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Note

High-performance liquid chromatographic assay for the metabolites of nitrofurantoin in plasma and urine

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We have previously published a high-performance liquid chromatographic assay for the quantitation of the urinary tract anti-infective nitrofurantoin in plasma and urine [1]. Subsequently, we isolated and identified, following anaerobic metabolism, two metabolites of nitrofurantoin [2]. These were the amino, 1-[[(5-amino-2-furanyl)methylene]amino]-2,4-imidazolidinedione, and the open-chain nitrile, 1-[[(3-cyano-1-oxopropyl)methylene]amino]-2,4-imidazolidinedione [2].

The purpose of the present investigation was to develop an assay for both the amino and cyano metabolites of nitrofurantoin. Since the assay was to be used for our bioavailability and pharmacokinetic studies, we wanted to develop a rapid, sensitive method for measuring low levels of both metabolites in plasma and urine. The high-performance liquid chromatographic assay we describe here is rapid, sensitive and accurate. Furthermore no prior extraction and/or derivatization of the plasma or urine samples is required.

EXPERIMENTAL

Apparatus

We used a high-pressure liquid chromatograph, Model ALC/GPC 244, Waters Assoc. (Milford, Mass., U.S.A.) characterized by a constant solvent flow at working pressures up to 420 kg/cm^2. This model includes a U6K universal injector and a dual-channel, fixed-wavelength ultraviolet absorption detector. The instrument was fitted with a 250 mm × 3.2 mm I.D. 5-μm particle size, LiChrosorb C_{18} reversed-phase column (Altex, Berkeley, Calif., U.S.A.). The chromatograph was operated isocratically at a flow-rate of 2 ml/min. The wavelengths of detection were fixed at 280 and 365 nm. A dual-pen recorder was used

(OmniScribe Model A5211-1; Houston Instruments, Austin, Texas, U.S.A.). Chart speed was 2.5 cm/min and full-scale response was 1 mV.

Reagents

Chemicals. The amino and cyano metabolites were prepared by catalytic hydrogenation of nitrofurantoin over 5% palladium on charcoal (ICN Pharmaceuticals, Plainview, N.Y., U.S.A.) [2]. After lyophilization, the amino was stored as the dry powder at −20°. Since the cyano tends to polymerize when the crystals are exposed to air, it was stored in a solution of methanol at −20°. The purity of each was >99.5% as confirmed by UV and chemical ionization mass spectroscopy [2]. The 5-nitro-2-furoic acid, Lot 090447, was purchased from Aldrich (Milwaukee, Wisc., U.S.A.).

The methanol (glass-distilled, Burdick and Jackson Labs., Muskegon, Mich., U.S.A.), and distilled water (glass-redistilled and stored in glass) were filtered through a 0.45-μm filter (FHLPO 4700 and HAWPO 4700; Millipore, Bedford, Mass., U.S.A.) before use. The acetonitrile (glass-distilled, Burdick and Jackson Labs.), and other chemicals were ACS reagent grade or better.

Mobile phase. The mobile phase consisted of methanol—0.02 *M* acetic acid (2.5:97.5). It was prepared by mixing 25 ml of methanol with 975 ml of water and adding 1.2 ml of glacial acetic acid. This mobile phase was degassed under vacuum before use.

Biological fluids. Human plasma that had been stored at −20° was obtained from the blood bank of the University of California, San Francisco. The plasma was thawed to room temperature before use. Human urine from a female donor was collected daily.

Procedure

Plasma. Add 5 μl of a 520 μg/ml solution of 5-nitro-2-furoic acid, the internal standard, to 100 μl of plasma. Shake well, add 500 μl of acetonitrile and shake to mix. Centrifuge for 10 min at 3000 *g* to precipitate the proteins. Pour the supernatant into a 4-ml glass tube. Evaporate to dryness under nitrogen. Dissolve the residue in 200 μl of distilled water and inject onto the chromatograph. Injection volumes of 30—120 μl were satisfactory for the entire range of plasma concentrations. The sensitivity of the 365-nm detector was set at 0.01 a.u.f.s. for detection of the amino metabolite and the 5-nitro-2-furoic acid. The 280-nm detector was set at 0.01 a.u.f.s. for detection of the cyano metabolite. Operate the chromatograph at 2 ml/min at room temperature using the methanol—0.01 *M* acetic acid (2.5:97.5) mobile phase. Retention times for the 5-nitro-2-furoic acid, cyano metabolite and amino metabolite are 2, 6 and 8 min respectively.

Urine. Add 5 μl of the 520 μg/ml solution of 5-nitro-2-furoic acid to 50 μl of urine and 50 μl of distilled water. Shake well and inject onto the chromatograph. Injection volumes of 30—75 μl were satisfactory for the entire range of urine concentrations when the detector sensitivity was set at 0.01 a.u.f.s. at 365 nm and 0.05 a.u.f.s. at 280 nm. All other chromatographic conditions were identical to those described above for plasma.

Standard curves were prepared by adding the cyano metabolite, the amino metabolite and the 5-nitro-2-furoic acid to plasma or urine. The concentration

of each metabolite is determined by comparing the metabolite:internal standard peak height ratio to its standard curve of peak height ratios versus metabolite concentration. With all curves we made a straight-line fit of the data by least squares linear regression analysis using the PROPHET system, a specialized computer resource developed by the Chemical/Biological Information Handling Program of the National Institutes of Health, U.S.A.

Stability study

Plasma. Prepare plasma samples containing 20 μg/ml of the amino metabolite and 20 μg/ml of the cyano metabolite and store at −20°. We performed analyses on six separate days for one month. Thaw the solution on the day of analysis, add the internal standard and proceed as described above for the plasma standard curve. On each day of analysis construct a standard curve and determine the metabolite concentrations of the stability study by comparing the peak height ratios with those of the standard curve.

Urine. Prepare urine samples containing 15 μg/ml of the amino metabolite or 120 μg/ml of the cyano metabolite and store at −20°. We performed analyses on six separate days over a period of three weeks. Thaw the solutions on the day of analysis, add internal standard and proceed as described above for urine standard curves. On each day of analysis construct a standard curve and determine the concentration of each metabolite by comparing the peak height ratios with those of the standard curve.

RESULTS AND DISCUSSION

Pharmacokinetic studies of nitrofurantoin following intravenous and oral administration have shown that only 30—50% of the nitrofurantoin is recovered unchanged in the urine [3—11]. At the time of these studies none of the metabolites had been identified in man. Products of the metabolic reduction of nitrofurantoin, which could account for up to 50—70% of the administered dose, have been implicated in the polyneuropathy, and possibly the pulmonary hypersensitivity that have been reported following long-term administration of nitrofurantoin [12—14]. These metabolites are probably also the end products of the metabolic pathway responsible for the mutagenic activity of nitrofurantoin in bacterial and mammalian tissue culture systems [12, 15, 16]. Since we wanted to study the disposition of nitrofurantoin and its reduced metabolites in man, we have developed a high-performance liquid chromatographic assay for quantitating the amino and cyano metabolites of nitrofurantoin in plasma and urine. The assay is rapid, sensitive and accurate. No extraction and/or derivatization is required. Fig. 1 shows a chromatogram for the quantitation of both metabolites in plasma. The retention times for the internal standard, the cyano metabolite and the amino metabolite are 2, 6 and 8 min respectively. The peak height ratio indicates a cyano concentration of 12 μg/ml and an amino concentration of 12 μg/ml. Standard curves were constructed by adding known amounts of each metabolite and the internal standard to plasma and plotting the peak height ratio versus concentration in μg/ml. Over a period of two months we constructed six plasma standard curves each containing five concentrations of both the amino and cyano metabolites. With 30 points the

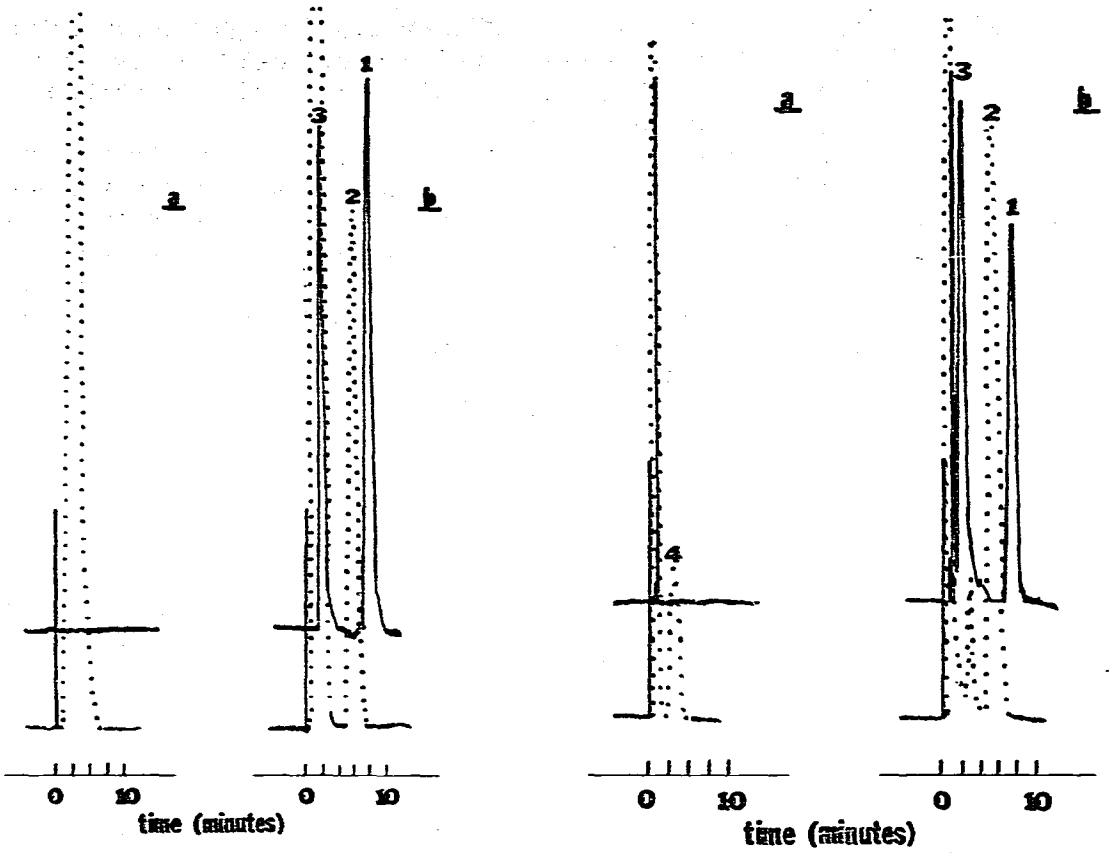

Fig. 1. Chromatograms of (a) blank plasma and (b) plasma with the amino metabolite (1), the cyano metabolite (2), and 5-nitro-2-furoic acid, the internal standard (3). The detector was set at 365 nm (0.01 a.u.f.s.) (——), and 280 nm (0.01 a.u.f.s.) (· · ·). The peak height ratios indicate an amino concentration of 12 μg/ml and a cyano concentration of 12 μg/ml.

Fig. 2. Chromatograms of (a) blank urine and (b) urine with the amino metabolite (1), the cyano metabolite (2), and 5-nitro-2-furoic acid, the internal standard (3). The detector was set a 365 nm (0.01 a.u.f.s.) (——), and at 280 nm (0.05 a.u.f.s.) (· · ·). The peak height ratios indicate an amino concentration of 12 μg/ml and a cyano concentration of 105 μg/ml. The peak labelled (4) is endogenous xanthines (see text for explanation).

regression line for the amino in plasma was $y = (0.138 \pm 0.009)\,x - (0.556 \pm 0.139)$ with a correlation coefficient of 0.93. With 30 points the regression line for the cyano was $y = (0.082 \pm 0.004)\,x - (0.040 \pm 0.073)$ with a correlation coefficient of 0.95. For both the amino and cyano the concentration range was 4—25 μg/ml.

Fig. 2 shows a chromatogram for the quantitation of both metabolites in urine. The retention times for the internal standard, the cyano metabolite and the amino metabolite are 2, 6 and 8 min respectively. The peak height ratios indicate a cyano concentration of 105 μg/ml and an amino concentration of 12 μg/ml. Over a period of two months we constructed six standard curves. With 29 points the regression line for the amino in urine was $y = (0.085 \pm 0.004)$

$x - (0.250 \pm 0.050)$ with a correlation coefficient of 0.97 over the concentration range of 4—25 μg/ml. With 29 points the regression line for the cyano was $y = (0.0127 \pm 0.0004)\ x - (0.186 \pm 0.029)$ with a correlation coefficient of 0.98 over the concentration range of 20—130 μg/ml.

The preliminary stability studies were performed over a period of one month for plasma and three weeks for urine. The results showed that the amino metabolite is not stable for longer than 2—3 days even when stored frozen at −20°. The cyano metabolite appears to be stable. However, it appears that some, although not all, of the amino breaks down to the cyano. Thus, we would advise that both plasma and urine samples be analyzed for nitrofurantoin metabolites as soon as possible after collection.

Both metabolites were analyzed at wavelengths close to their absorption maxima. The cyano has a $\lambda_{max} = 278$ nm and the amino a $\lambda_{max} = 348$ nm [2]. As shown in Fig. 2 the only potential interference from endogenous substances appears to be in urine at 3 min on the 280-nm detector. We have tentatively identified this peak as "xanthines" since caffeine, theophylline and several dimethylpurines co-chromatograph with this peak under the assay conditions.

With urine samples, where no precipitation is necessary, analyses can be performed in less than 10 min per sample. In the case of plasma, centrifugation after protein precipitation is necessary because direct injections of plasma onto the chromatograph result in increases in operating pressure caused by the build-up of proteins in the column. The evaporation to dryness also requires additional time, usually about 15 min. When plasma and urine samples are collected from volunteers or patients we divide them into two fractions. We analyze the first fraction for nitrofurantoin using our previously published method [1]. The second fraction is used to analyze for the amino and cyano metabolites. Both methods are simple, rapid and accurate. We are currently using them for bioavailability and pharmacokinetic studies on nitrofurantoin and its metabolites following intravenous and oral administration to normal volunteers.

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1 M.B. Aufrere, B. Hoener and M.E. Vore, Clin. Chem., 23 (1977) 2207.
2 M.B. Aufrere, B. Hoener and M. Vore, Drug Metab. Disp., 6 (1978) 403.
3 A.R. DiSanto, D.J. Chodos, J.P. Phillips, K.A. DeSante and R.G. Stoll, Int. J. Clin. Pharmacol., 13 (1976) 220.
4 J.D. Conklin and F.H. Hailey, Clin. Pharmacol. Ther., 10 (1969) 534.
5 G.L. Mattok, R.D. Hossie and I.J. McGilveray, Can. J. Pharm. Sci., 16 (1974) 63.
6 T.R. Bates, J.A. Sequeira and A.V. Tembo, Clin. Pharmacol. Ther., 16 (1974) 63.
7 D. Cadwallader, J. Amer. Pharm. Ass., NS15 (1975) 409.
8 H.A. Rosenberg and T.R. Bates, Clin. Pharmacol. Ther., 20 (1976) 227.
9 H.K. Reckendorf, R.G. Castringius and H.K. Spingler, Antimicrob. Ag. Chemother., 1961 (1962) 531.

10 J. Schirmeister, F. Stetani, H. Willmann and W. Hallaver, Antimicrob. Ag. Chemother., 1965 (1966) 223.
11 T.B. Vree, Y.A. Hekster, A.M. Baars, J.E. Damsma and E. van der Kleijn, J. Chromatogr., 162 (1979) 110.
12 S.M. Cohen, in G.T. Bryan (Editor), Carcinogenesis — A Comprehensive Survey, Vol. 4, Nitrofurans, Raven Press, New York, 1978, Ch. 6, p. 171.
13 M. Geller, D.K. Flaherty, H.A. Dickie and C.E. Reed, J. Allerg. Clin. Immunol., 59 (1977) 445.
14 A.I. Jacknowitz, J.L. LeFroch and R.A. Prince, Amer. J. Hosp. Pharm., 34 (1977) 759.
15 Y. Tazima, T. Kada and A. Murakami, Mutat. Res., 32 (1975) 55.
16 J.M. Klemencic and C.Y. Wang, in G.T. Bryan (Editor), Carcinogenesis — A Comprehensive Survey, Vol. 4, Nitrofurans, Raven Press, New York, 1978, Ch. 4, p. 99.