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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF NALIDIXIC ACID AND HYDROXYNALIDIXIC ACID IN PLASMA WITH A DYNAMIC ANION-EXCHANGE SYSTEM

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SUMMARY

A rapid high-performance liquid chromatographic method has been developed for monitoring plasma levels of patients treated intravenously with nalidixic acid. The major metabolite (in vitro also active) can be determined as well; 50-μl plasma samples are sufficient. Use is made of a dynamic anion-exchange system. Different parameters such as adsorption of the surfactant cetrimide onto the column; pH and ionic strength of the eluent, and the critical micelle concentration of the surfactant in the eluent have been studied.

INTRODUCTION

Nalidixic acid (1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid; NA) is occasionally used intravenously for the treatment of systemic infections. Renal failure is common amongst these patients and it is therefore necessary to monitor the plasma concentrations in order to prevent toxic or, on the other hand subtherapeutic levels. The major metabolite, hydroxynalidixic acid (1-ethyl-1,4-dihydro-7-hydroxymethyl-4-oxo-1,8-naphthyridine-3-carboxylic acid; HNA) is in vitro also active against Gram-negative micro-organisms and that is a reason for determining the plasma levels of this product too.

Several methods have been described for the assay of NA and HNA in biologic media. The original fluorescence method [1] and a modification thereof [2] are not suitable for monitoring plasma levels. Two high-performance liquid chromatographic (HPLC) methods have been reported; the first makes use of an ion-exchange column [3] and in the second method [4]

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NA is methylated and chromatographed on a reversed-phase system. A gas—liquid chromatographic (GLC) method [5] has also been described.

The aim of this study was to develop a procedure for the determination of NA and HNA in plasma, in which the advantages of the methods reported previously are combined, such as speed, selectivity, sensitivity and the use of small plasma samples. A dynamic ion-exchange system is used, which was introduced by Knox and co-workers [6, 7] under the name "soap chromatography". In this system a reversed-phase column is used and a surfactant such as cetrimide is added to the eluent. The cetrimide can be retained by the stationary phase, resulting in a "dynamically coated" column. The quaternary nitrogen groups of the bonded cetrimide molecules give the column ion-exchange properties, although ion-pair formation remains another possibility of the retention mechanism. The influence of several parameters on the chromatographic behaviour of NA, HNA and the internal standard used in the assay (pentothiobarbital) have been investigated. The critical micelle concentration (cmc) of the surfactant cetrimide in the mobile phase is a special point of interest in this study.

EXPERIMENTAL

Chemicals

Nalidixic acid and hydroxynalidixic acid were kindly supplied by Sterling Winthrop (Haarlem, The Netherlands). Pentothiobarbital was obtained by extraction with chloroform from an acidified aqueous solution of Pentothal® (Abbott, Campoverde, Italy). Methanol and chloroform were of analytical grade and were obtained from Merck (Darmstadt, G.F.R.). Cetrimonium bromide (cetrimide) was of European Pharmacopoean quality (OPG, Utrecht, The Netherlands).

Apparatus

The HPLC system consisted of a solvent delivery system 6000A, a universal injector U6K, a UV absorbance detector operated at 313 nm, a Model R401 differential refractometer and a μBondapak C_{18} column (particle size 10 μm), 30 cm × 3.9 mm I.D., all from Waters Assoc. (Milford, MA, U.S.A.). Peak areas were measured by means of a Spectra-Physics SP4000 data system (Santa Clara, CA, U.S.A.).

Conductance was measured with a Metrohm conductometer E518 in combination with a type EA645 titration cell (Metrohm, Herisau, Switzerland). Chromatographic and conductance measurements were carried out at 25°C.

Gas chromatography—mass spectrometry (GC—MS) analysis was performed on a Hewlett-Packard 5810 gas chromatograph; colum 2 m × 2.5 mm I.D., 3% OV-1 on Gas-Chrom Q (Chrompack, Middelburg, The Netherlands), 80—100 mesh; oven temperature 145°C; injection port temperature 300°C; carrier gas helium: flow-rate 25 ml min^{-1}; detection MS/total ion current. The mass spectrometer was a JEOL D300, with JMA-2000 data system; chamber voltage 70 eV; ion source temperature, 200°C; trap current 300 μA; accelerating voltage, 3 kV.

Adsorption of cetrimide onto the packing material (μBondapak C_{18})

The amount adsorbed was measured in the following way. A water—methanol (1:1, w/w) mixture containing an aqueous phosphate buffer pH 7.4 (final concentration of phosphate in the eluent, 0.016 mol kg^{-1}), was passed through the column. After equilibrium was reached, 0.12% (w/w) cetrimide was added to the eluent. The breakthrough volume of cetrimide was detected with a refractometric detector. Fractions were collected at the end of the chromatographic system and analyzed by GC—MS after evaporation of the solvent and reconstitution in methanol. The amount of cetrimide would then be $(V_R - V_0)C$, where V_R = breakthrough volume, V_0 = void volume and C = concentration of cetrimide in the eluent.

Determination of the critical micelle concentration

The cmc of cetrimide in the eluent was measured by titrating the eluent (without cetrimide), containing different percentages (0—50%, w/w) methanol, with a 50% (w/v) solution of cetrimide. Portions of 5 μl (at 0% methanol) up to 50 μl (at 50%, w/w, methanol) titrant were added stepwise to the eluent. After each addition the conductance was measured and plotted against the cetrimide concentration. The cmc is the concentration of cetrimide at the breakpoint of the titration curve.

Determination of NA and HNA in plasma

A 50-μl plasma sample was acidified with 50 μl 0.1 *M* hydrochloric acid and subsequently extracted with 1.50 ml chloroform containing the internal standard pentothiobarbital, 1.5 μg ml^{-1}. After mixing with a Vortex mixer for 30 sec and centrifugation at 2500 *g* for 2 min, the chloroform layer was transferred to another centrifuge tube and evaporated to dryness. The residue was dissolved in 50 μl eluent; 20 μl of this solution was injected into the chromatograph. The eluent consisted of a water—methanol (1:1, w/w) mixture, containing a phosphate buffer pH 7.4 (measured in water). The final concentrations of phosphate and cetrimide in the eluent are 0.016 mol kg^{-1} and 0.12% (w/w), respectively. The flow-rate was 1.5 ml min^{-1}; detection, UV absorbance at 313 nm.

RESULTS AND DISCUSSION

Adsorption of cetrimide onto the column

The method of measuring breakthrough volumes for the determination of the amount of cetrimide adsorbed onto the packing material has been described in the literature [6, 8]. Knox and Laird [6] collected 0.2-ml fractions of the eluate, which were added to a two-phase mixture of water, methylene chloride and propanol, containing the dye sunset yellow. Cetrimide, if present in the eluate, was extracted with the dye as ion-pair into the organic layer. However, the use of a differential refractometer, as described by Terweij-Groen et al. [8] is much more convenient.

In this study the latter method was used, but fractions of the eluate were still collected for GC—MS analysis.

Fig. 1 shows the refractometer response when 0.12% (w/w) cetrimide was added to the eluent.

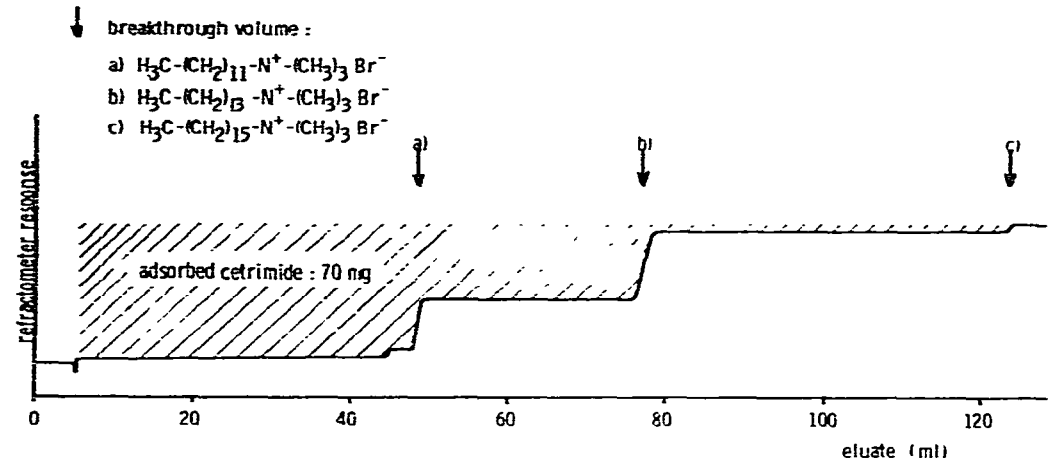
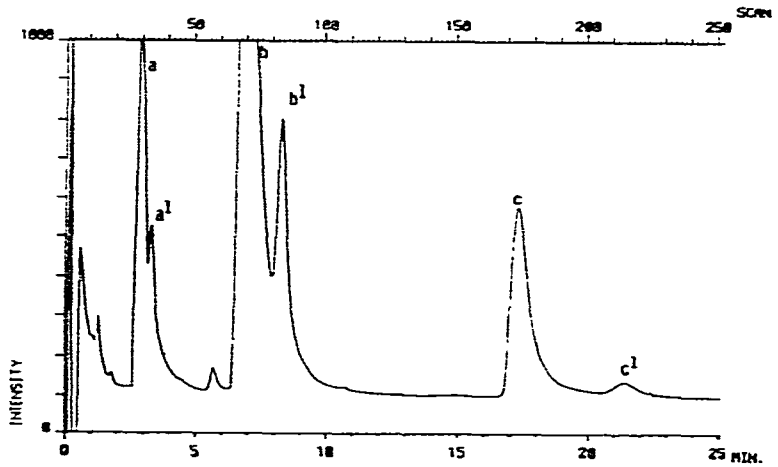


Fig. 1. Adsorption of cetrimide onto a μBondapak C_{18} column. Eluent: cetrimide, 0.12% (w/w); phosphate (pH = 7.4), 0.016 mol kg^{-1}; methanol—water (1:1, w/w).



peak	formula	m/e
a	$H_3C-(CH_2)_{11}-N(CH_3)_2$	213
a^1	$H_3C-(CH_2)_{11}Br$	248 (Br^{79}), 250 (Br^{81})
b	$H_3C-(CH_2)_{13}-N(CH_3)_2$	241
b^1	$H_3C-(CH_2)_{13}Br$	276 (Br^{79}), 278 (Br^{81})
c	$H_3C-(CH_2)_{15}-N(CH_3)_2$	269
c^1	$H_3C-(CH_2)_{15}Br$	304 (Br^{79}), 306 (Br^{81})

Fig. 2. GC—MS pyrogram of a cetrimide solution in methanol. For conditions, see Experimental.

Fig. 2 shows a pyrogram of cetrimide. The identity of the different compounds, as established by MS, is also displayed. When cetrimide is pyrolysed in the injection port of the gas chromatograph, two reactions predominate under the present conditions.

$$CH_3(CH_2)_nN^{\oplus}(CH_3)_3Br^{\ominus} \xrightarrow{\Delta T} CH_3(CH_2)_nN(CH_3)_2 + CH_3Br \quad (1)$$

$$CH_3(CH_2)_nN^{\oplus}(CH_3)_3Br^{\ominus} \xrightarrow{\Delta T} CH_3(CH_2)_nBr + N(CH_3)_3 \quad (2)$$

A comparable degradation pattern was observed for cetrimide in the presence of phosphate (same concentration as in the eluent). Choi et al. [10] described a method for the determination of cetrimide in pharmaceutical preparations, also based on GLC pyrolysis. They observed pyrolytic degradation according to pattern (1) only; however, their experimental conditions were different (a Carbowax KOH column was used and a much higher injection port temperature, 450°C, was applied).

Pyrolysis of different fractions of the eluate showed, that the three steps observed with the refractometer (Fig. 1) correspond with the breakthrough volumes of the three components of cetrimide, which differ in the length of the alkyl group.

Influence of the chromatographic conditions on the elution behaviour of NA, HNA and pentothiobarbital

In Fig. 3 the capacity ratio (k') is plotted versus the pH of the phosphate buffer, added to the eluent; the concentration of cetrimide and of phosphate were kept constant. On increasing the pH, the k' value of pentothiobarbital also increases. A phosphate buffer of pH 7.4 was chosen for the determination of NA and HNA; a good separation was then obtained.

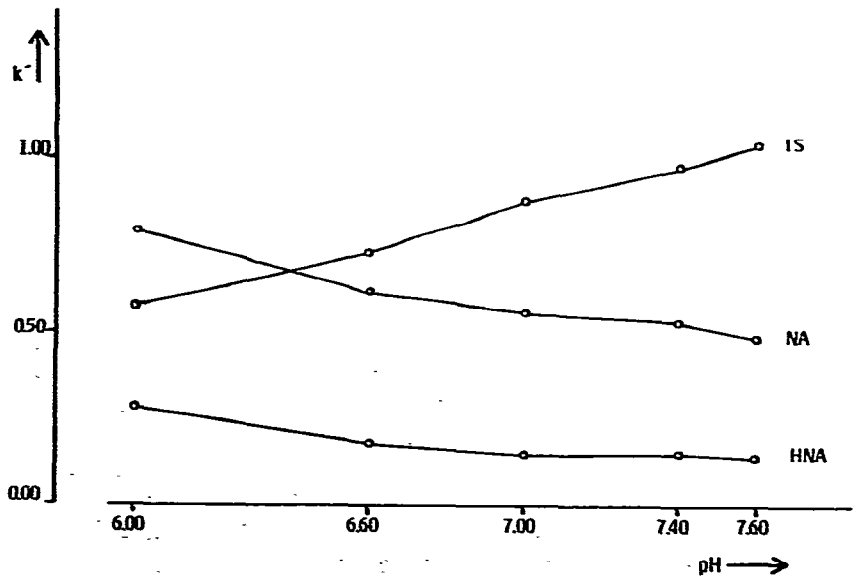


Fig. 3. Influence of the aqueous pH of the phosphate buffer on k'. Eluent: cetrimide, 0.12% (w/w); phosphate, 0.016 mol kg^{-1}; methanol—water (1:1, w/w).

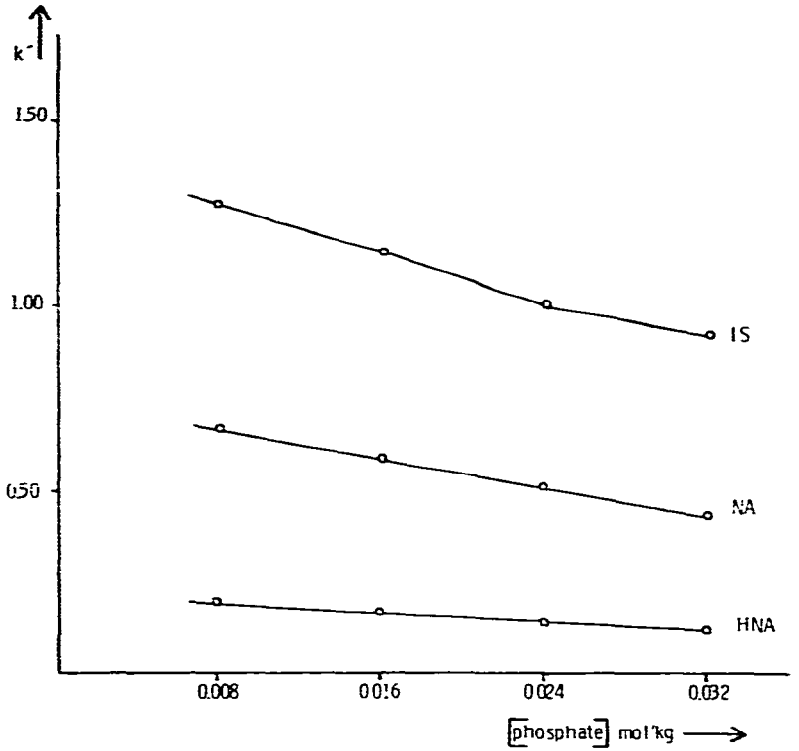


Fig. 4. Influence of the phosphate concentration at aqueous pH 7.4. Eluent: cetrimide, 0.12% (w/w); methanol—water (1:1, w/w).

Fig. 4 shows the relation between the phosphate concentration and k'. On increasing the phosphate concentration the retention times decrease. This is consistent with an ion-exchange process, which is thought to be the most important mechanism in these solvent-generated ion-exchange systems [7, 8]. With increasing phosphate concentration less solute anions will be retained, due to the competition with the phosphate ions, resulting in shorter retention times. Arbitrarily a concentration of 0.016 mol kg^{-1} phosphate was chosen for the determination of NA and HNA.

In Fig. 5 the cmc value, as calculated from the conductimetric titrations, is plotted versus the methanol concentration. For the determination of NA and HNA a concentration of 0.12% (w/w) cetrimide and 50% (w/w) methanol was chosen. In this eluent cetrimide micelles cannot be present, because the cmc in this eluent is about 1.5% (w/w) (see Fig. 5).

Fig. 6 shows the relationship between the cetrimide concentration and k' for NA, HNA and the internal standard. In the lowest cetrimide concentration region a small increase of the cetrimide concentration results in a relatively large increase of the retention times; indicating that partitioning of the undissociated acids between the eluent and the stationary phase is of secondary importance. With increasing cetrimide concentration in the mobile phase the amount of cetrimide adsorbed onto the stationary phase increases. Knox and Laird [6] showed that in their experiments the adsorption of cetrimide could

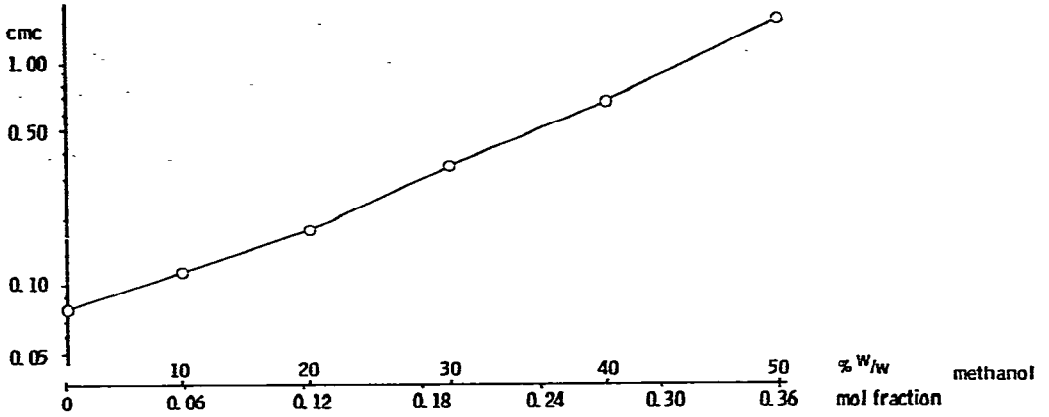


Fig. 5. Influence of the methanol concentration on the cmc of cetrimide in the eluent. Each solution contained a phosphate buffer, aqueous pH 7.4, with a final phosphate concentration of 0.016 mol kg^{-1}.

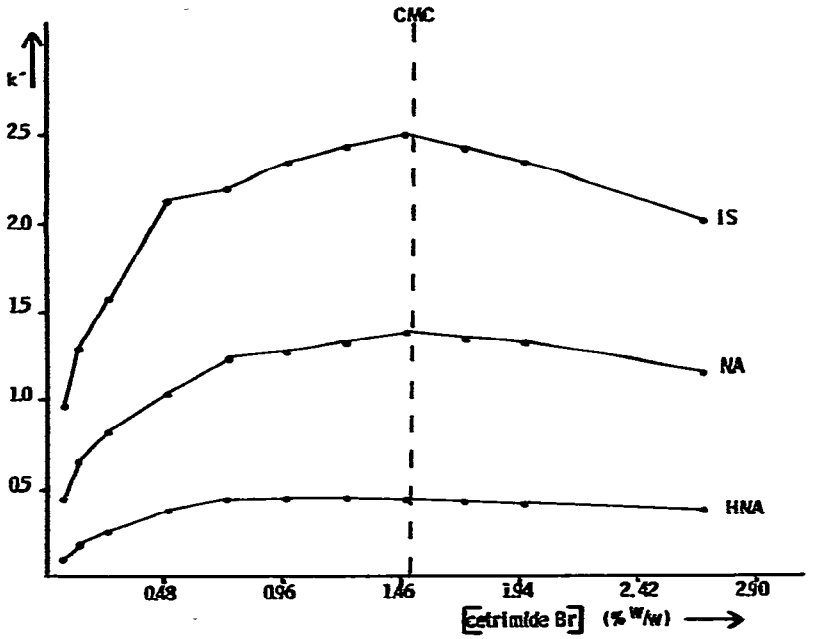


Fig. 6. Influence of the cetrimide concentration on k'. Eluent: phosphate (aqueous pH 7.4), 0.016 mol kg^{-1}; methanol—water (1:1, w/w).

be described by the Freundlich isotherm. The total amount of cetrimide adsorbed onto the stationary phase will hardly increase anymore at higher cetrimide concentrations in the mobile phase, resulting in an almost constant contribution to the retention mechanism by the ion-exchange process. The cetrimide concentration at which k' values are at a maximum, practically coincides with the cmc of cetrimide in the eluent. The decrease of k' values at cetrimide concentrations above 1.5% (w/w) can be explained by partitioning of

the solute anions between the mobile phase and the cetrimide micelles. A plot similar to Fig. 6 was obtained by Ghaemi and Wall [9]. Under the experimental conditions used in their investigations, the cmc of cetrimide* was also found to coincide with the cetrimide concentration at which maximal values for k' were obtained. It, therefore, appears that the role of cmc of cetrimide, and possibly of related compounds, in the mobile phase cannot be ignored as was suggested by Terweij-Groen et al. [8].

Determination of NA and HNA in plasma

Pentothiobarbital was chosen as the internal standard (IS), because it is extracted from acidified solutions, it can be detected at 313 nm and it has the required chromatographic properties. Following the procedure as described before, no decomposition of the internal standard was observed, even when after evaporation of the organic layer the residue was heated for 2 h at 60°C.

A calibration curve was constructed by analyzing 14 plasma samples (as described under Experimental) spiked with NA and HNA with concentrations ranging from 1 μg ml^{-1} up to 100 μg ml^{-1}. The peak area ratios (y), NA/IS and HNA/IS, were plotted against the concentration (x) of NA and HNA, respectively. The equations for the straight lines obtained for both species were calculated by the method of least squares and were found to be: $y = 0.0058 (\pm 0.0001)x + 0.0017 (\pm 0.0031)$ ($r^2 = 0.9995$) for NA, and $y = 0.0044 (\pm 0.0001)x + 0.0137 (\pm 0.0040)$ ($r^2 = 0.9987$) for HNA.

The reproducibility of the method was examined by analyzing two series of six samples each, to each of which was added NA and HNA, at concentrations for both compounds of 2 μg ml^{-1} and 20 μg ml^{-1}, respectively. The coefficients of variation were for NA and HNA 10% and 8%, respectively at 2 μg ml^{-1} and 2.7% and 3.5% at 20 μg ml^{-1}. The absolute recoveries of NA and HNA from plasma samples were determined following the procedure and calculated to be 90% for both species.

Using 50-μl plasma samples the detection limit for NA and HNA is about 1 μg ml^{-1}. Therapeutic levels for NA are in the range of 20—50 μg ml^{-1} plasma, so the method is sensitive enough.

Fig. 7 shows chromatograms obtained from blank plasma and from a plasma sample of a patient suffering from a complicated infection and renal failure, who was treated intravenously with 4 g NA per day. Apparently HNA levels in these patients are sufficiently high to be quantified by our method.

Recently Cuisinaud et al. [11] published a method comparable to the one described in this paper for the determination of NA, HNA and another metabolite, 1-ethyl-1,4-dihydro-4-oxo-1,8-naphtyridine-3,7-dicarboxylic acid (which has not been found in plasma). The influence of the various parameters on the chromatographic behaviour of NA and its metabolites was not reported. In this method [11] a double extraction procedure of 1-ml plasma samples was applied. This double extraction is necessary because a detection wavelength of 254 nm was chosen. In our experiments we observed that a number of plasma peaks interfere in the chromatogram at this wavelength after single

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*This brand of cetrimide has as its major component $H_3C-(CH_2)_{15}-N^{\oplus}(CH_3)_3Br^{\ominus}$.

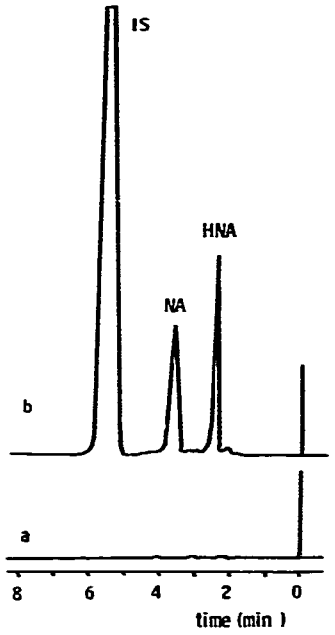

Fig. 7. Chromatograms obtained by analysis of 50 μl plasma. For conditions see Experimental. (a) Plasma blank; (b) plasma of a patient treated with nalidixic acid; NA, 17 $\mu g\ ml^{-1}$; HNA, 21 $\mu g\ ml^{-1}$.

extraction; these peaks are not detected at 313 nm. However, measuring at 254 nm has the advantage of lower detection limit.

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