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Sensitive determination of nitrofurantoin in human plasma and urine by high-performance liquid chromatography

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Abstract

A highly sensitive and selective HPLC method was developed and validated for the determination of nitrofurantoin in human plasma and urine. The method involves the liquid–liquid extraction of drug and internal standard from plasma with ethyl acetate followed by evaporation and reconstitution in mobile phase. Urine samples were simply diluted with purified water. UV detection was done at 370 nm. The limit of quantification for nitrofurantoin in plasma was 0.010 μg/ml. In urine nitrofurantoin could be quantified down to 0.380 μg/ml. Linearity was proven over the whole calibration range in plasma (2.48–0.0100 μg/ml) as well as in urine (187 μg/ml–0.380 μg/ml). The method was validated according to Good Laboratory Practice guidelines and its suitability was demonstrated by analysis of samples from a pharmacokinetic study

Keywords: Nitrofurantoin

1. Introduction

Nitrofurantoin (1 - [[(5 - nitro - 2 - furanyl)methylene]amino]-2,4-imidazolidinedione) is a nitrofuran-derivative antibacterial agent used in the therapy of urinary tract infections. It has been in clinical use since 1953. Consequently the analytical methods used for determination of nitrofurantoin range from colorimetric methods in the sixties to HPLC-methods in the seventies and eighties. In search of an HPLC method to assay samples from a pharmacokinetic study with Urospasmon®, an antibacterial drug containing nitrofurantoin and sulfadiazine, we reviewed the published methods. Published colorimetric [1,2] and spectrophotometric [3] methods did not offer a sufficient quantification limit, nor did they have the specificity needed for the determination of pharmacokinetic parameters. Published HPLC methods only reported sensitivities down to 0.02 μg/ml in plasma [4,5]. Additionally the pretreated samples were not stable and had to be analyzed within 5 min of preparation [5].

Therefore we developed an HPLC assay for the determination of nitrofurantoin in plasma that allows not only the use of an internal standard but also the measurement of sequences of samples with sufficient stability and that has a limit of quantification significantly lower than 0.02 μg/ml.

The method was extensively validated fulfilling international guidelines [6] and also adapted for measurement of nitrofurantoin in urine.

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2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents used were of HPLC grade or p.a. Nitrofurantoin was obtained from Heumann Pharma (Nürnberg, Germany). Furazolidone, used as internal standard, was purchased from Sigma (St. Louis, MO, USA). Sodium dihydrogen phosphate monohydrate, perchloric acid (70%) and *ortho*-phosphoric acid (85%) were purchased from Merck (Darmstadt, Germany). Solvents were of analytical and HPLC-grade. *n*-Hexane was of LiChrosolv quality and purchased from Merck as well as 0.1 *M* hydrochloric acid. Acetonitrile, methanol and ethyl acetate were of ChromAR quality (Promochem, Wesel, Germany). Water purified by Milli-Q® system (Millipore Corporation, Bedford, MA, USA) was used in all procedures involving water.

2.2. Apparatus and chromatographic conditions

The HPLC apparatus consisted of a Waters 510 pump (Waters, Eschborn, Germany), a Waters 490 variable wavelength UV detector (set at 370 nm) and a 710 B WISP autosampler (Waters, Eschborn, Germany). The integration was performed using the software Turbochrom 3 (Perkin Elmer Nelson, Cupertino, CA, USA) installed on a IBM compatible PC connected to a Star LC-10 printer. The analytical column (stainless steel, 250 × 4.6 mm I.D.) was packed with 5-μm particles of Spherisorb ODS II (M. Grom, Herrenberg, Germany). Refilled columns were used throughout the validation procedures. As a guard column we used cartridges manufactured by Merck, filled with Lichrospher RP 18, 5 μm (4 × 4 mm). Guard columns were replaced on a routine system after 60–90 injections (e.g. after one sequence) during the validation procedures. The temperature of columns was controlled at 30°C in a water bath. For analysis of plasma and urine samples the composition of the mobile phase was 5 m*M* NaH_2PO_4 buffer and acetonitrile (80:20, v/v). The pH was adjusted to 3.0 with *o*-phosphoric acid. The flow-rate of the mobile phase was set to 1.0 ml/min.

2.3. Preparation of stock solutions, calibration levels and validation samples

A stock solution of 250 μg/ml nitrofurantoin was prepared in acetonitrile. A stock solution of the internal standard furazolidone was prepared by dissolving an appropriate amount of furazolidone in acetonitrile to achieve a solution of 200 μg/ml furazolidone. Calibration levels in the appropriate drug-free matrix were prepared yielding a concentration range from 0.01 μg/ml up to 2.48 μg/ml. Validation samples (VS) at three different concentration levels were prepared leading to concentrations of 1.25 μg/ml, 0.25 μg/ml and 0.026 μg/ml. In the same way as the stock solutions, all calibration levels and validation samples were divided into aliquots anticipating the number needed for the validation experiments and stored frozen at −20°C until analysis. The calibration samples and validation samples were pretreated in exactly the same way as unknowns.

2.4. Sample pretreatment

Plasma.

During preparation samples were kept protected from light. Plasma samples were thawed, thoroughly vortexed for 15 s and centrifuged for 10 min at 2100 *g*. A 1000-μl volume of the diluted internal standard solution (1 μg/ml furazolidone) were pipetted into a brown glass tube and 1000 μl of the centrifuged plasma sample were added. No internal standard solution was used for blank samples. Then 5 ml of ethylacetate were added to the samples. The mixture was shaken for 10 min at 50 rpm. By centrifugation at 4800 *g* and 4°C for 10 min the organic and the plasma phase were separated. The organic phase was pipetted in another brown glass tube and evaporated under a gentle flow of nitrogen at room temperature. The residue was dissolved in 200 μl of mobile phase and vortexed for 30 s. The total solution was transferred to an Eppendorf 1.5-ml vial and 200 μl of *n*-hexane were added. After mixing for 15 s the samples were centrifuged for 3 min at 12 000 *g*. The organic phase was completely withdrawn by suction through a glass pasteur-pipette connected to the vacuum pump. 100 μl of the remaining sample were

transferred to an autosampler vial and 50 μl were injected onto the HPLC-system.

Urine

Urine samples were thawed under light protection and thoroughly vortexed. A 900-μl volume of Milli-Q-water was pipetted to a 1.5-ml reaction tube, 100 μl of the sample were added and thoroughly mixed (this dilution step was not performed for calibration standards and validation samples, as these solutions have already been weighed in diluted (1:10) urine during preparation of the standards). A 500-μl volume of the solution was transferred to a 1.5-ml reaction tube and 50 μl of the internal standard solution were added. After vortexing, an aliquot of 200 μl was pipetted into an autosampler vial and 40 μl were injected into the HPLC system.

2.5. Validation of the assay

Accuracy and precision of the assay as well as the linearity of the calibration curve were determined intra-day and inter-day on three different days. Recovery of the analyte and the internal standard following sample clean-up procedures relative to aqueous solutions were determined at different concentration levels. Stability of the analyte was tested at different light and temperature conditions in the appropriate biological matrix prior to and subsequent to the sample preparation procedures. All experiments were performed in a total of five sequences.

3. Results and discussion

3.1. Specificity, linearity and sensitivity

The specificity of the method was determined by screening blank plasma of six different healthy donors. Sulfadiazine, an antibacterial active compound of Urospasmon®, was investigated for interferences with nitrofurantoin or the internal standard furazolidone. No other compounds were investigated as the assay was developed for use in studies with healthy volunteers. Representative chromatograms of plasma samples derived from a healthy volunteer prior to administration of nitrofurantoin and 2 h after an oral dose of 50 mg nitrofurantoin (0.02 μg/ml nitrofurantoin) are shown in Fig. 1 and Fig. 2. Retention times for nitrofurantoin were between 7.9–10.1 min and between 10.0–13.3 min for the internal standard, furazolidon. Nitrofurantoin and the internal standard are completely resolved from each other and from any endogeneous peaks in plasma and urine. Sulfadiazine, the second compound in the pharmaceutical preparation to be tested, was not detected in the chromatographic system.

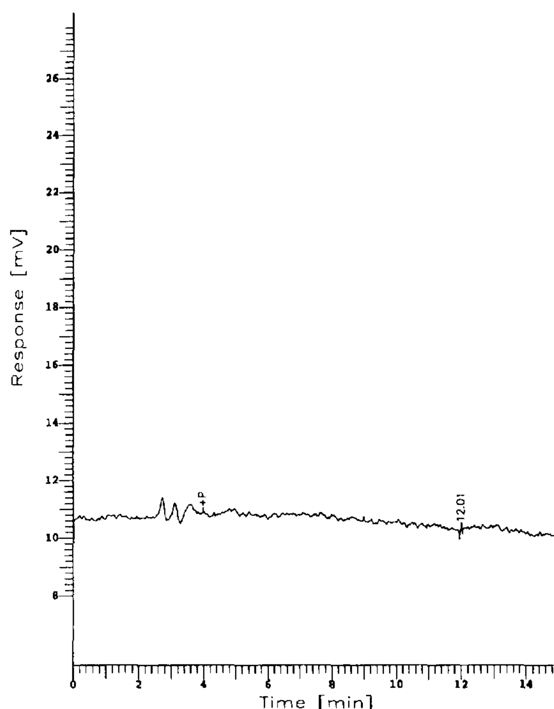

Fig. 1. Blank plasma of a healthy volunteer prior to drug administration.

For evaluation of the calibration graph a weighted linear regression (1/concentration) was performed with nominal concentrations of calibration levels and measured peak-height ratios (peak-height analyte/peak-height internal standard). The slope and intercept of the nine-point regression graph were determined according to standard equations. A total of six calibration curves were assayed and calculated on three different days. Linearity of the assay could be shown over a concentration range of 0.01–2.48 μg/ml. The coefficient of correlation (r^2) was above 0.999 in each case. The intercept relative to the

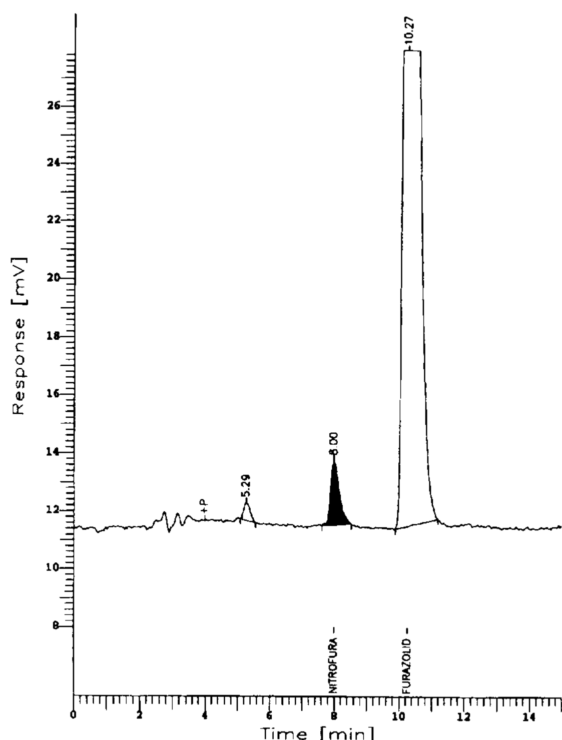

Fig. 2. Plasma sample of a healthy volunteer collected 2 h after an oral dose of 50 mg of nitrofurantoin (nitrofurantoin conc.: 0.0202 μg/ml)

response of the lowest calibration level ranged between 3.9 and 13.1% for the individual curves with a mean value of 9.8% (Table 1). The relative error of the individual calibration points was between −6.2 and 9.1%. The mean accuracy of the different calibration levels was between 96.2 and 105.0%.

The lowest calibration level used in the validation calibration curve yielded a concentration of 0.01 μg/ml of nitrofurantoin in plasma. This was set as the limit of quantification that can be measured with accuracy. The individual values of the relative error of this calibration level were between −4.2 and 8.8%.

3.2. Precision and accuracy

The intra-day precision and accuracy were determined by analyzing five aliquots of each validation sample within one sequence. Means, standard deviations, coefficients of variation (%) and relative errors (%) were determined. The intra-day precision was good and consistent with values of 4.0% (n=5) for a concentration of 1.25 μg/ml nitrofurantoin, 2.3% (n=5), and 2.3% (n=4) for a concentration of 0.253 μg/ml, and 0.0261 μg/ml nitrofurantoin, respectively (Table 2). Inter-day variation was determined by analyzing two calibration curves and two replicates of each validation sample on each of three different days. The respective values for the inter-day precision were in the same range as determined for intra-day variation. The mean value of the inter-day precision of the highest validation sample was 1.6%, the values for the medium and lower validation samples were 2.7% and 1.5%. The accuracy was calculated as the relative error by compiling the measured concentrations with the nominal concentrations of nitrofurantoin in validation samples. For the intra-day accuracy the individual deviations ranged from −7.2 to 2.4% for the high validation sample (1.25 μg/ml nitrofurantoin), −1.6 to 4.0% for the medium validation sample (0.25 μg/ml), and −0.8 to 3.8% for the low validation sample (0.026 μg/ml). The respective

Table 1
Calibration parameters of all sequences

Parameter	Day 1		Day 2		Day 3	
	Cal. curve 1	Cal. curve 2	Cal. curve 3	Cal. curve 4	Cal. curve 5	Cal. curve 6
Number of levels used	9	9	9	9	9	8
Slope	1.07	1.05	1.06	1.05	1.05	1.07
Intercept	−0.00134	−0.00105	−0.00130	0.000446	−0.00131	0.000626
Coefficient of correlation r^2	0.999 832	0.999 591	0.999 672	0.999 750	0.999 406	0.999 775
Response of lowest level	0.0102	0.0104	0.00992	0.0115	0.0100	0.0109
Intercept expressed as % of the response of the lowest level	13.1	10.1	13.1	3.9	13.1	5.7

Table 2
Intra-day precision and relative error (R.E.) of validation samples (VS)

	VS 1		VS 2		VS3	
	Concentration (μg/ml)	R.E. (%)	Concentration (μg/ml)	R.E. (%)	Concentration (μg/ml)	R.E. (%)
	1.25[a]		0.253[a]		0.0261[a]	
	1.28	2.4	0.249	−1.6	0.0345[b]	32.0[b]
	1.28	2.4	0.263	4.0	0.0262	0.4
	1.26	0.8	0.263	4.0	0.0259	−0.8
	1.16	−7.2	0.259	2.4	0.0271	3.8
	1.24	−0.8	0.261	3.2	0.0271	3.8
n	5		5		4	
Mean	1.24		0.259		0.0266	
S.D.	0.05		0.006		0.0006	
C.V.(%)	4.03		2.32		2.26	

[a] Nominal nitrofurantoin concentration (μg/ml).
[b] Outlier for unknown reason (not included in statistical evaluation).

values of the inter-day accuracy confirmed the range of the intra-day values. The individual values ranged from −0.6 to 3.6%, −1.7 to 6.4%, and 4.0 to 6.6% for the high, medium, and low validation samples, respectively.

3.3. Recovery

Recovery was measured by comparison of peak heights of non-extracted standards in mobile phase versus extracted standards of spiked plasma. The recoveries of the analyte and the internal standard were determined separately. The recovery of nitrofurantoin was determined as 86.2% (n=9) at 2.42 μg/ml, 84.2% (n=9) at 0.24 μg/ml and 86.4% (n=8) at 0.12 μg/ml. The recovery of the internal standard furazolidone was found to be 65.9% (n=5) at 0.94 μg/ml, 69.7% (n=4) at 0.19 μg/ml and 69.9% (n=5) at 0.02 μg/ml. These values indicate the reliability of the sample preparation over the concentration range tested.

3.4. Stability experiments

Evaluation of the stability of samples was in general based on the comparison of the mean of the respective sample at the respective condition with an initial value.

Light stability of nitrofurantoin in plasma (1 μg/ml) was determined under the following conditions:

A: not light protected, stored in direct sunlight at a lab window
B: not light protected, stored at ambient room (fluorescent) light in the lab
C: light protected

Nitrofurantoin as well as furazolidone were found to be unstable under exposure to light. Furazolidone was nearly totally decomposed by daylight during 6 h but was unchanged by fluorescent light in the same time period. Nitrofurantoin showed a degradation to 20% of the initial value during 6 h when stored in daylight. Contrary to the behaviour of furazolidone, storage under fluorescent light resulted also in a considerable decrease of 24% within 24 h (Table 3). All other conditions tested showed no influence on the stability of nitrofurantoin. For the determination of the counter stability (stability during sample preparation) validation samples were thawed at certain timepoints according to a time schedule (24 h, 6 h, 5 h, 4 h, 3 h, 2 h, and 1 h before sample clean-up). After thawing, the aliquots were stored at room temperature protected from light. At zero time all samples were prepared and measured. Stability of nitrofurantoin in native plasma was given over a 24-h period (Table 4). Stability was also tested after sample preparation at room temperature and +4°C after 24-h and 48-h storage. No instability of nitrofurantoin was observed over a time period of 48 h (Table 5). Three freeze-thaw cycles did not significantly alter the amount of nitrofurantoin analyzed

Table 3
Light stability of nitrofurantoin (1.00 μg/ml) and furazolidone (1.00 μg/ml) in plasma

Storage (h)	Nitrofurantoin		Furazolidone	
	Stability (%) during treatment		Stability (%) during treatment	
	A	B	A	B
2	51.3	101.9	40.1	101.1
4	30.1	93.7	10.6	94.8
6	20.2	92.8	7.2	99.6
24	–	76.2	–	102.4

A: not light protected, stored in direct sunlight at a lab window.
B: not light protected, stored at ambient room (fluorescent) light in the lab.
–: not performed.

Table 6
Freeze/thaw stability of nitrofurantoin in plasma

Cycle	Concentration (μg/ml)	Mean (μg/ml)	Stability (%)
	1.08[a]		
	1.09[a]	1.08	100.0
	1.06[a]		
1	1.72[b]	1.71[b]	158.3[b]
	1.70[b]		
2	1.02	1.07	99.1
	1.12		
5	1.01	1.06	98.1
	1.10		

[a]Baseline value.
[b] Outlier for unknown reason (not included in statistical evaluation).

in the spiked plasma samples (Table 6). Thus, it was concluded that nitrofurantoin is stable for a sufficient time period after thawing prior to sample preparation and after sample preparation without the need for cooling the samples. However, strict light protection is required during the whole process of analysis.

3.5. *Urine*

The HPLC method for measurement of nitrofuran-

Table 4
Counter stability of nitrofurantoin at room temperature (light protected)

Storage (h)	VS1		VS2	
	Concentration (μg/ml)	Stability (%)	Concentration (μg/ml)	Stability (%)
0	1.25[a]	100.0	0.253[a]	100.0
1	1.48	118.4	0.320	126.5
2	1.43	114.4	0.317	125.3
3	1.50	120.0	0.317	125.3
4	1.40	112.0	0.290	114.6
5	1.43	114.4	0.300	118.6
6	1.42	113.6	0.290	114.6
24	1.41	112.8	0.284	112.3

[a] Nominal nitrofurantoin concentration (μg/ml).

Table 5
Stability of nitrofurantoin after sample clean-up (light protected)

Time (h)	Storage at room temperature				Storage at +4°C			
	VS 1		VS 2		VS 1		VS2	
	Concentration (μg/ml)	Stability (%)	Concentration (μg/ml)	Stability (%)(μg/ml)	Concentration (%)	Stability (μg/ml)	Concentration (%)	Stability
	1.25[a]	100.0	0.253[a]	100.0	1.25[a]	100.0	0.253[a]	100.0
24	1.27	101.6	0.257	101.6	1.28	102.4	0.262	103.6
48	1.22	97.6	0.259	102.4	1.27	101.6	0.248	98.0

[a] Nominal nitrofurantoin concentration (μg/ml).

toin in human plasma was tested to see if it was suitable for the quantitation of nitrofurantoin in human urine. The only change in the assay conditions involves the sample work-up: Instead of deproteinizing by liquid–liquid-extraction, the urine samples were diluted with Milli-Q-water. As the validation in plasma samples has proved the good performance of the method in general, the validation procedure for measurement of urine samples was limited to the determination of precision, linearity and accuracy. The appropriate biological matrix, human drug-free (blank) urine was collected from human donors. For preparation of calibration standards and validation samples the native urine was prediluted 1:10 with Milli-Q water. Stock solutions of nitrofurantoin and furazolidone were prepared in acetonitrile.

The normalized responses of the calibration standards showed a small variation of 4.4%. The calculated concentrations of calibration levels yielded a comparable coefficient of variation of 4.5%. These numbers indicate a good precision and confirm the corresponding results of the validation of the plasma assay. The calibration graph exhibited an excellent linearity with a coefficient of correlation (r^2) of 0.998830 in the calibration range of 0.379–187 μg/ml. The relative errors of calibration standards ranged between −5.1% and +7.2%. The intercept expressed as the percentage of the response of the lowest level was 2.2%. Since level no. 10 had a relative error of −5.0% its nominal concentration of 0.379 μg/ml was accepted as the quantification limit. The analysis of the validation samples produced very small relative errors with +5.0% at 101 μg/ml nitrofurantoin, +3.5% at 9.48 μg/ml nitrofurantoin, and +14.5% at 4.93 μg/ml nitrofurantoin.

4. Application

4.1. Plasma

The validated method was used to analyze more than 650 plasma samples from a pharmacokinetic study in healthy volunteers. Calibration levels and validation samples were identical to the calibration levels and validation samples used during the validation procedures. Samples were analyzed during a two-month period in thirteen sequences. The values obtained for the calibration levels and the resulting calibration graphs were in a similar range observed during validation. The coefficient of correlation (r^2) was above 0.999 in each case. The intercept, expressed relative to the response of the lowest calibration level, ranged between 1.3–28.2%. The relative error of the individual calibration points was between −9.1 and 15.3%. The inter-day precision of the validation samples was very similar to those observed during validation. The precision of the highest validation sample (1.25 μg/ml nitrofurantoin) was 2.6% (n=26), the medium validation sample (0.253 μg/ml nitrofurantoin) yielded a value of 2.5% (n=25) and the low validation sample (0.0261 μg/ml nitrofurantoin) 5.0% (n=25).

4.2. Urine

More than 190 human urine samples were analyzed with the method described. Samples were analyzed in a two-week period in five sequences. Similar to the plasma assay, the quality of data obtained during routine work confirmed the findings of the validation procedure. The coefficient of correlation (r^2) of the calibration graphs was greater than 0.998 in each case. The precision of the validation samples was 3.5% (n=10) at 101 μg/ml nitrofurantoin, 5.7% (n=9) at 9.48 μg/ml, and 6.0% (n=10) at 4.93 μg/ml.

5. Conclusions

This paper describes a sensitive, selective and reliable HPLC assay for nitrofurantoin in human plasma and urine. The method includes a liquid–liquid extraction for plasma samples and a simple dilution step for urine samples. A linear quantification range from 0.01–2.48 μg/ml for plasma and 0.379–187 μg/ml for urine could be established. The method has been successfully used for measurement of samples derived from a human pharmacokinetic study after multiple dosing of Urospasmon. Plasma levels could be followed up until 26 h

after last dosing. A daily throughput of 80–100 plasma samples by manual sample preparation and an automated HPLC system is possible. The precision and accuracy found during the validation procedure for plasma was confirmed during the routine analysis. The values of the low validation sample were found to be somewhat more imprecise. This may reflect the more complex nature of sample preparation for plasma samples.

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