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Note

# Determination of urinary quinolinic acid by high-performance liquid chromatography

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Quinolinic acid is a compound of interest as a tryptophan metabolite and precursor of the nicotinamide moiety of NAD(H). Quinolinic acid has been quantitated in the urine of vitamin $B_6$-deficient humans [1], guinea pigs, hamsters and rats [2]. Elevated urinary excretion of quinolinic acid by humans and rats fed high levels of leucine in the diet has been reported [3]. Consequently, some workers have implicated excess dietary leucine as a causative factor in the pathogenesis of pellagra. Our laboratory is currently studying this problem and it was for this purpose that a simple and accurate method for the quantitation of urinary quinolinic acid using high-performance liquid chromatography (HPLC) was developed.

Methods for quantitating urinary quinolinic acid are numerous. Henderson's microbiological method [4] measured niacin-active compounds before and after autoclaving urine in glacial acetic acid. Autoclaving in acid decarboxylates quinolinic acid in the 2-carbon position yielding nicotinic acid. Therefore, quinolinic acid was estimated by difference. Quinolinic acid has also been directly determined microbiologically after separation by paper chromatography [5].

Heeley et al. [6] described a method which involves a partial separation of quinolinic acid from other urinary compounds by Dowex anion- and cation-exchange chromatography, decarboxylation by heating in acid, and quantitation spectrophotometrically by reacting the nicotinic acid with cyanogen bromide and *o*-toluidine. McDaniel et al. [7] described a similar method with a modification in the pre-cleanup of the urine which involved absorbing quinolinic acid onto Norit A followed by elution with ammonium hydroxide. Quino-

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linic acid has been derivatized to the diester with ethereal diazomethane and analyzed by gas–liquid chromatography [8]. Lastly, Crawford et al. [9] measured urinary quinolinic acid using thick-layer chromatography.

## MATERIALS AND METHODS

Quinolinic acid standard was purchased from Sigma (St. Louis, MO, U.S.A.). Other standards that were used were picolinic acid hydrochloride (E.H. Sargent & Co., Chicago, IL, U.S.A.) and nicotinic acid (Nutritional Biochemicals, Cleveland, OH, U.S.A.). All standards were dissolved in distilled water. All reagents used in the HPLC system were reagent grade. Methanol was glass distilled (Burdick & Jackson Labs., Muskegon, MI, U.S.A.).

The formate form of Dowex 1-X8, 200–400 mesh (Bio-Rad Labs., Richmond, CA, U.S.A.) was prepared by sequentially washing 1-lb. (450-g) batches of resin in a column of 10 cm diameter with 4.0 l of 6 *N* hydrochloric acid, 4.0 l of distilled water, 16 l of 3 *N* sodium formate, 4.0 l of distilled water, 8.0 l of 3 *N* formic acid and finally with 12 l of distilled water.

### *Sample preparation*

Twenty-four hour urine collections were made by normal human volunteers and the urine was preserved with a few milliliters of toluene. Aliquots of the urine were frozen at −4°C until analysis.

Two percent of the 24-h urine collection was pipetted in duplicate into 50-ml graduated tubes. To one duplicate was added 1 ml of standard quinolinic acid solution containing 0.5 μmoles/ml. The volumes of the samples were then brought up to 50 ml with distilled water.

Glass columns with reservoirs [10] were prepared by pipetting 15 ml of a 1:1 slurry (v/v) of Dowex 1-X8, 200–400 mesh (formate) resin into glass columns, 1.3 cm I.D. The resin bed heights were approximately 5.5 cm. The columns were washed with 30 ml of 3 *N* sodium formate in 3 *N* formic acid and then with 100 ml distilled water prior to loading samples.

Urine samples were poured onto the columns and the eluate discarded. The sample tubes were rinsed with 10 ml distilled water, the rinse poured onto the columns and the eluate discarded. The columns were then eluted with 50 ml of 0.08 *N* hydrochloric acid and the eluate discarded. About 97 ml of 0.15 *N* hydrochloric acid were passed through the columns and the eluate was collected in 100-ml graduated cylinders. The volumes of this eluate were brought up to 100 ml with distilled water. The urine samples and the 10 ml of water washings were eluted under $6.9 \times 10^3$ N $m^{-2}$ pressure, while the rest of the chromatography was done with gravity flow.

A 20-ml aliquot of the final 0.15 *N* hydrochloric acid eluate was evaporated under vacuum on a flash evaporator in a water bath at 50°C. The residue was dissolved in 12 ml of distilled water and again flash evaporated in order to rid the sample of hydrochloric acid. The final residue was redissolved in 2 ml of the buffer used in the HPLC system and filtered with a 0.22-μm Millipore filter (Millipore, Bedford, MA, U.S.A.).

*Apparatus and chromatographic conditions*

A Perkin-Elmer 601 liquid chromatograph (Norwalk, CT, U.S.A.) was used for chromatographic analysis. A strong anion-exchange column (25 cm × 4.6 mm I.D.) prepacked with Partisil-10 SAX (10-$\mu$m diameter particle) (Whatman, Clifton, NJ, U.S.A.) was used. The column was wrapped with a heating coil and insulation so that the column temperature could be varied by a variable transformer and monitored by a thermo-couple. The detectors used were a Model 250 fixed-wavelength detector (254 nm) and a LC-55 UV/VIS variable-wavelength spectrophotometer, both from Perkin-Elmer. The flow cells of the spectrophotometers were connected in series. The dual pen recorder was set at 0.015 a.u.f.s. for most samples. A Rheodyne syringe loading sample injector was used. Samples were injected onto the column by 22-gauge (0.071 cm) Hamilton syringes and sample volumes ranged from 10 to 100 $\mu$l.

The column was operated isocratically with 0.06 *M* potassium phosphate buffer (pH 2.2)—methanol (9:1) as solvent. The flow-rate was 2.0 ml/min and the column temperature was constant but was varied from 30 to 50°C depending on the run. Therefore, the column pressure ranged from 41 to 76 bar. All buffers were filtered on 0.22-$\mu$m Millipore filters.

Absorbance was recorded at 254 nm and 272 nm. Quantitation of compounds was determined by peak height measurements.

## RESULTS AND DISCUSSION

A representative chromatogram of a sample prepared from normal human urine is illustrated in Fig. 1A. Quinolinic acid added to the urine sample co-chromatographed with this peak (Fig. 1B) and had the same retention time as standard quinolinic acid which was directly injected (Fig. 1C and D). Also, standard quinolinic acid was directly added to a prepared urine sample and one peak resolved with a retention time equal to standard quinolinic acid. Quinolinic acid in distilled water was pretreated identically to urine samples. Only one peak resolved, thereby indicating that quinolinic acid was not decarboxylated by the sample preparation procedure.

Quinolinic acid peaks from a number of urine samples were collected and re-chromatographed. The major peak co-chromatographed with standard quinolinic acid. A peak also appeared at the solvent front due to tailing off of the initial unidentified compounds as seen in Fig. 1A. Because of the tailing off of the large initial peaks, baselines from which peak height measurements were made were drawn as shown in Fig. 1A.

The identity of the quinolinic acid peak was further established by comparing chromatographic and spectrophotometric properties of the peak of interest with standard quinolinic acid. The peak height ratios of urine samples and standards were determined by comparing absorbance at 272 nm (absorbance maximum of quinolinic acid) and 254 nm. Peak height ratios of absorbance at 272:254 nm for 13 analyses of samples (1.66 ± 0.036), recovery samples (1.64 ± 0.048) and standard quinolinic acid (1.64 ± 0.052) were identical. Urine samples and standard quinolinic acid behaved identically when column temperature and buffer strength were varied. Fig. 2A and B represent only a few of the many trials in which various solvents and temperatures were tested. Picolinic acid and nicotinic acid, two anionic pyridine compounds that

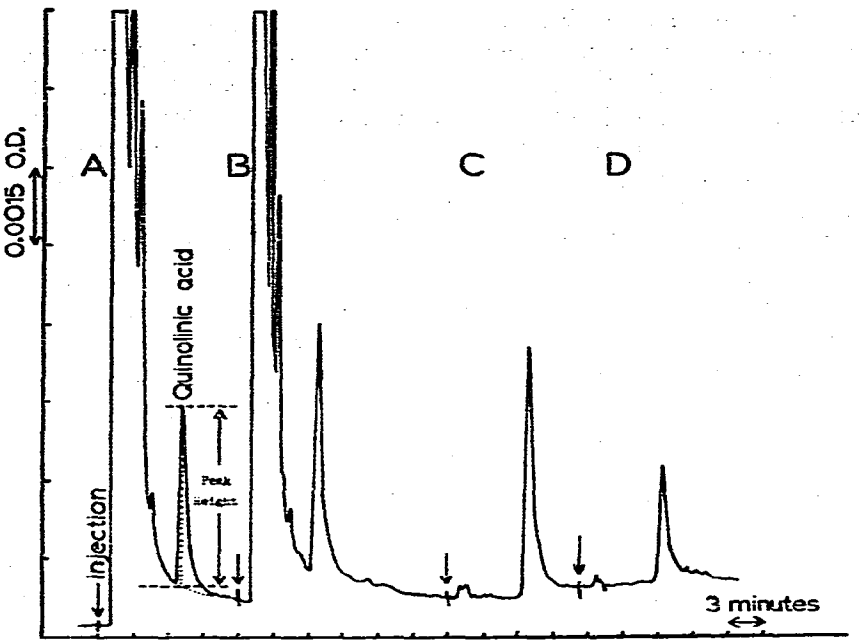


Fig. 1. Chromatograms of (A) normal urine sample and (B) urine sample with added quinolinic acid, both prepared as described in Sample preparation section; (C) 40 µl of quinolinic acid solution and (D) 20 µl of quinolinic acid solution (concentration = 0.1 µmole/ml). Conditions: column, Partisil-10 SAX, 25 cm × 4.6 mm I.D.; eluent, 0.06 *M* potassium phosphate buffer (pH 2.2)—methanol (9:1, v/v); flow-rate, 2 ml/min; column temperature, 38°C and pressure, 76 bar; detection, 272 nm.

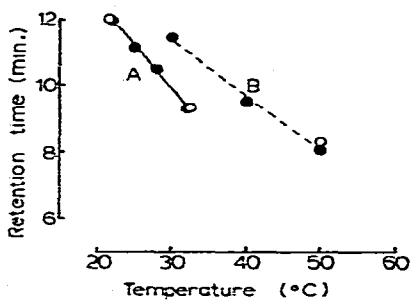


Fig. 2. Effect of temperature and buffer strength on retention time of quinolinic acid. (A) Conditions: column, Partisil-10 SAX; eluent, 0.08 *M* potassium phosphate buffer (pH 2.3)—methanol (9:1, v/v); flow-rate, 2 ml/min; detection, 272 nm. (B) Conditions, column, Partisil-10 SAX; eluent, 0.06 *M* potassium phosphate buffer (pH 2.35)—methanol (9:1, v/v); flow-rate, 2 ml/min; detection, 272 nm. Quinolinic acid peak from urine sample, •; peak from standard quinolinic acid, ○.

are excreted by humans, were tested. Both eluted at the solvent front.

Standard curves plotting peak height versus picolinate injected demonstrated excellent linearity (correlation coefficient = 0.999) in the range 0—10 nmoles. Urinary quinolinic acid was quantitated within the 2—8-nmole range by varying the volume of injected sample. Peak resolution, standard curves and the

quantitation or urinary quinolinic acid did not vary due to sample injection volumes. One ml of standard quinolinic acid solution (concentration = 0.5 μmoles/ml in distilled water) was added to the urine sample before treatment on Dowex 1 (formate) columns. More than 100 urine samples were analyzed and recoveries ranged between 95% and 105%. The percent of quinolinic acid recovered from urine was independent of the amount of quinolinic acid added to the urine sample as shown in Table I. Coefficient of variation (standard deviation × 100/mean) of replicate analyses was 7.2%. Freezing urine samples at −4°C and thawing them did not affect the quantitation of quinolinic acid. The same samples were analyzed four months apart with repeatable results.

This method was adapted for the analysis of rat urine. Rats fed a 10% casein diet and having a body weight of about 190 g excreted approximately 0.4 μmoles of quinolinic acid per 24 h.

TABLE I

RECOVERY OF QUINOLINIC ACID ADDED TO URINE SAMPLE

Conditions: column, Partisil-10 SAX; eluent, 0.06 *M* potassium phosphate buffer (pH 2.2)—methanol (9:1, v/v); flow-rate, 2 ml/min; column temperature, 37°C and pressure, 69 bar; detection, 254 nm; retention time, 10.2 min. Standard quinolinic acid was added to 2% of a 24-h urine collection from a normal male subject.

| Amount of quinolinic acid added (μmoles) | Amount of quinolinic acid recovered (μmoles) | Recovery (%) |
|---|---|---|
| 0 | 0.480 | |
| 0 | 0.506 | |
| 0.25 | 0.758 | 105 |
| 0.50 | 1.00 | 101 |
| 1.0 | 1.50 | 101 |
| 1.5 | 2.01 | 101 |

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REFERENCES

1 R.R. Brown, N. Yess, J.M. Price, H. Linkswiler, P. Swan and L.V. Hankes, J. Nutr., 87 (1965) 419.
2 J.K. Yeh and R.R. Brown, J. Nutr., 107 (1977) 261.
3 C. Gopalan and K.S. Jaya Rao, Vitam. Horm. New York, 33 (1975) 505.
4 L.M. Henderson, J. Biol. Chem., 181 (1949) 685.
5 W.B. Jakoby and D.M. Bonner, J. Biol. Chem., 191 (1951) 543.
6 A.F. Heeley, D.G. McCubbing and J. Shepherd, Arch. Dis. Childhood, 41 (1966) 562.
7 H.G. McDaniel, W.J. Reddy and R.R. Bosheil, Anal. Biochem., 49 (1972) 373.
8 P. Toseland, Clin. Chim. Acta, 25 (1969) 185.
9 M.A. Crawford, I.L. Hansen and A. Lopez, Brit. J. Cancer, 23 (1969) 644.
10 R. Levine and R. Luft, Advan. Met. Disord., 2 (1965) 159.