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Journal of Pharmaceutical and Biomedical Analysis
21 (1999) 1045–1051

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

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Liquid chromatographic determination of urinary 5-methyl-2′-deoxycytidine and pseudouridine as potential biological markers for leukaemia

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Received 16 April 1999; received in revised form 30 July 1999; accepted 21 August 1999

Abstract

A simple reversed-phase liquid chromatographic (LC) method for the determination of urinary 5-methyl-2′-deoxycytidine (m^5dCyd), recently claimed (on the basis of an imuno-technique) to be a potential marker for leukaemia, has been developed. Sample pre-treatment is based on a microcolumn clean-up step with an average recovery of 79% and a RSD of 3%. Detection limit was 0.2 μg/ml which is about tenfold lower than levels previously measured by an ELISA method in urine of healthy individuals. The creatinine (Cre) excretion, necessary for normalising the m^5dCyd excretion, was evaluated by ion-pair liquid chromatography which permitted the simultaneous determination of pseudouridine (Ψ), a modified nucleoside also potentially useful as a marker for leukaemia. The described LC procedures were applied to the analysis of urine samples from healthy individuals and leukaemia patients. While the urinary Ψ/Cre ratio was found significantly increased for leukaemia patients, the urinary m^5dCyd levels in healthy individuals were below the detection limits and did not increase in presence of the malignant disease. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Liquid chromatography; Biological markers; Leukaemia

1. Introduction

A marker for a malignant disease is defined as any substance that is elevated in the serum or urine, reflecting the presence of the malignant process. Ideally this substance should be present early enough and in sufficient quantity to allow screening for the disease; the quantity produced should also reflect the bulk of malignancy and finally the level of marker should reflect the success of therapy [1].

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PII: S0731-7085(99)00221-6

5-Methyl-2′-deoxycytidine (m^5dCyd) has been recently claimed to be a potential marker for leukaemia [2] as assessed by an immuno-technique based on the use of a monoclonal antibody (AMC) raised against 5-methylcytydine (m^5Cyd) [3,4] but, unexpectedly, found to have the highest reactivity towards m^5dCyd. The inhibition ELISA method for urinary m^5dCyd determination was linear in the range 0.05–10 μg/ml and showed a recovery (spiked urine samples experiments) ranging between 113 and 96%. Unspiked urine samples of three healthy individuals were found to have m^5dCyd concentrations of 2.0, 2.6 and 2.8 μg/ml. Urinary excretion of m^5dCyd (nmol of m^5dCyd/μmol creatinine (Cre)) in healthy individuals and cancer patients was also evaluated; among various type of cancer tested, elevated excretion levels of m^5dCyd were detected for leukaemia patients.

Since this was the first report about urinary occurrence of m^5dCyd, the authors suggested that generation of m^5dCyd might be caused by excision repair in DNA of eukariotic cells and that the origin of urinary m^5dCyd must be through DNA, though the turnover of the DNA is not so active as that of RNA.

It is in fact well known that urine excretion of modified nucleosides originates predominantly from tRNA [5]; among these nucleosides pseudouridine (Ψ) was the most abundant and its role as tumour marker is widely recognised [6–11].

The simultaneous determination of Ψ and Cre [12,13] and of Ψ, neopterine and Cre [14] in urine by HPLC has been described. Both methods were fast, accurate and sensitive. The precision was also significantly improved since a reduced sample pre-treatment was required and Cre was determined simultaneously in the same run.

The development of a simple chromatographic method for the determination of a potential leukaemia marker such as m^5dCyd, appears worthy of consideration. Such a method is described in this paper. Since the m^5dCyd excretion needs to be normalised to Cre excretion, each of the analysed samples were submitted to a second chromatographic run which permitted the simultaneous evaluation of the Ψ and Cre contents.

Results relevant to leukaemia patients are illustrated and discussed.

2. Experimental

2.1. Chemicals

Stock solution were prepared in tridistilled water and stored at 4°C in the dark. Solutions were stable for at least 6 months. More dilute solutions were prepared just before use.

Methanol (Carlo Erba, Milan, Italy), was HPLC grade. The HPLC mobile phase was filtered through a 0.45 μM membrane (Whatman Limited, Maidstone, UK) before use. Standards of m^5dCyd, Ψ and Cre were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

2.2. Apparatus

The HPLC system consisted of a Waters (Milford, MA) 600-MS multisolvent delivery system equipped with a Reodyne injector with a 20 μl loop and a 5 μm Supelcosil LC-18-S column (250 × 4.6 mm i.d.) (Supelco, Bellefonte, PA). A 5 μm Supelguard LC-18-S precolumn (20 × 4.6 mm i.d.) (Supelco) was used to protect the analytical column. The detector was an HP 1040A photodiode-array spectrophotometer (Hewlett–Packard, Palo Alto, CA) interfaced to an HP 85 computer equipped with an HP dual disk drive and an HP 7470A plotter.

2.3. Chromatographic and detection conditions

The optimised gradient elution program used for m^5dCyd determination was as follows: 0.05 M, pH 7 phosphate buffer/MeOH (12% v/v) for 10 min then linear to 30% of MeOH in 2 min and isocratic for 13 min; finally back to 12% MeOH in 2 min; equilibration time: 10 min. Detection wavelength was 280 nm (4 nm bandwidth).

A 0.017 M phosphate buffer (pH 5.8) containing 1.7 mM octanesulphonic acid as ion-pairing agent was used [14] as mobile phase for Ψ and Cre determination. Detection wavelength was 250

nm (4 nm bandwidth). The chromatographic column was as above.

In both cases the flow rate was 1 ml/min and temperature was ambient. Spectra were acquired in the 210–400 nm range at the apex and on the ascending or descending part of each peak. Peak purity could be checked by the technique of spectra overlaying after normalisation.

2.4. Sample collection and pre-treatment

Urine samples, collected from healthy donors and non-treated leukaemia patients in the early morning, were filtered through a 0.45 μm Millex-HV type filter (Millipore) and frozen at −20°C until analysis.

The key steps of the sample pre-treatment procedure by solid phase extraction (SPE), e.g. preconditioning, loading, washing and final elution, were optimised. Sample pre-treatment for m^5dCyd determination was as follows: a 500 μl aliquot of the filtered urine was loaded onto a previously conditioned (elution of 1 ml of MeOH and 2 ml of H_2O) Supelclean solid phase extraction tube (Supelco) containing 100 mg of a 40 μm particle size C_{18} packing. The SPE tube was connected to a syringe filter containing a 3 mm diameter, 0.45 μm pore size nylon membrane (Supelco). The SPE tube was then washed with 1 ml of a phosphate buffer (pH 7, 0.05 M)–methanol (97/3, v/v) mixture and eluted with 500 μl of a phosphate buffer (pH 7, 0.05 M)–methanol (70/30, v/v) mixture. Finally 20 μl of the eluate were injected.

Sample pre-treatment for Ψ and Cre was as already described [14]. Briefly, the filtered urine samples were diluted 1:2 with a phosphate buffer solution (pH 5.8, 0.017 M). Then, a 100 μl aliquot was loaded onto a SPE tube (see above). The SPE tube was then eluted with 900 μl of a phosphate buffer solution (pH 5.8, 0.017 M) and 20 μl were injected.

Table 1
Percent recovery of m^5dCyd from spiked urine samples

	Spiked (μg/ml)	% Recovery	RDS% ($n=3$)
m^5dCyd	0	n.d.[a]	–
	0.2	74	3
	2	73	3
	5	75	3
	10	85	3
	15	81	3

[a] n.d., not detected.

3. Results and discussion

The extraction efficiency of the pre-treatment procedure was evaluated on a pooled urine sample from healthy individuals spiked with known amount of m^5dCyd. Percentage recoveries and relevant coefficient of variation are summarised in Table 1. Note that m^5dCyd could not be recovered, nor detected, in the unspiked urine sample; this finding was further verified analysing 14 urine samples from healthy donors differing in age and sex. A calibration curve in urine could then be obtained using standards prepared by spiking different aliquots of a pooled urine sample from healthy donors with variable amounts of m^5dCyd in order to cover the concentration range from 0.2 to 200 μg/ml; spiked urine aliquots were then extracted and analysed as previously described. The calibration curve was linear over the investigated range with a correlation coefficient >0.999 and the intercept not significantly different from zero at the 95% confidence level. A typical calibration curve had the following equation:

$$y = (-6.4 \pm 10.2) + (120.9 \pm 2.0)x$$

where y represents the peak area (a.u.) and x the m^5dCyd concentration (μg/ml). The within day precision on a urine sample spiked at a concentration level of 2 μg/ml was 3% ($n=3$) and the day to day precision ($n=9$ over 3 days) was 4.7%.

Fig. 1 shows chromatograms relevant to (A) a healthy donor urine sample and (B) the same sample spiked with a known amount of m^5dCyd. 'Chemical noise', still present at the retention time of m^5dCyd ultimately limits the detection capability of the method; however, urine samples spiked at 0.2 μg/ml level gave (see later), a peak clearly distinguishable from the background. This value set a 'practical' detection limit of the technique which, as will be demonstrated in the following, was more than adequate for the purpose of this

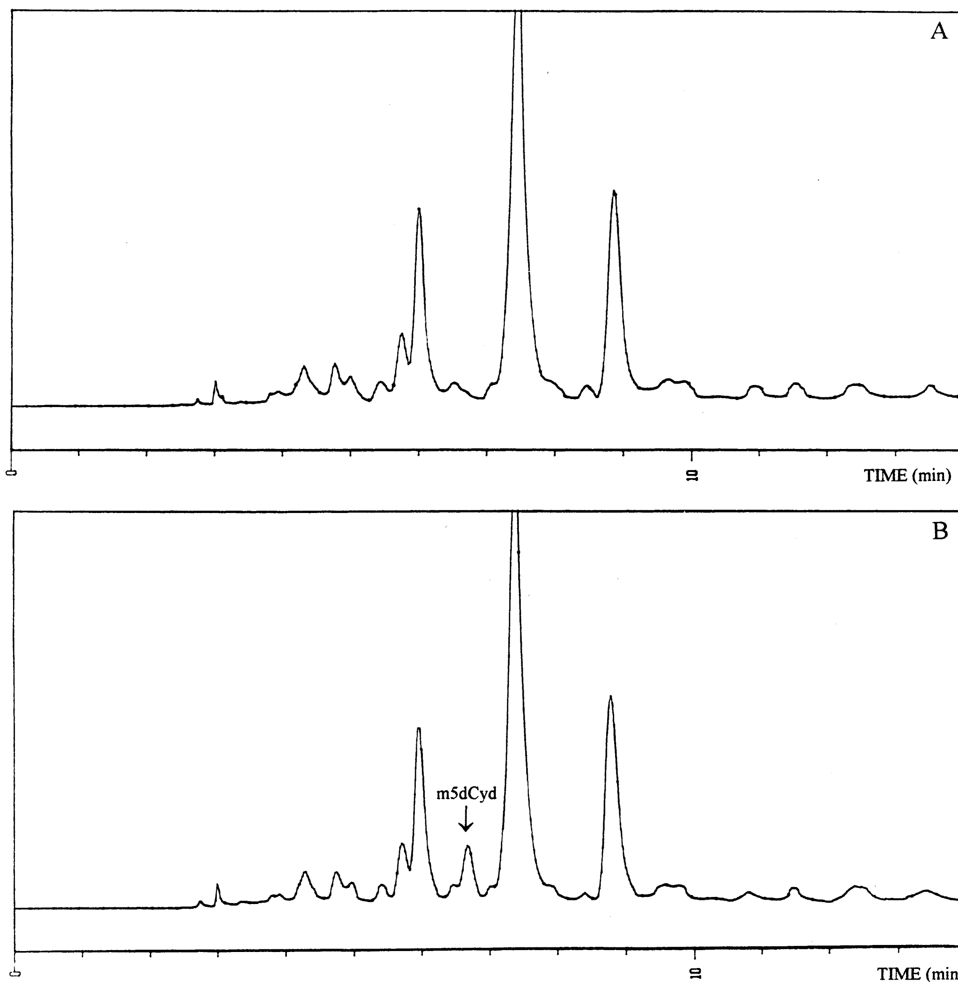

Fig. 1. Chromatograms relevant to (A) a healthy donor urine sample and (B) the same sample spiked with m^{5}dCyd (2 μg/ml). Detection wavelength: 280 nm. Absorbance axis: 40 mAU full scale.

work (note that injecting m^{5}dCyd standards a detection limit of 0.01 μg/ml could be estimated at a signal-to-noise ratio of three).

Having ascertained that urinary m^{5}dCyd could not be detected in healthy people, urine samples from 23 leukaemia patients were then analysed to see whether the malignant disease causes, as already claimed [2], increased m^{5}dCyd excretion.

Fig. 2 shows typical chromatograms relevant to extracts of an urine sample from a leukaemia patient (trace A) and the same sample spiked with m^{5}dCyd at 0.2 μg/ml level (trace B). Again it can be seen that m^{5}dCyd could not be detected even in the presence of leukaemia disease (this was verified for all the 23 analysed samples).

All these findings indicate that, urinary m^{5}dCyd, if present, is well below the level of 0.2 μg/ml in both healthy donors and leukaemia patients. Consequently urinary m^{5}dCyd does not appear to be a potentially useful marker for leukaemia in contrast with the claim of Itoh et al. [2]. A possible reason for this discrepancy is likely to be ascribed to a poor specificity of the monoclonal antibody, raised against m^{5}Cyd but, unexpectedly, found to have the highest reactivity towards the corresponding 2′-deoxyribonucleoside, i.e. m^{5}dCyd.

On the other hand, to justify their findings Itoh et al. [2] need to invoke turnover of the DNA which, however, is not so active as that of RNA. It is well known that turnover of transfer-RNA increases significantly in the presence of cancer, Ψ being the most abundantly excreted modified nucleoside. This should be the case also for leukaemia as it is demonstrated in the following.

Fig. 3 shows a typical chromatogram obtained for Ψ and Cre determination in a urine sample from a leukaemia patient. The Ψ/Cre (μmol/mmol) concentration ratios obtained for all the analysed samples are summarised in Fig. 4 and-grouped accordingly to sex and type of leukaemia: acute lymphoblastic leukaemia (ALL)and acute myeloid leukaemia (AML). The cut-off levels for the Ψ/Cre concentration ratio were taken as the average values for a normal population plus twice the standard deviation (16.76 ± 7.95 and 18.49 ± 8.50 μmol/mmol for male and female, respectively [14]). As it can beseen, urinary Ψ seems to possess, as a leukaemia marker, an excellent diagnostic sensitivity.

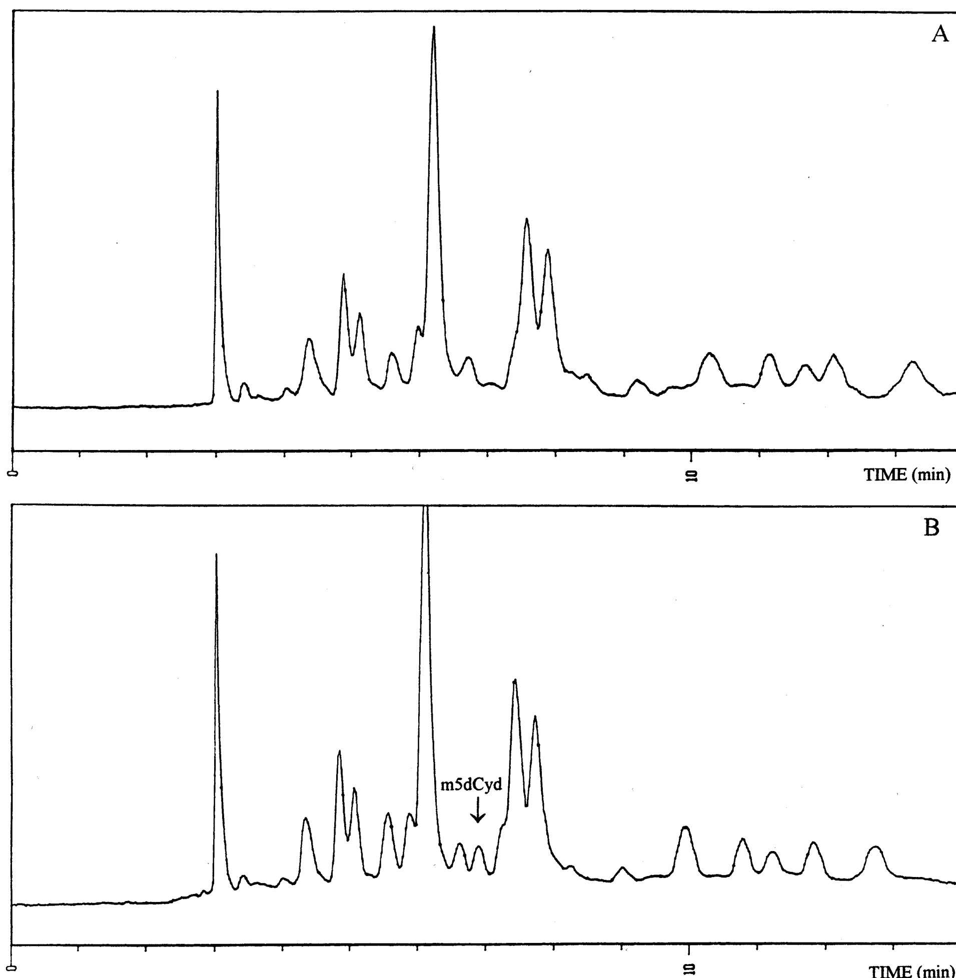

Fig. 2. Chromatograms relevant to (A) a leukaemia patient urine sample and (B) the same sample spiked with m^5dCyd at a concentration level (0.2 μg/ml) close to its detection limit. Detection wavelength: 280 nm. Absorbance axis: 10 mAU full scale.

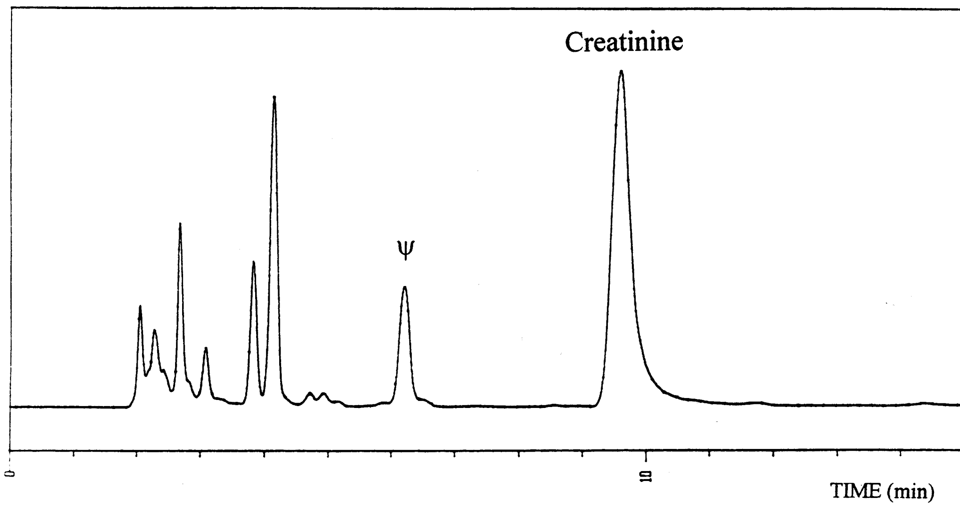

Fig. 3. Typical chromatogram relevant to Ψ and Cre determination in a urine specimen from a leukaemia patient. Detection wavelength: 250 nm. Absorbance axis: 70 mAU full scale.

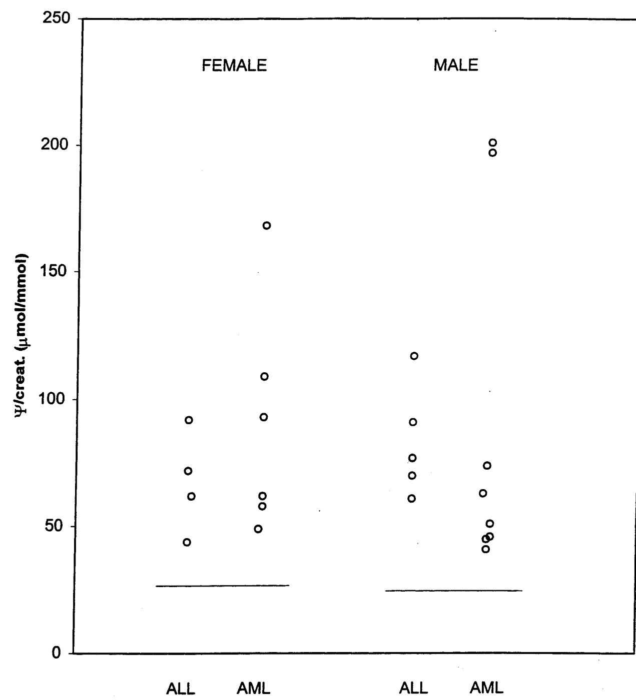

Fig. 4. Psu/Cre concentration ratios (μmol/mmol) in the urine of leukaemia patients. The horizontal lines represent the cut-off levels. Patients are grouped according to leukaemia type: acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML).

4. Conclusions

It has been shown for the first time that m^5dCyd can be determined in urine by reversed phase HPLC–UV detection after a microcolumn sample clean-up step. Cre content (usually required for normalising the urinary excretion of a tumour marker) can be assessed by a different chromatographic run which permitted the simultaneous determination of Ψ.

While Ψ demonstrates a clear potential as a marker for leukaemia, urinary m^5dCyd was not detected neither in healthy donors nor in leukaemia patients. This implies that m^5dCyd, if really occurring in urine as a consequence of DNA turnover, is present at concentration levels below 0.2 μg/ml and that, at least by the described HPLC method, it is not possible to ascertain whether leukaemia causes an increased excretion of this modified deoxynucleoside. It must be concluded that, at present, the potential of m^5dCyd as leukaemia marker appears doubtful.

In order to draw definite conclusions, the development of a more sensitive and specific technique for urinary m^5dCyd determination, e.g. HPLC–electrospray ionisation mass spectrometry, is required. Work in this direction is currently in progress in our laboratory.

Acknowledgements

Financial support from the MURST and the Associazione Italiana Ricerca sul Cancro (AIRC, Milan) are gratefully acknowledged. The authors would like to thank Giuseppe Laera for his experimental help.

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