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DETERMINATION OF ROXITHROMYCIN IN HUMAN URINE BY LIQUID CHROMATOGRAPHY WITH PHOTO DIODE ARRAY DETECTION

ODREĐIVANJE ROKSITROMICINA U URINU TEČNOM HROMATOGRAFIJOM SA UV SKENIRAJUĆIM DETEKTOROM

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Key words
roxitromycin, urine, HPLC

Ključne reči
roksitromicin, urin, HPLC

Apstrakt

Roxithromycin is a semi-sintetic macrolide antibiotic, which stops growth of bacteria. It is used to treat respiratory tract, urinary and soft tissue infections. After rapid absorption, it diffuses into most tissues and actively transport to the site of infection. Only a small portion of roxithromycin is metabolised. Most of roxithromycin is secreted unchanged into the bile and some in expired air. Less than 10% is excreted into the urine.
We described a simple and reproducible method for the determination of roxithromycin in human urine. Roxithromycin was extracted from urine by alkaline liquid–liquid extraction with dichlormethane. Extracts were analyzed by high performance liquid chromatography with photodiode array detection (HPLC/PDA). The mobile phase consisted acetonitrile and sodium dihydrogen phosphate (pH of the aqueous part of the mobile phase is 3.6) using gradient flow of 1-1.5 mL/min. Separation was performed on C8 column, operated at 30°C. Qualitative and quantitative analysis of roxithromycin was performed on λ = 200.5 nm. Relative retention time of roxithromycin was about 15.8 min. The limit of quantitation was 2 µg/mL and the calibration curve is linear up to 20 µg/mL, with correlation coefficient of 0.9913. Recovery from urine spiked by roxithromycin was in the range from 93.83 to 97.75 %. The assay could be used in monitoring of roxitromycin elimination by urine.

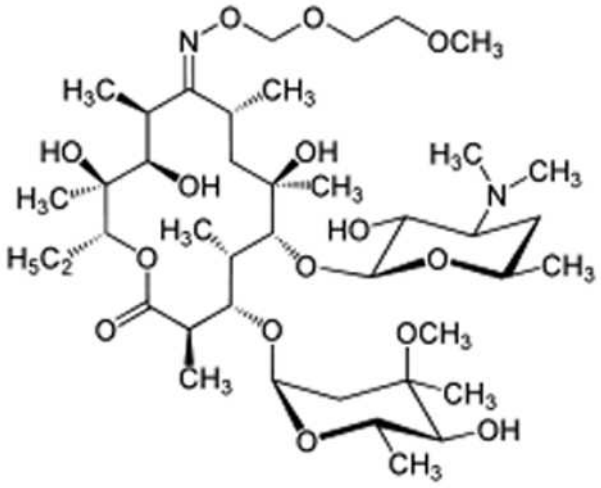
INTRODUCTION

Roxithromycin is a semi-synthetic macrolide antibiotic. It is used to treat respiratory tract, urinary and soft tissue infections. Roxithromycin is derived from erythromycin, containing the same 14-membered lactone ring, and has similar antimicrobial spectrum as erythromycin, but is more effective against certain gram-negative bacteria.

Roxithromycin prevents bacteria from growing, by interfering with their protein synthesis. It binds to the subunit 50S of the bacterial ribosome, and thus inhibits the translocation of peptides.

When taken before a meal, roxithromycin is very rapidly absorbed, and diffused into most tissues and phagocytes. Due to the high concentration in phagocytes, roxithromycin is actively transported to the site of infection.

Only a small portion of roxithromycin is metabolised. Most of roxithromycin is secreted unchanged into the bile



Picture 1 *Chemical structure of roxithromycin*

and some in expired air. Less than 10% is excreted into the urine.

The most applying methods for determination of roxithromycin in biological matrices use high performance liquid chromatography with ultraviolet (UV) [1-5] and mass spectrometric (MS) detection [6-7].

We described a simple, accurate and precise HPLC-UV method for determination of roxithromycin in urine samples.

MATERIALS

Analytical standard of roxithromycin (95 %), was obtained by Sigma aldrich.

Acetonitril, sodium hydrogen phosphate, phosphoric acid, methanol, ammonium hydroxide and dichlormethane were of HPLC or p.a. purity, obtained from MERCK. Water was purified by Millipore Milli-Q system.

Urine samples of patient treated by roxithromycin were analyzed.

Chromatography

High performance liquid chromatograph Waters Aliance 2695 XE Separations Module pump with Waters 2696 Photodiode Array Detector and Empower Login Software were used in this method.

The mobile phase was mixture of acetonitrile (A) and phosphate buffer pH 3.6 (B).

Ratio of mobile phases A and B and flow are given in gradient Table 1.

Table 1. Ratios of mibile phases A and B for HPLC-PDA method

time (min.)	flow (mL/min)	A %	B %	curve
	1.0	85	15	
3.0	1.0	65	35	6
9.0	1.0	20	80	6
28.0	1.5	20	80	6
31.0	1.5	20	80	6
31.5	1.5	85	15	6
35.0	0.3	85	15	6

Column Symmetry® C8 (waт 054270) 4,6 x 250mm (Waters) with guard column Sentry Guard Symmetry® C18, at the temperature of 30°C, with injector loop volume of 50 µL was used in this method. Detection of roxithromycin was performed on 200.5 nm. Retention time of roxithromycin was about 15.8 minutes.

Standard solutions and sample preparation

Stock standard solution of roxithromycin was prepared by dissolving 10 mg in 10 ml methanol and stored at +4ºC. Other concentrations of roxithromycin were made by diluting stock standard solutions with methanol to achieve calibration concentrations expected to meet in urine of treated patients.

In 1 mL of urine was added 0,1 mL of ammonium hydroxide and roxithromycin was extracted on mechanical shaker for 20 minutes with dichlormethane. After centrifugation on 3000 rpm, organic layer was separated and evaporated in stream of air. Dry extracts were reconstituted in methanol and analyzed by HPLC-PDA method on 200.5 nm.

Calibration and quality control samples were prepared by adding roxithromycin solution in blank ("drug-free") human urine. The amounts of roxithromycin in spiked urine ranged from 2.5 to 20 mg/L.

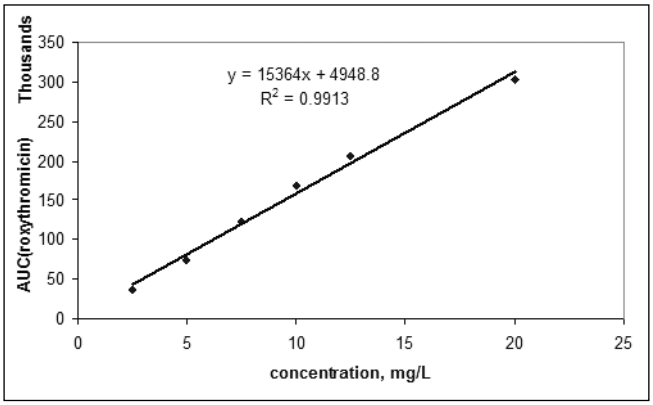
RESULTS

Roxithromycin concentrations were determined using weighed linear regression function. In Table 2 has shown calibration curve for roxithromycin in urine.

Table 2. Calibration curve for roxithromycin in urine

conc. (mg/L)	AUC for spiked urine
2.5	37026
5.0	74494
7.5	123936
10.0	168694
12.5	206564
20.0	302382

The correlation coefficient for urine spiked by roxithromycin was 0.9933. Picture 2 shows calibration curve for spiked urine.



Picture 2. Calibration curve for urine spiked by roxithromycin

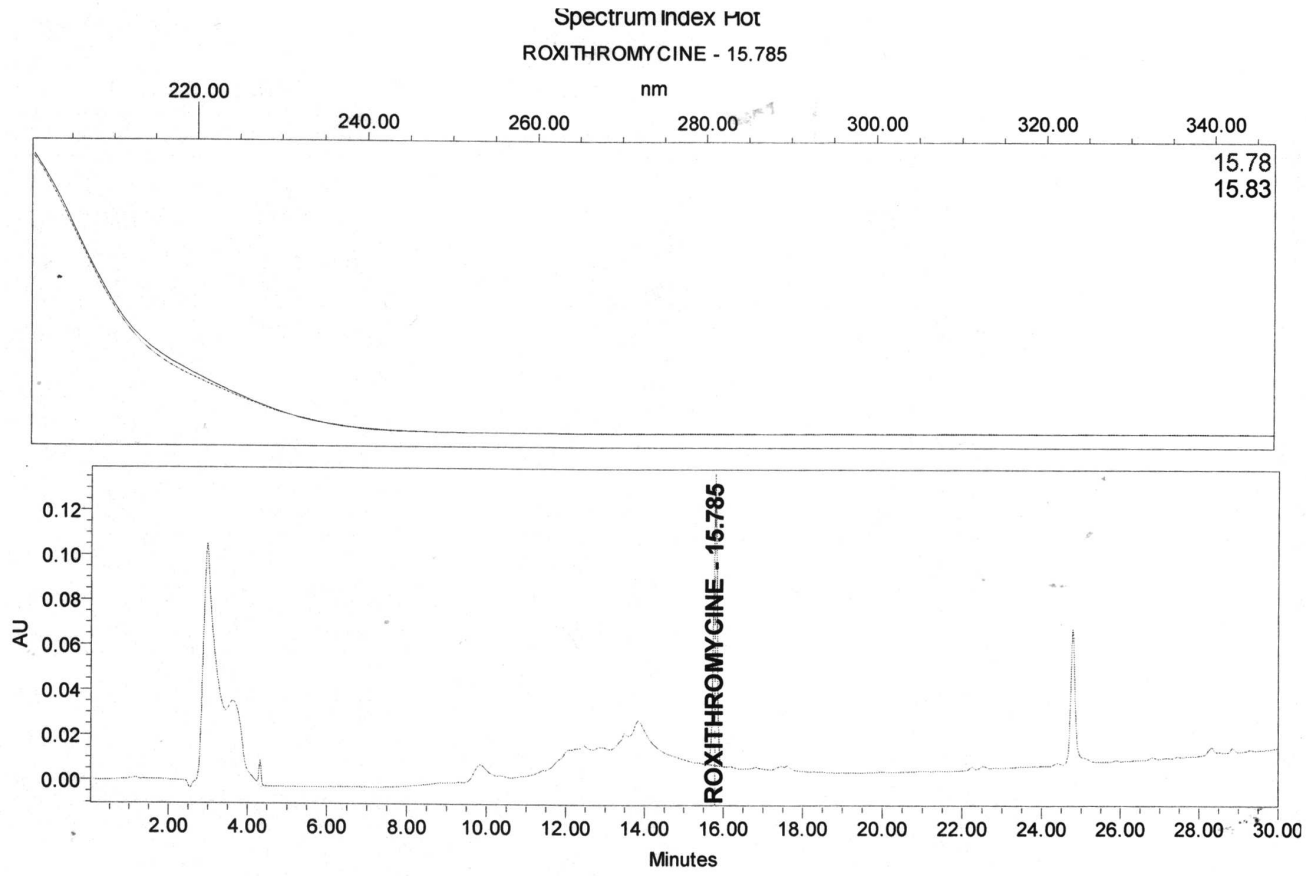
Recovery was determined, for each concentration, as the mean of three samples by comparing the peak areas of the extracted and non-extracted samples. The mean value was 95.50 %.

Table 3. Results for analytical recovery of roxithromycin in urine.

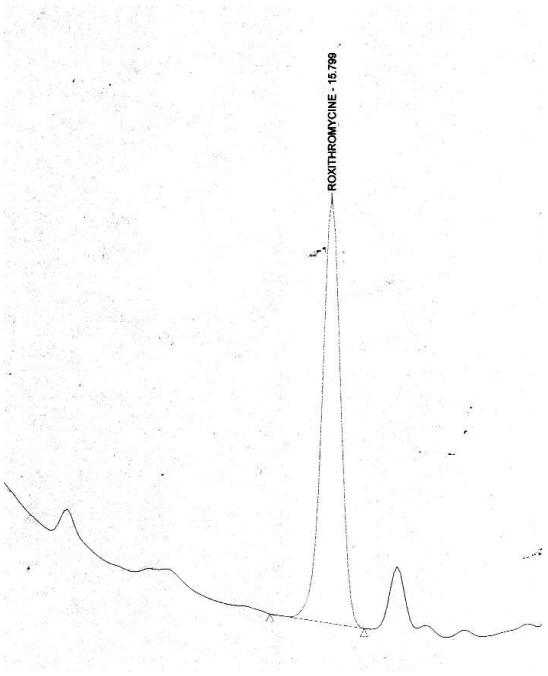
conc. of roxithromycin (mg/L)	recovery (%)
2.5	96.68
5.0	93.83
7.5	94.16
10.0	97.75
12.5	96.55
20.0	94.06

Retention time for roxithromycin in urine samples was 15,8 min.

Picture 3 shows UV spectrum and chromatogram of roxithrimycine standard solution concentration of 10 mg/L.



Picture 3. UV chromatogram of roxithromycin standard solution



Picture 4. Urine spiked by roxithromycin

Urine spiked by roxithromycin solution concentration of 10 mg/L has shown on picture 4.

DISCUSSION

Liquid chromatography is the most described method for determination of roxithromycin. New methods use mass spectrometric detection for monitoring drug concentrations in plasma for pharmacokinetic studies [6-7]. Due to the fact that LC/MS requires expensive equipment, we decided to use liquid chromatography with photodiode array detection.

There are different methods for sample preparation for determination of roxithromycin, such as solid-phase [2] or liquid-liquid extraction [1, 4]. We had performed liquid-liquid method for extraction of roxithromycin from urine samples by dichlormethane at pH about 10. This extraction gave better recoveries (mean value of 95.50%) than extraction by hexane-isoamylalcohol (98:2) (mean value of 90,0 ± 3%) described by Macek et al.

Identification of roxitrhromycin was performed by comparison of analytical standardžs relative retention time and UV spectrum and respectively peak in chromatogram of urine samples.

Roxithromycin has a weak absorbance at wavelength higher than 235 nm. So we determined it on 200.5 nm. It also could be determined at low wavelength such as 220 nm [1] or 215 nm [3] and 210 [4-5].

We used mobile phase which contained acetonitrile and phosphate buffer pH 3.6. These chromatographic conditions enablde good separation of roxithromycine from matrix compound. Other authors also used phosphate buffer in mobile phase, but, separation was performed at higher pH values [1-3].

Under described chromatographic conditions the retention time of roxithromycin was 15.8 min.

Calibration curve was linear in the range of 2.5 to 20 mg/L, which was in compliance with expected concentration in urine samples. The limit of quantitation (LoQ) was 1 mg/L, which was higher than LoQ of 0.5 mg/L (1), but, using of 5 mL of urine sample for analysis could decrease LoQ.

The proposed method was applied to the determination of roxithromycin in urine samples for monitoring of excretion unchanged drug by kidneys.

CONCLUSION

The described method allows monitoring elimination of roxithromycin via urine with basic chromatographic instrumentation in laboratory. The accuracy and precision of described method are comparable with other previously published HPLC-UV methods. Sensitivity of the HPLC-PDA method is sufficient for determination of roxithromycin in urine samples.

Apstrakt

Roksitromicin je polusintetski makrolidni antibiotik koji sprečava rast bakterija. Primenjuje se u lečenju infekcija respiratornog trakta, urinarnih i blagih infekcija tkiva. Nakon brze resorpcije difunduje u većinu tkiva i aktivnim transportom dospeva do mesta infekcije. Samo mali deo roksitromicina podleže metabolizmu. Najveći procenat leka se izlučuje u nepromenjenom obliku preko žuči i jedan deo preko izdahnutog vazduha. Manje od 10% roksitromicina izlučuje se urinom.

U radu je opisana jednostavna i reproduktivna metoda za određivanje roksitromicina u urinu. Roksitromicin je izolovan iz urina alkalnom tečno-tečnom ekstrakcijom dihlormetanom. Ekstrakti su zatim analizirani tečnom hromatografijom sa UV skenirajućim detektorom (HPLC/PDA). Protok mobilne faze koja se sastojala od acetonitrila i natrijum dihidrogenfosfata (pH vodenog rastvora koji je sastav mobilne faze je 3,6) je bio od 1 do 1,5 mL/min, uz korišćenje gradijent programa. Razdvajanje roksitromicina od komponenata matriksa izvršeno je na C8 koloni koja je zagrevana na 30°C.

Kvalitativna i kvantitativna analiza roksitromicina rađena je na talasnoj dužini od $\lambda = 200.5$ nm. Relativno retenciono vreme roksitromicina bilo je oko 15,8 min. Limit kvantifikacije iznosio je 2 μg/mL, a kalibraciona kriva je bila linearna u opsegu od 2 do 20 μg/mL, sa koeficijentrom korelacije od 0,9913. Prinos ekstrakcije iz urina opterećenog roksitromicinom bio je u opsegu od 93,83 do 97,75 %.

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