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SIMULTANEOUS DETERMINATION OF PSEUDOURIDINE AND CREATININE IN UNTREATED URINE BY ION-PAIR LIQUID CHROMATOGRAPHY WITH DIODE-ARRAY ULTRAVIOLET DETECTION

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SUMMARY

A simple procedure for the simultaneous determination of pseudouridine and creatinine in urine using ion-pair high-performance liquid chromatography with ultraviolet detection is described. It consists of simply diluting the filtered urine with mobile phase (1·20) followed by direct chromatographic injection. A single analysis takes only 10 min. This method has been applied to the analysis of urine samples from normal donors and patients with different types of cancer. The mean values, $\bar{x}$, of the peak-area ratio of pseudouridine to creatinine were $61.79 \cdot 10^{-3}$ and $81.92 \cdot 10^{-3}$ for male and female normal donors, respectively. Out of twenty-five urine samples of patients with cancer examined, nineteen (all the forteen males included) had values higher than $\bar{x}+2\sigma$.

INTRODUCTION

Pseudouridine (Ψ), a modified nucleoside [1,2] derived mostly from the degradation of t-RNA, has been studied by many researchers because of the important relationship between RNA metabolism and cancer [3,4]. The lack of a mechanism for the reincorporation of modified nucleosides into t-RNA and for their catabolic breakdown [5] makes the concentration of Ψ in urine a good source of information on the extent of the conformational and structural modifications of t-RNA and on the t-RNA turnover rate. The increase in t-RNA turnover rate observed in tumour tissues has suggested the use of Ψ as a tumoral marker [6]. In fact, amounts of Ψ above normal have been found in urine of patients with various malignant diseases [7–10].

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Several methods for the quantitation of Ψ in urine have been developed. These include gas chromatography [11], thin-layer chromatography [12] and reversed-phase high-performance liquid chromatography (HPLC) with UV [13–15], fluorescence [16] and electrochemical [17] detection. Most of these methods involve numerous time-consuming steps to specifically isolate the nucleoside fraction (and further Ψ) by affinity chromatography.

Currently, the pseudouridine/creatinine (Ψ/CR) ratio, rather than the Ψ content, is considered a more useful parameter to follow the progression of the neoplastic disease and to evaluate the response to the therapy [18–20]. In fact, it has been proved that urine samples taken randomly have the same Ψ/CR ratio of those collected for a 24-h period. The constancy of the Ψ/CR ratio implies a near quantitative renal excretion of both substances, independent of diet. This makes CR an optimal 'internal standard' for Ψ and frees the determination of Ψ from a rigid procedure for urine collection. However, both the accuracy and precision of the Ψ/CR ratio are lowered if Ψ and CR are determined with two independent methods on two sample aliquots. In fact the variance contributions originated from the numerous steps in each of the two procedures add when the two results are taken as a ratio.

The method proposed here is simple and rapid, as it requires the dilution of a filtered native urine sample and its direct analysis by ion-pair liquid chromatography. It is free from analyte losses because of minimal sample manipulation. Moreover, the simultaneous measurement of the Ψ and CR allows a more precise and reliable evaluation of the Ψ/CR ratio. Such a ratio is unaffected by possible errors resulting from sampling and dilution steps. The Ψ/CR value, expressed as a peak-area ratio, has proved to be as significant as the concentration ratio, but with the additional advantage of avoiding the need for calibration curves, and of being independent of the instability of the analytical system.

EXPERIMENTAL

Chemicals

Reference standards for Ψ and CR were purchased from Sigma (St. Louis, MO, U.S.A.) and used without any further purification. Stock solutions (2 g/l for ψ and 50 g/l for CR) were obtained by dissolving the pure reagents in bidistilled water, and stored in the dark at 4°C. Disodium hydrogenphosphate (Carlo Erba, Milan, Italy) and 1-octanesulphonic acid (sodium salt, Sigma) were analytical-reagent grade.

All solvents used were HPLC grade. Water used for the preparation of buffer and aqueous solutions was first deionized and then distilled twice from alkaline permanganate.

The buffer used in the mobile phase was adjusted to the appropriate pH by addition of phosphoric acid (Carlo Erba). The mobile phase was filtered

through a 0.45-μm membrane (HATF04700, Millipore, France) and vacuum-degassed.

Apparatus

The chromatographic system consisted of a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 3B pump module equipped with a Rheodyne 7125 injection valve and a (250 mm × 4.6 mm I.D.) reversed-phase column (Spherisorb ODS-II, 5 μm packing, Bishoff, Leonberg, F.R.G.). A Bishoff (20 mm × 4.6 mm I.D.) guard column with Spherisorb ODS-II (5 μm) packing was used to protect the analytical column. An HP1040A photodiode array (PDA) detector (Hewlett-Packard, Palo Alto, CA, U.S.A.) interfaced with an HP85 computer, equipped with an HP9121 dual disk drive and an HP7470 plotter, was used for data acquisition and processing. Peak areas and retention times were calculated with a Perkin-Elmer LCI-100 laboratory computing integrator directly connected to one analog output of the PDA detector.

Urine sample collection and treatment

The urine samples from patients with different types of cancer were furnished by local hospitals. The urine samples from healthy donors were collected from some of the personnel and students of our Department, with an age span of 20–50 years. Both the cancer and normal donors were not taking any type of drug at the time of the urine collection. The collected urines were filtered using 0.45-μm Millex-HV-type filters (Millipore), then stored at −50°C. Prior to the chromatographic separation and analysis, each urine sample was thawed and diluted 1:20 with mobile phase; a 20-μl aliquot was injected.

Chromatographic conditions

The mobile phase was 0.05 *M* phosphate buffer (pH 6.0)–methanol (99:1, v/v). Octanesulphonic acid (5 m*M*) was added as ion-pairing agent. The flow-rate was 1.0 ml/min, and the temperature was ambient. Unless otherwise specified, spectra were acquired in the 210–400 nm range on the apex as well as on the ascending or descending part of each peak using a pilot signal at 250 nm, a reference signal at 550 nm (100 nm band-width), and a threshold value of 1 mAU. Up to seven chromatograms at different wavelengths could be obtained and processed after the chromatographic analysis. The purity of the Ψ and CR urinary peaks was checked by the technique of spectra overlaying after normalization.

RESULTS AND DISCUSSION

Our first attempt to detect Ψ and CR simultaneously in an untreated urine sample by reversed-phase liquid chromatography was only partially successful, since Ψ was eluted on the trailing edge of the CR peak, which was strongly

tailed. The multisignal capability of the PDA detector, and the different spectral characteristics of Ψ and CR, permitted a partial solution to the problem. In fact, Ψ could be selectively determined at 279 ± 4 nm (reference signal at 550 ± 50 nm), where CR does not absorb. The Ψ peak could be almost suppressed, and CR selectively determined, by measurement of the chromatogram at 232 ± 4 nm versus a reference signal at 279 ± 4 nm. However, we abandoned this procedure, although we are convinced that, as a general method, it deserves further attention.

An alternative approach was a complete time separation of Ψ and CR, which was obtained by adding 5 m*M* octanesulphonic acid to the mobile phase. The ion-pairing agent left the retention time of Ψ almost unchanged, but delayed the elution of CR by formation of an ion pair. The CR retention time, at a given concentration of the ion-pairing agent, was strongly dependent on the pH of the mobile phase, which then represented a further factor that could be used to fine-tune the retention behaviour. A study of the dependence of the capacity factor of Ψ and CR peaks on the pH of the mobile phase was made, using both standard solutions and urine samples. The results are reported in Table I. A pH value of 6.0 was chosen since, at this pH, CR was eluted at a time when the concentration profile of urine did not show significant interfering peaks. Fig. 1A shows a typical spectrochromatogram obtained from the urine of a male patient with a colon cancer. The chromatogram at 250 nm (a wavelength suitable for the simultaneous determination of Ψ and CR) is shown in Fig. 1B. A purity test carried out on Ψ and CR peaks, by the technique of spectra overlay after normalization, is shown in Fig. 2. Out of more than 70 urine samples examined, two samples gave chromatograms with Ψ peaks incompletely resolved from other substances, and three other samples presented impure CR peaks. However, for at least two of these five samples it was ascertained that the donors had ingested some drugs before urine collection. These

TABLE I

CAPACITY FACTORS FOR DIFFERENT pH VALUES OF THE MOBILE PHASE

Mobile phase, phosphate buffer–1% methanol–5 m*M* octanesulphonic acid; flow-rate, 1.0 ml/min; column, Spherisorb ODS-II (5 μm).

pH	Capacity factor	
	Pseudouridine	Creatinine
6.2	1.17	3.29
6.0	1.18	4.06
5.8	1.19	5.07
5.6	1.19	6.61
5.4	1.14	8.18
5.2	1.01	10.41

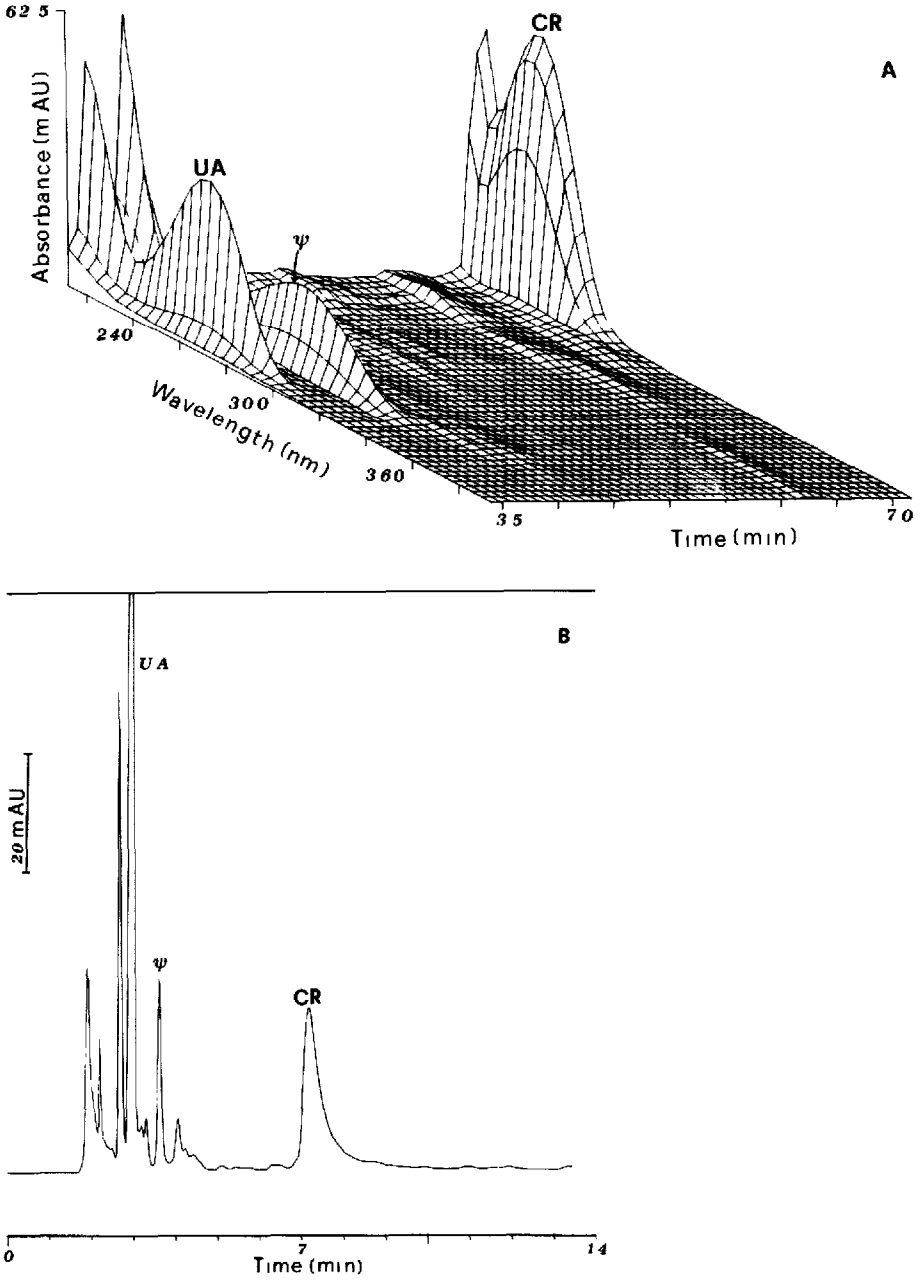

Fig. 1. (A) Typical spectrochromatogram obtained from the urine of a male patient with colon cancer. (B) Chromatogram at 250 nm (4 nm bandwidth) obtained from the same data set. Chromatographic conditions as specified in Experimental. Peaks: Ψ = pseudouridine; CR = creatinine; UA = uric acid.

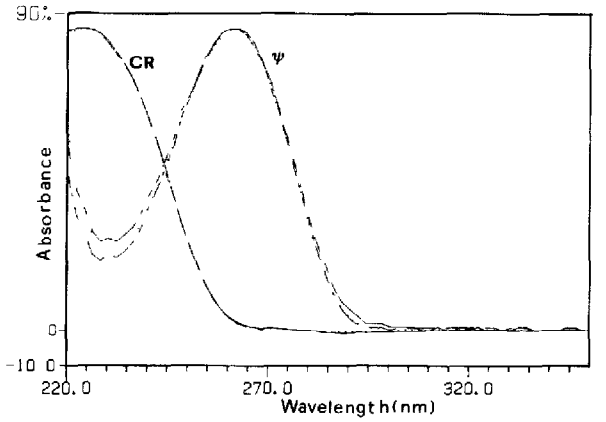

Fig. 2. Peak purity test for pseudouridine (Ψ) and creatinine (CR) obtained by overlaying the spectra after normalization. Data taken from the spectrochromatogram of Fig. 1A.

urine samples did not pass the purity test for the Ψ and CR peaks and were discarded in the following statistical analysis.

The linearity of the response (peak area) versus concentration was verified over the range 1–50 nmol for Ψ and 50–500 nmol for CR. The following regression lines were obtained respectively for Ψ and CR: $y = -8.2 \cdot 10^2 + 5.6 \cdot 10^6 x$ ($r^2 = 0.994$) and $y = -1.36 \cdot 10^3 + 8.92 \cdot 10^6 x$ ($r^2 = 0.999$), where y is expressed in integration units and x in nanomoles. The results obtained for the CR determination correlated well ($r^2 = 0.987$) with those of a colorimetric kinetic procedure [21] that is in routine use in clinical laboratories. This check was done by analysing eight urine samples by the two methods, each carried out in different laboratories. We did not check the correlation for Ψ because of the lack of a simple routine method for its determination.

The precision of our method for the evaluation of the Ψ/CR ratio was estimated both on a standard solution of Ψ and CR and on urine samples divided into four groups according to sex and pathological state of the donors. Ten replicate measurements on the Ψ and CR standard solution and three replicate measurements for each of the 69 urine samples were performed. The results of the one-way ANOVA calculations are summarized in Table II. In order to separate the different sources of variation on precision, the two-tailed F-test was performed on the within-sample relative standard deviation (R.S.D.) of the different groups. The F-test on the within-sample R.S.D. for the standard solutions and for the urine samples of normal male donors gave a value of $F = 2.9$ versus a critical value of $F_c = 3.3$ at the significance level of $P = 0.05$, indicating that the difference was not significant and that the precision of the method for these two groups was the same. Likewise, the above test applied to the within-sample R.S.D. of the normal female, female cancer and male cancer groups indicated homogeneity of precision of the results.

TABLE II

ANOVA ANALYSIS OF THE Ψ/CR PEAK-AREA RATIOS IN THE URINES OF NORMAL DONORS AND OF PATIENTS WITH DIFFERENT TYPES OF CANCER

Experiment	Ψ/CR ($\bar{x}\cdot10^3$)	Within-sample[a]		Between-sample[b]		n
		$\sigma\cdot10^3$	R.S.D. (%)	$\sigma\cdot10^3$	R.S.D. (%)	
Standard	52.30	0.43	0.82			1
Normal male urine	61.79	0.90	1.40	8.39	13.5	21
Normal female urine	81.92	1.99	2.43	16.34	19.9	24
Cancer urine (male)	163.21	4.89	3.01	71.09	43.5	14
Cancer urine (female)	122.40	4.23	3.46	64.14	52.4	10

[a]Calculated as $\sigma^2=\sum_i^n\sum_j^h(x_{ij}-\bar{x}_i)^2/n(h-1)$
[b]Calculated as $\sigma^2=h\cdot\sum_i^n(\bar{x}_i-\bar{x})^2/(n-1)$
where h, the number of replicate measurements of the ith sample, is 10 for the standard and 3 for all the urine samples.

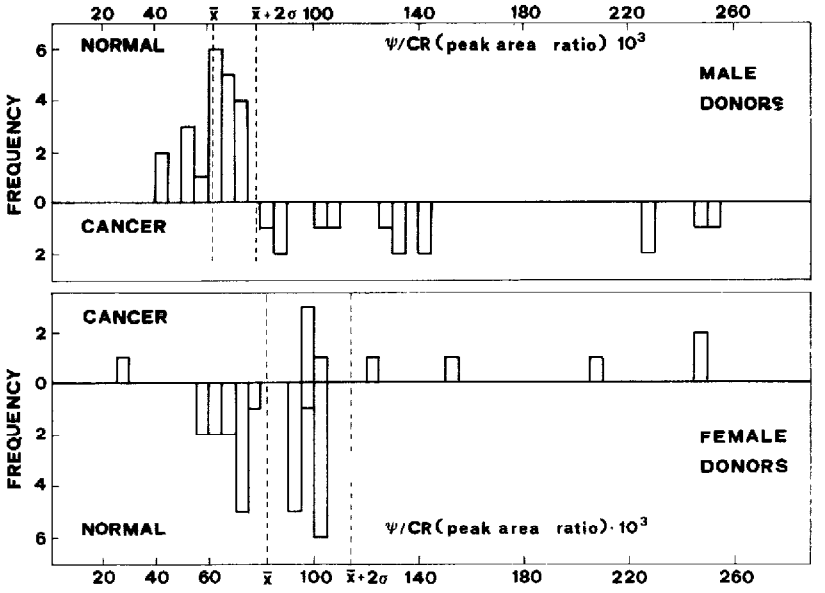

Fig. 3. Histograms of Ψ/CR peak-area ratios for the urine samples from normal donors and patients with different types of cancer.

The two-tailed F-test applied to the between-sample R.S.D. of the different urine groups indicated that, regardless of the sex, the Ψ/CR values for the normal donors and those for the patients with cancer derived from two different parent populations. These two parent populations were significantly different in terms of mean value and variance, as a consequence of the patholog-

ical state. In other words, the Ψ/CR value in urine is a significant parameter for the pathological state of cancer, at least at a 95% confidence level.

Fig. 3 shows histograms of the Ψ/CR urinary values for the four groups of Table II. A comparison between normal male and normal female urines shows a distribution closer to normal for the males, while for the females a tendency to a separation of the Ψ/CR values into two subgroups seems apparent. The peculiar behaviour observed for females (evidenced also by the different mean values of the Ψ/CR ratio and their higher R.S.D. values) might reflect, as already suggested [13], either a different excretion of Ψ and CR, or a control of Ψ excretion by hormonal activity. The Ψ/CR ratio for all the fourteen male patients with cancer were unambiguously higher than the $\bar{x}+2\sigma$ value of the normal male population. For the ten female patients with cancer, five samples were higher than $\bar{x}+2\sigma$, four samples fell in the interval from $\bar{x}$ to $\bar{x}+2\sigma$ and one sample was lower than $\bar{x}$ of the female normal population.

CONCLUSIONS

The described procedure, based on the measurement of the Ψ/CR peak-area ratio, allows one to obtain a parameter that is consistent and more precise than the Ψ/CR value obtained by ratioing the analyte concentrations measured with two independent methods on two different urine aliquots. The simple method consists of direct injection of the filtered and diluted urine sample and takes ca. 10 min per sample. The prognostic and diagnostic value of the Ψ/CR ratio seems to be particularly reliable for male patients.

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