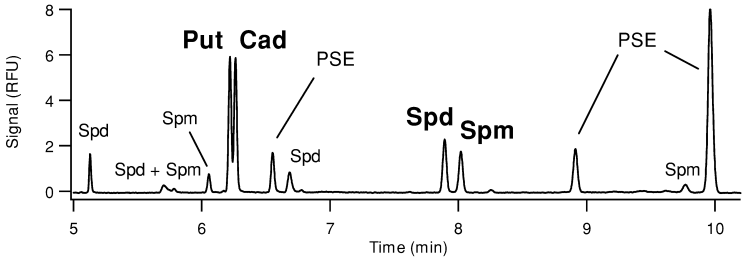

Fig. 3. Separation of PSE-labeled Put, Cad, Spm and Spd in under 10 min. A solution consisting of 50 μ*M* Put, 50 μ*M* Cad, 50 μ*M* Spm and 50 μ*M* Spd was derivatized according to the procedure described in the Experimental section, and diluted 100-fold with the separation buffer. The origin of each peak is identified on the figure. The peaks identified in bold were used for quantification. Separation buffer: 10 m*M* phosphate pH 7.2, 30 m*M* cholate, and 30% ACN. Separation conditions: 57-cm capillary (50 cm to detector), 30 kV applied, detection at 490 nm.

m*M*. These experiments were performed using 50% acetonitrile in the buffer, which Nohta et al. [36] had determined to be the optimal organic content for maximum excimer fluorescence. Peak 4 increased in intensity with increasing PSE, while the peak with the shortest migration time (peak 1) decreased in intensity. This implies that the later-eluting peaks correspond to more fully labeled analytes. This seems reasonable since more fully substituted polyamines will possess more pyrene groups, increasing their hydrophobicity and thus their partitioning into the cholate micelles. Peak 5 likely results from an impurity in the Spm sample. The peak heights were less dependent on reaction time. Upon increasing the reaction time from 20 to 80 min, the peak heights varied by less than 10%. Despite numerous attempts, the reaction could not be driven to completion so as to yield only the fully-labeled polyamines.

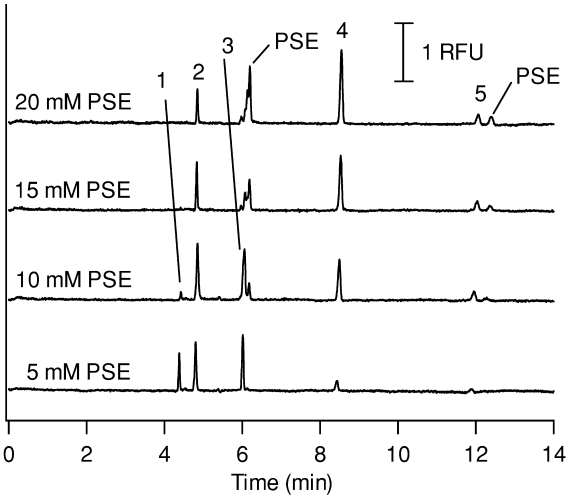

Fig. 4. Variation in the intensities of the PSE-labeled Spm peaks (peaks 1–5) as a function of PSE concentration. A 500 μ*M* Spm solution was derivatized according to the procedure described in the Experimental section, and diluted 1000-fold with the separation buffer. Separation buffer: 10 m*M* phosphate pH 7.2, 30 m*M* cholate, 50% ACN. Separation conditions: 37-cm capillary (30 cm to detector), 12 kV applied voltage, detection at 490 nm.

To confirm that the peaks of interest corresponded to multiply-labeled polyamines exhibiting *intra*molecular excimer fluorescence, and not mono-labeled polyamines or PSE degradation products exhibiting *inter*molecular excimer fluorescence, each polyamine was run using a 400 nm filter as well as the 490 nm filter. If the peaks observed at 490 nm result from intermolecular excimer formation, then the corresponding peaks at 400 nm (monomor fluorescence region) should have even greater intensities. The electropherograms obtained at each detection wavelength are shown in Fig. 5. As in Fig. 4, these studies were performed with 50% acetonitrile in the buffer. The polyamine peaks observed at 490 nm clearly result from intramolecular excimer fluorescence, since these peaks have a higher response in the 490 nm (excimer) region than in the 400 nm (monomer) region. The PSE degradation products give signals that are orders of magnitude higher in the 400 nm region than in the 490 nm region. Thus, the PSE degradation products observed at 490 nm result from *inter*molecular excimer formation.

The origin of the PSE peaks in Figs. 3–5 was determined by analyzing a fresh PSE standard solution. The resulting single peak (results not shown)

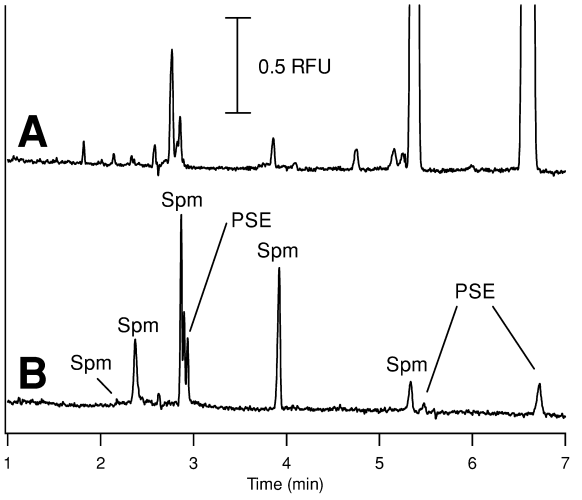

Fig. 5. Comparison of the fluorescence profile of PSE-labeled Spm with detection at (A) 400 nm and (B) 490 nm. A 500 μm Spm solution was derivatized according to the procedure described in the Experimental section, and diluted 1000-fold with the separation buffer. Separation buffer: 10 m*M* phosphate pH 7.2, 30 m*M* cholate, 50% ACN. Separation conditions: 37-cm capillary (30 cm to detector), 20 kV applied voltage.

did not correspond to any of the PSE peaks observed in Figs. 3–5. This indicates that following reaction and dilution with the separation buffer, the majority of the remaining PSE has been transformed into unidentified degradation products. Therefore, it is these degradation products, not native PSE, that are observed in Figs. 3–5.

3.3. Precision and limits of detection

The minimum possible dilution of the reaction mixture with separation buffer was determined to be 10-fold. Using this dilution factor, the PSE degradation product peaks became quite large. Nevertheless, the polyamine peaks of interest remained free from interferences. However, below this dilution factor, small impurity peaks began to interfere with the polyamine peaks. Therefore, a 10-fold dilution of the reaction mixture was used in all calibration studies.

The polyamine peaks used for quantification are identified in bold in Fig. 3. These peaks were chosen because they gave the highest response and were free from interferences with PSE peaks. Although the described method produces multiple peaks for spermidine and spermine, a single peak for each polyamine can be used for quantification owing to the high reproducibility of the technique. Using the peaks highlighted in Fig. 3, the inter-assay (repeated derivatization and injection of sample) peak height RSD was 7.9% for Put ($n=4$), 4.1% for Cad ($n=4$), 6.0% for Spd ($n=4$), and 3.1% for Spm ($n=5$). Further, the intra-assay (repeated injections of same derivatized sample) RSD's ($n=3$) for Put, Cad, Spd and Spm were 0.2, 0.8, 0.8 and 1.1%, respectively.

Using peak height, linear calibration curves were obtained for all four polyamines (Put $R=0.998$ ($n=6$), Cad $R=0.996$ ($n=6$), Spd $R=0.996$ ($n=5$), Spm $R=0.994$ ($n=5$)), with *y*-intercepts that were not statistically different from zero at the 95% confidence level. The detection limits for Put, Cad, Spd, and Spm were 6, 5, 13 and 15 n*M* injected, respectively. These correspond to concentrations of 125, 100, 270 and 310 n*M* in the original sample. These limits of detection are comparable or superior to those previously reported in the literature for fluorescence detection [26–30].

Sample stacking based on a method described by Palmer et al. [45] was achieved by diluting the reaction mixture 10-fold with a solution containing 10 m*M* phosphate pH 7.2, 30% acetonitrile, and 100 m*M* NaCl instead of the separation buffer. This enabled the injection time to be increased to 30 s without significant deterioration in peak shape for both Spd and Spm. This yielded a ~17-fold increase in signal for Spd and a ~10-fold increase in signal for Spm compared to the non-stacking experiments. Sample stacking can therefore be employed with this MEKC–LIF technique to improve the detection limits for Spd and Spm. The Put and Cad signals could not be improved through stacking, since the longer injection times resulted in peak overlap.

3.4. Selectivity of the method

A 10 μ*M* mixture of 17 amino acids was analyzed to demonstrate the selectivity of the method towards polyamines. The corresponding electropherogram is shown in Fig. 6. As expected, only those amino acids possessing more than one labeling site were detected at 490 nm. L-lysine gave a strong response, while L-arginine and L-histidine gave rather low responses

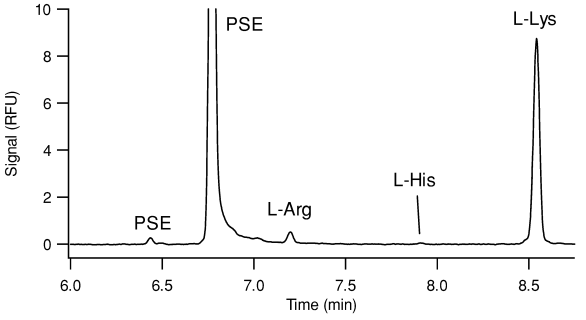

Fig. 6. Illustration of the selectivity of the method towards polyamines. A solution consisting of 17 amino acids (see Experimental section) was derivatized according to the procedure described in the Experimental section, and diluted 10-fold with the separation buffer. Before dilution, each amino acid was at 10 μ*M* except for L-cystine which was at 5 μ*M*. Only three PSE-labeled amino acids gave detectable responses at 490 nm, and are identified in the figure. Separation buffer and conditions as in Fig. 3.

within the time window of interest. Further, none of these amino acid peaks overlapped with the polyamine peaks of interest. This demonstrates the selectivity of the method, and its suitability for use with biological samples.

Until recently, it was believed that agmatine, a polyamine precursor for putrescine, was present only in bacteria. This ''bacterial polyamine'' is formed from the amino acid L-arginine in the metabolism of many bacteria [46]. However, the presence of agmatine in mammalian tissue has been recently reported [47,48]. Preliminary studies involving the present method showed that agmatine gave two peaks, the second of which overlapped with putrescine.

4. Conclusions

PSE-labeled putrescine, cadaverine, spermine and spermidine were separated and quantified in under 10 min using MEKC with LIF detection. Selective determination of the PSE-labeled polyamines was achieved using detection at 490 nm. Since polyamines possess several amine labeling sites, the multiple pyrene labels can form intramolecular excimers that emit at longer wavelengths (450–520 nm) than mono-labeled analytes (360–420 nm). The insensitivity of the technique towards amino acids containing a single amine group was demonstrated, thus showing that this method would be suitable for complex biological samples. PSE-labeled lysine, arginine, and histidine, which emitted excimer fluorescence detectable at 490 nm, did not interfere with the polyamine peaks of interest. The described technique provides polyamine detection limits that are superior or comparable to those previously reported in the literature using fluorescence detection. To our knowledge, this is the first use of PSE with a capillary electrophoretic technique.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and the University of Alberta. K.I. Roy gratefully acknowledges her NSERC Postgraduate Scholarship.

References

[1] L.D. Morrison, L. Becker, L.C. Ang, S.J. Kish, J. Neurochem. 65 (1995) 636.
[2] N. Romain, G. Dandrifosse, F. Jeusette, P. Forget, Pediatr. Res. 32 (1992) 58.
[3] L.M. Nairn, G.S. Lindsay, P.M. Woster, H.M. Wallace, J. Cell. Physiol. 182 (2000) 209.
[4] D.E. McCloskey, P.M. Woster, R.A.J. Casero, N.E. Davidson, Clin. Cancer Res. 6 (2000) 17.
[5] R.J. Bergeron, R. Müller, J. Bussenius, J.S. McManis, R.L. Merriman, R.E. Smith, H. Yao, W.R. Weimar, J. Med. Chem. 43 (2000) 224.
[6] S. Heimbecher, Y.-C. Lee, S.E. Tabibi, S.H. Yalkowsky, J. Chromatogr. B 691 (1997) 173.
[7] R. Draisci, L. Giannetti, P. Boria, L. Lucentini, L. Palleschi, S. Cavalli, J. Chromatogr. A 798 (1998) 109.
[8] K. Isobe, H. Yamada, Y. Soejima, S. Otsuji, Biochem. Med. Metab. Biol. 37 (1987) 110.
[9] I. Garthwaite, A.D. Stead, C.C. Rider, J. Immunol. Methods 162 (1993) 175.
[10] B. Dorhout, A.W. Kingma, E. de Hoog, F.A.J. Muskiet, J. Chromatogr. B 700 (1997) 23.
[11] S. Fu, X. Zou, X. Wang, X. Liu, J. Chromatogr. B 709 (1998) 297.
[12] H.M.H. van Eijk, D.R. Rooyakkers, N.E.P. Deutz, J. Chromatogr. A 730 (1996) 115.
[13] J. Rosier, C. Van Peteghem, Z. Lebensm.-Unters. Forsch. 186 (1988) 25.

[14] W.L. Whitmore, T.A. Slotkin, Experientia 41 (1985) 1209.
[15] K. Shimada, T. Oe, M. Tanaka, T. Nambara, J. Chromatogr. 487 (1989) 247.
[16] N. Seiler, B. Knodgen, J. Chromatogr. 221 (1980) 227.
[17] P.J. Oefner, S. Wongyai, G. Bonn, Clin. Chim. Acta 205 (1992) 11.
[18] H. Koning, H. Wolf, K. Venema, J. Korf, J. Chromatogr. 533 (1990) 171.
[19] J. Bontemps, J. Laschet, G. Dandrifosse, J.-L. van Cutsem, P.-P. Forget, J. Chromatogr. 311 (1984) 59.
[20] P. Koski, I.M. Helander, M. Sarvas, M. Vaara, Anal. Biochem. 164 (1987) 261.
[21] J.W. Jorgenson, K.D. Lukacs, Anal. Chem. 53 (1981) 1298.
[22] S. Wu, N.J. Dovichi, J. Chromatogr. 480 (1989) 141.
[23] D. Chen, N.J. Dovichi, Anal. Chem. 68 (1996) 690.
[24] H. Swerdlow, J.Z. Zhang, D.Y. Chen, H.R. Harke, R. Grey, S. Wu, N.J. Dovichi, C. Fuller, Anal. Chem. 63 (1991) 2835.
[25] J.Y. Zhao, N.J. Dovichi, O. Hindsgaul, S. Gosselin, M.M. Palcic, Glycobiology 4 (1994) 239.
[26] A. Kovács, L. Simon-Sarkadi, K. Ganzler, J. Chromatogr. A 836 (1999) 305.
[27] S. Oguri, S. Watanabe, S. Abe, J. Chromatogr. A 790 (1997) 177.
[28] S. Oguri, A. Tsukamoto, A. Yura, Y. Miho, Electrophoresis 19 (1998) 2986.
[29] K.B. Male, J.H.T. Luong, J. Chromatogr. A 926 (2001) 309.
[30] G. Nouadje, M. Nertz, P. Verdeguer, F. Couderc, J. Chromatogr. A 717 (1995) 335.
[31] I. Rodriguez, H.K. Lee, S.F.Y. Li, Electrophoresis 20 (1999) 118.
[32] P. de Montigny, J.F. Stobaugh, R.S. Givens, R.G. Carlson, K. Srinivasachar, L.A. Sternson, T. Higuchi, Anal. Chem. 59 (1987) 1096.
[33] B.K. Matuszewski, R.S. Givens, K. Srinivasachar, R.G. Carlson, T. Higuchi, Anal. Chem. 59 (1987) 1102.
[34] B.N. Jones, S. Pääbo, S. Stein, J. Liq. Chromatogr. 4 (1981) 565.
[35] H.N. Singh, W.L. Hinze, Analyst 107 (1982) 1073.
[36] H. Nohta, H. Satozono, K. Koiso, H. Yoshida, J. Ishida, M. Yamaguchi, Anal. Chem. 72 (2000) 4199.
[37] H. Yoshida, Y. Nakano, K. Koiso, H. Nohta, J. Ishida, M. Yamaguchi, Anal. Sci. 17 (2001) 107.
[38] R.O. Cole, M.J. Sepaniak, W.L. Hinze, J. High Resolut. Chromatogr. 13 (1990) 579.
[39] R.O. Cole, M.J. Sepaniak, W.L. Hinze, J. Gorse, K. Oldiges, J. Chromatogr. 557 (1991) 113.
[40] H. Nishi, T. Fukuyama, M. Matsuo, S. Terabe, J. Chromatogr. 498 (1990) 313.
[41] M. Aguilar, A. Farran, C. Serra, M.J. Sepaniak, K.W. Whitaker, J. Chromatogr. A 778 (1997) 201.
[42] S. Yang, M.G. Khaledi, Anal. Chem. 67 (1995) 499.
[43] P.G. Muijselaar, K. Otsuka, S. Terabe, J. Chromatogr. A 780 (1997) 41.
[44] K.D. Altria, B.J. Clark, M.A. Kelly, J. High Resolut. Chromatogr. 22 (1999) 55.
[45] J. Palmer, N.J. Munro, J.P. Landers, Anal. Chem. 71 (1999) 1679.
[46] G.J. Molderings, M. Burian, J. Homann, M. Nilius, M. Göthert, Dig. Dis. Sci. 44 (1999) 2397.
[47] G. Li, S. Regunathan, C.J. Barrow, J. Eshraghi, R. Cooper, D.J. Reis, Science 263 (1994) 966.
[48] W. Raasch, S. Regunathan, G. Li, D.J. Reis, Life Sci. 56 (1995) 2319.