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Note

Separation of cadaverine from putrescine, histamine and polyamines in rat kidney by phosphocellulose chromatography

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The aliphatic diamine cadaverine (1,5-diaminopentane) is a chemical analogue of putrescine (1,4-diaminobutane) and, like putrescine, the biosynthesis of cadaverine appears to be maximal during periods of rapid tissue proliferation [1].

For several years, numerous analytical methods have been devised for the separation and determination of the biogenic di- and polyamines [2]. Among these, high-performance liquid chromatography (HPLC) coupled with fluorometric detection presents strong sensitivity and good specificity as an analytical method for di- and polyamines [3, 4]. However, the superb resolution of HPLC must be offset, in part, by the inherent disadvantages including expensive equipment and limited loading capacity.

This paper describes a rapid and simple method of separating cadaverine from putrescine and other polyamines using phosphocellulose column chromatography. The applicability of the method is demonstrated on the kidney of a castrated rat treated with an anabolic steroid.

MATERIALS AND METHODS

Chemical reagents

Phosphocellulose was obtained from Brown (Berlin, NH, U.S.A.) (Lot No. M3G8190, capacity 1.04 mequiv./g). Fluorescamine [4-phenylspirofluran-2(3H),1′-phthalan-3,3′-dione, Lot No. 23F-0249] was obtained from Sigma (St. Louis, MO, U.S.A.). Durabolin (nandrolone phenpropionate, Lot No. 130755) was from Organon Teknika (Oss, The Netherlands). *o*-Phthalaldehyde

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(OPA, Lot No. M2M1045) was from Nakarai (Kyoto, Japan). All other reagents used were reagent grade and obtained from Wako (Tokyo, Japan).

Animal experiment and sample preparation

Male Wistar rats, weighing about 200 g and obtained from Shizuoka Lab-Animal Center (Shizuoka, Japan) were used. Castration was performed one week before the actual experiment. Durabolin, an anabolic steroid, was suspended in olive oil and injected subcutaneously 5 mg per 100 g daily for three days as reported previously [5]. Controls were given vehicle only. After the last injection, the experimental animals were sacrificed at different intervals and their kidneys were removed immediately and frozen on dry-ice. After homogenization in 5 vols. of ice-cold 0.4 *M* perchloric acid, the kidneys were centrifuged at 1600 *g* for 5 min at 4°C and the supernatants were neutralized to pH 5—6 with ice-cold potassium hydroxide. After neutralization the sample was centrifuged again and the supernatant was used for further analysis.

Column chromatography on phosphocellulose

The sample (0.5 ml of the above final supernatant) was applied to a phosphocellulose column (0.6 X 3.5 cm) equilibrated with phosphate buffer pH 6.2. First the column was washed stepwise with 1 ml of 0.01 *M* phosphate buffer (pH 6.2), 5 ml of 0.05 *M* phosphate buffer (pH 6.2) and 12 ml of 0.02 *M* borate buffer (pH 8). The diamines and polyamines were then eluted also in a stepwise manner: 6 ml of 0.1 *M* borate buffer (pH 8) were needed to elute histamine, 5 ml of 0.1 *M* borate buffer, pH 8, containing 0.015 *M* sodium chloride were needed for cadaverine, 9 ml of 0.1 *M* borate buffer, pH 8, containing 0.1 *M* sodium chloride for putrescamine, 3 ml of 0.2 *M* borate buffer, pH 8, containing 0.1 *M* sodium chloride for a contaminating compound (unknown), 9 ml of 0.2 *M* borate buffer, pH 8, containing 0.3 *M* sodium chloride for spermidine, and 9 ml of 0.2 *M* borate buffer, pH 8, containing 0.6 *M* sodium chloride for spermine. The mobile phase temperature was always maintained above 20°C. The content of putrescine, cadaverine, spermidine and spermine in the respective fractions was determined fluorometrically with fluorescamine using Endo's method [6]; the histamine fraction was quantitated using the method of Shore et al. [7]. All fluorescence measurements were made with a Shimadzu spectrofluorophotometer RF-500 (Shimadzu, Kyoto, Japan).

Recovery

To estimate the recoveries of biogenic amines, the tissue homogenate was divided into two fractions. Known amounts of the respective amines were added to the first fraction while the other fraction was used as control. The recoveries were determined by subtracting the values obtained for control. The recovery experiment was performed with a healthy kidney with quite satisfactory results as shown in Table I.

High-performance liquid chromatography

For the purpose of ascertaining the results, diamines and polyamines in the same samples were determined using an HPLC system. An aliquot of the final

TABLE I

PRECISION OF RECOVERY OF BIOGENIC AMINES USING THE EXTERNAL STANDARD METHOD

Amine standards were added to kidney homogenates prior to preparation of sample and separation.

Biogenic amine	Amount added (nmol)	Recovery (%) (Mean ± S.D., $n = 6$)
Histamine	1	90.6 ± 6.99
Cadaverine	10	92.8 ± 3.72
Putrescine	10	91.9 ± 7.25
Spermidine	50	91.7 ± 8.50
Spermine	50	92.0 ± 4.04

supernatant (0.5 ml) was applied to a phosphocellulose column (0.6 × 3.5 cm) equilibrated with pH 6.2 phosphate buffer. The column was washed in the same stepwise manner as described above. Then histamine was eluted by 6 ml of 0.1 *M* borate buffer (pH 8) and other amines were eluted with 12 ml of 0.3 *M* borate buffer (pH 9). The histamine fraction was reacted with OPA according to the method of Shore et al. [7] and was injected into the HPLC system. The fraction containing the other amines was reduced to 1 ml and reacted with Dns chloride [8]. The Dns amines were extracted with benzene and evaporated to dryness. The residue was dissolved in acetonitrile and injected into the HPLC system. A Shimadzu LC-3A liquid chromatograph equipped with a spectrofluorometer system (Shimadzu RF-530) was used for this purpose. The column was μBondapak C_{18} (Waters Assoc., Milford, MA, U.S.A.) and the mobile phase acetonitrile—water (70:30, v/v). The spectrofluorometer was set at 358/446 nm for histamine estimation and at 360/510 nm for the estimation of other amines.

RESULTS AND DISCUSSION

Fig. 1 shows the elution patterns of authentic amines. Several techniques have been described for the separation of cadaverine, putrescine and histamine [2]. In most cases, however, expensive equipment such as an HPLC system or amino acid analyser have been adopted. Low-pressure liquid chromatography uses simpler equipment and is cheaper to use than the previous systems; unfortunately it is not possible to separate cadaverine from putrescine in this way [6, 9, 10]. In the present study, cadaverine was separated clearly from other diamines, histamine and putrescine, using the phosphocellulose method. Our application of phosphocellulose to the analysis of cadaverine and other amines is based on the earlier work of others [6, 9, 10]. The resolution of cadaverine, putrescine and histamine was optimized by altering the ionic strength of the eluting buffers. For the fluorometric estimation, histamine was treated with OPA while other amines were treated with fluorescamine. The fluorescamine reaction is more sensitive, simple and gives a more stable

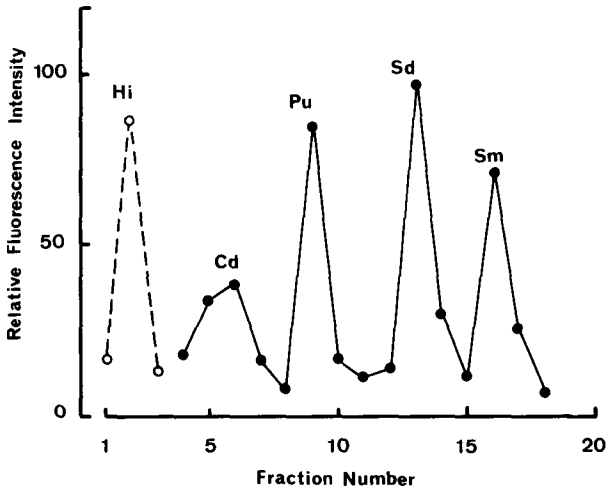

Fig. 1. Elution patterns of authentic diamines and polyamines. A standard solution (1 ml) containing histamine (Hi, 1 nmol), cadaverine (Cd, 10 nmol), putrescine (Pu, 10 nmol), spermidine (Sd, 50 nmol) and spermine (Sm, 50 nmol) was applied to the phosphocellulose column. Elution and estimation were carried out as described under Materials and methods; 3 ml of each fraction (except histamine and cadaverine fractions) were used for estimation. For histamine 2 ml were collected, for cadaverine 1 ml, and each made up to 3 ml with buffer and estimated. Control fluorescence intensities in the OPA and fluorescamine reactions were 50 for 0.5 nmol of histamine and 50 for 5 nmol of putrescine.

product than other methods [6]. Unknown amines, however, eluted near the region where histamine appears. Since OPA is known to react with histamine selectively and sensitively [7], this means of derivatization was used to avoid contamination of histamine with unknown amines. These considerations about the most suitable derivatives hold, however, for the stepwise elution during the phosphocellulose method. With HPLC, Dns derivatives of all polyamines (except histamine) are preferred [2].

Table II shows a comparison between the concentrations of diamines obtained by the phosphocellulose method and the HPLC method. The agreement between the two methods was acceptable with the sample set checked.

TABLE II

COMPARISON BETWEEN DIAMINE CONCENTRATIONS IN CASTRATED RAT KIDNEY UNDER THE INFLUENCE OF ANABOLIC STEROID AS DETERMINED BY THE PHOSPHOCELLULOSE AND HPLC METHODS

Animals received anabolic steroid subcutaneously (5 mg per 100 g, every day) for three days; 24 h after the last injection, they were sacrificed. Concentrations are expressed as nmol/g wet weight of tissue.

Biogenic diamine	Phosphocellulose	HPLC
Histamine	1.60 ± 0.15	1.68 ± 0.28
Cadaverine	8.74 ± 2.71	7.68 ± 1.60
Putrescine	54.62 ± 10.24	59.30 ± 22.14
Spermidine	655.6 ± 43.95	643.5 ± 33.99

The retention times with the phosphocellulose method were about 120 min, while with the HPLC method they were about 45 min; however, more than 30 samples could be separated at the same time by the phosphocellulose method using several columns side by side. Thus, the phosphocellulose method described here represents a very rapid and inexpensive procedure for separating the diamines and polyamines in biological samples.

Fig. 2 shows a typical chromatographic pattern of endogenous diamines and polyamines in the kidney of a castrated rat treated with an anabolic steroid, and indicates the presence of cadaverine in this sample. Henningsson et al. [5] reported that an anabolic steroid induced cadaverine synthesis in castrated

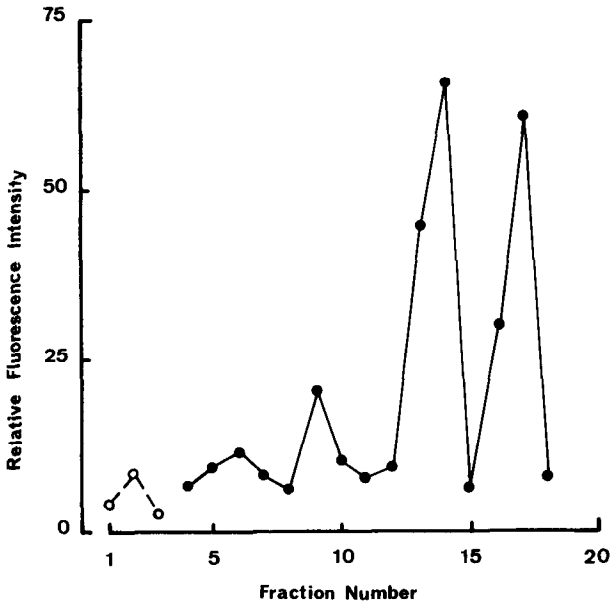

Fig. 2. Separation of endogenous diamines and polyamines in castrated rat kidney under the influence of anabolic steroid for three days. The separation and estimation procedures were described in Materials and methods.

TABLE III

RELATIONSHIP BETWEEN THE BIOGENIC AMINE CONCENTRATIONS IN CASTRATED RAT KIDNEY AND TIME AFTER THE LAST STEROID INJECTION

Concentrations are expressed as nmol/g wet weight of tissue. Each value is the mean ± S.D. of six or eight samples.

Time (h)	Histamine	Cadaverine	Putrescine	Spermidine	Spermine
0	1.06 ± 0.33	3.32 ± 1.16	41.7 ± 10.82	485.4 ± 48.17	681.9 ± 74.63
2	1.14 ± 0.20	5.71 ± 1.02*	47.8 ± 4.02	481.3 ± 41.12	719.4 ± 38.52
4	1.20 ± 0.11	5.46 ± 4.17	53.9 ± 4.71	509.3 ± 27.76	766.3 ± 17.98*
6	1.61 ± 0.35*	9.62 ± 4.36*	64.7 ± 6.82*	553.8 ± 28.60*	761.7 ± 19.56*
12	1.10 ± 0.16	7.84 ± 5.23	57.6 ± 5.00*	512.8 ± 20.78	696.6 ± 28.65

*Statistically significant difference between the mean of 0 h and experimental values, $p < 0.05$.

TABLE IV

CONCENTRATIONS OF DIAMINES AND POLYAMINES IN RAT KIDNEY AND DOSE OF ANABOLIC STEROID

Animals received anabolic steroid or vehicle every day for three days; 24 h after the last injection, they were sacrificed. Each value is the mean ± S.D. of six or seven rats. Concentrations are expressed as nmol/g wet weight of tissue.

Drug dose (mg per 100 g)	Histamine	Cadaverine	Putrescine	Spermidine	Spermine
Normal rats					
0	1.32 ± 0.15	2.57 ± 2.82	48.99 ± 4.15	617.8 ± 27.12	777.5 ± 56.61
2.5	1.12 ± 0.31	3.69 ± 3.70	41.85 ± 13.18	592.6 ± 19.36**	705.0 ± 45.47**
5	1.20 ± 0.29	5.93 ± 3.92	49.67 ± 6.97	614.8 ± 26.78	713.7 ± 57.56**
10	1.02 ± 0.11*	6.78 ± 5.42	47.41 ± 5.18	599.2 ± 27.09	701.8 ± 58.51**
Castrated rats					
0	1.30 ± 0.26	1.49 ± 1.86	43.99 ± 3.44	581.3 ± 43.44	729.6 ± 60.36
2.5	1.16 ± 0.29	6.12 ± 3.38*	48.11 ± 5.47	559.7 ± 56.84	756.7 ± 44.01
5	1.16 ± 0.15*	8.74 ± 2.71*	54.62 ± 10.24*	655.6 ± 43.95*	768.0 ± 122.22
10	0.94 ± 0.15*	8.94 ± 1.81*	49.27 ± 6.11**	617.9 ± 35.02	766.6 ± 55.41

*$p < 0.05$, ** $p < 0.1$: statistically significant difference between the means of control and steroid-treated rats

mouse kidney. The same phenomenon was observed in rat kidney. Table III shows the relationship between the content of renal polyamines and diamines and their time dependency after steroid injection. Six hours after the last injection, all the amines reached their maximum and decreased from then on. Cadaverine increased markedly. Table IV shows the relationship between the content of renal amines and the drug dose. The renal diamines and polyamines increased dose-dependently, and reached their maximum when 5 mg per 100 g were injected.

It is well known that anabolic steroids induce the rapid growth of kidneys of castrated animals. Salzman and Stepita-Klauco [1] suggested that the synthesis of cadaverine appears to be maximal during periods of rapid tissue proliferation and the major physiological role of cadaverine could be an endogenous modulator of polyamine metabolism. If this speculation is true, cadaverine must play an important role in mammalian cell proliferation. The present method appears to be a good technique for studying the role of diamines and polyamines in cell physiology.

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