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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PLASMA AND URINARY 1-ETHYL-1,4-DIHYDRO-4-OXO-1,8-NAPHTHYRIDINE-3,7-DICARBOXYLIC ACID

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SUMMARY

A high-performance liquid chromatographic method for the analysis of 1-ethyl-1,4-dihydro-4-oxo-1,8-naphthyridine-3,7-dicarboxylic acid (I) in plasma and urine is described. A statistical evaluation of the assay technique has shown acceptable accuracy and precision at concentrations as high as 2.0 μg/ml of plasma or 29.0 μg/ml of urine for samples augmented with I. As little as 0.08 μg/ml of I in plasma or 0.42 μg/ml of I in urine were quantitatively determined. The mean relative error for the assay of unknown concentrations of I in plasma and urine was ± 8% and ± 3%, respectively. This method was used for the analysis of I in the plasma and urine of rhesus monkeys following oral administration of 200 mg/kg of nalidixic acid.

INTRODUCTION

Nalidixic acid, 1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid[1], is an antibacterial agent which is widely used in the treatment of urinary tract infections and is effective against a variety of gram-negative pathogens. In man, nalidixic acid is converted to the 7-hydroxymethyl metabolite which is further oxidized to generate the dicarboxylic acid, I (ref. 2). In man, *ca.* 4% of a 1.0 g dose of nalidixic acid was excreted into the urine as unconjugated I; this figure was determined by a spectrophotofluorometric assay[3]. The metabolism of nalidixic acid has recently been reviewed[4].

This paper will describe a high-performance liquid chromatographic (HPLC) method, define its sensitivity, accuracy and precision, and demonstrate its utility for the analysis of I in biologic media.

EXPERIMENTAL

Materials

Nalidixic acid is a product of Winthrop Laboratories (New York, N.Y., U.S.A.). Both the title compound, I, and the internal standard for the assay, 1,4-

dihydro-4-oxo-1-propyl-1,8-naphthyridine-3,7-dicarboxylic acid, were synthesized at the Sterling-Winthrop Research Institute. Other chemicals were obtained commercially (reagent grade) and used without further purification.

Preparation of plasma standards

Plasma standards were prepared by supplementing 2 ml of control human plasma with 0.1 ml of appropriate stock solutions of I in water. Duplicate standards at each concentration of 0, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0 and 2.5 μg/ml of I were prepared.

Three sets of randomized and coded plasma samples to be analyzed under single blind conditions were prepared in the following manner:

Sets 1 and 2. Control human plasma was supplemented with aliquots of solutions of I as described above. Triplicate plasma samples for each set were prepared at each concentration level of 0, 0.22, 0.83, 1.8 and 4.1 μg I per 2 ml. Set 1 was analyzed immediately.

Set 2 was analyzed following a 12-day storage period in a −4° freezer.

Set 3. Triplicate plasma samples at concentrations of 0, 0.27 and 0.6 μg/ml of I per ml were prepared and allowed to stand at room temperature for a 12-day period before analysis.

Fresh plasma standards were prepared, extracted and analyzed concomitantly with each set of unknown samples.

Extraction and analysis. To 2 ml of human plasma, containing sodium oxalate as the anticoagulant, in a 50-ml centrifuge tube, were added 0.1 ml of the internal standard solution (20 μg/ml in water), 1 ml of 1 *N* hydrochloric acid and 12 ml of chloroform. The polyethylene stoppered tube was placed on a horizontal shaker for 10 min. After centrifugation, 10 ml of the organic phase were transferred to a clean 15-ml conical test-tube. The organic phase was extracted with 1 ml of 0.05 *N* sodium hydroxide for 10 min on a horizontal shaker. Following centrifugation, a 50-μl aliquot of the aqueous alkaline phase was analyzed with the following HPLC system: pump, Milton Roy Mini-pump equipped with a pulse dampening device; detector, Altex Model 153 UV detector operated at 254 nm; column (25 cm × 4.6 mm I.D.), Partisil PXS 10/25 PAC prepacked column (Whatman); mobile phase, methanol–0.1 *M* citrate buffer, pH 3 (95:15); flow-rate, 1.6 ml/min; retention time, 12 min (I), 10.5 min (internal standard).

Preparation of urine standards

Urine standards were prepared by supplementing control human urine with 0.1 ml of appropriate stock solutions of I in 0.1 *N* NaOH. Duplicate standards at the concentrations of 0, 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 25 and 30 μg/ml of I were prepared.

Fresh urine standards were prepared, extracted and analyzed along with each set of unknown samples.

Three sets of randomized and coded urine samples, to be analyzed under single blind conditions, were prepared in the following manner:

Sets 1 and 2. Control human urine was supplemented with aliquots of solutions of I as described above. Triplicate samples for each set were prepared at each concentration level of 0, 2.4, 9.8, 17.5, 23.0 and 29.0 μg/ml. Set 1 was analyzed immediately following sample preparation and set 2, following 16 days of storage at −4°.

Set 3. Triplicate urine samples at concentrations of 0, 17.5 and 29.0 μg/ml

were prepared and allowed to stand at room temperature for 2 weeks before analysis.

Extraction and analysis. Urine samples containing I were extracted and analyzed in a manner similar to plasma samples, except that 1 ml of human urine was used and 0.1 ml of internal standard solution (0.1 mg/ml in 0.05 *N* sodium hydroxide) was added.

Animal study

Two rhesus monkeys (weighing 4.1 and 4.7 kg) were fasted overnight before each received orally 200 mg/kg of nalidixic acid suspended in 4 ml of gum tragacanth. Water was available *ad libitum* throughout the experiment and solid food was available following medication.

Five ml of blood were withdrawn from the femoral vein of the monkeys prior to medication and at 1, 3, 5 and 7 h postmedication. The bloods were mixed with heparin to prevent coagulation. The blood samples were centrifuged and the plasma was separated and frozen (—4°) until the analysis for I was performed.

During the experiment, these animals were housed in individual metabolic cages so that urine samples could be collected at intervals. The volume of the urine samples was measured and they were frozen (—4°) until the analysis for I was performed.

The concentrations of I in plasma and urine were determined by the method described above. Those urine samples which contained concentrations of I greater than the highest standard were diluted with water and again analyzed.

RESULTS AND DISCUSSION

A recorder tracing from the HPLC analysis with UV (254 nm) detector of an extract from a control urine sample and from a urine sample supplemented with 5 μg/ml of I are shown in Fig. 1.

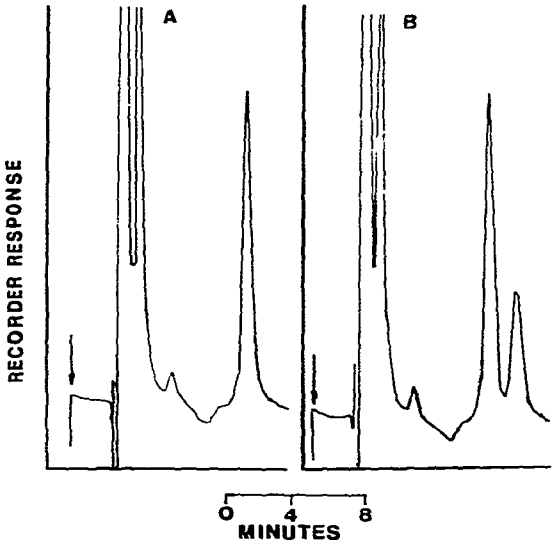

Fig. 1. Chromatogram of control urine containing the internal standard (A), and of the same sample containing 5 μg/ml of I (B).

A summary of the regression analysis of the plasma and urine standards analyzed with each set of samples that were assayed on a single-blind basis is presented on Table I. A linear model was employed to describe the relationship between the peak height ratio of I to the internal standard and the concentration of I in the plasma and urine standards.

TABLE I

SUMMARY OF LINEAR STANDARD CURVE DETERMINATIONS OVER TIME

Time of assay	*Set No.*	*No. of points*	*Slope* ± S.E.*	*Y_0** ± S.E.*	*MQL*** (μg/ml)*
Plasma					
Day 1	1	18	0.359 ± 0.006	0.070 ± 0.015	0.18
Day 12	2, 3	20	0.336 ± 0.004	0.068 ± 0.008	0.11
Urine					
Day 1	1	24	0.102 ± 0.001	0.042 ± 0.013	0.62
Day 14	2	24	0.077 ± 0.0004	0.013 ± 0.005	0.34
Day 17	3	24	0.079 ± 0.0003	0.026 ± 0.005	0.30

* Change in peak height ratio per unit change in concentration.

** *y*-Axis intercept of the least-squares regression line; units are peak height ratio.

*** Minimum quantifiable level based on least-squares error estimate where 80% confidence limit just encompasses zero.

To estimate assay sensitivity over time, sets of both plasma and urine samples were assayed after storage. Plasma parameters were in good agreement, while the first set of urine standards gave slightly higher ratios than the other two sets. The minimum quantifiable level (MQL) was defined as that concentration whose lower 80% confidence limit just encompasses zero[5]. The overall mean sensitivity for plasma was 0.08 μg/ml, while the mean sensitivity for urine was 0.42 μg/ml.

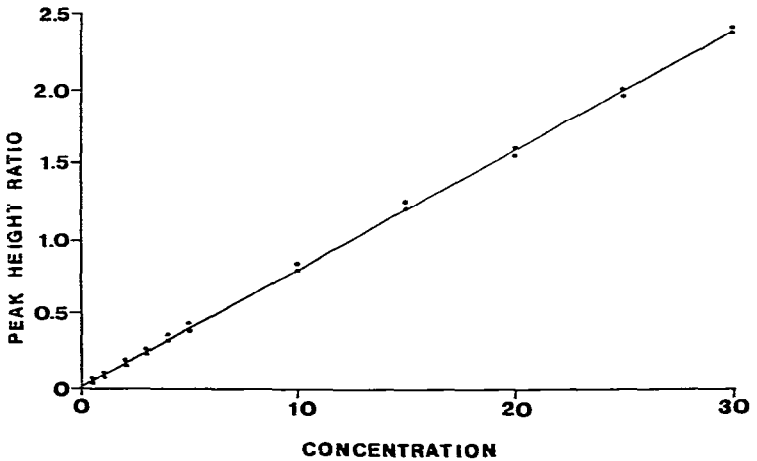

Fig. 2. Extracted standard curve of control urine augmented with I; each concentration was prepared and chromatographed in duplicate.

TABLE II

CONCENTRATIONS FOUND IN PLASMA SAMPLES SUPPLEMENTED WITH I*

Set 1			*Set 2*		*Set 3*		
Spiked level (μg/ml)	*Peak height ratio*	*Assayed level (μg/ml)*	*Peak height ratio*	*Assayed level (μg/ml)*	*Spiked level (μg/ml)*	*Peak height ratio*	*Assayed level (μg/ml)*
0	0.045	<MQL	0.042	<MQL	0	0.038	<MQL
0	0.057	<MQL	0.038	<MQL			
0	0.070	<MQL	0.042	<MQL			
0.11	0.136	0.09	0.161	0.14	0.275	0.266	0.295
0.11	0.150	0.11	0.144	0.11	0.275	0.248	0.269
0.11	0.126	0.08	0.129	0.09	0.275	0.270	0.300
Mean		0.09		0.11			0.288
S.D.		±0.02		±0.01			±0.02
Mean % difference		−15.2		+3.0			+4.7
0.415	0.320	0.349	0.341	0.407	0.60	0.455	0.58
0.415	0.335	0.370	0.371	0.452	0.60	0.443	0.56
0.415	0.324	0.355	0.300	0.346	0.60	0.443	0.56
Mean		0.358		0.40			0.57
S.D.		±0.01		±0.003			±0.007
Mean % difference		−13.7		−3.2			−5.6
0.90	0.696	0.87	0.706	0.95			
0.90	0.687	0.86	0.708	0.95			
0.90	0.717	0.90	0.698	0.94			
Mean		0.88		0.95			
S.D.		±0.01		±0.003			
Mean % difference		−2.6		+5.6			
2.05	1.47	1.95	1.540	2.19			
2.05	1.42	1.88	1.380	1.95			
2.05	1.52	2.02	1.360	1.92			
Mean		1.95		2.02			
S.D.		±0.07		±0.2			
Mean % difference		−4.9		−1.5			

* Standard curve parameters in Table I.

Precision was satisfactory for the range of concentrations used. Table II summarizes the results for the analysis of the unknown plasma concentrations. There was no statistically significant difference among concentration groups with respect to percentage differences and the overall mean relative error was ±8%.

A representative plot of an extracted urine standard curve is presented in Fig. 2. The recovery of I in the extracted standards, relative to direct injection of standard solutions, was 72.0 ± 1.0% (± S.E.) from plasma and 84.0 ± 1.2% from urine after correction for the aliquot of the final extract used for chromatography.

A logarithmic transformation was applied to the urine data (Table III) prior to statistical analysis to minimize the observed correlation between mean response

TABLE III

CONCENTRATIONS FOUND IN URINE SAMPLES SUPPLEMENTED WITH I*

Set 1			*Set 2*		*Set 3*		
Spiked level (μg/ml)	*Peak height ratio*	*Assayed level (μg/ml)*	*Peak height ratio*	*Assayed level (μg/ml)*	*Spiked level (μg/ml)*	*Peak height ratio*	*Assayed level (μg/ml)*
0	0.023	<MQL	0.016	<MQL	0	0.022	<MQL
0	0.024	<MQL	0.018	<MQL			
0	0.034	<MQL	0.015	<MQL			
2.4	0.296	2.48	0.210	2.56	17.5	1.45	17.9
2.4	0.277	2.30	0.215	2.63	17.5	1.52	18.8
2.4	0.285	2.38	0.208	2.54	17.5	1.46	18.1
Geometric mean		2.39		2.58			18.3
% S.E.		2		1			2
Mean % difference		+5.8		+8.1			+1.5
9.8	1.02	9.55	0.791	10.10	29.0	2.43	30.3
9.8	0.974	9.10	0.735	9.40	29.0	2.41	30.0
9.8	1.00	9.35	0.758	9.69	29.0	2.31	28.8
Geometric mean		9.33		9.73			29.7
% S.E.		1		2			2
Mean % difference		−2.0		−0.3			+0.7
17.5	1.92	18.3	1.38	17.8			
17.5	1.68	16.0	1.35	17.4			
17.5	1.68	16.0	1.33	17.1			
Geometric mean		16.7		17.4			
% S.E.		5		1			
Mean % difference		−1.6		0			
23.0	2.32	22.2	1.78	23.0			
23.0	2.38	22.8	1.82	23.5			
23.0	2.34	22.4	1.79	23.1			
Geometric mean		22.5		23.2			
% S.E.		1		1			
Mean % difference		−0.7		+0.7			
29.0	2.85	27.4	2.23	28.8			
29.0	3.21	30.9	2.17	28.1			
29.0	3.16	30.4	2.33	30.1			
Geometric %mean		29.5		29.0			
% S.E.		4		2			
Mean % difference		+0.5		0			

* Standard curve parameters in Table I. Data were analyzed on a logarithmic basis to minimize heterogeneity of group variances. Geometric mean = antilog (log mean).

TABLE IV
PLASMA CONCENTRATIONS OF I FOLLOWING ORAL MEDICATION OF MONKEYS WITH 200 mg/kg OF NALIDIXIC ACID

Time (h)	*Concentration (μg/ml)*		
	Monkey No. 7071	*Monkey No. 7074*	*Mean*
0	<MQL	<MQL	<MQL
1	0.76	0.15	0.46
3	1.56	1.55	1.56
5	1.48	1.77	1.63
7	1.81	1.54	1.68

and variance of the triplicate sets[6]. There was no significant difference among groups for the calculated percentage differences and the overall mean relative error was ±3%.

Monkey study

Table IV shows the plasma concentrations of I after oral administration of 200 mg/kg of nalidixic acid to rhesus monkeys. Between the 1- and 7-h plasma samplings, the mean plasma concentration of this metabolite increased from 0.46 to 1.68 μg/ml. The plasma pattern is closely followed by the urinary excretion rates (Table V), which range from 1.84 to 4.48 mg/h. From the plasma and urinary concentrations of I, the mean renal clearance of I was estimated as 2.37 l/h.

In summary, the liquid chromatographic assay is simple, rapidly reproducible and obviates the need for separation and purification required with the fluorescent

TABLE V
URINARY CONCENTRATIONS OF I FOLLOWING ORAL MEDICATION OF MONKEYS WITH 200 mg/kg OF NALIDIXIC ACID

Collection period (h postmed.)	*Concn.* (μg/ml)*	*Urine volume (ml)*	*Total amount in urine (mg)*	*% dose*	*Renal clearance (l/h)*	*Urinary excretion rate (mg/h)*
Monkey No. 7071						
−20– 0	<MQL	425	0	0		
0– 2	19.3	190	3.68	0.41	2.42	1.84
2– 4	31.3	270	8 45	0.94	2.71	4.23
4– 8	42.3	308	13.03	1.45		3.26
8–24	76.2	630	48.01	5.33		3.00
			total 73.16	total 8.13	Mean 2.56	
Monkey No. 7074						
−20– 0	<MQL	340	0	0		
0– 5.5	186.4	95	17.71	1.97	2.08	3.22
5.5–8	285.2	33	9.41	1.04	2.29**	3.76
8–24	162.8	440	71.63	7.96		4.48
			total 98.75	total 10.97	Mean 2.19	

* The urine samples were diluted with water before the assay was performed.
** Plasma concentration at 6.75 h was graphically estimated as 1.65 μg/ml.

method. Furthermore, more samples can be processed by a single technician in the same unit of time.

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