Journal of Chromatography, 582 (1992) 145–151
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6533

High-performance liquid chromatographic determination of nicotinamide and its metabolites in human and murine plasma and urine

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(First received June 10th, 1992; revised manuscript received August 5th, 1992)

ABSTRACT

A high-performance liquid chromatographic method is described which enables the determination of nicotinamide and eight of its possible metabolites in human and murine plasma and urine, using ion-pairing on a base-deactivated reversed-phase column. Calibration curves were linear up to 2 μmol/ml for nicotinamide and 200 nmol/ml for the metabolites; both the intra- and inter-assay relative standard deviations ranged between 1 and 8%. In murine plasma, the N-oxide was the major nicotinamide metabolite, but in man, formation of 1-methylnicotinamide and the 2- and 4-pyridones was also significant. In urine, nicotinuric acid was seen in the mouse, but no nicotinic acid metabolites were seen in man.

INTRODUCTION

It has recently been shown that nicotinamide sensitizes murine tumours to radiation, either alone [1] or in combination with carbogen breathing (95% oxygen, 5% carbon dioxide) [2–4]. Details of a pharmacokinetic study in normal volunteers have recently been published [5], but this only contained data on the parent nicotinamide and the N-methylnicotinamide metabolite; a phase 1 clinical trial is to begin shortly. However, little is known of the mechanism of action of nicotinamide, which is subject to metabolism by several pathways. It is possible that the active agent is not the parent compound itself, but a metabolite. Although there are a number of papers which describe methods for the analysis of nicotinamide and some metabolites [6–13], no one technique has been described which enables the measurement of all its metabolites simultaneously in both plasma and urine. We have therefore developed a novel high-performance liquid chromatographic (HPLC) technique using ion pairing which allows for the determination of nicotinamide, nicotinamide-N-oxide, 6-hydroxynicotinamide, nicotinic and nicotinuric acid, N-methylnicotinamide, and the 2- and 4-pyridones derived from N-methylnicotinamide. We have applied this to the analysis of murine and human plasma and urine.

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EXPERIMENTAL

Chemicals

Acetonitrile and methanol were from Rathburn (Walkerburn, UK), heptanesulphonic acid

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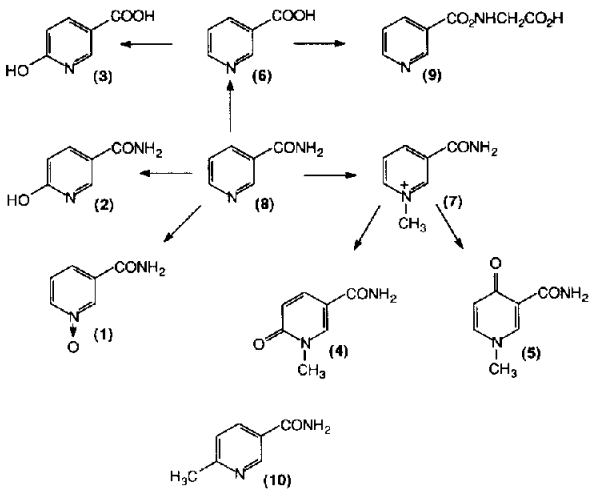

Fig. 1. Structures of the compounds investigated. 1 = Nicotinamide-N-oxide; 2 = 6-hydroxynicotinamide; 3 = 6-hydroxynicotinic acid; 4 = 1-methyl-2-pyridone-5-carboxamide; 5 = 1-methyl-4-pyridone-5-carboxamide; 6 = nicotinic acid; 7 = 1-methylnicotinamide; 8 = nicotinamide; 9 = nicotinuric acid; 10 = 6-methylnicotinamide (internal standard).

(sodium salt) and trimethylamine hydrochloride were from Fisons (Loughborough, UK), nicotinamide, nicotinamide-N-oxide, nicotinic and nicotinuric acids, 1-, 6- and N′-methylnicotinamide and 6-hydroxynicotinic acid were from Sigma (Poole, UK) and all other chemicals were from Merck (Poole, UK). 6-Hydroxynicotinamide was synthesised from 6-hydroxynicotinic acid using the method of Lee *et al.* [14]. The 2-pyridone of N-methylnicotinamide was prepared by ferricyanide oxidation of 1-methylnicotinamide [15] and the 4-pyridone enzymically [16] The structures of all the compounds studied are shown in Fig. 1.

Chromatography

Chromatography was carried out using a Waters 820 system (Millipore, Watford, UK) with a Waters 481 detector at 260 nm. Separation was achieved using gradient elution on a Hypersil 5-μm BDS column (Shandon Scientific, Runcorn, UK), 250 mm × 4.6 mm I.D., maintained at 30°C, with a Hypersil guard column (Hichrom), 10 mm × 2 mm I.D. The solvents used were: (A) 7 m*M* heptanesulphonic acid, 2 m*M* trimethylamine, 5 m*M* potassium dihydrogen orthophosphate, 5 m*M* orthophosphoric acid; (B) 55% acetonitrile, 25% methanol, 20% water for plasma or 75% acetonitrile and 25% water for urine. For plasma, the initial condition was 100% A held for 4 min, followed by a linear gradient from 0 to 12% B in 9 min. This was held for 1 min, then returned to the initial conditions. For urine, the initial conditions were the same, but the gradient was run over 7 min, with a 2-min hold. The flow-rate was 2 ml/min and the run time was 22 min.

Sample preparation

For analysis of plasma concentrations, 20 nmol of 6-methylnicotinamide (internal standard) were added to an aliquot, typically 100 μl; 1 ml of methanol was then added, with mixing after each addition, and the sample was centrifuged (1500 *g*, 10 min) and the supernatant taken to dryness in a Heto VR1 centrifugal evaporator (Heto Lab Equipment, Camberley, UK). The sample was then reconstituted in 250 μl of water, and 25 μl were injected onto the HPLC column. Urine samples were simply diluted 1:20 with the aqueous eluent prior to analysis.

RESULTS

A chromatogram obtained under the conditions used for plasma analysis, showing the separation of an aqueous standard of nicotinamide and its metabolites together with the 6-methylnicotinamide used as internal standard is shown in Fig. 2. Also shown is a methanol extract of a control plasma sample from a normal human volunteer. This illustrates both the good separation of the major metabolites of nicotinamide, and also the lack of interference from endogenous peaks in control plasma. The large unlabelled peak in the control plasma is uric acid; no other major interferences were observed. Fig. 3 shows a plasma extract from a normal volunteer, 8 h after taking 6 g of nicotinamide orally. The metabolites which were found have been identified according to Fig. 1; 6-hydroxynicotinamide has not previously been observed in man although Lee *et al.* [14] reported it in the urine of

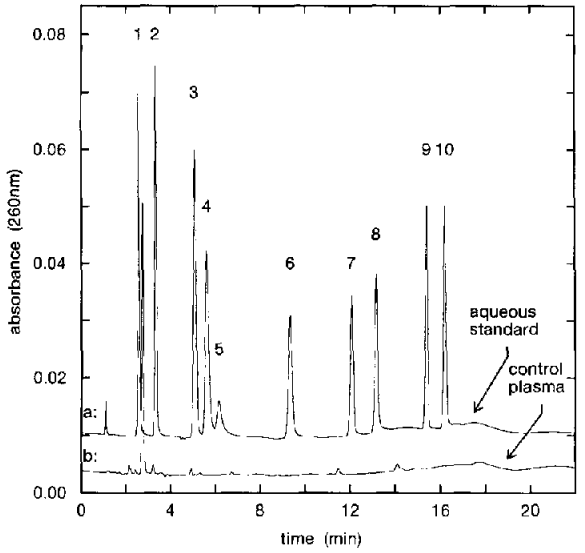

Fig. 2. Chromatograms of (a) aqueous standards of nicotinamide-related compounds and (b) control human plasma extract. For peak identification see Fig. 1. Amounts: 1 = 1 nmol; 2 = 1 nmol; 3 = 1 nmol; 4 = 1 nmol; 5 = 0.4 nmol; 6 = 2 nmol; 7 = 2 nmol; 8 = 2 nmol; 9 = 2 nmol; 10 = 2 nmol.

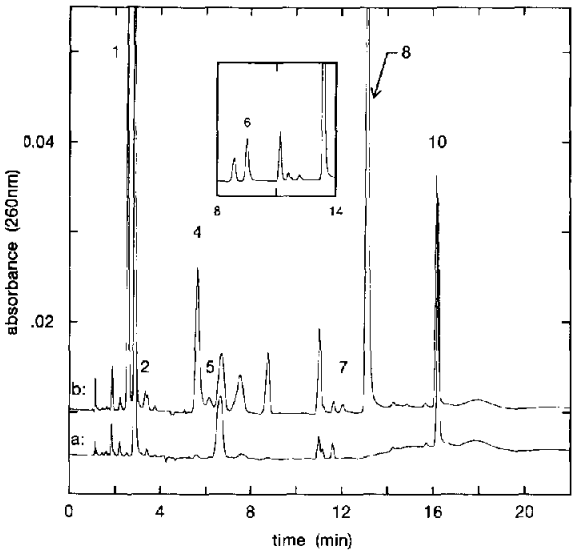

Fig. 4. Chromatograms of plasma extracts from a cancer patient (a) before and (b) 7.5 h after receiving 6 g of nicotinamide orally. The inset shows the appearance of nicotinic acid in the same sample rerun after three days. For peak identification see Fig. 1.

rats. 6-Hydroxynicotinic acid, nicotinic acid and nicotinuric acid were not seen in plasma. Plasma samples from cancer patients receiving nicotinamide have also been analysed and an example is shown in Fig. 4, together with a control plasma sample taken before administration of 6 g of nicotinamide. These patients are frequently prescribed concomitant medication which gives rise

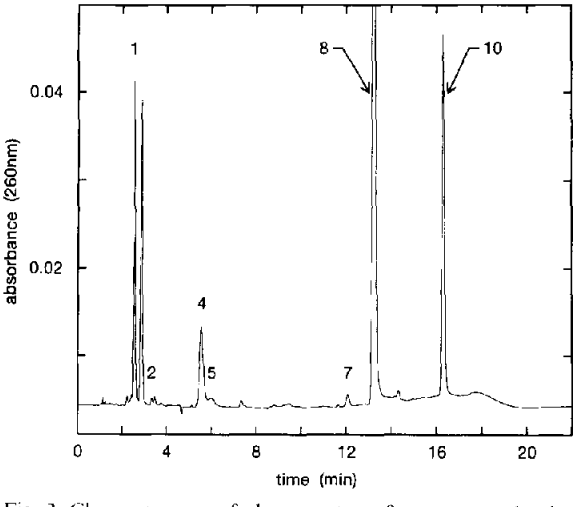

Fig. 3. Chromatogram of plasma extract from a normal volunteer 8 h after taking 6 g of nicotinamide orally. For peak identification see Fig. 1.

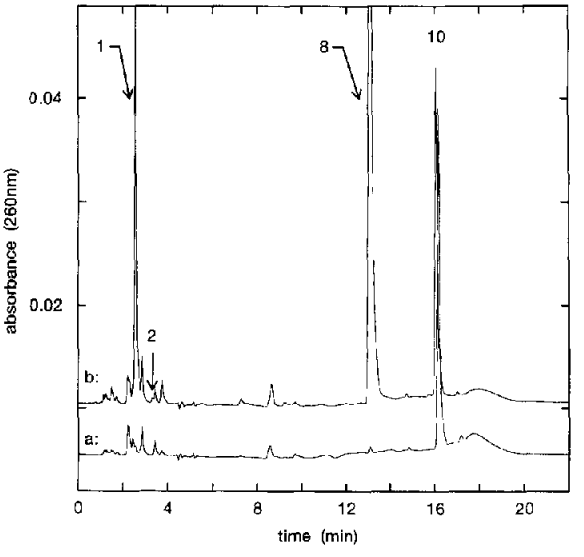

Fig. 5. Chromatograms of mouse plasma (a) before and (b) 1 h after an intraperitoneal injection of 100 mg/kg nicotinamide. For peak identification see Fig. 1.

to many additional peaks in plasma extracts as seen in the control sample compared to that shown in Fig. 2 for a normal volunteer control. Using the elution conditions described, this figure illustrates the lack of interference by these peaks.

We have never observed nicotinic acid in any samples when analysed immediately; however, the inset in Fig. 4 demonstrates the formation of nicotinic acid from nicotinamide in an extracted sample left at room temperature. We have attributed this to bacterial contamination since it does not occur in every sample; this phenomenon has also been observed in both diluted and undiluted urine samples.

We have also used this method for the analysis of nicotinamide and its metabolites in the mouse (Fig. 5), which shows that nicotinamide is metabolised mainly to the N-oxide, and also that control mouse plasma contains endogenous nicotinamide (~ 10 nmol/ml); in man, we could not detect nicotinamide prior to dosing.

For urine we used a slightly different elution solvent, primarily because of an interfering peak in control urine which co-chromatographed with nicotinuric acid. Using the acetonitrile eluent (see Experimental), nicotinuric acid was sufficiently separated from this peak to show that nicotinuric acid is not a significant metabolite of nicotinamide in man. Fig. 6 shows chromatograms of control human urine and of human urine collected between 6 and 8 h after 6 g of nicotinamide; the retention time of nicotinuric acid is marked by an arrow. Compared to plasma, 1-methylnicotinamide and the 2-pyridone form a much greater fraction of the total of nicotinamide-related material. In the mouse (Fig. 7), the N-oxide is a major excretion product [17], but no significant amounts of the pyridones were formed and 1-methylnicotinamide was only a minor product; however, nicotinuric acid formed a significant proportion of the total drug-related material.

The linearity, and intra- and inter-assay precision and accuracy of the method were assessed using control plasma spiked with known amounts of the metabolites of interest; these were then analysed as described above. For nicotinamide, concentrations up to 2 μmol/ml were used; for the metabolites, 200 nmol/ml was the highest plasma concentration investigated. All the curves were found to be linear using a com-

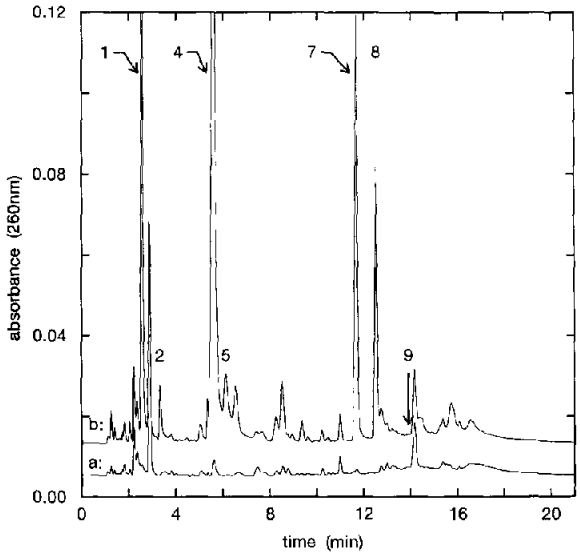

Fig. 6. Chromatograms of human urine (a) before and (b) 6–8 h after 6 g of nicotinamide orally. The elution position of nicotinuric acid is marked with a vertical arrow. For peak identification see Fig. 1.

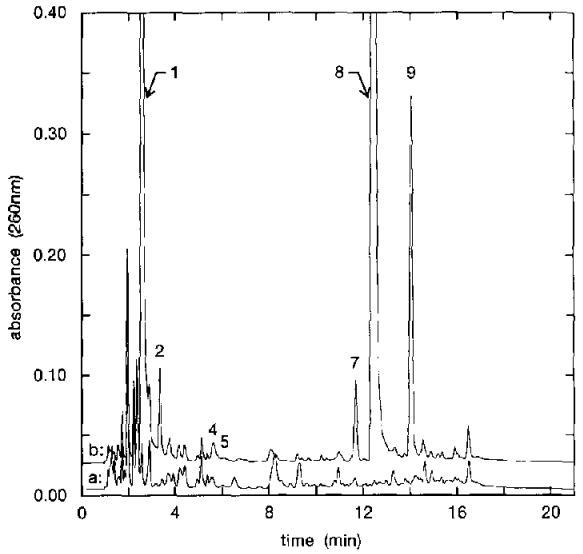

Fig. 7. Chromatograms of mouse urine (a) before and (b) 90 min after 500 mg/kg nicotinamide intraperitoneally. For peak identification see Fig. 1.

TABLE I

INTRA- AND INTER-ASSAY PRECISION AND ACCURACY OF THE HPLC PROCEDURE FOR NICOTINAMIDE (500 nmol/ml) AND ITS METABOLITES (50 nmol/ml)

Component	Intra-assay (n = 4)		Inter-assay (n = 5)	
	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)
Nicotinamide	0.9	95.7	1.4	96.6
Nicotinamide-N-oxide	1.0	94.9	1.3	95.1
6-Hydroxynicotinamide	1.1	94.4	1.4	95.1
2-Pyridone-5-carboxamide	0.9	96.1	1.8	97.0
4-Pyridone-5-carboxamide	5.7	92.2	5.2	91.7
Nicotinic acid	8.2	105.9	8.0	107.2
1-Methylnicotinamide	5.3	102.9	5.8	114.3
Nicotinuric acid	1.6	103.0	3.0	100.1

puter-based linear least-squares program (correlation coefficients, $r > 0.9997$). Endogenous levels of the 2- and 4-pyridones (~ 1 nmol/ml) resulted in a positive intercept for these compounds; all other compounds were fitted through the origin. Table I shows the precision and accuracy data. Only the 4-pyridone, nicotinic acid and 1-methylnicotinamide showed relative standard deviations (R.S.D.) > 5%; none of these are major metabolites in man, as illustrated in Table II, where replicate aliquots of a plasma sample from a normal volunteer were analysed. The limit of detection for nicotinamide was ~ 0.2 nmol/ml and in general it could not be detected in control human plasma. Detection limits for the other metabolites were similar except for the pyridones; the tailed peak shape for these two metabolites meant that the control levels referred to above were close to the minimum detectable.

TABLE II

MEAN AND STANDARD ERROR OF REPLICATE ANALYSES OF PLASMA FROM A NORMAL VOLUNTEER 7 h AFTER 6 g OF NICOTINAMIDE (n = 3)

Compound	Mean plasma concentration (nmol/ml)	S.E.M. (nmol/ml)
Nicotinamide	448	5.4
Nicotinamide-N-oxide	283	4.3
6-Hydroxynicotinamide	0.81	0.02
2-Pyridone-5-carboxamide	60.5	1.1
4-Pyridone-5-carboxamide	8.6	0.3
1-Methylnicotinamide	9.9	0.7

DISCUSSION

The separation of nicotinamide and its metabolites was attempted on a number of different columns but the analysis was made more difficult by several factors. All of the compounds contain at least one nitrogen atom, which, although it is only weakly basic (nicotinamide pK_a 4.2), causes severe tailing on many reversed-phase packings; other separation modes such as ion exchange were ruled out by the physicochemical differences between the metabolites. For example, both the pyridones and the N-oxide are neutral above pH 2 which is the practical pH limit for reversed-phase packings. This wide range of properties of the compounds of interest, which included acids, bases and neutral species, combined with the overall low lipophilicity, led us to exclude the use of solid-phase extraction, which has been employed for the analysis of nicotinamide alone [6]. We investigated a number of packing materials

which are claimed to be good for the efficient separation of bases. A YMC AQ column (Hichrom) initially seemed satisfactory but was prone to variable retention times, while a Hichrom RPB column gave a rather high back-pressure. The Hypersil BDS was found to be the most satisfactory of the three columns tested, although we found it necessary to run in a pseudo-equilibrated state. An initial "dummy" injection was used to give stable retention times, where the first sample was injected and chromatographed as described, but the resultant chromatogram was discarded.

In general, the limiting factor in the separation was the N-oxide which because of its high polarity combined with zero net charge was not amenable to ion pairing, and so was only slightly retained on any of the columns. It was also necessary to maintain resolution of the N-oxide from uric acid which is present at relatively high concentrations particulary in man. Certain of the metabolites are amenable to detection at higher wavelengths, such as the pyridones and the hydroxylated metabolites, which may help to minimise interfering peaks. However, for uric acid, which has a peak absorbance at 280 nm, increasing detection wavelength would actually increase the area of this component. We also wished to keep the method as simple as possible and therefore felt that it was preferable to avoid the introduction of wavelength changes or multi-channel detection. However, the identity of the 6-hydroxynicotinamide was confirmed by the use of peak-area ratios at 295 and 310 nm. Isonicotinamide has previously been described as an internal standard [6], but was found to elute too close to nicotinamide using this system, while N'-methylnicotinamide, which was also assessed as a possible internal standard, was found to contain small amounts of nicotinic acid.

A minor problem which limited the ultimate sensitivity of the method was that if the injection volume was increased above 25 μl, the 2-pyridone and nicotinic acid peaks became distorted. This appeared to be related to the ionic composition of the sample since, surprisingly, it was worse if the dried-down sample was reconstituted in eluent rather than water, and was not seen in purely aqueous standards. However, this did not significantly compromise the method since the levels to be measured were relatively high. It may arise because of the low ionic strength of the eluent, but this was necessary to achieve the separation required, particularly of the N-oxide.

Using the eluents described, no problems were encountered with endogenous plasma peaks or from peaks derived from concomitant medication. The method has proved robust (we have used it to analyse several hundred plasma and urine samples in both man and the mouse), and although each analysis is relatively long, with a run time of 22 min, up to nine components can be identified from each one. We believe this method will be of value for metabolic studies of nicotinamide, and may also help in the elucidation of the mechanism of the radiosensitizing action of nicotinamide.

ACKNOWLEDGEMENTS

We are grateful for the help and support of Professor S. Dische and all our clinical colleagues in the Marie Curie Research Wing at Mount Vernon Hospital. This work is supported by the Cancer Research Campaign.

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