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## Note

# Determination of nicotinamide in human plasma and urine by ion-pair reversed-phase high-performance liquid chromatography

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Nicotinamide is used in the prevention or treatment of dietary vitamin deficiencies as well as for other therapeutic purposes [1]. In connection with clinical pharmacological research with nicotinic acid and nicotinamide it was desirable to determine plasma and urine levels of nicotinamide in the presence of large amounts of nicotinic acid. Microbiological [2], colorimetric [3], fluorimetric [4] and ion-exchange [5] methods have been described for the analysis of nicotinamide in plasma and (or) urine, but lack specificity or are too lengthy for serial determinations.

A method combining sample pre-cleaning with a short column and the separation of nicotinamide with an ion-pair reversed-phase high-performance liquid chromatography (HPLC) system was developed using isonicotinamide as internal standard.

## MATERIALS AND METHODS

### *Apparatus*

An HPLC system consisting of a Model 6000A pump, a U6K injector, a Model 440 UV detector (all from Waters, Königstein, G.F.R.), a strip chart recorder Model BD 8 (Kipp Analytica, Solingen, G.F.R.) and an Autolab system I computing integrator (Spectra-Physics, Darmstadt, G.F.R.) were used for the chromatographic separation and quantitation. Centrifugations were effected at 2000 *g*.

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*Reagents*

All test substances were analytical grade. Methanol (for spectroscopy; Merck, Darmstadt, G.F.R.); distilled water (B. Braun, Melsungen, G.F.R.) and sodium dioctylsulfosuccinate (USP XII; Serva, Heidelberg, G.F.R.) for the mobile phase, were used without further purification. Sep-Pak $C_{18}$ cartridges were from Waters.

*Standard solutions*

Nicotinamide was dissolved in water in concentrations of 1000, 200 and 50 mg/l for the plasma calibration, and 5000, 2000 and 500 mg/l for the urine calibration; isonicotinamide was used at concentrations of 400 mg/l and 5000 mg/l as an internal standard for the plasma and urine analyses, respectively. Solutions were kept cold.

*Procedure*

Blood and urine samples were obtained from healthy volunteers at regular intervals after the administration of nicotinamide or nicotinic acid; blood was heparinized, immmediately centrifuged and stored at −23°C; urine was stored at the same temperature; before analysis they were again centrifuged.

*Plasma purification.* Plasma (1 ml) was mixed with 10 µl internal standard solution, 10 µl water, vortexed for 30 sec and then slowly pumped through a Sep Pak $C_{18}$ cartridge (previously washed with 2 ml methanol and 5 ml water); it was washed with 0.5 ml water (which was discarded) and eluted with 1.5 ml of the chromatographic mobile phase; this fraction was collected, centrifuged for 2 min and injected into the column (150 µl for concentrations below 2 mg/l and 100—25 µl for higher concentrations). For nicotinamide concentrations above 10 mg/l, the sample was diluted with water previous to the purification procedure. The calibration curve was obtained in the same way, but 10 µl of the standard nicotinamide solutions were mixed in instead of 10 µl of water.

*Urine purification.* The procedure was the same as for plasma, except that the concentrations of nicotinamide and internal standard were those indicated under *Standard solutions.*

*Chromatographic conditions*

The mobile phase was prepared by dissolving 4.446 g sodium dioctylsulfosuccinate in 1450 ml water, adjusting the pH to 2.5 with formic acid and mixing with 1050 ml methanol; the solution was passed through a 0.45-µm filter and degassed before use; the flow-rate was 2.0 ml/min and the pressure was 2000—3000 p.s.i.; the UV detector was set at 254 nm with a sensitivity of 0.01 a.u.f.s.; quantitation was effected through peak area integration; the calibration regression line was obtained using the relationship area nicotinamide/area isonicotinamide vs. added nicotinamide concentration (mg/l). The guard column (25 × 4 mm I.D.; filled with Bondapak $C_{18}$ Corasil (Waters) was renewed every day. Plasma was analyzed with a µBondapak $C_{18}$ (30 cm × 4 mm I.D.; Waters) or a LiChrosorb RP-18 10-µm (25 cm × 4 mm I.D.; Merck) column; for urine, two µBondapak $C_{18}$ columns were used in series. All chromatograms were run at room temperature.

## RESULTS

Fig. 1a shows the separation of a mixture of isonicotinic acid, nicotinic acid, nicotinuric acid, nicotinamide and the internal standard, isonicotinamide (retention times: 2.18, 2.63, 3.57, 4.60 and 6.00 min, respectively). Plasma extracts from blank samples show a very small peak from endogenous nicotinamide (20—50 μg/l) [4]. Fig. 1b displays a chromatogram from a volunteer's plasma, 40 min after the oral ingestion of 200 mg nicotinamide. Analysis time

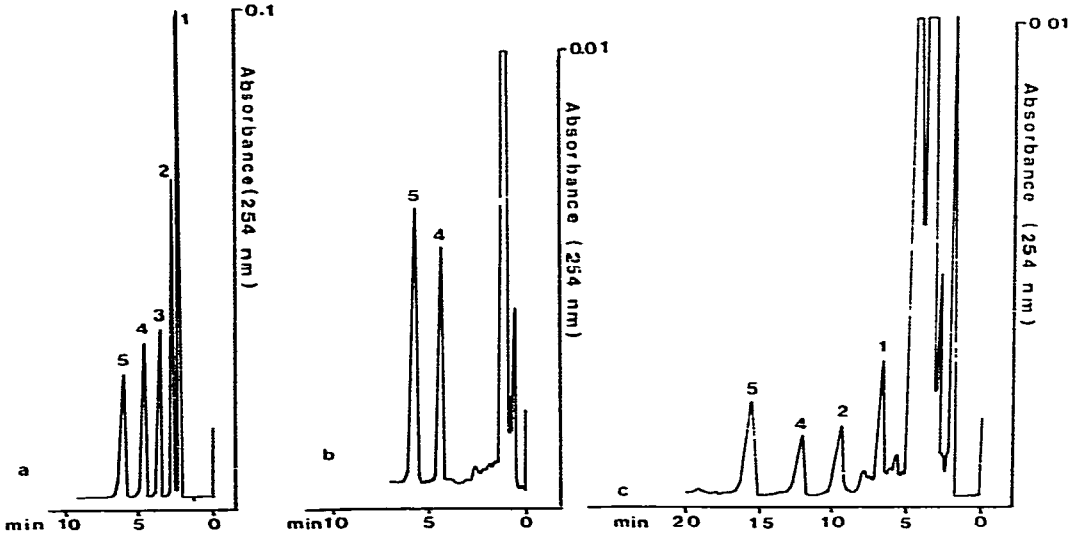


Fig. 1. Chromatograms of (a) mixture of isonicotinic acid (1), nicotinic acid (2), nicotinuric acid (3), nicotinamide (4) and the internal standard isonicotinamide (5) (each 1 μg); (b) plasma extract after nicotinamide ingestion; nicotinamide (4) (concentration, 2.10 mg/l), isonicotinamide (5) (concentration, 4 mg/l); 100-μl injection; (c) urine extract spiked with nicotinic acid (1), nicotinuric acid (2), nicotinamide (4) (each 20 mg/l) and isonicotinamide (5) (50 mg/l); 10-μl injection.

was 10—15 min per sample. The relationship, area nicotinamide/area isonicotinamide is linear in the range 0.1—10 mg/l and the statistical regression line can be represented as $y = 0.2924x + 0.0093$; $r = 0.999$. The recoveries of nicotinamide and isonicotinamide in plasma at a concentration of 10 mg/l were 91.8 ± 4.1% and 92.4 ± 4.1% ($n = 4$) respectively, and the same data were obtained from aqueous solutions. Urine analyses recoveries were in the range 1—50 mg/l with a linear regression line $y = 0.0227x + 0.0275$; $r = 0.999$; the analysis time was 20—30 min per sample. Fig. 1c shows the chromatogram of urine spiked with nicotinic acid, nicotinuric acid, nicotinamide and isonicotinamide (retention times were 6.02, 8.67, 10.91 and 14.29 min, respectively).

The sensitivity of the method is 0.1 mg/l for plasma and 1 mg/l for urine; precision and accuracy are shown in Table I.

The following substances showed no interference in the analysis: nicotinic acid; isonicotinic acid; nicotinuric acid; picolinic acid, 6-hydroxy nicotinic acid; nicotinic acid-$N^1$-oxide; nicotinamide-$N^1$-oxide; $N^1$-methyl nicotinamide

TABLE I

REPRODUCIBILITY OF PLASMA AND URINE ANALYSES FOR NICOTINAMIDE

| | Plasma (mg/l) | | | Urine (mg/l) | | |
|---|---|---|---|---|---|---|
| Concentration given | 0.5 | 2 | 10 | 5 | 20 | 50 |
| Concentration found | 0.53 | 1.98 | 10.0 | 5.1 | 19.8 | 50.0 |
| Coefficient of variation (%, $n$ = 10) | 3.4 | 4.5 | 4.8 | 6.8 | 5.1 | 5.6 |

chloride; N'-methyl nicotinamide; N'N'-diethyl nicotinamide; picolinamide; salicylic acid; acetylsalicylic acid; salicyluric acid; salicylamide; benzoic acid; hippuric acid; thiamine hydrochloride; biotin; ribofavine-5'-phosphate; D-panthenol; and pyridoxine hydrochloride.

## DISCUSSION

Several stationary phases, eluting solvents, modifiers and extraction procedures were tried [3, 4, 6—8]; although in many cases the separation of the nicotinamide and isonicotinamide peaks was satisfactory, interfering peaks from plasma and urine could not be eliminated; the use of dioctylsulfosuccinate [9] as an ion-pair in combination with reversed-phase chromatography with a $C_{18}$ modified stationary phase, preceded by purification through a small column circumvented these difficulties.

The concentration of nicotinamide could be determined even in the presence of a large excess of nicotinic and nicotinuric acid; other metabolites did not interfere, and plasma blanks showed no interfering peaks. The simultaneous determination of nicotinamide together with nicotinic and nicotinuric acid was not attempted as the latter showed analytical recoveries of 34% and 84%, respectively. Incubation and recovery experiments suggest that total plasma

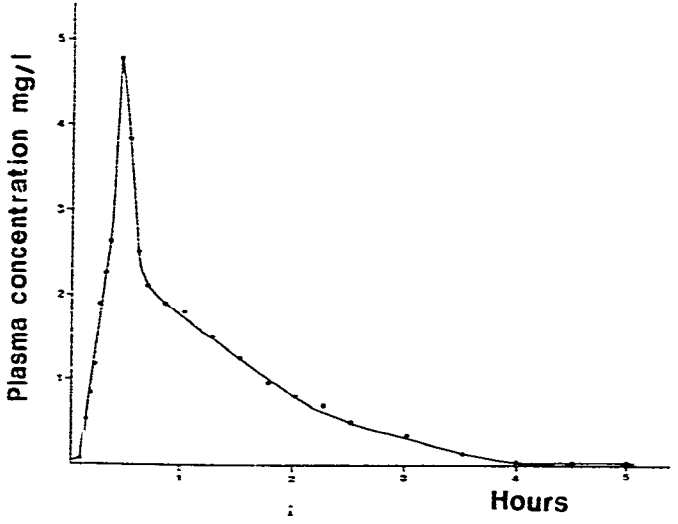


Fig. 2. Plasma concentration—time curve of nicotinamide after a single oral dose of a 200-mg tablet.

nicotinamide is determined. During a working day, 30—40 plasma or 10—15 urine analyses could be carried out, including the purification step. The method has been found to be rapid, simple, reproducible, specific and adequate for pharmacokinetic work, and has been used continuously over a period of a year. Fig. 2 shows a plasma concentration—time curve after nicotinamide ingestion. The results of these researches will be published elsewhere.

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REFERENCES

1 W.F. Körner and J. Völlm, in H.P. Kuemmerle, E.R. Garret and K.H. Spitzky (Editors), Klinische Pharmakologie und Pharmakotherapie, Urban & Schwarzenberg, München, 1976, 3rd ed., p. 361.
2 G.A. Goldsmith and O.N. Miller, in P. Gyorgy and W.N. Pearson (Editors), The vitamins, Chemistry, Physiology, Pathology, Methods, Vol. 7, Academic Press, New York, 1967, Ch. 5, p. 137.
3 L.A. Carlsson, Clin. Chim. Acta, 13 (1966) 349.
4 B.R. Clark, R.M. Halpern and A.R. Smith, Anal. Biochem., 68 (1975) 54.
5 J.E. Mrochek, R.L. Jolley, D.S. Young and W.J. Turner, Clin. Chem., 22 (1976) 1821.
6 R. Gloor and E.L. Johnson, J. Chromatogr. Sci., 15 (1977) 413.
7 E. Tomlinson, T.M. Jefferies and C.M. Riley, J. Chromatogr., 159 (1978) 315.
8 N. Hengen, V. Seiberth and M. Hengen, Clin. Chem., 24 (1978) 1740.
9 S.P. Sood, D.P. Wittmer, S.A. Ismael and W.G. Haney, J. Pharm. Sci., 66 (1977) 40.