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Simultaneous Determination of Nitrofurantoin and Phenazopyridine in Human Urine Samples By HPLC-UV

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Abstract

In this study, a sensitive, simple, and selective reversed HPLC-UV method has been developed for the quantitation of nitrofurantoin (NFT) and phenazopyridine (PHZ) in human urine samples. The target analytes were quantified using a gradient elution mobile phase consisting of 5 mmol/L sodium dihydrogen phosphate, pH 3 (A), and acetonitrile (B) at a flow rate of 1 mL/min and UV detection at 370 nm. Separation of NFT and PHZ was achieved using a reversed-phase C18 column (150 x 4.6 mm (i.d.), 5 µm particle size). The method was validated according to ICH guidelines and the calibration regression analysis for the calibration plot of PAP showed a good linear relationship over the concentration range of 0.5-40 µg/mL and 0.2-20 µg/mL for PHZ and NFT, respectively. The limit of detection (LOD) was 0.01 µg/mL for both PHZ and NFT, while the limit quantitation (LOQ) was 0.025 and 0.04 for PHZ and NFT, respectively. Intra- and Inter-day precisions calculated as the relative standard deviation %RSD values of the intra-day and inter-day were ≤5.5% at three concentration levels of the two analytes. the recovery expressed as percent relative error %RE ranged from -2.5% to 6%. The proposed method was applied for the analysis of volunteer human urine samples by the dilute and shoot technique.

Keywords: Phenazopyridine, Nitrofurantoin, HPLC-UV, Urinary tract infection, Urine Analysis.

1. Introduction

Nitrofurantoin [N-(5-nitro-2-furyldine)-1-aminohydantoin] (NFT) is a widely utilized urinary antimicrobial drug that has been associated with pulmonary fibrosis, neuropathy, hepatitis, and hemolytic anemia in patients with glucose-6-phosphate dehydrogenase deficiency (Sweetman 2005, Grayson and Whitby 2010, Sanchez, Baird et al. 2014). Although the molecular mechanism leading to nitrofurantoin-included cell toxicity is still uncertain, the antimicrobial activities, as well as other clinical toxicities of nitrofurantoin, may be due to the reductive metabolic activation of 5-nitro function to the anion radical, nitroso and hydroxylamine derivatives (Hoener, Noach et al. 1989). Several analytical methods have been described for the estimation of nitrofurantoin including colorimetry (Jain, Dwivedi et al. 2009), reductive flow injection amperometry (Fogg and Ghawji 1988), spectrophotometry (Hadi and Mouayed 2017, Abd-Alrassol, Sattar et al. 2020), high-performance liquid chromatography (Díaz, Cabanillas et al. 1997).

Aswakan and Surman (Surmann and Aswakun 1985) described a polarographic method for the analysis of the mixture of the two species in tablets.

Phenazopyridine hydrochloride (PHZ) is [3-Phenylazopyridine-2,6-diyldiamine hydrochloride] (Sweetman 2005). It has been used for a long time in conjunction with antibacterial agents for the treatment of urinary tract infections (Petri 2011, Hooton 2012). It exerts a local analgesic effect on the mucosa of the urinary tract and is used to provide symptomatic relief of pain in conditions such as cystitis and urethritis (Thomas, Whitehouse et al. 1990, Eastham and Patel 2022). It is absorbed from the gastrointestinal tract and is excreted mainly from the urine (Thomas, Whitehouse et al. 1990). A recent docking study showed that phenazopyridine forms strong hydrogen bonds with the hinge region of cyclin-G-associated kinase, and the two phosphatidylinositol kinases PI4KB and PIP4K2C ATP-binding pocket (Preynat-Seauve, Nguyen et al. 2021).

A literature survey reported several methods; including electrochemical methods (Vijaybhaskar and Ramachandraiah 2009, Ensafi, Arashpour et al. 2013, Yagmur, Yilmaz et al. 2013, Demirtas, Yilmaz et al. 2015, Pereira, da Silva et al. 2016) and spectrophotometry (Palabıyık and Onur 2004, Attia, El-Abasawi et al. 2016). Moreover, HPLC has been used to determine phenazopyridine in dosage forms (Belal 1988, Attia, El-Abasawi et al. 2017). Simultaneous determination of PHZ and sulfonamides in dosage forms has been also reported (Ni, Qi et al. 2006, Soudi, Hussein et al. 2020). Some researchers have used liquid chromatography-mass spectroscopy (LC-MS) (Shang, Xiang et al. 2005) and gas chromatography-mass spectrometry (GC–MS) (Chen, Li et al. 2007, Li, Chen et al. 2008) for this drug determination. The stability of PHZ has been studied using different techniques (Sabry 2008).

In vivo studies (Johnson and Chartrand 1976, Thomas, Whitehouse et al. 1993) of PHZ urinary metabolites in man revealed that drug goes through reductive metabolism by the cleavage of the azo linkage. The metabolites identified in man's urine included; aniline, N-acetyl-p-aminophenol,

p-aminophenol, and traces of o-aminophenol. About 45% of the drug is excreted unchanged (Thomas, Whitehouse et al. 1990).

NFT and PHZ are commonly formulated together in preparations used for urinary tract infections (Amábile-Cuevas and Arredondo-García 2013). A review of the literature revealed that reported methods for the analysis of mixtures of the two species include a gravimetric method (Bukowska and Bielowska 1962) based on precipitation of the two species as their silver salt in ammoniacal and acidic medium, respectively, and HPLC (Hollifield and Conklin 1970, Belal 1988, ONUR and ACAR 1991, Palabıyık and Onur 2004).

Quantitation of PHZ combination with NFT has been achieved by spectrophotometric methods (Berzas Nevado, Rodriguez Flores et al. 1993, Walash, EL-BRASHY et al. 1994).

Reviewing the literature, it was revealed that up to the present time, nothing has been published concerning the determination of NFT and PHZ together in human urine using HPLC with UV detection. This led us to search for a simple, rapid, accurate, and reliable method for the analysis of this mixture in urine. Chromatography seemed an ideal choice for the determination and the application of HPLC is particularly attractive due to its specificity and accuracy.

In this study, a simple reversed-phase gradient HPLC method was developed for the simultaneous determination of NFT and PHZ in urine by direct injection (dilute and shoot technique).

2. Experimental

2.1. Instrumentation

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series of LC-10 ADVP pump, SCL-10 AVP system controller, DGU-12A degasser, Rheodyne 7725i injector with a 5 µL loop, and an SPD-10AVP UV–Vis. detector.

2.2. Materials and reagents

NFT and PHZ of pharmaceutical grade were used and certified to contain 99.9% and 99.8% respectively. Acetonitrile and methanol used were HPLC grade (BDH, Poole, UK). Orthophosphoric (BDH Laboratory Supplies, Poole, England. Commercial Uricept® (Batch No. 1210600) used was manufactured by Kahira Pharmaceutical and Chemical industries Company, Cairo, Egypt and Mepafuran® used was manufactured by Arab Company for Pharmaceutical and Medicinal Plants (MEPACO) (Batch No. 1500413).

2.3. HPLC conditions

The HPLC separation and quantitation were made on a 150 x 4.6 mm (i.d.) (Luna, Phenomenex, Torrance, CA, USA) (5 µm particle size) reversed-phase C18 column. A gradient mobile phase system consisting of (A) 5 mmol/L sodium dihydrogen phosphate, pH 3, and (B) acetonitrile was used. The separation was achieved with a gradient program consisting of 0–7 min 15% mobile phase B and 7–10 min gradient up to 40% mobile phase B. After 10 min the gradient was returned to the initial conditions and the analytical column was reconditioned for 3 min. The flow rate was 1 mL/min. All determinations were performed at an ambient temperature of 25 ºC. The injection volume was 5 µL. The detector was set at 370 nm. Data acquisition was performed on class-VP software.

2.4. Standard solutions

The stock standard solutions were prepared by the

separately dissolving NFT and PHZ in methanol to obtain a concentration of 500 µg/mL. Further dilutions for NFT and PHZ with methanol were carried out to obtain concentration ranges of 0.5-40µg /mL and 0.2-20 µg/mL, respectively. These stock solutions were stored at 4ºC, in the absence of light.

2.5. Urine samples

Different volumes of NFT and PHZ stock standard were transferred to a 10 ml volumetric flask, 0.5 ml of blank urine was added and the solutions were diluted to 10 ml with the mobile phase to obtain a concentration range of 0.5-40 µg/mL for PHZ and 0.2–20 µg/mL for NFT. The solutions were filtered through 0.45µm disposable membrane filters. Triplicate 5 µl injections were made for each concentration of NFT and PHZ was chromatographed under the conditions described above.

2.6 Sample preparation (In vivo procedure)

This investigation conforms to the Egyptian Community guidelines for the use of humans in experiments. The Human Ethics Committee of the Faculty of Pharmacy, Suez Canal University, approved the study, with license number 201410H2. An excretion study of NFT and PHZ was carried out on a normal, healthy (normal liver, kidney functions, and electrocardiogram), male, informed adult volunteer (29 years, 85 kg, 178 cm height), with no history of allergic reaction to NFT or PHZ. The volunteer was instructed to abstain from all medications for 2 weeks before administration and also during the study. Also, the volunteer was instructed to be sure of evacuating his bladder as thoroughly as possible exactly before administration of 100 mg NFT capsules and 100 mg of PHZ tablets with food.

2.7 Analysis of real urine samples

This method was used to investigate the pattern of urinary excretion of NFT and PHZ. The 0-h urine sample was collected as blank. Urine samples were collected at intervals for up to 24 h. The volume of urine specimen was measured and recorded after each collection; 20 ml aliquots were stored at -20℃ until determination. Suitable dilution was carried out to 10 ml with the mobile phase to reach the calibration range. The solution was filtered through a 0.45µm membrane filter. A 20µl was injected into HPLC, in triplicate for each solution, and chromatographed under the conditions described above. The peak area ratios were determined for NFT and PHZ.

3. Results and discussion

3.1 Chromatographic conditions

Development of the liquid chromatographic method requires the careful combination of the polarity of the analyte, stationary phase, and mobile phase to obtain good separations in reasonable times. Furthermore, in analyses of biological fluids, especially with the complex matrix as in urine, it is necessary to have an efficient chromatographic separation between endogenous components and compounds of interest. Working with isocratic elution, using a mobile phase consisting of methanol and 5Mm sodium dihydrogen orthophosphate at different ratios, was insufficient to have good

separation concerning the resolution of peaks and retention time.

Using isocratic elution, the influence of acetonitrile concentration of the mobile phase on the separation efficiency between PHZ and NTF as well as endogenous compounds from urine samples was investigated at pH 3. It was found that decreasing the concentration of acetonitrile to less than 20% caused a severe increase in the retention time of PHZ peak that resulted in band broadening and excessive tailing while increasing the concentration of acetonitrile to more than 55% caused a decrease in retention time which led to the inadequate separation of the two peaks and biological matrices. So, it was shifted to gradient elution which gave the best separation, sharper peaks, and satisfactory results.

The influence of pH of the mobile phase on the retention behavior of NFT and PHZ as well as endogenous compounds of biological samples was investigated. It was found that variation of pH of the mobile phase resulted in maximum K\ value at pH 6 with loss of peak symmetry for PHZ and NFT. At lower pH values (less than 3) poor resolution for PHZ, NFT, and different biological matrices was observed. At pH 2.5-3.5, improved resolutions of the three peaks were observed; however, at pH 3 optimum resolution with reasonable retention time was affected.

The effect of concentration of sodium dihydrogen orthophosphate in the mobile phase was studied between 5 and 50 mM. The lowest concentration of sodium dihydrogen orthophosphate (5 mM) was selected because results were similar to those obtained at other concentrations and it was less damaging to the column.

Based on these investigations, a good chromatographic separation between NFT and PHZ in presence of biological matrices was achieved by the use of a gradient mobile phase system consisting of (A) 5 mmol/L sodium dihydrogen phosphate pH 3 and (B) acetonitrile. The separation was achieved with a gradient program consisting of 0–7 min 15% mobile phase B and 7–10 min gradient up to 40% mobile phase B. After 10 min the gradient was returned to the initial conditions and the analytical column was reconditioned for 3 min.

Chromatograms of blank human urine samples, urine samples spiked with 10 µg /mL NFT and 10 µg/mL PHZ, and real urine samples are taken after 1.5 h of oral 100mg per tablet Urisept® dose administration and oral 100 mg per capsule Mepafuran® dose administration (Fig.1a, 1b, 1c respectively) showed no interfering peaks at the retention times of PHZ and NTF. The average retention time ± SD for PHZ and NTF were found to be 6.09 ± 0.059 and 8.76 ± 0.042 min, respectively, for seven replicates. The peaks obtained were sharp and have clear baseline separation. The system suitability results are given in Table 1.

Performance of proposed method on real samples was demonstrated by via application to human urine samples taken from a male volunteer who received Urisept® and Mepafuran® medication. Figure 1 shows a typical HPLC chromatogram

Table 1: System suitability test results of the developed method for determining NFT and PHZ.

Comp.	Retention time (min)	Retention factor (K\)	Selectivity (α)	Resolution (R_s)	Tailing factor	%RSD of retention time	Plate count
NFT	6.09	3.68	1.56(a)	2.63(b)	1.02	0.08	59540
PHZ	8.76	5.75			1.04	0.10	62230

The retention time of unretained peak is 1.30 min.
a, b: are α and R_s calculated for PHZ and NFT.

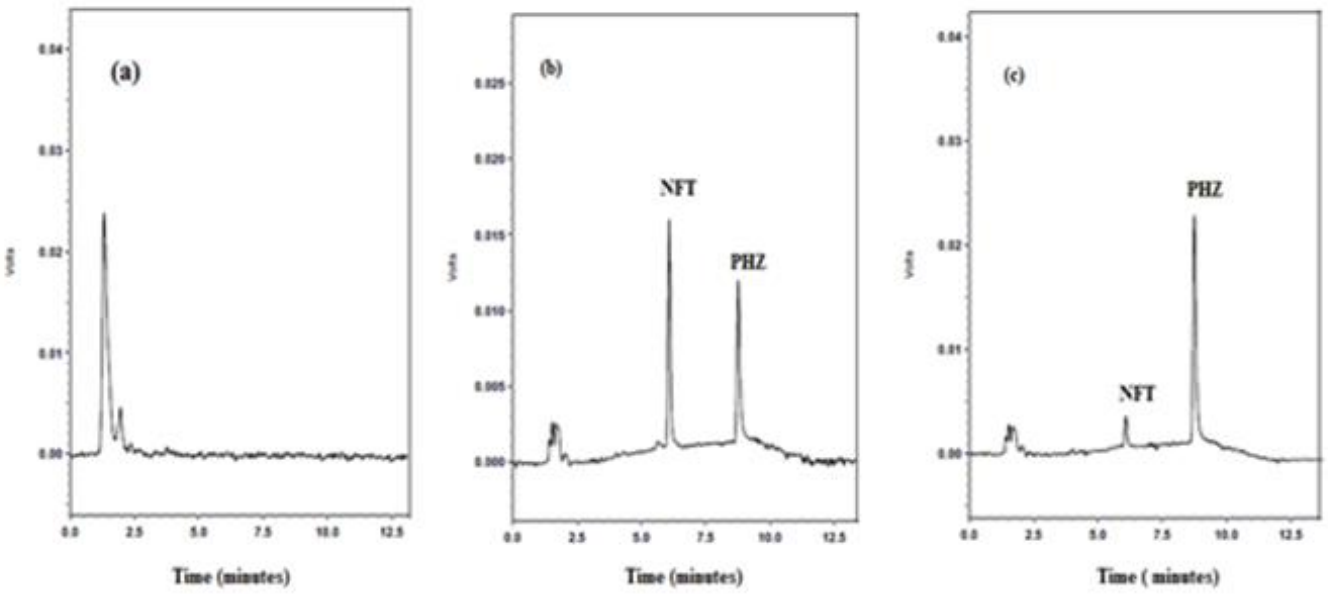

Figure 1. Typical HPLC chromatograms obtained from analysis of (a) blank urine sample (b) urine sample spiked with 10 µg /mL NFT and 10 µg /mL PHZ (c) real urine sample taken after 1.5 h of oral 100mg Urisept® dose administration and oral 100 mg Mepafuran® dose administration.

a real human urine sample taken after 1.5 h from receiving the drug. The concentrations of PHZ and NTF in urine were determined and found to be 20 µg/mL and 1.5 µg/mL, respectively after 1.5 h.

3.2 Validation

3.2.1 Linearity

The linearity of the proposed method was evaluated by analyzing seven concentrations of PHZ and NTF ranging between 0.5 and 40µg/mL and between 0.2 and 20µg/mL, respectively in human urine. Each concentration was repeated three times. The assay was performed according to experimental conditions previously established. The linearity of the calibration graphs was validated by the high value of the correlation coefficient and the intercept value, which was not statistically (p = 0.05) different from zero (Table 2). Characteristic regression data for the method, obtained by least squares treatment of the results, are in Table 2.

3.2.2 Accuracy and precision

The intra-day precision and accuracy were evaluated by analyzing five replicates at three concentration levels for samples on the same day. Inter-day precision and accuracy were evaluated by analyzing the three concentration levels for samples on five consecutive days. The criteria of acceptability of the data included accuracy expressed as the relative error (RE%) and precision expressed as relative standard deviation (RSD%), both should be within ± 15%. The intra-day and inter-day precision values (RSD%) and the accuracies (RE%) of both analytes were within ±15%. They met the requirements for the determination of biological sample concentration. The results obtained were shown in Table 3.

3.2.3 Range

The calibration range was established by consideration of the practical range necessary to give accurate, precise, and linear results. Calibration ranges for the proposed HPLC method are given in Table 2.

3.2.4 Detection and quantitation limits

According to ICH recommendations (Guideline 2005), the approach based on the S.D. of the response and the slope was used for determining the detection and quantitation limits. The theoretical values were assessed practically and given in Table 2.

3.2.5 Selectivity

The selectivity of the method was achieved via the addition of appropriate volumes of a standard solution of NFT and PHZ (different concentrations but within the linearity range concentration) to drug-free human urine samples in a ratio of 1:9. The spiked samples were analyzed according to the previous procedure described under sample analysis. Satisfactory results were obtained (Table 4) for PHZ and NFT, indicating the high selectivity of the proposed method. No interference resulted from biological matrices.

3.2.6 Robustness

Variation of the pH of the mobile phase (A) by ± 0.2 units, the ionic strength of sodium dihydrogen phosphate by ± 1mmol/L, organic strength of the mobile phase by ± 2%, and flow rate by± 0.2 ml/min did not have a significant effect on the chromatographic resolution of the HPLC method.

3.2.7 Stability

In anticipation of unexpected delays during analysis, it is important to have information about the stability of all solutions. When the stability of NFT and PHZ in the mobile phase was tested, it was found the samples were stable for at least 4 h at room temperature. Urine samples spiked with NFT and PHZ were evaluated for stability after freezing and thawing. The drug was stable through at least three freeze-thaw cycles. The stability of spiked Urine samples during storage for 4 weeks at –20ºC was also evaluated; no significant change was observed.

Table 2: Characteristic parameters of the calibration equations for the proposed HPLC method for the determination of NFT and PHZ in human urine.

Parameters	NFT	PHZ
Calibration range (μg ml^{-1})	0.2-20	0.5-40
Detection limit (μg ml^{-1})	0.011	0.010
Quantitation limit (μg ml^{-1})	0.04	0.025
Regression equation(Y)[a]: Slope (b)	14.78x 10^3	11.79 x 10^3
Standard deviation of the slope (S_b)	77.59	38.49
Relative standard deviation of the slope (%)	0.52	0.33
Confidence limit of the slope [b]	14.71 x 10^3 -14.86 x 10^3	11.75 x 10^3 -11.82 x 10^3
Intercept (a)	-37.37	233.29
Standard deviation of the intercept (S_a)	257.35	715.10
Confidence limit of the intercept [b]	(-698.91) - (624.17)	(-883.20) - (416.62)
Correlation coefficient (r)	0.9998	0.9998
Standard error of estimation	505.02	520.10

[a] Y = a + b C, where Y is the peak area, a is the intercept, b is the slope, and C is the concentration of NFT and PHZ (μgm/L) in human urine. [b] 95% confidence limit.

Table 3. Accuracy and precision of the proposed method for analysis of NFT and PHZ in urine.

Compound	Conc. (μg/mL)	Intra-day			Inter-day		
		Mean ± S.D.[a]	RSD%	RE%	Mean ± S.D.[a]	RSD%	RE%
NFT	0.2	0.19 ± 0.01	5.12	-2.50	0.21 ± 0.01	4.76	5.00
	10	9.91 ± 0.15	1.51	- 0.90	9.94 ± 0.36	3.62	-0.6
	20	20.05 ± 0.21	1.05	0.25	19.94 ± 0.21	1.05	-0.3
PHZ	0.5	0.47 ± 0.02	4.26	6.00	0.52 ± 0.02	5.50	3.85
	20	20.12 ± 0.31	0.60	1.54	19.79 ± 0.39	1.95	1.05
	40	39.69 ± 0.45	1.13	-0.78	40.33 ± 0.45	1.12	0.83

[a] Mean and S.D. for five determinations

Table 4: Recovery for NFT and PHZ from HPLC of directly injected spiked human urine samples.

Sample No	Conc. of NFT (µg/mL)	Conc. of PHZ (µg/mL)	% Recovery	
			NFT	PHZ
1	0.2	0.5	97.2	97.9
2	2	10	98.4	98.1
3	10	20	101.9	99.5
4	20	40	102.1	100.8
5	5	7	95.6	102.1
6	0.4	1	102.5	100.5
		Mean ± S.D.	99.82 ± 1.64	99.95 ± 1.88

4. Conclusion

An optimized reversed-phase HPLC method for direct quantitative analysis of NFT and PHZ in human urine without any extraction procedure before the separation. Also, a suitable procedure for the preparation of samples with the subsequent analytical method was developed for reliable drug determination in human urine. The proposed HPLC method provides simple, accurate, sensitive and robust determination of NFT and PHZ in human urine.

Conflict of interest

The authors declare a no conflict of interest nor receive any specific grant from any funding agencies in the public, commercial, or even not-for-profit sectors.

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