High-Performance Liquid-Chromatographic Assay for Nitrofurantoin in Plasma and Urine

Michel B. Aufrère,1 Betty-ann Hoener,2 3 and Mary E. Vore1

The high-performance liquid-chromatographic method described here for the quantitative analysis for nitrofurantoin in urine and plasma involves direct analysis of urine samples and analysis of plasma samples after protein precipitation by methanol. The assay, which requires only 0.2 ml of biological fluid and shows a linear relationship in the range 0.02 to 200 mg/liter, can be performed in 9 min and is reproducible (CV less than 2%). Results for nitrofurantoin so obtained correlate well with those obtained by the Hyamine 10-X spectrophotometric method (CV = 2%), but the present method is more sensitive. With no modification, the present procedure can also be used for nitrofurazone. The sensitivity, accuracy, and convenience of the method make it suitable for clinical monitoring and pharmacokinetic/bioavailability studies with 5-nitrofuram derivatives.

Additional Keyphrases: pharmacokinetics · nitrofurazone assay · monitoring therapy · milk and tissue analysis · sample storage conditions

Nitrofurantoin, an antibacterial agent, is used extensively in the treatment of urinary-tract infections. A review of nitrofurantoin includes ultraviolet, colorimetric, polarographic, and microbiological methods for its analysis in biological fluids (1). Each method has some disadvantages in specificity, sensitivity, or convenience. The preferred method of analysis is the nitromethane/Hyamine 10-X assay, which involves nitromethane extraction of nitrofurantoin from the biological fluid, followed by formation of a yellow complex when the quaternary ammonium compound Hyamine 10-X hydroxide is added (2-4). The concentration of nitrofurantoin is then determined spectrophotometrically from the absorbance of the complex at 400 nm. The Hyamine 10-X procedure has been found to be specific for nitrofurantoin with regard to certain antibacterials that may be coadministered with nitrofurantoin to patients with urinary tract infections (5). However, a urinary-tract analgesic, phenazopyridine hydrochloride, does interfere with the Hyamine 10-X determination, necessitating a time-consuming column-chromatographic separation before the Hyamine 10-X complex is formed (6). Moreover, as recommended by Conklin and Holllified (3) and Mattok et al. (4), the absorbance value of the samples should be determined rapidly after the Hyamine 10-X complex is formed, because there is a progressive increase in absorbance at 400 nm after Hyamine 10-X is added—an increase that is the result of the degradation of metha- zonic acid, which is formed on addition of Hyamine 10-X to nitromethane (7).

A gas–liquid chromatographic method has been described for detecting nitrofurantoin (8). However, we have been unable to duplicate this analysis, possibly because the high temperatures that are required (>200 °C) lead to rapid on-column decomposition of nitrofurantoin. A second gas–liquid chromatographic method has been developed for the detection of residues of 5-nitrofurans in animal tissues (9, 10). This method requires lengthy extraction procedures followed by the hydrolysis of the 5-nitrofurans to 5-nitro-2-furan dehyde, which is then measured by gas–liquid chromatography. This hydrolysis renders this method unsuitable for the determination of two or more different 5-nitrofurans or their metabolites in the same biological sample. This problem could be avoided by derivatization of the samples; however, in our laboratory, attempts to prepare nitrofurantoin derivatives that are suitable for gas–liquid chromatography were generally unsuccessful; we encountered nonlinear responses and poor reproducibility.

High-performance liquid chromatography appeared to be a suitable method for the rapid, sensitive quantitation of 5-nitrofurans in biological fluids without prior derivatization and extraction. In the method described here, peak heights and nitrofurantoin concentrations are linearly related in the range 0.02 to 200 mg/liter and the method is sufficiently accurate and sensitive for clinical monitoring of therapeutic concentrations in

Department of Pharmacology, School of Medicine,1 and Department of Pharmacy, School of Pharmacy,2 University of California, San Francisco, San Francisco, Calif. 94143.

To whom reprint requests should be addressed: School of Pharmacy, 926-S, University of California, San Francisco, Calif. 94143.

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patients and for pharmacokinetic/bioavailability studies.

**Materials and Methods**

**Instrumentation**

We used a high-pressure liquid chromatograph (Model ALC/GPC 244; Waters Associates, Inc., Milford, Mass. 01757), characterized by a constant flow of solvent at working pressures up to 420 kg/cm². This model includes a U6-K universal injector and a dual-channel ultraviolet absorption detector. The instrument was fitted with a 30 cm × 3.9 mm (i.d.) μBondapak C18 reversed-phase column (Waters Associates). The chromatograph was operated isocratically at a flow rate of 2.0 ml/min, at ambient temperature. The wavelength of detection was fixed at 365 nm. A dual-pen recorder was used (OmniScribe Model A5211-1; Houston Instruments, Austin, Tex. 78753).

A double-beam recording spectrophotometer (Shimadzu Model MPS-50L; Shimadzu Seisakusho Ltd., Japan) was used for the Hyamine 10-X assay.

**Reagents**

**Chemicals.** The purity of nitrofurantoin (Sigma Chemical Co., St. Louis, Mo. 63178) was assessed from melting point (260–262 °C), analysis by infra-red on NaCl disk (1343, 1435, 1525, 1724, 1785 cm⁻¹), ultraviolet spectroscopy (maxH₂O = 370, 266 nm; minH₂O = 307 nm), and thin-layer chromatography on Uniplate silica gel GF 250 μm (Analtech Inc., Newark, Del. 19711) with development in ether/acetone (3/2 by vol) or benzene/acetone (3/2). Powdered samples of nitrofurazone and furazolidone were used as obtained (Eaton Laboratories, Morton-Norwich Products, Norwich, N.Y. 13815; lot nos. P6335 and S3071, respectively).

The chromatographic solvents methanol (glass distilled; Burdick and Jackson Lab., Muskegon, Mich. 49442) and distilled water (glass-redistilled and stored in glass) were filtered through a 0.45-μm filter before use (cat. no. HAWPO 4700 and FHLP0 4700; Millipore Corp., Bedford, Mass. 01730). Spectrophotometric-grade dimethyl sulfoxide and nitromethane (Aldrich Chemical Co., Milwaukee, Wis. 53233) were used as received. Methanolic Hyamine 10-X hydroxide (benzylidemethyl-2-[2-(p-1,1,3,3-tetramethylbutylcresoxy)-ethoxy]ethylammonium hydroxide), 0.01 mol/liter, was prepared by diluting a 1.0 mol/liter solution (ICN Chemical and Radioisotope Div., Irvine, Calif. 92715; lot no. 2079).

**Mobile phase.** The mobile phase consisted of 20/80 by vol methanol/0.01 mol/liter Na acetate, pH 5.0, prepared by mixing 800 ml of water with 200 ml methanol, adding 0.6 ml of glacial acetic acid, and adjusting the pH of the solution to 5.0 with 4 mol/liter NaOH. The mobile phase was degassed before use by applying reduced pressure to the stirred solvent.

**Biological fluids.** Human plasma that had been stored frozen at −20 °C for four weeks 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 male donor was collected daily.

Urine was obtained from the collection bag of a catheterized male patient who was receiving nitrofurantoin. Blood from the same patient was collected in a heparinized tube (Vacutainer; Becton-Dickinson, Rutherford, N.J. 07070). It was centrifuged (3000 × g, 4 min) and the plasma fraction was decanted. This plasma and the urine were stored at −20 °C for four days before analysis.

**Stock solutions**

**Plasma.** A stock solution of nitrofurantoin was prepared by diluting 1 ml of a 100 mg/liter aqueous nitrofurantoin solution in a 10-ml volumetric flask to 10 ml with plasma. This 10 mg/liter stock solution was serially diluted with plasma to obtain nitrofurantoin concentrations of 5, 2, 1, and 0.5 mg/liter.

**Urine.** A stock solution containing 200 mg of nitrofurantoin per liter was prepared by dissolving 2 mg of nitrofurantoin in 10 ml of urine in a volumetric flask. The stock solution, wrapped in foil, was shaken overnight to ensure proper dissolution. Alternatively, solubilizing difficulties were avoided by dissolving 2 mg of nitrofurantoin in 0.5 ml of dimethyl sulfoxide in a 10-ml volumetric flask and diluting the solution to 10 ml with urine to yield a 200 mg/liter solution. This 200 mg/liter stock solution was serially diluted with urine to obtain nitrofurantoin concentrations of 100, 50, 20, and 10 mg/liter.

**Internal standards.** Two internal standard solutions of furazolidone were used in order to cover ranges of nitrofurantoin expected in both urine and plasma and still retain useful nitrofurantoin/furazolidone peak-height ratios. A 5 g/liter furazolidone solution in dimethyl sulfoxide was used as an internal standard for urine samples. For the plasma samples, a 50 mg/liter furazolidone solution was used, prepared by dissolving 5 mg of furazolidone in 3 ml of dimethyl sulfoxide in a 100-ml volumetric flask and diluting to 100 ml with doubly-distilled water.

**Preparation of Standard Curves**

**Plasma.** Add 10 μl of 50 mg/liter furazolidone internal standard solution to 200 μl of the nitrofurantoin plasma solution. Shake well, add 300 μl of methanol, mix the resulting solution thoroughly, and transfer to a 4-ml centrifuge tube. Centrifuge for 15 min at 10 000 × g to precipitate the proteins. Pour the supernate into a 4-ml glass tube and inject on chromatograph. Injection volumes of 15 to 60 μl, made with a 100-μl syringe, were satisfactory for the entire range of plasma concentrations when the sensitivity of the 365-nm detector was set at 0.01 A full scale. Operate the chromatograph at a flow rate of 2.0 ml/min at room temperature using the 20/80 methanol/Na acetate as the mobile phase. Retention times for nitrofurantoin and furazolidone are 6 and 8 min, respectively. The standard curves are constructed by plotting nitrofurantoin/furazolidone peak-height ratios vs. nitrofurantoin concentrations.
Urine. Add 3 μl of 5 g/liter furazolidone internal-standard solution to 200 μl of the nitrofurantoin urine solution. Shake well and inject directly into the chromatograph. Volumes of 4 to 10 μl, injected with a 10-μl syringe, were satisfactory for the entire range of urine concentrations when the sensitivity of the 365-nm detector was set at 0.1 A full scale. The chromatographic conditions are identical to those described above for the plasma standard curves. The urine standard curves are constructed by plotting nitrofurantoin/furazolidone peak-height ratios vs. nitrofurantoin concentrations.

Stability Studies

Plasma. Prepare solutions of nitrofurantoin (10 and 1 mg/liter of plasma) as described above. For each concentration, divide the solution among 20 vials, each containing 200 μl of nitrofurantoin solution, cap, and store at −10 °C, protected from light. We performed analyses on 10 separate days during 26 days: days 0 (sample not frozen), 1, 2, 4, 5, 7, 10, 15, 22, and 26. Thaw two vials of each concentration on the day of analysis, add 10 μl of 50 mg/liter furazolidone internal-standard solution to each vial, and proceed as described above for the plasma standard curves. On each day of analysis, construct a plasma standard curve and determine the nitrofurantoin concentrations of the stability study solutions by comparing their nitrofurantoin/furazolidone peak-height ratios with those of the standard curve.

Urine. Prepare solutions of nitrofurantoin (200 and 18 mg/liter of urine) as described above. For each concentration, divide the solution among 36 vials, each containing 200 μl of nitrofurantoin solution. Store the capped vials protected from light, half of them at −10 °C, the remainder at room temperature. We did analyses on nine separate days during 22 days: days 0 (sample not frozen), 1, 2, 4, 5, 7, 10, 15, and 22. Thaw two of the vials of each concentration on the day of analysis. Samples containing 200 mg of nitrofurantoin per liter crystallize on freezing; in this case, add 200 μl of dimethyl sulfoxide while thawing the solution. Samples that have been kept at room temperature are used directly. Add 3 μl of 5 g/liter furazolidone internal-standard solution to each vial and proceed as described above for the urine standard curves. On each day of analysis construct a urine standard curve and determine the nitrofurantoin concentrations of the stability solutions by comparing their nitrofurantoin/furazolidone peak-height ratios with those of the standard curve.

Other Analytical Variables

Repeated injections. Prepare a 2 mg/liter plasma solution and a 50 mg/liter urine solution of nitrofurantoin, then proceed as described above for the standard curves.

Effect of various solvents on the method. Prepare solutions of nitrofurantoin at concentrations of 100, 50, 20, 10, and 5 mg/liter in doubly-distilled water, dimethyl sulfoxide, plasma, and urine. Proceed as described above for the urine standard curves except that with the plasma solutions, proteins must be precipitated with methanol before injection into the chromatograph. Curves are constructed for each solvent by plotting nitrofurantoin/furazolidone peak-height ratios vs. nitrofurantoin concentrations.

Sensitivity. Prepare solutions of nitrofurantoin at concentrations of 0.4, 0.2, 0.1, 0.05, and 0.02 mg/liter in plasma. Add 10 μl of a 5 mg/liter furazolidone internal-standard solution to 400 μl of the nitrofurantoin solution. Add 600 μl of methanol to precipitate the proteins and mix thoroughly. Centrifuge for 15 min at 10 000 × g, pour the supernatant fluid into a 4-ml glass tube and inject into the chromatograph.

Comparison with Hyamine 10-X Assay

Prepare solutions of nitrofurantoin at concentrations of 20, 10, 5, 1, and 0.5 mg/liter in plasma. Subject each solution to both the present procedure for the standard curves and to the Hyamine 10-X method (3, 4). With the use of standard curves obtained for each method, determine the actual concentration of the solutions used in this comparison study. To obtain a correlation curve, plot the nitrofurantoin concentrations obtained by the present method vs. the nitrofurantoin concentrations obtained by the Hyamine 10-X method.

Nitrofurazone Plasma Standard Curve

Prepare solutions of nitrofurazone at concentrations of 10, 5, 2, 1, and 0.5 mg/liter in plasma. Add 10 μl of 50 mg/liter furazolidone internal-standard solution to 200 μl of nitrofurazone solution, then proceed as described above for the nitrofurantoin standard plasma curves, injecting five samples for each nitrofurazone concentration. The retention times for nitrofurazone and furazolidone are 7 and 8 min, respectively. Concentration curves are constructed by plotting nitrofurazone/furazolidone peak height ratios vs. nitrofurazone concentrations.

Data Handling

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. Results of this regression analysis are expressed throughout in the following form:

\[ y = (\text{slope of the line} \pm \text{standard deviation of the slope})x + (\text{intercept of the line} \pm \text{standard deviation of intercept}). \]

Results and Discussion

In human volunteers, a single 100-mg oral dose of nitrofurantoin produces nitrofurantoin concentrations near 1 mg/liter in the blood (4). When administered intravenously or intramuscularly, concentrations of nitrofurantoin in the blood reached 5 mg/liter. On the other hand, maximum urinary nitrofurantoin concentrations of 158 to 372 mg/liter were reported in nine normal individuals, each of whom received a 100-mg
tablet every 4 h (11). Since concentrations of the drug in plasma are several hundred times smaller than urinary concentrations, two standard curves were used; the urine standard curve covers the range 10–200 mg/liter, whereas the plasma standard curve covers the range 0.5–10 mg/liter. In addition, plasma nitrofurantoin concentrations ranging from 0.02 to 0.4 mg/liter were used to test the sensitivity of the present method.

The standard curves were constructed by adding known amounts of nitrofurantoin and internal standard (furazolidone) to urine and plasma and plotting the peak-height ratio of nitrofurantoin to furazolidone (y) against the concentration of nitrofurantoin in mg/liter (x). Over a period of 26 days, we constructed 10 plasma standard curves, each with five nitrofurantoin concentrations. With these 50 points, the regression line (±1 SD) for plasma was $y = (0.469 \pm 0.009)x + (0.097 \pm 0.047)$, with a CV of the slope of 1.9% and a correlation coefficient of 0.99. For urine, over a period of 22 days, nine standard curves were constructed, each with five nitrofurantoin concentrations. With these 45 points, the regression line for urine was $y = (0.0176 \pm 0.0001)x + (0.0127 \pm 0.0165)$, with a CV of the slope of 0.6% and a correlation coefficient of 0.99.

Stability studies were performed over a period of 26 days for plasma and 22 days for urine. Concentrations of nitrofurantoin in biological fluids were obtained by comparing their nitrofurantoin/furazolidone peak-height ratios with those of a standard curve obtained the same day. Plasma samples had been kept frozen at −10 °C and were thawed on the day of analysis. The two concentrations studied, 10 and 1 mg/liter, were stable during the 26 days, the values obtained being 10.06 ± 0.59 and 1.07 ± 0.08, respectively (Figure 1). Two concentrations of nitrofurantoin in urine were selected comparable to those typically encountered clinically, 200 and 18 mg/liter. Urine samples kept frozen at −10 °C and thawed on the day of analysis were stable over the period of 22 days tested, giving values of 198.42 ± 8.17 and 17.98 ± 0.54, respectively. If, however, samples were stored at room temperature, both urine solutions rapidly degraded in such a way that at day 22 the 18 mg/liter solution no longer contained any detectable nitrofurantoin. These results indicate that biological fluids awaiting nitrofurantoin analysis must be stored frozen.

Analysis of the samples was performed at 365 nm. Use of this wavelength increases the sensitivity of the assay, because the nitro group is determined near its absorption maximum (370 nm for nitrofurantoin). Moreover, at 365 nm most of the extraneous peaks resulting from the biological fluids were eliminated. As can be seen from examination of control human urine and plasma, we do not suspect any interference by endogenous substances with this assay because, under the conditions used for analysis, all the extraneous peaks have retention times of less than 3 min, whereas the retention times for nitrofurantoin and furazolidone are 6 and 8 min, respectively (Figures 2 and 3). As determined from various rat-tissue homogenates, nitrofurantoin metabolites do not interfere with detection of the parent compound (12). In all cases, by using a programmed gradient elution with the chromatograph, the metabolites eluted much earlier than did nitrofurantoin and they did not absorb at 365 nm (12).

With urine samples, where no precipitation is nec-
necessary, analyses can be performed in 9 min per sample. In the case of plasma, centrifugation after protein precipitation is necessary because direct injections of plasma into the chromatograph result in increases in operating pressures caused by build-up of proteins at the head of the column. Plasma samples require 15 min for protein precipitation, after which analyses can be performed in 9 min per sample. Neither the urine samples nor the methanol from the plasma samples need be filtered before injection into the chromatograph.

The reproducibility of the method was determined from repeated injection experiments. Two concentrations were used, 2 mg/liter in plasma and 50 mg/liter in urine, each injected nine times in the chromatograph. The 2 mg/liter plasma solution gave a CV of 1.5% (1.96 ± 0.03 mg/liter) whereas the 50 mg/liter urine solution gave a CV of 0.6% (51.00 ± 0.29 mg/liter).

The effect of various solvents on the present method was determined by preparing solutions of nitrofurantoin 100, 50, 20, 10, and 5 mg/liter in doubly distilled water, dimethyl sulfoxide, plasma, and urine. Concentration curves were constructed and the regression lines for each solvent were calculated: water, \( y = (0.0178 ± 0.0001) x - (0.0007 ± 0.0023) \); dimethyl sulfoxide, \( y = (0.0176 ± 0.0002) x + (0.0085 ± 0.0127) \); plasma, \( y = (0.0183 ± 0.0003) x - (0.0050 ± 0.0188) \); and urine, \( y = (0.0176 ± 0.0001) x + (0.0102 ± 0.0083) \); the correlation coefficients in all cases exceeded 0.99. The particular biological fluids or solvents used therefore had no effect on the quantitative aspect of the method. Moreover, it was concluded that use of dimethyl sulfoxide as a solubilizer in standard solutions having high nitrofurantoin concentrations (>100 mg/liter) did not interfere with the method. After protein denaturation and centrifugation, whole blood and milk were also used, without modification of the present assay, and were found to be suitable biological fluids for the determination of nitrofurantoin.

Chromatograms of urine and plasma samples from a male patient receiving nitrofurantoin (50 mg every 6 h) are shown in Figures 2 and 3, respectively. Samples were taken 90 min after administration of this patient's fifth 50-mg oral dose of nitrofurantoin. This patient was also receiving the following drugs: codeine, dextropropoxyphene, aluminum hydroxide gel, bisacodyl, ascorbic acid, a multiple-vitamin preparation, and oxycodone with aspirin, phenacetin, and caffeine. From the nitrofurantoin to furazolidone peak-height ratio in the patient's chromatograms, the nitrofurantoin concentration in urine was 26.1 mg/liter, whereas the concentration in plasma was 0.72 mg/liter.

To determine the sensitivity of the method, we used nitrofurantoin solutions in plasma over a concentration range from 0.02 to 4 mg/liter. For each of the five concentrations, two to three injections were made. The curve was constructed by plotting the peak-height ratios of nitrofurantoin to furazolidone vs. the concentration of nitrofurantoin. With 11 points, the regression line was \( y = (10.40 ± 0.18) x + (0.08 ± 0.04) \) with a correlation coefficient of 0.99.

For the comparison between the present method and the Hyamine 10-X method, solutions of nitrofurantoin in plasma covering the range of 0.5 to 20 mg/liter were used for both methods. Concentrations obtained by the present method, plotted vs. those obtained from the Hyamine 10-X method, gave a correlation line \( y = (1.030 ± 0.021) x + (0.340 ± 0.286) \), with a CV of the slope of 2% and a correlation coefficient of 0.99. Compared to the Hyamine 10-X method, the present method offers the advantage of easier sample preparation, because it requires only the addition of the internal standard and no extraction is necessary. The sensitivity is greater with the present method, 0.02 mg/liter being easily detected, whereas in the original Hyamine 10-X method (3) the sensitivity is reported to be 2 mg/liter and in the improved Hyamine 10-X method (4) the sensitivity is 0.2 mg/liter. In addition, solutions for the present analysis are stable; there is no progressive loss of sensitivity as is observed with the Hyamine 10-X method owing to the degradation of methazonic acid formed by the addition of Hyamine 10-X to nitromethane (6). However, when the absorbance of the Hyamine 10-X complex is measured rapidly after its formation, as is recommended by the authors of these publications, nitrofurantoin concentrations can be measured at the reported sensitivities (3, 4).
With no modification in the procedure, the present assay can be extended to other 5-nitrofurans. The regression line for the standard plasma curve with nitrofurazone is \( y = (0.730 \pm 0.005)x - (0.051 \pm 0.015) \), (CV = 0.7%, \( r = 0.99 \)). Since nitrofurazone is used in animal feeds for the prevention of a variety of diseases, the method lends itself to analysis of residues of 5-nitrofuran derivatives present in tissues of animals having been fed such a diet.

We believe that the present assay offers a sensitive, accurate, stable and convenient method for the analysis of nitrofurantoin and nitrofurazone in biological fluids.

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References