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Short communication

Determination of polyamines in human prostate by high-performance liquid chromatography with fluorescence detection

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Abstract

A high-performance liquid chromatographic method for the determination of polyamines in human prostate has been developed. This method is based on pre-column derivatization with dansyl chloride (Dns-Cl). The derivatives were separated on a μBondapak C_{18} column (250×4.6 mm I.D.; 10 μm), and eluted with methanol and distilled water using a one-step linear gradient. The column eluate was monitored by fluorescence detection (excitation, 370 nm; emission, 506 nm). The within-assay precision of the study (C.V.) was as follows: putrescine (PUT) 2.88%, spermidine (SPD) 2.94% and spermine (SP) 1.17%. The between-assay precision (C.V.) was: PUT 2.66%, SPD 3.06%, SP 2.79%. The recovery was greater than 97%. The detection limit for PUT, SPD and SP were 0.05, 0.08 and 0.06 nmol/ml, respectively. In contrast to other studies, sample or polyamine derivatives did not require extraction with an organic solvent such as ethanol, evaporation under vacuum or other condensation procedures. This is a simple, rapid and sensitive method that can be applied to the determination of polyamines in nearly all biological tissues and body fluids, such as urine and serum.

Keywords: Polyamines; Putrescine; Spermidine; Spermine

1. Introduction

The essential role of the natural polyamines, PUT, SPD and SP, in normal growth and proliferation of eukaryotic cells has been established [1,2]. The analytical determination of these three compounds in human tissues, urine and serum has generated much interest in recent years because elevated levels of these compounds have been associated with the rapid regeneration or regrowth of tissues [3,4]. Direct detection of polyamines is difficult because they do not exhibit any structural features that would allow their sensitive detection without derivatization. Dansyl chloride (Dns-Cl) is a well-known fluorogenic derivatizing agent for the determination of trace polyamines [5]. In this paper, we detail the optimum derivatization pH, temperature, time, concentration of Dns-Cl, concentration ratio of Dns-Cl to polyamines, efficient gradient programming and other chromatographic conditions.

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2. Experimental

2.1. Apparatus

The chromatographic apparatus consisted of a Hewlett-Packard (HP) 1090 M liquid chromatograph, an HP 106309 solvent delivery system, an HP 044 automatic sample injector and an HP 1046A fluorescence detector (excitation 370 nm, emission 506 nm). An HP 9153A integrator, an HP Think Jet printer and an HP Color Pro plotter were also employed in the study.

A μBondapak C_{18} column (250×4.6 mm I.D.; 10 μm) was used for the analytical separation procedure. Samples were separated at a flow-rate of 1.0 ml/min with a one-step linear gradient from 80 to 100% methanol in 11 min. The oven temperature was 50°C.

2.2. Reagents and chemicals

The three polyamines, PUT, SPD and SP, and the internal standard, hexamethylenediamine (HDA), were obtained as the hydrochloride salts from Sigma (St. Louis, MO, USA). Dns-Cl was purchased from Fluka (St. Louis, MO, USA). Other reagents were all of reagent grade and obtained from a Chinese company. Distilled water was prepared in our laboratory. Human normal prostate tissue and benign hyperplastic prostates (BPH) were gifts from Prof. Liu (Shandong Medical University).

2.3. Procedure

For sample preparation, tissues were homogenized with ten volumes of 0.05 *M* phosphate buffer (pH 7.2). Trichloroacetic acid (10%) was added to the supernatants to remove proteins. The supernatant was neutralized with saturated sodium carbonate and derivatized with Dns-Cl.

For derivatization, the reactant solution consisted of 100 μl of Dns-Cl dissolved in acetone (4 mg/ml), 400 μl of neutralized sample supernatant, 50 μl of HDA and 50 μl of saturated sodium carbonate. The reactant solution was incubated in a water bath for 30 min at 50°C. This solution was injected (the injection volume was 6 μl) and analyzed.

3. Results and discussion

A chromatogram of the standard mixture of the three polyamines and HDA is shown in Fig. 1, and a chromatogram of polyamines in BPH is shown in Fig. 2. It can be seen that the three polyamines and HDA derivatives were completely resolved from each other. The chromatographic analysis run time was 9 min. As far as we know, this is one of the most rapid methods available.

For recovery studies, the prostate sample was spiked with known amounts of polyamine standards and analyzed. The recoveries of the polyamines were greater than 97%. The within-assay precision test was accomplished by successively injecting standard Dns-polyamine eight times and recording the concentration (nmol/ml) of every peak. The within-assay precision (C.V.) was PUT, 2.88%; SPD, 2.94% and SP, 1.07%. The between-assay precision (C.V.) was PUT, 2.66%; SPD, 3.06 and SP, 2.79% ($n=8$). The limits of detection for this procedure were determined for each of the three polyamines. This was accomplished by setting the detector sensitivity at the maximum range and successively injecting decreasing quantities of each of the derivatized

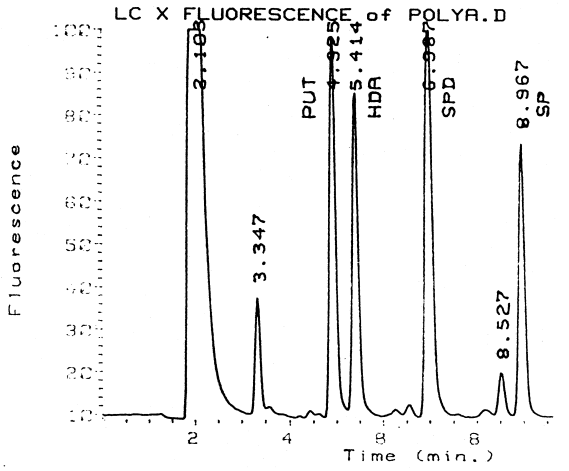

Fig. 1. Chromatogram of the separation of a standard mixture of three Dns-Cl-derivatized polyamines. PUT, HDA, SPD and SP stand for putrescine, hexamethylenediamine, spermidine and spermine, respectively.

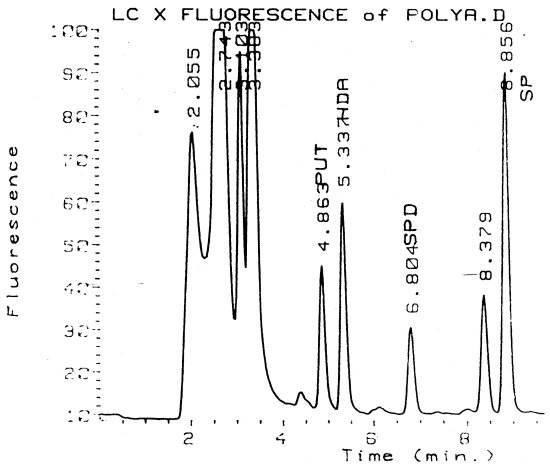

Fig. 2. Chromatogram of Dns-Cl-derivatized polyamines in a human BPH prostate sample. PUT, HDA, SPD and SP stand for putrescine, hexamethylenediamine, spermidine and spermine, respectively. For chromatography conditions, see Section 2.1.

polyamines. Each detection limit was obtained by recording the smallest amount of polyamine that still produced a peak at a signal-to-noise ratio of 3:1. Minimum detection limits were found to be 0.05, 0.08 and 0.06 nmol/ml for PUT, SPD and SP, respectively. The injection volume was 6 μl. For derivatization, the reaction of Dns-Cl with polyamine was found to be pH-dependent. This can be explained in terms of three competing reactions:

$$\text{R-NH}_2 + \text{Dns-Cl} \rightarrow \text{R-NH-Dns} + \text{HCl} \quad (1)$$

$$\text{H}_2\text{O} + \text{Dns-Cl} \rightarrow \text{Dns-OH} + \text{HCl} \quad (2)$$

$$\text{R-NH-Dns} + \text{Dns-Cl} \rightarrow \text{Dns-NH}_2 + \text{other products} \quad (3)$$

where R-NH_2 represents polyamine, Dns-OH is dansyl sulfonic acid and Dns-NH_2 is dansyl amide. The desirable reaction (Eq. (1)) is accelerated by high pH, but high pH also favors reaction 2 (Eq. (2)). Reaction 3 (Eq. (3)) leads to decomposition of R-NH-Dns (Dns-polyamine). Thus, an optimum pH must be found. The optimum reaction pH was determined by derivatizing each of the three polyamines at pH values ranging from 6.0 to 11.0 and measuring the fluorescence response for each eluted analyte as a function of pH. In this paper, we found that the optimum pH was 9.0.

Table 1
Polyamine concentrations in normal human prostate and BPH prostate (x±SD, nmol/mg protein)

Sample	*n*	PUT	SPD	SP
Normal	8	0.320±0.172	0.063±0.026	0.411±0.183
BPH	21	0.489±0.218	0.151±0.051[a]	1.880±0.650[a]

Compared with normal prostate. [a]$P<0.001$.

We also found that the concentration of Dns-Cl and the concentration ratio of Dns-Cl to polyamines were extremely important. At lower Dns-Cl concentrations, more Dns-Cl solution was needed for derivatization, leading to the dilution of samples. This would require concentration before analysis, using ether or ethyl acetate extraction, vacuum evaporation, etc. At high Dns-Cl concentrations, proline was needed to remove the excess Dns-Cl [6]. We found that the optimum concentration ratio of Dns-Cl to polyamine homogenate was 1:4 (v/v). The temperature was also found to be important; high temperature promotes the derivatization reaction, but too high a temperature may cause decomposition of polyamines. We found that 50°C was the optimum temperature, and only 30 min was needed for derivatization.

Table 1 shows the polyamine concentrations in normal human prostate and BPH. The concentration of polyamines in BPH prostate is markedly increased with respect to normal values.

In conclusion, this method is rapid, simple and sensitive, which should make it attractive for biomedical polyamine studies.

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