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THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE ANALYSIS OF NALIDIXIC ACID IN HUMAN PLASMA

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SUMMARY

A sensitive and highly selective thin-layer chromatographic method for determining plasma levels of nalidixic acid is presented. Plasma (50 μl) was acidified with 50 μl 1 *M* orthophosphoric acid and extracted with 100 μl toluene. A 40-μl aliquot of the extract was spotted onto the thin-layer plate with the aid of a Desaga Autospotter and, after irrigation, the nalidixic acid on the plate was converted into a fluorescent compound by exposing the plates to hydrogen chloride gas for 10 min and then to strong ultraviolet radiation from a mercury lamp for 10 min. The fluorescence was measured quantitatively using a spectrofluorimeter equipped with a thin-layer chromatogram scanning attachment.

INTRODUCTION

Nalidixic acid has been in use as an antibacterial agent in urinary tract infections since 1963. Numerous methods for the determination of nalidixic acid in pharmaceutical products and in biological specimens have been published. Among these are chemical methods based on acid—base titrations [1—3], microbiological [4], fluorimetric [5—7], UV spectrophotometric [8—10], gas—liquid chromatographic [11, 12], high-performance liquid chromatographic [13—16] and polarographic [17] methods. No quantitative thin-layer chromatographic (TLC) method has been established yet.

Whereas for previous assay methods at least 0.5 ml plasma was required we describe here a simple and specific TLC-densitometric method by means of which nalidixic acid can be determined in duplicate with good precision at therapeutic levels in a 100-μl plasma sample.

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EXPERIMENTAL

Reagents

All reagents used were of guaranteed reagent grade (Merck, Darmstadt, G.F.R.) and were used without further purification. Nalidixic acid was a gift from Winthrop (Durban, South Africa), and was used as received.

Apparatus

A Perkin-Elmer MPF3 spectrofluorimeter equipped with a TLC scanning attachment was used to measure the fluorescence of the spots on the plates using the following conditions: light source, xenon lamp; excitation wavelength, 375 nm; emission wavelength, 430 nm cut-off filter; excitation and emission slit widths, amplifier sensitivity and sample-adjustment were set so as to obtain approximately 80% of full-scale deflection on the recorder when the strongest spot was being scanned.

The other apparatus used consisted of silica gel 60 TLC plates (Merck), without fluorescent indicator; one 20 cm × 20 cm plate was cut into three plates of 6.7 cm × 20 cm; a Shandon S/P Chromatank; Hamilton dosing syringes (50 μl and 100 μl); 500-μl conical plastic microfuge tubes (Beckman Instruments, Johannesburg, South Africa); 10-μl disposable glass capillary micropipettes (Clay Adams division of Becton Dickinson, Parsippany NJ, U.S. A.); a Universal UV lamp, type TL/900 (Camag, Muttenz, Switzerland).

Plasma standards

An accurately weighed amount of nalidixic acid was dissolved in a weighed amount of fresh human plasma by shaking for 4 h. By using an average density of 1.027 for plasma [18] the nalidixic acid concentration can be calculated. Weighed amounts of this plasma stock solution were further diluted with weighed amounts of plasma to obtain standards with lower concentrations of nalidixic acid. Aliquots (0.5 ml) of the standard plasmas were stored frozen (−20°C) in 1-ml sealed glass ampoules.

Extraction

Volumes of plasma (50 μl), standard or unknown, were measured with the dosing syringe into the plastic conical microfuge tube followed by 50 μl 1 *M* orthophosphoric acid and 100 μl toluene. After shaking the stoppered microfuge tubes thoroughly by hand for 1 min, they were centrifuged briefly.

Spotting the plates

Aliquots of the toluene extracts (40 μl) were spotted 1 cm from the margin on the TLC plates using the Desaga Autospotter. Sixteen spots with unknown (*X*) and standard (*S*) amounts alternating in duplicate were applied to a 6.7 cm × 20 cm plate (*S*1 *X*1 *S*2 *X*2 *S*3 *X*3 *S*4 *X*4 etc.). The solutions were spotted at the maximum rate available (10 μl/min) with the autospotter while the fan speed was adjusted to evaporate the toluene at a rate which only just prevented the solvent fronts of the spots merging with each other.

Chromatography

Ascending development was carried out in an unsaturated tank up to the end of the plate at 6.7 cm with the mobile phase, dioxane—5 × diluted concentrated ammonia solution (3:1). The elution time was about 30 min at room temperature (ca. 23°C) and relative humidity of about 15%. After brief drying with a warm hairdryer the plates were dried by heating at 90°C for 5 min in a ventilated oven.

Induction of fluorescence and quantitation

The plates were allowed to cool down to room temperature and were then exposed for 10 min to hydrogen chloride gas generated in a chromatography tank by adding 2 ml concentrated hydrochloric acid to 10 ml concentrated sulphuric acid contained in a small beaker in the tank.

The hydrogen chloride treated plates were immediately exposed to UV radiation from an unfiltered mercury discharge lamp for 10 min. This procedure converted the nalidixic acid into a strongly fluorescent compound on the TLC plate ($R_F = 0.4$).

Each spot was scanned in the TLC scanning attachment of the MPF3 spectrofluorimeter in the direction of the solvent flow. Standard curves were constructed by plotting peak heights versus plasma concentrations of the known standards. The concentrations of unknown samples were then obtained by interpolation.

RESULTS AND DISCUSSION

Fig. 1 represents part of a densitogram showing peaks obtained for standard plasma containing the indicated amount of nalidixic acid, plasma samples of a

TABLE I

RECOVERY OF NALIDIXIC ACID FROM HUMAN PLASMA

In each case $n = 4$.

Method*	Nalidixic acid added (μg)	Mean recovery (μg)	C.V. (%)
a	2.34	2.32	9.5
	4.37	4.66	2.8
	8.72	9.08	2.1
	17.28	17.09	0.9
b	2.34	2.46	8.9
	4.37	4.88	6.6
	8.72	9.05	3.4
	17.28	16.50	6.0
c	2.34	2.18	11.9
	4.37	3.98	13.8
	8.72	8.85	6.1
	17.28	17.53	4.8

*(a) Spots scanned in direction of solvent flow; (b) spots scanned across direction of solvent flow; (c) spots applied manually (10 μl).

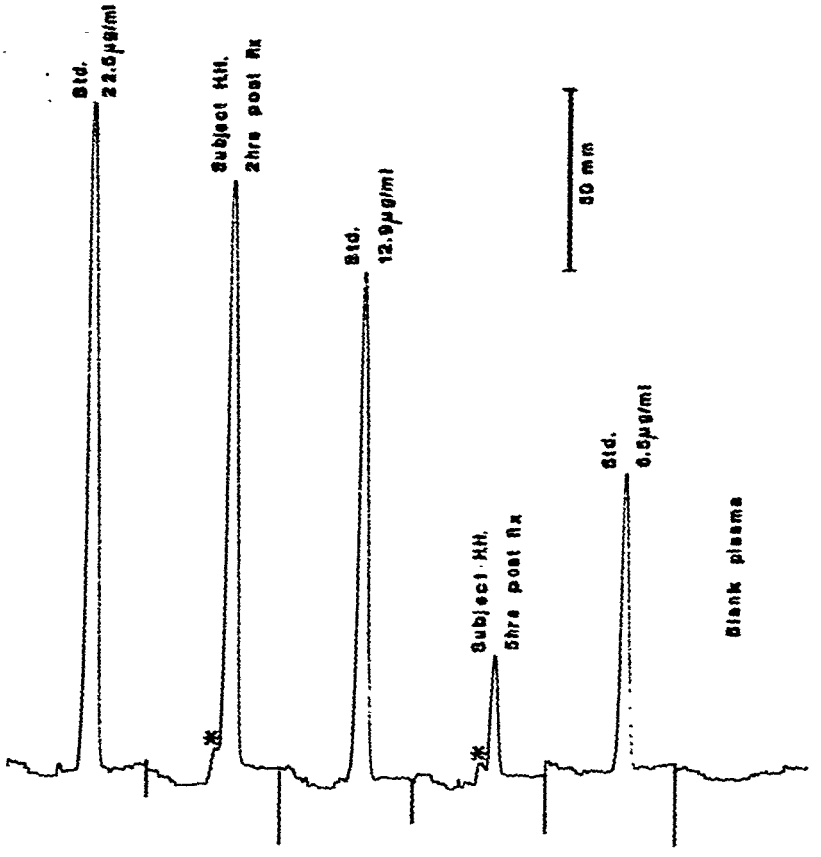

Fig. 1. Densitogram of nalidixic acid in plasma of a volunteer 2.5 h after ingestion of 1 g nalidixic acid; in plasma standards and blank plasma. Asterisk (*) indicates peak possibly due to hydroxynalidixic acid.

volunteer obtained 2 and 5 h after ingestion of 1 g of nalidixic acid as well as a blank plasma showing the absence of interfering endogenous compounds.

A summary of the recoveries of nalidixic acid added to normal human plasma is presented in Table I.

Little time could be saved by scanning the plates across the direction of solvent flow since the R_F values of the spots were found to be strongly dependent on the concentration of the nalidixic acid requiring frequent adjustment of the plate position while scanning. However, due to slight tailing producing elongated spots the detection limit is lower when the plates are scanned across the direction of solvent flow (characteristic of the slit geometry). A densitogram obtained of a plate scanned in such a way is shown in Fig. 2.

As an alternative to spotting the plates with the Desaga Autospotter, 10-µl aliquots of the toluene extracts were applied by means of calibrated 10-µl glass micropipettes allowing the whole volume to run into the plate by capillary action in a single application. Although the precision is lower using this method (see Table I) it is adequate for therapeutic monitoring. The main drawback of this manual spotting procedure is the time required to spot a large number of samples.

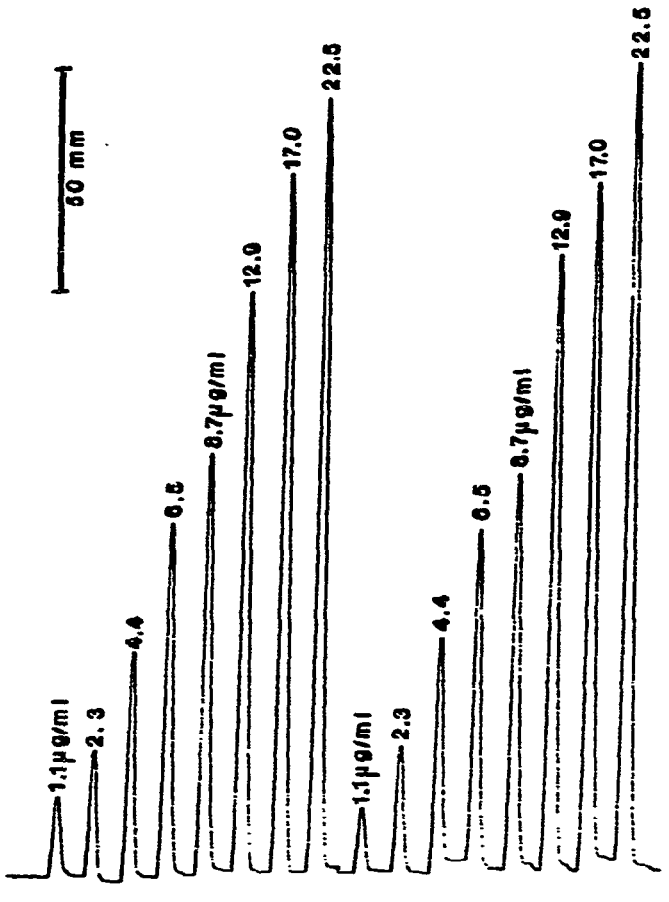

Fig. 2. Densitogram of spiked plasma samples scanned across the direction of solvent flow.

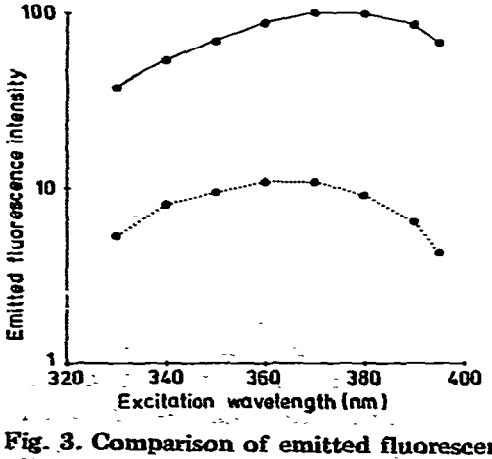

Fig. 3. Comparison of emitted fluorescence intensity obtained using two different types of silica gel plates. •——•, Silica gel 60 (without fluorescence indicator, Merck No. 5721); •.....•, silica gel F60 (with fluorescence indicator, Merck No. 5715).

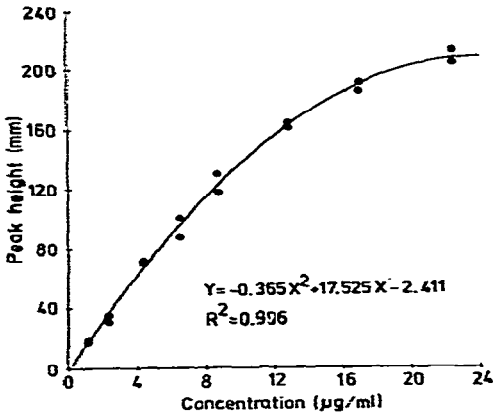

Fig. 4. Parabolic calibration curves obtained with spiked plasma standards.

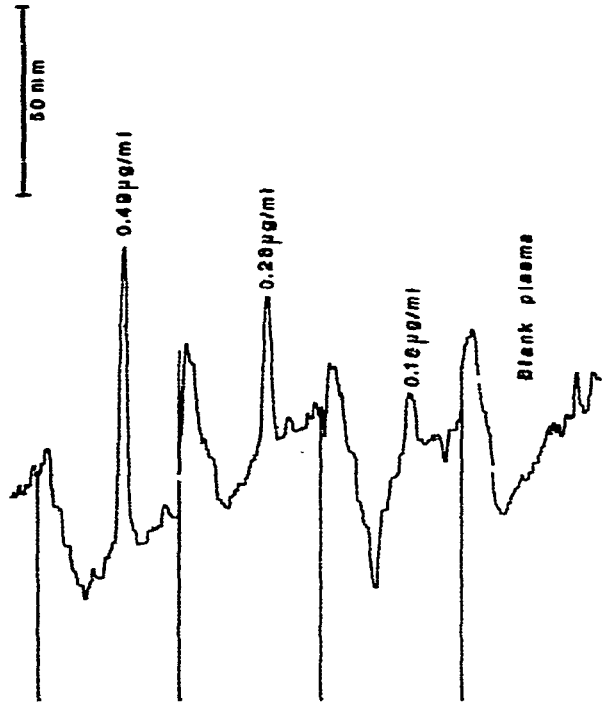

Fig. 5. Densitogram of plasma samples with very low concentrations of nalidixic acid.

The marked effect of the type of silica gel plates used on the fluorescence obtained is shown in Fig. 3 which shows the relative emitted fluorescence intensities obtained using silica gel plates F60 (with fluorescence indicator, Merck No. 5715) and silica gel 60 (without fluorescence indicator, Merck No. 5721). Note the logarithmic scale. These two types of plates were compared because, although fluorescence is being measured, we have in the case of other assays found it necessary on occasions to use plates with fluorescence indicator to be able to detect the presence of UV-absorbing compounds which may interfere

with the assay of the fluorescing compounds. In this case the marked difference in fluorescence intensity obtained was particularly noteworthy and is probably due to a relatively selective interaction between nalidixic acid and a component of the fluorescence indicator. The integrity of the fluorescence indicator is actually destroyed by the treatment with the hydrogen chloride gas.

Using the Autospotter to apply 40 μl of the toluene extract it was found by linear regression analysis that parabolic curves of the form $y = Ax^2 + Bx + C$ (y = peak height, x = plasma concentration) fitted the calibration data up to plasma concentrations of 22.5 μg/ml with correlation coefficients $r^2 = 0.99$ (n = 16) (Fig. 4). When concentrations higher than about 20 μg/ml are expected smaller volumes of the extracts should be spotted.

The lowest demonstrable concentration of nalidixic acid in plasma (spotting 40 μl of the toluene extract) was about 0.16 μg/ml. Fig. 5 shows a densitogram obtained for standard plasma samples with very low concentrations of nalidixic acid.

Selectivity

No interference with the determination was observed with the following compounds: mephenamic acid, flufenamic acid, niflumic acid, thiaprofenic acid, nalidixic acid, flubiprofen, fenoprofen, ketoprofen, ibuprofen, indoprofen, fenbufen, alclofenac, diclofenac, oxyphenbutazone, phenylbutazone, naproxen, probenecid, sulindac, indomethacin, carbamazepine and its two major metabolites carbamazepine epoxide and dihydroxycarbamazepine, penicillin G, furosemide, piretanide, all at a concentration of 20 μg/ml; paracetamol at 50 μg/ml; and salicylic acid at a concentration of 300 μg/ml.

Hydroxynalidixic acid, a known metabolite, was not available to be tested for possible interference although the small peak indicated by the asterisk in Fig. 1 which was obtained in the plasma of volunteers who had taken a single dose of 1 g of nalidixic acid in a bioavailability trial is probably the compound in question. In the trial this compound never attained concentrations which interfered with the nalidixic acid assay; however it is possible that during chronic treatment with nalidixic acid it may become troublesome.

This method was used to determine nalidixic acid in plasma samples obtained from volunteers participating in a comparative bioavailability trial. The results obtained in this trial are summarised in Table II.

TABLE II

PHARMACOKINETIC PARAMETERS FOR ORAL ABSORPTION OF 1 g OF NALIDIXIC ACID AS TABLETS IN THREE DIFFERENT FORMULATIONS

Mean (± S.D.) in six normal trial subjects.

Mean peak plasma concentration (μg/ml)	Mean time to peak plasma concentration (h)	Mean AUC* (0—32 h) (μg · h/ml)	Mean plasma halflife (min)
19.75 ± 6.14	2.25 ± 0.52	181 ± 65	
26.80 ± 11.57	2.58 ± 0.38	208 ± 96	93 ± 38
20.79 ± 16.21	2.08 ± 0.92	168 ± 101	

*AUC = Area under curve.

The halflife of 93 ± 38 min found is based on a one-compartment pharmacokinetic model and a blood sampling period of 10 h. This closely resembles the results obtained by other workers [5, 7]. However, with blood sampling time extended to include 24-h and 32-h samples, preliminary results indicate that the pharmacokinetics of nalidixic acid may be more accurately described by a two-compartment model with an alpha-phase constant of approximately 0.68 h^{-1} and a beta-phase constant of about 0.077 h^{-1}.

The main advantages of this TLC method lie in its simplicity, allowing a large number of samples to be processed per day, and its low sample-volume requirement which is a factor playing an increasingly important role in clinical trials where very often several parameters have to be monitored in each blood sample taken from the patient or volunteer.

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