Polyamine Metabolism III: Urinary Acetyl Polyamines in Human Cancer

MAHMOUD M. ABDEL-MONEM [x] **and KOSEI OHNO** *

Received February 6, 1978, from the *College of Pharmacy, University of Minnesota, Minneapolis, MN 55455.* Accepted for publication March 23, 1978. *Present address: Faculty of Pharmaceutical Sciences, Higashi Nippon Gakuen University, Ishikari-Tobetsu, Hokkaido, 061-02 Japan.

Abstract □ Polyamine levels were determined by high-pressure liquid chromatography in the unhydrolyzed 24-hr urine obtained from 15 cancer patients and nine normal subjects. N^1-Acetylspermine, N^4-acetylspermine, N^4-acetylspermidine, and N-(3-aminopropyl)acetamide were not detected in any samples. N^1-Acetylspermidine, N^8-acetylspermidine, acetylputrescine, and acetylcadaverine were present in all samples. Furthermore, acetylspermidine and acetylputrescine were excreted in much greater quantities than the respective amines in the urine of both cancer patients and normal subjects. The levels of N^1-acetylspermidine were considerably higher than the levels of N^8-acetylspermidine in the 24-hr urine of cancer patients, resulting in a ratio of N^1- to N^8-acetylspermidine in the urine of cancer patients significantly higher than that in normal subjects. The urinary levels of acetylputrescine were also significantly higher in cancer patients. The urinary polyamines in 13 of the 15 cancer patients were outside the 95% confidence limits of the normal mean for the ratio of N^1- to N^8-acetylspermidine and acetylputrescine. All cancer patients showed values outside the 95% confidence limits of the mean for either of these two parameters. Diurnal variation was observed in the urinary excretion of the acetyl polyamines but not for the free amines in two normal subjects.

Keyphrases □ Polyamines, various acetyl—high-pressure liquid chromatographic analyses in urine of normal humans and cancer patients □ High-pressure liquid chromatography—analyses, various acetyl polyamines in urine of normal humans and cancer patients

Since the initial report (1) describing elevated levels of polyamines in urine of cancer patients, interest in the possible use of urinary polyamine levels as biochemical markers of tumor presence and growth has increased. Independent studies involving large numbers of patients showed that the levels of these amines were elevated in the urine of a majority of the cancer patients (2–12). One comprehensive study indicated that the potential usefulness of the urinary polyamines as biological markers was less promising than originally anticipated (13). However, a recent study demonstrated the usefulness of urinary and serum polyamines as markers of response and disease activity in cancer chemotherapy (14, 15). Elevated polyamine concentration in the cerebrospinal fluid of patients with untreated malignant central nervous system tumors also was demonstrated (16).

Polyamines are present predominantly in human urine as conjugates that produce the free amines after hydrolysis. In most previous studies, the biological samples (urine, serum, and cerebrospinal fluid) were hydrolyzed prior to analysis. The data presented (1–15) pertain to the quantities of total polyamines excreted in urine and not to the nature of these amines.

In preliminary studies (17–19), the polyamines were examined in the unhydrolyzed urine of a limited number of cancer patients and normal subjects. The polyamines were present primarily in the form of the monoacetyl conjugates in all samples. This paper reports a more complete examination of the unhydrolyzed urine of additional subjects.

EXPERIMENTAL

Materials—All chemicals were the highest purity available or were purified before use. Chloroform (ACS grade) was stored over anhydrous calcium chloride overnight and distilled. 2-Propanol (ACS grade) was distilled from magnesium and a catalytic amount of iodine. Triethylamine[1] was distilled over potassium hydroxide and then over *p*-toluenesulfonyl chloride. Other high-pressure liquid chromatographic (HPLC) solvents were obtained from commercial sources[2]. All solvents were degassed before use by HPLC.

Urine Collection—Twenty-four-hour urine samples were obtained from nine healthy adults (six males and three females) as normal controls and from 15 patients with diagnosed cancer[3]. Urine was obtained from patients who had not received therapy except for one patient (DE) who had received intravenous fluorouracil weekly for 10 months. Samples for the study of diurnal variation were obtained from two normal males at 6-hr intervals.

Urine was collected in polyethylene bottles under toluene and kept refrigerated. The volume of urine was determined, and aliquots (2 ml each) were stored at −20° until analysis.

Analysis of Urine Samples—Each urine sample was analyzed in duplicate. Each urine aliquot (2 ml) was adjusted to pH 10–12 with 2 *N* NaOH (0.1 ml) and mixed with isoamyl alcohol (5 ml) on a vortex mixer for 2 min. The organic solvent extract was transferred into a centrifuge tube, and nitrogen gas was bubbled through the solvent for 30 min to remove ammonia. Concentrated hydrochloric acid (0.1 ml) was added, and the solvents were mixed on a vortex mixer for 30 sec.

The solvents were then evaporated[4] at 45° and 0.2 mm Hg. A 1-ml aliquot of sodium carbonate (50 mg/ml) and 2 ml of dansyl chloride (10.1 mg/ml in acetone) were added to the residue and mixed. Then the tubes were shaken at room temperature in the dark for 12 hr. Acetone was evaporated at 35° in a nitrogen stream, and the residue was mixed with water (1 ml) to dissolve the inorganic salts and then with benzene (5 ml). The solution was mixed on a vortex mixer for 30 sec. The benzene extract (4.8 ml) was transferred into a centrifuge tube, and the solvent was evaporated at 35° in a nitrogen stream. The residue was dissolved in chloroform (25–50 μl).

An aliquot of the chloroform solution (10 μl) was applied on a TLC silica gel GF plate[5] (20 × 20 cm, 250 μm), and the plate was developed as described previously (19). The spots on the plate corresponding to the position of N^1-acetylspermidine (VI), N^8-acetylspermidine (VII), acetylputrescine (III), acetylcadaverine (IV), putrescine (II), spermidine (V), and spermine (IX) were scraped separately. The adsorbent obtained from each spot was extracted immediately with a mixture of equal volumes of triethylamine and 2-propanol (10 ml). The extract was evaporated to dryness in a nitrogen stream at 40°, and the residue was stored at 2–4° until HPLC analysis.

The HPLC analysis was performed as described previously using two silica gel columns[6] (120 cm × 2.2 mm i.d.). One column was for the analysis of the dansyl derivatives of the monoacetyl polyamines with a solvent of chloroform–2-propanol (50:3). The flow rates were 0.3 ml/min for the analysis of VI and VII and 0.6 ml/min for the analysis of III and IV. The second column was for the analysis of the dansyl derivatives of the polyamines with a solvent of chloroform–triethylamine (50:1) at a flow rate of 0.4 ml/min.

Standard solutions of II, V, IX, and III, containing 1, 2, 3, or 4 nmoles

[1] Aldrich, Milwaukee, Wis.
[2] Burdick and Jackson Laboratories, Muskegon, Mich.
[3] Medical Oncology Service, University of Minnesota Hospitals, Minneapolis, Minn.
[4] Evapomix, Buchler Instruments, Fort Lee, N.J.
[5] Analtech, Newark, Del.
[6] Corasil II, Waters Associates, Milford, Mass.

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Table I—Pretreatment Levels of Polyamines in the 24-hr Urine of Cancer Patients and Normal Subjects

Patient	Micromoles per 24 hr							
	VI	VII	Ratio of VI to VII	III	IV	II	V	IX
FL	4.3	3.0	1.43	25.9	8.4	0.5	—[a]	—[a]
GB	2.2	1.6	1.34	17.1	—[b]	0.9	—[a]	—[a]
MH	26.5	3.9	6.76	41.0	2.5	1.3	—[c]	—[a]
CM	0.4	0.7	0.54	18.1	0.8	0.6	—[a]	—[a]
WM	27.2	8.0	3.41	65.5	—[b]	3.8	—[c]	—[c]
EN	4.9	1.5	3.16	17.4	—[b]	1.0	0.2	—[a]
WT	12.8	4.3	3.02	17.0	4.7	0.7	0.9	22.0
FL	2.2	1.0	2.21	5.5	—[b]	1.0	—[c]	—[c]
MV	5.3	4.4	1.20	34.3	—[b]	2.4	0.7	—[a]
FM	3.3	6.0	0.55	20.5	0.6	0.6	—[a]	—[a]
MB	3.4	3.4	1.02	17.5	3.1	1.1	—[c]	—[c]
LB	2.5	3.0	0.82	30.6	—[b]	—[c]	—[c]	—[c]
DE	3.7	5.2	0.70	18.3	0.4	2.0	—[a]	1.1
NL	3.0	2.1	1.47	14.6	—[b]	2.3	—[c]	—[c]
ET	4.8	4.2	1.17	14.8	0.6	0.83	0.5	3.3
Mean (*SE*)	7.1 (±2.2)	3.5 (±0.9)	1.92[d] (±0.41)	23.9[d] (±3.7)	2.6 (±1.0)	1.4	0.6	8.8
Normal mean (*SE*)	2.91 (±0.6)	2.84 (±0.5)	0.94[d] (±0.09)	11.7[d] (±1.5)	1.9 (±0.9)	1.6 (±0.4)	0.2 (±0.04)	2.1 (±1.0)

[a] The amine was detected by TLC and HPLC, but the levels were too low for quantitative measurement. [b] Acetylcadaverine was detected by TLC but was not determined by HPLC. [c] The sample was not analyzed for the amine. [d] Differences are significant at $p < 0.05$.

of each in water (2 ml), were analyzed concurrently with the urine samples. The percent recovery for each compound was calculated and used for correcting for losses during the analytical procedure. Since authentic samples of IV, VI, and VII were not available, the recovery of III was used for correcting the results.

$RHN(CH_2)_nNH_2$
I: R = $COCH_3$, $n = 3$
II: R = H, $n = 4$
III: R = $COCH_3$, $n = 4$
IV: R = $COCH_3$, $n = 5$

$R_1HN(CH_2)_3N(CH_2)_4NHR_3$
$|$
R_2
V: $R_1 = R_2 = R_3 = H$
VI: $R_1 = COCH_3$, $R_2 = R_3 = H$
VII: $R_1 = R_2 = H$, $R_3 = COCH_3$
VIII: $R_1 = R_3 = H$, $R_2 = COCH_3$

$R_1HN(CH_2)_3N(CH_2)_4NH(CH_2)_3NH_2$
$|$
R_2
IX: $R_1 = R_2 = H$
X: $R_1 = COCH_3$, $R_2 = H$
XI: $R_1 = H$, $R_2 = COCH_3$

RESULTS

Examination of the crude mixture of the dansyl derivatives of the polyamines obtained from unhydrolyzed human urine by TLC or HPLC alone indicated the complexity of the mixture. The combination of TLC and HPLC allowed the simultaneous determination of the free polyamines and the monoacetyl polyamines in the unhydrolyzed urine samples. Representative TLC and HPLC separations of the dansyl derivatives from the urine of normal subjects and cancer patients were described previously[7] (19).

The concentrations of each polyamine and monoacetyl polyamine in the urine samples were calculated by comparison of the HPLC peak height of its dansyl derivative with those obtained from known concentrations of the authentic sample of the derivative. The calculated values were corrected for losses during the analytical procedure, using the percent recovery determined concurrently for standard solutions. Fractions of HPLC column eluates corresponding to each polyamine and monoacetyl polyamine were collected and examined by two-dimensional TLC. All examined fractions contained only one fluorescent component with chromatographic mobility similar to the authentic compound.

Table I indicates the concentrations of the free polyamines and monoacetyl polyamines found in the 24-hr urine from 15 cancer patients as well as the mean values obtained for nine normal subjects. The free polyamines were not determined in all samples from cancer patients because these compounds were present in relatively low concentrations, which complicated the analytical procedure. The values reported in Table I have been corrected for losses during the analytical procedures and are accurate estimates of the actual urine concentrations.

N^1-Acetylspermine (X), N^4-acetylspermine (XI), N^4-acetylspermidine (VIII), and *N*-(3-aminopropyl)acetamide (I) were not detected in any urine sample obtained from the 24 subjects. On the other hand, N^1-acetylspermidine (VI), N^8-acetylspermidine (VII), acetylputrescine (III), and acetylcadaverine (IV) were present in all samples. Only a few samples were analyzed for the free amines, and some did not contain detectable levels of one or more of the free amines. The normal range for spermine (IX) was 0.7–5.1 μmoles/24 hr (mean = 2.1). The range for IX in the urine of three cancer patients was 1.1–22.0 μmoles/24 hr.

The levels of free spermidine (V) in the 24-hr urine of normal subjects were considerably lower than those of acetylspermidines VI and VII. The normal range for V was 0.1–0.3 μmole/24 hr (mean = 0.2). The normal range for the ratio of VI and VII to V was 14–89 (mean = 44). The range for V in the urine of the cancer patients was 0.2–0.9 μmole/24 hr (mean = 0.6), and the range for the ratio of VI and VII to V was 14–32 (mean = 21).

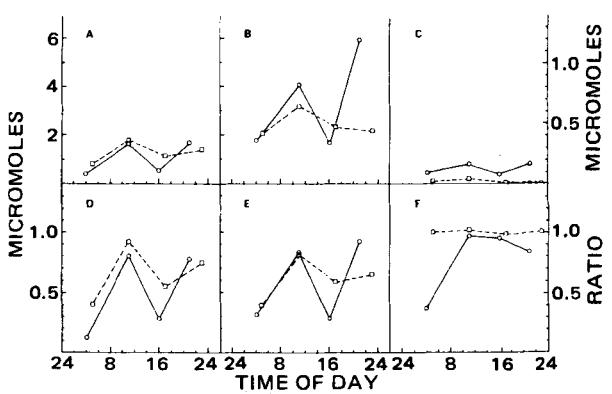

Figure 1—*Diurnal variation of urinary polyamines in two normal subjects, MA (—) and CC (- - -). Key: A, total acetylspermidines; B, acetylputrescine; C, acetylcadaverine; D,* N^1*-acetylspermidine; E,* N^8*-acetylspermidine; and F, ratio of* N^1*-acetylspermidine to* N^8*-acetylspermidine. On the abscissa, micromoles indicates the micromoles of polyamine/24 hr and ratio indicates the ratio of* N^1*-acetylspermidine to* N^8*-acetylspermidine.*

[7] Complete chemical names for compounds described in this article can be found in Ref. 19.

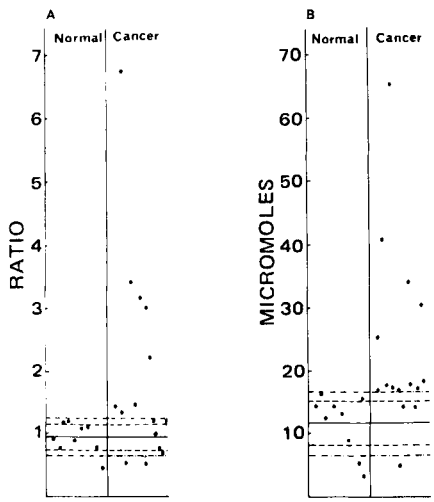

Figure 2—*Ratio of* N^1*-acetylspermidine to* N^8*-acetylspermidine (A) and urinary levels of acetylputrescine (B) in cancer patients and normal subjects. On the abscissa, ratio indicates the ratio of* N^1*- to* N^8*-acetylspermidine and micromoles indicates micromoles of acetylputrescine/24 hr.*

The concentration of II in the 24-hr unhydrolyzed urine of normal subjects was considerably higher than that for V. The normal range was 0.7–3.0 μmoles (mean = 1.6). The concentration of free II in the urine from the cancer patients was not significantly higher than that in the normal subjects. The range for free II in the urine of these patients was 0.5–3.8 μmoles/24 hr (mean = 1.4). The ratio of III to II in the urine of normal subjects was lower than that for VI and VII to V. The normal range for this ratio was 5–20 (mean = 11) in normal subjects and 5–50 (mean = 21) in cancer patients. These data indicate the much greater amounts of the acetylated II and V excreted in the urine of both normal and cancer patients relative to the amounts of the respective amines. This result is to be contrasted with the undetectable levels of X and XI in urine.

Compound IV appears to be a normal constituent of urine of both normal subjects and cancer patients. The normal range was 0.4–5.8 μmoles/24 hr (mean = 1.9), and the range in the urine of cancer patients was 0.4–8.4 μmoles/24 hr (mean = 2.6).

Both VI and VII were present in the unhydrolyzed 24-hr urine collected from the 24 subjects examined. The normal range for VI was 0.2–5.3 (mean = 2.9); for VII, it was 0.5–4.5 (mean = 2.8) μmoles/24 hr. The normal range for the ratio of VI to VII was 0.4–1.3 (mean = 0.9). The range for VI in the cancer patients was 0.4–27.0 μmoles/24 hr (mean = 7.1); for VII, it was 0.7–8.0 μmoles/24 hr (mean = 3.5). The ratio of VI to VII ranged from 0.5 to 6.8 (mean = 1.9) and was significantly higher than that in the normal subjects ($p < 0.05$).

The diurnal variation in the excretion of polyamines in the urine of two normal subjects is shown in Fig. 1. Periodicity was observed for the urinary excretion of VI, VII, and III for both subjects. One subject showed diurnal variation for urinary levels of IV. The second subject had considerably lower levels of acetylcadaverine in urine, and variation was not observed for this substance. One subject (MA) showed a relatively constant ratio of VI to VII. The second subject had a much lower ratio of VI to VII in the morning urine collection. The urinary excretion of the free polyamines did not show diurnal variation for either subject.

DISCUSSION

The results indicate that the differences in the urinary excretion of polyamines between normal subjects and cancer patients are not only in their levels but also in the nature of the polyamine conjugates. Cancer patients excreted considerably higher levels of VI than VII. The ratio of VI to VII in the urine of cancer patients was significantly higher than that in normal subjects (Fig. 2). The results obtained are in agreement with those observed earlier with only three cancer patients and three normal subjects (17, 19).

Compounds II and V were present primarily as acetyl conjugates in the urine of normal subjects and cancer patients. Compounds X and XI were not detected in any sample examined. In preliminary studies, III, VI, and VII were not detected in the serum of one normal subject, indicating that the acetyl compounds are not present in serum or that the serum concentration of the acetyl conjugates is much lower than that for the free polyamines. These preliminary findings suggest that either the acetylation of the polyamines takes place primarily in the kidneys or the renal clearance of the acetyl compounds is much greater than that for the free amines. The mechanisms of urinary excretion of the polyamines are not clear and are currently under investigation.

Diurnal variation was observed in the urinary excretion of the acetyl polyamines but not for the free amines in two normal subjects. Diurnal variation in polyamine excretion in normal and tumor-bearing rats was reported (20). However, diurnal variation of the urinary polyamines in humans has not been described.

Rosenblum *et al.* (21, 22) reported the formation of conjugates of radiolabeled V and II in rats and humans, but preliminary studies indicated that the conjugates in rat urine were not the acetyl compounds (22).

Comparison of the urinary profiles of polyamines in normal subjects and cancer patients indicates that the mean urinary levels for III and the ratio of VI to VII were significantly higher in cancer patients ($p < 0.05$). The levels of III and the ratio of VI to VII in the urine of cancer patients were outside the 95% confidence limits of the normal mean in 13 of 15 patients (Fig. 2). All cancer patients showed values outside the 95% confidence limits for either the ratio of VI to VII or levels of III.

REFERENCES

(1) D. H. Russell, *Nature New Biol.*, **233**, 144 (1971).

(2) D. H. Russell, C. C. Levy, S. C. Schimpff, and I. A. Hawk, *Cancer Res.*, **31**, 1555 (1971).

(3) F. Dreyfuss, R. Chayen, G. Dreyfuss, R. Dvir, and J. Ratan, *Isr. J. Med. Sci.*, **11**, 785 (1975).

(4) A. Lipton, L. M. Sheehan, and G. F. Kessler, Jr., *Cancer*, **35**, 464 (1975).

(5) D. C. Tormey, T. P. Waalkes, D. Ahmann, C. W. Gehrke, R. W. Zumwalt, J. Snyder, and H. Hansen, *ibid.*, **35**, 1095 (1975).

(6) M. Tsuji, T. Nakajima, and I. Sano, *Clin. Chim. Acta*, **59**, 161 (1975).

(7) Y. Takeda, T. Tominaga, M. Kitamura, T. Taguchi, T. Takeda, and T. Miwatani, *Gann*, **66**, 445 (1975).

(8) W. R. Fair, N. Wehner, and U. Brorsson, *J. Urol.*, **114**, 88 (1975).

(9) A. Lipton, L. Sheehan, and H. A. Harvey, *Cancer*, **36**, 2351 (1975).

(10) D. Bartos, R. A. Campbell, F. Bartos, and D. P. Grettie, *Cancer Res.*, **35**, 2056 (1975).

(11) A. Lipton, L. Sheehan, R. Mortel, and H. A. Harvey, *Cancer*, **38**, 1344 (1976).

(12) K. Fujita, T. Nagatsu, K. Maruta, M. Ito, H. Senba, and K. Miki, *Cancer Res.*, **36**, 1320 (1976).

(13) T. P. Waalkes, C. W. Gehrke, D. C. Tormey, R. W. Zumwalt, J. N. Hueser, K. C. Kuo, D. B. Lakingo, D. L. Ahmann, and C. G. Moertel, *Cancer Chemother. Rep.*, **59**, 1103 (1975).

(14) D. H. Russell, B. G. M. Durie, and S. E. Salmon, *Lancet*, **2**, 797 (1975).

(15) B. G. M. Durie, S. E. Salmon, and D. H. Russell, *Cancer Res.*, **37**, 214 (1977).

(16) L. J. Marton, O. Heby, V. A. Levin, W. P. Lubich, D. C. Crafts, and C. B. Wilson, *ibid.*, **36**, 973 (1976).

(17) M. M. Abdel-Monem, K. Ohno, I. E. Fortuny, and A. Theologides, *Lancet*, **2**, 1210 (1975).

(18) M. M. Abdel-Monem and K. Ohno, *J. Pharm. Sci.*, **66**, 1089 (1977).

(19) *Ibid.*, **66**, 1195 (1977).

(20) F. Halberg, C. W. Gehrke, H. H. Zinneman, K. Kuo, W. L. Nelson, D. P. Dubey, L. M. Cadotte, E. Haus, L. E. Scheving, R. A. Good, A. Rosenberg, L. Soong, H. Bazin, and M. M. Abdel-Monem, *Chronobiologia*, **3**, 309 (1976).

(21) M. G. Rosenblum, B. G. M. Durie, S. E. Salmon, S. Chang, and D. H. Russell, *Proc. Am. Assoc. Cancer Res.*, **17** (1976).

(22) M. G. Rosenblum and D. H. Russell, *Cancer Res.*, **37**, 47 (1977).

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