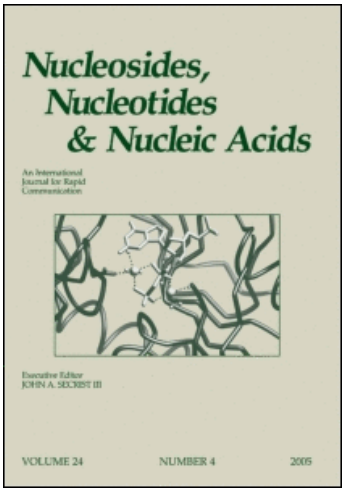

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E. A. Carrey[a]; R. T. Smolenski[b]; S. M. Edbury[a]; A. Laurence[c]; A. M. Marinaki[a]; J. A. Duley[a]; L. M. Zhu[d]; D. J. A. Goldsmith[e]; H. A. Simmonds[a]
[a] Purine Research Unit, Guy's Hospital, London, UK [b] Department of Biochemistry, Medical University of Gdansk, Gdansk, Poland [c] Department of Haematology, University College London, London, UK [d] Department of Health and Human Sciences, London Metropolitan University, London, UK [e] Department of Renal Medicine, Guy's Hospital, London, UK

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An Unusual Pyridine Nucleotide Accumulating in Erythrocytes: Its Identity and Positive Correlation with Degree of Renal Failure

E. A. Carrey,[1,*] R. T. Smolenski,[3] S. M. Edbury,[2] A. Laurence,[4] A. M. Marinaki,[2] J. A. Duley,[2] L. M. Zhu,[5] D. J. A. Goldsmith,[6] and H. A. Simmonds[2]

[1]University College London, Institute of Child Health, London, UK
[2]Purine Research Unit, Guy's Hospital, London, UK
[3]Department of Biochemistry, Medical University of Gdansk, Gdansk, Poland
[4]Department of Haematology, University College London, London, UK
[5]Department of Health and Human Sciences, London Metropolitan University, London, UK
[6]Department of Renal Medicine, Guy's Hospital, London, UK

ABSTRACT

We have investigated an unusual nucleotide that accumulates, with precursors, in the erythrocytes of patients in uraemia. This nucleotide is related chemically to the NAD breakdown product, N1-methyl-2-pyridone-5-carboxamide (Me2Py), found in high concentrations in the plasma of uraemic patients. Both Me2Py and the nucleotide accumulate to high concentrations in the blood during uraemia: our investigations of samples from renal out-patients have provided information on a plausible link between the two compounds.

Key Words: N1-methyl-2-pyridone-5-carboxamide (Me2Py); Uraemia; Pyridine Nucleotide; HPLC; LC/MS.

*Correspondence: E. A. Carrey, University College London, Institute of Child Health, 30 Guildford St., London WC1N 1EH, UK; E-mail: e.carrey@ich.ucl.ac.uk.

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INTRODUCTION

During our studies of nucleotides in metabolic disorders we observed that a novel nucleotide accumulates in the erythrocytes of patients with renal failure, approximately in parallel with the plasma concentrations of the pyridone base, Me2Py, that has been implicated in toxic events associated with uraemia.[1,2] We have investigated the chemical structure of the nucleotide and propose a possible identity related to Me2Py.

METHODS

Four categories of patients were invited to contribute to the study: mild renal failure (CRF), end-stage renal failure (ESRF), haemodialysis (HD), and continuous ambulatory peritoneal dialysis (CAPD). Blood samples were compared with healthy controls, and patients after successful kidney transplantation (Post-Tx). The serythrocytes were separated and washed as described,[1] and plasma was prepared from the same blood samples. TCA-soluble extracts of each were back-extracted with water-saturated diethyl ether, and frozen at −20°C if not analysed immediately by anion-exchange HPLC or RPLC as described.[1]

Chemical synthesis of Me2Py followed published methods;[3,4] its purity was confirmed by melting point, mass spectrometry, NMR and HPLC, and by its UV

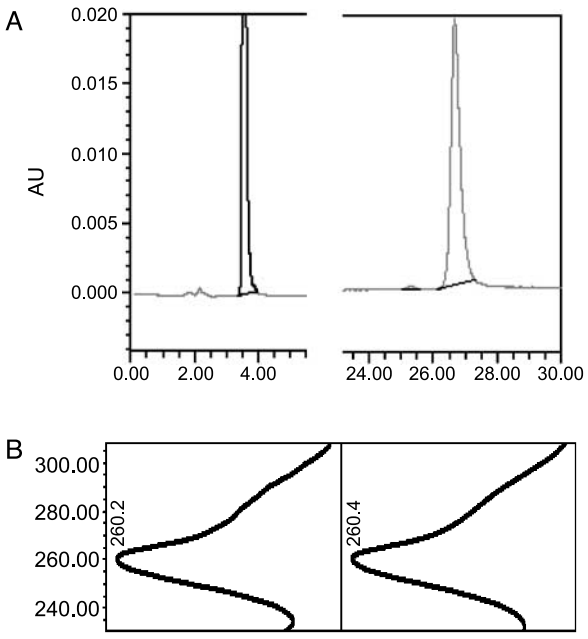

Figure 1. A: the novel nucleotide elutes at 26–27 min (right); the compound generated by digestion of pooled fractions elutes at 3.5 min (left). B: UV absorbance spectra between 230–310 nm for the digestion product (left) and the nucleotide (right).

absorbance spectrum.[4] The novel nucleotide was purified from erythrocyte extracts by repeated applications to the HPLC system, collecting the characteristic peak during each run. Pooled samples were incubated with snake venom nucleotidase and purine nucleoside phosphorylase[5] for 30 minutes at 37°C, and the reaction was stopped by adding TCA.

Purity was confirmed by HPLC, and the nucleotide and its base were also analysed by tandem LC-MS.

RESULTS

The nucleotide eluted later than ATP and GTP in anion exchange HPLC, and its UV absorption spectrum was unlike that of known purine or pyrimidine nucleotides. The purified nucleotide had the same retention time in HPLC and the same spectrum as the nucleotide in extracts, while the digestion product had the same spectrum but eluted after approximately 3.2 minutes, depending on buffer conditions (Fig. 1).

The mass spectrometric data from the digestion products generated values of 509 for the mass of the nucleotide and 137 for the base. The mass of Me2Py was 152, and its UV absorbance spectrum[4] differed from that of our isolated nucleotide or its base.

Anion-exchange HPLC profiles of erythrocyte extracts from healthy subjects and renal failure patients demonstrated that the novel nucleotide was normally present at low concentrations in healthy subjects, higher in those with mild renal disease, and highest in those who had progressed to end-stage disease and dialysis. When the nucleotide was found at high concentrations, a series of peaks with the same UV spectra were seen, eluting at positions appropriate for the nucleoside diphosphate and monophosphate. Nucleotide concentrations increased in parallel with the concentrations of Me2Py and creatinine in the plasma from the same patients. The erythrocyte extract from a subject lacking molybdenum cofactor (and hence deficient in aldehyde oxidase and xanthine oxidase activity) completely lacked the nucleotide and its related compounds, and also lacked Me2Py.

The data in Table 1 show the elevation of Me2Py and the novel nucleotide in parallel with increasing degree of renal failure. The plasma creatinine and Me2Py

Table 1. Nucleotides in the erythrocytes, Me2Py in plasma of renal failure patients: mean concentrations (μmol/l) ± standard deviation.

Patient group	N	ATP	2PyTP	M2Py
Control	28	1229 ± 184	8.1 ± 3.4	9.0 ± 4.5
CRF	30	1376 ± 299 *ns*	21.8 ± 17.2 *ns*	8.5 ± 4.8 *ns*
ESRF	34	1614 ± 409***	55.1 ± 30.6*	32.9 ± 13.8***
CAPD	28	1481 ± 355 *ns*	217 ± 145.8***	48.6 ± 21.2***
Pre-HD	27	1570 ± 325**	70.9 ± 30.6**	51.3 ± 23.6***
Post-HD	19	1508 ± 223 *ns*	62.1 ± 25.0*	22.2 ± 5.9*
Post-TX	30	1050 ± 315 *ns*	16.8 ± 13.1 *ns*	9.0 ± 5.3 *ns*

Significance compared with controls:
ns, no significant difference; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

concentrations were reduced to almost normal values by haemodialysis (pre-HD compared with post-HD) but the concentration of the nucleotide was unaffected by dialysis. The concentrations of all were close to normal in the post-transplant group. Study of a small number of patients receiving donor-related kidneys showed that Me2Py and creatinine concentrations returned to normal within the first few days after transplant (data not shown), while the novel nucleotide normalised with a half-life approx. 40 days, corresponding to the turnover time of erythrocytes.[6]

DISCUSSION

The molecular weight obtained by mass spectrometry is consistent with the structure of 2-pyridone-5-carboxamide ribonucleoside triphosphate (2PyTP). The UV absorbance spectra obtained by HPLC analysis of the nucleotide and of the digestion product (Fig. 1) are not the same as the published spectra of Me2Py or of 2-pyridone-5-carboxamide,[4] suggesting that digestion was incomplete and did not generate the base. Currently, we are synthesising authentic 2-pyridone-5-carboxamide to compare with our biological samples.

We have shown that both Me2Py and the nucleotide increase in concentration in parallel with the degree of renal failure, and both require aldehyde oxidase for their synthesis, thus they are both pyridones. We have found that Me2Py enters erythrocytes only slowly, and it is not converted to the nucleotide [Carrey et al, paper in preparation]. This is not surprising, since the N1-methyl group may prevent uptake through the nicotinamide transporter, and will also prevent attachment of ribose phosphate by a transferase enzyme within the cell.

Consistent with these findings, we suggest that nicotinamide is removed from circulation by more than one route. In the major route, nicotinamide is methylated at the N1 position; subsequent action by aldehyde oxidase produces Me2Py and, in humans, a smaller amount of Me4Py. These compounds are removed in the urine unless renal function is poor, when they accumulate in the plasma. We are currently investigating possible alternative routes through which the novel nucleotide is formed in the erythrocytes, where it accumulates in patients with renal failure.

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