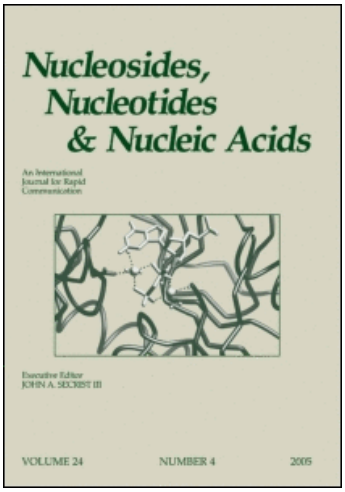

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LIQUID CHROMATOGRAPHIC/MASS SPECTROMETRIC PROCEDURE FOR MEASUREMENT OF NAD CATABOLITES IN HUMAN AND RAT PLASMA AND URINE

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□ *Monitoring level of the metabolites of the coenzyme NAD such as nicotinamide and its oxidized and methylated derivatives is important due to therapeutic applications of these compounds and monitoring of oxidative stress. We evaluated feasibility of using HPLC with electrospray ion-trap mass detection for single run separation and quantitation of all the NAD metabolites. We achieved good separation and retention of all the metabolites of interest using reversed-phase with ion-pairing. Single ion monitoring or tandem MS were used for detection and quantitation of the specific compounds with good linearity. The method was able to detect all the physiological metabolites in plasma samples of rats and humans or in urine. However, full validation is necessary before this method could be routinely applied.*

Keywords Nicotinamide; N-Methylnicotinamide; Poly(ADP-ribose) polymerase (PARP); Liquid chromatography/mass spectrometry

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) is an essential coenzyme for cellular red-ox reactions and also a substrate for poly(ADP-ribose) polymerase (PARP).[1] While red-ox reactions preserve NAD pool, poly(ADP-ribosyl)ation results in net loss of NAD. This later process plays a major role in cellular response to DNA damage and is a predominant mechanism of generation of NAD catabolites in the human body.[1] N-methyl-2-pyridone-5-carboxamide (M2PY) and N-methyl-4-pyridone-3-carboxamide (M4PY) are well established catabolites of NAD but number of other compounds such as N-methylnicotinamide (NMA) or nicotinamide

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N-oxide (NOX) also has been reported.[2] Nicotinamide (NA) or N-methylnicotinamide (NMA)—one of minor NAD catabolites has significant therapeutic potential.[3,4] However, no analytical method currently is available that allows comprehensive analysis of full spectrum of NAD catabolites. Our aim, therefore, was to develop such a procedure based on application of liquid chromatography mass spectrometry.

MATERIALS AND METHODS

Chemicals used for this study were obtained from Sigma with exception of 2PY, 4PY, M2PY, M4PY, and 4PYR that were chemically synthesized. Chromatographic separation was performed using 3 μm Hypersil C18-BDS 150 × 2.0 mm column. Buffer A was 10 mM nonafluoropentanoic acid (NFPA) in water and buffer B was 100% acetonitrile. Mobile phase was run at 0.2 ml/min in a gradient from 0% to 60% B in 12 minutes. The mass detector (Thermo-Finnigan LCQ Advantage, Waltham, MA, USA) with electrospray (ESI) ion source was operating in a positive single ion monitoring (SIM) mode for detection of $[M+H]^+$ species of NA, MNA, M2PY, M4PY, NOX, 2-pyridone-5-carboxamide (2PY), and 4-pyridone-3-carboxamide (4PY) and in positive MS^2 mode for detection of 4-pyridone-3-carboxamide riboside (4PYR) with the collision energy setting at 25%. Internal standard (2-chloroadenosine) signal was extracted from full MS mode. Electrospray cone voltage was set at 4.5 kV and heated capillary temperature was 275°C. Sheath gas flow was set at 35 arbitrary units. Ion optics was optimized using standard instrument procedures during infusion of nicotinamide. For assessment of the procedure in biological samples human or rat plasma or urine were deproteinized using 10% trichloracetic acid followed by ether extraction. Recovery of M2PY, M4PY and NA added to the samples with known concentration was 75–95%. Coefficient of variation was below 10% for repeated injections on the same day. However, much larger, >20%, variation was observed for between day analysis.

RESULTS AND DISCUSSION

We confirmed feasibility of single run comprehensive analysis of NAD metabolites using HPLC with electrospray ion-trap mass detection. All the metabolites were retained on the column and were detected under conditions established (Figure 1). The most important problem for bases separated here was lack of fragments that would allow detection in tandem MS mode. This would greatly increase sensitivity and improve specificity. It was only possible with nucleoside (4PYR, $m/z = 271$) that fragmented into ion at $m/z = 139$ corresponding to neutral loss of ribose. Another problem related to the reproducibility of the assays. Although the reproducibility of

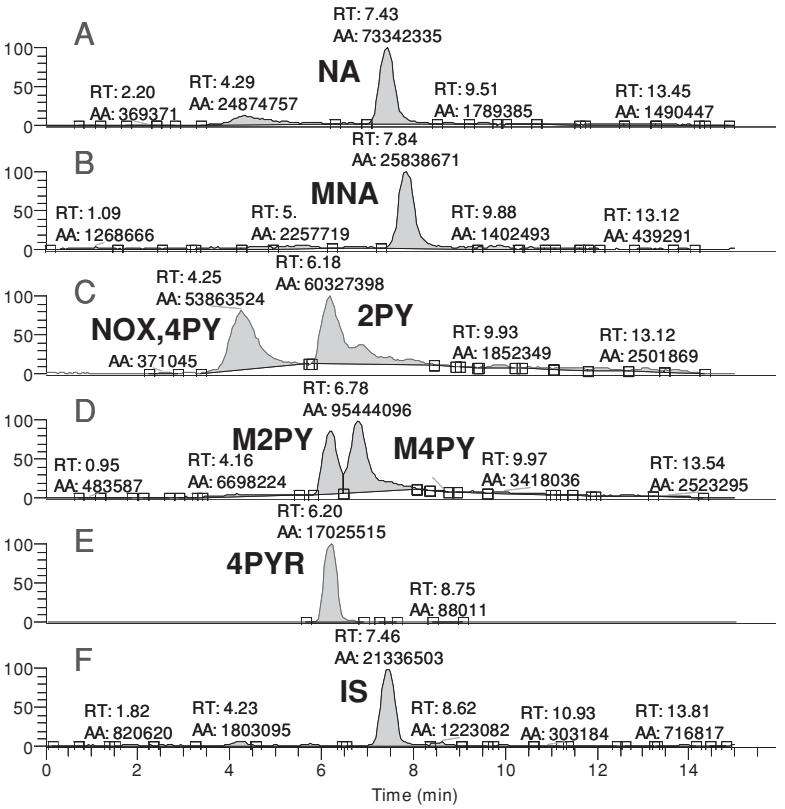

FIGURE 1 Ion chromatogram of the standards of nicotinamide metabolites: A) Nicotinamide (NA, SIM at m/z 123); B) N-methylnicotinamide (MNA, SIM at m/z 137); C) Nicotinamide N-oxide (NOX), 4PY (coeluting) and 2PY (SIM at m/z 139); D) M2PY and M4PY (SIM at m/z 153); E) 4PYR (MS2 m/z 139 from 271@25%); F) 2-Chloroadenosine (internal standard, m/z 302 from full MS).

the analyses in a single run was acceptable we noted much larger variation for assays carried out on different days even with application of internal standard. Perhaps application of stable isotope standards would resolve this problem, but this was not possible since stable isotopes are not commercially available for the compounds measured here. Any observations highlighted below were made by comparison of analyses conducted in a single run.

The results of preliminary application of this procedure indicated several interesting observations. We confirmed that M2PY is a major catabolite of NAD in human plasma but M4PY is predominant NAD catabolite in rats (Figure 2). Very interesting was demonstration of significant concentration of 4PYR in human plasma and much lower, undetectable level in rat plasma. The method worked particularly well with non-fractionated urine samples allowing detection of full spectrum of NAD catabolites. We also noted that both clinical and experimental renal failure not only leads to elevation of

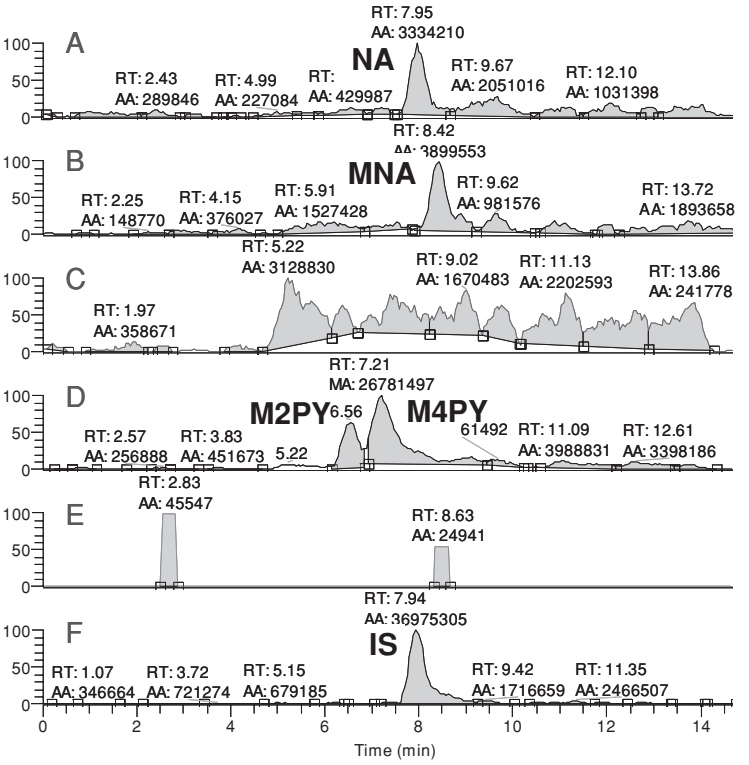

FIGURE 2 Ion chromatogram of rat plasma extract following treatment with N-methylnicotinamide. Traces A-F are as described in Figure 1. This chromatogram shows clear peaks of N-methylnicotinamide, nicotinamide, M4PY, and M2PY while nicotinamide N-oxide, 2PY, 4PY, or 4PYR were not detected. Traces C and E presents auto expanded chromatograms without clear peaks.

M2PY and M4PY plasma concentration as we have shown before[5] but also to elevation of NA, NMA, and 4PYR.

Our preliminary assessment of suitability of ion-trap LC/MS indicates that it could be a basis for development of analytical procedure for comprehensive monitoring of NAD catabolites in plasma and urine. This information could be useful for detection of DNA damage, recording changes in NAD metabolism in pathology or for monitoring of NAD metabolites during pharmacological treatment. However, some remaining issues such as poor reproducibility have to be resolved and full validation has to be conducted.

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