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Note

# High-performance liquid chromatographic analysis of nalidixic acid in plasma after alkylation with methyl iodide

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Nalidixic acid (1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid; NA) is used in the treatment of urinary-tract infections caused by Gram negative organisms other than Pseudomonas spp.

Most methods for its quantitative analysis are based on the fluorescence of NA [1, 2], but these methods have several disadvantages. They are non-specific, giving relatively high blank values and non-linear calibration curves and they need relatively large plasma volumes of 1—3 ml. A high-performance liquid chromatographic (HPLC) method using an ion-exchange column has been described [3], which needs only 1 ml of plasma, but in this method no internal standard is used. It is suggested that with this method the major metabolite, hydroxynalidixic acid (1-ethyl-1,4-dihydro-7-hydroxymethyl-4-oxo-1,8-naphthyridine-3-carboxylic acid; HNA) can also be determined, but this compound is not separated from the solvent peak in the system used.

In this paper we describe a method that can measure NA concentrations down to 0.6 μg/ml in a 100-μl plasma sample using a standard reversed-phase column. Because nalidixic acid gives very strongly tailing peaks in most chromatographic systems, the compound is methylated as described in the literature [4]. The calibration curve is linear up to at least 100 μg/ml and the intercept does not differ significantly from zero. Also the metabolite does not interfere with the assay. The method was used for determining plasma levels in patients treated with nalidixic acid.

## EXPERIMENTAL

### *Apparatus*

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000 A solvent delivery system, Model U 6 K injector and Model 440 absorbance detector operated at 313 nm. The two 30-cm columns used were μBondapak $C_{18}$ (Waters Assoc.) and LiChrosorb 10 RP-18 (Chrompack, Middelburg, The Netherlands), both $C_{18}$ bonded phases on 10-μm particles of silica gel. Peak areas were measured by means of a Spectra Physics SP 4000 data system.

### *Chemicals*

The solvents toluene, acetone, dimethylformamide (DMF) and the catalyst caesium carbonate were purchased from Merck (Darmstadt, G.F.R.); methyl iodide and nalidixic acid were from Fluka (Buchs, Switerland).

The propylester of NA (PNA), which was used as an internal standard, was prepared by refluxing 400 mg NA for 24 h in 200 ml of acetone to which had been added 500 mg caesium carbonate and 25 ml propyl iodide (Fluka). After the solution had been evaporated to dryness by means of a rotary evaporator, 200 ml of chloroform (Merck) were added to the residue. The insoluble inorganic catalyst was removed by filtration of the solution through filter paper. The chloroform was evaporated and the product obtained was purified by recrystallisation from cyclohexane (Merck), yielding 225 mg of PNA (m.p. 108—112°). The mass spectrum of this compound showed the parent peak to be at *m/e* 274, in accordance with the empirical formula $C_{15}H_{18}N_2O_3$ (PNA); unreacted NA should give a peak at *m/e* 232, but this peak was not present.

The metabolite HNA was isolated from urine of a patient who was treated with NA, according to the method of McChesney et al. [1]. The identity of this product was confirmed by its melting point and mass-spectral analysis.

### *Procedures*

*Extraction.* To 100 μl of plasma in a 7-ml glass centrifuge tube, 100 μl water and one drop of hydrochloric acid (2 *M*) were added. This solution was extracted with 2.00 ml of a stock solution of PNA in toluene (about 5 μg/ml). After mixing on a Vortex mixer for 1 min and subsequent centrifugation at 2500 *g* for 2 min, the toluene phase was transferred with a pasteur pipette into a 2-ml ampoule and evaporated to dryness under a stream of nitrogen at 80° in a metal heating block. Acetone (100 μl), caesium carbonate (about 5 mg) and methyl iodide (10 μl) were added, the ampoule was closed by melting after replacement of the air by nitrogen and shaken on a Vortex mixer for 5 sec. Esterification was completed after 15 min at 80°; the ampoule was opened at that time and evaporated to dryness under a stream of nitrogen at 50°. The residue was dissolved in 100 μl DMF; 20 μl of the clear solution was injected into the HPLC system.

*Chromatography.* The mobile phases used were 70% (v/v) methanol in water in combination with the LiChrosorb 10 RP-18 column and 63% (v/v) methanol in water for the μBondapak $C_{18}$ column. The flow-rate was 1.5 ml/min in both cases. All chromatograms were obtained at ambient temperature.

## RESULTS AND DISCUSSION

Toluene was used as extraction solvent as described by other authors [1, 2]; a very clear upper layer was obtained, which was easy to transfer. Because of the fact that underivatized NA gave very strongly tailing peaks on various stationary phases such as μBondapak $C_{18}$, Corasil Phenyl (Waters Assoc.) and μBondapak CN (Waters Assoc.) the methylester (MNA) was prepared and chromatographed.

For reasons of extraction, retention and detection, the propyl ester of NA was chosen as the internal standard. The ethyl ester is another potential internal standard but in this case there is a risk of the two peaks overlapping when the plate number of the column decreases.

Methyl iodide gave a peak very close to the MNA peak, so it was removed by evaporation. The residue was dissolved in DMF and not in the mobile phase or pure methanol, because by standing in the presence of caesium carbonate PNA was slowly transesterified to MNA by methanol; on column this phenomenon was not seen.

The stability of PNA during the derivatisation step was checked by heating 10 μg with methyl iodide and caesium carbonate in 100 μl acetone. Even after 18 h (72 × reaction time) at 80° no MNA could be detected.

Although the molar absorptivity of NA at 313 nm is lower than at 254 nm, the wavelength of 313 nm was chosen because at this wavelength no interfering plasma peaks are detected (Fig. 1).

The detection limit was about 10 ng MNA per injection. Calibration curves were made on a LiChrosorb 10 RP-18 column and a μBondapak $C_{18}$ column; the concentration range of NA was 0.66—99.2 μg/ml. The straight lines were calculated by the method of least squares and were, respectively, $y = -0.0009\ (\pm\ 0.0063) + 0.0100\ (\pm\ 0.0001)x$ ($r^2 = 0.9972$, $n = 22$), and $y = -0.0056\ (\pm\ 0.0129) + 0.0091\ (\pm\ 0.0002)x$ ($r^2 = 0.9887$, $n = 20$), where $y$ = peak area ratio MNA : PNA and $x$ = concentration of NA in μg/ml.

The accuracy of the method is shown in Table I. Different amounts NA were added to blank plasma and analysed by a technician to whom the concentrations had not been revealed.

The day-to-day reproducibility of the calibration curve on the same column (μBondapak $C_{18}$), using the same stock solution of PNA in toluene, is shown in the two following equations for, respectively, days 1 and 4: $y = 0.0106\ (\pm\ 0.0111) + 0.0115\ (\pm\ 0.0002)x$ ($r^2 = 0.9985$, $n = 10$); and $y = 0.0067\ (\pm\ 0.0097) + 0.0113\ (\pm\ 0.0002)x$ ($r^2 = 0.9990$, $n = 8$). There are no significant differences, which makes it possible to use the calibration curve for several days. The intercepts of the calibration curves did not differ significantly from zero, so only a few samples of the same concentration are sufficient to construct a calibration curve, passing through the origin. The absolute recovery for the whole method was 84% for NA and 88% for PNA.

The metabolite HNA was found not to interfere with the determination of NA. When treated as described under procedures, HNA gave some small peaks before the MNA peak. Even concentrations of HNA twice as high as NA (respectively, 100 μg/ml and 49.6 μg/ml) did not interfere with the assay.

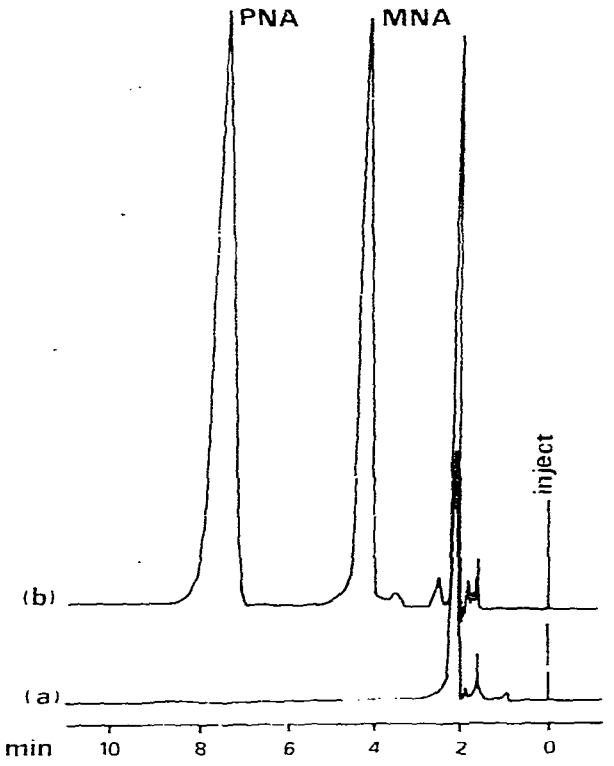


Fig. 1. Chromatograms obtained by the analysis of 100 µl plasma on a µBondapak $C_{18}$ column (for conditions *see text*). (a) *Plasma blank.* (b) *Plasma of a* 15-year-old boy who was *treated* for 3 days, every 6 h with an infusion of 1 g NA over a period of 20 min. Plasma was taken just before the next period of infusion. The plasma level was calculated to be 52.1 µg/ml.

TABLE I

RECOVERY OF NA FROM PLASMA

| NA (µg/ml) | | Recovery (%) |
|---|---|---|
| Added | Found | |
| 92.5 | 88.0 | 95.1 |
| 66.1 | 65.1 | 98.5 |
| 66.1 | 67.2 | 101.7 |
| 52.9 | 51.3 | 97.0 |
| 26.4 | 27.5 | 104.0 |
| 16.5 | 16.4 | 99.4 |
| 2.64 | 2.86 | 108.3 |
| | Mean ± S.D. | 100.6 ± 4.5 |

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