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Rapid UHPLC Method for Simultaneous Determination of Vancomycin, Terbinafine, Spironolactone, Furosemide and Their Metabolites: Application to Human Plasma and Urine

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The ultra high performance liquid chromatography (UHPLC)-UV method for the simultaneous determination of furosemide, saluamine (furosemide metabolite), spironolactone, carnenone (spironolactone active metabolite), terbinafine, *N*-desmethylcarboxy terbinafine (terbinafine metabolite) and vancomycin in human plasma and urine is proposed. Good separation of the analytes was achieved with the gradient RP-UHPLC-UV with the mobile phase composed as acetonitrile and 0.1% formic acid. The determined substances were eluted from a Hypersil GOLD C_{18}e (50 mm × 2.1 mm, 1.7 μm particles) column in 3.3 min. Good linear relationships were observed for all of the analytes (R^2 higher than 0.994). The limit of detection (LOD) values varied from 0.01 to 0.07 μg ml^{-1}, with vancomycin as an exception (0.11 μg ml^{-1}). After protein precipitation and solid-phase extraction, samples of plasma and urine were analyzed. Thanks to the short analysis time and small quantities of urine or plasma needed, this method can be applied to routine clinical analysis.

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Introduction

In this study, we propose the use of ultra high performance liquid chromatography (UHPLC) equipment for the fast simultaneous determination of vancomycin (VAN) (antibacterial drug), furosemide (FUR), spironolactone (SPR) (diuretics) and terbinafine (TER) (antifungal drug) along with their metabolites in human urine and plasma. The investigated metabolites were: saluamine (SE), furosemide metabolite; carnenone (CAR), active metabolite of spironolactone; *N*-desmethylcarboxy terbinafine (DMT), metabolite of terbinafine.

The proposed drug mixture was chosen intentionally, because these drugs are often administrated to patients on Intensive Care Units. Patients after cardiac surgery (*e.g.* bypass or valves implantation) are often treated with diuretics. In cases, where an infection occurs (especially with dangerous inter hospital bacteria strains), vancomycin is administrated. In the case of an antibiotic treatment and weakened patient organism, the most often observed complication is a consequential fungal infection, where terbinafine administration is advised. The possibility of the simultaneous monitoring of drugs and metabolites from different therapeutic groups allows to have hope for better therapy effects.

The literature survey revealed that no UHPLC methods for the simultaneous determination of the mentioned drugs were proposed. So far, FUR and SPR were determined by HPLC with UV-DAD[1] or MS[2-4] detection in human urine/plasma. SPR and its active metabolite were determined by HPLC with UV detection in plasma samples.[5]

Vancomycin, known for potential ear and kidney toxicity, when plasma concentrations reach the 50 μg ml^{-1} level[6] was determined by HPLC coupled with electrochemical,[6] fluorescence[7] or UV detectors.[8-10] The lowest limit of quantification (5 ng ml^{-1}) was achieved with fluorescence detection.

TER is mainly metabolized to *N*-desmethylcarboxy terbinafine (DMT), with liver metabolism and urine excretion. TER or TER and DMT were determined by RP-HPLC in pharmaceuticals,[11,12] plasma and urine.[13,14]

Last year we developed an HPLC-DAD method that allows us to determine VAN, TER and FUR with 19 other drugs in urine,[15] but the analysis time of that method was relatively long (VAN, TER and FUR concentrations could be established after 51 min).

In the proposed method, the analysis time was significantly shortened (to the 5.3 min). This method was applied not only to the urine, but also to plasma samples. Additionally, lower LOD values for TER and FUR were achieved, thanks to the UHPLC equipment usage. The proposed chromatographic system also allowed for metabolite determination, which was not examined before.

Thanks to the implementation of the method proposed in this paper, all of the analytes can be determined in one chromatographic system using one preconcentration procedure. Additionally, the shortest analysis time (5.3 min) can lower the labor cost and allow health workers to react rapidly to state changes of patients.

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Table 1 Gradient elution program

Step	Analysis time/ min	Solvent A, %	Solvent B, %	Flow/ ml min^{-1}
1	0	99	1	0.7
2	2	70	30	1.5
3	5	60	40	1.3

Experimental

Chemicals and solutions

VAN, SPR, TER, FUR and ketoprofen (IS) were purchased from Sigma-Aldrich (Schnelldorf, Germany) (97 – 99% purity). CAR, DMT, and SE (purity >98%) were bought from Toronto Research Chemicals (North York, Canada). Acetonitrile (ACN), methanol (MeOH), water acetic acid and formic acid (HPLC grade) were purchased from Merck (Darmstadt, Germany).

Stock solutions

Stock solutions of VAN, SPR, CAR, TER, DMT, FUR, SE and IS were prepared by dissolving the specified amount of drugs (or drugs salts, in the case of VAN and TER) in 10 ml of methanol to achieve the desired 1 mg ml^{-1} concentration of each drug free form. After preparation, stock solutions were stored at 4°C and were stable for at least four weeks. Working standard solutions were prepared daily by mixing individual stock solutions and diluting these mixtures with specified amounts of methanol. The working standard solutions were stable for at least 3 days at 4°C.

Apparatus and chromatographic conditions

A reversed-phase UHPLC system containing a UV detector Model L-2400U (Hitachi), L-2350 column oven (Hitachi, Merck), two L-2160U pumps and a reversed-phase Hypersil GOLD C_{18}e column (50 mm × 2.1 mm, 1.7 µm particles) (Thermo Scientific, Waltham, USA) was used. Samples were injected by an L-2200U autosampler (Hitachi). The solvent used for syringe wash was methanol. The human plasma/urine samples were centrifuged using a HERMLE 323K (Germany) centrifuge. Solid-phase extraction procedures were performed using a J.T. Baker SPE-12G (Deventer, Netherlands).

UHPLC analyses were carried out at 20°C with gradient elution. The best gradient program evaluated for the determination using the Hypersil GOLD C_{18}e column was received with 0.1% formic acid in water (solvent A) and ACN (solvent B). The gradient elution program is presented in Table 1. Before analysis, equilibration was performed. To equilibrate the initial conditions, the column was equilibrated for 2 min (mobile-phase composition the same as in the first step of the gradient program). After equilibration, injection was performed. After each injection the needle was washed with methanol. The eluate was monitored by UV detection at wavelengths specified for each drug. The analytical wavelengths and retention times are presented in Table 2.

Sample collection

Blank human urine or plasma were obtained from volunteers who have not taken any of the analyzed drugs for longer than one week. Additionally, plasma from patients treated with the drugs of interest (terbinafine, vancomycin and furosemide) were collected one and 4 h after drug administration. The anticoagulant agent used during sample collection was heparin.

Table 2 Monitoring wavelengths and retention time of the analytes (n = 6)

Analyte	Monitoring wavelength/nm	Retention time/ min	SD/min	RSD, %
SE	280	1.650	0.019	0.72
DMT	224	2.341	0.023	0.87
FUR	280	2.488	0.028	1.07
TER	224	2.746	0.007	0.27
VAN	215	2.960	0.010	0.38
SPR	245	3.037	0.021	0.80
CAR	280	3.215	0.027	1.05

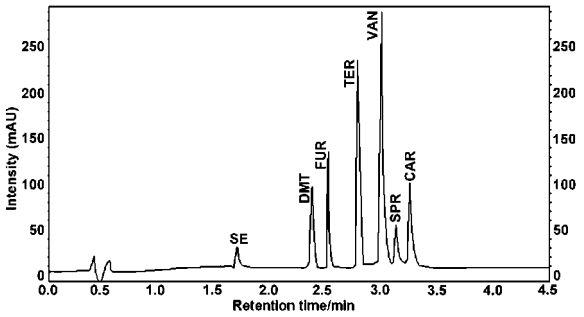

Fig. 1 Representative chromatogram of the standards mixture (10 µg ml^{-1}). Detections were performed at: λ = 280 nm for SE, FUR and CAR; λ = 224 nm for DMT and TER; λ = 215 nm for VAN; λ = 245 nm for SPR.

Solid phase extraction procedures

Samples were extracted on 500 mg, 3 ml Narc-2; 200 mg, 3 ml SDB (Baker Bond); 500 mg, 6 ml Bond Elut-PPL; 200 mg, 6 ml ABS Elut-Nexus (Varian) extraction columns, to find the best extraction conditions. The Narc-2 column was dedicated by the producer for drug extraction from body fluids. The other columns showed good recoveries in surface-water analysis, but have not been tested for mentioned drug preconcentration from plasma/urine. Therefore, we chose this group of columns for recovery tests.

All columns, apart from Nexus (a non-conditioned extraction column), were conditioned before extraction (one volume of MeOH and one volume of H_2O, pH 7).

Urine or plasma samples (1 ml) were transferred to 10 ml polypropylene tubes; 0.5 ml of MeOH and 0.5 ml of ACN were added for protein precipitation. After shaking, samples were centrifuged at 7500 rpm for 5 min to remove the precipitate; 2 ml of phosphate buffer pH 7 were added to the supernatant and put through the previously conditioned extraction columns. After column drying, elution with 2 ml of MeOH was performed. Methanol was evaporated and the residue was dissolved in 0.5 ml of MeOH. Such prepared samples were injected to a chromatograph. The optimal injection volume was 5 µl.

Results and Discussion

A new UHPLC analytical method for the simultaneous determinations of FUR, SPR, TER, VAN, SE, CAR and DMT was utilized. Various two-solvent gradient compositions and flow rates were tested. The first gratifying composition was 0.05% trifluoroacetic acid in water and ACN on a Chromolith

Table 3 Calibration curves, correlation coefficients, LOD and LOQ values for the analytes (n = 6)

Analyte	Linearity range/μg ml^{-1}	Calibration curve parameter					LOD/μg ml^{-1}	LOQ/μg ml^{-1}
		Slope, a	Sa[a]	Intercept, b	Sb[b]	R^2		
SE	0.16 - 20	8328	368	4255	3778	0.994	0.05	0.16
DMT	0.10 - 20	14348	239	1865	1218	0.999	0.03	0.10
FUR	0.04 - 5	28721	1041	18573	3066	0.998	0.01	0.04
	5 - 20	49927	5165	–57063	68332	0.995		
TER	0.03 - 20	161150	6587	–3218	67563	0.995	0.01	0.03
VAN	0.36 - 20	148272	8012	105555	82182	0.991	0.11	0.36
SPR	0.23 - 20	14583	472	10939	4848	0.996	0.07	0.23
CAR	0.17 - 20	81572	2350	14614	24111	0.997	0.05	0.17

a. Standard deviation of a slope. b. Standard deviation of intercept.

Fast Gradient® C_{18} monolithic column (Merck). Thanks to this, the gradient elution was finished after 6.5 min, with satisfactory separation of all analytes. Additional gradient and stationary phase modifications allow for a significant shortening of the analysis time. The optimal separation was achieved for a Hypersil GOLD C_{18}e column, with 0.1% formic acid and an ACN mixture as a mobile phase. Thanks to the sub 2 μm particle chromatographic column, the elution of analytes can be performed within 3.3 min. The following gradient was chosen for determining the analytes in urine/plasma and the recovery tests (Table 1). A sample chromatogram of standards (10 μg ml^{-1}) is presented on Fig. 1.

The very short time, selectivity and sensitivity of the presented method allow for use in routine clinical analysis.

Additionally, a solid-phase extraction procedure for analyte preconcentration was proposed. Firstly, the influence of a protein-precipitation step on the procedure was considered. When the proteins were not precipitated before the spe, matrix interferences with analytes were observed. Therefore, this part is necessary in the preparation procedure. Additionally, the influence of the pH values during column conditioning on the recoveries was tested. TER and DMT showed higher recoveries than the other drugs for pH 7 and 9. FUR, SE, SPR, CAR showed good recoveries while cartridges were being conditioned with water acidified to pH 2, with a not significant loss at pH 7. VAN, an amphoteric drug, showed similar recoveries in each case. Therefore a neutral pH was chosen for column conditioning.

Calibration curves

Calibration curves were determined as a linear function, $y = ax + b$, where y is the measured peak area and x is the drug concentration (μg ml^{-1}). The values a and b are constant. The limits of detection (LOD) were calculated as LOD = $3 \times s/a$, where s is the standard deviation of blank samples and a is the calibration curve slope. The limits of quantification (LOQ) values were determined based on the s/a ratio being multiplied by 10 (LOQ = $10 \times s/a$). The linearity range, LOD and LOQ values, and calibration curve parameters are detailed in Table 3. The estimated limit of the detection values are sufficient for the clinical analysis of the mentioned analytes. All of the calibration curves showed linearity for a wide range of standards concentrations. Only for FUR was the calibration curve separated for two linear ranges: low range (0.04 - 5 μg) and high range (5 - 20 μg), because of better linearization. While the whole range was considered in a single equation, the correlation coefficient (R^2) was 0.980. When the separation into two equations was made, the correlation coefficient increased to 0.998 (low range) and 0.995 (high range).

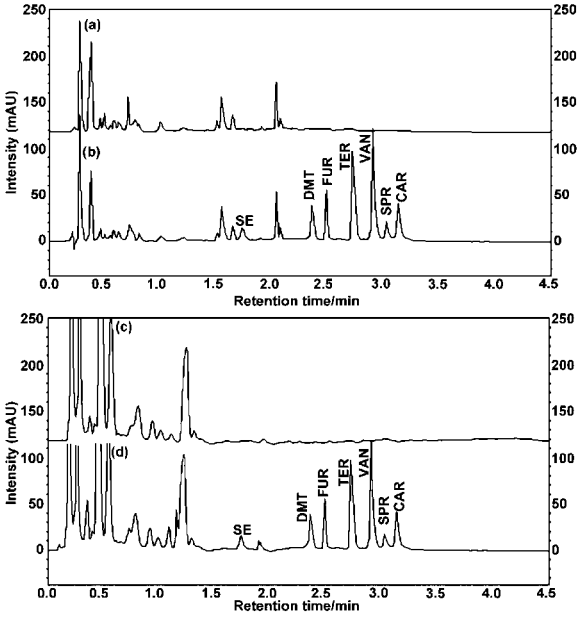

Fig. 2 Chromatograms after the extraction on Narc-2 cartridges: (a) best chromatogram of the blank human plasma, (b) plasma spiked with 5 μg of drugs, (c) best chromatogram of the blank human urine, (d) urine spiked with 5 μg of drugs. Spiking was performed before the preconcentration procedure. Specified monitoring wavelengths are presented in Table 2.

Selectivity and recoveries

Blank urine and plasma from 6 different middle-age volunteers was collected and tested for the matrix interferences with determined drugs. An example of blank human plasma and urine spiked with 5 μg of drugs (before SPE on Narc-2 cartridges) is presented on Fig. 2. As can be seen, the drugs are well resolved with no apparent interference from endogenous plasma/urine components.

The recoveries of analytes were tested on four extraction columns. Blank human urine or plasma was spiked with 5 μg of each substance and then extracted. The highest recoveries for the analytes were obtained using Narc-2 columns. The drug recoveries along with standard deviations (SD) on all of the tested columns are presented in Table 4.

For further analysis the procedure utilized on Narc-2 cartridges was chosen, because of the higher recoveries for all of the analytes.

Table 4 Drug recoveries on tested columns

Analyte	Recovery (SD), %							
	Narc-2		PPL		Nexus		SDB	
	Plasma	Urine	Plasma	Urine	Plasma	Urine	Plasma	Urine
SE	92.6 (3.7)	95.0 (5.1)	47.5 (6.1)	50.4 (2.1)	79.1 (5.6)	106.1 (7.2)	59.2 (4.6)	56.8 (4.2)
DMT	83.1 (2.5)	83.3 (3.2)	65.3 (4.1)	62.1 (3.2)	58.5 (2.5)	60.4 (3.4)	44.3 (3.8)	45.4 (3.2)
FUR	90.4 (2.0)	93.7 (2.9)	68.4 (2.5)	71.3 (5.1)	97.1 (3.2)	96.5 (3.7)	105.4 (5.21)	118.2 (7.2)
TER	85.1 (3.1)	86.6 (4.1)	20.0 (3.1)	19.4 (4.3)	51.4 (4.7)	52.0 (4.2)	45.2 (2.1)	49.5 (2.2)
VAN	85.4 (2.6)	89.1 (2.4)	47.2 (2.7)	46.3 (3.4)	46.1 (3.2)	46.1 (4.1)	67.0 (3.9)	67.6 (3.5)
SPR	75.5 (3.0)	71.9 (3.2)	62.0 (4.3)	66.5 (3.8)	75.1 (2.4)	71.1 (3.1)	73.2 (4.1)	77.4 (2.7)
CAR	87.3 (3.2)	91.0 (2.1)	<5	<5	51.2 (2.1)	50.8 (1.9)	<5	<5

Blank human urine/plasma spiked with a known amount of drug (5 μg ml^{-1}), then extracted (n = 6, pH 7).

Table 5 Accuracy values for determined drugs in plasma and urine (n = 6)

Analyte	Amount added/ μg ml^{-1}	Mean accuracy, %	
		Urine	Plasma
SE	0.5	97.8	103.3
	5.0	99.5	101.9
	10.0	100.2	99.8
DMT	0.5	102.7	102.1
	5.0	100.8	102.2
	10.0	100.5	100.9
FUR	0.5	102.2	102.9
	5.0	100.3	102.6
	10.0	99.9	100.3
TER	0.5	99.1	101.4
	5.0	99.5	99.8
	10.0	99.7	100.7
VAN	0.5	94.6	105.8
	5.0	99.1	99.3
	10.0	101.0	99.5
SPR	0.5	99.1	99.3
	5.0	99.6	98.9
	10.0	98.7	99.2
CAR	0.5	99.0	100.3
	5.0	99.3	99.9
	10.0	101.1	98.3

Accuracy

The accuracy was measured at low (0.5 μg ml^{-1}), medium (5.0 μg ml^{-1}) and high (10.0 μg ml^{-1}) concentrations for all of the analytes. For accuracy measurements, human plasma and urine samples were previously prepared on Narc-2 cartridges. The accuracy, expressed as a percentage, was determined by comparing the mean measured (calculated with the calibration curve equation) and target concentrations and multiplying by 100. The calculated accuracies (n = 6) are detailed in Table 5.

Plasma and urine stability tests

Four sets of quality control samples prepared by spiking blank human plasma (or urine) with 5 μg ml^{-1} of each substance were measured.

Table 6 Stability tests of plasma and urine control samples (5 μg ml^{-1})

Analyte	Test	Mean concentration difference, % (n = 6)	
		Urine	Plasma
SE	3 freeze/thaw cycles	3.3	4.1
	Post extraction, 24 h at 4°C	3.4	4.4
	In plasma, 24 h at room temperature	n/a	4.7
DMT	3 freeze/thaw cycles	3.1	2.6
	Post extraction, 24 h at 4°C	2.9	2.6
	In plasma, 24 h at room temperature	n/a	3.5
FUR	3 freeze/thaw cycles	3.2	3.5
	Post extraction, 24 h at 4°C	2.5	3.7
	In plasma, 24 h at room temperature	n/a	4.1
TER	3 freeze/thaw cycles	2.1	2.5
	Post extraction, 24 h at 4°C	2.6	2.6
	In plasma, 24 h at room temperature	n/a	3.0
VAN	3 freeze/thaw cycles	3.6	5.8
	Post extraction, 24 h at 4°C	3.7	4.6
	In plasma, 24 h at room temperature	n/a	5.6
SPR	3 freeze/thaw cycles	3.7	3.1
	Post extraction, 24 h at 4°C	4.0	3.5
	In plasma, 24 h at room temperature	n/a	3.9
CAR	3 freeze/thaw cycles	2.2	2.8
	Post extraction, 24 h at 4°C	2.6	3.2
	In plasma, 24 h at room temperature	n/a	3.7

The first set was measured directly after preparation, and the second after three freeze-thaw cycles. The next set was extracted directly after preparation. The extract was stored in 4°C for 24 h, and then measured. The fourth set (plasma samples) was stored at room temperature for 24 h. After 24 h the measurements were made. The concentration differences after the tests were placed in range of 2.1 – 5.8%, which is detailed in Table 6. Because of loss during storage, an internal standard addition was required. The internal standard proposed was ketoprofen (IS), because of a similar behavior during storage, preparation-similar recoveries (85.3%) and stable response factors for all of the analytes.

Plasma samples measurement

After method validation, plasma samples collected from patients taking TER (250 mg), FUR (40 mg) and VAN (125 mg) were measured. After SPE on Narc-2 cartridges, 5 μl of the sample was injected into UHPLC equipment. An example of the best chromatogram obtained from a plasma sample containing the above-mentioned drugs is presented on Fig. 3. Each drug was monitored at the proper wavelength. Because of the low quantity of the drug in plasma, the chromatogram is presented with a higher sensitivity. As a consequence, peaks from the matrix are higher. The measured concentrations for DMT, TER, FUR, SE and VAN were subsequent: 0.25 – 0.69 μg ml^{-1} for DMT, 0.36 – 0.59 μg ml^{-1} for TER, 0.21 – 0.95 μg ml^{-1} for FUR, 0.19 – 0.27 μg ml^{-1} for SE and 2.32 – 7.81 μg ml^{-1} for VAN.

Conclusion

A new and fast method has been proposed for the determination

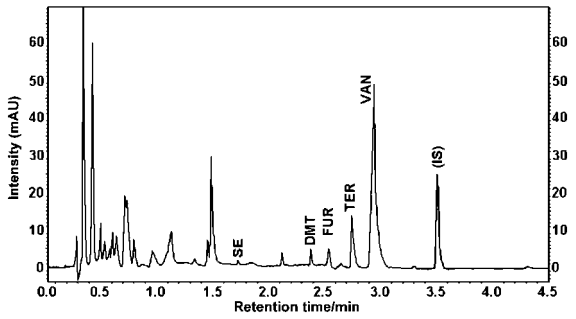

Fig. 3 Best chromatogram of a plasma sample from a patient taking FUR (40 mg), TER (250 mg) tablets and VAN injections (showed at $\lambda = 224$ nm for DMT and TER; $\lambda = 280$ nm for FUR and SE, $\lambda = 215$ nm for VAN and $\lambda = 254$ nm for the IS). Plasma spiked with ketoprofen (IS) (2 μg ml^{-1}) before the extraction on a Narc-2 SPE cartridge.

of pharmaceuticals and their metabolites in human urine and plasma using UHPLC equipment with UV detection with wavelength switching. Thanks to the presented chromatographic conditions, the separations of furosemide, terbinafine, spironolactone and vancomycin with the metabolites can be made in 5.3 min. The low LOD and LOQ values obtained for this method in relation to the short analysis time, allow for the implementation of this method to clinical analysis.

It should also be underlined that the evaluated procedure for the determination of drugs and their metabolites is also suitable for metabolic trace investigations. Therefore, it can be used in metabolomics and pharmacometabolimics.

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