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DETERMINATION OF NALIDIXIC ACID AND ITS TWO MAJOR METABOLITES IN HUMAN PLASMA AND URINE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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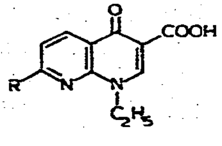
SUMMARY

This paper describes a precise and sensitive method for analysis of nalidixic acid and its two major metabolites in plasma and urine following the oral administration of a therapeutic dose in humans. After addition of an internal standard (oxolinic acid), 1-ml samples of plasma or urine are extracted at acidic pH with chloroform. The extracts are purified by re-extraction with sodium hydroxide solution and then chloroform. The final extracts are evaporated to dryness, reconstituted in mobile phase and injected into a high-performance liquid chromatograph equipped with RP-8 column and UV detector operating at 254 nm. The limit of sensitivity of the method is lower than 0.5 µg/ml of plasma or urine for each compound. The applicability of the method to pharmacokinetic studies of nalidixic acid in humans is demonstrated.

INTRODUCTION

Nalidixic acid, 1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid (NA) is an antibacterial agent [1,2] which is widely used in the treatment of urinary tract infections.

Contrarily to its metabolism which has been widely studied [3], the pharmacokinetics of NA in man are poorly described. This is probably due to the lack of an analytical method allowing to measure precisely NA concentrations in biological fluids separately from its two major metabolites, 7-hydroxynalidixic acid (HNA) and 7-carboxynalidixic acid (CNA).



R = CH_3 NA
= CH_2OH HNA
= COOH CNA

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Most procedures used for quantitation of NA are based on its fluorescence [2,4]. These techniques, which need large plasma volumes (1—3 ml), are non-specific towards the metabolites and give relatively high blank values and non-linear calibration curves.

A high-performance liquid chromatographic (HPLC) method using an ion-exchange column has been recently described [5], in which no internal standard was used, and the major metabolite in plasma, HNA, could not be determined as it was not separated from the solvent peak in the system used.

Two other HPLC methods have been developed. The first [6] was devoted to the determination of CNA only, and the second [7] to NA exclusively. On the other hand, two gas chromatographic methods have also been established, but their application was limited to the determination of NA alone in tablets [8] or in plasma [9].

In the present work, we describe a HPLC method which allows a precise and sensitive analysis of plasma and urine samples for NA, HNA and CNA simultaneously, using a standard reversed-phase column and, as internal standard, oxolinic acid (OXA), a compound whose structure is near to NA. An example of a pharmacokinetic study demonstrates its utility for the analysis of the three compounds in biological fluids.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 1081B high-performance liquid chromatograph was used, equipped with a Hewlett-Packard Model 1036A UV detector operating at 254 nm, and a RP-8 prepacked reversed-phase column, particle size 10 μm, (Hewlett-Packard), (250 × 4.6 mm I.D.).

Chemicals and reagents

NA (Negram), HNA and CNA were generously supplied by the Sterling-Winthrop Research Institute (Rensselaer, N.Y., U.S.A.) and OXA was a gift from Substantia Laboratories (Courbevoie, France). Methanol and chloroform (Merck, Darmstadt, G.F.R.) were distilled before use. Ion-exchange resin Dowex 1-X4, 200—400 mesh, Cl^- form was purchased from Roth (Karlsruhe, G.F.R.). All other reagents were of analytical grade (Merck).

The mobile phase consisted of 55% methanol and 45% phosphate buffer containing 7.5 g/l KH_2PO_4 and 2.5 g/l Na_2HPO_4 (adjusted to pH 8.2 with 1 *N* sodium hydroxide). N,N,N-Trimethylcetylammonium bromide (2 g/l) (Merck) was added to the mixture.

Stock solutions of internal standard (OXA) and the mixture of NA, HNA and CNA were prepared by dissolving 1 mg/ml of each compound in 0.03 *N* sodium hydroxide.

Working solutions were prepared by diluting the stock solutions in 0.03 *N* sodium hydroxide to obtain various concentrations of the three compounds studied, between 1 and 50 μg/ml for plasma and between 10 and 200 μg/ml for urine, and a fixed concentration of OXA, 10 and 50 μg/ml for plasma and urine, respectively.

Correlation curves were obtained after adding 1 ml of each of these diluted solutions to 1 ml of corresponding biological fluid.

Procedures

Plasma extraction. To 1 ml of human plasma in a 15-ml glass centrifuge tube, were added 1 ml of the internal standard solution (10 μg/ml), 0.5 ml of 1 *N* hydrochloric acid and 8 ml of freshly distilled chloroform. The tube was stoppered and placed on a rotating shaker (60 rpm) for 15 min. After centrifugation (15 min at 1200 *g*) 6.5 ml of the organic layer were transferred to another tube and extracted with 6 ml of 0.2 *N* sodium hydroxide for 15 min. Following centrifugation (10 min), 5 ml of the aqueous phase were transferred to a new tube containing 2 ml of 1 *N* hydrochloric acid and the mixture was extracted with 6 ml of chloroform for 15 min. After centrifugation (10 min), 5 ml of the organic phase were transferred to a 10 ml conical tube and evaporated to dryness under a stream of nitrogen on a 45° water-bath. The residue was dissolved in 0.25—5 ml of mobile phase and 20 μl of this solution were injected onto the column by injection loop at a flow-rate of 0.6 ml/min under a pressure of about 30 bars at room temperature.

Urine extraction. To 1 ml of human urine in a 15-ml glass centrifuge tube, were added 1 ml of the internal standard solution (50 μg/ml), 0.5 ml of 1 *N* hydrochloric acid and 8 ml of freshly distilled chloroform. The tube was stoppered and placed on a rotating shaker (60 rpm) for 15 min. After centrifugation (10 min at 1200 *g*) 7 ml of the organic phase were transferred to another tube and extracted with 6 ml of 0.2 *N* sodium hydroxide for 15 min. Following centrifugation (10 min), 5 ml of the alkaline aqueous phase were loaded on a short column (4 mm I.D.) packed with a glass wool plug and with ion-exchange resin Dowex 1-X4 (1 cm). The complete outflow was followed by two bidistilled water washings of 2 ml each. The elution of the four compounds was performed by washing the column twice with 2.5 ml of 1 *N* hydrochloric acid. The eluate was extracted with 6 ml of chloroform for 15 min. After centrifugation (10 min), 5 ml of the organic phase were transferred into a 10-ml conical tube and evaporated to dryness under a stream of nitrogen on a 45° water-bath. The residue was redissolved in 0.5—10 ml of mobile phase and this solution injected into the HPLC system.

RESULTS AND DISCUSSION

Specificity

Fig. 1A shows that NA is clearly separated from its two metabolites. Under the prescribed conditions, the retention times were 7.0, 8.8, 13.8 and 15.4 min for HNA, OXA, CNA and NA, respectively.

Because nalidixic acid and related compounds gave very strongly tailing peaks in most chromatographic systems, the paired-ion technique has been used. This procedure allowed the best separation. Symmetrical chromatographic peaks were obtained if the degree of ion-pair dissociation was kept constant. This was achieved when a high and constant concentration of the counter-ion was maintained in the mobile phase. Such a situation can be obtained by adding a cation to the aqueous mobile phase, which brings about a

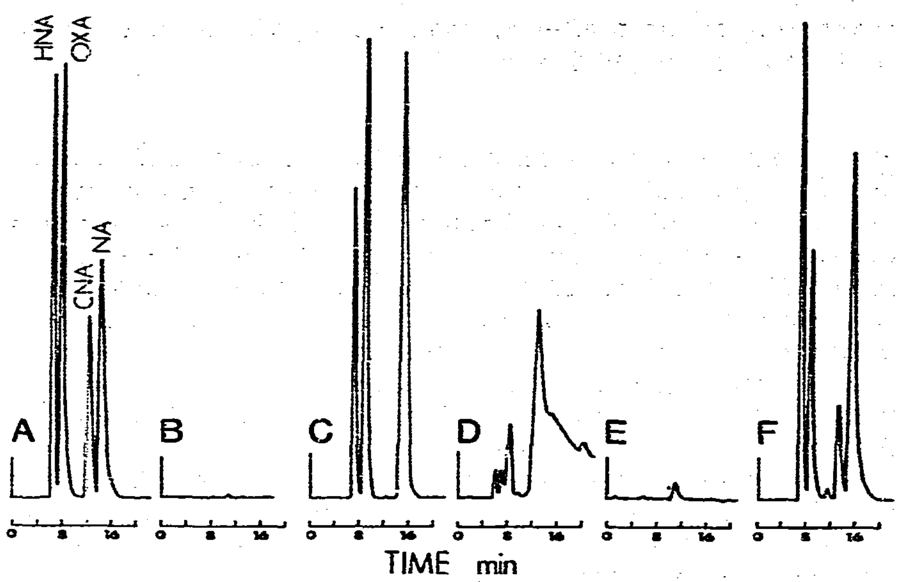


Fig. 1. Chromatograms of (A) a mixture of HNA, OXA, CNA and NA (50 ng of each compound injected); (B) a blank plasma extract; (C) plasma extract of a subject given an oral dose of nalidixic acid (HNA, OXA and NA; CNA is not present in plasma); a blank urine extract before (D) and after (E) the use of the ion-exchange resin; (F) urine extract of a subject given an oral dose of nalidixic acid (HNA, OXA, CNA and NA).

suitable ion-pair extraction of the counter-ion into the stationary phase [9,10]. Valid results were obtained with N,N,N-trimethylcetylammonium bromide at a concentration of 2 g/l in the mobile phase.

Plasma components did not interfere (Fig. 1B and C), but in the case of urine, it appeared necessary to clear the extracts from endogenous substances which gave a strong UV absorption. For this purpose, several resins were tested, and Dowex 1-X4 (strongly basic anion exchanger) was chosen because it gave the best recovery and cleaning. The residual peak observed in control urine has a retention time different from that of CNA (Fig. 1D, E and F).

Linearity and sensitivity

As shown in Fig. 2, the calibration graphs plotted as the peak height ratios of NA, HNA and CNA to the internal standard against the concentrations of each compound were straight lines. They were calculated by the least squares method and their equations are listed in Table I.

The concentration ranges studied in plasma (1—50 μg/ml) and urine (10—200 μg/ml) were chosen to correspond to the expected concentrations in biological fluids after a single therapeutic administration of NA in humans.

The detection limit, defined on the basis of the amount of compound injected which caused an absorption two times greater than the standard deviation of baseline noise, was found to be at 0.9, 2.1 and 1.8 ng, that is 10, 60 and 15 ng/ml of plasma for HNA, CNA and NA, respectively. In urine, the detection limit was higher than that observed in plasma, since endogenous

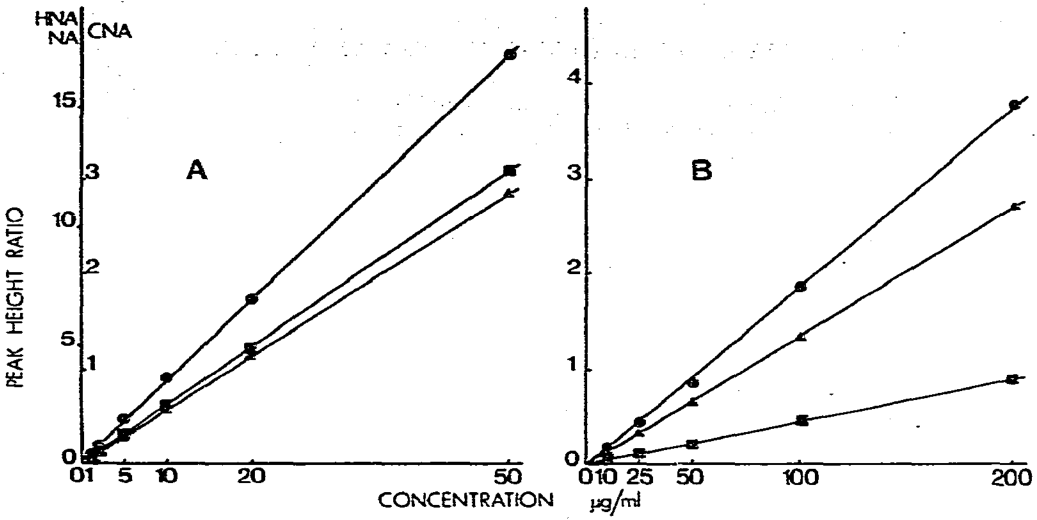


Fig. 2. Extracted standard curves of (A) plasma and (B) urine spiked with HNA (●), CNA (■) and NA (▲); each point represents the average of six determinations.

substances increased the background noise, and found to be at 80, 420 and 130 ng/ml for HNA, CNA and NA, respectively.

Reproducibility and recovery

In order to check the reproducibility of the extraction procedures used, various spiked plasmas and urines were repeatedly analysed. Table II shows the results obtained for several concentrations of HNA, CNA and NA. The overall mean coefficients of variation were ± 2.6%, ± 4.8% and ± 2.7% for HNA, CNA and NA, respectively.

TABLE I

LINEAR STANDARD CURVES DETERMINATION

Biological fluid	Compound	No. of points	Slope ± S.E.	Intercept ± S.E.	r^2
Plasma	HNA	6	0.340 ± 0.074	0.098 ± 0.0072	0.9987
	CNA	6	0.061 ± 0.007	0.011 ± 0.0007	0.9936
	NA	6	0.227 ± 0.026	0.049 ± 0.0026	0.9996
Urine	HNA	5	0.019 ± 0.003	− 0.042 ± 0.0003	0.9993
	CNA	5	0.0046 ± 0.0005	− 0.010 ± 0.00005	0.9997
	NA	5	0.014 ± 0.0012	− 0.004 ± 0.0001	0.9998

TABLE II

REPRODUCIBILITY OF THE ASSAY IN HUMAN PLASMA AND URINE

Compound	Biological fluid (1 ml)	Amount of compound added (μg)	Average of 6 assays (μg)	Coefficient of variation (%)
HNA	Plasma	1	0.99	2.5
		2	2.04	2.3
		5	5.13	2.9
	Urine	10	10.2	3.1
		25	24.8	2.0
		50	50.5	2.7
CNA	Plasma	1	0.93	8.8
		2	1.97	7.2
		5	5.12	3.9
	Urine	10	9.69	4.4
		25	25.9	1.0
		50	49.7	3.7
NA	Plasma	1	0.97	3.7
		2	1.92	3.6
		5	5.04	0.6
	Urine	10	10.4	4.7
		25	24.3	1.8
		50	49.6	1.8

TABLE III

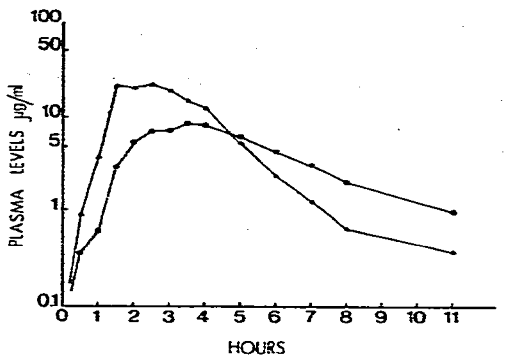
RECOVERY OF HNA, CNA AND NA FROM PLASMA AND URINE

Biological fluid	Compound	Added (μg/ml)	Found (μg/ml)	Recovery (%)	Mean ± S.E.
Plasma	HNA	2	1.64	82.0	83.4
		5	4.14	82.8	±1.8
		10	8.55	85.5	
	CNA	2	0.65	32.5	33.3
		5	1.69	33.8	±0.7
		10	3.37	33.7	
	NA	2	1.89	94.5	97.9
		5	4.96	99.2	±3.0
		10	10.02	100.2	
Urine	HNA	25	18.8	75.2	75.3
		50	38.2	76.4	±1.1
		100	74.3	74.3	
	CNA	25	7.8	31.2	30.3
		50	15.0	30.0	±0.8
		100	29.8	29.8	
	NA	25	22.9	91.6	93.1
		50	46.9	93.8	±1.3
		100	93.8	93.8	

The peak heights measured after extraction were compared to those obtained after injecting precise volumes of a solution of known concentration onto the HPLC column by means of the injection loop. The results, listed in Table III, show that HNA and NA were correctly extracted, whereas only 30% of CNA was recovered. This poor recovery for CNA was due to its low solubility in chloroform. For the three compounds, the recoveries observed were similar for plasma and urine, indicating that the use of ion-exchange resin in the urine procedure did not alter the extraction phase. It must be noted that the internal standard was extracted with a recovery of 97.5% and 95.5% for plasma and urine, respectively.

Kinetic studies

The method was applied to pharmacokinetic studies of NA in humans. Figs. 3 and 4 show the log plasma levels and cumulative urinary excretion curves versus time after administration of a single oral dose of 1 g of NA to a healthy volunteer.



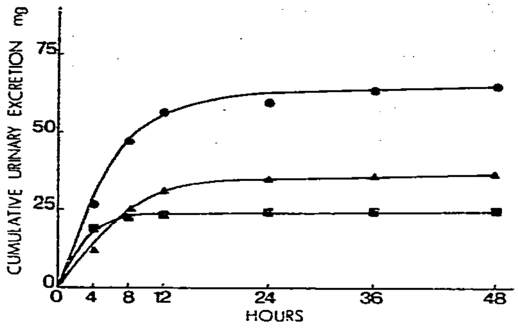


Fig. 3. Plasma level time curve obtained for NA (▲) and HNA (●) following a single oral administration of 1 g of nalidixic acid to a healthy volunteer (CNA was present in urine only).

Fig. 4. Cumulative urinary excretion of non-conjugated NA (▲), HNA (●) and CNA (■) observed after a single oral administration of 1 g of nalidixic acid to a healthy volunteer.

It must be noted that CNA could only be detected in urines. In plasma, NA concentration reached a maximum (25 µg/ml) within 2 h and then decreased rapidly to 0.2 µg/ml 11 h following the administration. Metabolite HNA exhibited a similar evolution but at lower levels than NA, and after a delay due to its formation from the parent drug. The cumulative urinary excretion curves for the compounds indicated that nalidixic acid was mainly eliminated as HNA and CNA.

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REFERENCES

1 G.Y. Lesher, E.J. Froelich, M.D. Gruett, J.H. Bailey and R.P. Brundage, J. Med. Pharm. Chem., 5 (1962) 1063.
2 E.W. McChesney, E.J. Froelich, G.W. Lesher, A.V.R. Crain and D. Rosi, Toxicol. Appl. Pharmacol., 6 (1964) 292.
3 J. Edelson, C. Davison and D.P. Benziger, Drug Metab. Rev., 6 (1977) 105.
4 P. Brühl, G. Gundlach, K. Wintjes, W. Eichner and H.P. Bastian, Arzneim.-Forsch., 23 (1973) 1311.
5 L. Shargel, R.F. Koss, A.V.R. Crain and V.J. Boyle, J. Pharm. Sci., 62 (1973) 1452.
6 F.H. Lee, R. Koss, S.K. O'Neil, M.P. Kullberg, M. McGrath and J. Edelson, J. Chromatogr., 152 (1978) 145.
7 R.H.A. Sorel and H. Roseboom, J. Chromatogr., 162 (1979) 461.
8 H.L. Wu, T. Nakagawa and T. Uno, J. Chromatogr., 157 (1978) 297.
9 H. Roseboom, R.H.A. Sorel, H. Lingeman and R. Bouwman, J. Chromatogr., 163 (1979) 92.
10 K.G. Wahlund, J. Chromatogr., 115 (1975) 411.
11 S. Eksborg and G. Schill, Anal. Chem., 45 (1973) 2092.