(11) G. V. R. Born, R. J. Haslam, M. Goldman, and R. D. Rowe, *Nature*, **205**, 678(1965).
(12) E. Mihich, D. A. Clarke, and F. S. Philips, *J. Pharmacol. Exp. Ther.*, **111**, 335(1954).
(13) D. A. Clarke, J. Davoll, F. S. Philips, and G. B. Brown, *ibid.*, **106**, 291(1952).
(14) J. A. Angus, L. B. Cobbin, R. Einstein, and M. H. Maguire, *Brit. J. Pharmacol.*, **41**, 592(1971).
(15) R. F. Furchgott and S. Bhadrakom, *J. Pharmacol. Exp. Ther.*, **108**, 129(1953).
(16) M. E. Reid, "The Guinea Pig In Research," Human Factors Research Bureau, Inc., Washington, D. C., 1958, p. 19.

ACKNOWLEDGMENTS AND ADDRESSES

Received March 12, 1973, from the *Department of Pharmacology and Toxicology, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907*

Accepted for publication April 6, 1973.

Supported by National Institutes of Health Grant GM 1500J and by a General Research Support Grant.

The authors thank Dr. Heinz Floss and Dr. R. Suhadolnik for supplying the toyocamycin. They also thank Dr. J. Wells for his advice and helpful suggestions.

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Nalidixic Acid and Hydroxynalidixic Acid Analysis in Human Plasma and Urine by Liquid Chromatography

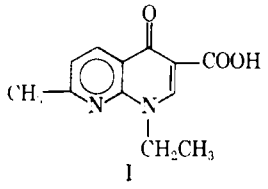
L. SHARGEL▲, R. F. KOSS, A. V. R. CRAIN, and V. J. BOYLE

Abstract □ A high pressure liquid chromatographic method was developed for the assay of nalidixic acid and hydroxynalidixic acid in human plasma and urine. This procedure measures both compounds in the same sample. The lower limits of detection for nalidixic acid and hydroxynalidixic acid were 0.25 mcg./ml. of each in plasma and 2.5 mcg./ml. of each in urine. Correlation coefficients for the analysis of nalidixic and hydroxynalidixic acids in urine of humans medicated with two different tablet formulations were 0.83 and 0.94 in comparison with a microbiological assay.

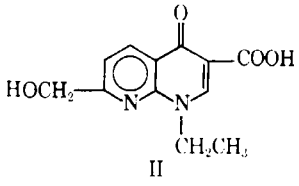
Keyphrases □ Nalidixic and hydroxynalidixic acids—analysis in human plasma and urine, high pressure liquid chromatography □ Hydroxynalidixic and nalidixic acids—analysis in human plasma and urine, high pressure liquid chromatography □ High pressure liquid chromatography—analysis, nalidixic and hydroxynalidixic acids in human plasma and urine

Nalidixic acid[1] (I) is currently used as an antibacterial agent in urinary tract infections (1–3). A major metabolite is hydroxynalidixic acid (II). This metabolite has an *in vitro* antibacterial spectrum similar to that of nalidixic acid (4). Both compounds have similar chemical structures and fluorescent spectra.

The original fluorescent method for the assay of nalidixic acid and hydroxynalidixic acid (4) involved differential extraction, which provided only partial separation of nalidixic acid and hydroxynalidixic acid. Thus, simultaneous equations were employed for the determination of the two drugs. Furthermore, blank values were occasionally high and erratic, giving unsatisfactory values for nalidixic acid.



I



II

The use of liquid chromatography for the separation and quantitation of many compounds has increased in recent years. The availability of a modern, high speed, liquid chromatograph allows the measurement of both nalidixic acid and hydroxynalidixic acid simultaneously. This report describes a new liquid chromatographic assay procedure for nalidixic acid and hydroxynalidixic acid which is more rapid and precise than previously published methods.

EXPERIMENTAL

Materials—Nalidixic acid[2] and hydroxynalidixic acid[2] were routinely prepared in the same standard solution of 0.03 *N* NaOH. Chloroform, analytical reagent grade[3], was used for all extractions. All other reagents were purchased from commercial sources.

Plasma Assay—To a 15-ml. glass-stoppered centrifuge tube were added 1.0 ml. of plasma[4] and 1.0 ml. of a standard solution containing both nalidixic acid and hydroxynalidixic acid in concentrations ranging from 0 to 40 mcg./ml. For unknown plasma samples, 1.0 ml. of sample was added to 1.0 ml. of 0.03 *N* NaOH. To this mixture was added 0.5 ml. of 0.6 *N* HCl (final pH approximately 1.0) and 5 ml. of chloroform. The tubes were stoppered, shaken 5 min., and centrifuged. The upper aqueous phase was then aspirated. A 4.0-ml. aliquot of the chloroform phase was transferred to a clean 15-ml. glass-stoppered centrifuge tube, and 1.0 ml. of 0.03 *N* NaOH was added. The tubes were shaken for 5 min. and centrifuged. The aqueous phase containing nalidixic acid and hydroxynalidixic acid was then subjected to high pressure liquid chromatography.

Urine Assay—To a 50-ml. glass-stoppered extraction tube were added 1 ml. of urine and 1 ml. of a standard solution containing 0–250 mcg./ml. each of nalidixic acid and hydroxynalidixic acid. For unknown urine samples, a 1.0-ml. aliquot was added to 1.0 ml. of 0.03 *N* NaOH. The glucuronides of nalidixic acid and hydrox-

[1] NegGram, Winthrop Laboratories, New York, N. Y.

[2] Supplied by Sterling-Winthrop Research Institute, Rensselaer, NY 12144

[3] Mallinckrodt Chemical Works, St. Louis, Mo.

[4] Since fresh human plasma caused less emulsion formation during extraction than outdated Red Cross plasma, the former was more desirable to use for processed standards.

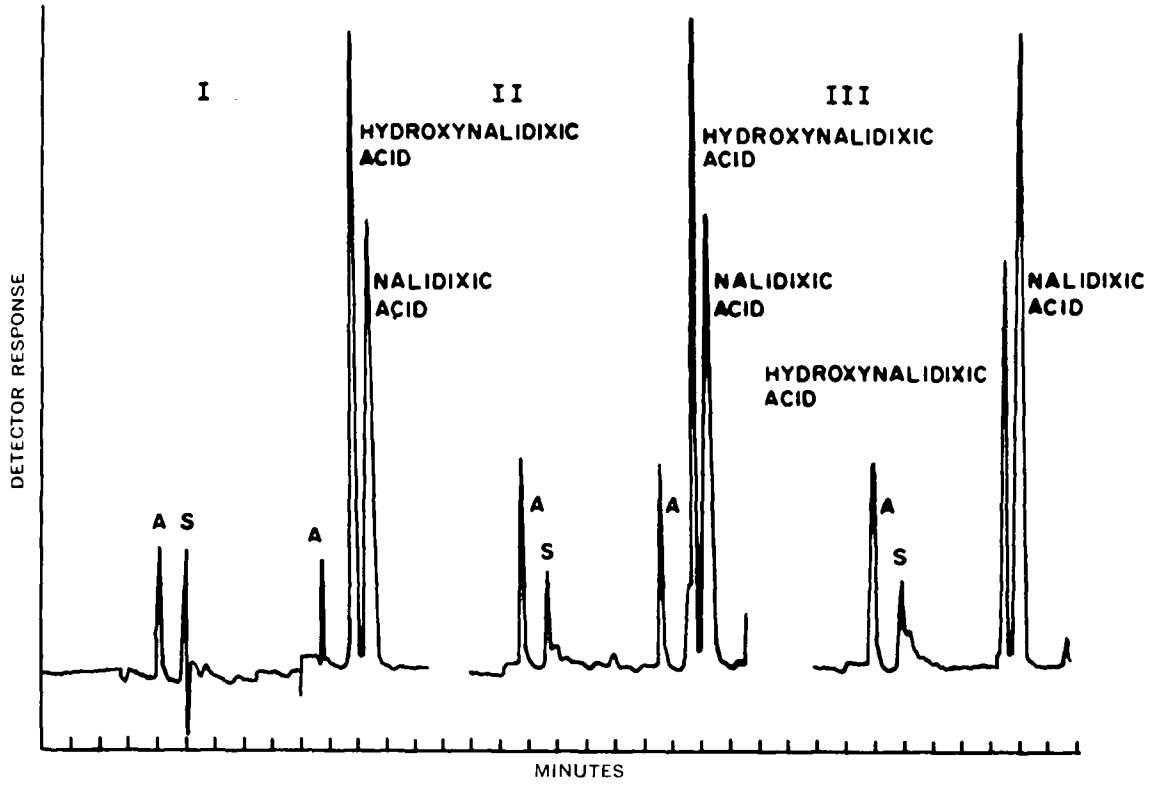


Figure 1—*Liquid chromatograms of processed standards of nalidixic acid and hydroxynalidixic acid in plasma and urine. Key: I, plasma blank (attenuation 0.02), and plasma containing 10 mcg./ml. each of nalidixic acid and hydroxynalidixic acid (attenuation 0.02); II, nonhydrolyzed urine blank (attenuation 0.02), and nonhydrolyzed urine containing 25 mcg./ml. each of nalidixic acid and hydroxynalidixic acid; III, hydrolyzed urine blank (attenuation 0.02), and hydrolyzed urine (attenuation 0.16) containing 280 mcg./ml. nalidixic acid and 125 mcg./ml. hydroxynalidixic acid; A, sample injection; and S, solvent front. Liquid chromatographic conditions are described in the* Experimental *section.*

ynalidixic acid were hydrolyzed by adding 0.5 ml. of 0.6 *N* HCl (final pH approximately 1.0) and heating the extraction tube on a steam bath for 30 min. The mixture was then neutralized with 0.5 ml. of 0.6 *N* NaOH. To the extraction tubes containing nonhydrolyzed urine samples was added 0.5 ml. of 0.6 *N* NaOH to keep the ionic strength and pH constant.

To both the hydrolyzed and nonhydrolyzed urine mixtures were added 1 ml. of a 0.2 *N* sodium phosphate buffer, pH 6.0, and 1.0 ml. of water. Nalidixic acid and hydroxynalidixic acid were extracted into 25 ml. of chloroform. The tubes were shaken for 5 min., followed by centrifugation. The aqueous upper phase was aspirated, and a 4.0-ml. aliquot of the lower chloroform phase was placed into a clean 15-ml. glass-stoppered centrifuge tube containing 1.0 ml. of 0.03 *N* NaOH. The tube was shaken for 5 min. and centrifuged. Then the aqueous phase containing nalidixic acid and hydroxynalidixic acid was subjected to high pressure liquid chromatography.

Liquid Chromatography[5]—Solutions were chromatographed at room temperature on a 0.5-m. stainless steel column (2.1 mm. i.d.) packed with Zipax SAX[6] strong anion-exchange resin. The eluting mobile phase was a solution containing 0.05 *M* sodium sulfate and 0.02 *M* boric acid (pH 9). Column pressure was maintained at 600 p.s.i. by compressed nitrogen gas. The flow rate of the eluate was 0.8 ml./min.

A 10-μl. aliquot of the extracted urine or plasma sample was chromatographed by a modified stop-flow procedure[7]. Direct standards of nalidixic acid and hydroxynalidixic acid were made in 0.03 *N* NaOH and chromatographed similarly. Recovery of nalidixic acid and hydroxynalidixic acid was calculated by comparing the peak heights of the processed standards to standards made directly in base. Concentrations of the unknown urine or plasma samples were calculated by comparing the peak heights of the extracted samples to processed standards.

Correlation with Microbiological Assay—Two tablet formulations containing 1000 mg. of nalidixic acid[8], but differing in tablet excipients, were administered orally to 16 adults of both sexes. The drug was administered with water after an 8-hr. fast. A premedication urine sample and a 0–24-hr. postmedication urine sample were obtained. After measurement of the volume and pH, the urine samples were frozen until analyzed for free and conjugated nalidixic and hydroxynalidixic acids by the liquid chromatographic assay. Only nonconjugated, biologically active nalidixic acid and hydroxynalidixic acid were analyzed by the microbiological assay.

Microbiological Assay—Urinary levels of nalidixic and hydroxynalidixic acids were determined by a modified, four-point, agar-diffusion method (5). Nalidixic acid standards were prepared in pooled, normal human urine to give final concentrations of 10 and 20 mcg./ml. Urine samples from medicated subjects were diluted appropriately to give estimated concentrations corresponding to those of the standards. By means of forceps, 1.3-cm. (0.5-in.) filter paper disks were saturated by immersion into the standard or sample solutions and excess fluid was removed. Saturated disks from the standard and sample solutions were placed on the surface of each agar plate seeded with the test organism, *Klebsiella pneumoniae* (ATCC 10872). Five plates containing standard and sample disks at constant dilution intervals were employed for each assay.

Following a prediffusion period of 2 hr. at 4°, all plates were incubated at 37° for 16–18 hr. and the zones of inhibition (diameters in millimeters) were recorded. The levels of biologically active drug (nalidixic acid and hydroxynalidixic acid) in the samples were calculated by extrapolation from the standard dose responses (6).

[5] Performed on a duPont model 840 liquid chromatograph equipped with a UV (254 nm.) photometric detector and a Hewlett-Packard model 7128A strip-chart recorder.

[6] Instrument Product Division, E. I. duPont de Nemours & Co., Wilmington, Del.

[7] The modified stop-flow procedure is as follows. The liquid chromatograph is maintained at 600 p.s.i. The valve is turned to the "Flow Off" position. After 30 sec., 10 μl. of the sample is injected on the column. The valve is then placed on the "Flow On" position and the sample is chromatographed. This method provided sharper peaks, possibly due to less diffusion of the drug prior to being chromatographed through the column and less "blowback" of sample from the syringe.

[8] NegGram Caplet, Winthrop Laboratories, New York, N. Y.

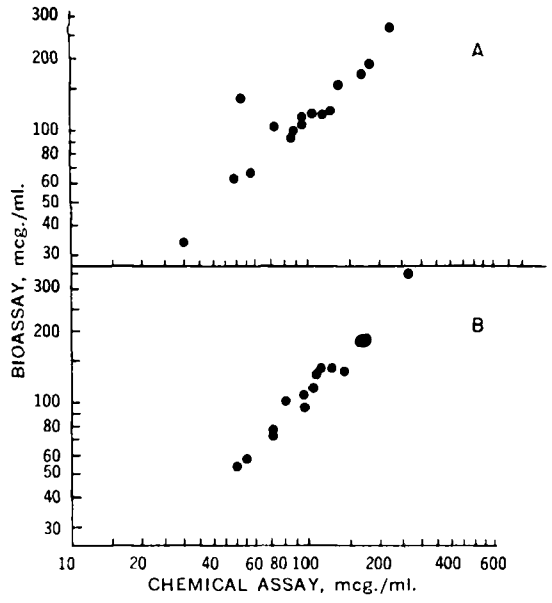


Figure 2—*Correlation of the liquid chromatographic and microbiological assays for nalidixic acid and hydroxynalidixic acid in urine. Human volunteers were administered 1000 mg. of nalidixic acid contained in tablet Formulation A or Formulation B. Nalidixic acid and hydroxynalidixic acid in the 24-hr. urine samples were assayed by both methods. Correlation coefficients for Formulations A and B were 0.83 and 0.94, respectively.*

RESULTS

Nalidixic acid and hydroxynalidixic acid were measured simultaneously by liquid chromatography. The retention times for hydroxynalidixic acid and nalidixic acid were 1.2 and 1.6 min., respectively. Chromatograms from control plasma and control urine yielded a small blank which did not interfere with the chromatograms from processed standards (Fig. 1). Plasma extracts demonstrated an "on-column" lower detection limit of 2 ng. each of nalidixic acid and hydroxynalidixic acid, corresponding to a plasma concentration of 0.25 mcg./ml. for each drug. Urine extracts demonstrated an on-column lower detection limit of 4 ng. each of nalidixic acid and hydroxynalidixic acid, corresponding to a urine concentration of 2.5 mcg./ml. for each drug. The sensitivity of this assay was comparable to that of the fluorescent method (4). Processed standards in plasma and urine were linear in concentrations up to 250 mcg./ml. One advantage of this method is that high urine levels need not be reprocessed; the sensitivity of the instrument is attenuated upward and the sample is reinjected.

Recoveries of processed standards of nalidixic acid from plasma and urine were 99.2 and 101%, respectively; the recoveries of processed standards of hydroxynalidixic acid were 91.0 and 88.8%, respectively (Table I). The lower recovery of hydroxynalidixic acid is possibly due to partitioning between the aqueous and chloroform phases. The small standard errors shown for the recovery data in Table I demonstrate that these compounds can be measured with high precision.

Urine from patients medicated with two different oral tablet formulations of nalidixic acid were subjected to both the microbiological and liquid chromatographic assays (Fig. 2). The mean ($\pm SE$) urine levels of nalidixic acid and hydroxynalidixic acid after administration of Formulation A were 121 ± 55.1 mcg./ml. (biological) and 109 ± 53.2 mcg./ml. (liquid chromatographic). The mean ratio of biological to liquid chromatographic values was 1.20. Only one major discrepancy was noted between the two assays (ratio 2.50). The mean ($\pm SE$) urine levels of nalidixic acid and hydroxynalidixic acid after administration of Formulation B were 129 ± 71.9 mcg./ml. (biological) and 110 ± 55.6 mcg./ml. (liquid chromatographic). The mean ratio of biological to chemical values was 1.04. Correlation coefficients for Formulations A and B were 0.83 and 0.94, respectively.

Table I—Recovery of Nalidixic Acid and Hydroxynalidixic Acid from Plasma and Urine[a]

Concentration, mcg./ml.	Percent Recovery	
	Nalidixic Acid	Hydroxynalidixic Acid
	Plasma	
1.0	98.9	76.9
2.5	95.0	91.1
5.0	101	94.4
10	102	93.8
20	98.9	94.2
30	99.4	94.5
Mean ± *SE*	99.2 ± 1.0	91.0 ± 3.1
	Urine	
12.3	101	89.8
25	101	89.7
50	100	87.7
100	99.7	87.2
150	104	89.5
Mean ± *SE*	101 ± 0.6	88.8 ± 0.5

[a] Standards were made in 0.03 *N* NaOH. Dilutions were added to human urine or plasma and processed according to the procedure described in the *Experimental* section.

DISCUSSION

The advantages of the liquid chromatographic assay of nalidixic acid and hydroxynalidixic acid include: (*a*) simple, rapid, and reproducible analyses of a multicomponent system without the need for an involved separation and purification method; and (*b*) more precision and specificity than the fluorescent method used previously. Moreover, 30–40 samples/day (8 hr.) can be determined by a single technician compared to 6–8 samples/day by the fluorescent method.

Microbiological assays of antibacterial agents such as nalidixic acid and hydroxynalidixic acid have been used frequently in the clinic. Although the bioassay is not specific (both nalidixic acid and hydroxynalidixic acid have similar antibacterial activity), the high degree of correlation between these methods demonstrates that either assay can be used routinely in the clinical laboratory.

REFERENCES

(1) G. Y. Lesher, E. J. Froelich, M. D. Gruett, J. H. Bailey, and R. P. Brundage, *J. Med. Chem.*, **5**, 1063(1962).

(2) I. V. Lishman and J. Swinney, *Brit. J. Urol.*, **35**, 116(1963).

(3) T. A. Stamey, N. J. Nemoy, and M. Higgins, *Invest. Urol.*, **6**, 582(1969).

(4) F. W. McChesney, E. J. Froelich, G. Y. Lesher, A. V. R. Crain, and D. Rosi, *Toxicol. Appl. Pharmacol.*, **6**, 292(1964).

(5) W. A. Goss and W. H. Deitz, *Bacteriol. Proc.*, **1963**, 93.

(6) C. I. Bliss, "The Statistics of Bioassay," Academic, New York, N. Y., 1952.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 20, 1973, from the *Sterling-Winthrop Research Institute, Rensselaer, NY 12144*

Accepted for publication April 4, 1973.

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