Development and validation of a fast and sensitive UHPLC-DAD assay for the quantification of nitrofurantoin in plasma and urine

Rixt A. Wijma[a,*], Karin E. J. Hoogtanders[b,*], Sander Croes[b,c], Johan W. Mouton[a] and Roger J. M. Brüggemann[d]

[a]*Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center, Rotterdam, The Netherlands;*

[b]*Department of Clinical Pharmacy and Toxicology, Maastricht University Medical Center, Maastricht, the Netherlands;*

[c]*CAPHRI-Care and Primary Health Research Institute, Maastricht University Medical Center, Maastricht, the Netherlands;*

[d]*Department of Pharmacy and Center of Expertise in Mycology, Radboud University/CWZ, Nijmegen, The Netherlands*

**Authors contributed equally.*

Corresponding author: Roger J. M. Brüggemann, PharmD, PhD, Department of Pharmacy and Center of Expertise in Mycology, Radboud University/CWZ, P.O. Box 9101, Nijmegen 6525 HB, The Netherlands, email address: roger.bruggemann@radboudumc.nl

ABSTRACT

Nitrofurantoin is an antimicrobial drug that has been used in the treatment of lower urinary tract infections for more than 50 years. Despite its long use, surprisingly little is known of the pharmacokinetics of nitrofurantoin, whereas this is essential to optimize patient treatment. We developed a novel analytical method for the quantification of nitrofurantoin in plasma and urine using ultra-high performance liquid chromatography and diode array detection to allow pharmacokinetic studies in these two matrices. The sample preparation method consisted of protein precipitation for plasma and liquid-liquid extraction for urine. 100 µL was needed for the sample preparation. Furazolidone was used as internal standard. Gradient chromatographic separation was performed on a HSS-T3 column. UV detection was performed at a wavelength of 369 nm. The analysis time was 5 minutes. The method was successfully validated according to the FDA-guidelines (2018). Linearity was confirmed over a concentration range from 50 to 1250 µg/L in plasma and from 4 to 200 mg/L in urine (r^2 > 0.95). Validation results of five QC concentrations for plasma and urine, respectively, are for within-day accuracy <± 13% in both matrices, for between-day accuracy <± 7% and <± 9%, for within-day precision <10% and <4% and for between-day precision <10% and <5%. Plasma samples are stable for seven days at 4°C, and for 2 years at -20°C and -80°C. Urine samples are stable for at least seven days at 4°C and at room temperature and for 2 years at -20°C and at -80°C, except from the lower concentrated samples, which are only stable at -80°C. All samples were kept from daylight using amber colored glassware. The presented method meets all validation requirements and was successfully used in a clinical study where the pharmacokinetics of nitrofurantoin were investigated in healthy volunteers. The easy sample preparation method and the short analysis time make this method suitable for use during routine clinical practice to study the pharmacokinetics of nitrofurantoin.

Keywords: Nitrofurantoin; Pharmacokinetics/Pharmacodynamics; UHPLC-DAD; Antimicrobial drug; Urinary tract infections

INTRODUCTION

Urinary tract infections (UTIs) are one of the most common infections worldwide (1). These infections became more difficult to treat due to the increasing prevalence of multi-drug resistance (including β-lactam resistance and quinolone-resistance) among Gram-negative uropathogens (2–4). In the search for alternative treatment options, there is a renewed interest in old antimicrobial drugs. However, an important disadvantage of these old drugs is that important pharmacokinetic (PK) information is lacking. It is well known that this information is important for dosing optimization and therefore maximizing treatment effectivity and minimizing the risk of emergence of resistance among pathogens (5).

One of these old antimicrobial drugs is nitrofurantoin. The drug is a member of the nitrofuran group and was marketed in the early 1950s for the treatment of uncomplicated UTIs. The chemical structure of nitrofurantoin is demonstrated in figure 1a. Its spectrum of activity includes (extended spectrum beta-lactamase-producing (ESBL)) *Enterobacteriaceae* and (vancomycin-resistant) *Enterococci*. Nitrofurantoin is registered for the treatment of UTIs in the standard dose of 50 mg q6h (regular release capsule) or 100 mg q8h or q12h (slow release formulation), depending on the geographical location. A single daily dose of 50-100 mg is registered for UTI prophylaxis. Maximum plasma concentrations after a dose of 50 mg q6h were never reported, but are expected to be low (around 700 μg/L) and therefore sub therapeutic, based on plasma concentrations after other dosages of nitrofurantoin (6–8). Around 40% of the dose is excreted unchanged in the urine after oral administration (9–11). Microbiological effective urine concentrations are expected to be around 200 mg/L (9, 12, 13).

The current body of PK knowledge of nitrofurantoin in UTI patients is poor and mainly based on decades-old studies using comparative, archaic laboratory and analytical techniques (14). Development of a sensitive analytical method for the quantification of nitrofurantoin in human plasma and urine is an important first step to fill in this PK knowledge gap.

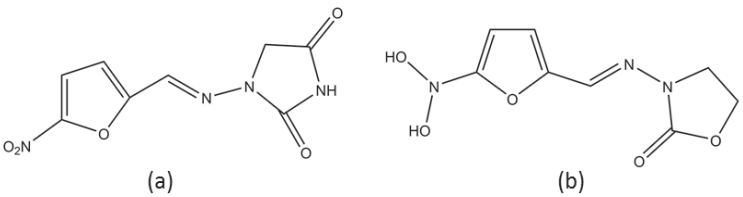

Figure 1. (a) The chemical structure of nitrofurantoin and (b) the internal standard furazolidone.

The first analytical method for nitrofurantoin in urine was the chromatographic method with spectrophotometric or colorimetric detection described by Bender, Nohle and Paul in 1956 (15). This method served as the base for the first methods using high performance liquid chromatography (HPLC) with UV-detection for the quantification of nitrofurantoin in plasma and/or urine (9, 16–20). LC methods with mass-spectrometry (LC-MS/MS) detection followed thereafter (8, 21). These methods are in general more specific compared to the HPLC-UV methods. Limits of quantification in plasma range from 5 to 20 µg/L with LC-MS and from 10 to 500 µg/L with LC-UV. No LC-MS methods were described for the quantification of nitrofurantoin in urine. The only methods describing the quantification of nitrofurantoin in both matrices are based on UV-detection where total analysis times up to 15 minutes are reported (20). One method was designed for the quantification of (some of) its metabolites because it is known that also metabolites are responsible for its antibacterial activity (18, 22). We aimed to develop an analytical method for the quantification of nitrofurantoin in plasma and urine using ultra-high performance liquid chromatography and diode array detection (UHPLC-DAD) to allow pharmacokinetic studies in these two matrices which can be used in daily clinical practice as well as for research purposes.

MATERIAL AND METHODS

Chemicals and reagents

Nitrofurantoin and the internal standard (IS) furazolidone (figure 1b) were purchased from *Sigma Aldrich* (Zwijndrecht, the Netherlands, purity 99.90% and 99.60%, respectively). Methanol was purchased from *Biosolve B.V.* (Valkenswaard, the Netherlands). Acetonitrile, dimethylsulfoxide (DMSO), acetic acid, hydrochloric acid (HCl), phosphoric acid (25%), zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), ammonium acetate (CH_3COONH_4), potassium chloride (KCl) and sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$) were purchased from *Merck Millipore* (Darmstadt, Germany). The water was purified using a Millipore Advantage A10 System also from *Merck Millipore*. All chemicals were of LC-MS quality.

Drug free plasma was obtained from volunteers who donated blood in the national blood donation center. The blood was centrifuged after donation and plasma was pooled and stored at -20°C prior to analysis. Drug free urine was obtained from drug-free subjects who donated urine voluntary. The urine was pooled and stored for maximum 3 days at 4°C after collection.

Table 1. The used gradient for the mobile phases A and B.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0-2.5	96.0	4.0
2.5-4	33.3	66.7
4-5	96.0	4.0

Preparation of calibration curve, quality controls samples and internal standard.

Stock solutions of nitrofurantoin and furazolidone of 1000 mg/L were prepared in Methanol-DMSO (1:1, v:v). The working solutions of 10 mg/L were prepared by diluting these stock solutions 100 times with purified water. Calibration standards and the quality control (QC) samples were prepared by diluting this working solution with drug free urine or plasma. The final concentrations of these samples are demonstrated in table 2. The stock solutions and working solutions were stored at 4°C and were brought to room temperature prior to use. Amber color glassware was used to protect the content from daylight.

Table 2. Concentrations of the six calibration standards and the quality control samples (QC-L, QC-M, QC-H, LLOQ and ULOQ).

	Calibrations standards						**Internal quality control samples (μg/L)**				
	1	2	3	4	5	6	QC-L	QC-M	QC-H	LLOQ	ULOQ
Plasma (μg/L)	50	100	200	500	750	1250	100	600	1000	50	1250
Urine (mg/L)	4	24	40	60	100	200	10	80	150	4	200

Sample preparation

Mobile phase A was prepared by dissolving 3.85 mg CH_3COONH_4 in 1000 mL purified water so that the molarity of the final solution was 0.05M. pH was adapted to 5.8 with acetic acid and the solution was mixed with acetonitrile (90:10, v:v). Mobile phase B consisted of acetonitrile. The buffer of pH 2 was a 6.57g/L solution of KCl in HCl and water and was prepared by dissolving 0.657 g KCl in 11.9 mL of 0.1 M HCl (conform The Dutch Pharmacopoeia, 8th edition, part I). Purified water was then added to the final volume of 100 mL. The 0.05 M phosphate buffer (pH 4.8) was prepared by dissolving 0.89 g Na_2HPO_4 . $2H_2O$ in 100 mL of purified water. The pH was adjusted to 4.8 with 25% phosphoric acid. The protein precipitation solution was prepared by mixing methanol with a $ZnSO_4$ solution and the 10 mg/L internal standard solution (20:20:1, v:v). The $ZnSO_4$ solution was prepared by dissolving 178 mg $ZnSO_4$. $7H_2O$ in 1000 mL purified water (stored in portions of 35 mL at -20°C, freshly prepared every week). 100

µL of a plasma sample was needed to perform the sample preparation using protein precipitation. The glass tubes were vortexed for one minute and thereafter centrifuged for five minutes at 16,1 **g**. 50 µL of the 0.05 M phosphate buffer (pH 4.8) and 100 µL of the protein precipitation solution were added. 100 µL of the supernatant was then transferred to an 1.5 mL tube (Greiner Bio-One™ Reaction tube) and mixed with 100 µL purified water. 40 µL of the final mixture was injected in the LC-apparatus.

Urine samples were prepared using liquid-liquid extraction. 100 µL of an urine sample was mixed with 900 µL purified water, 200 µL of the pH 2 buffer, 50 µL of the 100 mg/L internal standard solution, and 3 mL dichloromethane. The 100 mg/L internal standard solution was freshly prepared each time by ten-fold dilution of the stock solution with purified water. The mixture was vortexed for five minutes and centrifuged for five minutes at 6,0 **g**. The lower organic layer was separated from the upper aqueous layer by removing the aqueous layer using vacuum and then transferring the organic layer into a clean tube. After centrifugation during five minutes at 6,0 **g**, the organic layer was again transferred to a clean tube. The solvent was evaporated under nitrogen flow at room temperature and the residue was reconstituted in 200 µL of the mobile phase A. The mixture was vortexed for five minutes and then transferred to an ultrasonic bath for two minutes operating at room temperature. The mixture was then vortexed for five minutes and 5 µL of the final mixture was injected in the LC apparatus.

All samples were brought to room temperature one hour before use and were protected from light during the preparation using amber colored glass ware and plastic disposables.

Chromatography and detection

The UHPLC system consisted of a UHPLC-pump from *Waters Acquity Quaternary Solvent Manager,* an auto sampler operating at 15°C with flow through needle injection mode, a column compartment and a DA detector (all *Waters Chromatography B.V.*, Etten-Leur, the Netherlands). The software program *Empower version 3.0* (all *Waters Chromatography B.V*) was used for data processing.

Chromatographic separation was achieved using an Acquity UHPLC HSS-T3, 100 x 2.1 mm, 1.8 µm column (*Waters Chromatography B.V.*). Gradient elution was performed using mobile phases A and B. The used gradient is described in table 1 where the composition changed linear over time. The total analysis time was five minutes and the flow rate was stable at 0.6 mL/min. The retention times of nitrofurantoin and the internal standard were 1.7 and 2.3 minutes, respectively. Detection was performed at 369 nm and the column temperature was 45°C.

Analytical validation

UHPLC-UV settings and the sample preparation procedure were adapted from in-house methods for other compounds. Validation was performed according to the Food and Drug Administration (FDA) guideline for bioanalytical method validation, 2018 (23).

Selectivity

The interference from endogenous compounds was investigated by analyzing blank plasma and urine of six different individuals who did not use nitrofurantoin. Furthermore, we tested the following frequently used co-administered drugs: acenocoumarol, acetylsalicylic acid, enalapril, ethinyl estradiol, furosemide, ibuprofen, metoprolol, paracetamol, simvastatin, and metformin. No interfering peaks were allowed.

Accuracy, precision and limits of quantification

Five quality control (QC) samples of each level (25 samples in total) were prepared and the deviations of the measured concentrations were compared to the theoretical concentrations of the samples (table 2). To define the within-day accuracy, the mean of five replicates was calculated. For the between-day accuracy, all QC samples were prepared in triplicate (15 samples in total) and were measured on three different days. The claim for the accuracy parameters is that the measured concentrations should be within the acceptance criteria of ± 20% for the lower limit of quantification (LLOQ) concentration and ± 15% of the nominal concentration for the other QC concentrations.

The same 25 samples were used to investigate the within-day precision and the same 15 samples were used to investigate the between-day precision. For these purposes, the coefficient of variation was calculated for each concentration level. The claim for the precision parameters is that the coefficient of variation should be under 20% (LLOQ) or 15% (other QCs).

The LLOQ of the method was investigated by calculating the coefficient of variation for the analysis of six LLOQ samples and should be less than 20%. Additionally to the aforementioned FDA guidelines, the lower limit of detection (LOD) was investigated by analyzing five drug free samples of each matrix. The LOD was defined as the difference between the minimal and the maximum background signal at the retention time of nitrofurantoin and could be calculated with the following formula: LOD = (3 x background signal/LLOQ signal) x concentration of LLOQ sample.

Linearity

Linearity across the therapeutic range was evaluated in order to confirm the presence of the linear relationship between the concentration of the calibration standards and the response (correlation coefficient (r^2) > 0.95). Therefore, six calibration standards were prepared in duplicate (table 2) together with two blank samples. Concentrations

of these standards were based on the expected concentrations of nitrofurantoin in the two matrices, reported in previous publications (6–9, 12, 13). The specification of the correlation coefficient is part of the in-house aims used for method validation. The calibration line was not forced to the origin.

Recovery

Recovery was tested by analyzing two sets of QC samples in duplicate for each level. The first set was prepared using the standard sample preparation method as described in section 2.3. The second set was prepared by spiking drug free urine just before injection into the LC-apparatus at the same concentration level as the first set. The recovery was then calculated as the ratio of the response from the samples in set one and two. The response was normalized for the response of the internal standard.

Stability

Stability of stock solutions and working solutions was investigated by quantifying nitrofurantoin and furazolidone concentrations in the solutions just after preparation and after storages in the fridge (4°C) for successive periods of three months, up to 2 years. The time periods were chosen based on recommendations by the World Health Organization and are part of in-house procedures (24). The concentrations after three months should not deviate more than 10% from the original concentrations. Additionally, the following four stability forms of spiked samples during storage were investigated as part of the validation. Stability of plasma samples was tested at two different QC levels (low and high) and stability of urine samples was tested at all three different QC levels:

Short-term stability: QC samples were prepared in duplicate. Short-term stability was investigated by placing the samples on the workbench at room temperature (± 18°C) and in the fridge at 4°C during seven days without protecting them from light.

Long-term stability: Several sets of the QC samples were prepared in duplicate and were stored at -20°C or at -80°C. Stability was then confirmed every three months afterwards for up to 2 years.

Freeze-thaw stability: Stability during three freeze-thaw cycles was tested for the QC samples in duplicate.

Auto sampler stability: Stability in the auto sampler was tested by preparing all five QC samples in duplicate and by storing them in the auto sampler (15°C) during one week.

The measured concentration of the QC samples should not deviate more than 15% from the theoretical concentration for all stability forms.

Clinical Validation

The method was developed in order to quantify nitrofurantoin concentrations in plasma and urine samples from a clinical study. The study was approved by the local ethical committee (CER 13-036) and registered with FDA number (2014DR1008). Participation was voluntary and enrollment occurred after informed written consent had been obtained. The study aimed to investigate the PK of nitrofurantoin in twelve healthy, female volunteers after administering a standard dose of 50 mg q6h (normal release capsule) or 100 mg q8h (slow release capsule) with food at steady state. Plasma samples were collected during six or eight hours after administration, depending on the dose and were immediately centrifuged after taking the sample. Urine samples were also collected during the same time. All samples were immediately kept from light using amber colored glassware and disposables. Samples were stored at -80°C and were analyzed within three months after storage, taking into account the stability testing results. 100 µL of each sample was used for analysis as described in section 2.3.

RESULTS

Analytical validation

Selectivity

Selectivity of the method was confirmed because no interfering peaks from endogenous compounds or co-medication were found around the retention times of nitrofurantoin or the internal standard [data not shown]. Blank samples were used to determine matrix effect. No matrix effects were found since the response of the blank samples was negligible compared to the nitrofurantoin response in the LLOQ standard. The chromatograms of five drug free samples did not show any peaks after injection of a ULOQ sample so no carry-over was observed.

Accuracy, precision and limits of quantification

Accuracy and precision data are demonstrated in table 3. All values were within the accepted range of 15%.

The LOD was calculated with the equation described in section 2.5.2 and was found to be 27 µg/L in plasma and 0.046 mg/L in urine. Calibration standard 1 and 6 were considered to be the LLOQ and ULOQ in both matrices.

Table 3. Within-day accuracy and precision and between-day accuracy and precision results of the assay.

Matrix	[a] Sample	Within-day [b] accuracy (%)	Between-day [b] accuracy (%)	Within-day [c] precision (%)	Between-day [c] precision (%)
Plasma	LLOQ	110.3	99.9	6.7	9.3
	QC-L	86.6	96.3	9.6	8.8
	QC-M	105.7	102.5	5.4	3.8
	QC-H	105.1	103.2	5.4	1.9
	ULOQ	113.6	106.6	5.6	5.5
Urine	LLOQ	113.0	109.0	1.0	4.1
	QC-L	108.7	103.0	2.4	4.8
	QC-M	104.3	101.6	3.2	2.2
	QC-H	107.6	103.2	1.4	3.7
	ULOQ	108.6	104.9	2.2	2.9

[a] The sample concentrations are presented in table 2.
[b] The accuracy values represent the deviation of the measured concentrations compared to the theoretical concentrations, expressed as a percentage of the theoretical concentrations.
[c] The precision values are expressed as coefficients of variation. All values should be within the acceptance criteria of ± 20% for the LLOQ samples and ± 15% for the other QC samples.

Linearity

The method was successfully validated over a range from 50 to 1250 µg/L in plasma ($r^2 > 0.95$) and the clinically relevant range from 4 to 200 mg/L in urine ($r^2 > 0.95$) with a weighting factor of 1/x so the target for linearity was reached in both matrices. All calibration standards (singular) and QCs of five levels (duplicate) were prepared when the method was used for routine analysis. Patient samples were prepared in singular.

Recovery

The following percentages were measured when testing the recovery of the method in urine: 60% (RSD = 5.1%) for nitrofurantoin and 105% (RSD = 3.3%) for the internal standard. Recovery of nitrofurantoin was low, but consistent. No recovery was tested for plasma samples since this is not customary when using protein precipitation.

Stability

The two stock solutions and the two working solutions were found to be stable at 4°C for 2 years since the concentrations were still within the 10% range of the initial concentrations (+6.4% and +6.0% for the stock solutions and -1.5% and +3.1% for the working solutions).

Stability data of the assay is presented in table 4. QCs in plasma are stable for seven days at 4°C, for 2 years at -20°C and at -80°C (only QC-H), but for less than

three hours at room temperature (± 18°C). Plasma samples of all levels are stable for seven days in the auto sampler (15°C). There is however a stability problem for low concentrated plasma samples when stored for more than three months and for low concentrated urine samples in general, based on the stability testing results for QC-L samples in both matrices. Long-term stability of low concentrated urine samples was only confirmed for samples stored at -80°C (stable for at least 2 years). On the contrary, QC-M and QC-H samples in urine are stable at 4°C and at room temperature for at least 7 days, and at -20°C and -80°C during at least 2 years. Only higher concentrated samples in both matrices are stable during three freeze-thaw cycles.

Table 4. Stability data of the assay.

Matrix	Condition	Time	QC-L	QC-M	QC-H
Plasma	[b] Short-term 4°C	7 days	97%	-	91%
	[b] Short-term room temp. (± 18°C)	3h	**80%**	-	**84%**
	[c] Long-term -20°C	3 months	115%	-	99%
		6 months	**119%**		113%
		2 years	-		115%
	[c] Long-term -80°C	3 months	114%	-	100%
		6 months	**116%**		108%
		2 years	-		110%
	Freeze-thaw cycles	3 cycles	**133%**	-	114%
	[a] Auto sampler (15°C)	7 days	All QC samples meet requirement		
Urine	[b] Short-term 4°C	7 days	**71.6%**	87.2%	90.1%
	[b] Short-term room temp. (± 18°C)	3 h	**67.3%**	85.3%	91.7%
		7 days	**64.8%**	89.4%	88.8%
	[c] Long-term -20°C	3 months	**73.9%**	88.7%	91.6%
		6 months	**-**	88.0%	87.0%
		2 years	**-**	87.1%	86.1%
	[c] Long-term -80°C	3 months	94.1%	113.8%	103.2%
		6 months	92.0%	114.1%	104.0%
		2 years	90.1%	114.6%	106.5%
	Freeze-thaw cycles	3 cycles	**84.9%**	90.9%	91.5%
	[a] Auto sampler (15°C)	24 hours	All QC samples meet requirement		

[a] tested for QC-L, QC-M, QC-H, LLOQ and ULOQ
[b] Short-term stability was tested for stored samples.
[c] Long-term stability was tested for at least three months.
The values represent the mean recovery (%) of two (plasma) or three (urine) QC samples of each level.

Clinical validation

A chromatogram obtained after injection of a plasma sample and a urine sample of a volunteer is demonstrated in figure 2. The concentration of the plasma sample was 199 µg/L and 56.4 mg/L for the urine sample. No interfering peaks were observed in both matrices when analyzing the samples. Maximum plasma concentrations ranged from 209 to 450 µg/L after a dose of 50 mg q6h and from 222 to 1255 µg/L after 100 mg q8h. Urine concentrations after these dosages ranged from 26.8 to 176.3 mg/L and from 40.1 to 209.4 mg/L, respectively. Concentrations were comparable with those found in literature (6, 8, 9, 12, 13). The samples of which the initial concentrations exceeded the validated concentrations ranges were re-analyzed after diluting the samples with drug free plasma or urine. This dilution step did not affect the quality of the method, as confirmed during additional analysis as part of the method validation.

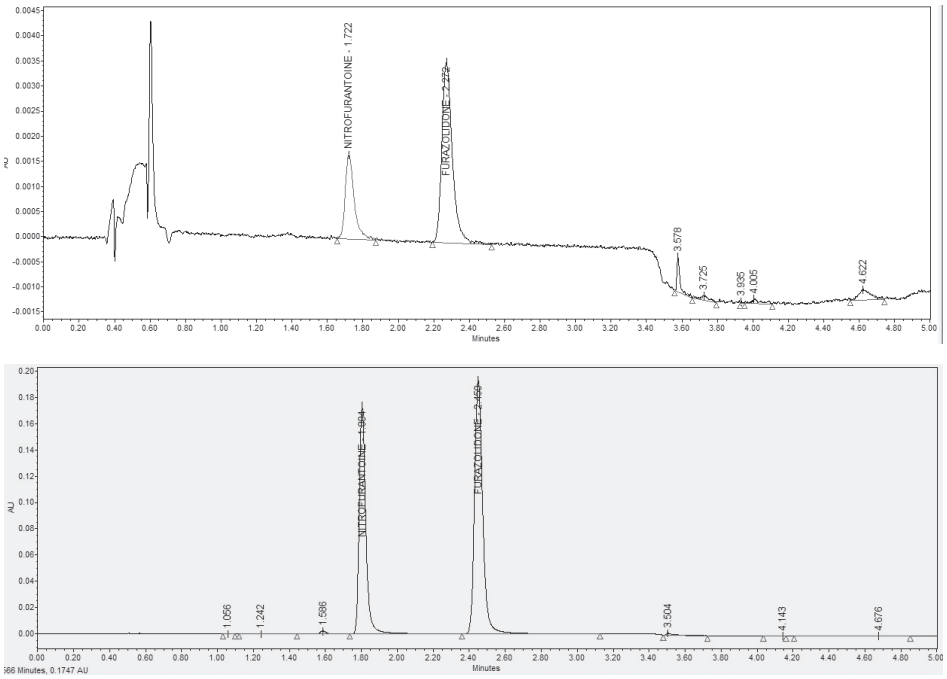

Figure 2. Chromatograms of a volunteers' plasma sample (upper) and urine sample (bottom). The retention time is presented on the horizontal axis and the absorption at 369 nm is demonstrated on the vertical axis. The number above the individual peaks represents the retention time of nitrofurantoin (first peak) and furazolidone (second peak).

DISCUSSION

Recent guidelines for drug registration include PK/PD research to provide the rationale for dose and dose frequency selection (5, 25, 26). This information is missing for old antimicrobial drugs as this was not required at the time of registration. This is an important and worrisome knowledge gap since it is known that this information is necessary for therapy optimization and the prevention of emergence of drug resistance (5).

We developed this method to quantify nitrofurantoin concentrations in plasma and urine. Based on these concentrations, we will be able to study the PK after administration of nitrofurantoin in different dosages and in different dosing regimens. This is of specific interest because the registered dose, frequency, duration and formulation of nitrofurantoin is different per indication and/or per country (27). A scientific base for dose justification for nitrofurantoin is lacking which is alarming, especially for an old drug which has been in clinical use for decades. We believe that it is highly important to investigate the effectivity and safety of the dosing regimens which are currently used in clinical daily practice in order to keep nitrofurantoin as a possible oral treatment option for uncomplicated UTIs in an era of emergence of resistance among common uropathogens (2–4).

An important advantage of the method we present is the short analysis time of only five minutes in comparison with other published methods (19, 20). This advantage ensures that the method can be integrated easily in daily lab routine for research and therapeutic drug monitoring purposes. The method is sensitive enough in order to quantify nitrofurantoin concentrations in the clinically relevant concentration areas, but cannot match the sensitivity of a LC-MS/MS method that can achieve a quantification limit of 5 µg/L in plasma (21). Although the majority of the nitrofurantoin dose is excreted unchanged in urine, it is important to mention that it cannot be ruled out whether other drugs and/or metabolites with coincidently the same retention time and/or UV absorption spectrum as nitrofurantoin are detected too (28). To overcome this limitation as much as possible, peak height and shape of the internal standard in the sample needs to be judged for every analysis and compared to those from the QC samples, which are measured simultaneously. This is a common strategy to rule out the presence of interfering peaks. Any deviation from the peak pattern of the internal standard must be mentioned when reporting the measured concentrations when using the method during clinical daily practice. This is a general limitation of the published analytical methods using UV based detection methods for nitrofurantoin and is of course closely related to the fact that the metabolic pattern of nitrofurantoin has still not been fully elucidated (18, 22). In order to make the measured concentrations be more translatable to treatment effectivity, selecting which metabolites to include in the method would have relied on guesswork and was therefore considered not war-

ranted for inclusion. This factor should to be taken into account when interpreting the concentrations and translating them to antibacterial activity, clinical effectivity and/or toxicity, since the measured concentrations may not be fully 'responsible' for these effects.

Several steps had to be taken to optimize the analytical assay before final validation. These steps will be discussed. We started using an unbuffered protein precipitation solution for the plasma sample preparation. Measured calibration standard concentrations and QC concentrations were different for different batches of drug free plasma. Based on this finding, we concluded that the pH differences between plasma batches caused this deviation and we started to use a buffered protein precipitation solution. pH 4.8 was found to be the optimal. We aimed to apply the plasma sample preparation method to the urine samples, but this resulted in varying concentrations of the calibration standards and disruptions in the chromatogram due to pollution of the urine matrix. We therefore decided to use liquid-liquid extraction with dichloromethane instead of nitromethane, based on a previous method (29). The final sample preparation method for urine samples consists of extraction with a buffer with KCl and HCl of pH 2 since HCl has no buffering effect by itself.

CONCLUSIONS

This is the first UHPLC-DAD method suitable for the quantification of nitrofurantoin concentrations in plasma and urine with a small sample volume and a short analysis time. The method was found to be selective ad sensitive with low LLOQ concentrations. These properties ensure that the method is highly suitable for use during the daily routine for analyzing patients' samples in the context of clinical care and research where it can serve as a base for therapy evaluation and optimization. The applicability of the method was demonstrated during its use in a clinical study.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

This work was in part supported by the European Commission FP7 AIDA project (Preserving old antibiotics for the future, Health-F3-2011- 278348).

REFERENCES

1. Tandogdu Z, Wagenlehner FME. 2016. Global epidemiology of urinary tract infections. Curr Opin Infect Dis 29:73–79.
2. Naber KG, Schito G, Botto H, Palou J, Mazzei T. 2008. Surveillance Study in Europe and Brazil on Clinical Aspects and Antimicrobial Resistance Epidemiology in Females with Cystitis (ARESC): Implications for Empiric Therapy. Eur Urol 54:1164–1178.
3. Gupta K, Hooton TM, Naber KG, Wullt B, Colgan R, Miller L. 2011. International clinical practice guidelines for the treatment of acute uncomplicated cystitis and pyelonephritis in women: A 2010 update by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases. Clin Infect Dis 52:103–120.
4. Garau J. 2008. Other antimicrobials of interest in the era of extended-spectrum β-lactamases: Fosfomycin, nitrofurantoin and tigecycline. Clin Microbiol Infect 14:198–202.
5. Mouton JW, Ambrose PG, Canton R, Drusano GL, Harbarth S, MacGowan A, Theuretzbacher U, Turnidge J. 2011. Conserving antibiotics for the future: New ways to use old and new drugs from a pharmacokinetic and pharmacodynamic perspective. Drug Resist Updat 14:107–117.
6. Felts JH, Hayes DM, Gergen JA, Toole JF. 1971. Neural, hematologic and bacteriologic effects of nitrofurantoin in renal insufficiency. Am J Med 51:331–339.
7. Albert KS, Sedman AJ, Wilkinson P, Stoll RG, Murray WJ, Wagner JG. 1974. Bioavailability Studies of Acetaminophen and Nitrofurantoin. J Clin Pharmacol 14:264–270.
8. Adkison KK, Vaidya SS, Lee DY, Koo SH, Li L, Mehta AA, Gross AS, Polli JW, Lou Y, Lee EJD. 2008. The ABCG2 C421A polymorphism does not affect oral nitrofurantoin pharmacokinetics in healthy Chinese male subjects. Br J Clin Pharmacol 66:233–239.
9. Mason WD, Conklin JD, Hailey FJ. 1987. Between-Lot and Within-Lot Comparisons of Bioavailability of Macrocrystalline Nitrofurantoin Capsules. Pharm Res An Off J Am Assoc Pharm Sci 4:499–503.
10. Conklin J. 1978. The pharmacokinetics of nitrofurantoin and its related bioavailability. Antibiot Chemother 25.
11. Meyer MC, Slywka GWA, Dann RE, Whyatt L. 1974. Bioavailability of 14 Nitrofurantoin Products. J Pharm Sci 63:1693–97.
12. Lippman RW; Wrobel CJ; Rees R; Hoyt R. 1958. A theory concerning recurrence of urinary infection: prolonged administration of nitrofurantoin for prevention. J Urol 80:77–81.
13. Jawetz E; Hopper J; Smith DR. 1957. Nitrofurantoin in Chronic Urinary Tract Infections. Intern Med 100:549–557.
14. Cunha BA. 2006. New Uses for Older Antibiotics: Nitrofurantoin, Amikacin, Colistin, Polymyxin B, Doxycycline, and Minocycline Revisited. Med Clin North Am 90:1089–1107.
15. Bender RC, Nohle EG, Paul MF. 1956. Nitrofurantoin Estimation in Urine with the Aid of Chromatography. Clin Chem 2:420–426.
16. Aufrere MB, Hoener B, Vore ME. 1977. High-Performance Liquid-Chromatographic Assay for Nitrofurantoin in Plasma and Urine. Clim chem 23:2207–2212.

17. Roseboom H., Koster HA. 1978. The determination of nitrofurantoin and some structurally related drugs in biological fluids by high-pressure liquid chromatography. Anal Chim Acta 101:359–365.

18. Hoener B, Wolff JL. 1980. High-performance liquid chromatographic assay for the metabolites of nitrofurantoin in plasma and urine. J Chromatogr 182:246–251.

19. Muth P, Metz R, Siems B, Bolten WW, Vergin H. 1996. Sensitive Determination of Nitrofurantoin in Human Plasma and Urine by High-Performance Liquid Chromatography. J Chromatogr A 729:251–258.

20. Arancibia V, Valderrama M, Madariaga A, Zuniga MC, Segura R. 2003. Extraction of nitrofurantoin and its toxic metabolite from urine by supercritical fluids. Quantitation by high performance liquid chromatography with UV detection. Talanta 61:377–383.

21. Patel DS, Sharma N, Patel MC, Patel BN, Shrivastav PS, Sanyal M. 2013. Quantitation of nitrofurantoin in human plasma by liquid chromatography tandem mass spectrometry. Acta Pharm 63:141–58.

22. Mc Osker CC, Fitzpatrick PM. 1994. Nitrofurantoin: Mechanism of action and implications for resistance development in common uropathogens. J Antimicrob Chemother 33:23–30.

23. Food and Drug Administration (FDA). 2018. Bioanalytical Method Validation Guidance for Industry.

24. WHO. 2018. Annex 10: Stability testing of active pharmaceutical ingredients and finished pharmaceutical products No. 1010:309–352.

25. Muller AE, Theuretzbacher U, Mouton JW. 2015. Use of old antibiotics now and in the future from a pharmacokinetic/pharmacodynamic perspective. Clin Microbiol Infect 21:881–885.

26. Zayyad H, Eliakim-Raz N, Leibovici L, Paul M. 2017. Revival of old antibiotics: needs, the state of evidence and expectations. Int J Antimicrob Agents 49:536–541.

27. Huttner A, Verhaegh EM, Harbarth S, Muller AE, Theuretzbacher U, Mouton JW. 2015. Nitrofurantoin revisited: A systematic review and meta-analysis of controlled trials. J Antimicrob Chemother 70:2456–2464.

28. Hoener B, Patterson SE. 1981. Nitrofurantoin disposition. Clin Pharmacol Ther 29:808–16.

29. Tromp F, Dankert J, Remmelts R, Huizinga T. 1976. De concentratie en de antimicrobiële activiteit van nitrofurantoïne en hydroxymethylnitrofurantoiune in de urine. Ned Tijdschr voor Geneeskd 120:911–8.