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Analytical Biochemistry 339 (2005) 191–197
ANALYTICAL BIOCHEMISTRY
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Simultaneous determination of free and N-acetylated polyamines in urine by semimicro high-performance liquid chromatography using 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride as a fluorescent labeling reagent

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Received 7 August 2004
Available online 2 February 2005

Abstract

We have developed a simple and highly sensitive semimicro high-performance liquid chromatographic method for the simultaneous determination of free and N-acetylated polyamines in urine. Polyamines and N-acetylated polyamines were derivatized with 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride to produce fluorescent sulfonamides. The labeling reaction was carried out at 50 °C for 15 min at pH 9. The fluorescent derivatives were separated on a reversed-phase column with a gradient elution using water–acetonitrile–methanol at 50 °C and detected by fluorescence measurement at 318 nm (excitation) and 406 nm (emission). The detection limits (signal-to-noise ratio = 3) of the polyamines and N-acetylated polyamines were 0.7–4.5 fmol/injection. The within-day and day-to-day relative standard deviations were 3.2–7.9 and 3.0–7.7%, respectively. Significant differences were found in the urinary excretion of polyamines between cancer patients and normal subjects.

Keywords: Semimicro HPLC; Fluorometric detection; 4-(5,6-Dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride; Polyamine; N-Acetylated polyamine; Urine

Polyamines, found in all living organs, play important roles in cell growth, proliferation, and differentiation. Polyamines are mainly excreted as their acetyl conjugates in human urine [1,2], though they exist as free and conjugated forms in physiological fluids. Since a report stating that the urinary excretion of polyamines increased in cancer patients in comparison with normal subjects [3] was published, much research on the relationship between polyamines and cancer has been carried out. In these studies, as urinary free and N-acetylated polyamine levels were increased in various types of cancers, the role of urinary polyamines as a marker to screen cancers or to monitor the efficacy of therapy was investigated [4–10]. It is recognized that urinary polyamines are useful markers for monitoring the efficacy of therapy [5–9]. However, although the usefulness of polyamines as a marker for the diagnosis of cancer has been discussed, clinical results have not yet been obtained. In a recent study, it was shown that significant differences between cancer patients and normal subjects were found in the urinary excretion of polyamines and that the various types of cancers had specific patterns of urinary polyamines [10]. Therefore, to understand the relationship of polyamines with various types of cancers, it is necessary for the urinary free and N-acetylated polyamines to be simultaneously determined with highly sensitive detection.

At present, some methods for the simultaneous determination of free and N-acetylated polyamines in urine by gas chromatography (GC) [10,11] and high-perfor-

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0003-2697/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2005.01.008

mance liquid chromatography (HPLC) [12,13] have been reported. Unfortunately, the HPLC and GC methods are technically demanding and time consuming; therefore we sought to develop a more efficient method based on precolumn derivatization with 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride (DMS-Cl),[1] which reacts quantitatively with amino acids to form stable and highly fluorescent sulfonamides with a labeling yield of about 100% [14–16].

In this paper, a highly sensitive, simple semimicro HPLC method for the simultaneous determination of free and N-acetylated polyamines in urine with fluorescence detection after precolumn derivatization with DMS-Cl is described.

Materials and methods

Chemicals and solvents

All chemicals were of analytical–reagent grade, unless stated otherwise. DMS-Cl was prepared as described in a previous paper [14]. Putrescine (Put) dihydrochloride and cadaverine (Cad) dihydrochloride were purchased from Nacalai (Kyoto, Japan), and spermidine (Spd) trihydrochloride, spermine (Spm) tetrahydrochloride, *N*-acetylputrescine (AcPut) hydrochloride, *N*-acetylcadaverine (AcCad) hydrochloride, N^1-acetylspermidine (N^1-AcSpd) dihydrochloride, N^8-acetylspermidine (N^8-AcSpd) dihydrochloride, and *N*-acetylspermine (AcSpm) trihydrochloride were purchased from Sigma (St. Louis, MO, USA). The HPLC-grade acetonitrile and Creatinine-Test Wako were obtained from Wako (Osaka, Japan). Dowex 1-X8 was obtained from Bio-Rad (Richmond, CA, USA). Deionized-distilled water was purified with the Milli-QII system (Yamato, Tokyo, Japan) prior to use. The vials were silanized with dimethyldichlorosilane followed by successive thorough rinsings with absolute toluene and methanol prior to each use to avoid losses due to the adsorption of amines on the glass surface.

Instrumental conditions

The HPLC low-pressure gradient system (Hitachi, Tokyo, Japan) consisted of an L-7100 HPLC pump, an L-7300 column oven, an L-7200 auto sampler, and an L-7480 fluorescence detector, and a D-7500 integrator was used with a DGU-14A on-line degasser (Shimazu, Kyoto, Japan). A TSK gel ODS-80Ts column (150 × 2 mm, I.D., 5 μm, Tosoh) was employed at 50 °C with an elution program using three solvents, water, acetonitrile, and methanol, as shown in Table 1. The flow rate was 0.2 ml/min. The fluorescence intensities were monitored at excitation and emission wavelengths of 318 and 406 nm, respectively. Uncorrected fluorescence spectra of the eluate corresponding to the peak due to Spm on the HPLC were measured with a Shimadzu RF-530 spectrofluorimeter using a quartz cell (optical path length, 10 mm).

Table 1
Elution program for separation of polyamines and N-acetylated polyamines labeled with DMS-Cl

Time (min)	Ratio of solvent[a] (%)			Elution mode
	A	B	C	
0	72	23	5	
				Isocratic
15	72	23	5	
				Linear gradient
40	45	35	20	
				Linear gradient
50	20	55	25	
				Stepwise
50	72	23	5	
				Isocratic (equilibration of column)
60	72	23	5	

[a] Solvent: A, water; B, acetonitrile; C, methanol.

Sample collection

Urine from normal subjects (seven healthy volunteers selected from the Japanese staff and students in our laboratory) who were eating self-selected diets was collected at around 10:30 a.m. and used within 1 h or stored at −20 °C until use. The urine from the patients was frozen and then transported to our laboratory.

Analytical procedure

Dowex 1-X8 (about 40 mg) was added to the urine (100 μl) which was diluted with water (400 μl) and mixed thoroughly. After standing for 3 min, an aliquot of the supernatant (50 μl) was mixed with borate buffer (0.2 M, pH 9, 50 μl) and DMS-Cl (10 mM, in acetonitrile, 100 μl). The labeling reaction was carried out at 50 °C for 15 min and then proline (0.1 M, in water/acetonitrile(1/1), 200 μl) was added to the reaction mixture. After standing for more than 2 min at room temperature, an aliquot of the supernatant (2 μl) was subjected to semimicro HPLC.

Results and discussion

Semimicro HPLC separation and detection limits

The derivatives of polyamines and N-acetylated polyamines labeled with DMS-Cl were successfully separated

[1] *Abbreviations used:* DMS-Cl, 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride; Put, putrescine; Cad, cadaverine; Spd, spermidine; Spm, spermine; AcPut, *N*-acetylputrescine; AcCad, *N*-acetylcadaverine; N^1-AcSpd, N^1-acetylspermidine; N^8-AcSpd, N^8-acetylspermidine; AcSpm , *N*-acetylspermine.