# Determination of Polyamines in Urine of Normal Human and Cancer Patients by Capillary Gas Chromatography†

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**A capillary gas chromatographic method is described for the determination of polyamines (putrescine, spermidine and spermine) in the urine of normal human and cancer patients. Morning urine after acid hydrolysis is cleaned up on a silica gel column and derivatized with trifluoroacetic-anhydride. Creatinine in human urine is used as internal standard. Recoveries of polyamines are 96.7% putrescine, 102.6% spermidine (Spd), and 98.7% spermine. SD of the method for Spd is 1.949 ± 0.041 (μg/mg creatinine, mean ±SD, $n = 5$). The results show that the mean level of polyamines in cancer patients urine is much higher than that in normal human urine. The mean of total polyamines in the normal human and the cancer patients is 2.01 and 44.74, respectively (g/mg creatinine).**

## INTRODUCTION

The polyamines putrescine (Put), spermidine (Spd) and spermine (Spm) are metabolites of cell growth. They have been found in many bacteria, plants and bacteriophages as well as in animal tissue and body fluids. The concentration of polyamines in human body fluids has a close relationship with the process of normal cell growth and tumour cell growth. The possible role of polyamines as clinical biochemical markers for malignancy was first suggested by a report indicating that polyamines are present in increased amounts in the urine of cancer patients (Russell *et al.*, 1971). Since then, many papers and reviews about the relationship between polyamines and cancer, the function of polyamines in biology, and the clinical utility of polyamine determination have been published (Russell, 1977; Heby and Andersson, 1980; Sunkara *et al.*, 1981). It is usually considered that the polyamine level which is low in normal human body fluid, increases greatly in cancer patients and returns to the initial low level in patients on recovery. Determination of total polyamines in biological fluids is especially important for estimating the extent of chemo- and radiotherapeutically induced tumor cell death in cancer patients (Russell, 1982). Increased concentrations of extracellular polyamines before therapy are related to rapid cell growth, spontaneous cell death, or both (Russell, 1977, 1982). Determination of intracellular polyamines (Heby and Andersson, 1980) and detailed monitoring of extracellular polyamines in patients being treated for cancer may yield clinically useful information on the kinetics of therapeutically induced recruitment (an increase in the number of cells moving from the nonproliferating to the proliferating cell compartment) and cell death processes (Russell, 1977, 1982).

Nowadays, the most common methods for determining polyamines are ion exchange column chromatography in modified amino acid analyzers (Milano *et al.*, 1980; Mach *et al.*, 1981), ion exchange chromatography (Prussak and Russell, 1982), and normal phase and reversed phase high performance liquid chromatography (Abdel-Monem and Merdink, 1981; Seiler and Knodgen 1980). The chief advantage of such methods usually is their minimal requirement for sample pretreatment. Few methods have been described for the determination of polyamines, primarily because of difficulties in obtaining derivatizable salt-free extracts of polar analytes in biological fluids. Current pretreatment methods include extraction with alkaline butanol (Beninati *et al.*, 1977), cation exchange chromatography (Ohki *et al.*, 1982), extraction after derivatization (Bakowski *et al.*, 1981) and adsorption with silica gel (Yamamoto *et al.*, 1982; Muskiet *et al.*, 1984). As there is a low recovery with liquid/liquid extraction, extraction with alkaline butanol is not generally used. The ratio of polyamines and creatinine (Crt) is usually taken as an appraisal standard, and creatinine was usually measured by a picric acid method. In this paper, however, creatinine was measured directly by gas chromatography (GC) which shortens the analysis time and makes the method easier.

## EXPERIMENTAL

**Instruments.** Sigma 115 gas chromatograph with flame ionization detector (FID) was used (Perkin-Elmer Corp., Norwalk, CT, USA). The column was 27 m × 0.25 mm I.D. fused silica capillary coated with SE-54, gas flow (nitrogen) 1.07 mL/min, split ratio 1:14, detector temperature 300 °C, and injector temperature 300 °C. The oven temperature was programmed from 100 °C to 250 °C at 10 °C/min, and then held for 15 min at 250 °C.

**Standards and reagents.** Putrescine.2HCl, spermidine.3HCl, spermine.4HCl and creatinine were obtained from Fluka (Buchs, Switzerland); trifluoroacetic anhydride (TFAA) was from Eastman Kodak Co., Rochester, NY USA; derivative reagents consisted of acetonitrile + TFAA (30:70 v/v); buffer, $NH_4Cl + NH_3.H_2O$, pH 10.5, eluent, 0.1 mol HCl in methanol. The other reagents and materials were obtained from commercial sources.

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**Samples.** Specimens of first morning urine were obtained from ostensibly normal persons and cancer patients. The specimens were acidified without delay to pH 1-2, with HCl and samples were stored at −20 °C until analysis.

### Analytical Procedure for polyamines

**Acid hydrolysis.** 1.0 ml 12 M HCl was added to 5 mL acidified urine in 10 mL ampoule, the ampoule was sealed and heated for 5 h at 100 °C, then left to cool to room temperature.

**Sample clean-up.** The hydrolysate was centrifuged at 2000 × *g* for 10 min. The supernatant was transferred to a tube and adjusted to pH 9 with 4 M aqueous NaOH then 10 mL pH 10.5 buffer was added. The supernatant was centrifuged and passed through a 2 g silica gel column, which was washed previously with 10 mL 0.1 M methanolic hydrochloric acid solution (12 M HCl in water diluted to 0.1 M with methanol) and then washed with 20 mL water to pH 7. The silica gel column was washed with 10 mL water and the polyamines eluted into a 20 mL tube with 15 mL of a 0.1 M solution of HCl in methanol. To regenerate the columns they were washed with 10 mL methanolic hydrochloric acid and 25 mL of water.

**Derivatization.** The eluate from the silica gel columns was evaporated at 60 °C, under a stream of air. The residue was dehydrated completely by putting the tube in the dryer overnight. To prepare the trifluoroacetic anhydride derivatives (TFAA), 0.9 mL of reagent was added to the tube, the tube was capped tightly, and heated at 80 °C for 15 min and then evaporated at room temperature under a gentle stream of nitrogen. The residue was redissolved in 200 μL acetone then 1.0 μL aliquots of the solution were injected into the gas chromatograph.

**Analytical procedure for creatinine.** 0.5 mL urine was evaporated under a stream of air at 90 °C, 0.9 mL of TFAA was added and the tube capped tightly and heated at 80 °C for 15 min, cooled to room temperature and evaporated under a stream of air. 200 μL acetone was added and 1.0 μL aliquots of the solution injected into the gas chromatograph.

## RESULTS

### Determination of polyamines in urine of normal humans and cancer patients

Figure 1 shows the chromatogram of the standard derivatives of polyamines. Figures 2 and 3 give the chromatograms of polyamines in urine from a normal person and a cancer patient respectively. Figure 4 shows the chromatogram of urinary creatinine in a normal human. The results are listed in Tables 1 and 2.

### The recovery and the standard deviation of the methods.

Table 3 shows the level and straight line range of the method for the determination of Put, Spd, Spm and Crt. The polyamine standards were added to the urine prior to hydrolysis. As shown in Table 4, the average recovery was 96.7% for Put, 102.6% for Spd, and 98.7% for Spm. The average polyamine concentrations in urine were 3.489 ± 0.018 for Put and 1.949 ± 0.041 for Spd (μg/mg creatinine, mean ± SD, $n = 5$), therefore the standard deviation of the method was 0.041.

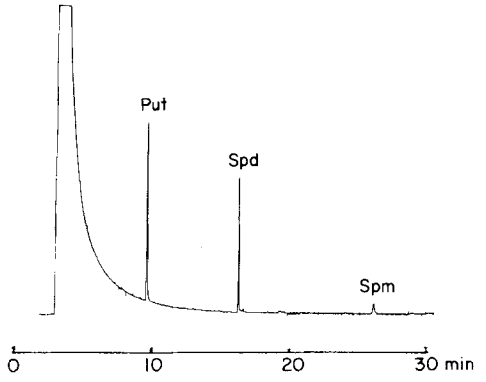


**Figure 1.** GC of polyamines as TFAA derivatives. GC conditions: initial temp; 100 °C; rate, 10 °C/min; final temp; 250 °C; hold, 15 min; SE-54 capillary column; inj. temp. and det. temp; 300 °C; split ratio, 1:14; 300 ng/injection. Derivatization procedure. To the dry polyamines as hydrochlorides was added 0.9 mL of TFAA (30% acetonitrile + 70% TFAA, v/v). The solution was heated at 80 °C for 15 min and, when cooled, the solution was evaporated by air, and the residue was redissolved with acetone for GC analysis.

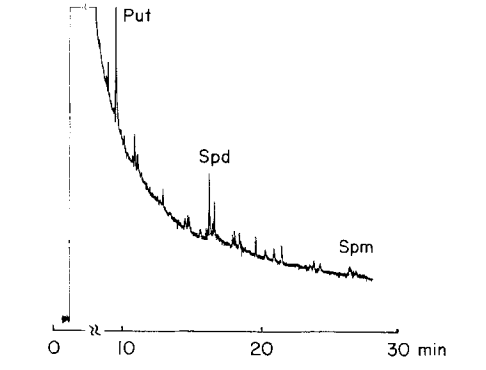


**Figure 2.** GC of polyamines as TFAA derivatives in human urine (normal). 5 mL urine was hydrolyzed in 2 M HCl at 100 °C for 5 h; once cooled and neutralized, the solution was cleaned up with a silica column, and the eluant was evaporated and derivatized. The derivatization procedure was as for Fig. 1.

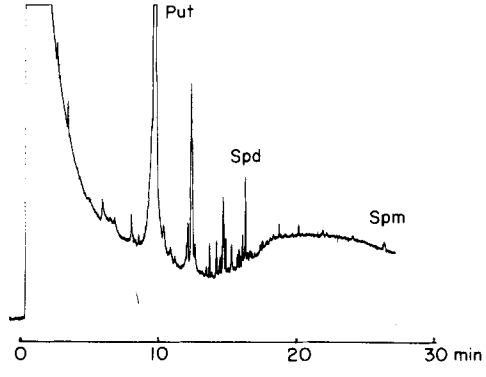


**Figure 3.** GC of polyamines as TFAA derivatives in the urine of a cancer patient. Analysis procedure and chromatography conditions are as for Figs 1 and 2.

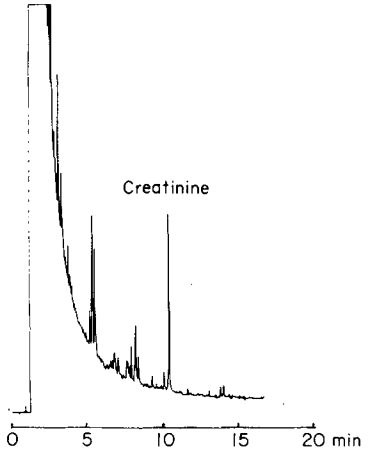


**Figure 4.** GC of creatinine as its TFAA derivative in human urine (normal). 0.5 mL urine was evaporated by air at 90 °C and the residue was derivatized. Derivative procedure and chromatography conditions are as for Fig. 1.

**Table 1. Determination of polyamines in normal human urine**

| Age (yr) of normals | Sex | Creatinine (mg/mL) | Polyamines (μg/mg c reatinine) Put | Spd | Spm |
|---|---|---|---|---|---|
| 33 | M | 1.465 | 1.787 | 0.303 | 0.881 |
| 26 | M | 1.419 | 0.912 | 0.307 | 0.426 |
| 53 | M | 1.600 | 0.540 | 0.266 | 0.499 |
| 29 | F | 1.364 | 0.431 | 0.288 | 0.438 |
| 20 | M | 1.958 | 0.479 | 0.747 | 0.777 |
| 40 | F | 1.905 | 0.940 | † | 0.299 |
| 13 | F | 1.638 | 2.116 | 0.055 | 0.322 |
| 25 | M | 1.389 | 2·422 | 0.175 | † |
| 48 | M | 1.097 | 2.112 | 0.025 | 0.559 |
| | | Average | 1.304 | 0.241 | 0.467 |
| | | SD | 0.799 | 0.227 | 0.262 |

†, Under the level of determination

**Table 2. Determination of polyamines in urine from cancer patients**

| Age (yr) normals | Sex | Diagnosis | Polyamines (μg/mg c reatinine) Put | Spd | Spm |
|---|---|---|---|---|---|
| 27 | F | breast cancer | 2.910 | 1.521 | 1.056 |
| 49 | M | lung cancer | 8.063 | 1.312 | † |
| 53 | F | melanoma | 5.509 | 1.809 | † |
| 44 | M | ovary tumour | 10.27 | 1.092 | † |
| 53 | M | lung cancer | 12.49 | 4.632 | 10.45 |
| 73 | M | lung cancer | 40.88 | 5.862 | † |
| 58 | M | lung cancer | 8.80 | 2.62 | † |
| 63 | F | lung cancer | 10.15 | 2.84 | 6.33 |
| 60 | F | lung cancer | 288.0 | 8.80 | † |
| 56 | M | lung cancer | 2.10 | † | † |
| 66 | M | lung cancer | 1·70 | 1.46 | † |
| 29 | M | lung cancer | 1.90 | 0.19 | † |
| 51 | M | lung cancer | 29.0 | 13.0 | 8.71 |
| 64 | M | lung cancer | 9.29 | † | † |
| 53 | M | lung cancer | 21.60 | 43.8 | † |
| 50 | M | lung cancer | 4.86 | † | † |
| $n=16$ | | Average | 28.60 | 5.56 | 1.66 |
| | | SD | 70.0 | 10.7 | 3.3 |

†, Under the level of determination.

**Table 3. Straight line range and the inferior limit**

| Poly-amines | Line range (μg/μL) | Regression equation[a] | Correlation coefficient | The level for determination[b] |
|---|---|---|---|---|
| Put | ≤1 | $Y=1.28x-0.01$ | 0.9979 | 2 |
| Spd | ≤1 | $Y=1.48x-0.01$ | 0.9987 | 5 |
| Spm | ≤0.5 | $Y=2.66x+0.01$ | 0.9919 | 25 |
| Crt | ≤4 | $Y=12.97x+0.03$ | 0.9983 | 50 |

[a] $n=8$, [b] ng/μL

**Table 4. Recovery of polyamines in urine**

| Polyamines | Added (ng) | Recovery (%) | Average |
|---|---|---|---|
| Put | 40 | 98.4 | |
| | 100 | 94.9 | 96.7 |
| Spd | 40 | 97.8 | |
| | 100 | 107.0 | |
| | 100 | 107.6 | |
| | 200 | 98.1 | 102.6 |
| Spm | 50 | 94.2 | |
| | 100 | 102.9 | |
| | 100 | 97.3 | |
| | 150 | 100.4 | 98.7 |

## DISCUSSION

### Effect of the amount of derivatization reagent.

0.15 mg polyamines and 0.8 mg creatinine were reacted with various amounts of TFAA reagent at 80 °C for 20 min. As shown in Fig. 5, the products reached their maximal levels at 0.25 mL and 0.5 mL, respectively, for polyamines and creatinine. 0.9 mL TFAA was adopted as a routine quantity.

### Effect of derivatization time

0.3 mg polyamines and 1.0 mg creatinine were reacted with excess TFAA at 80 °C for various time periods. As shown in Fig. 6, reaction products were stable within

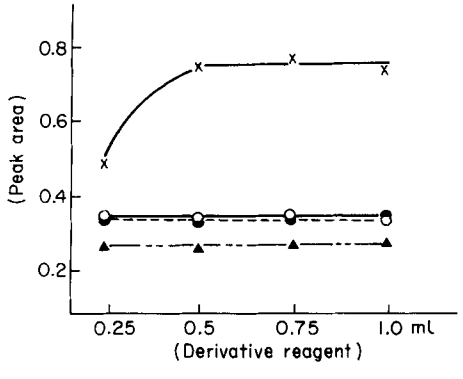


**Figure 5.** Effect of the amount of reagent on peak area of polyamines as TFAA derivatives. 0.15 mg polyamines and 0.8 mg of creatinine were reacted with various amounts of derivative reagent at 80 °C for 20 min. GC conditions are as for Fig. 1. ●, Put; ▲, Spd; ○, Spm; and ×, Crt.

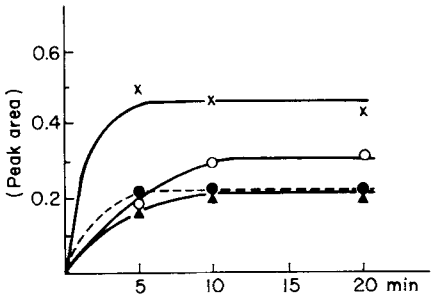


**Figure 6.** Effect of the derivatization time on peak area of polyamines as TFAA derivatives. 0.3 mg polyamines and 1.0 mg of creatinine were reacted with excess TFAA at 80 °C for various time periods. GC conditions are as for Fig. 1. ●, Put; ▲, Spd, ○, Spm; ×, Crt.

10–20 min, therefore 80 °C and 15 min were chosen as the temperature and time of the derivatization.

### Stability of the derivatives

The derivatives of polyamines and creatinine were stored at 4 °C for various time periods. The results in Fig. 7 show that they were stable in acetone at 4 °C for 30 h.

### Effect of buffer pH on adsorption of polyamines

Equivalent amounts of the polyamines were mixed with 10 mL buffer at different pH. The mixtures were passed through 2 g of silica gel column, then eluted, evaporated, converted into TFAA derivatives and measured. The results in Fig. 8 show that there was no obvious variation from pH 9 to 11. Therefore pH 10.5 for the buffer was adopted.

### Effect of acid concentration and hydrolysis time on hydrolysis of polyamines in urine

Acid hydrolysis of urine will help release most of the polyamines in urine. 6 M HCl and 120 °C for 16–20 h were adopted in most of the reports concerning the analysis of polyamines from urine. Aqueous 6 M HCl has a very high partial pressure of HCl vapour at 120 °C

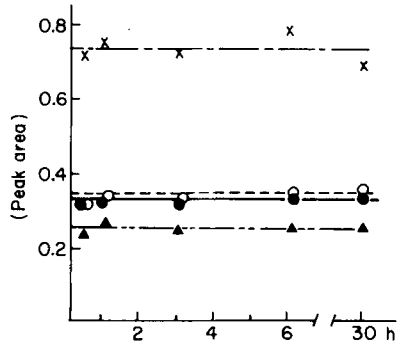


**Figure 7.** The stability of derivatives of polyamines and creatinine as TFAA derivatives at 4 °C in acetone. ●, Put; ▲, Spd; ○, Spm; ×, Crt;.

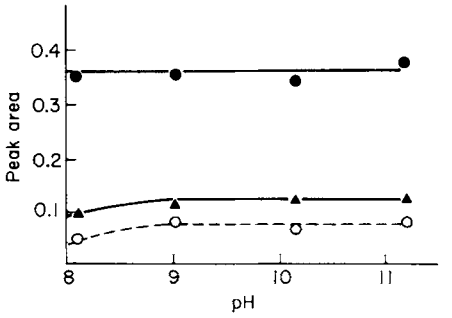


**Figure 8.** Effect of buffer pH on adsorption of polyamines. The same amount of each polyamine was mixed with 10 mL buffer at differing pH. The mixture was passed through 2 g of silica gel column, then eluted and evaporated. Procedure and GC conditions are as for Fig. 1. ●, Put; ▲, Spd; ○, Spm.

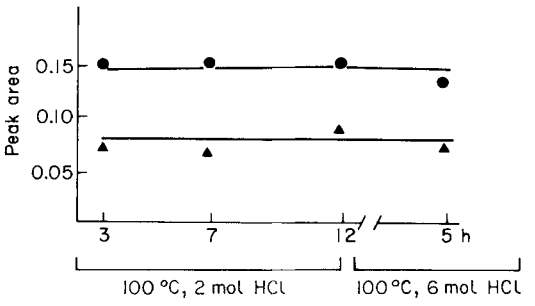


**Figure 9.** Effect of HCl concentration and hydrolysis time on human urine. The analysis procedure and GC condition are as for Figs 1 and 2. ●, Put; ▲, Spd.

and an ordinary ampoule may not be suitable for the task. 100 °C and 2 M HCl are mild conditions, and the ordinary ampoule can be used. These two conditions were compared, and the results given in Fig. 9 shows that there was no obvious difference between them. Hydrolysis conditions of 2 M HCl and 100 °C for 5 h were adopted.

### Effect of the procedure of drying the urine on determination of creatinine

0.5 mL urine was taken for creatinine determination. The result given in Table 5 using student's *t*-test showed that there was no obvious difference between the three drying procedures, and 90 °C was adopted. Isobutyl chloroformate and silylation reagents (TMCS, BSA, BSTFA) had been tested: the results were not as good

**Table 5. Comparison of three procedures of urine drying**

| | A. Freeze-drying | B. Evaporation by air acidified 90 °C | C. Evaporation by air not acidified 90 °C |
|---|---|---|---|
| | 0.6398 | 0.5427 | 0.5287 |
| | 0.6702 | 0.5334 | 0.5330 |
| | 0.4633 | 0.4823 | 0.5658 |
| | 0.4287 | 0.5667 | 0.7356 |
| Mean | 0.5505 | 0.5318 | 0.5908 |
| SD | 0.1221 | 0.0355 | 0.0980 |

$(|t_{AB}|=0.34, |t_{AC}|=0.52, |t_{BC}|=1.25) < t_{0.05,6}, t_{0.05,6}=2.45$

**Table 6. Comparision of polyamines in urine of normal and cancer patients**

| Polyamines | | n | Range[a] | Average |
|---|---|---|---|---|
| Put | normal | 9 | 0.43–2.42 | 1.30 |
| | normal[b] | 52 | 0.80–3.39 | 1.85 |
| | cancer patients | 16 | 1.7–288.0 | 28.60 |
| Spd | normal | 9 | 0.02–0.31 | 0.24 |
| | normal[b] | 52 | 0.23–0.72 | 0.45 |
| | cancer patients | 16 | 0.19–43.8 | 5.56 |
| Spm | normal | 9 | 0.30–0.88 | 0.47 |
| | normal[b] | 52 | 0.02–0.32 | 0.07 |
| | cancer patients | 16 | trace–10.25 | 1.66 |
| Total | normal | 9 | 1.16–2.97 | 2.01 |
| | normal[b] | 52 | 1.05–4.44 | 2.42 |
| | cancer patients | 16 | 2.1–296.8 | 44.74 |

[a] μg/mg creatinine, [b] come from (Muskiet *et al.*, 1984)

as expected. TFAA was obtained easily and the derivatization was complete. The same result was also obtained by milder conditions of room temperature overnight. Cation exchange resin was compared to the silica column for sample clean-up with the hydrochloride aqueous solution taken as eluate. It was difficult to evaporate to dryness the eluate from the ion exchange resin. The recovery of Put was on the low side. It may be the case that the Put derivative volatilized (since the Put molecule is smaller in comparison with Spd and Spm) when the derivative sample was evaporated by air.

The amount of urinary Crt excreted reflects human renal function, and the Crt production rate remains approximately the same from day to day unless the muscle mass changes. It is not altered significantly by illness, sepsis, trauma of fever , or by the state of hydration (Spencer, 1986). The ratio of polyamines and Crt was taken as an appraisal standard in most papers about the determination of polyamines in human body fluid. After interference by amino acids was eliminated by ion exchange chromatography, the urinary creatinine was measured by a picric acid method. In this paper, urinary creatinine levels were measured directly by GC, quickly and easily. The polyamine levels in the urine of normal human and cancer patients were compared. As shown in Table 6, the mean level of polyamines in the urine of cancer patients was significantly higher than those in normal humans. Up to now many papers have reported different results for urinary polyamines excreted from normal subjects, because of differences in instruments and in methods used. Although there is thus far no authoritative threshold of urinary polyamines excreted, determination of urinary polyamines is useful as a biochemical marker for the diagnosis of cancers, for prognosis and for follow-up. We are aware that the pretreatment method is rather laborious precluding prompt laboratory results, and we believe that the development of new assay procedures should predominantly focus on this important aspect.

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